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COMPREHENSIVE INVITED REVIEW

The Disulfide Proteome and Other Reactive Cysteine Proteomes: Analysis and Functional Significance

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Abstract

Ten years ago, proteomics techniques designed for large-scale investigations of redox-sensitive proteins started to emerge. The proteomes, defined as sets of proteins containing reactive cysteines that undergo oxidative post-translational modifications, have had a particular impact on research concerning the redox regulation of cellular processes. These proteomes, which are hereafter termed "disulfide proteomes," have been studied in nearly all kingdoms of life, including animals, plants, fungi, and bacteria. Disulfide proteomics has been applied to the identification of proteins modified by reactive oxygen and nitrogen species under stress conditions. Other studies involving disulfide proteomics have addressed the functions of thioredoxins and glutaredoxins. Hence, there is a steadily growing number of proteins containing reactive cysteines, which are probable targets for redox regulation. The disulfide proteomes have provided evidence that entire pathways, such as glycolysis, the tricarboxylic acid cycle, and the Calvin-Benson cycle, are controlled by mechanisms involving changes in the cysteine redox state of each enzyme implicated. Synthesis and degradation of proteins are processes highly represented in disulfide proteomes and additional biochemical data have established some mechanisms for their redox regulation. Thus, combined with biochemistry and genetics, disulfide proteomics has a significant potential to contribute to new discoveries on redox regulation and signaling. *Antioxid. Redox Signal.* 14, 2581–2642.

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I. Introduction

A. The concepts of redox regulation and redox signaling

Redox regulation has been defined according to Buchanan and Balmer (39) as "a reversible posttranslational alteration in the properties of a protein, typically the activity of an enzyme, as a result of change in its oxidation state." These authors made a clear distinction between redox regulation and terminal oxidation, which they defined as "an irreversible reaction that marks proteins for degradation" (39) and is implicitly understood to impair protein function. While sometimes used interchangeably with "redox regulation," the term "redox signaling" extends the meaning to include entire chains involving cascades of redox reactions, eventually leading to changes in gene expression. However, there is so far no consensus definition that unambiguously discriminates between the terms "redox regulation" and "redox signaling." Behind the research ventures reported in this review article lies the firm conviction of many researchers that our current knowledge on redox regulation reflects merely the tip of the iceberg and that many more processes remain to be discovered.

The aim of this review is to give an overview of the recent contributions of proteomics to our understanding of redox biology involving cysteine modifications. Due to the extension of the field, it has not been possible to go into details of individual research areas, and many excellent nonproteomic studies on individual proteins could not be covered. Instead, we attempt to present a broad overview of proteome research on disulfides and reactive cysteines through all kingdoms of life, highlighting some of its opportunities and limitations.

B. Cysteine reactivity and cysteine modifications

Cysteine is a rarely used amino acid that accounts for about 2% of the amino acids in eukaryotic proteins and about 1% in proteins from eubacteria and archaea (251). Redox signaling often implies a post-translational protein modification of cysteine residues (56), and a particular cysteine residue may be differentially modified in response to diverse stimuli. Post-translationally modified cysteines are not necessarily directly involved in the catalytic activities of enzymes, but may function at an allosteric site and, thus, regulate the enzymatic activities or other protein functions through structural changes. Reactive oxygen and nitrogen species (reactive oxygen species [ROS] and reactive nitrogen species [RNS], respectively) can induce redox signals by means of oxidative modifications of cysteine residues (63, 85, 86). Numerous excellent and exhaustive reviews, of which we just mention a few, have covered the production and fate of ROS and RNS in plants (7, 90), bacteria (146, 349), and animal cells (44, 300).

The large, polarizable sulfur atom in a thiol group is electron-rich and highly nucleophilic (263); hence, cysteines can undergo a broad range of chemical reactions (148). Figure 1 shows some of the most common chemical modifications of cysteine thiols in proteins. The oxidation states of sulfur in these cysteines range from $-$II in the thiol group to $+$IV in sulfonic acid. Typically, the reactive cysteine thiol (Cys-SH) of a protein is in equilibrium with its deprotonated thiolate form ($Cys\text{-}S^-$). This equilibrium depends both on the pK_a of the thiol and the pH of the environment, which makes thiols well suited to function as pH-sensitive molecular switches. Thiolate anions are susceptible to oxidation by, for example, hydrogen peroxide, organic hydroperoxides, and hypochlorous acid. A product of such oxidation is the sulfenic acid (Cys-SOH) (257). In many cases, the cysteine sulfenate form undergoes rapid condensation with other cysteine thiols to form disulfide bonds. The resulting disulfides can be intramolecular, with another cysteine from the same protein, or intermolecular, with a cysteine from another protein or a small molecule, such as glutathione or free cysteine (260). The covalent attachment of glutathione to protein cysteines through a mixed disulfide bond is known as glutathionylation (61) or S-thiolation. The disulfide bonds resulting from cysteine oxidation play important roles in protein structure and oligomerization, as well as enzyme activity regulation.

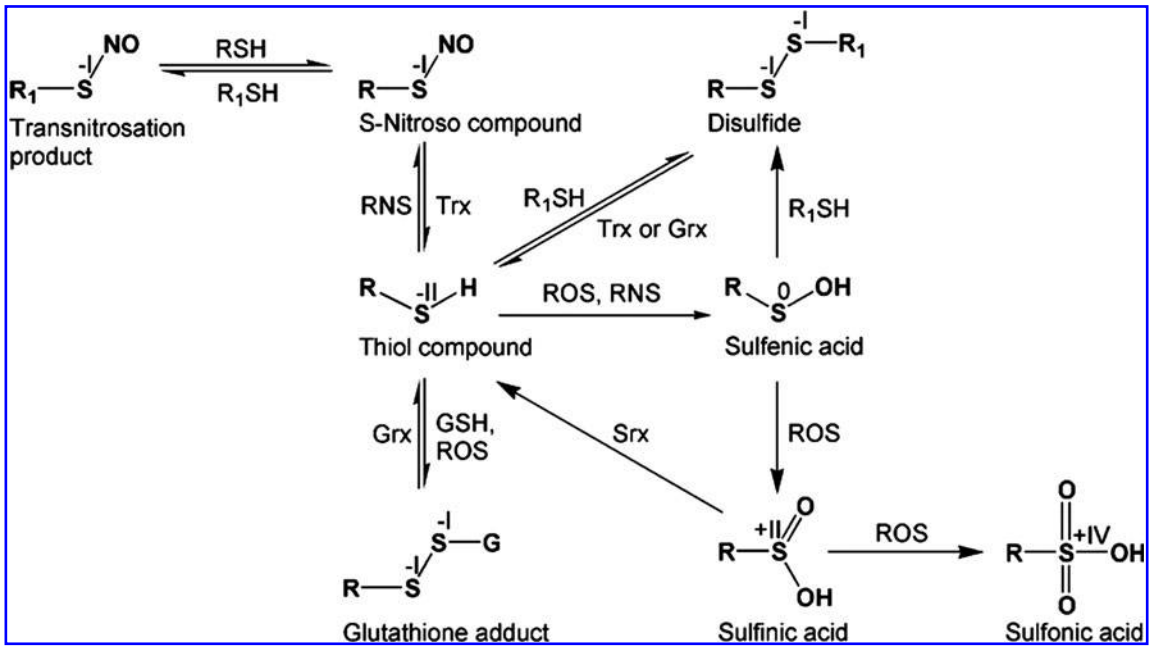

FIG. 1. Relevant redox reactions of protein thiols under physiological conditions. Protein cysteine thiols (R-SH) can undergo a broad range of chemical reactions. One of the most common reactions of thiol groups is disulfide formation with another thiol, which changes the oxidation state of sulfur from −2 to −1. In a similar reaction protein-bound thiol groups can form mixed disulfides with glutathione, which is referred to as glutathionylation. Both disulfides and glutathione adducts can be reduced back to the thiol form by thioredoxin (Trx) and glutaredoxin (Grx), respectively. In the presence of hydrogen peroxide or other reactive oxygen species (ROS), thiol groups can be oxidized to sulfenic acid, in which sulfur has the oxidation state 0. Sulfenic acid can either be reduced by other thiols to the disulfide state, or become further oxidized to sulfinic acid where sulfur exists in the oxidation state +2. Sulfinic acid can be reduced to the thiol state by sulfiredoxin (Srx). However, oxidation of the sulfinic acid to sulfonic acid, where sulfur has the oxidation state +4, is irreversible. Thiol groups can also be oxidized by reactive nitrogen species (RNS) and form S-nitrosothiols, where sulfur has the oxidation state −1. S-nitrosothiols can nitrosylate other cysteine thiols by a transnitrosylation reaction or they can be denitrosylated by reduced Trx.

However, contrary to popular belief, intramolecular disulfides do not always stabilize proteins (331). The RNS, including nitric oxide (NO) and peroxynitrite (ONOOH), can mediate S-nitrosylation yielding S-nitrosothiol (Cys-SNO) and disulfides (74, 124). Denitrosylation catalyzed by reduced thioredoxin (Trx) can restore the thiol state (25, 26, 130, 284). In addition, NO can be transferred from S-nitrosothiols in other thiol groups by transnitrosylation (124, 155, 332). Many oxidative cysteine modifications are reversible through reduction catalyzed by oxidoreductases, such as Trxs or glutaredoxins (Grxs), in prokaryotic as well as eukaryotic organisms (193, 230). The discoveries that bacterial and mammalian Trx are able to reduce disulfides in some plant chloroplast enzymes (36, 126) and that bacterial Trx reduces insulin (127) raised the awareness of Trx as being a universal catalyst for protein disulfide reduction.

However, in certain proteins, the microenvironment may stabilize the cysteine sulfenic acid and this form could in turn be sensitive to hyperoxidation yielding sulfinic ($R\text{-}SO_2H$) and sulfonic acid ($R\text{-}SO_3H$) (56, 260). While cysteine sulfinic acid can be reverted to the thiol form by sulfiredoxin (Srx) (28) or sestrins (41), hyperoxidation to sulfonic acid is believed to be irreversible. Details of the chemistry of oxidative cysteine thiol modifications have been excellently reviewed in ref. (263).

Protein cysteines differ in their reactivity, and not all cysteines are susceptible to modification. For a comparison, redox potentials in thiol-disulfide oxidoreductases range from −95 to −330 mV, whereas structural disulfides may have potentials as low as −470 mV (331 and references therein). In other words, such structural disulfides would never be found as dithiols under normal physiological conditions. The properties of the protein microenvironment, which control cysteine reactivity, are not completely understood, though the cysteine pK_a is known to play an important role. Any cysteine modification, which involves an initial thiol deprotonation at physiological pH, would require a lowered sulfhydryl pK_a for the modifiable cysteine (275). Lowering the pK_a of a cysteine increases its nucleophilicity and thereby its ability to undergo modifications.

As for sulfenic acid modifications, the mean pK_a for modifiable cysteine residues, as compared to nonmodifiable ones, was calculated to be 6.9 for the reactive cysteines and 8.14 for the control cysteines (275). The decrease in pK_a is influenced by the presence of titratable, polar, but not necessarily charged, residues in the cysteine microenvironment. The histidines are highly represented in this microenvironment and, in addition, the presence of threonine residues has been related with such a pK_a shift (275). The cysteine sulfenic acid (Cys-SOH) plays an important role in redox signaling and the protein structure may ensure the stability of this cysteine form to maintain its reactivity and, hence, its signaling capacity. In this case, the Cys-SOH microenvironment is characterized by the lack of nearby reduced cysteines and the presence of local hydrogen-bonding residues, which stabilize the sulfenate form (257).

As for S-nitrosylation, a comparison of the pK_a of S-nitrosylated and unmodified cysteines within individual liver proteins did not reveal any significant differences (73).

On the other hand, the impact of the pK_a of cysteine residues for their susceptibility toward in S-nitrosylation has been shown by a quantification of individual S-nitrosylation sites (348). The level of S-nitrosylation of different cysteines varied within individual proteins, and the upregulation of S-nitrosylation was more pronounced for cysteine residues with a lower pK_a than for those with a higher pK_a (348). However, the pK_a of a cysteine residue is not a parameter that alone can be used to define the specificity of S-nitrosylation. The modification of cysteine thiols by S-nitrosylation is likely to involve multiple mechanisms (73, 215). Basic and acidic amino acids in the close neighborhood of the cysteine are considered to play an important role in the transfer of NO^+ to the thiolate group. For instance, the site of S-nitrosylation in hepatic methionine adenosyltransferase is Cys121 that is flanked by Arg 357 and Arg 363, and by Asp355. The basic groups of amino acids Arg 357 and Arg 363 are suggested to facilitate deprotonation by lowering the pK_a of the cysteine, whereas the carboxyl group of Asp 355 is considered to increase the pK_a of the thiol group of *S*-nitroso-glutathione (GSNO), which assists in the donation of NO^+ (118, 252).

C. Disulfide proteomes

The identification of proteins with redox-active cysteines has conventionally involved tedious biochemical characterization of proteins with conserved cysteines after previous selection based on genetic experiments. However, in the past decade proteomic approaches that aimed at large-scale identification of proteins with modified cysteines have provided tools for unraveling new redox-regulated processes. Thus, the endeavor to establish disulfide proteomes could be considered an effort to accelerate discovery, though with no intention of eliminating the requirements for validation. The term "disulfide proteome" might be used in the strict sense as a set of proteins containing disulfide bonds. However, it is often understood implicitly that the "disulfide sub-proteome" of interest consists of those proteins containing disulfides, which are susceptible to reduction by oxido-reductases, for example, Trx. Such proteomes started to appear in the scientific literature in the late 1990s and the term "disulfide proteome" was used already in 2002 (339). "Disulfide proteomes" have also been widely defined as sets of proteins that contain cysteines, which exhibit any kind of change in their redox state (198). Elsewhere, these proteomes have been referred to as the "reactive cysteine proteome" (197).

II. Methods

The majority of the reported studies on disulfide proteomes can be divided into two categories. Those of the first category are based on the question, "Which of the proteins in this organism, tissue, or organelle contain cysteines susceptible to oxidative modifications under certain conditions?" The second category includes studies raising the question, "Which proteins in this organism, tissue, or organelle contain oxidized cysteines, such as disulfides and sulfenic acids that are reduced by Trx or Grx?" or, in other words, "What are the functions of Trx or Grx in this organism, tissue, or organelle?" The latter disulfide proteomes are therefore generally referred to as Trx (or Grx) target proteomes. Although each category of proteomes is addressed using different methodologies, these studies often represent two sides of the same coin, since it would be expected that many proteins undergoing cysteine oxidation become rereduced by Trx or Grx.

A. Trapping the cysteine redox state

The reactive nature of cysteine thiols is often an experimental challenge when determining the *in vivo* cysteine oxidation state of proteins (115). In samples from tissues or cell cultures, postlysis thiol-disulfide exchange between proteins may lead to misinterpretations of data; if molecular oxygen is present in the buffers, oxidation of cysteine thiols may occur during isolation. Hence, one of the critical steps when working in redox biology is quenching the samples to trap the thiol-disulfide status. This can be achieved by either acidification followed by alkylation, or by a direct alkylation. Acidification is usually performed using trichloroacetic acid, which precipitates the proteins and keeps the free cysteine thiols protonated until the protein pellet is dissolved in a buffer containing an alkylating reagent to block the free thiols. Acid quenching of thiols is reversible, though, and thiol/disulfide exchange might continue during the analytical procedure for determining the thiol/disulfide ratio (279). An alternative approach involves direct alkylation of free thiols during the lysis of cells (115, 188, 290). Anaerobic conditions during sample handling and alkylation help preventing thiol oxidation artifacts (29).

Widely used reagents for blocking free protein thiols are 2-iodoacetamide (IAM), *N*-ethylmaleimide (NEM), and methyl methanethiosulfonate (MMTS) (115, 188). IAM reacts with thiols in alkaline solutions (pH 8–8.5) in a nucleophilic substitution, in which the iodine group is replaced and a carbamidomethylated thiol is formed (Fig. 2B). NEM reacts specifically with thiols to form a thioether (Fig. 2A). Thiol alkylation by IAM and NEM is not reversible, but the reaction of MMTS with thiols results in the formation of mixed disulfides and can be reversed by reducing agents (Fig. 2C). However, there are potential pitfalls in the use of these reagents, and their labeling efficiency and specificity *in vivo* is difficult to assess. Side reactions of IAM and NEM with, for example, methionine, histidine, and peptide bonds have been observed (170, 298), and the formation of artificial disulfides after MMTS labeling has been detected (157). These side reactions are favored by high pH and high concentrations of the alkylating reagent (115), whereas, for example, NEM is usually thiol-specific at pH 7 and at concentrations up to 20 m*M* (35).

In the following steps of the labeling protocol, the disulfides or sulfenic acids are usually reduced to the thiol form using dithiothreitol (DTT) (54) or tris(2-carboxyethyl)phosphine (42). Finally, the newly formed thiols are commonly labeled with reagents that allow detection through fluorescence, radioactivity, or differential mass tags (115, 188, 290). To specifically label the thiols that are to be detected and quantified, common labeling reagents carry a functional group that is classified as a primary halogene alkane (Fig. 3), *N*-maleimide (Fig. 4), or a disulfide (Fig. 5). The reactions of these classes of compounds with thiols follow the principal scheme shown in Figure 2.

B. Thiol labeling

The catalog of thiol labeling reagents includes a large number of different compounds, and we can only mention

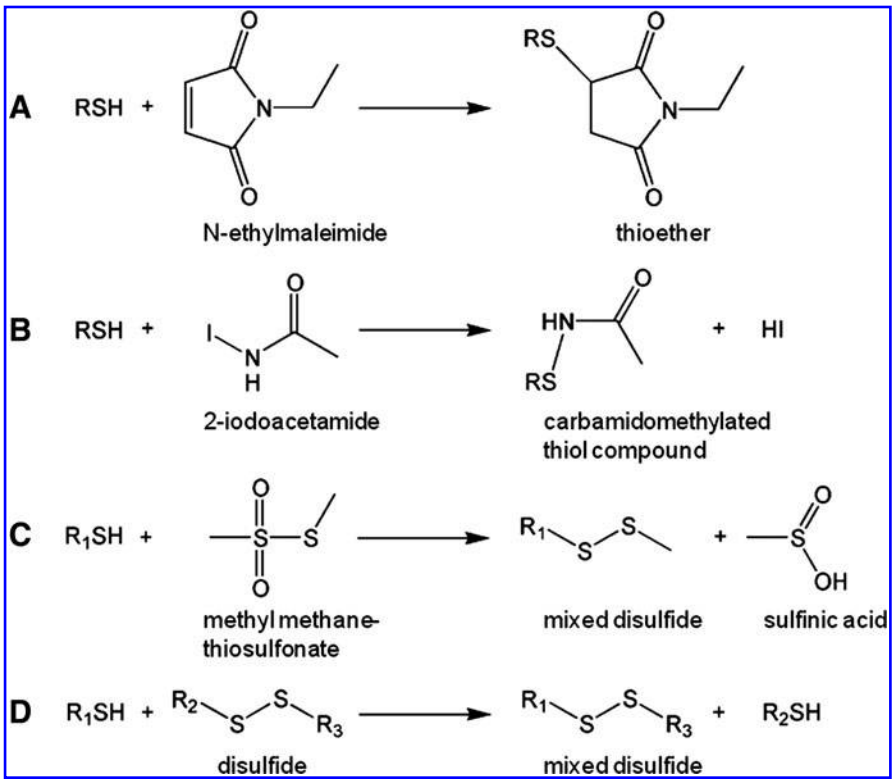

FIG. 2. The chemistry of thiol labeling reactions. The principal reactions of widely used thiol labeling reagents with thiols are shown. *N*-ethylmaleimide (NEM) **(A)** reacts with thiols in a Michael addition to form a stable thioether. Iodoacetamide **(B)** reacts with thiols in a nucleophilic substitution that also results in the formation of carbamidomethylated thiols. Methyl methanethiosulfonate **(C)** forms mixed disulfides with free thiol groups. Finally, disulfide compounds **(D)** label thiols *via* a thiol/disulfide exchange.

some selected thiol probes that have been used in recent studies of disulfide proteomes. The molecular design of these compounds includes bifunctional reagents that consist of a thiol-reactive group and a fluorophore such as, for instance, monobromobimane (mBBr) (Fig. 3B) (4, 112, 163, 338), 5-iodacetamidofluorescein (Fig. 3D) (20, 21, 226), Bodipy FL C1-IA (Fig. 3E) (121), and the Cy3 and Cy5 maleimide dyes (Fig. 4D, E) (95, 317). Instead of a fluorophore, a biotin affinity tag or a triphenylphosphine group can be included into the molecular architecture of the probe. Examples of this design are *N*-biotinoyl-*N*′-(6-maleimidohexanoyl)hydrazide (biotin-maleimide) (Fig. 4B) (276, 302), *N*-(6-(biotinamido)hexyl)-3′-(2′-pyridyldithio)propionamide (biotin-HPDP) (Fig. 5B) (150–152), and (4-iodobutyl)triphenylphosphonium (IBTP) (Fig. 3F) (194, 217, 315). In addition, the thiol-labeling reagents may include linker regions to facilitate affinity purification. This type of molecular design is exemplified by *N*′-iodoacetyl-*N*-biotinylhexylenediamine (IAB) (Fig. 3C) (328), 1-biotinamido-4-(4′[maleimidoethylcyclo-hexane]-carboxyamido)-butane (BMCC) (Fig. 4C) (328), and the cleavable isotope coded affinity tag (cICAT) reagent (Fig. 3G) (109, 114, 287, 288). A new class of thiol reactive probes is represented by the bromomaleimides (297). In contrast to reagents based on maleimide chemistry, labeling by bromomaleimides is reversible. Besides, dibromomaleimides have the useful characteristic that they can become inserted into an intact disulfide bond, which opens new perspectives for cysteine labeling strategies (297). The specificity of the thiol-labeling reagent and efficiency of the labeling reaction have considerable impact on the biological information that can be extracted when using the reagent. The choice of a reagent is therefore a crucial parameter in experimental designs aimed at taking snapshots of a disulfide proteome under a given physiological condition.

C. Enrichment techniques

The broad dynamic range of biological samples is a well-known problem that limits the coverage of proteome studies on cellular extracts, and if low abundance proteins are to be analyzed, methods to enrich these proteins are usually required. This can be achieved by subcellular fractionation, techniques to remove highly abundant proteins such as serum albumin and ribulose bisphosphate carboxylase/oxygenase (RuBisCo), or by affinity purification steps that are designed to specifically enrich the proteins of interest.

1. Activated thiol sepharose chromatography. Activated thiol sepharose (ATS) (Fig. 6) has long been known as a tool for protein purification, but it has rarely been applied in studies on disulfide proteomes. Its potential to facilitate purification of disulfide proteins takes advantage of the specific reactivity of the pyridyldithiol group, which is also found in the thiol reagent biotin-HPDP (Figs. 5B and 6). To minimize nonspecific binding, free thiols of the protein sample are blocked. Before binding to the ATS matrix, the protein disulfides are reduced, and the exposed new thiol groups bind to ATS. During this reaction mixed disulfides between the protein thiols and the reactive groups of ATS are formed and pyridine-2-thione is released. The fraction of disulfide proteins is finally eluted using DTT. The potential for ATS to facilitate purification of disulfide proteins has been shown in a study of global protein disulfides in Arabidopsis (177), in a study of the differential expression of protein in response to arsenite treatment of cultured human epidermal cells (176), and in global studies of disulfide proteins in *Escherichia coli* (137, 138). However, the binding efficiency for ATS might differ between individual proteins, which could lead to biased

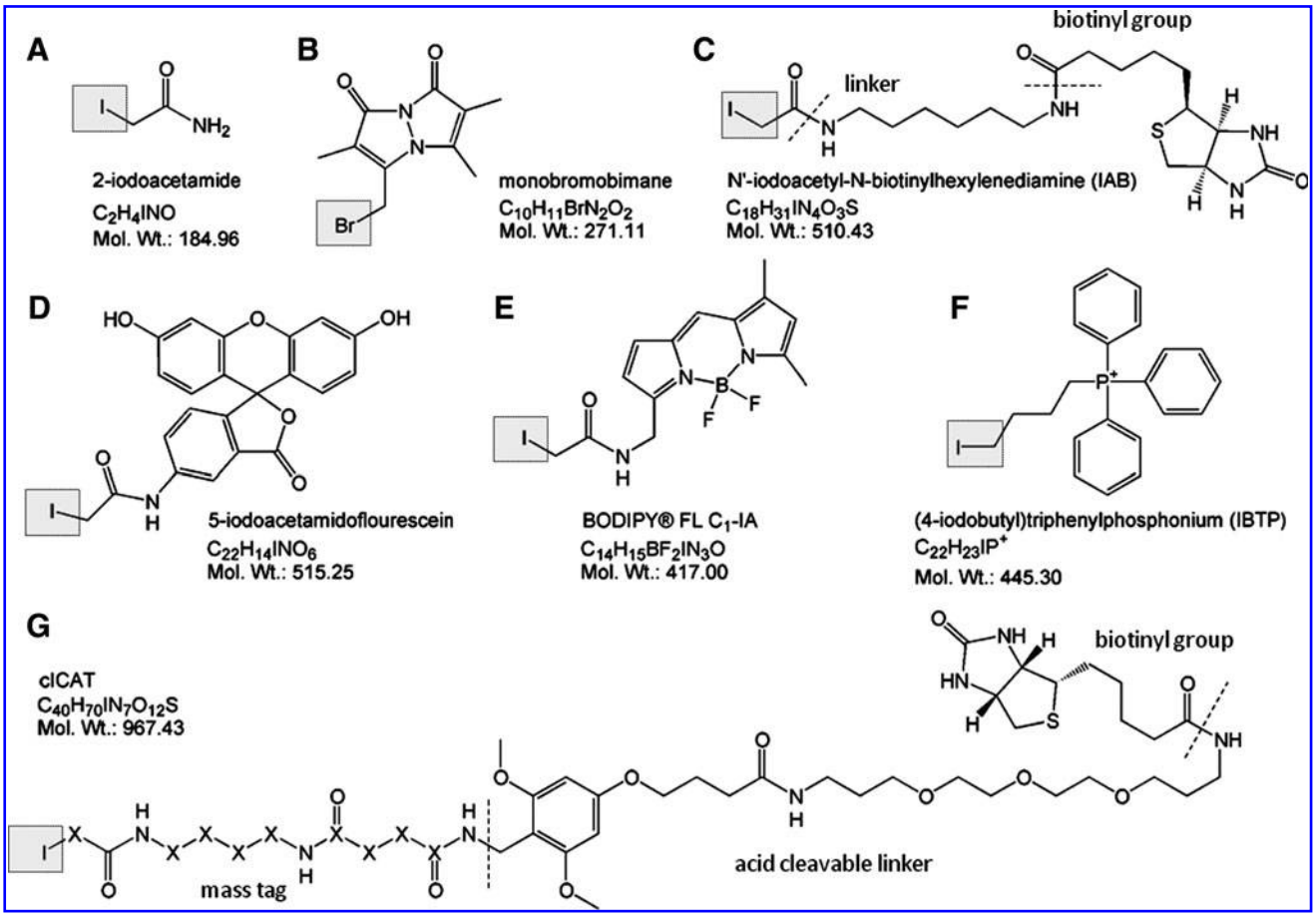

FIG. 3. Thiol labeling reagents of the haloalkane type. The reactive group for thiol labeling is marked by a gray box. The reagents documented in the literature include **(A)** 2-iodoacetamide (IAM) (182, 210, 211), **(B)** the fluorescent dye monobromobimane (mBBr) (4, 112, 163, 338), **(C)** the biotin-tagged probe *N*′-iodoacetyl-*N*-biotinylhexylenediamine (328), **(D)** the fluorescent dye 5-iodoacetamidofluorescein (20, 21, 226), **(E)** the fluorescent dye BODIPY FL-C_1-IA (121), **(F)** the probe (4-iodobutyl)triphenylphosphonium (IBTP) (194, 217, 315), **(G)** the cleavable isotope coded affinity tag (cICAT) reagent consisting of a biotin tag, an acid cleavable linker, and a mass tag that is labeled with either the light (^{12}C) or the heavy (^{13}C) isotope on the positions marked with X (109, 114, 287, 288).

results. Binding to ATS is therefore carried out under denaturing conditions in the presence of urea, which also prevents the copurification of intact protein complexes that contain ATS-binding subunits.

2. Trx affinity chromatography. This approach takes advantage of the catalytic mechanism of Trx, which proceeds through a mixed disulfide intermediate between the first (and most reactive) cysteine of the Trx active site (-WCGPC-) and the substrate protein (129, 156, 333). This short-lived mixed disulfide is subsequently broken by a nucleophilic attack of the second cysteine of the active site. The key feature of Trx affinity chromatography is hence a monocysteinic Trx, in which the second cysteine residue is replaced with a serine (-WCGPS-) or alanine (-WCGPA-) residue through site-directed mutagenesis. The first cysteine of the catalytic site in the recombinant Trx remains active and reacts with disulfides and sulfenic acids in substrate proteins, usually termed "target proteins." Consequently, Trx target proteins are trapped by the formation of stable mixed disulfides with the immobilized monocysteinic Trx (Fig. 7) and can be eluted with DTT. Separation and identification of the eluted disulfide proteins is usually performed by one-dimensional electrophoresis (1-DE) or two-dimensional electrophoresis (2-DE) combined with an analysis of the individual protein spots or bands by matrix-assisted laser desorption ionization mass spectrometry (MALDI-MS) or ESI-liquid chromatography coupled on-line to tandem mass spectrometry (LC-MS/MS). Originally developed for screening of disulfide proteins in yeast (316), this method has been widely used in screening studies *in vitro* for targets of Trx and Grx in plants, algae, and cyanobacteria (120). It should be kept in mind that protein disulfides are frequently inaccessible to Trx and that, for instance, only 5 of the 29 disulfides in fibrinogen are reduced by Trx, and none of the four disulfides in lysozyme are reduced by Trx (128). Further, the affinities of different disulfide proteins for a monocysteinic Trx can differ considerably. This method, therefore, is usually unsuitable for global quantitative experiments.

The copurification of proteins forming complexes with the trapped disulfide proteins also has to be considered. A further development of this technique involves the use of a His-tagged monocysteinic Trx, immobilized on an Ni affinity matrix (Fig. 8) (196, 197). This provides the benefit that the

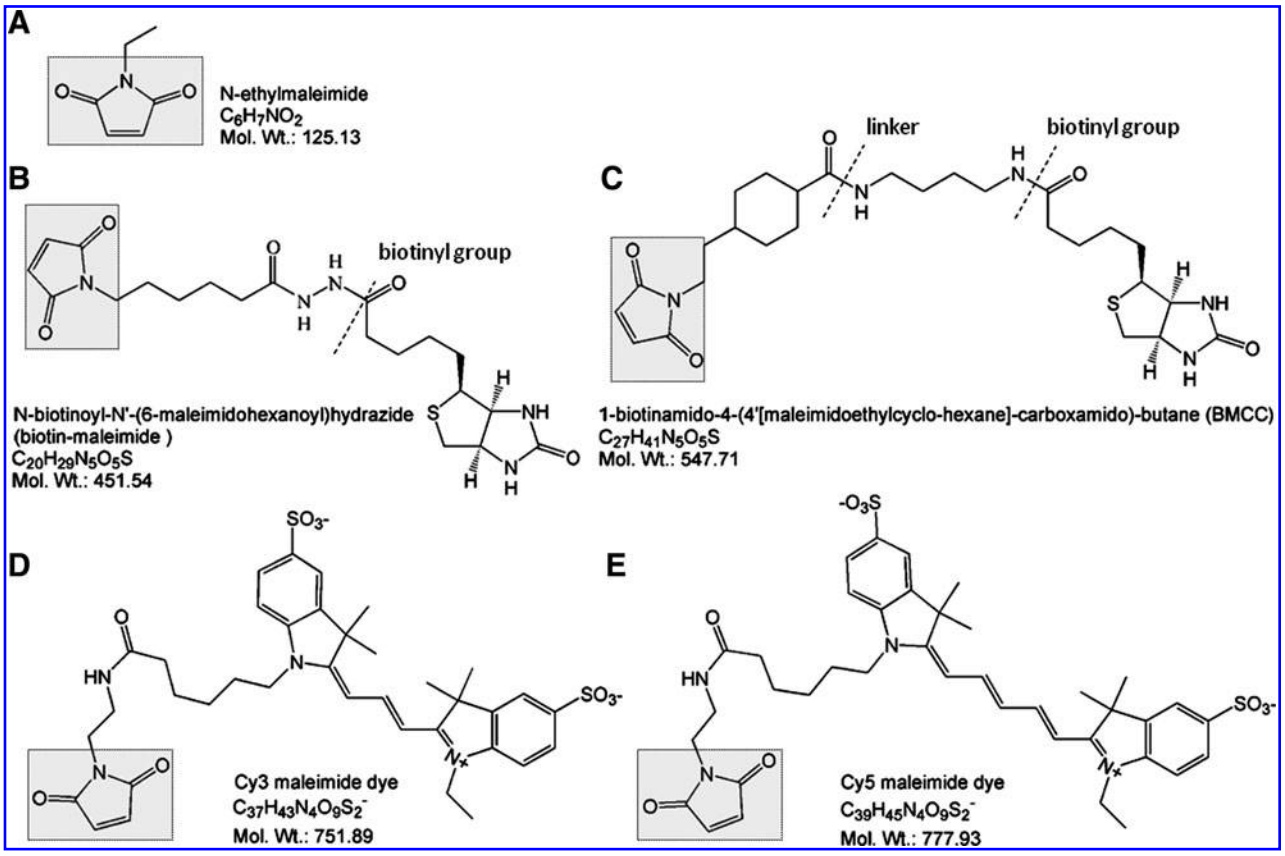

FIG. 4. Thiol labeling reagents of the maleimide type. The reactive group for thiol labeling is marked by a gray box. The reagents documented in the literature include **(A)** NEM (112, 143, 144, 226, 278), **(B)** biotin–maleimide (276, 302), **(C)** 1-biotinamido-4-(4′[maleimidoethylcyclo-hexane]-carboxyamido)-butane (BMCC) (328), **(D)** the fluorescent CyDye Cy3 maleimide (95, 140, 317), and **(E)** the fluorescent CyDye Cy5 maleimide (95, 140, 206, 317).

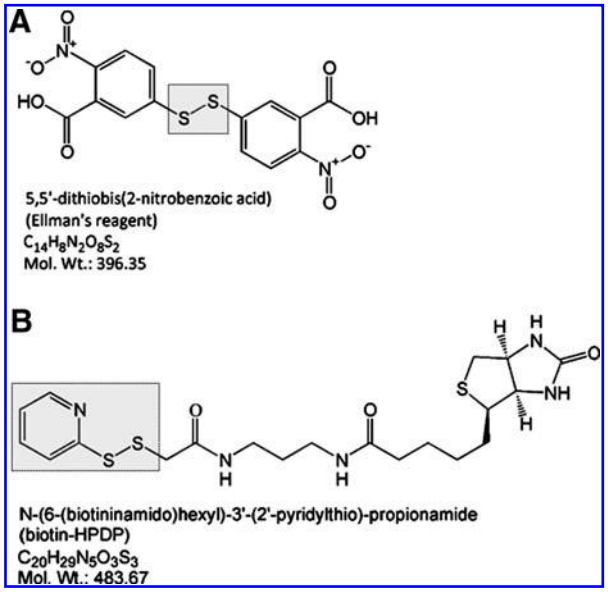

FIG. 5. Thiol labeling reagents of the disulfide type. The reactive group for thiol labeling is marked by a gray box. The reagents documented in the literature include **(A)** 5, 5′ dithiobis (2-nitrobenzoic acid) (Ellman's reagent) (171, 172, 269) and **(B)** *N*-(6-(biotinamido)hexyl-3′-(2′-pyridyldithio)-propionamide (biotin-HPDP) (150–152).

target-Trx complexes can be eluted with imidazole and analyzed by nonreducing/reducing SDS-PAGE, which separates direct targets from other proteins that bind noncovalently to the targets. This sorting is achieved because proteins carrying one or more monocysteinic Trx ligands migrate only slowly in the first dimension of the nonreducing/reducing SDS-PAGE, and are therefore detected under the diagonal of the 2-D gel (Fig. 8). An advantage of this method is that it has proven suitable for the analysis of membrane proteins, and has enabled the identification of 50 membrane-associated Trx-targets of the cyanobacterium *Synechocystis* sp. PCC 6803 (223). Other studies of membrane-located Trx-targets using conventional Trx-affinity chromatography were less successful in terms of coverage (19, 241).

3. Detection of S-nitrosylated proteins. Protein modification by S-nitrosylation of cysteine thiol groups has been studied in many tissues and cell types. The most commonly used technique to analyze this post-translational modification is the so-called biotin switch method (150–152, 204). This method is based on the observation that ascorbate specifically reduces S-nitrosylated cysteines, but not disulfides or sulfenic acids. Hence, the principal steps of the biotin switch approach (Fig. 9) consist of blocking of free protein thiols, for example, with MMTS, which is followed by a reduction step with ascorbate to convert S-nitrosocysteines to cysteines. These cysteines are in turn labeled by the formation of stable

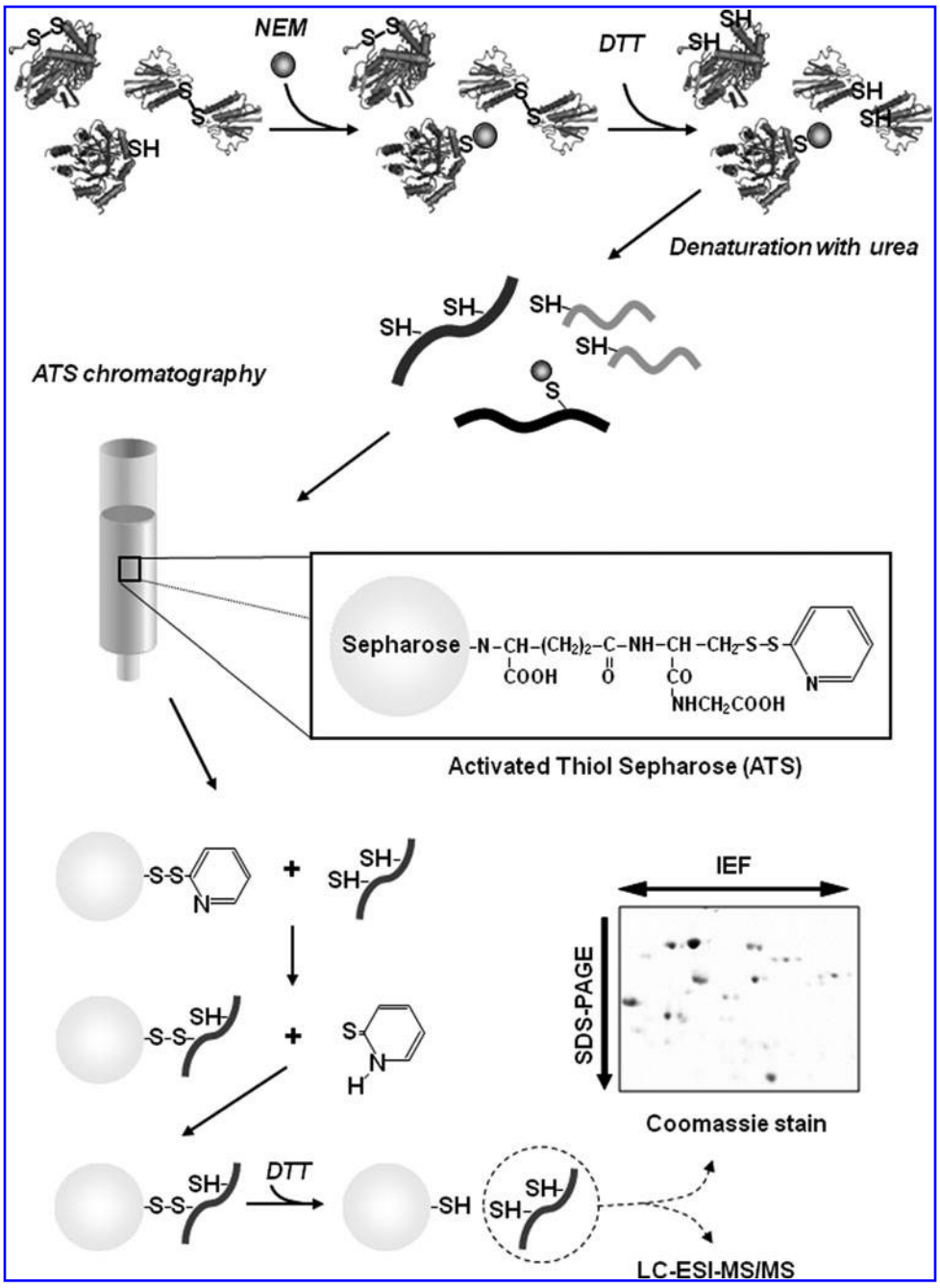

FIG. 6. Disulfide proteins can be enriched by affinity chromatography on activated thiol sepharose (ATS). This method takes advantage of the chemistry of the pyridyldithiol group that specifically reacts with thiols to form mixed disulfides. Therefore, proteins that contain newly formed cysteine thiols after alkylation and reduction bind to the ATS matrix under denaturing conditions, whereas other proteins are removed from the ATS-column by washing. The bound disulfide proteins are eluted with dithiothreitol (DTT) and identified using gel- or chromatography-based proteomics techniques.

disulfides with biotin-HPDP (Figs. 5B and 9). The biotin-labeled proteins can then be purified by streptavidin affinity chromatography for a further analysis by 1-DE or 2-DE and mass spectrometry (141, 166, 180, 203, 335).

Although reduction using ascorbate is considered to be specific for S-nitrosothiols (87, 150), it has been argued that the selectivity of this method might be compromised by the reduction of disulfides by ascorbate. Further, it has been pointed out that its sensitivity is still too low to track S-nitrosylated proteins at physiological levels (100). On the other hand, many efforts have been made to improve the biotin switch approach. To minimize the identification of false-positives, a strict assessment of the individual MS/MS spectra of potential S-nitrosylated peptides is applied. Only peptides that are identified by high quality MS/MS spectra that clearly show cysteine labeling by biotin-HPDP are accepted, which ensures the specific identification of S-nitrosylation sites (107). To adapt the biotin-switch method to high-throughput LC-MS/MS analysis, the biotinylated proteins are digested using trypsin before the affinity chromatography on streptavidin. Elution of the biotinylated peptides from the column using β-mercaptoethanol results in a fraction of unmodified peptides that can be analyzed by standard LC-MS/MS methods. This strategy for SNO site identification, called SNOSID, provides the advantage that only biotinylated peptides that carry an S-nitrosylation site are analyzed. The contribution of false positives resulting from inter- and intramolecular disulfides of S-nitrosylation targets is in this way minimized (67).

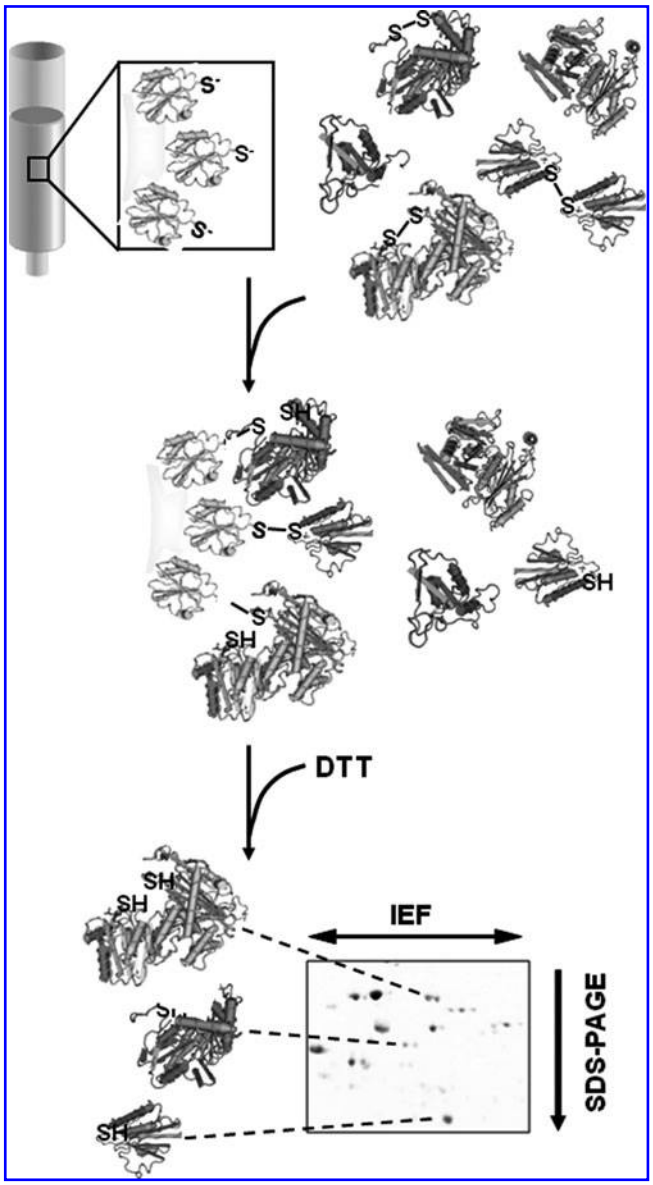

FIG. 7. Proteins with reactive disulfides are efficiently trapped by Trx-affinity chromatography. This technique is characterized by the formation of mixed disulfides between a monocysteinic Trx and a selection of the disulfide-containing proteins in a sample of a cellular fraction. Thus, the disulfide proteins bind to the immobilized monocysteinic Trx, whereas unbound proteins are removed by washing and finally the disulfide proteins are eluted after reduction with DTT. Identification of the Trx target proteins present in eluates can be performed using two-dimensional electrophoresis (2-DE) combined with matrix-assisted laser desorption ionization (MALDI)- or electrospray mass spectrometry. The modified Trx used as ligands in this technique include Trx *h*3 (C41S) (211), Trx *f* (C49S), and Trx *m* (C40A) (12) and Hcf164 (C153S) (241).

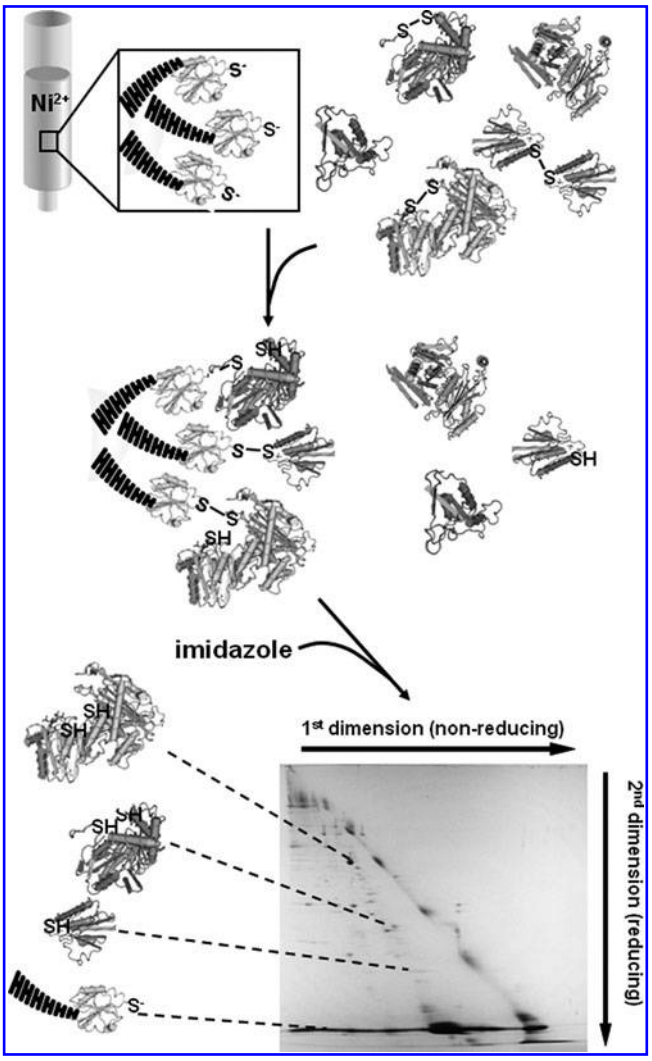

FIG. 8. Trx-affinity chromatography using a His-tagged monocysteinic Trx improves specificity and confidence of disulfide protein detection. An improved version of the Trx-affinity chromatography (Fig. 7) uses a His-tagged monocysteinic Trx that can be reversibly immobilized on a Ni-affinity column (196, 197). This feature provides the advantage that the target-Trx mixed disulfide-complexes, including the His-tagged Trx ligand, can be eluted using imidazole and thereafter resolved by nonreducing/reducing SDS-PAGE. Hence, Trx target proteins can be separated from contaminants and nontarget subunits of complexes brought along with the target proteins participating directly in mixed disulfides. Proteins bound to one or more monocysteinic Trx molecules migrate slower in the first dimension of the diagonal SDS-PAGE, whereas proteins that bind to the complexes through electrostatic or hydrophobic interactions are equally retained in both dimensions and found at the diagonal.

Another alternative technique for improving the selectivity of the biotin switch method involves replacing labeling by biotin-HPDP, with irreversible alkylation using biotin-maleimide. This modification allows the removal of proteins that form intermolecular disulfides with S-nitrosylated proteins before the affinity purification using streptavidin (139). Another alternative to labeling with biotin-HPDP is the SNO resin-specific capture technique (SNO-RAC) (88). The design of this method combines the thiol labeling and the pulldown of the biotin switch method in one step. After reduction with ascorbate, the thiol groups are labeled using 2- or 4-dipyridyl disulfide coupled to a resin (Fig. 9). Peptides for analysis by LC-MS/MS can be generated by direct on-resin digestion, which shortens sample preparation time (88).

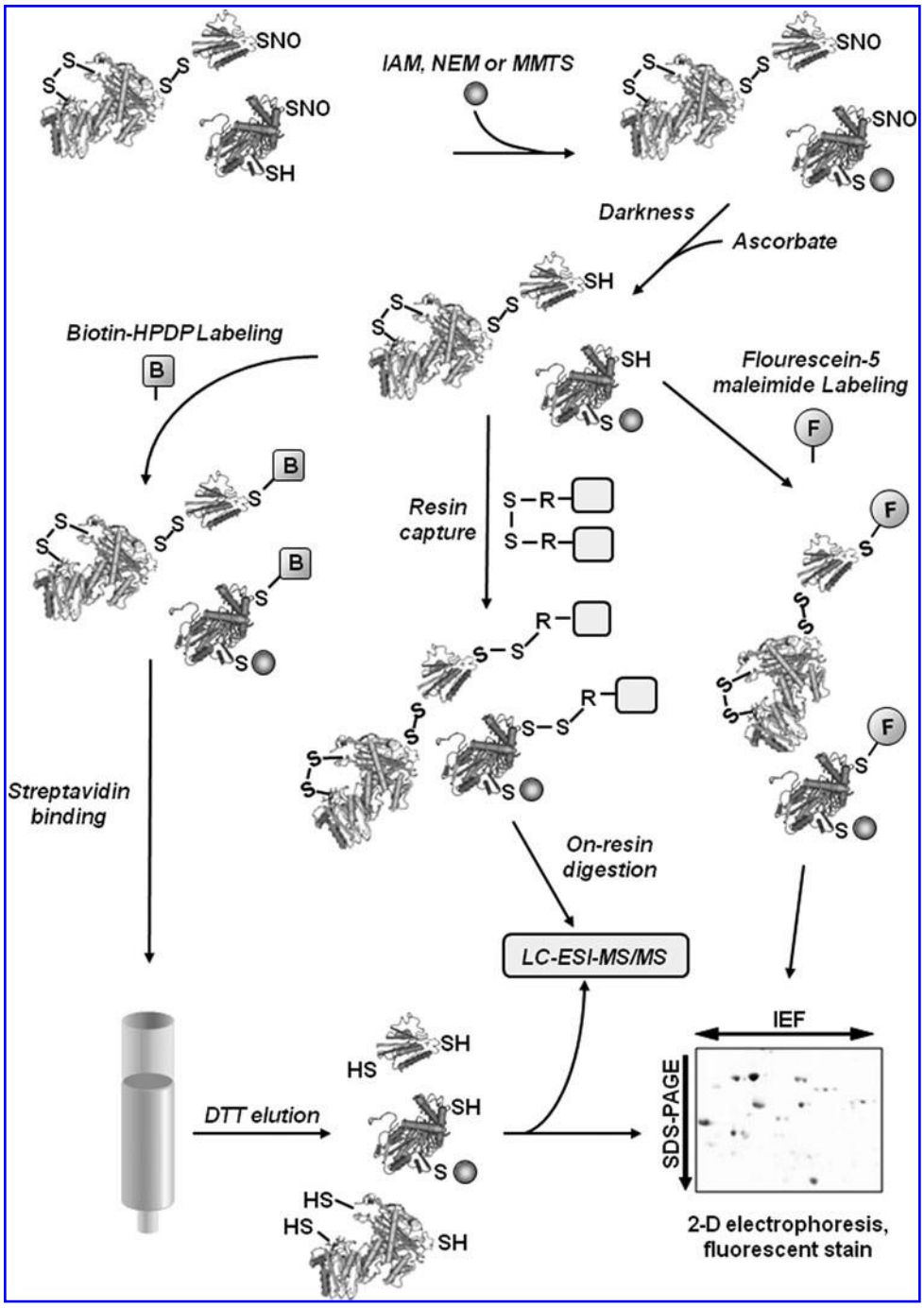

FIG. 9. The biotin switch—a method for the identification of proteins that are modified by S-nitrosylation or sulfenation. A key step of this method involves the selective reduction of S-nitrosothiols by ascorbate to thiols that in turn are biotinylated using the thiol-specific probe biotin-HPDP. Several modifications of this method have been reported that aim to simplify the protocol and increase the sensitivity for the detection of S-nitrosothiols. The principal steps of the biotin-switch technique (150) involve (i) blocking of all free thiols under denaturing conditions using, for example, methyl methanethiosulfonate (MMTS); (ii) selective reduction of S-nitrosothiols using ascorbate followed by biotinylation using biotin-HPDP; (iii) biotinylated proteins can be detected by immunoblotting or they can be purified by affinity chromatography on streptavidin beads and identified by proteomics techniques including nano-reversed phase chromatography, 2-DE, and electrospray mass spectrometry. A simplified version of the biotin switch method replaces the labeling using biotin-HPDP and the streptavidin affinity purification by a resin-assisted capture of S-nitrosothiols (SNO-RAC) that combines these two steps in one. S-nitrosylated proteins are labeled using a thiol reactive resin and can be directly digested on resin for further analysis by mass spectrometry (88). The fluorescence switch approach (307) replaces labeling of S-nitrosylated thiols by biotin-HPDP by labeling with flourescein-5 maleimide, which enables detection of S-nitrosylated proteins by two-dimensional difference gel electrophoresis.

The design of the fluorescence switch employs a similar concept to that of the biotin switch method, but replaces the MS based detection of S-nitrosylated proteins by fluorescence labeling and 2-DE (307). Instead of biotin-HPDP, flourescein-5 maleimide is used to label the thiol groups formed after reduction by ascorbate. S-nitrosylated proteins are detected by analysing images of the 2-D gels from control and treated samples, and identified by mass spectrometry. The benefits of the fluorescence switch method include a higher sensitivity compared to the classical biotin switch method, which permits the analysis of scarce samples. Moreover, the 2-DE analysis allows, for instance, the detection of protein isoforms that are easily missed in LC-MS/MS (307).

4. Detection of proteins containing cysteine sulfenic acids. Sulfenic acids are particular in that they exhibit both nucleophilic and electrophilic properties, and several methods based on their electrophilic character have been designed for the exclusive labeling of cysteine sulfenic acids in complex protein mixtures and even in intact cells. One of the first re-

ports on proteomic screening for proteins containing sulfenic acids applied a biotin switch-like method based on the principle that arsenite specifically reduces sulfenic acids to thiols, and led to the identification of 17 proteins from rat hearts (276).

More than 35 years ago, it was discovered that dimedone (Fig. 10A) specifically and irreversibly modifies the sulfenic acid form of glyceraldhyde 3-phosphate dehydrogenase (GAPDH), thereby inhibiting its acyl phosphatase activity (24). Dimedone-based chemical probes have therefore been widely used to label sulfenic acids exclusively. A biotinylated dimedone derivative has been synthesized and used to detect and purify protein sulfenic acids (49). However, this identified only a few more proteins from rat heart tissue than were identified in the study mentioned above (276). The principal drawback with this method is perhaps that the dimedone-biotin conjugates are relatively large and might have difficulty entering intact cells and accessing partially buried protein sulfenic acids. The synthesis of three different biotin conjugates of a dimedone analog, 1,3-cyclohexadione, has been reported in a different study, though no data on their potential proteomics applications were included (259).

An alternative to labeling with the bulky biotin-dimedone was recently devised, which involves the synthesis of the small bifunctional chemical probe *N*-(3-azidopropyl)-3,5-dioxocyclohexanecarboxamide, referred to as DAz-1 (Fig. 10A) (285) and the closely related probe DAz-2 (189). These compounds are dimedone analogs provided with an azide

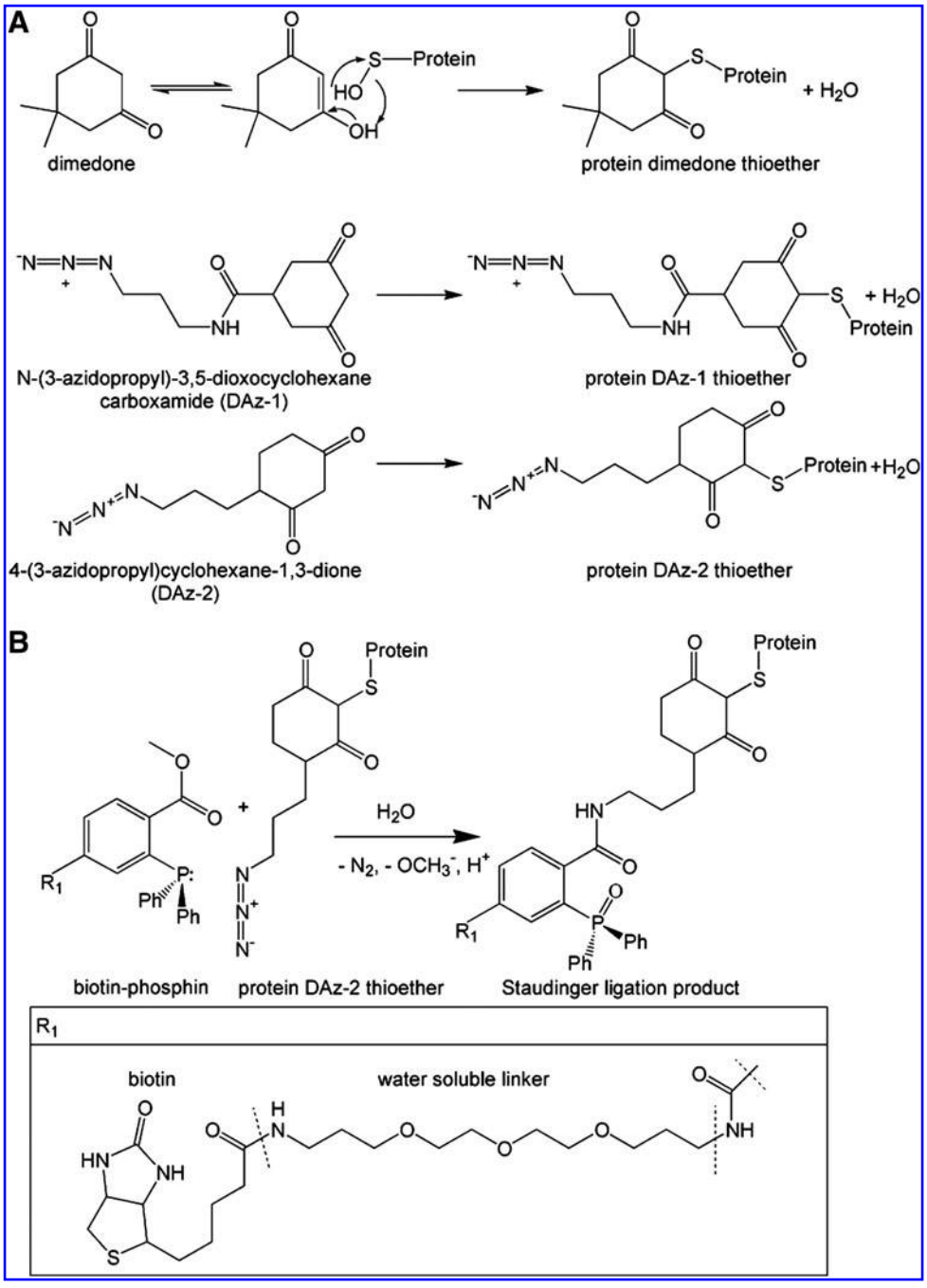

FIG. 10. Dimedone and dimedone-based probes react specifically with sulfenic acid. (A) The reaction product of dimedone and sulfenic acid is a stable thioether. While the dimedone-thioether does not have any functional groups for a further reaction, the dimedone-based probes DAz-1 and DAz-2 carry a tag with an azido group that can be used to link DAz-1/DAz-2-labeled proteins in a modified Staudinger ligation with biotin-phosphine (189, 285). **(B)** Biotin-phosphine reacts with azides under physiological conditions to a stable amide (277). The reaction is rapid and gives high yields. In addition, the biotin-phosphine and the azido group of the DAz-2 ligand are essentially unreactive toward biomolecules in the cell or on the cell surface. These features make the modified Staudinger ligation of biotin-phosphine with azides a highly selective reaction and a useful tool for the analysis of DAz-2-labeled sulfenic acid groups.

"chemical handle," which enables subsequent labeling with phosphine-conjugated biotin (p-biotin) through a so-called Staudinger ligation (Fig. 10B) (277). Such a procedure, which combines labeling *in vivo* with a small functional tag and the posterior covalent binding to this tag of an exogenously delivered probe, is commonly referred to as "bioorthogonal" (262). DAz-1 is membrane permeable and labeling has been tried out in intact cells using the human T lymphoma cell line Jurkat (264). After p-biotinylation of DAz-1-labeled proteins and western blot analysis using streptavidin-HRP, about 30 bands were observed. DAz-2 (Fig. 10A) has been applied to the detection of protein sulfenic acids in HeLa cells using a similar approach (189). Biotinylated proteins were affinity-purified on a NeutrAvidin matrix, resolved on 1-DE and identified by LC-electrospray ionization tandem mass spectrometry (ESI-MS/MS). This procedure led to the confirmation of 14 known sulfenated proteins and about 175 new candidates for proteins undergoing sulfenic acid formation *in vivo* (189). Another procedure that involves dimedone labeling *in vivo* for the detection of protein sulfenic acids is the immunochemical method, which uses specific antibodies raised against a synthetic hapten with similarity to dimedone-derivatized cysteine (286). However, the method has so far only been applied in the determination of levels of protein sulfenic acids in tumour cells; no attempts to identify the sulfenated proteins have yet been made (286). Another alternative immunological method has recently been devised that involves antibodies raised directly against dimedone-derivatized sulfenic acids of denatured keyhole limpet hemocyanin (208). These antibodies, which are commercially available, were applied in studies of sulfenated proteins in intact rat myocytes (208).

D. Gel-based detection of proteins with reactive disulfides

The potential of nonreducing/reducing diagonal electrophoresis to detect cross-linked proteins has long been recognized (299), and there are numerous studies where this technique also has been used to detect intra- and intermolecular protein disulfides. The method takes advantage of the fact that proteins without disulfides have approximately the same mobility in both dimensions and migrate on the diagonal of the gel. By contrast, proteins containing intramolecular disulfides have a higher electrophoretic mobility in their oxidized than in their reduced forms, and therefore migrate above the diagonal. Proteins complexes that are cross-linked by intermolecular disulfides migrate slower in the first dimension than in the second one, and are hence displayed under the diagonal (235, 338). Diagonal electrophoresis has, for instance, been used to study disulfide formation in the cytoplasm of neuronal HT22 cells (58), and in chloroplast fractions (301).

The combination of diagonal electrophoresis with thiol labeling with the fluorescent dye mBBr (Fig. 3B) has proven to be a useful method for the detection of disulfide proteins. In addition, thiol labeling with mBBr has been shown to be equally useful for detecting disulfide proteins resolved by conventional 2-DE (Fig. 11) (338). Thiol-specific protein detection by 2-D mBBr fluorescence electrophoresis has been used in many studies on Trx-interacting proteins in plants (15, 112, 326, 327, 340). The background label in 2-D mBBr fluorescence electrophoresis can easily be minimized by blocking free thiols with NEM (112). Higher sensitivity can be achieved if reduced disulfides are labeled using either the Cy5 maleimide dye (Fig. 4E) (206) or the BODIPY FL C_1-IA (Fig. 3E) (121). Disulfide proteins in 2-DE experiments have also been detected using the fluorescent dye 5-iodacetamido-fluorescein (Fig. 3D) (20, 21, 226). An alternative approach to gain higher sensitivity in disulfide detection involves differential thiol labeling using IAM and radioactive [^{14}C]IAM (Fig. 11) (182, 210, 211).

The 2-D-DIGE technique is widely used to quantify differences in protein expression between two different samples, but it can also be used to quantify the oxidation state of cysteines. This variant of two-dimensional difference gel electrophoresis (2-D DIGE) has also been referred to as "redox-DIGE." The principal difference as compared to traditional 2-D DIGE is that proteins are labeled using the thiol-reactive Cy3 and Cy5 maleimide dyes instead of the amino-reactive Cy2, Cy3, and Cy5 minimal dyes. To study the redox-sensitive proteome, control and hydrogen peroxide-treated samples are labeled using the Cy5 maleimide dye, whereas the internal standard is labeled using the Cy3 maleimide dye (95). Another form of redox-DIGE was recently used as part of a modified biotin-switch method to quantify the relative levels of protein S-nitrosylation (140). The relative levels of S-nitrosylation of individual proteins were calculated from the ratios of Cy5 to Cy3 labeling of individual protein spots that were detected in the comparison of two samples by 2-D DIGE (140). A similar strategy was employed to quantify the levels of thiol oxidation in Jurkat T lymphocytes (205). A drawback of redox-DIGE is the lack of commercially available Cy2 maleimide dye, since without a set of three Cy dyes, a complete 2-D DIGE experiment including an internal standard cannot be performed. However, other thiol-reactive dyes, such as the rhodamine based thiol-reactive dyes Dy505, Dy535, and Dy635 (317), and the DY maleimide dyes (68, 270) might be an alternative to the Cy3 and Cy5 maleimide dyes.

E. Mass spectrometry-based detection of proteins with reactive disulfides

Differential cysteine alkylation using isotope-coded reagents has long been known as a method to quantify the relative expression levels of proteins (282), and is the underlying principle of the ICAT technique that provided a tool for the relative quantification of proteins in complex samples (108). However, differential isotope labeling has been introduced relatively recently for the quantification of the redox state of cysteine residues in individual proteins (278, 287, 288). The cICAT reagents have since proved to be a useful tool in disulfide proteomics (95, 96, 109, 183, 288).

The structure of the cICAT reagent is shown in Figures 3G and 12A; Figure 12B summarizes the workflow of the cICAT method. In the beginning of the labeling protocol equal amounts of the oxidized and control samples are labeled separately with the light and heavy cICAT reagent. Subsequently, the samples are mixed and digested using trypsin. In the following steps the peptides of the digest are purified and analyzed by LC-MS/MS. The relative ratios of the light and heavy forms of individual peptides are determined from the intensities of their signals in the MS survey scans, and

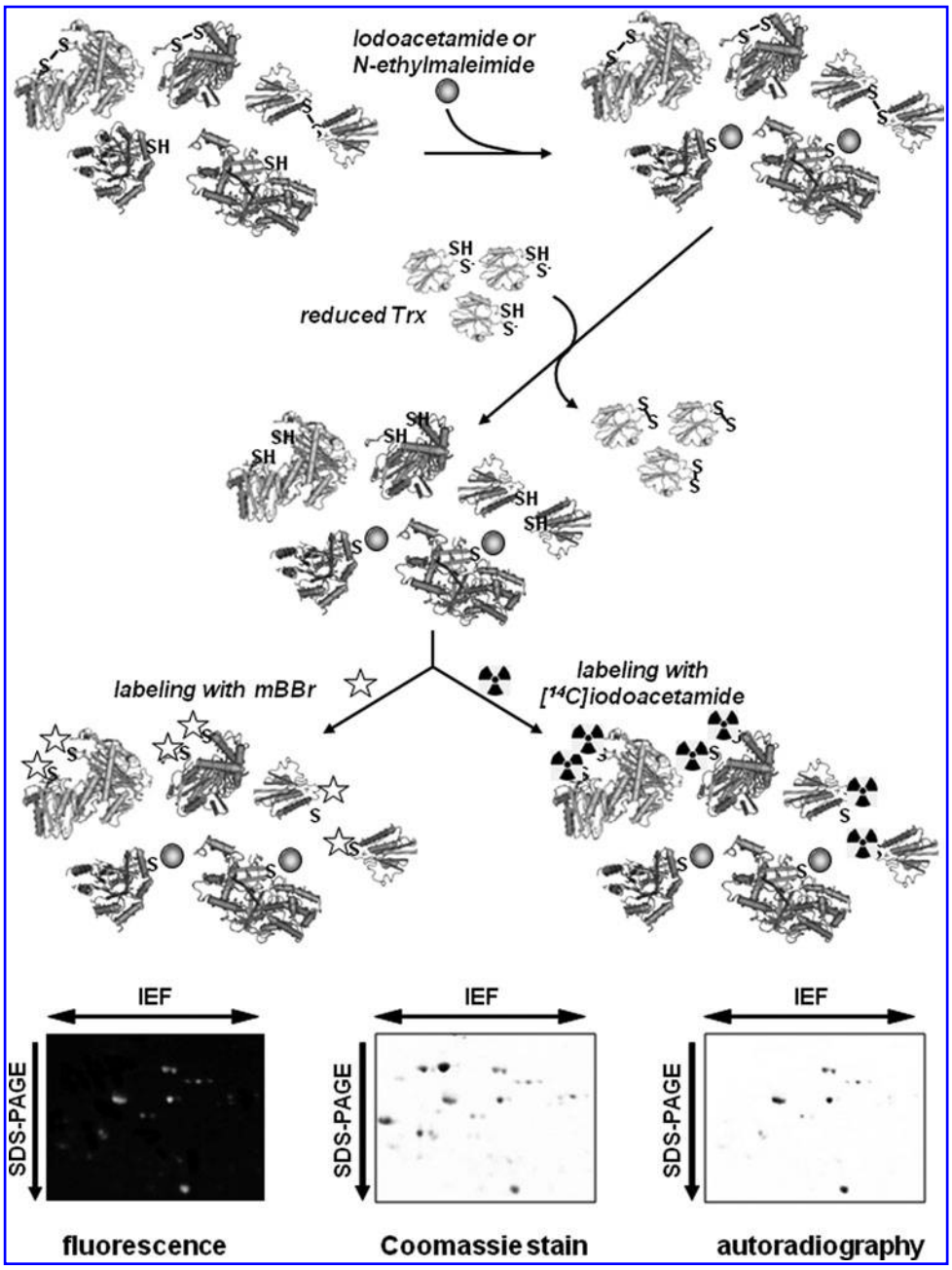

FIG. 11. Detection of Trx targets by thiol-specific alkylation combined with 2-DE. This technique involves three principal steps. First, the free thiols of a protein sample are blocked under native conditions using, for instance, IAM (Fig. 3A) or NEM (Fig. 4A). Second, the proteins are incubated with reduced Trx, which reduces accessible and reactive disulfides. The newly formed thiols are susceptible to labeling by alkylating reagents. Typical thiol probes are mBBr (Fig. 3B) and [^{14}C]iodoacetamide (Fig. 3A). Proteins with labeled thiols are separated by 2-DE and either observed by UV light or by autoradiography, respectively. Finally, the images of the 2-D gels displaying the reactive protein disulfides are matched to a Coomassie-stained 2-D gel and the corresponding protein spots are identified by MALDI or electrospray mass spectrometry.

their identification is achieved by a subsequent MS/MS analysis.

To determine the thiol redox state of disulfide proteins using the cICAT reagents, both forward and reverse labeling strategies have been used. In the forward approach the control sample is labeled with the light cICAT reagent, whereas the oxidized test sample is labeled with the heavy cICAT reagent. The samples are then mixed and subjected to the following steps of the cICAT protocol (95, 96, 287, 288). By contrast, in the reverse labeling, the free thiols are blocked using iodoacetamide followed by a reduction of the disulfides. The thiols arising from the reduced disulfides are then labeled using the light and heavy cICAT reagents for control and test samples, respectively (95, 109). As an alternative, the free thiols of the control and oxidized test sample can be labeled with the light cICAT reagent. Subsequently, the disulfides are reduced and the newly formed thiols labeled using the heavy cICAT reagent. This approach is also termed OxICAT (183, 184).

Analysis of disulfide peptides by tandem mass spectrometry is difficult and not usually feasible when complex samples are to be analyzed. However, the future development of software tools might facilitate the interpretation of MS/MS spectra of disulfide peptides. The recent DBond algorithm employs characteristic fragments of disulfide peptides to discover disulfide bonds (52), which may become a routine analysis of disulfide-containing peptides by tandem mass spectrometry.

III. Disulfide Proteomes in All Kingdoms of Life

In this section we attempt to give a comprehensive account of the currently available reports on "disulfide proteomes" in

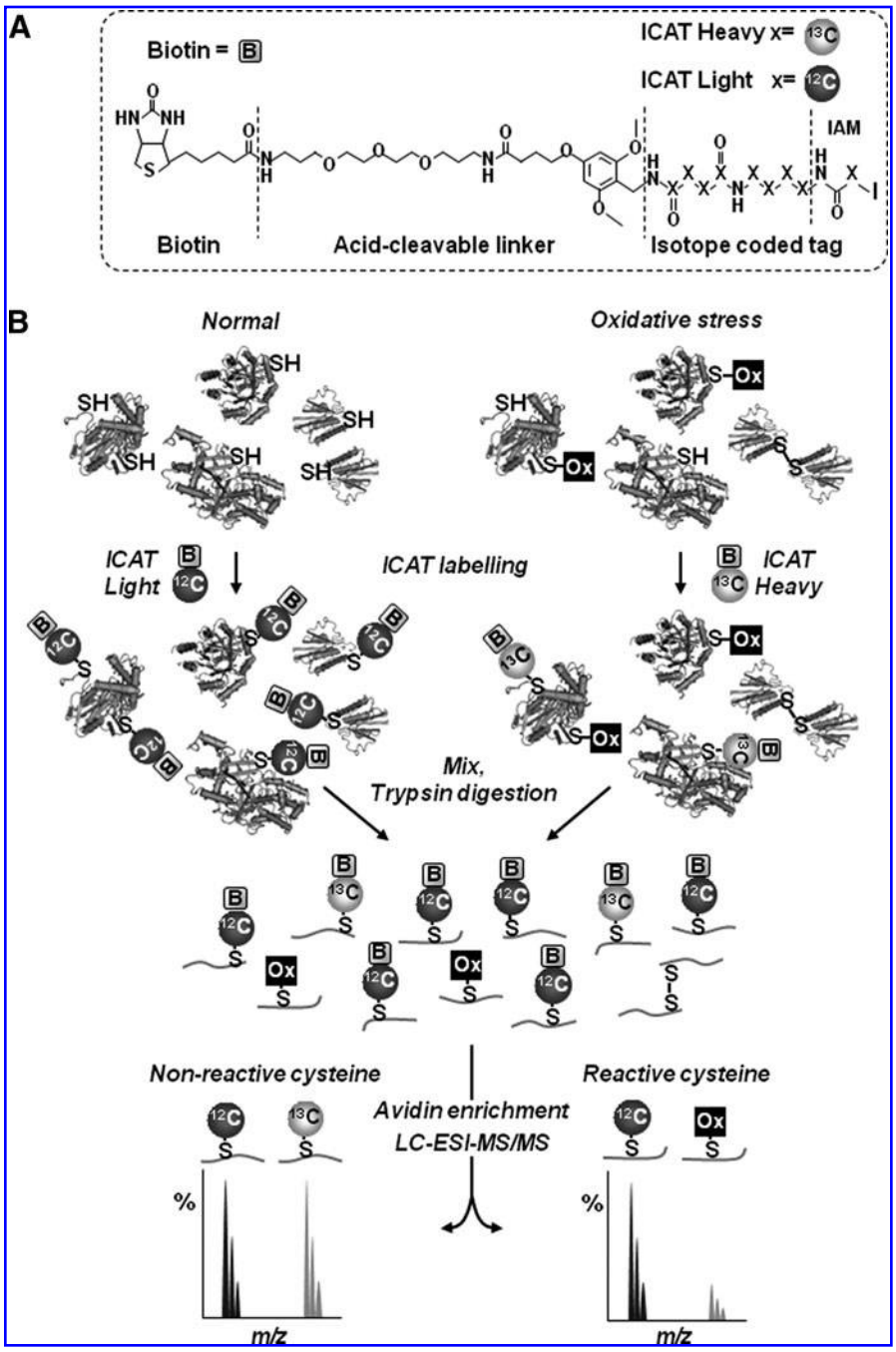

FIG. 12. Quantifying the cysteine redox state of proteins using cICAT. The cICAT reagent was originally developed as a tool for the identification and quantification of differentially expressed proteins. A key feature of the ICAT technology is that the reagent labels cysteine thiols. Therefore, only cysteine-containing peptides are analyzed and quantified, which considerably reduces the complexity of the samples. This feature also makes the ICAT technology a suitable tool for quantifying changes in the cysteine redox state of individual proteins. The cICAT reagent shown in **A** has four functional parts: (i) a reactive iodoacetamido group for binding to thiols; (ii) the isotope coded tag that contains nine ^{12}C (light reagent) or nine ^{13}C atoms (heavy reagent); (iii) an acid cleavable linker; and (iv) a biotin affinity tag. The steps of the cICAT protocol summarized in **B** involve preparation of the protein samples, labeling the samples with the light and heavy reagents, mixing the samples, and digestion with trypsin followed by a clean-up on an ion exchange column. In the next steps the cICAT-labeled peptides are separated from the unlabeled peptides on an avidin affinity column, after which the biotin portion of the tag is cleaved. The labeled peptides are then separated by reversed phase chromatography and identified and quantified by either on-line electrospray mass spectrometry or off-line by MALDI-TOF/TOF mass spectrometry.

the widest sense of the term. The cited studies include disulfide proteomes from animals, fungi, plants, and bacteria, with brief descriptions of the goals, the methodology, and the most prominent proteins or classes of proteins identified in each study. For a quick overview, Tables 1–3 give a summary of studies on disulfide proteomes in eukaryotes, including subcellular compartments, and in the field of prokaryotes.

A. Disulfide proteomes in eukaryotes

1. Animals.

a. Mammalian cell cultures. Cultured cells have served as a model system for studying the effects of different forms oxidative stress on cytoplasmic proteins, and for understanding the role of surface thiols of proteins of the plasma membrane. These surface thiols are considered to play a role as redox sensors and to participate in the disulfide mediated transduction of signals between the extracellular environment and intracellular space. Studies in cultured human peripheral blood mononuclear cells and Chinese hamster ovary cells have shown that the cysteines of plasma membrane proteins exist largely in the oxidized state of the disulfide (171, 172). A quantification of surface thiols using 5,5′ dithiobis(2-nitrobenzoic acid) (Fig. 5A) has shown that the exofacial disulfides of peripheral blood mononuclear cells are reduced by extracellular *N*-acetyl-L-cysteine (NAC) in a dose-dependent manner. Four surface proteins have been identified as a target for NAC, of which one was integrin alpha-4 (VLA-4). In agreement with this finding, NAC was able to increase the integrin alpha-4-mediated adhesion of Jurkat cells to

fibronectin (171). In Chinese hamster ovary cells, reductions of two cell-surface proteins by NAC have been detected. The first one is vimentin, which is a component of the type III filament of the cytoskeleton. The second protein is ERp57, which belongs to the family of protein disulfide isomerases (PDIs) and has a vicinal dithiol in the CXXC motif of the active site. These findings suggest that disulfides of these proteins might be involved in the transmission of redox signals (172).

The oxidation state of exofacial thiols is not only affected by extracellular redox agents, such as NAC, but also by a change in the intracellular glutathione level (171, 172). However, a quantitative analysis of the surface thiols of peripheral blood mononuclear cells has indicated that the local redox environment is more important for the *in vivo* redox status of the cell surface thiols than the internal redox status of the cells (274). The importance of cell surface protein thiols has also been highlighted by the effect of the anticancer drug parthenolide on lymphoma cells (296). A treatment of six different lymphoma cell lines with parthenolide caused a dose-dependent decrease of their viability and a loss of their surface thiols. Consistent with the observation that parthenolide binds to exofacial protein thiols, a pretreatment of Granta cells with the thiol antioxidants reduced glutathione (GSH), GSHee (glutathione monoethyl ester) and NAC inhibited the cytotoxic effect of parthenolide and increased cell viability (296).

In contrast to cysteines on the cellular surface, protein cysteines in the cytoplasm have been generally considered to be in the reduced state. However, many cytosolic proteins form disulfides under oxidative stress. A treatment of cultures of the mammalian neuronal cell line HT22 with the oxidants hydrogen peroxide and diamide (Fig. 13) led to the increased glutathionylation of proteins (58). An analysis of the cytosolic proteins from these cells by nonreducing/reducing diagonal electrophoresis showed that the oxidant treatment also induced the formation of intramolecular disulfides (58, 60). Altogether, 32 spots were identified, of which 6 were detected above the diagonal of the gel indicating formation of intramolecular disulfides. The other spots were found under the diagonal of the gel and displayed proteins that formed one or more intermolecular disulfides. The proteins forming intramolecular disulfides included the chaperones, calreticulin, and the θ-subunit of T-complex protein-1. In addition, intramolecular disulfides were formed by subunit 3 of the eukaryotic translation initiation factor 2 (eIF-2γ), and the enzymes lactate dehydrogenase and enolase. The proteins forming intermolecular disulfides included chaperones, translation elongation factors, and aminoacyl-tRNA synthetases. Mixed disulfides were also formed by the glycolytic enzyme GAPDH, by α- and β-tubulin, and by inosine-5′-monophosphate dehydrogenase 2 (58). The neuronal HT22 disulfide proteome also included the well-known enzyme ribonucleotide reductase m1, the catalytic mechanism of which depends on reversible disulfide formation (344). Finally, intermolecular disulfide formation was found in signal transduction proteins, including the NF-κB p100 subunit and in the redox proteins Trx reductase 1 and peroxiredoxin (Prx)1 and 2. To define some of the protein-protein interactions observed in this study, FLAG-tagged Hsp70 expressed in HT22 was immunoprecipated under oxidizing conditions. An analysis of the Hsp70 complex by diagonal electrophoresis revealed the presence of intermolecular disulfides with β4-spectrin and the APC protein (58).

The response of the cellular disulfide proteome to oxidative stress has also been studied in Jurkat cells (20, 21). Proteins that formed disulfides in the presence of hydrogen peroxide, or mixed disulfides with glutathione, were labeled using 5-iodoacetamidofluorescein (IAF) (Fig. 3D) and resolved by 2-DE. A short treatment for 10 min in the presence of 200 μM hydrogen peroxide caused pronounced changes in the cysteine redox state of 54 protein spots. This finding shows that protein thiol oxidation under oxidative stress is selective and affects only a small group of cytoplasmic proteins. GAPDH and Prx2 were among the most peroxide-sensitive proteins (21).

Human T lymphocytes isolated from blood samples have been examined for protein S-glutathionylation induced by addition of 1 m*M* hydrogen peroxide or 1 m*M* diamide (92). The method used to detect glutathionylated proteins involved labeling of intact cells with [^{35}S]cysteine in the presence of cycloheximide and the subsequent separation of proteins on 2-DE under nonreducing conditions. A total of 38 glutathionylated proteins were identified by peptide mass fingerprinting (PMF). Notably, all of these were labeled in the presence of diamide (Fig. 13), whereas only eight were labeled in the presence of hydrogen peroxide. Cytoskeletal proteins were dominant among the glutathionylated proteins. Further, proteins with functions in protein synthesis and folding included a translation elongation factor, a ribosomal subunit, a PDI, and Hsp70 and Hsp60 (92). A few glutathionylated proteins were identified in a similar study of primary rat hepatocytes and the human hepatoma cell line HepG2 (93). However, their identities were essentially the same as the most abundant glutathionylated proteins in lymphocytes. This indicates that there is considerable overlap in the glutathionyl-protein disulfide proteomes from different cell types (92, 93).

In an intact organism, organs and cells are normally exposed to very low oxygen levels. Therefore, cells probably experience oxidative stress when cultured under standard conditions in a humidified atmosphere consisting of 95% air and 5% carbon dioxide (205). These circumstances could alter the function of proteins by oxidation of redox-sensitive thiol groups, and such cells might activate ROS-dependent signaling pathways that are not usually operative *in vivo*. The degree of oxidation of cytoplasmic protein thiols has been quantified using a 2-D-DIGE approach that employed sequential labeling of reduced protein thiols by BODIPY dyes. It was observed that the cytoplasmic proteins of Jurkat T lymphocytes displayed different thiol redox ratios when cells were cultured under standard conditions, mild oxidative stress and low oxygen conditions. Under mild oxidative stress, some proteins, including Hsp70 and the Prx2 and 6, became more oxidized than under standard growth conditions, whereas under low oxygen conditions many proteins became more reduced than under standard growth conditions. The oxidative stress in cultured cells might affect studies on cell functions and should be considered if ROS-dependent signaling pathways are studied (205). A different technique was used to monitor S-glutathionylated proteins in HeLa cells treated with hydrogen peroxide or TNF-α (303). This method involved incubation of cells with biotinylated glutathione ethyl ester, which permeates the plasma membrane, and subsequent purification of the glutathionylated proteins on streptavidin-agarose. Yet another method was employed to

Table 1. Disulfide Proteomes in Eukaryotes

Aim	*Experimental system*	*Proteomics approach*	*Outcome/highlights*	*Reference*
Animals				
Mammalian cell cultures				
Identification of redox active surface thiols of the plasma membrane	Blood mononuclear cells and Chinese hamster ovary cells	Thiol labeling using the membrane impermeable dye BIAM combined with 2-DE	Reduction of the surface protein integrin alpha-4 mediates adhesion of Jurkat cells to fibronectin	171
Identification of redox active surface thiols of the plasma membrane	Chinese hamster ovary cells	Thiol labeling using the membrane impermeable dye BIAM combined with 2-DE	Reactive surface thiols are present in cytoskeleton protein vimentin and the PDI ERp57	172
Identification of cytoplasmic proteins that form disulfide bonds	Mammalian neuronal cell line HT22	Nonreducing/reducing diagonal electrophoresis	Disulfide formation of aminoacyl-tRNA transferases, translation initiation and elongation factors, and chaperones indicates redox-control of protein translation and folding	58
Detection of proteins with oxidant-sensitive cysteine thiols	Jurkat T lymphocytes	2-DE combined with labeling by IAF	2-DE combined with labeling by IAF is a suitable approach for detection of oxidant-sensitive protein thiols	20
Detection of proteins with oxidant-sensitive cysteine thiols	Jurkat T lymphocytes	2-DE combined with labeling by IAF	Thiol oxidation under oxidative stress was identified in 54 cytoplasmic proteins including 14-3-3 zeta/delta and six other cell signaling proteins.	21
Detecting changes in the thiol redox state of cytoplasmic proteins under oxidizing and low oxygen conditions	Jurkat T-lymphocytes	2-D DIGE using BODIPY-dyes	Under low oxygen conditions many proteins became more reduced than under standard growth conditions	205
Identificaiton of S-glutathionlyated proteins in T-cell blasts under oxidative stress	Human T-lymphocytes	Labeling of intact cells with [^{35}S]cysteine and nonreducing 2-DE	Many proteins can undergo S-glutathionlyation under oxidative stress; the activities of enolase 1 alpha and 6-phosphogluconolactonase were inhibited after S-glutathionlyation	92
Identification of S-glutathionlyated proteins in hepatocytes	Primary rat hepatocytes and human HepG2 hepatoma cells	Labeling of intact cells with [^{35}S]cysteine and 2-DE combined with autoradiography	New targets for S-glutathionlyation under oxidative stress include ubiquitin thioesterase L3, mitochondrial histidine triad nucleotide binding protein 2, and Ran specific GTPase activating protein	93
Identification of S-glutathionlyated proteins that are related to TNF alpha signaling	HeLa cells	Labeling using biotinylated glutathione ethyl ester flowed by streptavidin chromatography, microsequencing and MS	Trx peroxidase II and annexin II are glutathionylated in response to TNF alpha singaling	303
Identification of S-glutathionlyated proteins in response to diamide treatment	Immortalized ECV304 endothelial-like cells, derived from umbilical vein	Selective reduction of S-glutathionylated proteins using the mutant C14S, C65Yof *Escherichia coli* Grx 3; NEM-biotin labeling followed by 2-DE and MS	22 targets of S-glutathionylation comprise PDIs, chaperones and signaling proteins including 14-3-3 zeta	195

(*continued*)

Table 1. (Continued)

Aim	*Experimental system*	*Proteomics approach*	*Outcome/highlights*	*Reference*
Identification of S-nitrosylated proteins during alteration of SNO homeostatis in hepatocytes	Human hepatocytes	Biotin-switch assay	Treatment of human hepatocytes with *S*-nitroso-L-cycteine is accompanied by S-nitrosylation of 20 proteins including Raf kinase inhibitor protein PEBP-1	203
Identification of target proteins for S-nitrosylation in mesangial cells	Mouse mesangial cells (ATCC CLR-1927)	Biotin-switch assay combined with 2-DE	A set of 32 novel *in vitro* targets for S-nitrosylation includes signaling proteins (*e.g.*, 14-3-3), receptors (*e.g.*, interfereon gamma) and transcription factors (*e.g.*, PPAR gamma)	166
Identification of the S-nitrosoproteome of endothelial cells	EA.hy293 hybridoma cells	Biotin-switch assay combined with 1-DE	A set of 12 targets of endogenous S-nitrosylation includes the τ- and ζ/δ 14-3-3 proteins	218
Identification of the S-nitrosoproteome of endothelial cells	Human aortic endothelial cells, bovine aortic endothelial cells	Modified biotin-switch assay	The S-nitrosoproteome of bovine aortic endothelical cells colocalizes with mitochondria; glyceraldhyde 3-phosphate dehydrogenase is the most abundant S-nitrosylated protein in untreated human aortic endothelial cells	335
Facilitate identification of S-nitrosylated proteins by mass spectrometry	Human embryonic kidney (HEK293) cells	Resin-assisted capture of S-nitroylated proteins (SNO-RAC)	The method is suitable for quantitative proteome studies of S-nitrosylated proteines. Most S-nitrosylated proteins underwent rapid denitrosylation	88
Identification of the S-nitrosoproteome of endothelial cells	Endothelial cells derived from EAhy 926 hybridoma cells	Modified biotin-switch assay combined with 2-DE/Western blotting	A set of 28 S-nitrosylated proteins was identified; of which the largest group had functions in the cytoskeleton	141
Impact of flow conditions in the surrounding on S-nitrosylation in endothelial cells	Endothelial cells derived from umbilical human cords	Redox 2-D DIGE employing thiol labeling with Cy3 and Cy5 maleimide for relative quantification of S-nitrosylated proteins	Endothelial cells that experienced shear flow displayed higher levels of S-nitrosylation than cells under steady state conditions	140
Higher sensitivity of the detection of S-nitrosylated proteins in proteome studies	Endothelial cells (EA.hy296 and HUVEC), macrophage cells (Raw264.7)	Fluorescence switch method using thiol labeling by fluorescein-5 maleimide combined with 2-DE	The sensitivity of detection of S-nitrosylated proteins is much higher than that of the biotin-switch method	307
Systematic analysis of potential targets for protein S-nitrosylation in human spermatozoa	Human spermatozoa	Biotin-switch assay	S-nitrosylation colocalizes with the postacrosomal region of the head and the flagellum; 240 potential S-nitrosylation targets include eight transcription factors and three protein kinase A anchoring proteins	180

(*continued*)

Table 1. (Continued)

Aim	*Experimental system*	*Proteomics approach*	*Outcome/highlights*	*Reference*
Detection of sulphenic modified proteins in living cells	Jurkat T-lymphocytes	1-DE combined with Western blotting	The cell permeable azido-probe DAz-1 is selective for sulphenic acid modifications *in vitro* and able to detect sulphenic-acid modified proteins *in vivo*	264
Improved synthesis of the cell permeable sulfenic acid probe DAz-1	HeLa cells	1-DE combined with Western blotting	Labeling by DAz-1 detects sulfenic acid modification in the active site of the cell cycle regulatory phosphatase Cdc25A	285
Identification of sulfenic acid modifications of the thiol proteome	HeLa cells	*In vivo* labeling using DAz-2 followed by a Staudinger ligation	DAz-2 is a tool for global proteomics studies of sulphenic acid modifications	189
Mammalian tissues				
Identification of cardiac proteins *S*-thiolated during ischemia and reperfusion	Intact rat hearts	*S*-thiolation with biotinylated cysteine	The majority of the S-thiolated proteins are cytoskeletal or from myofilaments.	76
Identification of renal proteins *S*-thiolated during ischemia and reperfusion	Intact rat kidneys	*S*-thiolation with biotinylated cysteine	Triose phosphate isomerase was found to be S-thiolated, as in heart tissue	78
Detection of cardiac proteins glutathionylated *in vitro* upon treatment with rose bengal	Soluble rat heart protein extracts	Glutathionylation with reduced glutathione-agarose	Only malate dehydrogenase was identified	77
Identification of glutathionylated cardiac proteins	Ventricular myocytes isolated from rat hearts	Glutathionylation with biotin-GSSG	Many myofilament proteins become glutathionylated	34
Identification of cardiac proteins with inter- and intramolecular disulfides	Ventricular myocytes isolated from rat hearts	Diagonal nonreducing/reducing 2-D SDS-PAGE	Several glycolytic and TCA-cycle enzymes form disulfides upon diamide treatment	33
Identification of cardiac membrane proteins with specific cysteines oxidized by H_2O_2	Rabbit heart muscle cellular membranes	Alkylation with IAM-linked ICAT reagent	VDACs and mitochondrial electron transport proteins have redox-sensitive cysteines	288
Comparison of ICAT and fluorescence-DIGE in detection of protein cysteine oxidation	Soluble murine heart protein extracts treated with H_2O_2	ICAT and fluorescence-DIGE using Cy3- and Cy5-maleimide	The methods are complementary, but ICAT is more sensitive than fluorescence-DIGE	95
Identification of cardiac proteins containing cysteines reduced by Trx1	Transgenic mice with heart-specific overexpression of Trx1	ICAT and independent analysis with iTRAQ for protein quantification	78 cysteines within 55 heart proteins are significantly reduced by Trx1 overexpression	96
Identification of cardiac proteins containing sulfenic acids after H_2O_2 treatment	Intact rat hearts	Arsenite-specific reduction of sulfenic acids and labeling with biotin-maleimide	The majority of the sulfenated proteins are cytoskeletal, from myofilaments and mitochondria	276
Identification of cardiac proteins containing sulfenic acids: roles of H_2O_2 and O_2	Intact rat hearts	Specific labeling of sulfenic acids with biotin-dimedone	Hypoxia suppresses drastically basal cysteine sulfenation.	49
Identification of renal proteins containing cysteine sulfenic acids	Kidneys from spontaneously hypertensive rats (SHR) and control rats	Arsenite-specific reduction of sulfenic acids and labeling with biotin-maleimide	Spontaneously hypertensive rats (SHR) have elevated levels of renal protein sulfenation.	311

(*continued*)

Table 1. (Continued)

Aim	*Experimental system*	*Proteomics approach*	*Outcome/highlights*	*Reference*
Other animals				
Identification of mussel gill proteins with disulfides and other oxidized cysteines	Gill proteins extracted from menadione-treated mussels and control mussels	Diagonal nonreducing/reducing 2-D SDS-PAGE combined with immunoblotting, 2-DE combined with IAF labeling	PDIs, glutathione-S-transferase pi and cytoskeletal proteins have cysteines oxidized by menadione treatment	226
Fungi				
Identification of yeast Trx target proteins *in vivo*	A yeast *trx* double knockout mutant expressing a plant monocysteininc Trx *h*3	Trx affinity chromatography *in vivo* using the monocysteininc Arabidopsis Trx *h*3	The only target identified, a type II Prx, defines a new family of Prx	316
Identification of yeast proteins *S*-thiolated in response to H_2O_2 treatment	Wild type yeast cells	*S*-thiolation with radioactive [^{35}S]cysteine in the presence of cycloheximide	Enzymes participating in glycolysis and protein synthesis become S-thiolated and, thus, reversibly inactivated during oxidative stress	292
Identification of cytosolic yeast proteins with oxidized cysteines	Wild type yeast and mutants lacking either Trx- or reduced glutathione-based reductive pathways	Labeling of protein thiols with biotin-HPDP or radioactive [^{14}C]NEM + 2-DE	Proteins with oxidized cysteines are present in the cytosol of unstressed yeast cells under aerobic, but not anaerobic conditions.	187
Identification of yeast proteins with oxidized cysteines under oxidative stress	Wild type yeast cells	Labeling of protein thiols with biotin-HPDP, trypsin cleavage, LC-electrospray ionization tandem mass spectrometry	Treatment with H_2O_2 induces reversible cysteine oxidation of glycolytic and TCA-cycle enzymes	227
Identification of redox-reactive surface exposed cysteines under native conditions	Wild type yeast cells	BIAM labeling after reduction by TCEP, TRX1 and TTR1; 1-DE combined with MS/MS and LC-MS/MS	185 redox-active cysteines identified; the largest group of proteins with redox-active cysteines had functions in protein synthesis and degradation including many ribosomal proteins	216
Plants (*Seeds*)				
Identification of Trx targets in seeds	Peanut extract	mBBr labeling combined with nonreducing/reducing electrophoresis and 2-DE	Labeling by mBBr reveals interactions of Trx with a seed maturation protein and a desiccation related protein	338
Identification of Trx-linked metabolic processes of cereal endosperm	Wheat endosperm	mBBr labeling combined with 2-DE	23 potential Trx targets in the endosperm of wheat seeds link Trx to functions in metabolism, protein degradation and folding, and antioxidant defense	325
Identification of Trx-targets in germinating barley seeds	Extracts from embryo and endosperm proteins of barley seeds	mBBr labeling combined with 2-DE	The redox state of Trx *h* targets in barley seeds changes during seed development	222
Solubility of Trx-targeted proteins	Wheat endosperm	mBBr labeling combined with 2-DE	Reduction by Trx alters solubility of seed proteins	327
Identification of Trx-targets in developing wheat seeds	Wheat endosperm	mBBr labeling combined with 2-DE, and Trx affinity chromatography *in vitro* using a monocysteinic Trx *h*1 of poplar combined with 2-DE	The methods are complementary and show that the endosperm of young and of mature seeds have their distinct sets of Trx targets; 68 potential Trx targets were detected	326

(*continued*)

Table 1. (Continued)

Aim	*Experimental system*	*Proteomics approach*	*Outcome/highlights*	*Reference*
Trx-linked reactions in seed germination	Germinating rice seeds	mBBr labeling combined with 2-DE	The degradation of seed storage proteins during germination of rice seeds is accompanied by a reduction of their disulfides	337
Trx-linked reactions in seed germination	Rice bran extract	mBBr labeling combined with 2-DE	Evidence for a regulatory role of disulfides in proteolysis	340
Identification of seed proteins targeted by Trx *h* isoforms	Barley seed extracts	Cy5-maleimide labeling combined with 2-DE	Evidence that Trx *h*1 and *h*2 control defense proteins including alpha amylase/trypsin inhibitors and the alpha amylase/subtilisin inhibitor BASI	206
Identification of seed proteins targeted by Trx *h* isoforms	Barley seed extracts	Differential alkylation combined with 2-DE and matrix assisted laser desorption ionization mass spectrometry	Barley Trx *h*1 reduces specific classes of disulfides	207
Identification of seed proteins targeted by Trx *h*1	Barley seed embryos	Redox ICAT	More than 100 potential disulfide targets of Trx *h*1 were quantified in embryos of germinated barley seeds including a seryl-tRNA synthetase and 19 ribosomal proteins	109
Protein expression profiles of wheat seeds under drought	Extracts of seeds of the wheat varieties Afghani, Arvand, and Khazar-1	2-DE and MS/MS	Trx *h* is up-regulated in the drought-tolerant variety Khazar-1	110
Identification of seed proteins targeted by Trx	Extracts of seeds of *Medicago truncatula*	mBBr labeling combined with 2-DE, and Trx affinity chromatography *in vitro* using a monocysteinic Trx *h*3 of pea combined with 2-DE	The disulfide proteome of the seeds of the dicotyledon *Medicago truncatula* has many similarities to that of the monocotyledon wheat	4
Leaves and roots				
Trx-targets of cytosolic Trx *h* isoforms	Cell lysates from etiolated Arabidopsis seedlings	Trx affinity chromatography *in vitro* using a monocysteinic Trx *h*1 of Arabidopsis combined with 2-DE	15 potential Trx targets identified including type II Prx, EF-2 and Hsp70	334
Trx-targets of cytosolic Trx *h* isoforms	Arabidopsis leaves, RuBisCo depleted extracts	2-DE combined with autoradiography	More than 40 potential targets of Trx *h*3 covered a broad range of cellular functions including defense against pathogens	210
Trx-targets of cytosolic Trx *h* isoforms	Arabidopsis leaves, RuBisCo depleted extracts	(i) 2-DE combined with autoradiography; (ii)biotin labeling combined with avidin chromatography and 2-DE; (iii) Trx affinity chromatography *in vitro* using a monocysteinic Trx *h*3 of Arabidopsis	Methods (i) and (iii) are complementary	211
Trx-targets of roots	Arabidopsis roots, extracts of soluble proteins	Trx affinity chromatography *in vitro* using a monocysteinic Trx *y*2 of Arabidopsis combined with 2-DE	The potential Trx targets in roots indicate a role of Trx in many metabolic pathways, detoxification and defense	212

(*continued*)

Table 1. (Continued)

Aim	*Experimental system*	*Proteomics approach*	*Outcome/highlights*	*Reference*
Grx-targets of poplar secreted GrxC4	Leaves of Arabidopsis and poplar, chloroplast and mitochondria from pea, potato tubers	Trx affinity chromatography *in vitro* using a monocysteinic GrxC4 from poplar	Identification of 94 potential Grx targets shows that there is no obvious difference in substrate specificities between Trx and Grx *in vitro*	273
Response of the disulfide proteome of Aradidopsis to jasmonate signals	Arabidopsis seedlings	2-DE combined with mBBr labeling and SyproRuby staining	Roots and shoots clearly displayed different responses to the jasmonate treatment in their metabolic pathways; 12 redox-sensitive cysteine residues were identified.	6
Plants (cell cultures)				
Identification of S-glutathionylated proteins *in vitro*	Arabidopsis cell suspension cultures	Immunoblotting, chromatography, N-terminal microsequencing	Cytosolic triose phosphate isomerase and plastidic fructose bisphosphate aldolase isoform 1 are S-glutathionylated *in vitro*	147
Identification of S-glutathionylated proteins *in vivo* and in vitro	Arabidopsis cell suspension cultures	GSSG-biotin labeling combined with strepatavidin chromatography and 2-DE	*In vivo* S-glutathionylation of nine proteins including a transducin; *in vitro* S-glutathionylation of 74 proteins including 20 proteases and six members of the glutathione-S-transferase superfamily	71
Identification of S-nitrosylated proteins *in vitro*	Leaves and cell suspension cultures of Arabidopsis	Biotin switch combined with 1-DE and 2-DE	A set of 38 candidates for S-nitrosylation includes four translation elongation and three translation initiation factors. The S-nitroslyation targets overlap largely with those reported from animal studies.	199
Detection of protein S-nitrosylation in response to pathogen attack	Arabidopsis leaf extracts	Biotin switch combined with 2-DE	The hypersensitive response is accompanied by protein S-nitrosylation	272
Algae				
Identification of Trx-targeted proteins of *C. reinhardtii*	Soluble fraction of *C. reinhardtii*	Trx affinity chromatography *in vitro* using monocysteinic Trx *h*1 of *C. reinhardtii*	Potential Trx-targets were found in many central cytoplasmic and plastidal pathways; the nuclear Ran protein is reduced by Trx	185
Identification of S-thiolated proteins of *C. reinhardtii*	Soluble fraction of *C. reinhardtii*	^{35}S-labeling, 2-D electrophoresis, autoradiography	25 potential S-Thiolation targets were identified, and S-thiolation of Hsp70B, chloroplastic 2-Cys Prx and isocitrate lysase was confirmed *in vitro*	232

1-DE, one-dimensional electrophoresis; 2-DE, two-dimensional electrophoresis; 2-D DIGE, two-dimensional difference gel electrophoresis; BIAM, *N*-(biotinoyl)-*N*-(iodoacetyl) ethylendiamine; biotin-HPDP, *N*-(6-(biotinamido)hexyl)-3′-(2′-pyridyldithio)propionamide; biotin-maleimide, *N*-biotinoyl-*N*′-(6-maleimidohexanoyl)hydrazide; GSSG, oxidized glutathione; Grx, glutaredoxin; IAF, 5-iodoacetamidofluorescein; IAM, 2-iodoacetamide; LC-MS/MS, liquid chromatography coupled on-line to tandem mass spectrometry; mBBr, monobromobimane; NEM, *N*-ethylmaleimide; PDI, protein disulfide isomerase; Prx, peroxiredoxin; RuBisCo, ribulose bisphosphate carboxylase/oxygenase; TCA, tricarboxylic acid; TCEP, tris(2-carboxyethyl)phosphine; Trx, thioredoxin.

Table 2. Subcellular Disulfide Proteomes

Aim	*Experimental system*	*Proteomics approach*	*Outcome/highlights*	*Reference*
Mammalian mitochondria				
Design of a method for specific labeling of mitochondrial protein thiols within intact cells	Intact human fibroblasts, Jurkat cells or purified rat liver mitochondria	Labeling with IBTP and immunological detection of the IBTP reagent	IBTP accumulates more than 100-fold in the mitochondrial matrix and labels specifically protein cysteine thiols	194
Identification of proteins with changes in the cysteine redox state after ethanol intake	Liver mitochondria isolated from ethanol-fed and control rats	Labeling with IBTP and immunological detection of the IBTP reagent	Liver aldehyde dehydrogenase and the glucose-regulated protein 78, GRP78, have their cysteines more oxidized after ethanol intake	315
Identification of mitochondrial proteins *S*-nitrosylated upon treatment with *S*-nitroso-glutathione	Purified rat liver mitochondria	Ascorbate-specific reduction and labeling with biotin-HPDP	S-nitrosylation was observed equally under both aerobic and anaerobic conditions, though only six proteins were identified	89
Identification of proteins with changes in the cysteine redox state after ethanol exposure	Human hepatoma HepG2 cell cultures	Labeling with biotin-maleimide	Oxidation of cysteines in several mitochondrial proteins, for example, Hsp60, aldehyde dehydrogenases and GRP78, is enhanced by ethanol exposure	302
Design of a mass tagging approach for specific labeling of mitochondrial protein thiols	Purified rat heart mitochondria	Labeling with stable isotope-coded IBTP: IBTP-d_{15} and IBTP-d_0	This method proved to be highly reproducible and useful for the determination of the cysteine redox state of mitochondrial proteins	217
Identification of mitochondrial proteins with oxidized cysteines under oxidative stress	Purified rat heart mitochondria	Fluorescence-DIGE using Cy3- and Cy5-maleimide	Endogenous reactive oxygen species production caused by reverse electron transport leads to cysteine oxidation in 6 enzymes involved in fatty acid oxidation	143
Identification of mitochondrial proteins with cysteines alkylated with IAB or BMCC	Purified mitochondria from HEK 293 cells	Labeling with the biotinylated reagents IAB and BMCC	Of the 809 proteins identified, 209 were labeled only with IAB, 238 only with BMCC and 362 with both reagents.	328
Plant mitochondria				
Identification of plant mitochondrial Trx target proteins *in vitro*	Mitochondrial matrix from potato tubers and leaves from pea and spinach	Trx affinity chromatography using a monocysteininc Arabidopsis Trx-*m*	Seven enzymes of the TCA cycle were identified as Trx targets	14
Identification of mitochondrial proteins with inter- and intramolecular disulfides	Mitochondria isolated from *Arabidopsis thaliana* cell cultures	Diagonal nonreducing/reducing 2-D SDS-PAGE	Citrate synthase and several subunits of the ATP synthase participate in intermolecular disulfides	321
Identification of S-nitrosylated proteins in plant mitochondria	Mitochondria isolated from *Arabidopsis thaliana* leaves	Ascorbate-specific reduction and labeling with biotin-HPDP	Glycine decarboxylase, which functions in photorespiration, is inhibited by S-nitrosylation and glutathionylation	250
Chloroplasts				
Identification of chloroplast Trx target proteins *in vitro*	Chloroplasts from spinach, stromal fraction	Affinity chromatography using a monocysteininc Trx-*m* (spinach)	The method is suitable to study chloroplast Trx networks	239
Identification of chloroplast Trx target proteins *in vitro*	Chloroplasts from spinach, stromal fraction	Affinity chromatography using a monocysteininc Trx *f* and Trx *m* (spinach)	Ten novel processes in chloroplasts are potentially regulated by Trx	12

(*continued*)

Table 2. (Continued)

Aim	*Experimental system*	*Proteomics approach*	*Outcome/highlights*	*Reference*
Identification of chloroplast Trx target proteins *in vitro*	Chloroplasts from spinach, stromal fraction	Electrostatic binding to immobilized monocysteininc Trx *f* and Trx *m* (spinach)	Ten novel potential Trx targets including four ribosomal proteins	13
Identification of chloroplast Trx target proteins *in vitro*	Chloroplasts from potato, soluble fraction	Affinity chromatography using a monocysteininc CDSP32 (potato)	The Trx CDSP32 binds to PrxQ and BAS1 peroxiredoxins *in vivo*.	265
Identification of chloroplast Trx targeted membrane proteins *in vitro*	Chloroplasts from spinach, thylakoid fraction	mBBr labeling followed by 2-D electrophoresis	ATP synthesis and photosynthetic electron transport are potentially controlled by Trx	15
Mechanism of transfer of redox equivalents across the e thylakoid membrane	Thylakoid membranes from Arabidopsis chloroplasts	Affinity chromatography using a monocysteininc mutant of the soluble part of Hcf164	Hcf164 can receive reducing thiol equivalents from stromal Trx *m* and reduce lumenal proteins *in vitro*	241
Identification of chloroplast disulfide proteins *in vitro*	Chloroplasts from Arabidopsis, RuBisCo depleted soluble fraction	Nonreducing/reducing 2-D electrophoresis	The photosynthetic reaction center proteins PsbA (D1) and PsaA (subunit 1a) are potential targets for redox regulation *via* reactive cysteines	301
Identification of chloroplast Trx target proteins *in vitro*	Envelope membranes from barley chloroplasts	Affinity chromatography using a monocysteininc Trx *f* and Trx *m* (spinach)	Three potential Trx targets of the envelope membrane function in protein import and chlorophyll metabolism	19
Identification of chloroplast Trx target proteins *in vitro*	Chloroplasts from Arabidopsis, lumenal fraction	mBBr labeling followed by 2-D electrophoresis and affinity chromatography using a monocysteininc mutant of cyanobacterial TrxA	More than 40% of the known lumenal chloroplast proteins can be reduced by Trx *in vitro*; the redox state of three lumenal proteins is decisive for their degradation *in vitro*	112
Amyloplasts				
Mapping the metabolic functions of amyloplasts	Amyloplasts from wheat endosperm	Analysis of the soluble and membrane fraction of purified amyloplasts by 1-D and 2-DE combined with LC-MS/MS	Evidence for a ferredoxin/Trx system in amyloplasts	16
Identifying a ferredoxin/Trx system in amyloplasts and the corresponding Trx targets	Amyloplasts from wheat endosperm	mBBr labeling combined with 2-DE and affinity chromatography using a monocysteininc Trx *m* (spinach)	Trx *m* is a potential regulatory link between photosynthesis and metabolic processes in amyloplasts	17

IAB, *N*′-iodoacetyl-*N*-biotinylhexylenediamine; IBTP, (4-iodobutyl)triphenylphosphonium.

detect protein S-glutathionylation in endothelial-like cells derived from umbilical vein (195). A bacterial Grx was used to reduce S-glutathionylated proteins, which were then alkylated with biotin-NEM and purified on avidin-agarose.

Protein modification by S-nitrosylation has been studied in many different kinds of cells. The targets for S-nitrosylation in human hepatocytes indicate that NO probably controls proteins that have important functions in metabolism, signal transduction, and redox response (203). In additon, the *in vitro* profile of S-nitrosylated proteins of glomerular mesangial cells from mouse suggested that NO might have a potential role in many cellular processes (166). Extracted proteins from mesangial cells were nitrosylated using GSNO and S-nitrosylated proteins purified using the biotin switch method and analyzed by 2-DE combined with MALDI-MS. The identified proteins included 32 novel targets of NO that were sorted into five groups. The largest group consisted of 15 proteins with assigned functions in signal transduction. For instance, S-nitrosylation of the GTP binding protein $G\alpha_{i2}$ linked NO to the network of G-protein coupled receptor signaling. The NO targets also included 14-3-3 ε and ζ that are key players in cellular signaling (313). Moreover, the members of this group included the natriuretic peptide uroguanylin, and an NADPH cytochrome P450 oxidoreductase. The second largest group of NO-targets contained nine members that represented receptors and membrane proteins. The other

Table 3. Disulfide Proteomes in Prokaryotes

Aim	*Experimental system*	*Proteomics approach*	*Outcome/highlights*	*Reference*
Nonphotosynthetic prokaryotes				
Identification of *in vivo* substrates of DsbA and TrxA and proteins sensitive to H_2O_2	Wild type *E. coli* and *dsbA* and *trxA* knockout strains	Labeling of protein thiols with radioactive [^{14}C]IAM + 2-DE	Many periplasmic proteins are subject to cysteine oxidation by DsbA and several TCA-cycle enzymes are substrates for TrxA	182
Identification of protein cysteines oxidized by treatment with *S*-nitroso-glutathione or DEANO *in vivo*	Wild type *E. coli*	Labeling of protein thiols with radioactive [^{14}C]IAM + 2-DE	Glutamate synthase activity is inhibited in response to reactive nitrogen species due to cysteine oxidation of GltD, but is insensitive to H_2O_2	30
Identification of protein cysteines oxidized by treatment with H_2O_2 or NaOCl *in vivo*	Wild type *E. coli*	OxICAT: thiol blocking with light ICAT, new thiols are labeled with heavy ICAT	The method allows absolute quantification of cysteine oxidation and precise identification of the modified cysteines	183
Identification of bacterial proteins containing cysteine sulfenic acids	*E. coli* expressing a His-tagged C-terminal fragment of yeast Yap1	His-tagged C-terminal domain of Yap1 used as bait *in vivo*	About 30 *E. coli* proteins were found to contain cysteine sulfenic acids after treatment with H_2O_2	305
Identification of proteins containing cysteines oxidized by treatment with menadione	Wild type *E. coli*	ATS chromatography	Most *E. coli* proteins displaying cysteine oxidation are involved in translation, glycolysis and the TCA cycle	138
Identification of proteins containing oxidized cysteines after oxidative stress treatment	*Bacillus subtilis*	Labeling with the BODIPY FL-C_1-IA fluorescent dye and 2-DE	Enzymes involved in amino acid metabolism are highly represented among the proteins with oxidized cysteines	121
Identification of bacterial proteins *S*-thiolated with cysteine upon diamide treatment	*Bacillus subtilis*	Labeling *in vivo* with [^{35}S]cysteine in the presence of chloramphenicol	Three of the six identified S-cysteinylated proteins are known to be S-glutathionylated in other organisms	122
Identification of proteins containing oxidized cysteines after oxidative stress treatment	*Staphylococcus aureus*	Labeling with the BODIPY FL-C_1-IA fluorescent dye and 2-DE	Various proteins participating in intermediary metabolism undergo cysteine oxidation after treatment with H_2O_2	324
Comparison of protein expression and thiol oxidation in response to NO treatment in *B. subtilis* and *S. aureus*	*Bacillus subtilis* and *Staphylococcus aureus*	Labeling with the BODIPY FL-C_1-IA fluorescent dye and 2-DE	Treatment with NO causes distinct changes in protein expression in *B. subtilis* and *S. aureus*, but it does not change the overall thiol oxidation state.	123
Identification of bacterial proteins containing oxidized cysteines induced by diamide	*Bacillus subtilis* and *Staphylococcus aureus*	Labeling with fluorescent BODIPY FL-C_1-IA and diagonal electrophoresis	Diamide treatment results mainly in protein S-cysteinylation in these gram-positive species, which lack glutathione biosynthesis	261
Identification of proteins S-nitrosylated in response to sodium nitrite exposure	*Mycobacterium tuberculosis*	Ascorbate-specific reduction and labeling with biotin-HPDP	The majority of the 29 S-nitrosylated proteins identified participate in intermediary metabolism	266

(*continued*)

Table 3. (Continued)

Aim	*Experimental system*	*Proteomics approach*	*Outcome/highlights*	*Reference*
Photosynthetic prokaryotes				
Identification of cyanobacterial Trx target proteins *in vitro*	*Synechocystis* sp. PCC 6803 cytosolic extract	Trx affinity chromatography using a His-tagged monocysteininc Trx *m*	The identified proteins were quite different from chloroplast Trx targets, though the same processes were represented	196
Identification of cyanobacterial proteins, which are targets for different Trx *in vitro*	*Synechocystis* sp. PCC 6803 cytosolic extract	Trx affinity chromatography using His-tagged monocysteinic Trx *m*, *x* and *y*	The Trx target proteomes for the three different *Synechocystis* Trx are largely overlapping	253
Identification of cyanobacterial Grx target proteins *in vitro*	*Synechocystis* sp. PCC 6803 cytosolic extract	Grx affinity chromatography using a monocysteininc Grx2061 (GrxA)	Out of the 42 identified proteins, 18 had been previously identified as targets for Trx in *Synechocystis*	190
Identification of cyanobacterial membrane proteins, which are Trx targets *in vitro*	Isolated total membranes from *Synechocystis* sp. PCC 6803	Binding of a His-tagged monocysteininc Trx *m* to membrane proteins *in situ*	Membrane-associated Trx target proteins include ATP-dependent proteases and transporters of ions and preproteins	223
Identification of cyanobacterial Trx target proteins *in vivo*	*Synechocystis* sp. PCC 6803 expressing a His-tagged monocysteininc Trx *x*	*In vivo* Trx affinity chromatography using the His-tagged monocysteininc Trx *x*	*In vivo* targets for Trx *x* include 6 ribosomal subunits that had not been identified as Trx targets *in vitro*	254
Identification of green sulfur bacterial Trx target proteins *in vitro*	*Chlorobaculum tepidum*	Trx affinity chromatography using monocysteininc Trx1 and Trx2	Six Trx target proteins belong to the reductive TCA cycle for carbon dioxide fixation of *Chlorobaculum tepidum*	135

ATS, activated thiol sepharose; BMCC, 1-biotinamido-4-(4′[maleimidoethylcyclo-hexane]-carboxyamido)-butane.

groups included proteins of the cytoskeleton and the cell matrix, and cytoplasmic proteins. Finally, three transcription factors were found, including the peroxisome proliferator activated receptor γ, the zinc finger proteins 297B, and the TFIIA-α/β-like factor. However, it should be kept in mind that these results may not be relevant to the physiological situation (166).

The S-nitrosoproteome of endothelial aorta cells colocalizes with the mitochondria and the peri-mitochondrial compartment. The short life-time of the S-nitrosoproteome implies a high dynamics of denitroslyation (335), which is also indicated by recent findings that show that most S-nitrosylated proteins of human embryonic kidney cells (HEK293) are rapidly denitrosylated (88). To define the S-nitrosylated proteins of endothelial cells, EA.hy926 hybridoma cells were treated with S-nitroso-L-cysteine, and endogenously s-nitrosylated proteins were purified using the biotin switch method (218). The major targets of S-nitrosylation consisted of

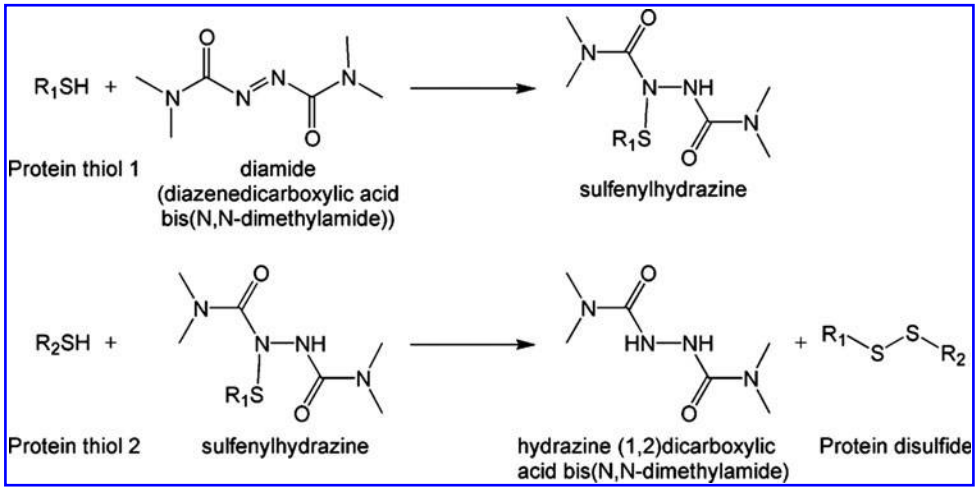

FIG. 13. Thiol oxidation by diamide. The oxidizing reagent diamide mediates the oxidation of thiols to disulfides and changes the redox status of a biological system. Diamide oxidizes protein thiols *via* a sulfenylhydrazine intermediate to disulfides. By an analogous mechanism diamide also rapidly depletes the glutathione pool of a biological system and mediates the formation of protein-glutathione mixed disulfides, that is, glutathionylation (164).

12 proteins that included β- and γ-actin, Prx1, GADPH, and the τ- and ζ/δ 14-3-3 proteins that play important roles in cellular signal transduction (218). The proteins β-actin, Prx1, and GADPH have also been identified among the targets of endogenous S-nitrosylation in human aorta endothelial cells (HAEC). In addition, the S-nitrosylated proteins of HAEC include the cytoskeleton-related protein vimentin and the ubiquitin conjugating enzyme UbcH7 (335). To increase the sensitivity of the detection of S-nitrosoproteins in endothelial cells, the streptavidin affinity chromatography of the biotin switch method was replaced by a western blotting protocol, in which the proteins that carried biotin-labeled cysteine residues were detected using streptavidin horseradish peroxidase (HRP) conjugate. To identify these proteins, they were resolved on 2-DE and the corresponding protein spots were analyzed by mass spectrometry. This strategy considerably reduced the amount of cultured cells needed for the detection of the S-nitrosoproteins. A set of 28 protein spots were identified from 2-D gels of umbilical vein endothelial cells (EA.hy926, a hybridoma cell line). In agreement with earlier studies, the S-nitrosoproteins of endothelial cells had functions related to the cytoskeleton. In addition, they included molecular chaperones, mitochondrial ATPase F1, and proteins involved in mRNA processing and elongation (141).

Recent modifications of the biotin switch technique replaced thiol labeling with biotin-HPDP (Fig. 5B) by labeling with cysteine-specific fluorescence dyes. The detection of S-nitrosylated proteins is performed by 2-DE instead of mass spectrometry (140, 307). In the fluorescence switch approach thiols are labeled using fluorescein-5 maleimide, and the S-nitrosylated samples and controls are compared by 2-DE. This technique enabled identification of 21 S-nitrosylated proteins in CysNO-treated endothelial cells (EA.hy926 hybridoma cells) using 200 μg extracted proteins as starting material (307). In the redox 2-D DIGE technique, thiols are labeled using the cysteine-specific fluorescence dyes Cy3 and Cy5 maleimide (Fig. 4D, E) (140). Proteins with S-nitrosylated cysteine residues are then detected in a 2-D DIGE experiment and identified by mass spectrometry. Using this strategy, more than 100 S-nitrosoproteins were identified in endothelial cells. In addition, the impact of the flow conditions in the surrounding medium on S-nitrosylation was highlighted. Endothelial cells that experienced shear flow displayed higher levels of S-nitrosylation than cells under steady state conditions. A group of 12 proteins displayed increased levels of S-nitrosation under shear flow. These proteins included heat-shock proteins, PDI, nucleophosmin, and a transitional endoplasmic reticulum ATPase. This observation is consistent with the shear flow-induced stimulation of endothelial NO synthase through phosphorylation by the Akt kinase (69),

One of the largest studies to date on S-nitrosylation addressed target proteins of NO in human spermatozoa (180). Using the biotin switch assay combined with 1-DE and ESI-MS/MS techniques 240 S-nitrosylated proteins were identified that included a considerable number of novel proteins (198) that had not been documented in studies of S-nitrosylation in other cell types. The novel S-nitroso proteins in human spermatozoa included three kinase-anchoring proteins that were not known to be targets of S-nitrosylation and might play a role in mediating the effect of NO on sperm mobility. Moreover, the S-nitroso proteins included the ryanodine receptor RyR2 that plays a role in Ca^{2+} signaling and a sperm-specific isoform of GAPDH. In addition, S-nitrosylation of nine heat-shock proteins was found. It is also noteworthy that 4% of the S-nitroso proteins had functions related to transcription, although sperms were considered to be transcriptionally inactive. A large group representing 25% of the identified S-nitroso proteins did not have a known subcellular location, and for 23% of the NO-target proteins no gene ontology was assigned. Hence, post-translational modification of spermatozoan proteins by S-nitrosylation probably reflects specific properties of this highly specialized cell type that enable them to sense and respond to NO signals generated by cells of the female tract (180).

As for proteins specifically undergoing sulfenic acid modifications, the most successful labeling attempts have been carried out using the compounds DAz-1 (264, 285) and DAz-2 (189), both of which are dimedone analogs linked to an azide moiety (Fig. 10A). DAz-2 proved to be highly membrane permeable and reactive toward protein sulfenic acids and was therefore used in a screening *in vivo* of cultured unstressed HeLa cells. After protein labeling with DAz-2 and cell lysis, phosphine-conjugated biotin was ligated to the DAz-2 azide functional groups and used to pull down the labeled proteins (Fig. 10B) (189). Among the 193 proteins identified with LC-ESI-MS/MS, 14 had previously been found to undergo sulfenation and another 71 proteins had been reported to harbor redox-active cysteines subject to other types of modification, such as glutathionylation or nitrosylation. Notably, sulfenated proteins were found in most cellular compartments, including cytoplasm, nucleus, Golgi, endoplasmic reticulum, mitochondria, and plasma membrane. When sorted according to protein function, the largest groups of sulfenated proteins consisted of cytoskeletal proteins (22%), enzymes involved in protein synthesis (21%) and metabolic enzymes (16%), though other significant groups comprised proteins involved in signaling (8%), protein trafficking (9%) and nuclear transport (7%). Certain protein families were represented by several members, for example, 5 translation elongation factors, as many as 16 ribosomal subunits, 4 aminoacyl-tRNA synthetases and 9 chaperones. Interestingly, the sulfenome included 10 Rab proteins, which belong to the Ras superfamily of small GTPases involved in vesicle transport. It is also worth mentioning that sulfenation has been observed of the phosphoinositide-3-kinase and the serine/threonine-protein phosphatase PP1-α catalytic subunit, both of which participate in signal transduction (189).

b. Mammalian tissues. Reperfusion after an incident of ischemia leads to the production of ROS and oxidative stress involved in cardiovascular disease (32, 133). The occurrence of *S*-thiolation in the myocardium of intact rat hearts after ischemia and reperfusion was studied by labeling with biotinylated cysteine (76). Biotin-cysteine added to the perfusion buffer was able to penetrate the plasma membranes and form mixed disulfides with a number of cellular proteins from cytosolic, membrane, and myofilament/cytoskeletal fractions. These *S*-thiolated proteins were affinity-purified using streptavidin-agarose and identified by western blotting using specific antibodies or by MALDI-MS analysis. Western blot analysis indicated that proteins that are *S*-thiolated during cardiac reperfusion include actin, GAPDH, Hsp27, protein-tyrosine phosphatase 1B, protein kinase Cα, and the small G-protein ras. Treatment of intact hearts with diamide (Fig. 13)

increased several-fold the formation of mixed disulfides between biotin-cysteine and the cellular protein thiols, but no further proteins were identified (76). The same technique was applied in a study of protein S-thiolation in rat kidneys during reperfusion after ischemia (78). Renal proteins labeled with biotin-cysteine were purified on streptavidin agarose, resolved by 1-DE and identified by PMF. In both hearts and kidneys, the glycolytic enzyme triose phosphate isomerase (TPI) was found to be S-thiolated after ischemia and reperfusion (76, 78). Evidence for glutathionylation of mammalian heart proteins comes from a study *in vitro*, in which soluble rat heart protein extracts were subjected to oxidative treatment with rose bengal (77). Subsequent isolation on GSH-agarose selected the proteins that formed mixed disulfides with GSH, which would mimic *in vivo* glutathionylation. However, the only glutathionylated protein identified was malate dehydrogenase (MDH). A later report described protein glutathionylation in ventricular myocytes isolated from rat hearts using *N*,*N*-biotinyl glutathione disulfide, or "biotin-oxidized glutathione (GSSG)" for short (34). The intact myocytes were incubated with biotin-GSSG and, thereafter, glutathionylated proteins were bound to streptavidin agarose. Finally, the proteins eluted with DTT were separated by 1-DE and analyzed by LC-ESI-MS/MS. Identified proteins included GAPDH, TPI, creatine kinase, and trifunctional enzyme α. A number of myofilament proteins were also identified. Interestingly, protein labeling with biotin-GSSG has also been observed in tissue homogenates from lungs, kidneys, liver, brain, and skeletal muscles, although no attempts were made to identify these proteins (34).

The formation of intermolecular disulfides in intact ventricular myocytes isolated from rat hearts has been studied by nonreducing/reducing diagonal electrophoresis (33). Since slower migration in the first dimension is indicative of intermolecular disulfides, CBB-stained proteins that presented this type of migration were identified by LC-MS/MS. It was found that the level of mixed protein–protein disulfides is low under control conditions, but that treatment of myocytes with 5 m*M* diamide (Fig. 13) increases substantially the proportion of proteins interlaced with disulfide bonds. In contrast, treatments with *S*-nitroso-*N*-acetylpenicillamine (SNAP) and doxorubicin, which might induce oxidation, and with the reducing agent *N*-acetylcysteine failed to increase or decrease interprotein disulfide bonds detected in control preparations (33). Several subunits of actin and tubulin have been found, as well as the enzymes GAPDH, MDH, isocitrate dehydrogenase, succinate dehydrogenase, TPI, and a subunit of pyruvate dehydrogenase. Complementary analysis of the myocytes using western blotting has shown that the regulatory subunit of protein kinase A forms disulfide-linked dimers due to oxidative treatment (33). Interestingly, no formation of intermolecular disulfides by Hsp70 has been observed, although this protein had been reported to form mixed disulfides with β4-spectrin and the APC protein in hippocampal HT22 cells (58).

A membrane preparation from rabbit heart muscle cells has been examined for redox-sensitive cysteines using a variant of the ICAT thiol-labeling procedure (288). In brief, the isolated total cellular membranes were treated with 10 m*M* hydrogen peroxide for 10 min and thereafter the cysteine thiols were labeled with the heavy ICAT reagent, whereas untreated control samples were labeled using the light ICAT reagent. In this manner, the reactive cysteine residues were identified, and, the degree of oxidation quantified. Among the proteins found to contain redox-sensitive cysteines were three voltage-dependent anion-selective channels (VDAC) of the mitochondrial outer membrane. The VDACs have been reported to function in the control of mitochondrial membrane transport and play a role in apoptosis. Also worth mentioning are the flavoprotein subunit of the mitochondrial succinate dehydrogenase (complex II), and an accessory subunit of the NADH dehydrogenase (complex I), which are both located in the mitochondrial inner membrane and function in respiratory electron transport. Finally, creatine kinase, which has been reported to be peripherally associated to mitochondrial inner membranes, was found to contain oxidized cysteines. Interestingly, oxidation of cysteines by the peroxide treatment described was relatively limited; only 10% of the thiols present in the cysteine-containing peptides recovered were oxidized in a proportion higher than 50% (288).

The gel-free ICAT method (288) and a DIGE-based method using Cy3- and Cy5-maleimide (Fig. 4D, E) were applied in parallel in a study of cysteine oxidation *in vitro* in extracts of soluble murine heart protein (95). Oxidative cysteine modifications induced by a treatment with 0.5 m*M* hydrogen peroxide were found in 63 different proteins, of which 37 were only identified with ICAT, 13 only with DIGE, and another 13 with both methods. The authors noted that many metabolic proteins, especially those involved in glycolysis, the pyruvate dehydrogenase complex and the tricarboxylic acid (TCA) cycle, were found to be susceptible to oxidation by hydrogen peroxide (95). Further, Prx5, superoxide dismutase 1 (SOD1), Hsp60, and the transcription factor NRF, as well as different forms of actin and myosin were found.

The role of Trx1 as a negative regulator of cardiac hypertrophy induced by ROS has been extensively studied, though the molecular mechanisms are not completely understood (1). A physiological assessment of the function of Trx1 in the mammalian heart tissue was accomplished through a proteomic study of transgenic mice with cardiac-specific overexpression of Trx1 (96). In this study transgenic (Tg-Trx1) and control mice were subjected to surgery, which induced oxidative stress and hypertrophy through transverse aortic constriction. The redox state of individual cysteine residues was compared between the Tg-Trx1 and control mice by ICAT labeling. Taking into account the fact that Trx1 could also affect the expression levels of proteins, protein levels were analyzed independently using the redox-insensitive iTRAQ method for protein quantification. The authors found that 78 cysteines within 55 proteins were significantly reduced by Trx1 overexpression. In contrast, all these proteins showed very similar expression levels in Tg-Trx1 and control mice. The majority of the Trx1-responsive proteins included metabolic enzymes from the glycolytic pathway, TCA-cycle, oxidative phosphorylation, and β-oxidation of fatty acids. In addition, the list of Trx1 targets contained proteins with function in muscles and the cytoskeleton, ion channels, chaperones, and stress response proteins (96).

Protein cysteines oxidized specifically to sulfenic acids have been studied in rat hearts using a method based on the fact that arsenite selectively reduces sulfenic acids to sulfhydryls but is inert toward disulfides or sulfinic acids (276). *A priori*, it might be thought that protein sulfenic acids are scarcely observed *in vivo*, since these are normally short-lived

and tend to form disulfides with neighboring cysteine thiols. Nevertheless, little was known about the true prevalence of sulfenic acids and the authors aim was to determine the extent of this post-translational modification in proteins from heart tissue under oxidative stress. Intact rat hearts were therefore treated with hydrogen peroxide. The thiols of the heart protein extracts were blocked with maleimide under denaturing conditions and arsenite was added to reduce the sulfenic acids. Labeling of the newly formed thiols was performed by the addition of biotin-maleimide and labeled proteins were purified using streptavidin-agarose. Notably, the occurrence of sulfenic acids depended strongly on the peroxide concentration and showed a maximum at around 0.1 m*M* hydrogen peroxide for all sulfenated proteins. Higher peroxide concentrations abolished labeling, probably due to sulfinic and sulfonic acid derivatization of the reactive cysteines. The study resulted in the identification of 17 biotinylated proteins, among which were the cytoskeletal tropomyosin, actin, and heavy and light chains of myosin, all of which are implicated in the contraction of heart muscle. Several mitochondrial proteins were also found. The authors remarked that the proteins identified are generally of high abundance in heart cells, and that there are probably some low abundance sulfenic acid-containing proteins still to discover (276).

A different experimental approach was therefore designed in an attempt to improve the method for the detection and identification of sulfenated proteins in heart tissue. This method involved the synthesis of a reagent, biotin-dimedone, which reacts directly and specifically with sulfenic acids without the need for reduction using arsenite (49). As in the previous study (276), the biotin moiety served as a handle for the purification of sulfenated proteins using streptavidin-agarose and detection by western blot analysis using streptavidin-horseradish peroxidase conjugates and enhanced chemiluminescence (ECL). Purified proteins were resolved on 1-DE and identified by LC-ESI-MS/MS analysis. The authors found that the basal level of protein sulfenation observed in isolated rat hearts under aerobic conditions was drastically reduced under hypoxia. However, reoxygenation was able to restore the initial levels of protein sulfenation, and the addition of 50 μM hydrogen peroxide further enhanced the formation of protein sulfenic acids (49). This highlights protein sulfenation as a potential mechanism of oxygen sensing. Among the sulfenated proteins identified, the heavy and light chains of cardiac muscle α- and β-myosin were still predominant, along with actin and tropomyosin. Moreover, as in the preceding study, the mitochondrial enzymes aconitase and MDH were found along with a few more newly identified cytoplasmic metabolic enzymes (49). However, the authors recognized that the identified proteins were still typically highly abundant cardiac myocyte proteins and the dynamic range of the method did not seem to allow for the detection of putative regulatory proteins.

The elevated sulfenation of renal tissue is a characteristic of the physiological differences between rats with normal and high blood pressure. The renal medulla, the innermost part of the kidneys, from spontaneously hypertensive rats (SHR) displayed significantly enhanced levels of protein sulfenation as compared to normotensive Wistar rats (311). Medulla tissue from SHR also had a lower content of free thiols and a lower activity of Trx reductase. Using the modified biotin switch method (276) combined with 2-DE and LC-MS/MS, 32 sulfenated proteins were identified in medulla tissue extracts from SHR. A prominent group of sulfenated proteins had functions in protecting against oxidative stress and were represented by the Prx2, Prx3, Prx5, and Prx6, Cu-Zn SOD, and aflatoxin B1 aldehyde reductase. Many of the sulfenated proteins found in kidney medulla were known redox-sensitive proteins including, for instance, GAPDH and β-actin. Sulfenation of these proteins might result from higher concentrations of ROS in medulla tissue of SHR kidney and reflect a form of redox-regulation that is typical for the physiological conditions under hypertension (311).

c. Other animals. The blue mussel *Mytilus edulis* is a model for studying the ecotoxic effects of harmful compounds. To identify key proteins involved in oxidative stress response, extracts of gill proteins from *M. edulis* mussels treated with menadione (Fig. 14) have been analyzed for carbonylation and disulfide formation (226). It was found that disulfide proteins associated with actin and PDI were preferentially carbonylated. The PDI-associated proteins were isolated by immunoprecipation and their thiol oxidation status was assayed by labeling using IAF (Fig. 3D). The IAF-labeled proteins were subsequently separated by 2-DE and analyzed by LC-MS/MS. The formation of intermolecular disulfides was detected for PDI, and the cytoskeleton proteins tubulin and gelsondin. In addition, the disulfide forming proteins included the glycolytic enzyme enolase, and glutathione-S-transferase (GST) pi (226).

2. Fungi. Nearly all studies on disulfide proteomes in fungi have been carried out in baker's yeast. In an early study, Trx affinity chromatography was employed in a screening for

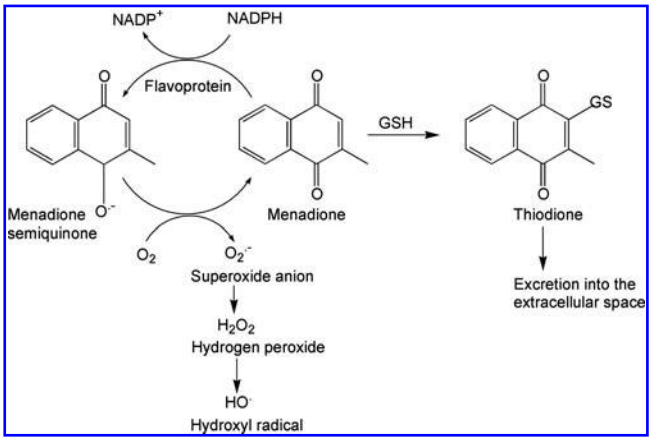

FIG. 14. Depletion of cellular thiols by menadione. The diquinone menadione can permeate the cell membrane and disturbs the intracellular redox status by generating ROS. The action of menadione as oxidant can deplete the cellular pool of protein thiols in four ways: (i) the ROS produced by menadione can initiate disulfide formation in proteins; (ii) menadione can deplete the cellular thiol pool by formation of oxidized glutathione and protein glutathionylation; (iii) menadione can also directly react with glutathione to form thiomedone that in turn is exported to detoxify the cells; and (iv) menadione can arylate proteins on their thiol groups (23, 167, 225). The mechanism of ROS production by menadione involves a semiquinone that is produced through the reduction of menadione by a flavoprotein, such as NADPH cytochrome P450 reductase (308).

new yeast target proteins *in vivo*, though the bait was actually a plant Trx. The Arabidopsis cytosolic Trx *h*3 lacking its second active site-cysteine was expressed heterologously in a ΔTrx yeast mutant (316). This attempt led to the discovery of only one target protein, YLR109, which turned out to be the yeast member of a new family of Type II Prx.

A recent study indicated that as much as about one-fourth of the surface exposed cysteines of the yeast proteome might be either functional sites that are regulated by oxidoreductases, or participate in structural disulfides and metal binding (216).

Protein S-thiolation in yeast under oxidative stress conditions has been studied by labeling with [^{35}S]cysteine in the presence of 2 m*M* hydrogen peroxide (292). Cycloheximide was added to prevent cytosolic protein synthesis and therefore radioactive incorporation into proteins was exclusively post-translational. Among the 15 radioactively labeled proteins identified, the four glycolytic enzymes, fructose bisphosphate aldolase (FBA), GAPDH, TPI, and enolase, were found. Further, the translation elongation factors EF-1α and EF-1β, a translation initiation factor, and a small ribosomal subunit were identified, indicating a role of oxidative cysteine modifications in protein synthesis (292).

Some years later, Le Moan *et al.* (187) set out to challenge the dogma that disulfides are not to be found in the highly reducing cytoplasm of unstressed yeast cells. To this end, they investigated the *in vivo* disulfide proteome in yeast using a standard procedure involving acid quenching, blocking sulfhydryls with IAM under denaturing conditions, and subsequent reduction of disulfides and sulfenic acids with DTT. Proteins containing newly formed cysteine thiols were then isolated by labeling with biotin-HPDP (Fig. 5B) and further purified using streptavidin-Sepharose. Newly formed thiols were also labeled with [^{14}C]NEM, instead of biotin-HPDP, to determine the relative levels of protein disulfides. The proteins eluted from the streptavidin-Sepharose matrix, as well as the proteins alkylated with radioactive NEM, were subjected to gel-based separation using isoelectric focusing/SDS-PAGE. In this manner, yeast cultures grown under normal aerobic conditions yielded 64 identified disulfide-containing proteins, 57 of which were cytoplasmic. Hence, it may be concluded that thiol oxidation does occur in the yeast cytosol under normal growth conditions without the imposition of any particular stress. The proteins identified in this study were sorted into several categories, including ROS scavengers and chaperones, carbohydrate and energy metabolism enzymes, amino acid biosynthetic enzymes, and protein translation factors. The largest group of proteins with oxidized cysteines that were identified in this study comprised the carbohydrate and energy metabolism enzymes, where the glycolytic GAPDH was particularly prominent (187).

To test the influence of molecular oxygen on disulfide formation, yeast cells have been grown anaerobically for a few hours before analysis (187). This treatment resulted in a dramatic decrease in the content of cellular protein disulfides, which was determined as being one-tenth that found under aerobic control conditions. Thus, cellular thiol oxidation depends to a great extent on the presence of molecular oxygen. In contrast, a brief exposure to 1 m*M* hydrogen peroxide under aerobic conditions affected only the redox state of a limited number of proteins that included, for instance, the Prx and GAPDH. These proteins were more oxidized, or even over oxidized to sulfinic acid forms. However, no new disulfide-containing proteins were detectable after peroxide treatment. A yeast triple-knockout mutant lacking the cytosolic Trx, Trx1, and Trx2, and the Trx reductase, Trr1, displayed strikingly high levels of disulfides. Interestingly, this mutant, devoid of its cytosolic Trx pathway, showed increased thiol oxidation not only of the three cytosolic Prx, but also of the mitochondrial Prx1. In addition, the glutathione peroxidase Gpx2, the methionine sulfoxide reductase Mxr1, and the Cu/Zn SOD1 were substantially more oxidized. This means that the Trx pathway is the probable physiological source of reducing equivalents for many of these ROS-detoxifying enzymes (187).

A recent study has addressed the reversible thiol oxidation in yeast under oxidative stress using a gel-free "shotgun" variant of disulfide proteomics (227). The aim of this study was to search for protein cysteines, which are protected from irreversible oxidation during oxidative stress by reversible thiol modifications. Thus, yeast cells treated with 10 m*M* hydrogen peroxide for 1 h, and untreated control cells, were lysed in the presence of NEM under denaturing conditions to block protein thiols. After reduction with DTT, newly formed thiols were labeled with biotin-HPDP (Fig. 5B) as described elsewhere (187). However, instead of purifying intact proteins, samples were digested directly with trypsin and the biotinylated tryptic fragments were isolated using a streptavidin-Sepharose resin and analyzed by LC-ESI-MS/MS (227). As a result, 27 proteins containing reversibly oxidized thiols were reproducibly identified. Under control conditions, the glycolytic enzymes GAPDH and 3-phosphoglycerate kinase were found, whereas under oxidative stress the pyruvate kinase was detected. FBA and TPI were found to harbor oxidized cysteines under both oxidative stress and control conditions. The TCA cycle enzymes aconitase and isocitrate dehydrogenase were only identified after peroxide-induced oxidative stress (227). An interesting hypothesis was put forward to explain the advantage of reversible thiol oxidation of glycolytic and TCA cycle enzymes. The fact that these enzymes, as well as others identified in the same study, are commonly inactivated by peroxide treatment led the authors to suggest that reversible inactivation by disulfide formation during oxidative stress might divert glucose from glycolysis and further metabolism toward the pentose phosphate pathway. This, in turn, would serve to replenish the NADPH consumed by the Trx pathway for reduction of ROS scavenging enzymes, such as Prx (227).

3. Plants. Trx and Grx are found in all kingdoms of life ranging from archeae to mammals. However, plants are particular in that they have a considerably higher number of Trx and Grx than other organisms. More than 40 Trx genes have been identified in Arabidopsis and in poplar (50, 229), which highlights the impact of thiol-mediated redox control in higher plants. Disulfide proteomics in plants includes two major research lines that have addressed (i) the functional roles of disulfides in seed development, and (ii) the regulation of chloroplast metabolic pathways by photosynthesis. Detailed overviews are given in recent reviews dealing with chloroplasts (186, 198, 236, 281) and seeds (289, 341). Other studies of the plant disulfide proteome and its redox modifications are still rare. However, there are many excellent studies that have addressed the role of thiol-dependent

regulation for the functioning of individual proteins (66, 80, 119, 136, 145, 174, 237, 240, 245). This section focuses on studies of disulfide proteomes in seeds, leaves and roots of green plants; it also gives a brief account of S-nitrosylation and glutathionylation in plant cells. The disulfide proteomes of chloroplasts, amyloplasts and plant mitochondria are covered in the section about organelles.

a. Seeds. Studies of the disulfide proteome have addressed changes occurring in seeds of cereals and other plants during grain filling, maturation, and germination. Trx has been an important tool used to define seed disulfide proteomes and their functions. The time-dependent changes of the oxidation state of cysteines in seed proteins suggest that disulfides are molecular switches that are used to time principal steps during different stages of seed development (83, 339, 341).

An early approach to detect Trx targets in seeds *in vitro* involved a combination of diagonal 2-DE and thiol labeling using the fluorescent dye mBBr (Fig. 3B). This technique enabled the detection of 20 targets of Trx in peanut seeds, including two novel targets that were related to desiccation and seed maturation (338). Using the same method, 23 proteins from wheat endosperm were linked to regulation by Trx *h*. The functions of these targets indicated a regulatory function of Trx *h* during the time-course of seed development (325). Consistent with a role of Trx *h* in timing, a study in barley seeds showed that the redox state of Trx *h* target proteins displayed clear changes during the course of seed development (222). Disulfide reduction by Trx also increased the solubility of the methanol-insoluble protein fraction of wheat endosperm, which suggested that the reduction of disulfides might facilitate the degradation of storage proteins during seed germination (327). This finding was consistent with the observation that the degradation of storage proteins in germinating rice seeds was accompanied by a reduction of their disulfides (337). Further support for a regulatory role of disulfides in proteolysis comes from a study of the disulfide proteome of rice bran extracts (340). Three fragments of the embryo-specific protein 2 (ESP2) displayed redox-dependent proteolysis, which appeared to be the result of two independent functions of Trx. First, reduced Trx activated a cysteine protease responsible for degradation of the ESP2 fragments; second, the direct reduction of the ESP2 fragments by Trx led to their unfolding and, hence, facilitated complete degradation (340).

Labeling with the fluorescent dye Cy5-maleimide (Fig. 4E) instead of mBBr (Fig. 3B) increased the sensitivity of thiol detection (206). This technique allowed the detection of 16 putative targets of HvTrx1 and HvTrx2 in germinated barley seeds and provided evidence that Trx controls the regulation of defense proteins, including α-amylase/trypsin inhibitors, the α-amylase/subtilisin inhibitor BASI, chitinase isoenzymes, and lipid transfer protein LPT1 (206). Large-scale profiling of seed protein disulfides became feasible when 2-DE-based analysis of disulfides was combined with Trx affinity chromatography using monocysteinic wheat Trx *h* as a ligand (326). The combination of these techniques enabled the identification of more than 60 potential Trx targets in the endosperm of wheat seeds and provided an overview of the biological pathways that were likely to be regulated by Trx. The putative and known functions of the target proteins suggested that Trx was involved in the control of carbohydrate metabolism and nitrogen metabolism. In addition, the target proteins had functions in cell structure and division, and in the synthesis and degradation of proteins. Finally, the targets of Trx *h* were involved in protein storage and stress-related reactions. The profile of Trx targets displayed clear differences during the time-course of seed development. The disulfide proteome of young wheat seeds (10 days postanthesis) highlighted the impact of cell division and protein synthesis, whereas storage proteins and enzymes involved in protein degradation were characteristic of mature seeds (36 days postanthesis) (326).

A more detailed analysis of the role of individual disulfide bonds became possible when differential thiol alkylation was monitored by MALDI-MS (207). This technique revealed that barley Trx *h*1 reduces disulfides selectively and recognizes specific classes of disulfides. For instance, the α-amylase/subtilisin inhibitor BASI of barley has two disulfides, of which Cys144-Cys148 was reduced to a much greater extent by Trx *h*1 than Cys43-Cys90 (207). Quantitative mass spectrometry using redox-ICAT (287, 288) further improved sensitivity and accuracy in the detection of disulfides that were targeted by Trx *h*1 (109). More than 100 disulfide targets of Trx *h*1 were quantified in embryos of germinated barley seeds, indicating functions of Trx *h*1 in redox control, metabolism, translation, protein folding, and development. A remarkable result was the identification of a seryl-tRNA synthetase and 19 ribosomal proteins. These proteins had not been found in earlier 2-DE-based studies and highlighted the potential impact of Trx-mediated regulation in translation (109).

The impact of proteins containing redox-active cysteines in response to environmental changes was also highlighted by the protein expression profiles of wheat seeds under drought (110). Although this study did not address changes in the redox state of individual disulfide proteins, it clearly showed the significance of Trx *h* that was upregulated in the drought-tolerant variety Khazar-1 (110). It is also worth noting that the disulfide proteome of the seeds of the cereal wheat, which is a monocotyledon, revealed many similarities to the Trx targets found in the seeds of the legume *Medicago truncatula*, which is a dicotyledon (4). However, the disulfide proteome of *M. truncatula* seeds also revealed many new members that included enzymes involved in the synthesis of vitamins, DNA and cell wall proteins, metal-binding proteins, and proteins involved in response to hypoxia (4).

b. Leaves and roots. Early studies of the cytosolic disulfide proteome of green plants employed Trx affinity chromatography to gain insights into the functions of the cytosolic Trx *h* (210, 211, 334). Screening *in vitro* for targets of Trx *h*1 in cell lysates from etiolated Arabidopsis seedlings resulted in the identification of 15 putative Trx targets, of which four were located in chloroplasts (334). The target proteins were sorted into three groups. The first groups of targets had functions in stress response and included type II Prx and the ascorbate peroxidase APX1. The second group participated in protein synthesis, folding, and degradation. The third group comprised metabolic enzymes, such as alcohol dehydrogenase and GAPDH (334). A more comprehensive analysis of targets of Trx *h*3 has been accomplished by a combination of complementary methods (210, 211). Protein extracts from Arabidopsis leaves were depleted of RuBisCo before target screening to improve the sensitivity of the method. The ana-

lytical tools to detect targets of Trx h3 included differential alkylation, radioactive labeling combined with 2-DE and autoradiography, and Trx affinity chromatography. More than 40 target proteins were found that had subcellular locations in the cytosol, mitochondria, and chloroplasts. The Trx *h*3 targets covered a broad range of cellular functions, such as photosynthesis, protein synthesis, folding, and degradation, and the biosynthesis of amino acids and pigments. In addition, the targets of Trx *h*3 also had functions in the oxidative stress response and in defense responses against pathogens and herbivores (210, 211). There are few examples of plant disulfide proteomes unrelated to Trx activity, but in two global studies of the Arabidopsis leaf disulfide proteome *in vivo* more than 80 disulfide-containing proteins were detected (5, 177). However, no attempts were made to link these proteins to dynamic changes of the cysteine redox state.

The targets of the chloroplastic Trx of Arabidopsis have been addressed in many proteome studies (12, 13, 15, 19, 239, 241). However, it is now known that the genes encoding plastidal Trx are also expressed in nonphotosynthetic tissues (55, 310). As for plastidal Trx *y*, two isoforms are known that display different gene expression patterns. Trx *y1* mRNA is found in seeds and roots, whereas Trx *y2* is expressed in leaves (55). A recent study revealed 72 putative targets of Trx *y* in the roots of Arabidopsis, of which 24 were not previously known (212). The target proteins contained many metabolic proteins, including members of the biosynthesis of isoprenoids and phenylpropanoids. A large group of proteins indicated Trx to have an important defensive role against oxidative stress in roots tissues (212).

Apart from studies on disulfide proteomes using Trx as a tool, the related oxidoreductase Grx has also been employed for screening plant proteins. A comprehensive study of *in vitro* targets for Grx in a variety of plant tissues and subcellular compartments has been performed using a monocysteinic poplar GrxC4 as affinity ligand for Grx affinity chromatography (273). In this study, whole cell soluble extracts from poplar and Arabidopsis leaves, pea chloroplast stroma, and mitochondrial protein extracts from potato tubers and Arabidopsis leaves were analyzed. The vast majority of the proteins identified had already been found as Trx targets in other studies on plant mitochondria (14), plant chloroplasts (12), whole cell extracts from leaves (334), and the green alga *Chlamydomonas reinhardtii* (185). It may therefore be concluded that, *in vitro*, there is no obvious difference in substrate specificities between Trx and Grx. A similar lack of specificity had already been observed between two different types of Trx, the chloroplast Trx *m* and Trx *f* (12).

An investigation into disulfide switches in jasmonate signaling is one of the few studies that has addressed the role of oxidative cysteine modifications in another signal transduction pathway (6). Jasmonates control gene expression in response to wounding and pathogenesis and are important for plant fertility (98). The treatment of Arabidopsis seedlings using methyl jasmonate (MeJA) caused the production of hydrogen peroxide in leaves and roots, and changed both the protein expression levels and the redox state of disulfide proteomes. Roots and shoots clearly displayed different responses to the jasmonate treatment in their metabolic pathways. Proteins involved in defense mechanisms represented the largest group of proteins that were affected in either their cysteine redox state or in their expression levels. Thus, in shoots, the oxidation of β-glucosidase PYK10 and a coronatine/jasmonic acid-induced protein were observed. Roots displayed oxidation of a trypsin protease inhibitor and oxidation of major latex-protein related/RNA-binding protein 1. A noteworthy detail is the identification of 12 redox-sensitive cysteine residues within the proteins that were found in this study (6).

c. Plant cell cultures. Arabidopsis cell cultures have served as a model system for studying the functions of S-glutathionylation and S-nitrosylation in plants. There is evidence that glutathione regulates many cellular processes in plants, including flowering, cell death, and pathogen resistance (247). However, compared to mammals, relatively little is known about the S-glutathionylation of plant proteins and the regulatory functions of this post-translational modification (97). When cell suspensions generated from Arabidopsis seedlings were incubated with biotinylated GSH ethylester, followed by incubation with hydrogen peroxide, glutathionylation of numerous proteins was observed (147). The glutathionylated proteins were purified chromatographically and further analysis by N-terminal microsequencing revealed that two of the glutathionylated proteins were cytosolic TPI and plastidic FBA (isoform1), which are enzymes of the carbohydrate metabolism of the cytoplasm and the chloroplast, respectively. Treatment with GSSG inactivates recombinant TPI in a dose-dependent manner (147). By contrast, chloroplastic FBA1 is activated by GSH in a pH-dependent way, but inactivated by DTT and Trx (224, 247), which is noteworthy, as many other enzymes of the Calvin Benson cycle are activated by Trx (281). As identification of S-glutathionylation in cultured Arabidopsis cells *in vivo* is compromised by proteolysis, an *in-vitro* strategy was used in another study, in which crude extracts from Arabidopsis cell cultures were treated with GSSG-biotin after reduction with DTT (71). The thiolated proteins were then purified by streptavidin affinity chromatography and analyzed by 2-DE combined with MALDI-MS. Compared to only nine thiolated proteins being identified after *in vivo* S-glutathionylation, the *in vitro* experiment resulted in the identification of 79 proteins that were either thiolated or formed stable complexes with thiolated proteins. The two largest groups of potential thiolated proteins had functions in metabolism and protein turnover. Sixteen subunits of the proteasome and four other proteases were identified. Nineteen metabolic enzymes were detected that had functions in amino acid and carbohydrate metabolism and included several members of the TCA cycle. In addition, six members of the GST superfamily were found, as well as a group of proteins with different functions, among which were the oxidative defense proteins 2-Cys Prx, Grx, monodehydroascobate reductase, and Hsp60. Observations of the *in vitro* S-glutathionylation of recombinant S-glutathionylation targets suggested two different mechanisms for the reaction with GSSG-biotin. Dehydroascorbate reductase and a ζ-class glutathione transferase were able to undergo spontaneous glutathionylation in the presence of GSSG-biotin. By contrast, alcohol dehydrogenase and methionine synthase required the presence of a cofactor from the crude protein extracts of the cell suspension (71).

The functions of S-nitrosothiols in signal transduction, and their role as modulators of enzyme activities, have become fields of increasing interest (124), and the importance of NO

signals in plants has also been recognized. NO appears to be a universal signaling molecule in plants and plays an important role in pathogen defense (65, 200). However, little is known about how S-nitrosylation regulates the function of individual plant proteins. To define a map of S-nitrosylation in Arabidopsis, Lindermayr *et al.* (199) applied the biotin switch method to detect S-nitrosylated proteins in total extracts from cell cultures and from NO-treated plants. A list of 38 selected candidates for S-nitrosylation was reported, which included proteins with functions in metabolism and photosynthesis, the cytoskeleton, and stress response. This dataset contained glycolytic enzymes, TCA cycle enzymes, and the α- and β-subunits of ATP synthase. The list of S-nitrosylated proteins also included six translation elongation and translation initiation factors. The cytoskeleton was represented by α- and β-tubulin, actin and annexin. Moreover, the chaperone Hsp90, a GST, and Cu/Zn SOD were detected. Finally, the candidates for S-nitrosylation included two putative Prx, a putative Grx, and a putative glutathione peroxidase. Biochemical analysis showed that the activity of GAPDH in extracts from cell cultures was inhibited in the presence of GSNO and sodium nitroprusside, and restored by DTT (199). Five of these proteins, including GAPDH, PrxIIE, and RuBisCo, were also detected recently as being targets for S-nitrosylation in the hypersensitive response to pathogen attack (272). The hypersensitive response is a form of controlled cell death at the site of an infection that is used to prevent the pathogen from spreading. In the study of the hypersensitive response to pathogen attack mentioned above (272), Arabidopsis leaves were infiltrated with *Pseudomonas syringae* pv. tomato (Pst), and leaves of a control group were infiltrated with Pst carrying the *avrB* avirulence gene (Pst avrB). S-nitrosylated proteins from infected leaves in controls were purified using the biotin-switch method and compared by 2-DE. A set of 16 proteins was identified that displayed differential S-nitrosylation during hypersensitive response. These proteins included new S-nitrosylation targets such as phosphoribulokinase and RNA-binding proteins, and known targets such as the large subunit of RuBisCo, the glycolytic enzymes TPI and GAPDH, and mitochondrial MDH (272).

4. Algae. The unicellular alga *Chlamydomonas reinhardtii* has been examined for Trx target proteins *in vitro* using Trx affinity chromatography with a monocysteinic version of the cytosolic Trx *h1* as ligand (185). Since total cellular extracts were applied on the affinity columns, the 55 Trx targets identified originated from several compartments, including plastids, mitochondria, nuclei, and vacuoles. As judged by spot intensities in the Coomassie-stained 2-DE gels, the most abundant Trx targets were the RuBisCo large and small subunits, enolase, aconitase, chloroplastic, and cytosolic forms of 2-Cys Prx and the chloroplastic glutamine synthetase. Thus, the processes linked to putative redox regulation in this organism comprise the Calvin-Benson cycle, glycolysis, the TCA cycle, oxidative stress response, and nitrogen metabolism among others (185). A complementary study addressed *in vivo* glutathionylation of proteins in C. *reinhardtii* (232). To label glutathionylated proteins *in vivo*, cytosolic and organellar protein synthesis were first inhibited by cycloheximide and chloramphenicol, respectively. The cells were incubated with [^{35}S]cysteine, which was incorporated into newly synthesized glutathione. Subsequently, the cells were treated with diamide (Fig. 13) to induce glutathionylation. Glutathionylation was detected by autoradiography combined with MALDI-MS. In total, 25 glutathionylated proteins were identified that had functions in photosynthesis, and in different metabolic pathways, including amino acid synthesis, ATP synthesis, acetate assimilation, and the pentose phosphate pathway. The largest group of glutathionylated proteins that were found included six chaperones. 2-Cys Prx, two RNA-binding proteins, and EF-Tu were also found. Although 18 of the 25 glutathione-targets had also been identified as Trx- or Grx targets in studies on other organisms, there was relatively little overlap with the previously identified Trx-targets of *C. reinhardtii* (185). Only three proteins found among the glutathionylated proteins were also found among the Trx targets. These proteins included isocitrate lysase of the glyoxylate cycle, and as glutathionylation inhibited its activity *in vitro*, a regulatory role for protein glutathionylation in *C. reinhardtii* was suggested (22, 232).

B. Subcellular disulfide proteomes

1. Mammalian mitochondria. Mitochondria as ROS producers have a remarkable impact on the redox biology of the entire cell and, surprisingly, the mitochondrial activity has been shown to affect disulfide formation in cell surface proteins (336). The effects of ROS on endogenous mitochondrial proteomes, regarding both protein levels and post-translational modifications, have attracted interest in the context of disease and alcohol abuse (9). For more than a decade mitochondria have been known to possess their own NADPH Trx reductase (NTR)/Trx system (234). The mammalian mitochondria-specific Trx-2 is an essential protein involved in apoptosis (306) and many other mitochondrial proteins with reactive cysteine thiols have recently been linked to apoptosis (328). Mitochondrial proteins containing redox-modified cysteines are occasionally identified in proteomic studies of whole cells, notably from heart tissues (49, 96, 276, 288) and stressed yeast cells (227). Nevertheless, one might expect higher sensitivity and better coverage if the studies were performed directly on the mitochondrial proteomes. However, during the time-consuming isolation of organelles, changes of the physiological cysteine modifications may take place and under aerobic conditions new cysteine oxidation might occur after cell lysis.

In an attempt to circumvent some of these pitfalls, changes of the cysteine redox state induced by endogenous ROS production in isolated mitochondria has been studied using the 2-D-DIGE technique combined with the cysteine-specific saturation dyes Cy3- and Cy5-maleimde (Fig. 4D, E) (143, 144). The cysteine redox states of eight proteins from purified rat mitochondria were found to change drastically in response to endogenous ROS, which were induced by stimulation of the reverse electron transport and by activation of complex III. These proteins included a pyruvate dehydrogenase kinase and six enzymes participating in the β-oxidation of fatty acids. Indeed, during the endogenous production of ROS induced by reverse electron transport, the cysteine redox status of these proteins not only changed, but their activities were also inhibited. This finding indicates that changes in the redox state of protein cysteines due to endogenous ROS production in isolated mitochondria are related to the activities of the corresponding proteins (143).

The reactive thiols of mitochondrial proteins and their impact on apoptosis were investigated in a study of the thiol proteome of mitochondria of human embryonic kidney 293 cells (HEK293) (328). The question of whether thiol alkylation by reactive electrophiles might induce apoptosis was investigated in that study by using the thiol probes IAB (Fig. 3C) and BMCC (Fig. 4C). When purified mitochondria from HEK293 cells were incubated with IAB and BMCC respectively, IAB induced apoptosis, whereas BMCC did not. This observation is noteworthy for two reasons. First, the thiol probe used can be physiologically active and considerably disturb the biological system that is studied. Second, the only difference between IAB and BMCC is the reactive group. While IAB has an iodoacetamide group, BMCC has a maleimidoethylcyclohexane group. However, the proteins detected with IAB and BMCC, respectively, were largely different. The overlap included only 362 of the 809 proteins identified in this study. In the IAB dataset, 44 apoptosis-related proteins were identified that included subunits of the permeability transition core complex, the TOM and TIM translocases for protein import, heat shock proteins, and Prx, Trx, and Trx reductases (328).

The new compound, IBTP (Fig. 3F), has been synthesized to label mitochondrial protein cysteine thiols in intact cells (194). This lipophilic cation was able to penetrate the membranes of intact cells and accumulated more than 100-fold in the mitochondrial matrix. IBTP alkylates free cysteine thiols and is unreactive toward oxidized cysteines. To detect IBTP labeling, an antibody was raised against the triphenylphosphonium (TPP) moiety and used for western blot analysis. Labeling of mitochondrial protein thiols has thus been shown in human fibroblasts, Jurkat cells, and bovine aortic endothelial cells. Additions of diamide (Fig. 13) or tert-butyl hydroperoxide in the micromolar range were found to significantly reduce IBTP labeling (194). This method has been applied in studies on the effect of chronic alcohol consumption on the redox state of protein cysteines in hepatic mitochondria of ethanol-fed rats (315). Among the few liver mitochondrial proteins observed using the anti-TPP antibody, an aldehyde dehydrogenase and the glucose-regulated protein 78, GRP78, were significantly less labeled with IBTP in mitochondria from ethanol-fed rats. The authors therefore concluded that alcohol consumption might lead to the oxidation of cysteines in proteins from liver mitochondria (315).

A mass tagging approach using stable isotope-coded IBTP reagents, termed IBTP-d_0 and IBTP-d_{15}, has been designed and tested on isolated rat heart mitochondria under non-denaturing conditions (217). The principle was similar to that of the ICAT method, and the heavy IBTP-d_{15} reagent was synthesized using deuterium-substituted triphenylphosphine, whereas the light IBTP-d_0 was synthesized using normal phosphine. The reproducibility of the method was tested by labeling mitochondrial preparations in parallel with heavy and light IBTP reagents. However, the goal of the study was merely to evaluate the technical aspects of the method; hence, no attempts to study cysteine modifications in a proteome-wide scale were made (217).

Human hepatoma HepG2 cell cultures have been used as a model to study the influence of ethanol on oxidative cysteine modifications in mitochondria of human liver cells (302). The major isoform of the ethanol-inducible cytochrome P450, CYP2E1, has been implicated in the generation of ROS during ethanol metabolism. A HepG2 cell line with stable transfection of human CYP2E1, termed E47 HepG2, and a control cell line, C34 HepG2, were therefore compared in a series of experiments in which the cells were exposed to 100 m*M* ethanol for up to 8 h before isolation of mitochondria and analysis of the oxidation of protein thiols. The proteomic strategy involved blocking free thiols with NEM, reduction with DTT, labeling of the newly formed thiols with biotin-*N*-maleimide (biotin-NM), and purification of biotinylated proteins using streptavidin-agarose. Ethanol exposure increased biotin-NM labeling of mitochondrial HepG2 proteins—an increase that was particularly pronounced in the CYP2E1-transformed E47 cell line (302). The relatively few purified proteins were resolved by 2-DE and seven of these were identified either through PMF or LC-MS/MS analysis. The most abundant protein containing oxidized cysteines was found to be the chaperone Hsp60; others included the aldehyde dehydrogenases ALDH2 and ALDH5 and glucose-regulated protein 78.

Whereas protein tyrosine nitration in mitochondria has been examined in mammals and yeast (27, 202), the occurrence of protein *S*-nitrosylation in these organelles has not been studied to the same extent. However, nitrosylation of protein cysteines in isolated rat liver mitochondria has been investigated using a typical biotin-switch approach, including ascorbate-mediated reduction and labeling with biotin-HPDP (Fig. 5B) (89). The incubation of isolated mitochondria or mitochondrial extracts with GSNO as nitric oxide donor resulted in the S-nitrosylation of several proteins, even under anaerobic conditions. Two of the six nitrosylated proteins identified were catalase and dihydrolipoamide dehydrogenase, which is a subunit of the pyruvate dehydrogenase supercomplex. Since the subcellular localization of catalase is controversial, the mitochondria were subfractionated and the catalase was proven to be localized in the outer mitochondrial membrane, whereas the dihydrolipoamide dehydrogenase was found in the inner membrane fraction (89).

2. Plant mitochondria. The proteome of plant mitochondria is predicted to consist of 2000–3000 proteins (233), and various proteome studies in Arabidopsis have resulted in the detection of 517 mitochondrial proteins (117). However, mitochondrial disulfide proteomes of plants and algae have received less attention than those of chloroplasts. Nevertheless, mitochondria in photosynthetic eukaryotes contain a complete NTR/Trx system, and it is anticipated that redox regulation involving changes in the cysteine redox state should occur in these organelles (11, 169). The first study of disulfide proteomes in plant mitochondria comprised a comprehensive analysis of Trx target proteins in mitochondria isolated from pea and spinach leaves, and potato tubers (14). Trx affinity chromatography and mBBr-fluorescence electrophoresis were applied in series, thus selecting the proteins susceptible to identification by both procedures. After Trx affinity chromatography on either monocysteinic Trx *m* or Trx *h*, the target proteins eluted with DTT were extensively dialyzed to remove this reductant and to allow disulfides to form again. Thereafter, the remaining free thiols were blocked with NEM and samples were incubated with *E. coli* NTR and Trx in the presence of NADPH. Finally, the reduced proteins were detected by 2-D mBBr-fluorescence electrophoresis and identified by ESI-MS/MS (14). The Trx target proteomes were highly similar, regardless of whether Trx *m* or Trx *h* were used as ligands in the Trx

affinity chromatography step. Among the most conspicuous results were the identifications of seven enzymes belonging to the TCA cycle, catalase, type II Prx, aldehyde dehydrogenase, alcohol dehydrogenase, Hsp60, and Hsp70. The relative amounts of the target protein clearly differed between mitochondria from leaves and potato tubers. Some Trx targets were only found in mitochondria from potato tuber, *i.e.*, nonphotosynthetic tissue. Tuber-specific targets included several proteins involved in electron transport: two subunits of cytochrome c oxidase (Complex IV), one subunit of NADH-ubiquinone oxidoreductase (Complex I), and one subunit of ubiquinol-cytochrome c reductase (Complex III). Finally, four target proteins involved in amino acid metabolism were found exclusively in tuber, and not in leaf mitochondria (14).

A later study of the plant mitochondrial disulfide proteome was performed using mitochondria isolated from *Arabidopsis thaliana* cell cultures (321). These proteins were analyzed by nonreducing/reducing diagonal electrophoresis. Proteins migrating below the diagonal were deduced to participate in intermolecular disulfides and included α-, β-, and γ-subunits of the ATP synthase, alternative oxidase, and the adenine nucleotide translocator, ANT. Interestingly, citrate synthase, which is one of the few enzymes from the TCA-cycle that were not identified as Trx targets in the previous study (14), was also found to migrate below the diagonal. Among the few proteins migrating just above the diagonal, and so assumed to possess intramolecular disulfides, were subunit E1 β of the pyruvate dehydrogenase complex, and adenylate kinase (321), both of which were previously identified as Trx targets (14). The phosphate carrier PiC was also reported for the first time as a member of a plant mitochondrial disulfide proteome (321), but taken together the results obtained by diagonal electrophoresis did not shed much new light on the issue.

While there is evidence that S-nitrosylation serves a regulatory function in animal mitochondria (89), little is known about the role of S-nitrosylation in plant mitochondria. A recent study demonstrated, however, that NO probably plays a role in the regulation of the mitochondrial part of photorespiration in Arabidopsis (250). S-nitrosylated proteins were purified using the biotin switch method after incubation of partially purified mitochondria from Arabidopsis with GSNO and GSH. Further analysis of the S-nitrosylated proteins by SDS-PAGE combined with LC-MS/MS resulted in the identification of 11 proteins that included the H, P1, and P2 subunits of Gly decarboxylase and serine hydroxymethyltransferase of the mitochondrial photorespiratory pathway. The activity of Gly decarboxylase in purified mitochondria was inhibited in the presence of GSNO and increased in the presence of DTT. GSNO also inhibited the activity of Gly decarboxylase in leaf discs, whereas incubation of the leaf discs with DTT after the GSNO treatment restored the activity of Gly decarboxylase. However, mass spectrometry analysis of partially purified P-protein showed the presence of S-glutathionylated peptides but was not able to detect any S-nitrosylation. Therefore, as a control, partially purified P-protein was nitrosylated using sodium nitroprusside that is not able to induce S-glutathionylation. As a result of this treatment the Gly decarboxylating activity of the P-protein was inhibited, supporting the hypothesis that S-nitrosylation of the P-protein inactivates mitochondrial Gly decarboxylase (250).

3. Chloroplasts. The ferredoxin/Trx system of chloroplasts enables photosynthetic organisms to regulate biosynthetic pathways in response to their photosynthetic activity. In the light, ferredoxin receives electrons from photosynthetic electron transport, and the ferredoxin Trx reductase reduces Trx, which in turn reduces its target proteins. The reduction by Trx may induce a conformational change that turns the enzymatic activities of the target proteins on or off (38, 281). As a rule, enzymes belonging to biosynthetic pathways become activated by Trx-catalyzed reduction in the light. This group includes, for example, the classical Trx targets MDH, the ATP synthase γ-subunit, and several enzymes of the Calvin-Benson cycle for carbon dioxide fixation, such as fructose-1,6-bisphosphatase and sedulohеptulose-1,7-bisphosphatase. In contrast, other proteins may be deactivated by Trx in the light and activated through cysteine oxidation in the dark. A well-known example is glucose-6-phosphate dehydrogenase that plays a key role in the oxidative pentose phosphate pathway (245, 281).

For a long time, it was generally believed that the chloroplastic ferredoxin/Trx system includes two types of Trx, Trx *m* and Trx *f*, which control the enzymatic activities of a relatively small group of stromal enzymes. During the last decade this picture has changed. Arabidopsis has more than 20 known and putative plastid Trx (229). Currently, there is evidence that 15 Trx of Arabidopsis are targeted to chloroplasts. All of these Trx, except the thylakoid-bound Hcf164, have been found in the stroma (45). The functions of chloroplast Trx have revealed a higher level of complexity than anticipated and the number of target proteins has increased considerably. Proteome studies of plastid Trx targets in plants and in the alga *Chlamydomonas reinhardtii* succesfully identified more than 130 potential Trx targets (198, 236).

Using classical diagonal electrophoresis, 49 disulfide proteins have been identified in chloroplast fractions from Arabidopsis (301). However, Trx affinity chromatography using monocysteinic Trx as ligands has played a key role in unraveling the Trx targets of the individual chloroplast compartments (12, 13, 19, 112, 239, 241). The initial studies of novel targets of stromal Trx *m* and *f* revealed that Trx control was an intrinsic feature of many metabolic pathways of chloroplasts, including not only the Calvin-Benson cycle (Fig. 17), but also the biosynthesis of tetrapyrroles and vitamins. Trx targets have also been found in the pathways for the assimilation of sulfur and nitrogen. In addition, the novel stromal target proteins have been found to have functions in transcription and translation, protein folding and assembly, and in oxidative stress response (12, 239). Apart from studies using the canonical Trx *m* and *f*, a monocysteinic version of the Trx-like chloroplast protein CDSP32 has been employed for Trx affinity chromatography (265). One important result from these studies is that chloroplast Trx appears to regulate the activities of all enzymes of the Calvin-Benson cycle (Fig. 17) (12, 13, 15, 210, 211, 239, 265). Different lines of research have suggested that the redox-sensitive disulfide proteome of chloroplasts is not limited to the stroma, but is present in all chloroplast compartments ranging from the envelope to the thylakoid lumen (15, 19, 105, 210, 211, 241). As for the envelope, the Tic55 subunit of the translocon of the inner envelope membrane of chloroplasts (Tic) has been identified as a potential target of Trx *m* and *f* (19). Trx-dependent redox control of protein import into chloroplasts has also been suggested by the finding that the stromal part of the channel protein, Tic110, has a redox-active disulfide, which is reduced by Trx

in vitro (18). The disulfide proteome of the envelope of Arabidopsis also includes the pPORA translocon protein PTC52 and a pheophorbide A oxygenase (19). In summary, the known disulfide proteome of the chloroplast envelope of Arabidopsis includes four proteins. This number is low considering that about 700 proteins have been identified in the envelope of Arabidopsis chloroplasts (81). There are likely to be many more redox-sensitive disulfide proteins in the chloroplast envelope that have not yet been found, and Trx might well play a more important role in the regulation of metabolite and protein trafficking though the envelope, than is currently thought to be the case.

The thylakoid membrane, which resides in the chloroplast stroma, generates the electron current that keeps the cascade of ferredoxin, ferredoxin Trx reductase, and Trx activated. To identify redox-sensitive disulfide proteins of the thylakoid membrane, the Trx targets of thylakoids from spinach have been studied using 2-D mBBr-fluorescence electrophoresis (15). Unfortunately, the hydrophobicity of the membrane proteins restricted this approach, mainly to the detection of soluble extrinsic membrane proteins. However, the potential Trx targets of thylakoids included the intrinsic membrane proteins LHCIIb and the PsaK subunit of Photosytem I (15).

In the light, when photosynthetic electron transport occurs, the thylakoid lumen is acidified as the trans-thylakoid pH gradient is formed. Further, the oxygen evolved from the luminal side of Photosystem II creates an oxidizing environment. These conditions do not favor transmission of Trx-mediated redox signals, which depend on the formation of a thiolate anion in the Trx active site. However, not long after the proteome studies of stromal Trx targets (12, 239), evidence emerged that proteins with redox-sensitive disulfides are also present in the lumen. First, the thiol-dependent peroxidase PrxQ of Arabidopsis was found in the chloroplast lumen (159, 256). In addition, the lumenal proteins PsbO and TL17 were found in studies screening for targets *in vitro* of the cytosolic Trx *h* (210, 211). The structure of the lumenal immunophilin FKBP13 revealed two disulfides, which were experimentally proven to be reduced by Trx from *E. coli*, suggesting that this protein might undergo redox regulation (105). Taken together, these data indicate that Trx-linked signal transduction is not only a feature of the chloroplast stroma, but is present in all chloroplast compartments, including the thylakoid lumen (40). It was recently discovered that the thylakoid lumen is the chloroplast compartment with the highest percentage of potential Trx targets known so far (112). Nineteen lumenal Arabidopsis proteins, representing more than 40% of the known lumenal proteome, were found to be reduced by Trx *in vitro*. While the functions of most of these Trx targets remain elusive, some Trx interactions of the lumenal proteins indicate them to have a role in the redox regulation of the xanthophyll cycle, and a decisive role in the degradation of the extrinsic photosystem II subunit PsbO (112).

So far, the pathways leading to the reduction of the redox-sensitive disulfide proteome in the lumen are not well understood. The Trx-like Hcf164, which is anchored in the thylakoid membrane and faces the lumen (241), and the membrane-bound CcdA (242, 249) have been suggested to function in a redox cascade transporting reducing equivalents through disulfide/dithiol exchange from the chloroplast stroma to the thylakoid lumen. On the other hand, the ability of the thylakoid lumen to form disulfides has been documented (10, 309) and a homolog of the DsbA/B-like oxidoreductase found in cyanobacteria (295) might be involved in oxidative protein folding of lumenal proteins.

4. Amyloplasts. Amyloplasts are considered to be plastids that are specialized for the production of starch. Interestingly, a proteome study of amyloplasts from wheat endosperm revealed the presence of a complex metabolic network (16), and a recent evaluation of the metabolic pathways of amyloplasts from wheat endosperm has suggested an essential role for these plastids in endosperm metabolism (75). The potential impact of thiol-dependent redox control was indicated by the discovery of ferredoxin, ferredoxin-NADP reductase, and ferredoxin-Trx reductase. The presence of these proteins implied that amyloplasts possess a ferredoxin/Trx system similar to that of chloroplasts (16), since which study, conclusive evidence has been provided (17). Using 2-D mBBr fluorescence electrophoresis and Trx affinity chromatography, 42 potential Trx targets have been found in amyloplasts. The functions of these proteins include starch and sugar metabolism, amino acid metabolism, biosynthesis of lipids, and protein folding and assembly. These findings suggest that Trx might control the major metabolic pathways of amyloplasts (17). It is noteworthy that ADP-glucose pyrophosphorylase, a key enzyme in starch synthesis, has been identified in Trx- or Grx target proteomes of chloroplasts (13, 273), amyloplasts (17), and cyanobacteria (196). Recently, it was demonstrated *in vivo* that ADP-glucose pyrophosphorylase is controlled by light and sucrose through the NADPH-dependent Trx-like protein NTRC in both chloroplasts and amyloplasts (231). NTRC is exclusively localized in plastids and consists of two domains, NADPH-Trx reductase and Trx, and thus represents a functional Trx system in a single polypeptide chain (255).

C. Disulfide proteomes in prokaryotes

1. Nonphotosynthetic prokaryotes. Most of the disulfide proteomic studies in nonphotosynthetic prokaryotes have been performed on the gram-negative model bacterium *E. coli* and the gram-positive model bacterium *Bacillus subtilis*. Despite the fact that Trx was originally identified in *E. coli* in 1964 (173), Trx-dependent disulfide proteomic approaches have been developed and applied principally in photosynthetic organisms (39, 198). Although Trxs and Grxs play fundamental roles as electron donors in *E. coli*, they are largely redundant (230). While none of the *trx* genes is required for viability in *E. coli*, Trx1 is essential in several other bacteria, for example, *Rhodobacter sphaeroides*, *Bacillus subtilis*, *Anacystis nidulans*, and *Synechocystis* sp. PCC 6803 (345).

Long before the proteomics era, biochemical and genetic studies were able to describe some targets of redoxins in *E. coli*. Initially, Trx was identified as an electron donor for ribonucleotide reductase, a key enzyme in DNA synthesis (173). Grx was discovered in an *E. coli* Trx-deficient mutant as an alternative source of reducing equivalents for ribonucleotide reductase (125). Since then, various other functions have been attributed to prokaryotic Trxs and Grxs as electron donors to enzymes reducing methionine sulfoxide or participating in reductive sulfate assimilation (102). For example, Trx1, Trx2, and Grx1 donate reducing equivalents to the *E. coli* PAPS reductase, the enzyme responsible for reduction of

3′-phosphoadenylylsulfate to sulfite (192), whereas Grx2 is an efficient electron donor for arsenate reductase (ArsC), which catalyzes the reduction of arsenate to arsenite (293).

An interesting example of redox regulation that was discovered in *E. coli* before disulfide proteomics is the oxidative activation of the molecular chaperone Hsp33, which contains four conserved cysteines prone to the formation of disulfide bridges (153). Another example is the bacterial transcription factor OxyR, which serves as a peroxide-sensitive thiol-based redox sensor and controls the expression of several genes involved in the antioxidant response (8, 347). Among the genes controlled by OxyR are a peroxidase, AhpC, and its reductase, AhpF, first described in *Salmonella typhimurium* (53). AhpC belongs to the family of Prx, which catalyzes the reduction of H_2O_2 and alkyl hydroperoxides through reversible disulfide formation (258).

The first global study of proteins interacting with Trx in *E. coli* was based on a Tandem Affinity Purification (TAP) strategy, expressing a TAP-tagged version of Trx1 in *E. coli* cells with a *trxA*$^-$ knockout genetic background (165). However, it should be noted that the TAP-tagged Trx1 used in this study possessed the wild-type active site, including both cysteines, and would not therefore form stable mixed disulfides. Consequently, the target proteins isolated and identified did not necessarily interact with the Trx through thiol chemistry, but rather through electrostatic and/or hydrophobic interactions, forming multiprotein complexes. In this context, it is worth mentioning that *E. coli* Trx1 was previously found to be an essential structural subunit of the bacteriophage T7 DNA polymerase (142, 294). Nevertheless, some of the targets identified in the study using a TAP-tagged Trx (165) were found later in studies of *E. coli* using different disulfide proteome approaches (30, 182, 183).

One method used to analyze the disulfide proteome in *E. coli* involves differential thiol-trapping combined with 2-DE gel analysis, in which the cysteines that were oxidized in the cell are reduced and thereafter carbamidomethylated by radioactively labeled [^{14}C]IAM (30, 182). A quantitative thiol proteome study was carried out combining this method with ICAT chemistry (183). These approaches were used to monitor the cysteine redox status of cellular protein under normal growth conditions as well as under different oxidative stress conditions. The redox-sensitive cysteine proteome identified in *E. coli* was classified into three categories: antioxidant mechanisms, intermediary metabolism, and regulatory processes; the majority of these proteins have a role in the intermediary metabolism. GAPDH (GapA) has been detected reproducibly in these various studies. Notably, the amino acid metabolism and related processes are represented by many enzymes. Interestingly, the reversible cysteine thiol oxidation of MetE (cobalamin-independent methionine synthase) after oxidative stress has been previously shown to be responsible for the inhibition of methionine biosynthesis (131, 132). The Prx AhpC and Tpx, as well as the reductase of AhpC (AhpF), were also found in these studies. Finally, regulatory proteins, such as translation elongation factor EF-Tu, ProQ (regulator of ProP, involved in osmoregulation), and YhiF (a transcriptional regulator) and various ribosomal subunits were also identified.

In an unusual study of the reactive cysteine proteome in *E. coli*, a histidine-tagged C-terminal domain of the redox-sensitive yeast transcription factor Yap1 was expressed and used as bait *in vivo* for affinity purification of selected proteins (305). Yap1 has been described as the yeast equivalent to the bacterial OxyR in the sense that it regulates a typical H_2O_2-responsive operon (63). The activation of Yap1 occurs through intramolecular disulfide formation catalyzed by the sulfenic acid intermediate of the oxidant receptor peroxidase 1 (Orp1), also known as Gpx3 (64). Thus, the reduced Cys 36 of Orp1 reacts with hydrogen peroxide to form a sulfenic acid intermediate, which, in turn, forms a transient mixed disulfide with one of the cysteines present in the Yap1 C-terminal domain, Cys 598. Finally, the products of the reaction are re-reduced Orp1 and oxidized Yap1 carrying an intramolecular disulfide between Cys 598 and Cys 303 (63). This suggests that the heterologous expression in *E. coli* of a C-terminal fragment of Yap1, which contains Cys 598 but lacks Cys 303, might lead to the formation of stable mixed disulfides with a variety of sulfenic acid-containing *E. coli* proteins (305). Indeed, expression of the histidine-tagged Yap1 C-terminal fragment followed by purification using Ni-affinity chromatography led to the identification of about 30 bacterial proteins, most of which had already been shown to possess redox-active cysteines. Among these identified *E. coli* proteins were the Prx Bcp, Tpx, and AhpC, a glutathione peroxidase, the chaperones Hsp33 and DnaK, which is the bacterial homolog of Hsp70, TrxA, and the methionine sulfoxide reductase MsrB. Further, three ribosomal subunits and two translation elongation factors were identified. The addition of hydrogen peroxide or alkyl hydroperoxides during expression enhanced mixed disulfide formation between *E. coli* proteins and the Yap1 fragment, whereas addition of dimedone (Fig. 10) had an inhibitory effect. This was in agreement with the proposal that sulfenic acid-containing proteins would react with Yap1 (305).

A recent study describes the application of ATS chromatography for analyzing the *E. coli* disulfide proteomes in control- and menadione-treated cells (138). To isolate disulfide-containing proteins, the soluble extracts were subjected to ATS chromatography after NEM and DTT treatments. Thiol-containing proteins were isolated by direct binding to the ATS matrix. Eight ribosomal proteins were found to form disulfides in response to menadione (Fig. 14). The translation elongation factors EF-Tu and EF-G also displayed disulfides after menadione treatment. Another interesting observation was that four of the aminoacyl-tRNA synthetases, glycyl-, aspartyl-, leucyl-, and isoleucyl-tRNA synthetase, have cysteine thiols that are lost upon menadione treatment, but apparently do not give rise to new disulfides. As for metabolic enzymes, disulfides were detected after menadione treatment in, for example, aldolase, phosphoglycerate kinase, enolase, aconitase, and isocitrate lyase. In contrast, disulfides were found in GAPDH and pyruvate kinase in both control and menadione-treated samples (138).

To study the disulfide proteome in the gram-positive bacterium *Bacillus subtilis* an approach similar to that used for *E. coli* (182) has been applied. The principal difference was that the thiols arising from reduced disulfides were not detected using radioactive IAM, but with the BODIPY FL-C_1-IA fluorescent dye (Fig. 3E) (121). The cysteine redox state of cytoplasmic *B. subtilis* proteins was analyzed under normal growth conditions and after oxidative stress caused by diamide (Fig. 13), hydrogen peroxide, superoxide, and the antibiotic nitrofurantoin (121). The proteins identified under normal growth were, among others, the antioxidant system

consisting of the typical 2-Cys Prx AhpC and its reductase (AhpF), TrxA, and the phosphoadenosine phosphosulfate (PAPS) reductase. The set of proteins that formed disulfides were found to be specific for the oxidant used, but six proteins were identified under all conditions of oxidative stress, including the Prx AhpC and Tpx, and enzymes belonging to amino acid metabolism. Some proteins formed disulfides under more than one of the oxidative conditions tested. Finally, some proteins formed disulfides only under one type of oxidative stress condition, which is consistent with observations made in *E. coli*. For example, the enzymes IlvC and GlyA, and the translation elongation factor EF-Tu were only oxidized in the presence of diamide (Fig. 13). In contrast to the oxidative stress induced by diamide and ROS (121), the addition of benzoquinone depleted the intracellular thiol pool of *B. subtilis* and caused irreversible damage to the members of the disulfide proteome (191). The cytotoxic effect of benzoquinone was accompanied by protein precipitation that was probably a consequence of adduct formation of thiol groups with benzosemiquinone. The only redox-sensitive protein of *B. subtilis* identified *in vivo* after benzoquinone treatment was GAPDH (191). A different technique was developed in *B. subtilis* for the analysis of the protein S-thiolation by cysteine (here called S-cysteinylation) after diamide treatment (122). Thus, proteins were labeled *in vivo* with [^{35}S]cysteine in the presence of chloramphenicol to inhibit protein synthesis. Thereafter, proteins were resolved using 2-D isoelectric focusing/SDS-PAGE under nonreducing conditions and six proteins were shown to undergo S-cysteinylation in response to diamide (122). Four of these proteins had already been reported in the previous study using BODIPY fluorescence (121). Proteins modified by S-cysteinylation have also been found in *Staphylococcus aureus* after diamide treatment (261). Two S-cysteinylated proteins, identified by MS/MS analysis, where the modified tryptic fragments carried a characteristic additional mass of 119 Da, were the acetoine reductase and a putative lipoprotein (261).

The same fluorescence-labeling approach described for *B. subtillis* (121) was used to study the reversibly oxidized thiols of cytoplasmic proteins in *S. aureus* during normal growth and under oxidative stress induced by diamide (Fig. 13) and hydrogen peroxide treatments (324). Among the proteins found to possess reversibly oxidized cysteines under normal growth conditions were the Prx system AhpC/AhpF, dihydrolipoyl dehydrogenase of the pyruvate dehydrogenase complex, and a probable cysteine desulfurase. Diamide treatment resulted in a global oxidation of many cysteine residues in intracellular proteins of *S. aureus*. Several of these proteins had been previously detected in *B. subtilis* as well as in *E. coli*. Eighteen different proteins displaying thiol oxidation after hydrogen peroxide-induced stress were identified. Of these proteins, the largest number belongs to the intermediary metabolism, for example, aldehyde dehydrogenase, MetE, and the CTP synthase. Moreover, proteins implicated in oxidative stress tolerance and peroxide detoxification were identified, such as the Trx system consisting of TrxA and its reductase TrxB, Tpx, and a glutathione peroxidase homolog. The regulatory protein Spx has also been observed to contain redox-sensitive cysteines in *S. aureus* (324).

In addition to oxidative stress caused by hydrogen peroxide and diamide, *B. subtilis* and *S. aureus* were compared for their response to nitric oxide (NO). Although NO induced distinct changes in the protein expression levels of both bacteria, it did not significantly alter the thiol oxidation levels of proteins. Instead, a treatment with NO appeared to protect the proteins of *B. subtilis* and *S. aureus* from thiol oxidation by hydrogen peroxide (123).

The host immune response to *Mycobacterium tuberculosis* involves the expression of nitric oxide synthase, the products of which can kill this pathogen with a molar potency greater than that of many conventional antitubercular agents (266). However, the protein targets of the NOS remained to be identified. Proteins with S-nitrosylated cysteines were therefore analyzed in an *in vivo* study of *M. tuberculosis* using the biotin switch method (266). The majority of proteins identified were implicated in the intermediary metabolism, particularly in the amino acid metabolism, for example, asparagine synthase, 3-phosphoglycerate dehydrogenase, glutamine synthetase, or glutamate synthase. Antioxidant proteins, for example, the catalase KatG, were also identified among the S-nitrosylated proteins.

2. Photosynthetic prokaryotes. The existence of Trx in cyanobacteria was revealed not long after their discovery in chloroplasts (101, 342). Cyanobacterial Trx (*m*-, *x*-, and *y*-types) are highly similar to those of chloroplasts, which is not surprising in the light of their common origin (84). However, it soon became clear that several chloroplast enzymes that were subject to light-dependent redox regulation through Trx reduction (37, 38) could not be predicted as Trx substrates in cyanobacteria (149). For instance, NADPH-MDH does not have a homolog in cyanobacteria and the Calvin-Benson cycle enzyme FBA lacks both sequence motifs containing the regulatory cysteines found in chloroplasts (Fig. 15). The cyanobacterial homolog of the well-known chloroplast substrate for Trx γ- ATP synthase also lacks the entire sequence motif containing two regulatory cysteines (Fig. 15).

Deletion of the cyanobacterial *m*-type Trx is lethal (243, 244), which indicates its fundamental importance in these organisms. Nevertheless, indications of the function of Trx in cyanobacteria did not appear until the first proteomic analyses of Trx target proteins in the unicellular model cyanobacterium *Synechocystis* sp. PCC 6803. Initially, a modified Trx affinity chromatography protocol was developed for the isolation of Trx target proteins *in vitro* (197). Thus, a monocysteinic version of the *Synechocystis m*-type Trx, TrxA, was immobilized on a nickel affinity matrix through its His-tag and cytosolic proteins were screened for their capacity to form mixed disulfides with this Trx. Thereafter, the intact target-TrxA complexes were eluted with imidazole and resolved using 2-D SDS-PAGE under nonreducing/reducing conditions and, finally, proteins were identified by PMF (196). The *Synechocystis* Trx target proteome was remarkably different from the reported chloroplast Trx target proteomes (84, 196, 198). Although most of the processes associated with Trx-dependent regulation in chloroplasts (39) were represented in the *Synechocystis* Trx target proteome, less than one-fourth of the cyanobacterial targets had chloroplast homologs that have been identified as Trx targets (84, 198). The relatively few targets common to *Synechocystis* and chloroplasts included GAPDH, transketolase, ADPglucose pyrophosphorylase, and two Calvin-Benson cycle enzymes: the RuBisCo large subunit and phosphoribulokinase (84). Other common targets were the Prx II (type II Prx), Hsp60 (GroEL), and the translation

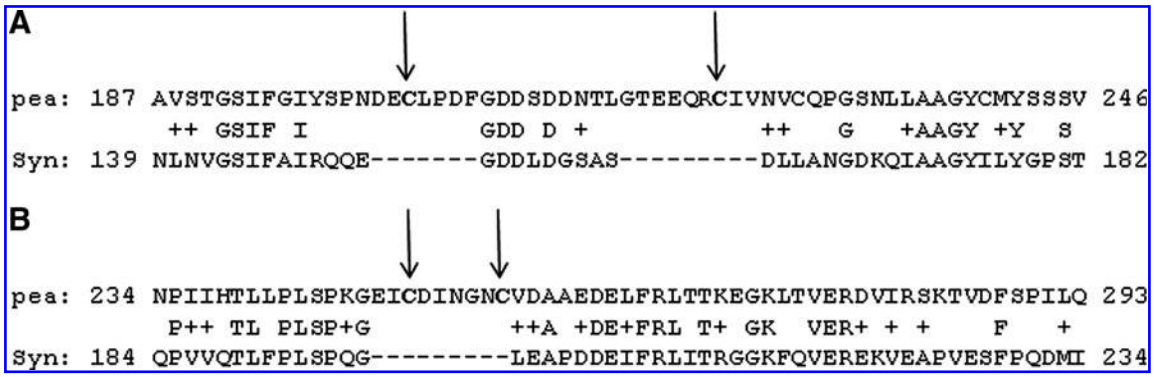

FIG. 15. Cyanobacterial enzymes lacking regulatory cysteines identified in chloroplast homologs. The sequence alignments compare **(A)** the fructose 1,6-bisphosphatase from pea (*Pisum sativum*) (chloroplast precursor, P46275) to that of *Synechocystis* sp. PCC 6803 (P74324), and **(B)** the ATP synthase γ-subunit of pea (*Pisum sativum*) (chloroplast precursor, P28552) to that of *Synechocystis* sp. PCC 6803 (P17253).

elongation factors EF-G and EF-Tu (84). A comparative study was performed using monocysteinic versions of the *Synechocystis m-*, *x-*, and *y*-type Trx, denoted TrxA, TrxB, and TrxQ, respectively, as ligands for Trx affinity chromatography (253). This direct comparison of affinity ligands showed that there is very little variation between the target proteomes of the three different Trx *in vitro*. The interaction of the *Synechocystis x*-type Trx with 1-Cys Prx and type II Prx was confirmed in an independent study using Trx affinity chromatography (134). The cytosolic *Synechocystis* proteome has also been screened for proteins interacting with Grx using the monocysteinic GrxA as bait in a standard affinity chromatography protocol, followed by 1-DE and LC-MS/MS analysis (190). The *Synechocystis* proteins forming mixed disulfides with GrxA included 18 proteins that had previously also been found to be targets for Trx, indicating a high degree of overlap in the target proteomes of these enzymes.

Unlike other prokaryotes, cyanobacteria possess three membrane systems. In addition to the outer membrane and plasma membrane, they contain thylakoid membranes, where the light-dependent photosynthetic reactions take place (312). A method was designed to search for Trx targets among the *Synechocystis* membrane proteins, and was applied on a total membrane preparation including thylakoid and plasma membranes (223). Intact membranes were incubated with histidine-tagged monocysteinic *Synechocystis* TrxA and, thus, mixed disulfides with the target proteins were formed *in situ*. After fractionation and solubilization with the detergent dodecyl maltoside, the membrane-bound Trx targets were isolated by nickel affinity chromatography and nonreducing/reducing 2-D SDS-PAGE, as in the previously reported procedure (196, 197). Fifty membrane-associated Trx target proteins were identified, which means that there are almost twice as many unique targets associated with membranes as compared to those present exclusively in the cytosol in *Synechocystis* (196, 223, 253). Interestingly, the ATP-binding subunits of several transporters, such as SecA (preprotein translocase), ArsA (oxyanion translocase), UrtD (urea transporter), and NrtD (nitrate/nitrite transporter) have been identified as potential Trx targets in cyanobacteria (223).

A recent study included an *in vivo* screening for TrxB target proteins in *Synechocystis* and involved expression of a monocysteinic histidine-tagged version of TrxB in a *Synechocystis trxB* knockout mutant (254) since, unlike TrxA, TrxB is not essential in *Synechocystis*. Hence, the target-TrxB mixed disulfides were formed *in vivo* and subsequently isolated and identified. Eleven of the 24 TrxB targets (254) had been previously identified as Trx targets *in vitro* (196, 223, 253). However, six different ribosomal subunits were identified for the first time in this study of *in vivo* targets for TrxB, which might suggest their having a role in redox-regulation of translation (254).

The green sulfur bacterium *Chlorobaculum tepidum* lives in anaerobic environments and performs anoyxgenic photosynthesis. The genome of *C. tepidum* encodes two Trx, termed Trx1 and Trx2. To study their functions, potential target proteins were captured *in vitro* by Trx affinity chromatography using the monocysteinic mutants Trx1 (C28S) and Trx2 (C36S) (135). The proteins that bound to the Trx ligands *via* mixed disulfides were separated by 2-DE and analyzed by MALDI-MS. This approach resulted in the identification of 37 proteins, which bound both to Trx1 and Trx2. The potential Trx targets were sorted into seven groups. Notably, the reductive TCA cycle for carbon dioxide fixation was represented by six enzymes. The group of proteins with functions in amino acid metabolism had 10 members, and 3 of the target proteins were linked to sulfur assimilation. In addition, the target proteins included members of the pathways for glycolysis, and the biosynthesis of sugar, purine, pyrimidine, and thiamine. Finally, the stress defense proteins 2-Cys Prx and Prx Q, Hsp70 (DnaK), and the translation elongation factor EF-Tu were also found (135). Taken together, these findings display clear similarities to the Trx targets in chloroplasts and cyanobacteria. Although *C. tepidum* lacks the Calvin-Benson cycle for carbon dioxide fixation, the other processes linked to redox regulation in this sulfur bacterium have also been found in chloroplasts (198). Some of the target proteins of *C. tepidum* are homologous to chloroplast Trx target proteins in Arabidopsis and poplar, and include, for instance MDH, the thiamine biosynthesis protein ThiC, 2-Cys Prx and Prx Q, EF-Tu, and the chaperones GroEL and Hsp70. Notably, EF-Tu and GroEL of *C. tepidum* are also homologous to Trx target proteins of the cyanobacterium *Synechocystis* sp. PCC 6803.

IV. Biological Processes Associated with Disulfide Proteomes

Many proteins have been described that possess reactive cysteines and, hence, might undergo putative redox

regulation. Nevertheless, the vast majority of the reported studies on disulfide proteomes are mainly descriptive and do not include discussions on the possible significance of the discoveries, let alone experimental verification. Therefore, it is no easy task to make sense of the large quantity of information resulting from disulfide proteomics. However, some processes are particularly rich in enzymes associated with disulfide proteomes and deserve attention.

A. Glycolysis and the TCA cycle

GAPDH, which catalyzes one of the key steps in glycolysis, is probably the single most common enzyme found in disulfide proteomes. The explanation lies in its high abundance in most organisms and the fact that its activity relies on a reactive catalytic cysteine residue. In mammals GAPDH has been described as a multifunctional protein and a mediator for cell death, which involves S-nitrosylation of its catalytic cysteine (116). The roles of eukaryotic GAPDH in signal transduction and apoptosis have been discussed (31). Moreover, an Arabidopsis plastid GAPDH appears to be regulated through S-glutathionylation of the catalytic cysteine (343) similarly to the human enzyme (57). GAPDH is also found among the sulfenated proteins in HeLa cells (189); in *S. aureus*, the catalytic cysteine of GAPDH is converted to a sulfonic acid under conditions of severe oxidative stress (319). In conclusion, GAPDH from many organisms may undergo a variety of oxidative modifications leading to the inactivation of glycolysis. It is noteworthy, however, that not all isoforms of GAPDH are susceptible to post-translational thiol modification. Among the three isoforms of GAPDH in yeast, only the Tdh3 isoenzyme was able to undergo S-thiolation, which protected this enzyme under oxidative stress. The Thd1 and Thd2 isoenzymes of GAPDH could not be S-thiolated and were therefore readily irreversibly inactivated under oxidizing conditions (291).

In fission yeast peroxide-dependent oxidation of the GAPDH active site, cysteine enhances the association of this enzyme to the response regulator Mcs4 and a stress-responsive MAP kinase kinase kinase, which promotes peroxide-dependent signaling through the MAP kinase cascade (238). Redox modifications of human GAPDH have been found to be involved in a mechanism controlling the expression of endothelin-1 (ET-1), an endogenous vasoconstrictor, through binding to its mRNA (271). Thus, unmodified native GAPDH binds the 3′ untranslated region of the ET-1 mRNA, thereby accelerating its degradation. Under oxidizing conditions GAPDH is glutathionylated at the active site cysteine, which renders the enzyme unable to bind the ET-1 mRNA, hence leading to its stabilization. During progression of Alzheimer's disease, disulfide-linked inactive forms of GAPDH accumulate in the nuclei of neuronal cells and form insoluble aggregates (59). After apoptosis of these cells GAPDH is found in extracellular amyloid plaques.

Apart from GAPDH, other glycolytic enzymes, including aldolase, TPI, phosphoglycerate kinase, enolase, and pyruvate kinase, are frequently found in proteomic studies of oxidative cysteine modifications (99). Biochemical studies have confirmed that several of these enzymes are reversibly inactivated under oxidative stress conditions (292).

The TCA cycle is one of the most extensively covered processes in disulfide proteomes from all kinds of organisms, ranging from bacteria to plants and animals (Fig. 16). In *E. coli*, five enzymes from the TCA cycle—citrate synthase, aconitase B, isocitrate dehydrogenase, succinyl-CoA synthetase (α-subunit), and succinate dehydrogenase—were sensitive to oxidative stress and significantly more oxidized in a TrxA knockout mutant (182). Citrate synthase from *E. coli* contains a cysteine in a cofactor-binding site, C206, the oxidation of which renders the enzyme inactive (72). Aconitase B contains three cysteines in a redox-sensitive iron-sulfur cluster (314). The α-subunit of the succinyl-CoA synthetase has a cysteine in a cofactor-binding site, C123 (91), and succinate dehydrogenase subunit SdhB contains an iron-sulfur cluster (62). Therefore, these enzymes appear indeed to be reversibly redox-regulated and their activities depend on the balance between ROS levels and the activity of TrxA in *E. coli*.

Seven enzymes from the TCA cycle have been identified as *in vitro* targets for Trx in plant mitochondria (14), five of which were also identified as targets for Grx (273). Arabidopsis citrate synthase was found to participate in intermolecular disulfides (321). Aconitase and α-ketoglutarate dehydrogenase (subunit E2) were isolated by Trx affinity chromatography from the unicellular alga *C. reinhardtii* (185). As for the cyanobacterium *Synechocystis* sp. PCC 6803, three subunits from the pyruvate dehydrogenase complex have been identified as Trx targets (223, 253). However, so far no cyanobacterial enzymes belonging to the TCA cycle have been found in disulfide proteomes. In this context it should be mentioned that *Synechocystis* sp. PCC 6803 does not have a complete TCA cycle and that α-ketoglutarate dehydrogenase is missing from this cyanobacterium (178).

Reversible cysteine oxidation of aconitase, isocitrate dehydrogenase (227), and MDH (187) occurs in yeast after exposure to hydrogen peroxide *in vivo*. Aconitase has been reported to be severely inhibited *in vivo* after the addition of hydrogen peroxide or menadione to yeast cultures (43) and isocitrate dehydrogenase has been found to be reversibly inhibited by glutathionylation at cysteine 269 (160). An examination of proteins from rat heart myocytes exposed to diamide revealed the presence of intermolecular disulfides in the flavoprotein subunit of succinate dehydrogenase, isocitrate dehydrogenase, and MDH (33). Incubation of isolated rat liver mitochondria with GSNO led to S-nitrosylation of MDH and the dihydrolipoamide dehydrogenase subunit of succinyl-CoA synthase (89). Human spermatozoa display S-nitrosylation of succinate dehydrogenase, fumarase, MDH, citrate synthase, and pyruvate dehydrogenase (180). Further, succinate dehydrogenase is among the cysteine-sulfenated proteins detected in HeLa cells (189).

The reversible inactivation of glycolysis and the TCA-cycle under oxidative stress conditions favors a transient diversion of the reduced carbon flux toward the oxidative pentose phosphate pathway (OPPP) with a concomitant production of NADPH (227, 292). The functional significance of such a metabolic rerouting has been suggested to be that the NADPH might be utilized to combat the oxidative stress *via* the NTR/Trx and Prx system for peroxide decomposition (227) or *via* glutathione reductase for the regeneration of GSH.

B. Amino acid metabolism

A considerable number of enzymes identified in studies of disulfide proteomes are implicated in the metabolism of

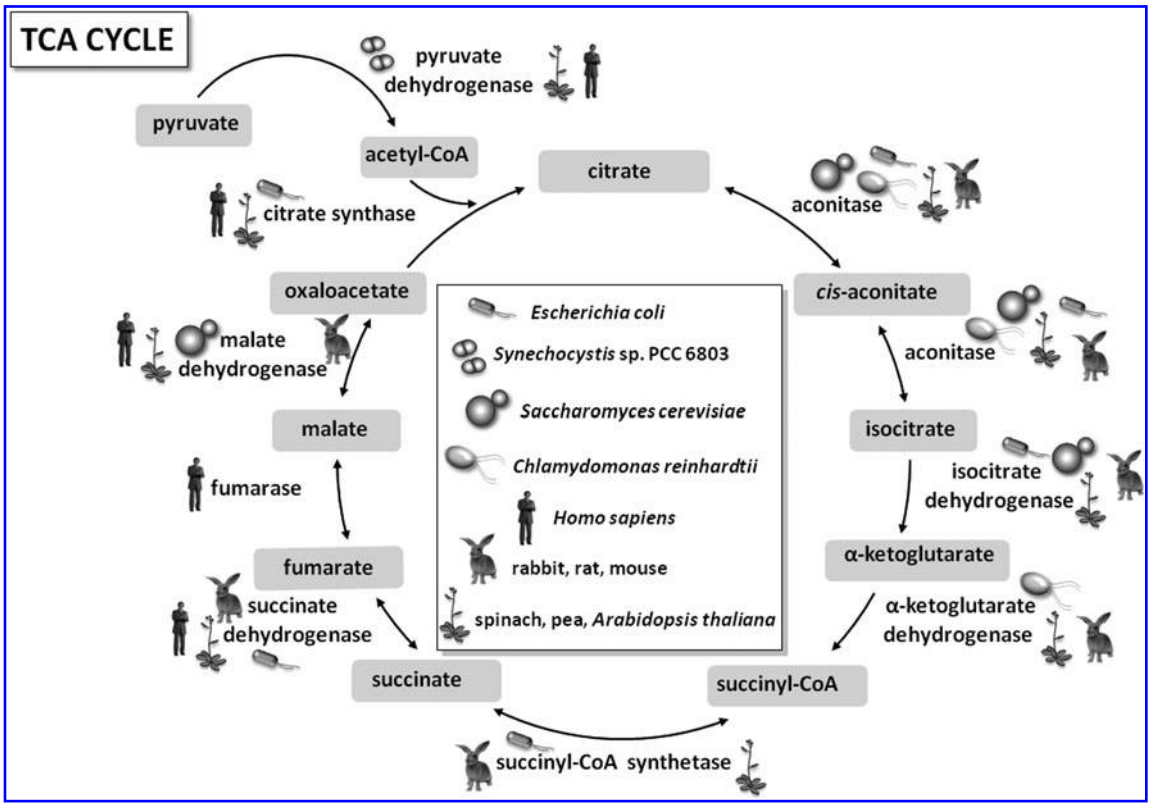

FIG. 16. Enzymes from the tricarboxylic acid (TCA) cycle in all kingdoms of life are found in disulfide proteomes. Enzymes participating in the TCA cycle have been detected in nearly all studies on disulfide proteomes in either whole cells or isolated mitochondria from organisms ranging from bacteria to humans. The organisms, in which these enzymes were found, are indicated by the corresponding symbols. Biochemical analyses have confirmed that in most of these enzymes cysteine oxidation leads to inhibition of catalytical activity. Thus, oxidative stress downregulates the TCA cycle.

amino acids and related molecules, particularly in prokaryotes. Bacteria have low-molecular-weight (LMW) thiols that act as thiol redox buffers and protect the protein cysteines against over-oxidation by S-thiolation. Gram-negative bacteria use GSH as a redox buffer, but many gram-positive bacteria lack genes involved in GSH biosynthesis. *B. subtilis* and *S. aureus* rely on different LMW thiols, such as bacillithiol (BSH), or even the free cysteine (261). Thus, it has been suggested that under oxidative stress it is necessary to increase the cysteine pool to protect a cell from damage. A metabolome study carried out in *B. subtillis* revealed significant modifications in the cysteine- and branched-chain amino acid biosynthesis pathways in response to diamide (261). A drastic depletion of cysteine and its precursors supports the idea of S-thiolation of protein thiols after exposure to diamide and, hence, that cysteine must be regenerated. Under these stress conditions, cysteine biosynthesis is induced by derepression of the CymR regulon (181). The pool of glycine, which is a precursor of serine, is reduced under oxidative stress. Further, the synthesis of the glycyl-tRNA synthetase is decreased, which means that less glycine becomes incorporated into proteins (181, 261). This might indicate an enhanced provision of glycine for the synthesis of serine, which is a cysteine precursor. The CymR-controlled MccA (YrhA) and MccB (YrhB) proteins participate in the conversion of methionine to cysteine and are induced under disulfide stress (181). In contrast, the methionine synthase MetE is inactivated by S-thiolation in *E. coli* under diamide stress (122, 131, 132), and thus the methionine pool is decreased in diamide-treated cells. It might also be added that the plant methionine synthase is modified by glutathionylation (71). All these findings indicate a metabolic net flux from glycine *via* serine to cysteine, and from methionine to cysteine, under oxidative stress conditions in gram-negative as well as gram-positive bacteria; these findings also highlight the significant role of cysteine in stress protection (261).

C. The Calvin-Benson cycle

The Calvin-Benson cycle for the fixation of carbon dioxide is one of the first metabolic pathways known to be regulated by Trx (38). The 13 reactions of the Calvin-Benson cycle are catalyzed by 11 enzymes (94, 113). The light-dependent activation of the enzymes fructose 1,6-bisphosphatase, sedulohеptulose 1,7-bisphosphatase, GAPDH, and phosphoribulokinase are classical examples of regulation mediated by Trx-catalyzed reduction (186, 281). In the dark, these enzymes are oxidized and inactive, whereas in the light, Trx-mediated reduction of critical disulfides induces conformational changes, which lead to activation. RuBisCo is not directly activated by Trx, but indirectly *via* RuBisCo activase, which is controlled by Trx *f* (346). The reducing equivalents needed to drive the Trx-mediated activation of the Calvin-Benson cycle are delivered by photosynthetic electron transport *via* the cascade of ferredoxin, ferredoxin-Trx-reductase, and Trx (186, 281). Recent proteomics studies indicate that not some, but all enzymes of the Calvin-

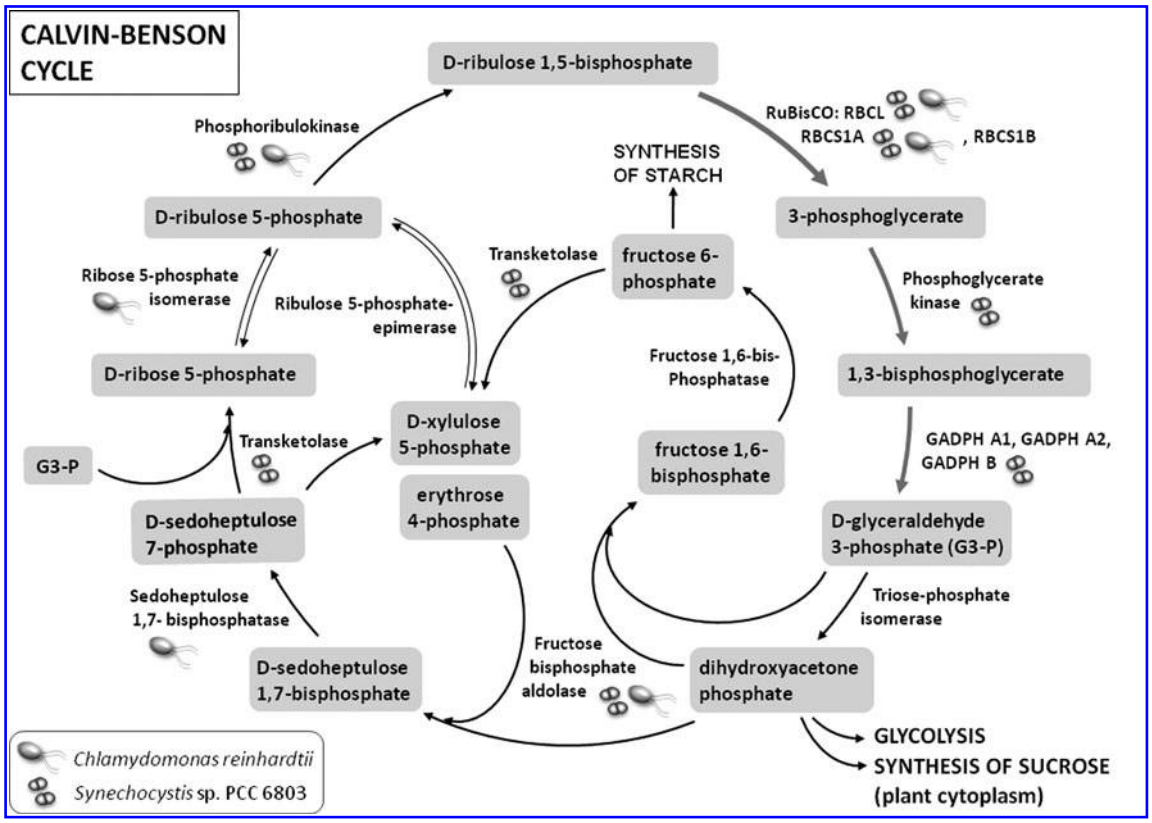

FIG. 17. The entire chloroplast Calvin-Benson cycle for carbon fixation is under the control of Trx. The Calvin-Benson cycle has 11 enzymes that catalyze 13 reactions. The first metabolite is 3-phosphoglycerate that is formed after the incorporation of carbon dioxide into ribulose 1,5-bisphosphate. The following reactions convert 3-phosphoglycerate into glyceraldehyde 3-phosphate and its tautomeric form dihydroxyacetone phosphate. These metabolites are in turn used by the chloroplast for the synthesis of starch and for the regeneration of the Calvin-Benson cycle. A part of the dihydroxyacetone phosphate produced is also exported into the cytoplasm where it is used for the synthesis of sucrose. It has long been known that the activities of some enzymes of the Calvin-Benson cycle are controlled by Trx that receives the reducing equivalents from photosynthesis *via* the cascade of ferredoxin and ferredoxin Trx reductase. Proteomics analysis has shown that not some, but all enzymes of the Calvin-Benson cycle of Arabidopsis are *in vitro* targets for Trx and/or Grx, which suggests that the Calvin-Benson cycle is completely turned off in the dark, when photosynthesis ceases. Notably, all Calvin-Benson cycle enzymes of Arabidopsis, except phosphoglycerate kinase, have conserved cysteines (198). *In vitro* interactions with Trx have also been found for some Calvin-Benson cycle enzymes from the alga *Chlamydomonas reinhardtii* and the cyanobacterium *Synechocystis* sp. PCC 6803, which is indicated by the corresponding symbols.

Benson cycle are regulated by Trx (198). This suggests that Trx could function as a main switch to turn on the Calvin-Benson cycle in the light and turn it off in the dark, which would prevent waste of NADPH and ATP. However, the redox regulation of the Calvin-Benson cycle by Trx and reactive cysteines appears to be more complex. Apart from the indirect control of RuBisCo by Trx *via* RuBisCo activase, this enzyme is also subject to direct modifications on conserved redox-sensitive cysteines (237). Modulation of the conformational changes of RuBisCo by redox-sensitive cysteines has also been found in the alga *C. reinhardtii* (214) and in the cyanobacterium *Synechocystis* sp. PCC 6803 (213).

Many chloroplast proteins are now known to form multiprotein complexes (248), a fact that had already been recognized for enzymes of the Calvin-Benson cycle some time ago (304). The small protein CP12 mediates the formation of a super-complex that assembles CP12 with GAPDH and phosphoribulokionase (PRK) (320). CP12 contains two disulfides and has to be fully oxidized to form a complex with GAPDH and PRK (106). Both GAPDH and PRK are inactive in the complex with CP12 (320). A fraction of PRK has been found to be reduced in the dark, although the enzyme was inactive because it was assembled in the complex with CP12 (136). After reduction by Trx *f* the complex of CP12, PRK, and GAPDH dissociates, and releases active PRK. This mechanism has been suggested as an alternative to the activation of PRK through direct reduction by Trx (136).

The A- and B-subunits of plastid GAPDH assemble into different isoforms, of which the major isoforms A_2B_2 and A_8B_8 contain two redox-active disulfides per tetramer and are controlled by Trx. Theses reactive disulfides are located at the C-termini of the B-subunits. By contrast, the minor A_4-isoform does not have any disulfide and is independent from regulation by Trx (80). Instead, the catalytic cysteine of the A_4-isoform of GAPDH forms a mixed disulfide with glutathione, which inactivates the enzymatic activity and might be a mechanism to protect the A_4-isoform of GAPDH from irreversible oxidation under stress (343). Glutathionylation has also been found for the enzymes phosphoglycerate kinase and ribose-5-phosphate isomerase in *C.*

reinhardtii (232), but the impact of this modification on their enzymatic activities is not yet known. The FBA of Arabidopsis was found to be S-glutathionylated in cultured suspension cells (147). It has been observed that the FBA1 isoform is activated by GSH, but inactivated by DTT and Trx (224, 247), which appears to be in disagreement with the current model for Trx-controlled redox regulation of the Calvin-Benson cycle. In addition, the activity of chloroplastic FBA from rapeseed was stimulated by formation of a complex with 2-Cys Prx. The activation of FBA by 2-Cys Prx involved the 170's loop of FBA but by a different mechanism than in the activation of FBA by Trx *f* (46). The functional impact of these observations remains to be shown.

Studies of the disulfide proteomes in the alga *C. reinhardtii* and the cyanobacterium *Synechocystis* sp. PCC 6803 have so far failed to provide evidence for a complete control of the Calvin-Benson cycle by Trx. However, the known Trx-targets include six proteins of the Calvin-Benson in *C. reinhardtii* and seven in *Synechocystis* sp. PCC 6803 (Fig. 17). Notably, the Trx-targets in *Synechocystis* sp. PCC 6803 cover the initial steps of carbon dioxide fixation, the synthesis of glyceraldehyde 3-phosphate, and the control of PRK and GAPDH (198). This suggests an ancient origin of the light-dependent redox regulation of carbon dioxide fixation.

D. Oxidative stress response

Nearly 20 years ago, thiol-specific antioxidant (TSA) enzymes from mammals and yeast, and the alkyl hydroperoxide reductases (AhpC) from bacteria, were found to belong to the same phylogenetic family of peroxidases (48). Whereas the bacterial AhpC uses the flavoprotein AhpF as the immediate electron donor (258), a yeast member of the family was proven to be reduced by a system consisting of NADPH/NTR/Trx, and was therefore called TPx for Trx peroxidase (47). The entire family of peroxidases was named "peroxiredoxins" (47, 48) and this name (Prx) is still used today. While there are subgroups within the Prx family with different properties and catalytic mechanisms, all Prx depend on a catalytically active cysteine residue, termed the peroxidatic cysteine, which is oxidized to a sulfenic acid by the peroxide substrate (330). Eukaryotic 2-Cys Prx are reversibly inactivated by over-oxidation of the peroxidatic cysteine to sulfinic acid under oxidative stress conditions (329) and this feature is thought to contribute to the hydrogen peroxide-dependent signal transduction (111, 267). Hence, Prx has a function in peroxide detoxification as well as in peroxide-induced signaling. The relationships between Prx, glutathione peroxidases (Gpx), Trx, Grx, and their reductases in mammalian cells have been beautifully illustrated (2). Prx are extraordinarily abundant enzymes, for instance, AhpC, is the seventh most abundant protein in *E. coli* (201). Therefore, given the high Prx levels and the reactivity of the peroxidatic cysteine, it is not surprising that these enzymes are omnipresent in disulfide proteomes. Apart from sulfenic and sulfinic acid derivatives of Prx, proteomic studies have shown that these enzymes also undergo glutathionylation (92), S-nitrosylation (180, 335), and intermolecular protein–protein disulfide formation (33). While the disulfide-linked homodimers of typical 2-Cys Prx are part of their catalytic cycle (330), the significance of glutathionylation and S-nitrosylation of Prx is less certain. Various members of the Prx family have been identified as targets for Trx *in vitro*, for example, in plants and cyanobacteria (12, 112, 196, 239), and *in vivo* in mice and yeast (96, 187).

Other enzymes that function in the oxidative stress response are also found in some disulfide proteomes, though to a lesser extent than the Prx. In yeast, the glutathione peroxidase Gpx2, the methionine sulfoxide reductase Mxr1 and the Cu/Zn SOD1 displayed elevated levels of reversible cysteine oxidation in a Trx knockout mutant (187). The most straightforward interpretation of this result is that Trx would be the physiological electron donor for the Gpx2 and Mxr1 enzymes sustaining their catalytic activities. The wheat seed GSH dehydroascorbate reductase and ascorbate peroxidase have been found to be Trx targets *in vitro* (325). GST have been reported to undergo sulfenic acid formation in HeLa cells (189), and a GST from *M. edulis* forms disulfides in response to oxidative stress induced by menadione (Fig. 14) (226). Five different Arabidopsis GSTs appear to be glutathionylated (71). Two forms of rat heart SOD have been found to participate in intermolecular disulfides (33) and a Cu-Zn SOD from renal tissue undergoes sulfenation (311), whereas a plant Cu-Zn SOD forms disulfides (177). In most cases, the significance of these modifications remains to be established, since biochemical data concerning the effects on enzymatic activities are not yet available. However, biochemical analysis of a catalase identified as a target for Trx *in vitro* in the alga *C. reinhardtii* showed that this catalase is inactivated by reduction catalyzed by Trx (185). In contrast, the plant root Trx target monodehydroascorbate reductase is activated several-fold by reduced Trx y *in vitro*, which suggests that Trx *y* plays a role in the regeneration of ascorbate for peroxide detoxification in root plastids (212).

E. Protein synthesis, folding, and degradation

Enzymes involved in different processes of protein metabolism account for a significant part of the disulfide proteomes known so far (Fig. 18). Translation elongation factors are among the most frequent and abundant proteins in disulfide proteomes from eukaryotes as well as prokaryotes. EF-Tu is by far the most abundant protein in *E. coli* (201) and has been shown *in vitro* to possess PDI activity (268). Eukaryotic elongation factors have been described as multifunctional proteins involved in many more processes than protein translation, and, for example, EF-1γ has been reported to have GST activity (79). The comprehensive list of sulfenated HeLa proteins includes four different forms of EF-1 and one form of EF-2 (189). Several translation elongation factors have also been found in the neuronal HT22 disulfide proteome (58). EF-1γ and EF-2 are among the S-nitrosylated proteins identified in human spermatozoa (180). Two yeast elongation factors, EF-1α and EF-1β, have been found to be S-thiolated in response to hydrogen peroxide (292), and protein synthesis has been reversibly inhibited after the addition of hydrogen peroxide to yeast cultures. Interestingly, a knockout mutant for γ-glutamylcysteine synthetase, the first enzyme of the glutathione synthesis pathway, was unable to recover protein synthesis after peroxide treatment, indicating irreversible damage to the translational apparatus (292).

Regarding photosynthetic organisms, the cytosolic EF-1 and EF-2, as well as chloroplast EF-Tu, have been found to be targets for Trx in algae (185), and the elongation factors EF-1-

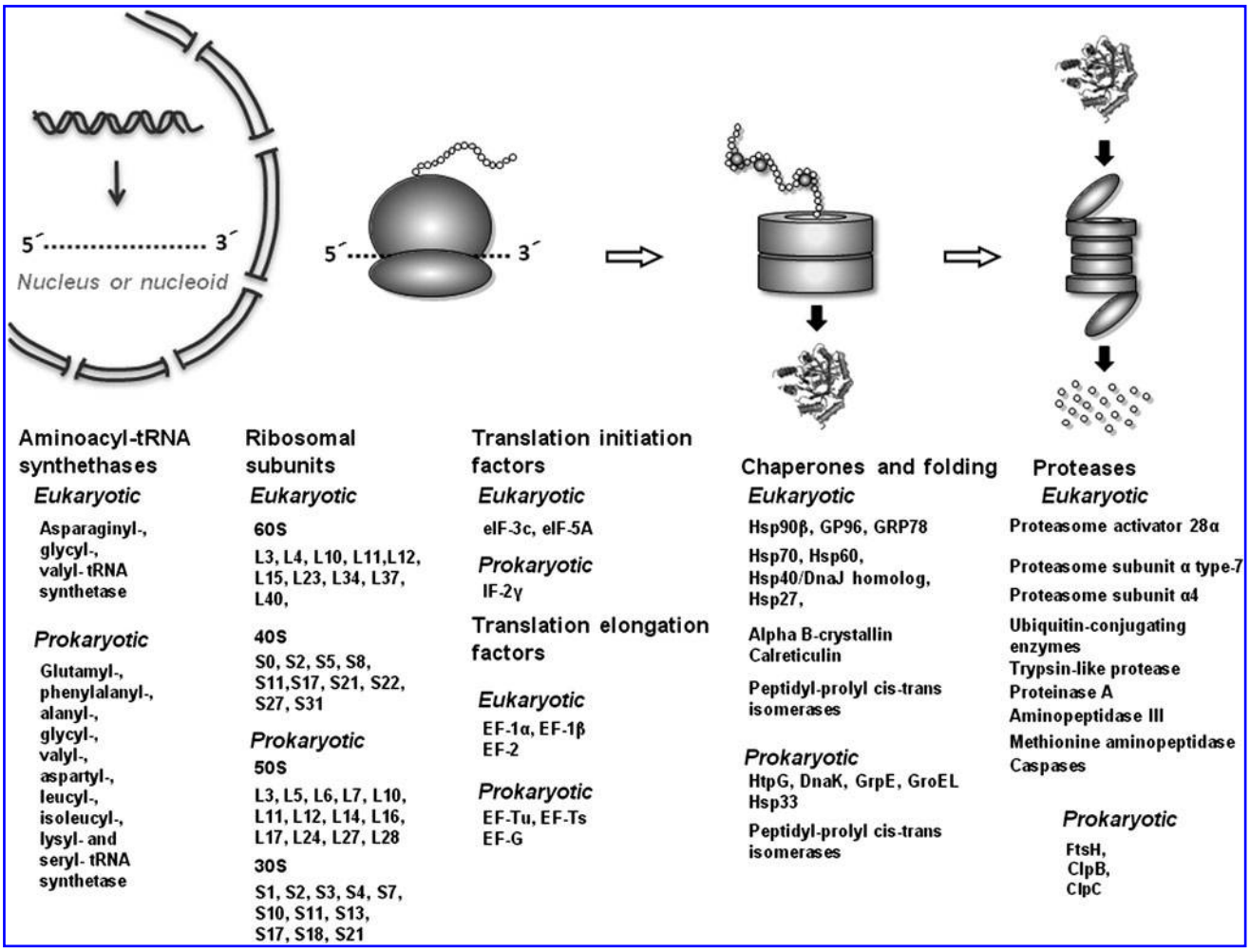

FIG. 18. Enzymes involved in principal steps of the protein metabolism are found in the disulfide proteomes of both prokaryotes and eukaryotes. These proteins have functions in translation, protein folding, and proteolysis. Translation is represented by t-RNA synthetases, ribosomal subunits, and translation initiation and elongation factors. Redox control of protein folding and assembly is indicated by the identification of many heat-shock proteins and chaperones in the disulfide proteomes of animals, plants, and bacteria. In addition, different proteome studies also suggested a thiol-mediated redox control of proteolysis. Due to space restrictions the lists of proteins in this figure are not complete but only show some representative members of their protein families.

α, EF-2, B1-α, and B1-γ have been identified among S-nitrosylated proteins from Arabidopsis cells (199). Activation of chloroplast EF-G by light was reported many years ago (3), though, at that time, no mechanism for activation was suggested. However, the fact that EF-G was later found to be a target for chloroplast Trx *in vitro* (12) would strongly suggest that this protein is subject to light-dependent redox control catalyzed by Trx. Further, EF-G has been identified as a target for Trx in cyanobacteria (196). The translation of photosynthetic proteins in cyanobacteria had been known to be sensitive to ROS, although the targets for oxidative modifications were then yet to be established (246). Subsequently, cyanobacterial EF-G was found to be inhibited by ROS (161) in a mechanism, which involved the formation of an intramolecular disulfide (162). Reduction of this specific disulfide catalyzed by Trx restored translational activity *in vitro* (162). In conclusion, a solid basis for a mechanism of redox-dependent translation in photosynthetic organisms has drawn on a combination of biochemical, genetic, and proteomic approaches.

The presence of a large variety of ribosomal subunits in different studies of disulfide proteomes is intriguing. For example, a total of six subunits from the chloroplast ribosome were identified in different studies on chloroplast disulfide proteomes (198) and another six ribosomal subunits were identified as *in vivo* targets for Trx in a cyanobacterium (254). Eight ribosomal proteins from *E. coli* form disulfides after treatment with menadione. Importantly, the application of denaturing conditions during the isolation of these *E. coli* proteins by ATS-chromatography ruled out the possibility of copurification of the entire ribosome (138). The impressive number of 16 different ribosomal subunits among the sulfenic acid-containing proteins in HeLa cells (189) raises the question whether all these proteins are indeed functionally modified as a means of regulation of translation. It should therefore be kept in mind that peripheral ribosomal proteins can potentially dissociate from mature ribosomes and some of these proteins have been reported to exert other cellular functions (70). A possible role for the thiol redox state in the binding of peripheral ribosomal subunits to the mature ribosome remains to be investigated.

Table 4A. Members of Disulfide Proteomes Involved in Regulation of Transcription

	Accession	*Experimental system*	*Cysteine modification*	*Proteomics approach*	*Reference*
Animals					
Peroxisome proliferator-activated receptor-γ	NCBI: 514307	Extracted soluble and membrane proteins from mouse mesangial cells	S-nitrosylation	Biotin-switch assay combined with 2-DE and MS	166
Zinc finger protein 297B	NCBI: 23397003M	″	″	″	″
TFIIA-α/β-like factor	NCBI: 12963759	″	″	″	″
Centromere protein F	Uniprot: P49454	Intact human spermatozoa lysed after treatment with different NO donors	S-nitrosylation	Biotin-switch assay combined with 1-DE and LC-MS/MS	180
HIV Tat-specific factor 1	Uniprot: O43719	″	″	″	″
Protein bassoon	Uniprot: Q9UPA5	″	″	″	″
SW1/SNF-related matrix-associated	Uniprot: Q92922	″	″	″	″
Actin-dependent regulator of chromatin subfamily C member 1	—	″	″	″	″
Zinc finger protein 395	Uniprot: Q9H8N7	″	″	″	″
Zinc finger protein 512	Uniprot: Q96ME7	″	″	″	″
Zinc finger protein 696	Uniprot: Q9H7X3	″	″	″	″
Zinc finger protein 740	Uniprot: Q8NDX6	″	″	″	″
Transcription factor NRF	—	Soluble murine heart protein extracts treated with H_2O_2	Reversible cysteine oxidation, unspecified	ICAT and fluorescence-DIGE using Cy3- and Cy5-maleimide	95
NF-κB-repressing factor (transcriptional silencer protein)	—	″	″	″	″
Fungi					
Transcription factor STP1	Uniprot: Q00947	Wild type yeast cells	Redox-active surface-exposed cysteine	BIAM labeling after reduction by TCEP, TRX1 and TTR1; 1-DE combined with MS/MS and LC-MS/MS	216
Transcription elongation factor S-II (DST1)	Uniprot: A6ZUV3	Wild type yeast cells	*S*-thiolation	*S*-thiolation with radioactive [^{35}S]cysteine in the presence of cycloheximide	292
Bacteria					
Transcriptional regulator YhiF	Uniprot: Q1R5A9	Wild type *E. coli*	Cysteines oxidized by hypochlorite	OxICAT: thiol blocking with light ICAT, new thiols are labeled with heavy ICAT	183

(*continued*)

Table 4A. (Continued)

	Accession	*Experimental system*	*Cysteine modification*	*Proteomics approach*	*Reference*
Transcriptional regulator H-NS	—	*E. coli* expressing a His-tagged C-terminal fragment of yeast Yap1	Sulfenation	His-tagged C-terminal domain of Yap1 used as bait *in vivo*	305
RNA polymerase sigma factor RpoS	NCBI: 83272624	Wild type *E. coli*	Reversible cysteine oxidation, unspecified	ATS chromatography	138
Alternative sigma factor	″	″	″	″	
Trp repressor-binding protein	NCBI: 148264	″	″	″	″
Catabolite gene activator protein	NCBI: 157159326	″	″	″	″
DNA gyrase, subunit A	NCBI: 157161714	″	″	″	″
DNA-directed RNA polymerase, beta subunit	NCBI: 110644322	″	″	″	″
Transcriptional regulator AbrB	—	*Bacillus subtilis*	Reversible cysteine oxidation, unspecified	Labeling with the BODIPY FL-C_1-IA fluorescent dye and 2-DE	121
Transcriptional regulator TenA	—	″	″	″	″
Transcriptional regulator Spx	NCBI: 57284376	*Staphylococcus aureus*	Reversible cysteine oxidation, unspecified	Labeling with the BODIPY FL-C_1-IA fluorescent dye and 2-DE	324
Cyanobateria					
Transcription termination factor NusA	Uniprot: P72688	Isolated total membranes from *Synechocystis* sp. PCC 6803	Oxidized cysteines reduced by TrxA	Binding of a His-tagged monocysteininc Trx *m* to membrane proteins *in situ*	223
Transcription antitermination factor NusG	Uniprot: P36265	″	″	″	″
Response regulator Rre1	Uniprot: slr1783	″	″	″	″

Molecular chaperones, such as Hsp70 and calreticulin, were found to harbor intramolecular disulfides in a study of neuronal HT22 cells using diagonal electrophoresis (58). Hsp 70 and Hsp60 are glutathionylated in response to oxidative stress in human T lymphocytes (92), and Hsp90, mt-Hsp70, and Hsp60 are S-nitrosylated in endothelial aorta cells (141). These are only a few examples of all the disulfide proteomic studies in which molecular chaperones have been identified. Oxidative stress may cause protein denaturation and compromise protein folding. That chaperones are commonly found in disulfide proteomes would therefore, at a first glance, suggest extensive redox control of protein folding catalyzed by molecular chaperones. However, few molecular chaperones have been demonstrated to be regulated through changes in the redox state of their cysteines. The effect of NO on the activity of Hsp90 has been studied, and it has been shown that S-nitrosylation inhibits the intrinsic ATPase activity of Hsp90, which blocks its ability to function as a chaperone and as a coactivator of endothelial nitric oxide synthase (eNOS) (219). The human homolog of the DnaJ cochaperone is inhibited by hydrogen peroxide treatment and reactivated by Trx-catalyzed reduction (51). In contrast, the bacterial Hsp33 is activated by the oxidation of cysteines (322, 323). GroEL, the bacterial homolog of the eukaryotic Hsp60, is relatively insensitive to hydrogen peroxide, although treatment with hypochlorous acid or peroxynitrite leads to the formation of methionine sulfoxide and cysteine sulfonic acid with concomitant inactivation (158).

Nine subunits of the 26S proteasome have been found to undergo S-nitrosylation in human spermatozoa (180). In an *in vitro* study of protein glutathionylation, as many as 16 subunits from the 20S proteasome were identified in extracts

Table 4B. Members of Disulfide Proteomes Involved in Signal Transduction

	Accession	Experimental system	Cysteine modification	Proteomics approach	Reference
Animals					
Mammalian cell cultures					
Annexin	Uniprot P08133	Jurkat T lymphocytes treated with H_2O_2	Reversible cysteine oxidation, unspecified	2-DE combined with labeling by IAF	21
PKA type II, α regulatory chain	Uniprot: P13861	″	″	″	″
Mitotic checkpoint kinase BUB1 β	Uniprot: O60566	″	″	″	″
Coronin-like protein P57	Uniprot: P31146	″	″	″	″
Deoxycytidin kinase	Uniprot: P27707	″	″	″	″
14-3-3 protein ζ/δ	Uniprot: P29312	″	″	″	″
Nucleosid diphosphate kinase B	Uniprot: P22392	″	″	″	″
CALM3 protein	Uniprot: Q9BRL5	Jurkat T-lymphocytes	Reversible cysteine oxidation, unspecified	2-D DIGE using BODIPYdyes	205
Cyclophiln A	Uniprot: P05092	Primary rat hepatocytes and human HepG2 hepatoma cells	*S*-thiolation	Labeling of intact cells with [^{35}S]cysteine and 2-DE combined with autoradiography	93
14-3-3 protein ζ	Uniprot: P29312	Immortalized ECV304 endothelial-like cells, derived from umbilical vein	*S*-thiolation	Selective reduction of S-glutathionylated proteins using the mutant C14S, C65Yof *E. coli* Grx 3; NEM-biotin labeling followed by 2-DE	195
Glutathione transferase omega-1	Uniprot: P78417	Human hepatocytes	S-nitrosylation	Biotin-switch assay	203
Interferon-γ receptor	NCBI: 124475	Mouse mesangial cells (ATCC CLR-1927)	S-nitrosylation	Biotin-switch assay combined with 2-DE	166
Myotonic dystrophy kinase	NCBI: 2143501	″	″	″	″
MBL associated serine protease	NCBI: 16040962	″	″	″	″
Very low density liporotein receptor	NCBI: 609533	″	″	″	″
14-3-3 protein τ	NCBI: 5803227	EA.hy293 hybridoma cells	S-nitrosylation	Biotin-switch assay combined with 1-DE and MS	218
14-3-3 protein ζ/δ	NCBI: 112695	″	″	″	″
DNA-dependent protein kinase; catalytic subunit	Uniprot: P78527	Human embryonic kidney (HEK293) cells	S-nitrosylation	Resin-assisted capture of S-nitroylated proteins (SNO-RAC)	88

(*continued*)

TABLE 4B. (CONTINUED)

	Accession	*Experimental system*	*Cysteine modification*	*Proteomics approach*	*Reference*
Serine/threonine-protein kinase WNK1	Uniprot: Q9H4A3	″	″	″	″
A-kinase anchor protein 12	Uniprot: Q02952	CysNO-treated RAW264.7 macrophages	″	″	″
Mitogen-activated protein kinase 3/1	Uniprot: P63085	″	″	″	″
Phosphatidylethanolamine-binding protein	Uniprot: P17918	″	″	″	″
Serine/threonine-protein phosphatase 2A	Uniprot: Q76MZ3	″	″	″	″
14-3-3 protein α/β	Uniprot: P31946	Endothelial cells (EA.hy296 and HUVEC), macrophage cells (Raw264.7)	S-nitrosylation	Fluorescence switch method using thiol labeling by fluorescein-5 maleimide combined with 2-DE	307
14-3-3 protein ζ/δ	Uniprot: P63104	″	″	″	″
14-3-3 protein ε	Uniprot: P62258	″	″	″	″
Leucine-rich repeat protein SHOC-2	Uniprot: Q9UQ13	Intact human spermatozoa lysed after treatment with different NO donors	S-nitrosylation	Biotin-switch assay combined with 1-DE and LC-MS/MS	180
Phosphoinositide 3-kinase regulatory subunit 5	Uniprot: Q8WYR1	″	″	″	″
Protein kinase A-anchoring protein 3	Uniprot: O75969	″	″	″	″
Protein kinase A-anchoring protein 4	Uniprot: Q5JQC9	″	″	″	″
Protein kinase A-anchoring protein 9	Uniprot: Q99996	″	″	″	″
14-3-3 protein ζ/δ	Uniprot: P63104	″	″	″	″
FKBP12-rapamycin complex associated protein	Uniprot: P42345	″	″	″	″
Ras GTPase-activating-like protein IQGAP2	Uniprot: Q13576	″	″	″	″
Ras GTPase-activating protein 4	Uniprot: O43374	″	″	″	″
Ras-related protein Rab-2A	Uniprot: P61019	″	″	″	″
Ras-related protein Rab-14	Uniprot: P61106	″	″	″	″
Ribosome-binding protein 1	Uniprot: Q9P2E9	″	″	″	″
Signal-regulatory protein gamma precursor	Uniprot: Q9P1W8	″	″	″	″

(*continued*)

Table 4B. (Continued)

	Accession	*Experimental system*	*Cysteine modification*	*Proteomics approach*	*Reference*
Aspartyl-hydroxylase	—	HeLa cells	Sulfenation	*In vivo* labeling using DAz-2 followed by a Staudinger ligation	189
Cardiotrophin-like cytokine factor 1	—	"	"	"	"
IQGAP1	—	"	"	"	"
G protein, polypeptide 2	—	"	"	"	"
PI-3-kinase, catalytic subunit	—	"	"	"	"
Protein GPR107	—	"	"	"	"
Protein phosphatase PP1, catalytic subunit	—	"	"	"	"
SH2 domain-containing adapter protein F	—	"	"	"	"
Mammalian tissues					
protein-tyrosine phosphatase 1B	—	Intact rat hearts	*S*-thiolation	*S*-thiolation with biotinylated cysteine.	76
protein kinase Cα	—	Intact rat hearts	*S*-thiolation	*S*-thiolation with biotinylated cysteine.	76
Ser/thr protein phosphatase 2A	Uniprot: P36876	Ventricular myocytes isolated from rat hearts	Intermolecular disulfide	Diagonal nonreducing/reducing 2-D SDS-PAGE	33
phosphorylase B kinase beta regulatory	Uniprot P12798	Rabbit heart muscle cellular membranes	Reversible cysteine oxidation, unspecified	Alkylation with IAM-linked ICAT reagent	288
Rab GDP dissociation inhibitor beta chain	Uniprot: Q61598	Soluble murine heart protein extracts treated with H_2O_2	Reversible cysteine oxidation, unspecified	ICAT and fluorescence-DIGE using Cy3- and Cy5-maleimide	95
Rab GDP dissociation inhibitor beta	Uniprot: Q61598	Transgenic mice with heart-specific over-expression of Trx1	Oxidized cysteines reduced by Trx1	ICAT and independent analysis with iTRAQ for protein quantification	96
WD repeat domain 1	NCBI: NP_001014157	Rat kidney medulla	Sulfenation	Arsenite reduction and biotin-maleimide labeling	311
Mammalian organelles					
FEM-1-like death receptor binding protein	Uniprot: Q9UK73	Purified mitochondria from HEK 293 cells	Cysteines susceptible to alkylation with IAB or BMCC	Labeling with the biotinylated reagents IAB and BMCC	328
FK506-binding protein 8variant	Uniprot: Q53GU3	"	"	"	"
38 kDa FK-506 binding protein homolog	Uniprot: Q14318	"	"	"	"
programmed cell death 6-interacting protein	Uniprot: Q8WUM4	"	"	"	"

(*continued*)

TABLE 4B. (CONTINUED)

	Accession	*Experimental system*	*Cysteine modification*	*Proteomics approach*	*Reference*
programmed cell death protein 8, mitochondrial precursor	Uniprot: O95831	"	"	"	"
Fungi					
regulatory subunit B of protein phosphatase 2A	—	Wild type yeast cells	Redox-active surface-exposed cysteine	BIAM labeling after reduction by TCEP, TRX1 and TTR1; 1-DE combined with MS/MS and LC-MS/MS	216
BMH1 14-3-3 protein	—	"	"	"	"
CYR1 adenylate cyclase	—	"	"	"	"
FPR1 (rapamycin-binding)	—	"	"	"	"
GIS2 (zinc-finger protein)	—	"	"	"	"
SHE3 (mRNA-binding protein)	—	"	"	"	"
Plants					
Arg-methyltransferase	—	Extracts of seeds of *Medicago truncatula*	Reversible cysteine oxidation, unspecified	mBBr labeling combined with 2-DE, and Trx affinity chromatography *in vitro* using a monocysteinic Trx *h*3 of pea	4
ADP-ribosyltransferase	—	"	"	"	"
GTP binding protein	—	"	"	"	"
RNA binding protein	—	"	"	"	"
14-3-3 protein	—	"	"	"	"
Transducin (putative)	TAIR: At5g19920	Arabidopsis cell suspension cultures	S-thiolation	GSSG-biotin labeling combined with strepatavidin chromatography and 2-DE	71
Guanine nucleotide-binding protein, β subunit-like protein	TAIR: At1g18080	Arabidopsis roots, extracts of soluble proteins	Reversible cysteine oxidation, unspecified	Trx affinity chromatography *in vitro* using a monocysteinic Trx *y*2 of Arabidopsis combined with 2-DE	212
Major latex protein 34	TAIR: At1g70850				
14-3-3 Like protein	TAIR: At5g10450				
Translationally controlled tumor protein-like protein	TAIRAt3g16640				

(*continued*)

Table 4B. (Continued)

	Accession	*Experimental system*	*Cysteine modification*	*Proteomics approach*	*Reference*
14-3-3 protein	TAIR: At5g65430	Leaves of Arabidopsis and poplar, chloroplast and mitochondria from pea, potato tubers	Cysteines susceptible to formation of mixed disulfides	Trx affinity chromatography *in vitro* using a monocysteinic GrxC4 from poplar	273
nucleoside diphosphate kinase 1	—	Arabidopsis seedlings	Reversible cysteine oxidation, unspecified	2-DE combined with mBBr labeling and SyproRuby staining	6
Annexin	NCBI: CAA67608	Leaves and cell suspension cultures of Arabidopsis	S-nitrosylation	Biotin switch combined with 1-DE and 2-DE	199
Allene oxide cyclase	NCBI: 34391982	Arabidopsis leaf extracts	S-nitrosylation	Biotin switch combined with 2-DE	272
Bacteria					
cAMP-regulatory protein		Wild type *E. coli*	Reversible cysteine oxidation, unspecified	ATS chromatography	138

from Arabidopsis cell cultures (71). However, since nondenaturing conditions were applied, the authors mention that thiolation of one subunit might well result in the purification of the entire complex. The ATP-dependent subunits of the prokaryotic Clp protease, ClpC, and ClpB, have been identified as targets for Trx or Grx in plant chloroplasts (12, 273) and in cyanobacteria (223). Yet another prokaryotic ATP-dependent protease belonging to the AAA-family of ATPases, the membrane-bound FtsH, has also been identified as a Trx target protein in chloroplasts (241) and cyanobacteria (223). These findings suggest a conservation of redox-controlled proteolysis in photosynthetic organisms (198). The proteases Deg1 and Deg5 in the plant chloroplast thylakoid lumen were found to be targets for Trx (112). Interestingly, experiments have shown that reducing conditions favor the degradation of certain proteins in this compartment, whereas other proteins remain stable (112). Redox-dependent proteolysis has also been observed during different stages of seed development (337, 340). Hence, redox-dependent protein degradation appears to be a well-extended phenomenon in plants.

V. Concluding Remarks

The regulation of key enzymes in metabolic pathways through intricate mechanisms is usually illustrated by numerous examples in the standard biochemical textbooks. However, the disulfide proteomes have taught us that entire pathways, such as glycolysis, the TCA cycle, and the Calvin-Benson cycle, are controlled by mechanisms involving changes in the cysteine redox state of each enzyme implicated. A new paradigm is thus emerging, in which the cysteine redox state of these enzymes functions as a master switch to turn the process on and off, probably to prevent wasteful use of certain compounds, for example, NADPH or ATP. It has been demonstrated that the ambient oxygen is critical for the redox state of protein cysteines *in vivo* on a global scale in many organisms, such as Jurkat T lymfocytes (205), yeast cells (187), and *E. coli* (182), and that under low-oxygen conditions cysteine oxidation is hardly observed.

Frequently, the same proteins are found in more than one kind of disulfide proteome. This has previously been observed for a number of proteins, for example, Prx, cytoskeletal proteins, chaperones, and glycolytic enzymes (99), and most likely reflects the fact that some reactive cysteines may undergo more than one type of oxidative modification, such as S-nitrosylation, glutathionylation, sulfenic acid formation, and disulfide formation. However, the analytical procedures used in most studies reported, for example, the biotin switch method and labeling with fluorescent or radioactive probes, do not usually allow the identification of the cysteine residues that undergo modification. Only differential alkylation with mass-coded tags, such as the ICAT-based methods, can pinpoint the precise cysteine residues that are modified.

An attractive possibility for future studies would be the search for biomarkers of diseases among the members of disulfide proteomes. In this context it is worth mentioning that patients suffering from schizophrenia have higher expression levels of some glycolytic enzymes in different brain tissues, and yet the pyruvate levels are lower than in healthy controls (220, 221). This might be explained by oxidative inactivation of these enzymes and would encourage the analysis of the cysteine redox state of glycolytic enzymes in patients and controls.

So far, the disulfide proteomes have not been successfully employed in defining new redox signaling pathways, in the sense of "cascades of redox reactions, eventually leading to

changes in gene expression," as stated at the beginning of this review. Although, a relatively large number of proteins that fulfil functions in cellular signaling have been identified (Table 4), proteins such as protein kinases, protein phosphatases, and nucleotide-binding proteins are under-represented in individual studies of disulfide proteomes, and are often missing altogether. Nevertheless, protein phosphorylation and transcriptional regulation have long been known to be governed by redox modifications of cysteines (283). For example, the protein kinase C family of protein kinases contains redox-active cysteines in both the regulatory domain and the catalytic domain (104), and the protein tyrosine phosphatase (PTP) 1B is reversibly inactivated by cysteine oxidation (179). The reason why these enzymes are rarely found in disulfide proteomes relates to their low abundance and the limited dynamic range in most of the reported studies on disulfide proteomes. Indeed, the studies that include proteins involved in signal transduction are also those with the highest numbers of proteins identified. For example, of the 240 S-nitrosylated proteins in human spermatozoa treated with GSNO, 9 were protein kinases and 13 were other proteins involved in signal transduction (180). In the complete list of 189 cysteine sulfenic acid-containing HeLa proteins, 15 proteins were related to signaling (189). Despite the extensive coverage in these studies, proteins that function in cellular signaling were a minority. Among the sulfenated HeLa proteins, 8% were involved in signaling, as compared to 22% cytoskeletal proteins, 21% involved in protein synthesis, and 16% metabolic enzymes (189). Thus, future studies on disulfide proteomes aimed at the discovery of new mechanisms for redox signaling might benefit from the selective enrichment of certain classes of proteins before applying disulfide proteomics procedures. For example, kinases might be enriched on a γ-phosphate-linked ATP resin (154) and nucleotide-binding proteins could be enriched using heparin Sepharose chromatography (280) to tackle the problems related to the dynamic range and detection limits. To our knowledge, isolated nuclei have not so far had their disulfide proteomes examined, despite evidence for extensive redox regulation in this compartment (103). Neither has the plastid organellar genome, the nucleoid, yet been subjected to disulfide proteome analysis. However, the presence of a new chloroplast Trx (228) in the plastid RNA polymerase supercomplex isolated from heparin Sepharose chromatography (280) is encouraging. We therefore believe that in the near future reports on disulfide proteomes from animals as well as plant sources will shed new light on the redox regulation of transcription, and perhaps chromatin organization. Table 4 gives an overview of currently identified members of disulfide proteomes involved in signal transduction and transcriptional regulation.

Apart from improvement of disulfide proteomics techniques, to find the exciting proteins that we believe should be present, ongoing biochemical and genetic research continues the validation of those proteins already identified and the testing of their possible physiological redox regulation. However, there are classes of proteins that are reproducibly found in disulfide proteomes, but which are surprisingly overlooked when it comes to validation. Despite the widespread prevalence of cytoskeletal proteins in disulfide proteomes, these proteins have only just started to attract the attention of scientists in the context of redox control. Thus, the redox regulation of actin polymerization and the glutathionylation-dependent disassembly of the actinomyosin complex during cell adhesion have only recently been reported (82, 318).

The title of section III in this review "Disulfide proteomes in all kingdoms of life" is not entirely accurate. Archaea have been predicted to be rich in intracellular proteins forming disulfide bonds based on a combination of genomic and bioinformatic analyses involving structural modeling (209); moreover, Trx are ubiquitous in these organisms as well (175). The existence of a Trx-like protein disulfide oxidoreductase (PDO) in archaea, such as *Pyrococcus furiosus* and *Sulfolobus solfataricus*, further raises the expectations that reversible cysteine modifications might occur in these organisms (168). However, to our knowledge, disulfide proteomic studies have not been performed in this third superkingdom. Among the eukaryotes, some common model organisms have not yet been analyzed with respect to their disulfide proteomes, for example, *Schizosaccharomyces pombe* (fission yeast), *Caenorhabditis elegans* and Zebrafish. Hence, it is likely that in the future we will learn about the disulfide proteomes in other organisms, some of which share similarities with us, whereas others are extremely distantly related.

Disulfide proteomics, in conjunction with classical biochemistry and genetics, has a significant potential to contribute to new discoveries on redox regulation and redox signaling. In conclusion, the disulfide proteomes have come to stay and will accompany us in our continuous efforts to learn how cells function and adapt to their environment.

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References

1. Ago T and Sadoshima J. Thioredoxin 1 as a negative regulator of cardiac hypertrophy. *Antioxid Redox Signal* 9: 679–687, 2007.
2. Ahsan MK, Lekli I, Ray D Yodoi J, and Das DK. Redox regulation of cell survival by the thioredoxin superfamily: an implication of redox gene therapy in the heart. *Antioxid Redox Signal* 11: 2741–2758, 2009.
3. Akkaya MS and Breitenberger CA. Light regulation of protein synthesis factor EF-G in pea chloroplasts. *Plant Mol Biol* 20: 791–800, 1992.
4. Alkhalfioui F, Renard M, Vensel WH, Wong J, Tanaka CK, Hurkman WJ, Buchanan BB, and Montrichard F. Thioredoxin-linked proteins are reduced during germination of *Medicago truncatula* seeds. *Plant Physiol* 144: 1559–1579, 2007.
5. Alvarez S, Wilson GH, and Chen S. Determination of *in vivo* disulfide-bonded proteins in Arabidopsis. *J Chromatogr B* 877: 101–104, 2009.
6. Alvarez S, Zhu M, and Chen S. Proteomics of Arabidopsis redox proteins in response to methyl jasmonate. *J Proteomics* 73: 30–40, 2009.
7. Apel K and Hirt H. Reactive oxygen species: metabolism, oxidative stress and signal transduction. *Annu Rev Plant Biol* 55: 373–379, 2004.

8. Åslund F, Zheng M, Beckwith J, and Storz G. Regulation of the OxyR transcription factor by hydrogen peroxide and the cellular thiol–disulphide status. *Proc Natl Acad Sci USA* 96: 6161–6165, 1999.
9. Bailey SM, Landar A, and Darley-Usmar V. Mitochondrial proteomics in free radical research. *Free Radic Biol Med* 38: 175–188, 2005.
10. Bally J, Paget E, Droux M, Job C, Job D, and Dubald M. Both the stroma and thylakoid lumen of tobacco chloroplasts are competent for the formation of disulphide bonds in recombinant proteins. *Plant Biotechnol* J 6: 46–61, 2008.
11. Balmer Y and Buchanan BB. Yet another plant thioredoxin. *Trends Plant Sci* 7: 191–193, 2002.
12. Balmer Y, Koller A, del Val G, Manieri W, Schürmann P, and Buchanan BB. Proteomics gives insight into the regulatory function of chloroplast thioredoxins. *Proc Natl Acad Sci USA* 100: 370–375, 2003.
13. Balmer Y, Koller A, Val GD, Schürmann P, and Buchanan BB. Proteomics uncovers proteins interacting electrostatically with thioredoxin in chloroplasts. *Photosynth Res* 79: 275–280, 2004.
14. Balmer Y, Vensel WH, Tanaka CK, Hurkman WJ, Gelhaye E, Rouhier N, Jacquot J-P, Manieri W, Schürmann P, Droux M, and Buchanan BB. Thioredoxin links redox to the regulation of fundamental processes of plant mitochondria. *Proc Natl Acad Sci USA* 101: 2642–2647, 2004.
15. Balmer Y, Vensel WH, Hurkman WJ, and Buchanan BB. Thioredoxin target proteins in chloroplast thylakoid membranes. *Antioxid Redox Signal* 8: 1829–1834, 2006.
16. Balmer Y, Vensel WH, DuPont FM, Buchanan BB, and Hurkman WJ. Proteome of amyloplasts isolated from developing wheat endosperm presents evidence of broad metabolic capability. *J Exp Bot* 57: 1591–1602, 2006.
17. Balmer Y, Vensel WH, Cai N, Manieri W, Schürmann P, Hurkman WJ, and Buchanan BB. A complete ferredoxin/thioredoxin system regulates fundamental processes in amyloplasts. *Proc Natl Acad Sci USA* 103: 2988–2993, 2006.
18. Balsera M, Goetze TA, Kovács-Bogdán E, Schürmann P, Wagner R, Buchanan BB, Soll J, and Bölter B. Characterization of Tic110, a channel-forming protein at the inner envelope membrane of chloroplasts, unveils a response to Ca(2+) and a stromal regulatory disulfide bridge. *J Biol Chem* 284: 2603–2616, 2009.
19. Bartsch S, Monnet J, Selbach K, Quigley F, Gray J, von Wettstein D, Reinbothe S, and Reinbothe C. Three thioredoxin targets in the inner envelope membrane of chloroplasts function in protein import and chlorophyll metabolism. *Proc Natl Acad Sci USA* 105: 4933–4938, 2008.
20. Baty JW, Hampton MB, and Winterbourn CC. Detection of oxidant sensitive thiol proteins by fluorescence labeling and two-dimensional electrophoresis. *Proteomics* 2: 1261–1266, 2002.
21. Baty JW, Hampton MB, and Winterbourn CC. Proteomic detection of hydrogen peroxide-sensitive thiol proteins in Jurkat cells. *Biochem J* 389: 785–795, 2005. Erratum in: *Biochem J* 390: 791–792, 2005.
22. Bedhomme M, Zaffagnini M, Marchand CH, Gao XH, Moslonka-Lefebvre, Michelet L, Decottignies P, and Lemaire SD. Regulation by glutathionylation of citrate lyase from *Chlamydomonas reinhardtii*. *J Biol Chem* 284: 36282–36291, 2009.
23. Bellomo G, Thor H, and Orrenius S. Modulation of cellular glutathione and protein thiol status during quinone metabolism. *Methods Enzymol* 186: 627–635, 1990.
24. Benitez LV and Allison WS. The inactivation of the acyl phosphatase activity catalyzed by the sulfenic acid form of glyceraldehyde 3-phosphate dehydrogenase by dimedone and olefins. *J Biol Chem* 249: 6234–6243, 1974.
25. Benhar M, Forrester MT, Hess DT, and Stamler JS. Regulated protein denitrosylation by cytosolic and mitochondrial thioredoxins. *Science* 320: 1050–1054, 2008.
26. Benhar M, Forrester MT, and Stamler JS. Protein denitrosylation: enzymatic mechanisms and cellular functions. *Nat Rev Mol Cell Biol* 10: 721–732, 2009.
27. Bhattacharjee A, Majumdar U, Maity D, Sarkar TS, Goswami AM, Sahoo R, and Ghosh S. *In vivo* protein tyrosine nitration in *S. cerevisiae*: identification of tyrosine-nitrated proteins in mitochondria. *Biochem Biophys Res Commun* 388: 612–617, 2009.
28. Biteau B, Labarre J, and Toledano MB. ATP-dependent reduction of cysteine-sulphinic acid by S. cerevisiae sulphiredoxin. *Nature* 425: 980–984, 2003.
29. Boivin B, Zhang S, Arbiser JL, Zhang Z-Y, and Tonks NK. A modified cysteinyl-labeling assay reveals reversible oxidation of protein tyrosine phosphatases in angiomyolipoma cells. *Proc Natl Acad Sci USA* 105: 9959–9964, 2008.
30. Brandes N, Rinck A, Leichert LI, and Jakob U. Nitrosative stress treatment of *E. coli* targets distinct set of thiol-containing proteins. *Mol Microbiol* 66: 901–914, 2007.
31. Brandes N, Schmitt S, and Jakob U. Thiol-based redox switches in eukaryotic proteins. *Antioxid Red Signal* 11: 997–1014, 2009.
32. Brandes RP, Weissmann N, and Schröder K. NADPH oxidases in cardiovascular disease. *Free Radic Biol Med* 49: 687–706, 2010.
33. Brennan JP, Wait R, Begum S, Bell JR, Dunn MJ, and Eaton P. Detection and mapping of widespread intermolecular protein disulphide formation during cardiac oxidative stress using proteomics with diagonal electrophoresis. *J Biol Chem* 279: 41352–41360, 2004.
34. Brennan JP, Miller JIA, Fuller W, Wait R, Begum S, Dunn MJ, and Eaton P. The utility of *N*,*N*-biotinyl glutathione disulfide in the study of protein *S*-glutathiolation. *Mol Cell Proteomics* 5: 215–225, 2006.
35. Brewer CF and Riehm JP. Evidence for possible nonspecific reactions between *N*-ethylmaleimide and proteins. *Anal Biochem* 18: 248–255, 1967.
36. Buchanan BB and Wolosiuk RA. Photosynthetic regulatory protein found in animal and bacterial cells. *Nature* 264: 669–670, 1976.
37. Buchanan BB. Role of light in the regulation of chloroplast enzymes. *Annu Rev Plant Physiol* 31: 341–374, 1980.
38. Buchanan BB, Schürmann P, Wolosiuk RA, and Jacquot JP. The ferredoxin/thioredoxin system: from discovery to molecular structures and beyond. *Photosynth Res* 73: 215–222, 2002.
39. Buchanan BB and Balmer Y. Redox regulation: a broadening horizon. *Annu Rev Plant Biol* 56: 187–220, 2005.
40. Buchanan BB and Luan S. Redox regulation in the chloroplast thylakoid lumen: a new frontier in photosynthesis research. *J Exp Bot* 56: 1439–1447, 2005.
41. Budanov AV, Sablina AA, Feinstein E, Koonin EV, and Chumakov PM. Regeneration of peroxiredoxins by p53-regulated sestrins, homologs of bacterial AhpD. *Science* 304: 596–600, 2004.
42. Burns JA, Butler JC, Moran J, and Whitesides GM. Selective reduction of disulfides by tris(2-carboxyethyl)phosphine. *J Org Chem* 56: 2648–2650, 1991.

43. Cabiscol E, Piulats E, Echave P, Herrero E, and Ros J. Oxidative stress promotes specific protein damage in *Saccharomyces cerevisiae*. *J Biol Chem* 275: 27393–27398, 2000.
44. Cadenas E and Davies KJ. Mitochondrial free radical generation, oxidative stress, and aging. *Free Radic Biol Med* 29: 222–230, 2000.
45. Cain P, Hall M, Schröder WP, Kieselbach T, and Robinson C. A novel extended family of stromal thioredoxins. *Plant Mol Biol* 70: 273–281, 2009.
46. Caporaletti D, D'Alessio AC, Rodriguez-Suarez RJ, Senn AM, Duek PD, and Wolosiuk RA. Non-reductive modulation of chloroplast fructose-1,6-bisphosphatase by 2-Cys peroxiredoxin. *Biochem Biophys Res Commun* 355: 722–727, 2007.
47. Chae HZ, Chung SJ, and Rhee SG. Thioredoxin-dependent peroxide reductase from yeast. *J Biol Chem* 269: 27670–27678, 1994.
48. Chae HZ, Robison K, Poole LB, Church G, Storz G, and Rhee SG. Cloning and sequencing of thiol-specific antioxidant from mammalian brain: alkyl hydroperoxide reductase and thiol-specific antioxidant define a large family of antioxidant enzymes. *Proc Natl Acad Sci USA* 91: 7017–7021, 1994.
49. Charles RL, Schröder E, May G, Free P, Gaffney PR, Wait R, Begum S, Heads RJ, and Eaton P. Protein sulfenation as a redox sensor: proteomics studies using a novel biotinylated dimedone analogue. *Mol Cell Proteomics* 6: 1473–1484, 2007.
50. Chibani K, Wingsle G, Jacquot JP, Gelhaye E, and Rouhier N. Comparative genomic study of the thioredoxin family in photosynthetic organisms with emphasis on *Populus trichocarpa*. *Mol Plant* 2: 308–322, 2009.
51. Choi HI, Lee SP, Kim KS, Hwang CY, Lee YR, Chae SK, Kim YS, Chae HZ, and Kwon KS. Redox-regulated cochaperone activity of the human DnaJ homolog Hdj2. *Free Radic Biol Med* 40: 651–659, 2006.
52. Choi S, Jeong J, Na S, Lee HS, Kim HY, Lee KJ, and Paek E. New algorithm for the identification of intact disulfide linkages based on fragmentation characteristics in tandem mass spectra. *J Proteome Res* 9: 626–635, 2010.
53. Christman MF, Morgan RW, Jacobson FS, and Ames BN. Positive control of a regulon for defenses against oxidative stress and some heat-shock proteins in Salmonella typhimurium. *Cell* 41: 753–762, 1985.
54. Cleland WW. Dithiothreitol, a new protective reagent for SH groups. *Biochemistry* 3: 480–482, 1964.
55. Collin V, Lamkemeyer P, Miginiac-Maslow M, Hirasawa M, Knaff DB, Dietz KJ, Issakidis-Bourguet E. Characterization of plastidial thioredoxins from Arabidopsis belonging to the new y-type. *Plant Physiol* 136: 4088–4095, 2004.
56. Cooper CE, Patel RP, Brookes PS, and Darley-Usmar VM. Nanotransducers in cellular redox signaling: modification of thiols by reactive oxygen and nitrogen species. *Trends Biochem Sci* 27: 489–492, 2002.
57. Cotgreave IA, Gerdes R, Schuppe-Koistinen I, and Lind C. S-glutathionylation of glyceraldehyde-3-phosphate dehydrogenase: role of thiol oxidation and catalysis by glutaredoxin. *Methods Enzymol* 348:175–182, 2002.
58. Cumming RC, Andon NL, Haynes PA, Park M, Fischer WH, and Schubert D. Protein disulphide bond formation in the cytoplasm during oxidative stress. *J Biol Chem* 279: 21749–21758, 2004.
59. Cumming RC and Schubert D. Amyloid-β induces disulfide bonding and aggregation of GAPDH in Alzheimer's disease. *FASEB J* 19: 2060–2062, 2005.
60. Cumming RC. Analysis of global and specific changes in the disulphide proteome using redox two-dimensional polyacrylamide gel electrophoresis. *Methods Mol Biol* 476: 165–179, 2008.
61. Dalle-Donne I, Rossi R, Colombo G, Giustarini D, and Milzani A. Protein S-glutathionylation: a regulatory device from bacteria to humans. *Trends Biochem Sci* 34: 85–96, 2009.
62. Darlison MG and Guest JR. Nucleotide sequence encoding the iron-sulphur protein subunit of the succinate dehydrogenase of *Escherichia coli*. *Biochem J* 223: 507–517, 1984.
63. D'Autréaux B and Toledano MB. ROS as signalling molecules: mechanisms that generate specificity in ROS homeostasis. *Nat Rev Mol Cell Biol* 8: 813–824, 2007.
64. Delaunay A, Pflieger D, Barrault, MB, Vinh J, and Toledano MB. A thiol peroxidase is an H_2O_2 receptor and a redox-transducer in gene activation. *Cell* 111: 471–481, 2002.
65. Delledonne M. NO news is good news for plants. *Curr Opin Plant Biol* 8: 390–396, 2005.
66. Delobel A, Graciet E, Andreescu S, Gontero B, Halgand F, and Laprévote O. Mass spectrometric analysis of the interactions between CP12, a chloroplast protein, and metal ions: a possible regulatory role within a PRK/GAPDH/CP12 complex. *Rapid Commun Mass Spectrom* 19: 3379–3388, 2005.
67. Derakhshan B, Wille PC, and Gross SS. Unbiased identification of cysteine S-nitrosylation sites on proteins. *Nat Protoc* 2: 1685–1691, 2007.
68. Dietz L, Bosque A, Pankert P, Ohnesorge S, Merz P, Anel A, Schnölzer M, and Thierse HJ. Quantitative DY-maleimide-based proteomic 2-DE-labeling strategies using human skin proteins. *Proteomics* 18: 4298–4308, 2009.
69. Dimmeler S, Fleming I, Fisslthaler B, Hermann C, Busse R, and Zeiher AM. Activation of endothelial nitric oxide synthase in endothelial cells by Akt-dependent phosphorylation. *Nature* 399: 601–605, 1999.
70. Dinman JD. The eukaryotic ribosome: current status and challenges. *J Biol Chem* 284: 11761–11765, 2009.
71. Dixon DP, Skipsey M, Grundy NM, and Edwards R. Stress-induced protein S-glutathionylation in Arabidopsis. *Plant Physiol* 138: 2233–2244, 2005.
72. Donald LJ, Crane BR, Anderson DH, and Duckworth HW. The role of cysteine 206 in allosteric inhibition of *Escherichia coli* citrate synthase. Studies by chemical modification, site-directed mutagenesis and 19F NMR. *J Biol Chem* 266: 20709–20713, 1991.
73. Doulias PT, Greene JL, Greco TM, Tenopoulou M, Seeholzer SH, Dunbrack RL, and Ischiropoulos H. Structural profiling of endogenous S-nitrosocysteine residues reveals unique features that accommodate diverse mechanisms for protein S-nitrosylation. *Proc Natl Acad Sci USA* 107: 16958–16963, 2010.
74. Ducrocq C, Blanchard B, Pignatelli B, and Ohshima H. Peroxynitrite: an endogenous oxidizing and nitrating agent. *Cell Mol Life Sci* 55: 1068–1077, 1999.
75. Dupont FM. Metabolic pathways of the wheat (*Triticum aestivum*) endosperm amyloplast revealed by proteomics. *BMC Plant Biol* 8: 39, 2008.
76. Eaton P, Byers HL, Leeds N, Ward MA, and Shattock MJ. Detection, quantitation, purification, and identification of cardiac proteins S-thiolated during ischemia and reperfusion. *J Biol Chem* 277: 9806–9811, 2002.
77. Eaton P and Shattock MJ. Purification of proteins susceptible to oxidation at cysteine residues: identification of

malate dehydrogenase as a target for S-glutathiolation. *Ann N Y Acad Sci* 973: 529–532, 2002.
78. Eaton P, Jones ME, McGregor E, Dunn MJ, Leeds N, Byers HL, Leung K-Y, Ward MA, Pratt JR, and Shattock MJ. Reversible cysteine-targeted oxidation of proteins during renal oxidative stress. *J Am Soc Nephrol* 14: S290–S296, 2003.
79. Ejiri S. Moonlighting functions of polypeptide elongation factor 1: From actin bundling to zinc finger protein R1-associated nuclear localization. *Biosci Biotechnol Biochem* 66: 1–21, 2002.
80. Fermani S, Sparla F, Falini G, Martelli PL, Casadio R, Pupillo P, Ripamonti A, and Trost P. Molecular mechanism of thioredoxin regulation in photosynthetic A2B2-glyceraldehyde-3-phosphate dehydrogenase. *Proc Natl Acad Sci USA* 104: 11109–11114, 2007.
81. Ferro M, Brugière S, Salvi D, Seigneurin-Berny D, Court M, Moyet L, Ramus C, Miras S, Mellal M, Le Gall S, Kieffer-Jaquinod S, Bruley C, Garin J, Joyard J, Masselon C, and Rolland N. AT_CHLORO, a comprehensive chloroplast proteome database with subplastidial localization and curated information on envelope proteins. *Mol Cell Proteomics* 9: 1063–1084, 2010.
82. Fiaschi T, Cozzi G, Raugei G, Formigli L, Ramponi G, and Chiarugi P. Redox regulation of β-actin during integrin-mediated cell adhesion. *J Biol Chem* 281: 22983–22991, 2006.
83. Finnie C and Svensson B. Barley seed proteomics from spots to structures. *J. Proteomics* 72: 315–324, 2009.
84. Florencio FJ, Pérez-Pérez ME, López-Maury L, Mata-Cabana A, and Lindahl M. The diversity and complexity of the cyanobacterial thioredoxin systems. *Photosynth Res* 89: 157–171, 2006.
85. Forman HJ, Torres M, and Fukuto J. Redox signalling. *Mol Cell Biochem* 234/235: 49–62, 2002.
86. Forman HJ, Maiorino M, and Ursini F. Signaling functions of reactive oxygen species. *Biochemistry* 49: 835–842, 2010.
87. Forrester MT, Foster MW, Stamler JS. Assessment and application of the biotin switch technique for examining protein S-nitrosylation under conditions of pharmacologically induced oxidative stress. *J Biol Chem* 282: 13977–13983, 2007.
88. Forrester MT, Thompson JW, Foster MW, Nogueira L, Moseley MA, and Stamler JS. Proteomic analysis of S-nitrosylation and denitrosylation by resin-assisted capture. *Nat Biotechnol* 27: 557–579, 2009.
89. Foster MW and Stamler JS. New insights into protein *S*-nitrosylation. Mitochondria as a model system. *J Biol Chem* 279: 25891–25897, 2004.
90. Foyer CH and Noctor G. Redox regulation in photosynthetic organisms: signaling, acclimation and practical implications. *Antioxid Redox Signal* 11: 861–905, 2009.
91. Fraser ME, James MN, Bridger WA, and Wolodko WT. A detailed structural description of *Escherichia coli* succinyl-CoA synthetase. *J Mol Biol* 285: 1633–1653, 1999.
92. Fratelli M, Demol H, Puype M, Casagrande S, Eberini I, Salmona M, Bonetto V, Mengozzi M, Duffieux F, Miclet E, Bachi A, Vandekrckhove J, Gianazza E, and Ghezzi P. Identification by redox proteomics of glutathionylated proteins in oxidatively stressed human T lymphocytes. *Proc Natl Acad Sci USA* 99: 3505–3510, 2002.
93. Fratelli M, Demol H, Puype M, Casagrande S, Villa P, Eberini I, Vandekerckhove J, Gianazza E, and Ghezzi P. Identification of proteins undergoing glutathionylation in oxidatively stressed hepatocytes and hepatoma cells. *Proteomics* 3: 1154–1161, 2003.
94. Fridlyand LE, Scheibe R. Regulation of the Calvin cycle for CO2 fixation as an example for general control mechanisms in metabolic cycles. *Biosystems* 1999 51: 79–93, 1999.
95. Fu C, Hu J, Liu T, Ago T, Sadoshima J, and Li H. Quantitative analysis of redox-sensitive proteome with DIGE and ICAT. *J Proteome Res* 7: 3789–3802, 2008.
96. Fu C, Wu C, Liu T, Ago T, Zhai P, Sadoshima J, and Li H. Elucidation of thioredoxin target protein networks in mouse. *Mol Cell Proteomics* 8: 1674–1687, 2009.
97. Gao XH, Bedhomme M, Veyel D, Zaffagnini M, and Lemaire SD. Methods for analysis of protein glutathionylation and their application to photosynthetic organisms. *Mol Plant* 2: 218–235, 2009.
98. Gfeller A, Liechti R, and Farmer EE. Arabidopsis jasmonate signaling pathway. *Sci Signal* 3: cm4, 2010.
99. Ghezzi P, Bonetto V, and Fratelli M. Thiol-disulfide balance: from the concept of oxidative stress to that of redox regulation. *Antioxid Redox Signal* 7: 964–972, 2005.
100. Giustarini D, Dalle-Donne I, Colombo R, Milzani A, and Rossi R. Is ascorbate able to reduce disulfide bridges? A cautionary note. *Nitric Oxide* 19: 252–258, 2008.
101. Gleason FK and Holmgren A. Isolation and characterization of thioredoxin from the cyanobacterium, *Anabaena* sp. *J Biol Chem* 256: 8306–8309, 1981.
102. Gleason FK and Holmgren A. Thioredoxin and related proteins in prokaryotes. *FEMS Microbiol Rev* 4: 271–297, 1988.
103. Go Y-M and Jones DP. Redox control systems in the nucleus: mechanisms and functions. *Antioxid Redox Signal* 13: 489–509, 2010.
104. Gopalakrishna R and Jaken S. Protein kinase C signalling and oxidative stress. *Free Radic Biol Med* 28: 1349–1361, 2000.
105. Gopalan G, He Z, Balmer Y, Romano P, Gupta R, Héroux A, Buchanan BB, Swaminathan K, and Luan S. Structural analysis uncovers a role for redox in regulating FKBP13, an immunophilin of the chloroplast thylakoid lumen. *Proc Natl Acad Sci USA* 101: 13945–13950, 2004.
106. Graciet E, Gans P, Wedel N, Lebreton S, Camadro JM, and Gontero B. The small protein CP12: a protein linker for supramolecular complex assembly. *Biochemistry* 42: 8163–8170, 2003.
107. Greco TM, Hodara R, Parastatidis I, Heijnen HF, Dennehy MK, Liebler DC, and Ischiropoulos H. Identification of S-nitrosylation motifs by site-specific mapping of the S-nitrosocysteine proteome in human vascular smooth muscle cells. *Proc Natl Acad Sci USA* 103: 7420–7425, 2006.
108. Gygi SP, Rist B, Gerber SA, Turecek F, Gelb MH, and Aebersold R. Quantitative analysis of complex protein mixtures using isotope-coded affinity tags. *Nat Biotechnol* 17: 994–999, 1999.
109. Hägglund P, Bunkenborg J, Maeda K, and Svensson B. Identification of thioredoxin disulphide targets using a quantitative proteomics approach based on isotope-coded affinity tags. *J Proteome Res* 7: 5270–5276, 2008.
110. Hajheidari M, Eivazi A, Buchanan BB, Wong JH, Majidi I, and Salekdeh GH. Proteomics uncovers a role for redox in drought tolerance in wheat. *J Proteome Res* 6: 1451–1460, 2007.
111. Hall A, Karplus PA, and Poole LB. Typical 2-Cys peroxiredoxins–structures, mechanisms and functions. *FEBS J* 276: 2469–2477, 2009.
112. Hall M, Mata-Cabana A, Åkerlund HE, Florencio FJ, Schröder WP, Lindahl M, and Kieselbach T. Thioredoxin targets of the plant chloroplast lumen and their implications for plastid function. *Proteomics* 10: 987–1001, 2010.

113. Halliwell B. *Chloroplast Metabolism*. Oxford: Clarendon Press, 1981, pp. 89–104.
114. Hansen KC, Schmitt-Ulms G, Chalkley RJ, Hirsch J, Baldwin MA, and Burlingame AL. Mass spectrometric analysis of protein mixtures at low levels using cleavable 13C-isotope-coded affinity tag and multidimensional chromatography. *Mol Cell Proteomics* 2: 299–314, 2003.
115. Hansen RE and Winther JR. An introduction to methods for analyzing thiols and disulphides: reactions, reagents and practical considerations. *Anal Biochem* 394: 147–158, 2009.
116. Hara MR, Cascio MB, and Sawa A. GAPDH as a sensor of NO stress. *Biochim Biophys Acta* 1762: 502–509, 2006.
117. Heazlewood JL, Verboom RE, Tonti-Filippini J, Small I, and Millar AH. SUBA: the Arabidopsis Subcellular Database. *Nucleic Acids Res* 35: D213–D218, 2007.
118. Hess DT, Matsumoto A, Kim SO, Marshall HE, and Stamler JS. Protein S-nitrosylation: purview and parameters. *Nat Rev Mol Cell Biol* 6: 150–166, 2005.
119. Hicks LM, Cahoon RE, Bonner ER, Rivard RS, Sheffield J, and Jez JM. Thiol-based regulation of redox-active glutamate-cysteine ligase from *Arabidopsis thaliana*. *Plant Cell* 19: 2653–2661, 2007.
120. Hisabori T, Hara S, Fujii T, Yamazaki D, Hosoya-Matsuda N, and Motohashi K. Thioredoxin affinity chromatography: a useful method for further understanding the thioredoxin network. *J Exp Bot* 56: 1463–1468, 2005.
121. Hochgräfe F, Mostertz J, Albrecht D, and Hecker M. Fluorescence thiol modification assay: oxidatively modified proteins in *Bacillus subtilis*. *Mol Microbiol* 58: 409–425, 2005.
122. Hochgräfe F, Mostertz J, Pöther DC, Becher D, Helmann JD, and Hecker M. S-cysteinylation is a general mechanism for thiol protection of *Bacillus subtilis* proteins after oxidative stress. *J Biol Chem* 282: 25981–25985, 2007.
123. Hochgräfe F, Wolf C, Fuchs S, Liebeke M, Lalk M, Engelmann S, and Hecker M. Nitric oxide stress induces different responses but mediates comparable protein thiol protection in *Bacillus subtilis* and *Staphylococcus aureus*. *J Bacteriol* 190: 4997–5008, 2008.
124. Hogg N. The biochemistry and physiology of S-nitrosothiols. *Annu Rev Pharmacol Toxicol* 42: 585–600, 2002.
125. Holmgren A. Hydrogen donor system for *Escherichia coli* ribonucleoside-diphosphate reductase dependent upon glutathione. *Proc Natl Acad Sci USA* 73: 2275–2279, 1976.
126. Holmgren A, Buchanan BB, and Wolosiuk RA. Photosynthetic regulatory protein from rabbit liver is identical with thioredoxin. *FEBS Lett* 82: 351–354, 1977.
127. Holmgren A. Thioredoxin catalyzes the reduction of insulin disulfides by dithiothreitol and dihydrolipoamide. *J Biol Chem* 254: 9627–9632, 1979.
128. Holmgren A. Enzymatic reduction-oxidation of protein disulfides by thioredoxin. *Methods Enzymol* 107: 295–230, 1984.
129. Holmgren A. Thioredoxin. *Annu Rev Biochem* 54: 237–271, 1985.
130. Holmgren A. SNO removal. *Science* 320: 1019–1020, 2008.
131. Hondorp ER and Matthews RG. Oxidative stress inactivates cobalamin-independent methionine synthase (MetE) in *Escherichia coli*. *PLoS Biol* 2: e336, 2004.
132. Hondorp ER and Matthews RG. Oxidation of cysteine 645 of cobalamin-independent methionine synthase causes a methionine limitation in *Escherichia coli*. *J Bacteriol* 191: 3407–3410, 2009.
133. Hoshino Y, Shioji K, Nakamura H, Masutani H, and Yodoi J. From oxygen sensing to heart failure: role of thioredoxin. *Antioxid Redox Signal* 9: 689–699, 2007.
134. Hosoya-Matsuda N, Motohashi K, Yoshimura H, Nozaki A, Inoue K, Ohmori M, and Hisabori T. Anti-oxidative stress system in cyanobacteria. Significance of type II peroxiredoxin and the role of 1-Cys peroxiredoxin in *Synechocystis* sp. strain PCC 6803. *J Biol Chem* 280: 840–846, 2005.
135. Hosoya-Matsuda N, Inoue K, and Hisabori T. Roles of thioredoxins in the obligate anaerobic green sulfur photosynthetic bacterium *Chlorobaculum tepidum*. *Mol Plant* 2: 336–343, 2009.
136. Howard TP, Metodiev M, Lloyd JC, and Raines CA. Thioredoxin-mediated reversible dissociation of a stromal multiprotein complex in response to changes in light availability. *Proc Natl Acad Sci USA* 105: 4056–4061, 2008.
137. Hu W, Tedesco S, Faedda R, Petrone G, Cacciola SO, O'Keefe A, and Sheehan D. Covalent selection of the thiol proteome on activated thiol sepharose: a robust tool for redox proteomics. *Talanta* 80:1569–1575, 2010.
138. Hu W, Tedesco S, McDonagh B, Bárcena JA, Keane C, and Sheehan D. Selection of thiol- and disulphide-containing proteins of *Escherichia coli* on activated thiol-Sepharose. *Anal Biochem* 398: 245–253, 2010.
139. Huang B and Chen C. Detection of protein S-nitrosation using irreversible biotinylation procedures (IBP). *Free Radic Biol Med* 49: 447–456, 2010.
140. Huang B, Chen SC, and Wang DL Shear flow increases S-nitrosylation of proteins in endothelial cells. *Cardiovascular Res* 83: 536–546, 2009.
141. Huang B, Liao CL, Lin YP, Chen SC, and Wang DL. S-nitrosoproteome in endothelial cells revealed by a modified biotin switch approach coupled with Western blot-based two-dimensional gel electrophoresis. *J Proteome Res* 8: 4835–4843, 2009.
142. Huber HE, Russel M, Model P, and Richardson CC. Interaction of mutant thioredoxins of *Escherichia coli* with the gene 5 protein of phage T7. *J Biol Chem* 261: 15006–15012, 1986.
143. Hurd TR, Prime TA, Harbour ME, Lilley KS, and Murphy MP. Detection of reactive oxygen species-sensitive thiol proteins by redox difference gel electrophoresis: implications for mitochondrial redox signaling. *J Biol Chem* 282: 22040–22051, 2007.
144. Hurd TR, James AM, Lilley KS, and Murphy MP. Measuring redox changes to mitochondrial protein thiols with redox difference gel electrophoresis (redox-DIGE). *Methods Enzymol* 456: 343–361, 2009.
145. Ikegami A, Yoshimura N, Motohashi K, Takahashi S, Romano PG, Hisabori T, Takamiya K, and Masuda T. The CHLI1 subunit of Arabidopsis thaliana magnesium chelatase is a target protein of the chloroplast thioredoxin. *J Biol Chem* 282: 19282–19291, 2007.
146. Imlay JA. Cellular defenses against superoxide and hydrogen peroxide. *Annu Rev Biochem* 77: 755–776, 2008.
147. Ito H, Iwabuchi M, and Ogawa K. The sugar-metabolic enzymes aldolase and triose-phosphate isomerase are targets of glutathionylation in *Arabidopsis thaliana*: detection using biotinylated glutathione. *Plant Cell Physiol* 44: 655–660, 2003.
148. Jacob C, Giles, GI, Giles, NM, and Sies, H. Sulfur and selenium: the role of oxidation state in protein structure and function. *Angew Chem Int Ed* 42: 4742–4758, 2003.
149. Jacquot J-P, Lancelin JM, and Meyer Y. Thioredoxins: structure and function in plant cells. *New Phytol* 136: 543–570, 1997.

150. Jaffrey SR, Erdjument-Bromage H, Ferris CD, Tempst P, and Snyder SH. Protein S-nitrosylation: a physiological signal for neuronal nitric oxide. *Nat Cell Biol* 3: 193–197, 2001.
151. Jaffrey SR and Snyder SH. The biotin switch method for the detection of S-nitrosylated proteins. *Sci STKE* 86: pl1, 2001.
152. Jaffrey SR. Detection and characterization of protein nitrosothiols. *Methods Enzymol* 396:105–118, 2005.
153. Jakob U, Muse W, Eser M, and Bardwell JCA. Chaperone activity with a redox switch. *Cell* 96, 341–352, 1999.
154. Jaleel M, Saha S, Shenoy AR, and Visweswariah SS. The kinase homology domain of receptor guanylyl cyclase C: ATP binding and identification of an adenine nucleotide sensitive site. *Biochemistry* 45: 1888–1898, 2006.
155. Ji Y, Akerboom TP, Sies H, and Thomas JA. S-nitrosylation and S-glutathiolation of protein sulfhydryls by S-nitroso glutathione. *Arch Biochem Biophys* 362: 67–78, 1999.
156. Kallis GB and Holmgren A. Differential reactivity of the functional sulfhydryls groups of cysteine-32 and cysteine-35 present in the reduced form of thioredoxin from *Escherichia coli*. *J Biol Chem* 255: 10261–10265, 1980.
157. Karala A-R and Ruddock LW. Does S-methyl methanethiosulfonate trap the thiol-disulfide state of proteins? *Antioxid Redox Signal* 9: 527–532, 2007.
158. Khor HK, Fisher MT, and Schöneich C. Potential role of methionine sulfoxide in the inactivation of the chaperone GroEL by hypochlorous acid (HOCl) and peroxynitrite ($ONOO^-$). *J Biol Chem* 279: 19486–19493, 2004.
159. Kieselbach T and Schröder WP. The proteome of the chloroplast lumen of higher plants. *Photosynth Res* 78: 249–264, 2003.
160. Kil IS and Park J-W. Regulation of $NADP^+$-dependent isocitrate dehydrogenase activity by glutathionylation. *J Biol Chem* 280: 10846–10854, 2005.
161. Kojima K, Oshita M, Nanjo Y, Kasai K, Tozawa Y, Hayashi H, and Nishiyama Y. Oxidation of elongation factor G inhibits the synthesis of the D1 protein of photosystem II. *Mol Microbiol* 65: 936–947, 2007.
162. Kojima K, Motohashi K, Morota T, Oshita M, Hisabori T, Hayashi H, and Nishiyama Y. Regulation of translation by the redox state of elongation factor G in the cyanobacterium *Synechocystis* sp., PCC 6803. *J Biol Chem* 284: 18685–18691, 2009.
163. Kosower EM and Kosower NS. Bromobimane probes for thiols. *Methods Enzymol* 251:133–148, 1995.
164. Kosower NS and Kosower EM. Diamide: an oxidant probe for thiols. *Methods Enzymol* 251: 123–133, 1995.
165. Kumar JK, Tabor S, and Richardson CC. Proteomic analysis of thioredoxin-targeted proteins in *Escherichia coli*. *Proc Natl Acad Sci USA* 101: 3759–3764, 2004.
166. Kuncewicz T, Sheta EA, Goldknopf IL, and Kone BC. Proteomic analysis of S-nitrosylated proteins in mesangial cells. *Mol Cell Proteomics* 2: 156–163, 2003.
167. Kyle ME, Nakae D, Sakaida S, Serroni A, and Farber JL. Protein thiol depletion and the killing of cultured hepatocytes by hydrogen peroxide. *Biochem Pharmacol* 38: 3797–3805, 1989.
168. Ladenstein R and Ren B. Protein disulfides and protein disulfide oxidoreductases in hyperthermophiles. *FEBS J* 273: 4170–4185, 2006.
169. Laloi C, Rayapuram N, Chartier Y, Grienenberger J-M, Bonnard, and Meyer Y. Identification and characterisation of a mitochondrial thioredoxin system in plants. *Proc Natl Acad Sci USA* 98: 14144–14149, 2001.
170. Lapko VN, Smith DL, and Smith JB. Identification of an artefact in the mass-spectrometry of proteins derivatized with iodoacetamide. *J Mass Spectrom* 35: 572–575, 2000.
171. Laragione T, Bonetto V, Casoni F, Massignan T, Bianchi G, Gianazza E, and Ghezzi P. Redox regulation of surface protein thiols: identification of integrin alpha-4 as a molecular target by using redox proteomics. *Proc Natl Acad Sci USA* 100: 14737–14741, 2003. Erratum in: *Proc Natl Acad Sci USA* 101: 4718, 2004.
172. Laragione T, Gianazza E, Tonelli R, Bigini P, Mennini T, Casoni F, Massignan T, Bonetto V, and Ghezzi P. Regulation of redox-sensitive exofacial protein thiols in CHO cells. *Biol Chem* 387: 1371–1376, 2006.
173. Laurent TC, Moore EC, and Reichard P. Enzymatic synthesis of deoxyribonucleotides. IV. Isolation and characterization of thioredoxin, the hydrogen donor from *Escherichia coli* B. *J Biol Chem* 239: 3436–3444, 1964.
174. Laxa M, König J, Dietz KJ, and Kandlbinder A. Role of the cysteine residues in Arabidopsis thaliana cyclophilin CYP20–3 in peptidyl-prolyl cis-trans isomerase and redox-related functions. *Biochem J* 401: 287–297, 2007.
175. Lee DY, Ahn B-Y, and Kim K-S. A thioredoxin from the hyperthermophilic archaeon Methanococcus jannaschii has a glutaredoxin-like fold but thioredoxin-like activities. *Biochemistry* 39: 6652–6659, 2000.
176. Lee C, Lee YM, and Rice RH. Human epidermal cell protein responses to arsenite treatment in culture. *Chem Biol Interact* 155: 43–54, 2005.
177. Lee K, Lee J, Kim Y, Bae D, Kang KY, Yoon SC, and Lim D. Defining the plant disulphide proteome. *Electrophoresis* 25: 532–541, 2004.
178. Lee S, Ryu J-Y, Kim SY, Jeon J-H, Song JY, Cho H-T, Choi S-B, Choi D, Tandeau de Marsac N, and Park Y-I. Transcriptional regulation of the respiratory genes in the cyanobacterium *Synechocystis* sp. PCC 6803 during the early response to glucose feeding. *Plant Physiol* 145: 1018–1030, 2007.
179. Lee S-R, Kwon K-S, Kim S-R, and Rhee SG. Reversible inactivation of protein-tyrosine phosphatase 1B in A431 cells stimulated with epidermal growth factor. *J Biol Chem* 273: 15366–15372, 1998.
180. Lefièvre L, Chen Y, Conner SJ, Scott JL, Publicover SJ, Ford WC, and Barratt CL. Human spermatozoa contain multiple targets for protein S-nitrosylation: an alternative mechanism of the modulation of sperm function by nitric oxide? *Proteomics* 7: 3066–3084, 2007.
181. Leichert LI, Scharf C, and Hecker M. Global characterization of disulfide stress in *Bacillus subtilis*. *J Bacteriol* 185: 1967–1975, 2003.
182. Leichert LI and Jakob U. Protein thiol modifications visualized *in vivo*. *PLoS Biol* 2: e333, 2004.
183. Leichert LI, Gehrke F, Gudiseva HV, Blackwell T, Ilbert M, Walker AK, Straler JR, Andrews PC, and Jakob U. Quantifying changes in the thiol redox proteome upon oxidative stress *in vivo*. *Proc Natl Acad Sci USA* 105: 8197–8202, 2008.
184. Leichert LI. Quantitative detection of the cysteine redox state *in vivo*–the OxICAT method. In: *Methods in Redox Signaling*, edited by Das D. New Rochelle, NY: Mary Ann Liebert, Inc., 2010, pp. 63–70.
185. Lemaire SD, Guillon B, Le Maréchal P, Keryer E, Miginiac-Maslow M, Decottignies P. New thioredoxin targets in the unicellular photosynthetic eukaryote *Chlamydomonas reinhardtii*. Proc Natl Acad Sci USA 101: 7475–74780, 2004.

186. Lemaire SD, Michelet L, Zaffagnini M, Massot V, and Issakidis-Burguet E. Thioredoxins in chloroplasts. *Curr Genet* 53: 643–665, 2007.
187. Le Moan N, Clement G, Le Maout S, Tacnet F, and Toledano MB. The *Sacharomyces cerevisiae* proteome of oxidised protein thiols. Contrasted functions for the thioredoxin and glutathione pathways. *J Biol Chem* 281: 10420–10430, 2006.
188. Le Moan N, Tacnet F, and Toledano MB. Protein-thiol oxidation, from single proteins to proteome-wide analyses. *Methods Mol Biol* 476: 181–198, 2008.
189. Leonard SE, Reddie KG, and Carroll KS. Mining the thiol proteome for sulfenic acid modifications reveals new targets for oxidation in cells. *ACS Chem Biol* 4, 783–799, 2009.
190. Li M, Yang Q, Zhang L, Li H, Cui Y, and Wu Q. Identification of novel targets of cyanobacterial glutaredoxins. *Arch Biochem Biophys* 458: 220–228, 2007.
191. Liebeke M, Pöther DC, van Duy N, Albrecht D, Becher D, Hochgräfe F, Lalk M, Hecker M, and Antelmann H. Depletion of thiol-containing proteins in response to quinones in *Bacillus subtilis*. *Mol Microbiol* 69: 1513–1529, 2008.
192. Lillig CH, Prior A, Schwenn JD, Åslund F, Ritz D, Vlamis-Gardikas A, and Holmgren A. New thioredoxins and glutaredoxins as electron donors of 3′-phosphoadenylylsulfate reductase. *J Biol Chem* 274: 7695–7698, 1999.
193. Lillig CH and Holmgren A. Thioredoxin and related molecules–From biology to health and disease. *Antioxid Redox Signal* 9: 25–47, 2007.
194. Lin T-K, Hughes G, Muratovska A, Blaikie FH, Brookes PS, Darley-Usmar V, Smith RAJ, and Murphy MP. Specific modification of mitochondrial protein thiols in response to oxidative stress. A proteomics approach. *J Biol Chem* 277: 17048–17056, 2002.
195. Lind C, Gerdes R, Hamnell Y, Schuppe-Koistinen I, Brockenhuus von Löwenhielm H, Holmgren A and Cotgreave IA. Identification of S-glutathionylated cellular proteins during oxidative stress and constitutive metabolism by affinity purification and proteomic analysis. *Arch Biochem Biophys* 406: 229–240, 2002.
196. Lindahl M and Florencio FJ. Thioredoxin-linked processes in cyanobacteria are as numerous as in chloroplasts, but targets are different. *Proc Natl Acad Sci USA* 100: 16107–16112, 2003.
197. Lindahl M and Florencio FJ. Systematic screening of reactive cysteine proteomes. *Proteomics* 4: 448–450, 2004.
198. Lindahl M and Kieselbach T. Disulphide proteomes and interactions with thioredoxin on the track towards understanding redox regulation in chloroplasts and cyanobacteria. *J Proteomics* 72: 416–438, 2009.
199. Lindermayr C, Saalbach G, and Durner J. Proteomic identification of S-nitrosylated proteins in Arabidopsis. *Plant Physiol* 137: 921–930, 2005.
200. Lindermayr C and Durner J. S-Nitrosylation in plants: pattern and function. *J Proteomics* 73: 1–9, 2009.
201. Link AJ, Robison K, and Church GM. Comparing the predicted and observed properties of proteins encoded in the genome of *Escherichia coli* K-12. *Electrophoresis* 18: 1259–1313, 1997.
202. Liu B, Tewari AK, Zhang L, Green-Church KB, Zweier JL, Chen YR, and He G. Proteomic analysis of protein tyrosine nitration after ischemia reperfusion injury: mitochondria as the major target. *Biochim Biophys Acta* 1794: 476–485, 2009.
203. López-Sánchez LM, Corrales FJ, González R, Ferrín G, Muñoz-Castañeda JR, Ranchal I, Hidalgo AB, Briceño J, López-Cillero P, Gómez MA, De La Mata M, Muntané J, and Rodríguez-Ariza A. Alteration of S-nitrosothiol homeostasis and targets for protein S-nitrosation in human hepatocytes. *Proteomics* 8: 4709–4720, 2008.
204. López-Sánchez LM, Muntané J, de la Mata M, and Rodríguez-Ariza A. Unraveling the S-nitrosoproteome: tools and strategies. *Proteomics* 2009 9: 808–818, 2009.
205. Lui JK, Lipscombe R, and Arthur PG. Detecting changes in the thiol redox state of proteins following a decrease in oxygen concentration using a dual labeling technique. *J Proteome Res* 9: 383–392, 2010.
206. Maeda K, Finnie C, and Svensson B. Cy5 maleimide labelling for sensitive detection of free thiols in native protein extracts: identification of seed proteins targeted by barley thioredoxin *h* isoforms. *Biochem J* 378: 497–507, 2004.
207. Maeda K, Finnie C, and Svensson B. Identification of thioredoxin h-reducible disulphides in proteomes by differential labelling of cysteines: insight into recognition and regulation of proteins in barley seeds by thioredoxin h. *Proteomics* 5: 1634–1644, 2005.
208. Maller C, Schröder E, and Eaton P. Glyceraldehyde 3-phosphate dehydrogenase is unlikely to mediate hydrogen peroxide signaling: studies with a novel anti-dimedone sulfenic acid antibody. *Antioxid Redox Signal* 14, 49–60, 2011.
209. Mallick P, Boutz DR, Eisenberg D, and Yeates TO. Genomic evidence that the intracellular proteins of archaeal microbes contain disulfide bonds. *Proc Natl acad Sci USA* 99: 9679–9684, 2002.
210. Marchand C, Le Marechal P, Meyer Y, Miginiac-Maslow M, Issakidis-Bourguet E, and Decottignies P. New targets of Arabidopsis thioredoxins revealed by proteomic analysis. *Proteomics* 4: 2696–2706, 2004.
211. Marchand C, Le Marechal P, Meyer Y, and Decottignies P. Comparative proteomic approaches for the isolation of proteins interacting with thioredoxins. *Proteomics* 6: 6528–6537, 2006.
212. Marchand CH, Vanacker H, Collin V, Issakidis-Bourguet E, Maréchal PL, and Decottignies P. Thioredoxin targets in Arabidopsis roots. *Proteomics* 10: 2418–2428, 2010.
213. Marcus Y, Altman-Gueta H, Finkler A, and Gurevitz M. Dual role of cysteine 172 in redox regulation of ribulose 1,5-bisphosphate carboxylase/oxygenase activity and degradation. *J Bacteriol* 185: 1509–1517, 2003.
214. Marín-Navarro J and Moreno J. Cysteines 449 and 459 modulate the reduction-oxidation conformational changes of ribulose 1.5-bisphosphate carboxylase/oxygenase and the translocation of the enzyme to membranes during stress. *Plant Cell Environ* 29: 898–908, 2006.
215. Marino SM and Gladyshev VN. Structural analysis of cysteine S-nitrosylation: a modified acid-based motif and the emerging role of trans-nitrosylation. *J Mol Biol* 395: 844–859, 2010.
216. Marino SM, Li Y, Fomenko DE, Agisheva N, Cerny RL, and Gladyshev VN. Characterization of surface-exposed reactive cysteine residues in *Saccharomyces cerevisiae*. *Biochemistry* 49: 7709–7721, 2010.
217. Marley K, Mooney DT, Clark-Scannell G, Tong TT-H, Watson J, Hagen TM, Stevens JF, and Maier CS. Mass tagging approach for mitochondrial thiol proteins. *J Proteome Res* 4: 1403–1412, 2005.
218. Martínez-Ruiz A and Lamas S. Detection and proteomic identification of S-nitrosylated proteins in endothelial cells. *Arch Biochem Biophys* 423: 192–199, 2004.

219. Martinez-Ruiz A, Villanueva L, González de Orduña C, López-Ferrer D, Higueras MA, Tarin C, Rodríguez-Crespo I, Váquez J, and Lamas S. S-nitrosylation of Hsp90 promotes the inhibition of its ATPase and endothelial nitric oxide synthase regulatory activities. *Proc Nat Acad Sci USA* 102: 8515–8530, 2005.
220. Martins-de-Souza D, Dias-Neto E, Schmitt A, Falkai P, Gormanns P, Maccarrone G, Turck CW, and Gattaz WF. Proteome analysis of schizophrenia brain tissue. *World J Biol Psychiatry* 11: 110–120, 2010.
221. Martins-de-Souza D, Maccarrone G, Wobrock T, Zerr I, Gormanns P, Reckow S, Falkai P, Schmitt A, and Turck CW. Proteome analysis of the thalamus and cerebrospinal fluid reveals glycolysis dysfunction and potential biomarkers candidates for schizophrenia. *J Psychiatr Res* 44, 1176–1189, 2010.
222. Marx C, Wong JH, and Buchanan BB. Thioredoxin and germinating barley: targets and protein redox changes. *Planta* 216: 454–460, 2003.
223. Mata-Cabana A, Florencio FJ, and Lindahl M. Membrane proteins from the cyanobacterium *Synechocystis* sp. PCC 6803 interacting with thioredoxin. *Proteomics* 7: 3953–3963, 2007.
224. Matsumoto M, Ito H, Henmi K, Sugimoto I, and Ogawa K. Regulation of fructose-1,6-bisphosphate aldolase via glutathionylation in Arabidopsis chloroplasts. Supplement to *Plant Cell Physiol* 47: 221, 2006.
225. Mauzeroll J, Bard AJ, Owhadian O, and Monks TJ. Menadione metabolism to thiodione in hepatoblastoma by scanning electrochemical microscopy. Proc Natl Acad Sci USA 101: 17582–17587, 2004.
226. McDonagh B and Sheehan D. Effect of oxidative stress on protein thiols in the blue mussel *Mytilus edulis*: proteomic identification of target proteins. *Proteomics* 7: 3395–3403, 2007.
227. McDonagh B, Ogueta S, Lasarte G, Padilla CA, and Bárcena JA. Shotgun redox proteomics identifies specifically modified cysteines in key metabolic enzymes under oxidative stress in *Saccharomyces cerevisiae*. *J Proteomics* 72: 416–438, 2009.
228. Meng L, Wong JH, Feldman LJ, Lemaux PG, and Buchanan BB. A membrane-associated thioredoxin required for plant growth moves from cell to cell, suggestive of a role in intercellular communication. *Proc Natl Acad Sci USA* 107: 3900–3905, 2010.
229. Meyer Y, Reichheld JP, and Vignols F. Thioredoxins in Arabidopsis and other plants. *Photosynth Res* 86: 419–433, 2005.
230. Meyer Y, Buchanan BB, Vignols F, and Reichheld J-P. Thioredoxins and glutaredoxins: unifying elements in redox biology. *Annu Rev Genet* 43: 335–367, 2009.
231. Michalska J, Zauber H, Buchanan BB, Cejudo FJ, and Geigenberger P. NTRC links built-in thioredoxin to light and sucrose in regulating starch synthesis in chloroplasts and amyloplasts. *Proc Natl Acad Sci USA* 106: 9908–9913, 2009.
232. Michelet L, Zaffagnini M, Vanacker H, Le Maréchal P, Marchand C, Schroda M, Lemaire SD, and Decottignies P. *In vivo* targets of S-thiolation in *Chlamydomonas reinhardtii*. *J Biol Chem* 283: 21571–21578, 2008.
233. Millar AH, Heazlewood JL, Kristensen BK, Braun HP, and Møller IM. The plant mitochondrial proteome. *Trends Plant Sci* 10: 36–43, 2005.
234. Miranda-Vizuete A, Damdimopoulos AE, and Spyrou G. The mitochondrial thioredoxin system. *Antioxid Redox Signal* 2: 801–810, 2000.
235. Molinari M and Helenius A. Analyzing cotranslational protein folding and disulfide formation by diagonal sodium dodecyl sulfate-polyacrylamide gel electrophoresis. *Meth Enzymol* 348: 35–42, 2002.
236. Montrichard F, Alkhalfioui F, Yano H, Vensel WH, Hurkman WJ, and Buchanan BB. Thioredoxin targets in plants: the first 30 years. *J Proteomics* 72: 452–474, 2009.
237. Moreno J, García-Murria MJ, and Marín-Navarro J. Redox modulation of Rubisco conformation and activity through its cysteine residues. *J Exp Bot* 59: 1605–1614, 2008.
238. Morigasaki S, Shimada K, Ikner A, Yanagida M, and Shiozaki K. Glycolytic enzyme GAPDH promotes peroxide stress signaling through multistep phosphorelay to a MAPK cascade. *Mol Cell* 30: 108–113, 2008.
239. Motohashi K, Kondoh A, Stumpp MT, and Hisabori T. Comprehensive survey of proteins targeted by chloroplast thioredoxin. *Proc Natl Acad Sci USA* 98: 11224–11229, 2001.
240. Motohashi K, Koyama F, Nakanishi Y, Ueoka-Nakanishi H, and Hisabori T. Chloroplast cyclophilin is a target protein of thioredoxin. Thiol modulation of the peptidyl-prolyl cis-trans isomerase activity. *J Biol Chem* 278: 31848–31852, 2003.
241. Motohashi K and Hisabori T. HCF164 receives reducing equivalents from stromal thioredoxin across the thylakoid membrane and mediates reduction of target proteins in the thylakoid lumen. *J Biol Chem* 281: 35039–35047, 2006.
242. Motohashi K and Hisabori T. CcdA Is a Thylakoid Membrane Protein Required for the Transfer of Reducing Equivalents from Stroma to Thylakoid Lumen in the Higher Plant Chloroplast. *Antioxid Redox Signal* 13: 1169–1176, 2010.
243. Muller EG and Buchanan BB. Thioredoxin is essential for photosynthetic growth. The thioredoxin *m* gene of *Anacystis nidulans*. *J Biol Chem* 264: 4008–4014, 1989.
244. Navarro F and Florencio FJ. The cyanobacterial thioredoxin gene is required for both photoautotrophic and heterotrophic growth. *Plant Physiol* 111: 1067–1075, 1996.
245. Née G, Zaffagnini M, Trost P, and Issakidis-Bourguet E. Redox regulation of chloroplastic glucose-6-phosphate dehydrogenase: a new role for f-type thioredoxin. *FEBS Lett* 583: 2827–2832, 2009.
246. Nishiyama Y, Yamamoto H, Allakhverdiev SI, Inaba M, Yokota A, and Murata N. Oxidative stress inhibits the repair of photodamage to the photosynthetic machinery. *EMBO J* 20: 5587–5594, 2001.
247. Ogawa K. Glutathione-associated regulation of plant growth and stress responses. *Antioxid Redox Signal* 7: 973–981, 2005.
248. Olinares PD, Ponnala L, and van Wijk KJ. Megadalton complexes in the chloroplast stroma of *Arabidopsis thaliana* characterized by size exclusion chromatography, mass spectrometry, and hierarchical clustering. *Mol Cell Proteomics* 9: 1594–1615, 2010.
249. Page ML, Hamel PP, Gabilly ST, Zegzouti H, Perea JV, Alonso JM, Ecker JR, Theg SM, Christensen SK, and Merchant S. A homolog of prokaryotic thiol disulfide transporter CcdA is required for the assembly of the cytochrome b6f complex in Arabidopsis chloroplasts. *J Biol Chem* 279: 32474–32482, 2004.
250. Palmieri MC, Lindermayr C, Bauwe H, Steinhauser C, and Durner J. Regulation of plant glycine decarboxylase by s-nitrosylation and glutathionylation. *Plant Physiol* 152: 1514–15128, 2010.
251. Pe'er I, Felder CE, Man O, Silman I, Sussman JL, and Beckmann JS. Proteomic signatures: amino acid and oli-

gopeptide compositions differentiate among phyla. *Proteins* 54: 20–40, 2004.
252. Pérez-Mato I, Castro C, Ruiz FA, Corrales FJ, and Mato JM. Methionine adenosyltransferase S-nitrosylation is regulated by the basic and acidic amino acids surrounding the target thiol. *J Biol Chem* 274: 17075–17079, 1999.
253. Pérez-Pérez ME, Florencio FJ, and Lindahl M. Selecting thioredoxins for disulphide proteomics: target proteomes of three thioredoxins from the cyanobacterium *Synechocystis* sp. PCC 6803. *Proteomics* 6: S186–S195, 2006.
254. Pérez-Pérez ME, Martín-Figueroa E, and Florencio FJ. Photosynthetic regulation of the cyanobacterium *Synechocystis* sp. PCC 6803 thioredoxin system and functional analysis of TrxB (Trx *x*) and TrxQ (Trx *y*) thioredoxins. *Mol Plant* 2: 270–283, 2009.
255. Pérez-Ruiz JM, Spínola MC, Kirchsteiger K, Moreno J, Sahrawy M, and Cejudo FJ. Rice NTRC is a high-efficiency redox system for chloroplast protection against oxidative damage. *Plant Cell* 18: 2356–2368, 2006.
256. Petersson UA, Kieselbach T, García-Cerdán JG, and Schröder WP. The Prx Q protein of Arabidopsis thaliana is a member of the luminal chloroplast proteome. *FEBS Lett* 580: 6055–6061, 2006.
257. Poole LB, Karplus PA, and Claiborne A. Protein sulfenic acids in redox signaling. *Annu Rev Pharmacol Toxicol* 44: 325–347, 2004.
258. Poole LB. Bacterial defenses against oxidants: mechanistic features of cysteine-based peroxidases and their flavoprotein reductases. *Arch Biochem Biophys* 433: 240–254, 2005.
259. Poole LB, Klomsiri C, Knaggs SA, Furdui CM, Nelson KJ, Thomas MJ, Fetrow JS, Daniel LW, and King SB. Fluorescent and affinity-based tools to detect cysteine sulfenic acid formation in proteins. *Bioconjugate Chem* 18: 2004–2017, 2007.
260. Poole LB and Nelson KJ. Discovering mechanisms of signalling-mediated thiol oxidation. *Curr Opin Chem Biol* 12: 18–24, 2008.
261. Pöther DC, Liebeke M, Hochgräfe F, Antelmann H, Becher D, Lalk M, Lindequist U, Borovok I, Cohen G, Aharonowitz Y, and Hecker M. Diamide triggers mainly S-Thiolations in the cytoplasmic proteomes of *Bacillus subtilis* and *Staphylococcus aureus*. *J Bacteriol* 191: 7520–7530, 2009.
262. Prescher JA and Bertozzi CR. Chemistry in living systems. *Nat Chem Biol* 1: 13–21, 2005.
263. Reddie KG and Carroll KS. Expanding the functional diversity of proteins through cysteine oxidation. *Curr Opin Chem Biol* 12: 746–754, 2008.
264. Reddie KG, Seo YH, Muse III WB, Leonard SE, and Carroll KS. A chemical approach for detecting sulfenic acid-modified proteins in living cells. *Mol Biosyst* 4, 521–531, 2008.
265. Rey P, Cuiné S, Eymery F, Garin J, Court M, Jacquot J-P, Rouhier N, and Broin M. Analysis of the proteins targeted by CDSP32, a plastidic thioredoxin participating in oxidative stress responses. *Plant J* 41: 31–42, 2005.
266. Rhee KY, Erdjument-Bromage H, Tempst P, and Nathan CF. S-nitroso proteome of *Mycobacterium tuberculosis*: enzymes of intermediary metabolism and antioxidant defense. *Proc Natl Acad Sci USA* 102: 467–472, 2005.
267. Rhee SG. H_2O_2, a necessary evil for cell signaling. *Science* 312: 1882–1883, 2006.
268. Richarme G. Protein-disulfide isomerase activity of elongation factor EF-Tu. *Biochem Biophys Res Commun* 252: 156–161, 1998.
269. Riddles PW, Blakeley RL, and Zerner B. Reassessment of Ellman's reagent. *Methods Enzymol* 91: 49–60, 1983.
270. Riederer IM, Herrero RM, Leuba G, and Riederer BM. Serial protein labeling with infrared maleimide dyes to identify cysteine modifications. *J Proteomics* 71: 222–230, 2008.
271. Rodríguez-Pascual F, Redondo-Horcajo M, Magán-Marchal N, Lagares D, Martínez-Ruiz A, Kleinert H, and Lamas S. Glyceraldehyde-3-phosphate dehydrogenase regulates Endothelin-1 expression by a novel, redox-sensitive mechanism involving mRNA stability. *Mol Cell Biol* 28: 7139–7155, 2008.
272. Romero-Puertas MC, Campostrini N, Mattè A, Righetti PG, Perazzolli M, Zolla L, Roepstorff P, and Delledonne M. Proteomic analysis of S-nitrosylated proteins in *Arabidopsis thaliana* undergoing hypersensitive response. *Proteomics* 8: 1459–1469, 2008.
273. Rouhier N, Villarejo A, Srivastava M, Gelhaye E, Keech O, Droux M, Finkemeier I, Samuelsson G, Dietz K-J, Jacquot J-P, and Wingsle G. Identification of plant glutaredoxins targets. *Antioxid Redox Signal* 7: 919–929, 2005.
274. Sahaf B, Heydari K, Herzenberg LA, and Herzenberg LA. Lymphocyte surface thiol levels. *Proc Natl Acad Sci USA* 100: 4001–4005, 2003.
275. Salsbury FR Jr., Knutson ST, Poole LP, and Fetrow JS. Functional site profiling and electrostatic analysis of cysteines modifiable to cysteine sulfenic acid. *Protein Sci* 17: 299–312, 2008.
276. Saurin AT, Neubert H, Brennan JP, and Eaton P. Widespread sulphenic acid formation in tissues in response to hydrogen peroxide. *Proc Natl Acad Sci USA* 101: 17982–17987, 2004.
277. Saxon E and Bertozzi CR. Cell surface engineering by a modified Staudinger reaction. *Science* 287: 2007–2010, 2000.
278. Schilling B, Yoo CB, Collins CJ, and Gibson BW. Determining cysteine oxidation status using differential alkylation. *Int J Mass Spectrom* 236: 177–127, 2004.
279. Schöneich C. Kinetics of thiol reactions. *Methods Enzymol* 251: 45–55, 1995.
280. Schröter Y, Steiner S, Matthäi K, and Pfannschmidt T. Analysis of oligomeric protein complexes in the chloroplast sub-proteome of nucleic acid-binding proteins from mustard reveals potential redox regulators of plastid gene expression. *Proteomics* 10: 2191–2204, 2010.
281. Schürmann P and Buchanan BB. The ferredoxin/thioredoxin system of oxygenic photosynthesis. *Antioxid Redox Signal* 10: 1235–1273, 2008.
282. Sechi S and Chait BT. Modification of cysteine residues by alkylation. A tool in peptide mapping and protein identification. *Anal Chem* 70: 5150–5158, 1998.
283. Sen CK. Cellular thiols and redox-regulated signal transduction. *Curr Top Cell Regul* 36: 1–30, 2000.
284. Sengupta R, Ryter SW, Zuckerbraun BS, Tzeng E, Billiar TR, and Stoyanovsky DA.Thioredoxin catalyzes the denitrosation of low-molecular mass and protein S-nitrosothiols. *Biochemistry* 46: 8472–8483, 2007.
285. Seo YH and Carroll KS. Facile synthesis and biological evaluation of a cell-permeable probe to detect redox-regulated proteins. *Bioorg Med Chem Lett* 19: 356–359, 2009.
286. Seo YH and Carroll KS. Profiling protein thiol oxidation in tumor cells using sulfenic acid-specific antibodies. *Proc Natl Acad Sci USA* 106: 16163–16168, 2009.
287. Sethuraman M, McComb ME, Heibeck T, Costello CE, and Cohen RA. Isotope-coded affinity tag approach to identify and quantify oxidant-sensitive protein thiols. *Mol Cell Proteomics* 3: 273–278, 2004.
288. Sethuraman M, McComb ME, Huang H, Huang S, Heibeck T, Costello CE, and Cohen RA. Isotope-coded affinity tag

(ICAT) approach to redox proteomics: identification and quantitation of oxidant-sensitive cysteine thiols in complex protein mixtures. *J Proteome Res* 3: 1228–1233, 2004.
289. Shahpiri A, Svensson B, and Finnie C. From proteomics to structural studies of cytosolic/mitochondrial-type thioredoxin systems in barley seeds. *Mol Plant* 2: 378–389, 2009.
290. Sheehan D, McDonagh B, and Bárcena JA. Redox proteomics. *Expert Rev Proteomics* 7: 1–4, 2010.
291. Shenton D, Perrone G, Quinn KA, Dawes IW, and Grant CM. Regulation of protein S-thiolation by glutaredoxin 5 in the yeast *Saccharomyces cerevisiae*. *J Biol Chem* 277: 16853–16859, 2002.
292. Shenton D and Grant CM. Protein S-thiolation targets glycolysis and protein synthesis in response to oxidative stress in the yeast *Saccharomyces cerevisiae*. *Biochem J* 374: 513–519, 2003.
293. Shi J, Vlamis-Gardikas A, Åslund F, Holmgren A, and Rosen BP. Reactivity of glutaredoxins 1, 2, and 3 from *Escherichia coli* shows that glutaredoxin 2 is the primary hydrogen donor to ArsC-catalyzed arsenate reduction. *J Biol Chem* 274: 36039–36042, 1999.
294. Singha NC, Vlamis-Gardikas A, and Holmgren A. Real-time kinetics of the interaction between the two subunits, *Escherichia coli* thioredoxin and gene 5 protein of phage T7 DNA polymerase. *J Biol Chem* 278: 21421–21428, 2003.
295. Singh AK, Bhattacharyya-Pakrasi M, and Pakrasi HB. Identification of an atypical membrane protein involved in the formation of protein disulfide bonds in oxygenic photosynthetic organisms. *J Biol Chem* 283: 15762–15770, 2008.
296. Skalska J, Brookes PS, Nadtochiy SM, Hilchey SP, Jordan CT, Guzman ML, Maggirwar SB, Briehl MM, and Bernstein SH. Modulation of cell surface protein free thiols: a potential novel mechanism of action of the sesquiterpene lactone parthenolide. *PLoS One* 4: e8115, 2009.
297. Smith ME, Schumacher FF, Ryan CP, Tedaldi LM, Papaioannou D, Waksman G, Caddick S, and Baker JR. Protein modification, bioconjugation, and disulfide bridging using bromomaleimides. *J Am Chem Soc* 132: 1960–1965, 2010.
298. Smyth DG, Blumenfeld OO, and Konigsberg W. Reactions of N-ethylmaleimide with peptides and amino acids. *Biochem J* 91: 589–595, 1964.
299. Sommer A and Traut RR. Diagonal polyacrylamide-dodecyl sulfate gel electrophoresis for the identification of ribosomal proteins crosslinked with methyl-4-mercaptobutyrimidate. *Proc Nat Acad Sci USA* 71: 3946–3950, 1974.
300. Stadtman ER. Protein oxidation and aging. *Free Rad Res* 40: 1250–1258, 2006.
301. Ströher E and Dietz KJ. The dynamic thiol-disulphide redox proteome of the Arabidopsis thaliana chloroplast as revealed by differential electrophoretic mobility. *Physiol Plant* 133: 566–583, 2008.
302. Suh S-K, Hood BL, Kim B-J, Conrads TP, Veenstra TD, and Song BJ. Identification of oxidized mitochondrial proteins in alcohol-exposed human hepatoma cells and mouse liver. *Proteomics* 4: 3401–3412, 2004.
303. Sullivan DM, Wehr NB, Fergusson MM, Levine RL, and Finkel T. Identification of oxidant-sensitive proteins: TNF-α induces protein glutathionylation. *Biochemistry* 39: 11121–11128, 2000.
304. Suss KH, Arkona C, Manteuffel R, and Adler K. Calvin cycle multienzyme complexes are bound to chloroplast thylakoid membranes of higher plants *in situ*. *Proc Natl Acad Sci USA* 90: 5514–5518, 1993.
305. Takanishi CL, Ma L-H, and Wood MJ. A genetically encoded probe for cysteine sulfenic acid protein modification *in vivo*. *Biochemistry* 46: 14725–14732, 2007.
306. Tanaka T, Hosoi F, Yamaguchi-Iwai Y, Nakamura H, Masutani H, Ueda S, Nishiyama A, Takeda S, Wada H, Spyrou G, and Yodoi J. Thioredoxin-2 (Trx-2) is an essential gene regulating mitochondria-dependent apoptosis. *EMBO J* 21: 1695–1703, 2002.
307. Tello D, Tarín C, Ahicart P, Bretón-Romero R, Lamas S, and Martínez-Ruiz A. A "fluorescence switch" technique increases the sensitivity of proteomic detection and identification of S-nitrosylated proteins. *Proteomics* 9: 5359–5370, 2009.
308. Thor H, Smith MT, Hartzell P, Bellomo G, Jewell SA, and Orrenius S. The metabolism of menadione (2-methyl-1,4-naphthoquinone) by isolated hepatocytes. A study of the implications of oxidative stress in intact cells. *J Biol Chem* 257: 12419–12425, 1982.
309. Tissot G, Canard H, Nadai M, Martone A, Botterman J, and Dubald M. Translocation of aprotinin, a therapeutic protease inhibitor, into the thylakoid lumen of genetically engineered tobacco chloroplasts. *Plant Biotechnol J* 6: 309–320, 2008.
310. Traverso JA, Vignols F, Cazalis R, Serrato AJ, Pulido P, Sahrawy M, Meyer Y, Cejudo FJ, and Chueca A. Immunocytochemical localization of Pisum sativum TRXs f and m in non-photosynthetic tissues. *J Exp Bot 59*: 1267–1277, 2008.
311. Tyther R, Ahmeda A, Johns E, McDonagh B, and Sheehan D. Proteomic profiling of perturbed protein sulfenation in renal medulla of the spontaneously hypertensive rat. *J Proteome Res* 9: 2678–2687, 2010.
312. van de Meene AML, Hohmann-Marriot MF, Vermaas WFJ, and Roberson RW. The three-dimensional structure of the cyanobacterium *Synechocystis* sp. PCC 6803. *Arch Microbiol* 184: 259–270, 2006.
313. van Heusden GP. 14–3-3 proteins: regulators of numerous eukaryotic proteins. *IUBMB Life* 57: 623–629, 2005.
314. Varghese S, Tang Y, and Imlay JA. Contrasting sensitivities of *Escherichia coli* aconitases A and B to oxidation and iron depletion. *J Bacteriol* 185: 221–230, 2003.
315. Venkatraman A, Landar A, Davies AJ, Ulasova E, Page G, Murphy MP, Darley-Usmar V, and Bailey SM. Oxidative modification of hepatic mitochondria protein thiols: effect of chronic alcohol consumption. *Am J Physiol Gastrointest Liver Physiol* 286: G521–G527, 2004.
316. Verdoucq L, Vignols F, Jacquot J-P, Chartier Y and Meyer Y. *In vivo* characterization of a thioredoxin *h* target protein defines a new peroxiredoxin family. *J Biol Chem* 274: 19714–19722, 1999.
317. Volke D and Hoffmann R. Quantitative proteomics by fluorescent labeling of cysteine residues using a set of two cyanine-based or three rhodamine-based dyes. *Electrophoresis* 29: 4516–4526, 2008.
318. Wang X, Ling S, Zhao D, Sun Q, Li Q, Wu F, Nie J, Qu L, Wang B, Shen X, Bai Y, Li Y, and Li Y. Redox regulation of actin by Thioredoxin-1 is mediated by the interaction of the proteins *via* cysteine 62. *Antioxid Redox Signal* 13: 565–573, 2010.
319. Weber H, Engelmann S, Becher D, and Hecker M. Oxidative stress triggers thiol oxidation in the glyceraldehyde-3-phosphate dehydrogenase of *Staphylococcus aureus*. *Mol Microbiol* 52: 133–140, 2004.
320. Wedel N, Soll J, and Paap BK. CP12 provides a new mode of light regulation of Calvin cycle activity in higher plants. *Proc Natl Acad Sci USA* 94: 10479–10484, 1997.

321. Winger AM, Taylor NL, Heazlewood JL, Day DA, and Millar AH. Identification of intra- and intermolecular disulphide bonding in the plant mitochondrial proteome by diagonal gel electrophoresis. *Proteomics* 7: 4158–4170, 2007.
322. Winter J and Jakob U. Beyond transcription–new mechanisms for the regulation of molecular chaperones. *Crit Rev Biochem Mol Biol* 39: 297–317, 2004.
323. Winter J, Linke K, Jatzek A, and Jakob U. Severe oxidative stress causes inactivation of DnaK and activation of the redox-regulated chaperone Hsp33. *Mol Cell* 17: 381–392, 2005.
324. Wolf C, Hochgräfe F, Kusch H, Albrecht D, Hecker M, and Engelmann S. Proteomic analysis of antioxidant strategies of *Staphylococcus aureus*: diverse responses to different oxidants. *Proteomics* 8: 3139–3153, 2008.
325. Wong JH, Balmer Y, Cai N, Tanaka CK, Vensel WH, Hurkman WJ, and Buchanan BB. Unraveling thioredoxin-linked metabolic processes of cereal starchy endosperm using proteomics. *FEBS Lett* 547: 151–156, 2003.
326. Wong JH, Cai N, Balmer Y, Vensel WH, Hurkman WJ, and Buchanan BB. Thioredoxin targets of developing wheat seeds identified by complementary proteomic approaches. *Phytochemistry* 65: 1629–1645, 2004.
327. Wong JH, Cai N, Tanaka CK, Vensel WH, Hurkman WJ, and Buchanan BB. Thioredoxin reduction alters the solubility of proteins of wheat starchy endosperm: an early event in cereal germination. *Plant Cell Physiol* 45: 405–415, 2004.
328. Wong HL and Liebler DC. Mitochondrial protein targets of thiol-reactive electrophiles. *Chem Res Toxicol* 21: 796–804, 2008.
329. Wood ZA, Poole LB, and Karplus PA. Peroxiredoxin evolution and the regulation of hydrogen peroxide signaling. *Science* 300: 650–653, 2003.
330. Wood ZA, Schröder E, Harris JR, and Poole LB. Structure, mechanism and regulation of peroxiredoxins. *Trends Biochem Sci* 28: 32–40, 2003.
331. Wouters MA, Fan SW, and Haworth NL. Disulphides as redox switches: from molecular mechanisms to functional significance. *Antioxid Redox Signal* 12: 53–91, 2010.
332. Wu C, Liu T, Chen W, Oka S, Fu C, Jain MR, Parrott AM, Baykal AT, Sadoshima J, and Li H. Redox regulatory mechanism of transnitrosylation by thioredoxin. *Mol Cell Proteomics* 9: 2262–2075, 2010.
333. Wynn R, Cocco MJ, and Richards FM. Mixed disulfide intermediates during the reduction of disulfides by *Escherichia coli* thioredoxin. *Biochemistry* 34: 11807–11813, 1995.
334. Yamazaki D, Motohashi K, Kasama T, Hara Y, and Hisbori T. Targets of the cytosolic thioredoxins in *Arabidopsis thaliana*. *Plant Cell Physiol* 45: 18–27, 2004.
335. Yang Y and Loscalzo J. S-nitrosoprotein formation and localization in endothelial cells. *Proc Natl Acad Sci USA* 102: 117–122, 2005. Erratum in: *Proc Natl Acad Sci USA* 102: 4655, 2005.
336. Yang Y, Song Y, and Loscalzo J. Regulation of the protein disulfide proteome by mitochondria in mammalian cells. *Proc Natl Acad Sci USA* 104: 10813–10817, 2007.
337. Yano H, Wong JH, Cho MJ, and Buchanan BB. Redox changes accompanying the degradation of seed storage proteins in germinating rice. *Plant Cell Physiol* 42: 879–883, 2001.
338. Yano H, Wong JH, Lee YM, Cho M-J, and Buchanan BB. A strategy for the identification of proteins targeted by thioredoxin. *Proc Natl Acad Sci USA* 98: 4794–4799, 2001.
339. Yano H, Kuroda S, and Buchanan BB. Disulphide proteome in the analysis of protein function and structure. *Proteomics* 2: 1090–1096, 2002.
340. Yano H and Kuroda M. Disulphide proteome yields a detailed understanding of redox regulations: a model study of thioredoxin-linked reactions in seed germination. *Proteomics* 6: 294–300, 2006.
341. Yano H and Kuroda S. Introduction of the disulphide proteome: application of a technique for the analysis of plant storage proteins as well as allergens. *J Proteome Res* 7: 3071–3079, 2008.
342. Yee BC, de la Torre A, Crawford NA, Lara C, Carlson DE, and Buchanan BB. The ferredoxin/thioredoxin enzyme regulation in a cyanobacterium. *Arch Microbiol* 130: 14–18, 1981.
343. Zaffagnini M, Michelet L, Marchand C, Sparla F, Decottignies P, Le Maréchal P, Miginiac-Maslow M, Noctor G, Trost P, and Lemaire SD. The thioredoxin-independent isoform of chloroplastic glyceraldehyde-3-phosphate dehydrogenase is selectively regulated by glutathionylation. *FEBS J* 274: 212–226, 2007.
344. Zahedi Avval F and Holmgren A. Molecular mechanisms of thioredoxin and glutaredoxin as hydrogen donors for mammalian S phase ribonucleotide reductase. *J Biol Chem* 284: 8233–8240, 2009.
345. Zeller T and Klug G. Thioredoxins in bacteria: functions in oxidative stress response and regulation of thioredoxin genes. *Naturwissenschaften* 93: 259–266, 2006.
346. Zhang N and Portis AR Jr. Mechanism of light regulation of Rubisco: a specific role for the larger Rubisco activase isoform involving reductive activation by thioredoxin-f. *Proc Natl Acad Sci USA* 96: 9438–9443, 1999.
347. Zheng M, Åslund F, and Storz G. Activation of the OxyR transcription factor by reversible disulphide bond formation. *Science* 279: 1718–1721, 1998.
348. Zhou X, Han P, Li J, Zhang X, Huang B, Ruan HQ, and Chen C. ESNOQ, proteomic quantification of endogenous S-nitrosation. *PLoS One* 5: e10015, 2010.
349. Ziegelhoffer EC and Donohue TJ. Bacterial responses to photo-oxidative stress. *Nat Rev Microbiol* 7: 856–863, 2009.

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Abbreviations Used

1-DE = one-dimensional electrophoresis
2-D DIGE = two-dimensional difference gel electrophoresis
2-DE = two-dimensional electrophoresis
ATS = activated thiol sepharose
BIAM = *N*-(biotinoyl)-*N*-(iodoacetyl) ethylendiamine
biotin-HPDP = *N*-(6-(biotinamido)hexyl)-3′-(2′-pyridyldithio)propionamide
biotin-maleimide = *N*-biotinoyl-*N*′-(6-maleimidohexanoyl)hydrazide
BMCC = 1-biotinamido-4-(4′[maleimidoethylcyclo-hexane]-carboxyamido)-butane
cICAT = cleavable isotope coded affinity tag
DTT = dithiothreitol
ESI-MS/MS = electrospray ionization tandem mass spectrometry
FBA = fructose bisphosphate aldolase
GAPDH = glyceraldhyde 3-phosphate dehydrogenase
Grx = glutaredoxin
GSH = reduced glutathione
GSNO = S-nitroso-glutathione
GSSG = oxidized glutathione
GST = glutathione-S-transferase
IAB = *N*′-iodoacetyl-*N*-biotinylhexylenediamine
IAF = 5-iodoacetamidofluorescein
IAM = 2-iodoacetamide
IBTP = (4-iodobutyl)triphenylphosphonium
LC-MS/MS = liquid chromatography coupled on-line to tandem mass spectrometry
MALDI-MS = matrix assisted laser desorption ionization mass spectrometry
mBBr = monobromobimane
MDH = malate dehydrogenase
MMTS = methyl methanethiosulfonate
NAC = *N*-acetyl-L-cysteine
NEM = *N*-ethylmaleimide
NTR = NADPH thioredoxin reductase
Orp1 = oxidant receptor peroxidase 1
PDI = protein disulfide isomerase
PMF = peptide mass fingerprinting
Prx = peroxiredoxin
RNS = reactive nitrogen species
ROS = reactive oxygen species
RuBisCo = ribulose bisphosphate carboxylase/oxygenase
SOD = superoxide dismutase
TCA = tricarboxylic acid
TCEP = tris(2-carboxyethyl)phosphine
TPI = triose phosphate isomerase
Trx = thioredoxin

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1. Yves Meyer , Christophe Belin , Valérie Delorme-Hinoux , Jean-Philippe Reichheld , Christophe Riondet . 2012. Thioredoxin and Glutaredoxin Systems in Plants: Molecular Mechanisms, Crosstalks, and Functional Significance. *Antioxidants & Redox Signaling* **17**:8, 1124-1160. [Abstract] [Full Text HTML] [Full Text PDF] [Full Text PDF with Links]
2. M.-a. Sun, Y. Wang, H. Cheng, Q. Zhang, W. Ge, D. Guo. 2012. RedoxDB--a curated database for experimentally verified protein oxidative modification. *Bioinformatics* **28**:19, 2551-2552. [CrossRef]
3. Alicia Izquierdo-Álvarez, Elena Ramos, Joan Villanueva, Pablo Hernansanz-Agustín, Rubén Fernández-Rodríguez, Daniel Tello, Montserrat Carrascal, Antonio Martínez-Ruiz. 2012. Differential redox proteomics allows identification of proteins reversibly oxidized at cysteine residues in endothelial cells in response to acute hypoxia. *Journal of Proteomics* **75**:17, 5449-5462. [CrossRef]
4. Alejandro Mata-Cabana , Mario García-Domínguez , Francisco J. Florencio , Marika Lindahl . 2012. Thiol-Based Redox Modulation of a Cyanobacterial Eukaryotic-Type Serine/Threonine Kinase Required for Oxidative Stress Tolerance. *Antioxidants & Redox Signaling* **17**:4, 521-533. [Abstract] [Full Text HTML] [Full Text PDF] [Full Text PDF with Links] [Supplemental material]
5. Michael B. Sporn, Karen T. Liby. 2012. NRF2 and cancer: the good, the bad and the importance of context. *Nature Reviews Cancer* **12**:8, 564-571. [CrossRef]
6. Yong-Hak Kim, Myeong-Hee Yu. 2012. Overexpression of Reactive Cysteine-Containing 2-Nitrobenzoate Nitroreductase (NbaA) and Its Mutants Alters the Sensitivity of Escherichia coli to Reactive Oxygen Species by Reprogramming a Regulatory Network of Disulfide-Bonded Proteins. *Journal of Proteome Research* 120507110614008. [CrossRef]
7. Mirko Zaffagnini , Mariette Bedhomme , Christophe H. Marchand , Samuel Morisse , Paolo Trost , Stéphane D. Lemaire . 2012. Redox Regulation in Photosynthetic Organisms: Focus on Glutathionylation. *Antioxidants & Redox Signaling* **16**:6, 567-586. [Abstract] [Full Text HTML] [Full Text PDF] [Full Text PDF with Links]
8. Jeong-Won Park, Jae-Young Song, Hyang-Ran Hwang, Hee-Jin Park, Hee-Shang Youn, Ji-Hyun Seo, Hyung-Lyun Kang, Kon-Ho Lee, Seung-Chul Baik, Woo-Kon Lee, Myung-Je Cho, Kwang-Ho Rhee. 2012. Proteomic Analysis of Thiol-active Proteins of Helicobacter pylori 26695. *Journal of Bacteriology and Virology* **42**:3, 211. [CrossRef]
9. Tahira Farooqui, Akhlaq A. FarooquiPerspective and Directions for Future Studies 377-384. [CrossRef]
10. Alicia Izquierdo-Álvarez, Antonio Martínez-Ruiz. 2011. Thiol redox proteomics seen with fluorescent eyes: The detection of cysteine oxidative modifications by fluorescence derivatization and 2-DE. *Journal of Proteomics* . [CrossRef]
11. Wei Wang, Steven W. Barger. 2011. Roles of quaternary structure and cysteine residues in the activity of human serine racemase. *BMC Biochemistry* **12**:1, 63. [CrossRef]