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Thioredoxin Target Proteins in Chloroplast Thylakoid Membranes

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ABSTRACT

In recent years, impressive progress has been made in the identification of thioredoxin-linked proteins. However, due to technical difficulties inherent in working with hydrophobic proteins, identifications so far have been restricted to proteins in the soluble fraction. Thus, our knowledge of redox regulated membrane proteins is quite limited. To gain information in this area, the authors have applied an adaptation of the approach based on the fluorescent thiol probe monobromobimane (mBBr) to identify redox-linked proteins of chloroplast thylakoids. By application of this procedure, 14 potential membrane-bound thioredoxin target proteins were identified, including seven new candidates functional in processes associated with photosynthetic electron flow, ATP synthesis, and Photosystem II/Photosystem I state transitions. *Antioxid. Redox Signal.* 8, 1829–1834.

INTRODUCTION

During the past 5 years, unprecedented progress has been made in elucidating the redox regulatory network of plants. The development of approaches to single out redox-linked proteins in complex extracts, coupled with mass spectrometry, have led to the identification of almost 200 potential thioredoxin target proteins in a number of systems (6). Two methods were successfully applied, one based on affinity chromatography and the other on labeling with a thiol probe.

The affinity procedure traps proteins interacting covalently with thioredoxin via stabilization of the transient heterodisulfide formed during the reduction reaction (4). A mutated thioredoxin, in which the buried cysteine of the active site is replaced by serine or alanine, is used as bait to isolate target proteins selectively through the formation of a stable heterodisulfide derivative. Thus, this trap allows the selective enrichment of thioredoxin-linked proteins in crude extracts. This type of approach, developed by Motohashi *et al.* (28), was successfully applied to the soluble protein fraction of chloroplasts (stroma) (2, 13, 28), mitochondria (matrix) (3), cereal endosperm (33, 34) and Arabidopsis plants (35).

Monobromobimane (mBBr) is a thiol-specific probe that fluoresces after covalently reacting with an SH group. This property is exploited for labeling protein extracts following reduction by thioredoxin, itself reduced enzymatically with NADPH and NADP/thioredoxin reductase (NTR). After separation of the reduced and control (untreated) samples in 2-D gels, fluorescence is recorded and gels are stained for protein. The fluorescence of the two gels is compared, and those proteins reduced by thioredoxin are identified by an increase in fluorescence. This approach proved successful in initial experiments with the soluble fraction from several plant sources: peanut seeds (37), barley embryo (25), and wheat endosperm (33, 34). A modification, in which the highly sensitive probe, Cy5 maleimide, replaced mBBr, was successfully applied to barley grain in addition to the standard mBBr approach (22). It has been found, however, that the analysis of proteins with mBBr in more reducing environments requires an initial alkylation step to block the copious free cysteine residues and to decrease background fluorescence prior to reduction and labeling (3, 36). An alkylation procedure using ^{14}C-iodoacetamide was also successfully applied to identify thioredoxin targets in Arabidopsis seedlings (23).

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While the column affinity and fluorescent gel procedures are complementary (34), both were originally designed to study soluble fractions and neither is readily applicable to membrane proteins. To explore this area with chloroplasts, we have modified the original fluorescent gel procedure to include treating the preparations with a nonionic detergent. The addition of this solubilization step, together with recent improvements in separating hydrophobic proteins in 2-D gel electrophoresis (21), has enabled the identification of 14 potential thioredoxin targets. Of these, nine are membrane-bound and seven are previously unrecognized potential targets functional in electron flow and ATP synthesis. The results are consistent with an increased role of light in regulating reactions associated with processes of thylakoid membranes, including photosynthetic electron transport, ATP synthesis, and Photosystem II/Photosystem I state transitions.

MATERIALS AND METHODS

Thylakoid membranes were isolated from intact spinach chloroplasts and ruptured by osmotic shock (2). The membrane fraction was recovered by centrifugation (10,000 *g*, 4°C, 20 min). Following resuspension, the precipitate, containing the thylakoid fraction, was subjected to sonication (3 × 10 s) to rupture the membranes. The membrane fragments were pelleted by centrifugation (100,000 *g*, 4°C, 45 min) and then solubilized in buffer containing 50 m*M* Tris-HCl, pH 7.5, 1 m*M* EDTA, and 4% Triton-X100 (a nonionic detergent). After blocking the free cysteines with a mixture of 5 m*M* iodoacetmide/5 m*M* *N*-ethylmaleimide, the samples were extensively dialyzed against the above buffer at 4°C to remove excess blocking reagent. As seen below, for reasons not yet clear, the thioredoxin targets observed depended on the state of the membranes. Thus, certain targets were identified only in fresh or frozen preparations and others were found in both. It seems that this difference could, at least in part, be due to variability in topography of the membranes when analyzed fresh or after freezing.

After dialysis, membrane proteins were reduced by the NADP/thioredoxin system of *Escherichia coli* as previously described (34, 37), except that 4% Triton X-100 was included. After labeling newly formed thiol groups with mBBr, the protein fraction was precipitated with 4 volumes of cold acetone, resuspended in IEF rehydration buffer [7 *M* urea, 2 *M* thiourea, 2% beta-dodecyl maltoside, 0.5% ampholytes pH 3–10, 50 m*M* DTT (21)] and separated by 2-DE (3). Reduced protein spots were visualized by fluorescence imaging at 400 nm (34). Isoelectric focusing was accomplished with an IPG (Immobilized pH Gradient) strip in IEF rehydration buffer. After staining with colloidal Coomassie Blue (16), spots of interest were manually excised, digested with trypsin, and analyzed by electrospray ionization tandem mass spectrometry using a QSTAR Pulsar i quadrupole time-of-flight mass spectrometer (Applied Biosystems/MDS Sciex, Toronto, Ontario, Canada) (3). Matching of mass spectra resulting from three different sets of experiments to known protein or nucleotide sequences was carried out using the Open Source program X! Tandem [obtained from the Global Proteome Machine (GPM) organization] (8, 11). Further analysis of the data was carried out June 1, 2005 on the Global Proteome Machine (http://www.thegpm.org/). A locally installed copy of these programs was used for all of the analyses. Reported mass spectrometer identifications from the plant protein database search had expectation value of $<1 \times 10^{-3}$.

RESULTS

Owing to technical difficulties inherent in working with hydrophobic fractions, the identification of thioredoxin-linked proteins has been limited to soluble representatives. In a first approach to investigate the role of redox in the regulation of membrane processes, we modified the mBBr gel procedure to enable the identification of bound thioredoxin target proteins. Thylakoid membranes were disrupted by sonication, pelleted by ultracentrifugation, and then solubilized with a nonionic detergent. To minimize background fluorescence due to free SH groups, the sample was treated with a mixture of unlabeled thiol reagents (iodoacetamide and *N*-ethylmaleimide). Following this blocking step, the solubilized membranes were reduced with thioredoxin, itself reduced with NADPH and NTR (in the presence of the detergent). After mBBr labeling, the proteins were collected by precipitation with acetone and separated by 2-DE. While ongoing progress in separation procedures will undoubtedly lead to future improvements in the ability to resolve hydrophobic proteins, it is noted that, aside from the state of the membranes being analyzed, the present protocol gave reproducible results with the different chloroplast preparations used.

Figure 1 shows gels of an experiment with freshly prepared chloroplast thylakoids. Comparison of the fluorescence of the control (Fig. 1B) with the thioredoxin reduced sample (Fig. 1A) shows about 20 spots specifically reduced by thioredoxin. Of these, 11 yielded MS spectra sufficient for identification (Fig. 1C, Coomassie staining). Interestingly, despite treatment of the thylakoid fraction with iodoacetamide and *N*-ethylmaleimide, the control gel still showed significant fluorescence indicative of residual free cysteine residues (Fig. 1 B). Their presence could be due to the resistance of certain thiols to alkylation or, alternatively, to reaction of mBBr by nonthiol groups in the preparation, for example, chlorophyll (26).

The results from the three sets of experiments are shown in Table 1 (note that the five proteins observed only in frozen preparations are not shown in Fig. 1). In all, 14 different proteins were identified, of which nine were integral membrane components. Seven of these were not previously recognized as redox regulated proteins. The new candidates, each containing conserved cysteines, are linked to Photosystem I (subunits N and psaK), photosystem II (oxygen evolving enhancer protein 2), light harvesting complex (chlorophyll a/b binding protein or LHCIIb), the electron transport chain (plastocyanin, Rieske FeS protein), and ATP synthesis (ATP synthase beta subunit). Prior to our experiments, the only thioredoxin targets reported for thylakoid membrane were the gamma subunit of ATP synthase and oxygen evolving enhancer protein 1 (6, 23), both of which were confirmed in our experiments. Additionally, we confirmed the ability of thioredoxin to reduce four soluble proteins that were partly bound to thylakoids (rubisco large and small subunits, rubisco acti-

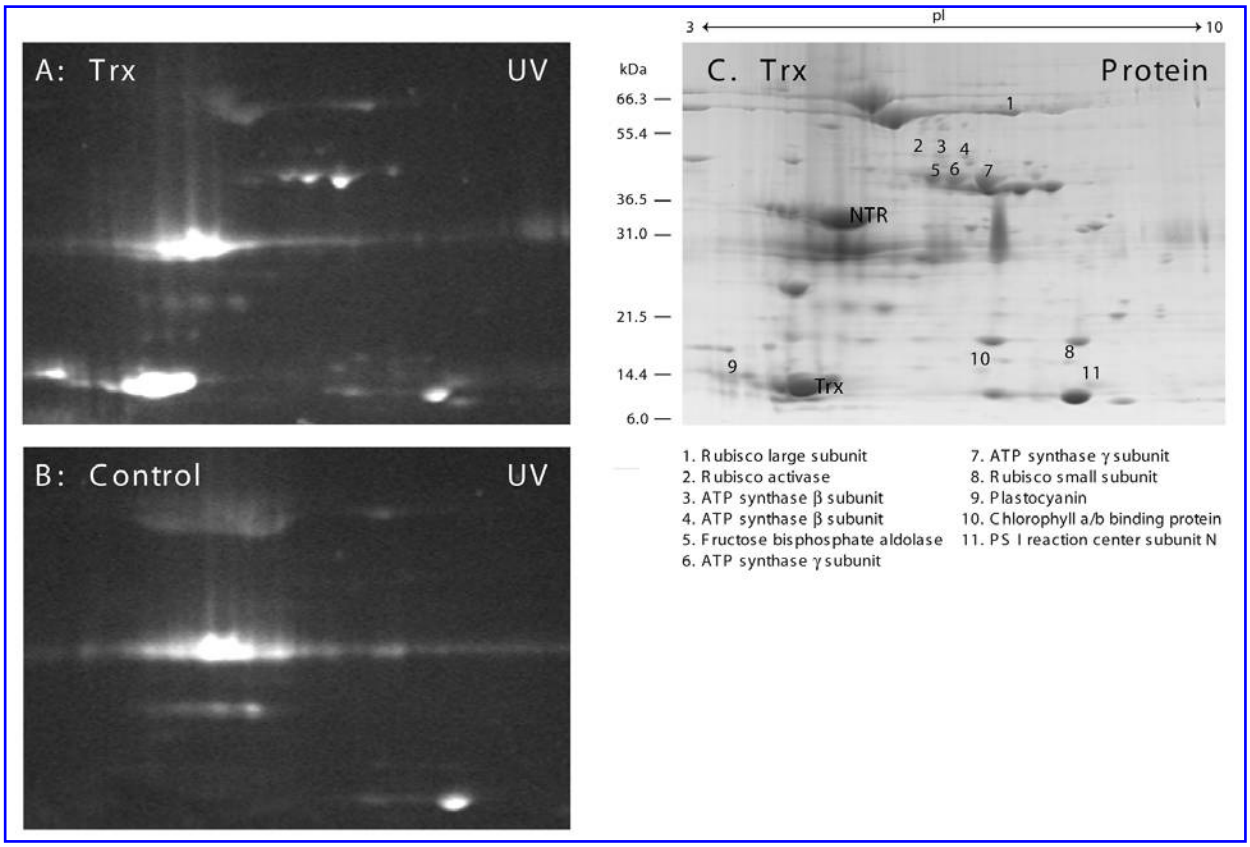

FIG. 1. Reduction of proteins of freshly prepared chloroplast thylakoid membranes by thioredoxin visualized using the mBBr 2-DE procedure. Alkylated thylakoid proteins were either reduced by thioredoxin using NADPH and NADP-thioredoxin reductase (**A**) or were not treated (**B**). Following reduction, the proteins were derivatized with mBBr, separated by 2-DE, and the fluorescence recorded. Proteins 1–11 in Coomassie Blue-stained gels (**C**) were identified by mass spectroscopy. Trx and NTR: added recombinant *E. coli* thioredoxin and NADP-thioredoxin reductase, respectively.

TABLE 1. THIOREDOXIN TARGET PROTEINS OF CHLOROPLAST THYLAKOID MEMBRANES

Protein	*Cys*	*Swiss-Prot*
Fresh Preparations		
Rubisco large subunit*†	8	P00875
Rubisco activase*†	2	P10871
ATP synthase, beta subunit	1	P00825
Rubisco small subunit* †	2	P00875
Plastocyanin	1	P00289
Frozen Preparations		
Photosystem II oxygen evolving enhancer (OEE 1)*	2	P12359
Photosystem II oxygen evolving enhancer (OEE 2)	3	P12302
Rieske FeS protein	5	P08980
Photosystem I reaction center, subunit psaK	1	P14627
Hypothetical protein	7	O65502
Both Preparations		
ATP synthase, gamma subunit*	3	P05435
Chlorophyll a/b binding protein (LHCIIb)	2	Q40247
Photosystem I reaction center, subunit N	4	P31093
Fructose bisphosphate aldolase* †	2	P16096

*Previously described target protein
† Bound soluble proteins

vase, and fructose 1,6-bisphosphate aldolase) (2, 20, 28). Finally, one hypothetical protein with homology to an NADPH-dependent reductase was also detected. The significance of these results is discussed below.

Photosystem I

The two photosystem I subunits identified, N and K, contain 4 and 1 conserved cysteines, respectively. The N subunit is localized on the lumenal side of the thylakoid and could be involved in the binding of the antenna complexes to the reaction center (17). This subunit was previously identified as a disulfide-containing protein in *Arabidopsis thaliana* (19). Also associated with Photosystem I is psaK—an intrinsic subunit of the reaction center complex where it is involved in Photosystem II/Photosystem I state transitions (32).

Photosystem II

Oxygen evolving enhancer protein 2 was identified as a potential thioredoxin target. Together with its oxygen evolving enhancer protein 1 counterpart, this protein is located on the lumenal side of the thylakoid membrane and seems to function in stabilizing the manganese cluster, the site of water oxidation. Interestingly, a soluble protein from green algae identical to oxygen evolving enhancer protein 1 shows thioredoxin-like activity (14).

Light harvesting complex

The chlorophyll a/b binding protein (specifically LHCIIb), contains two conserved cysteines and associates with chlorophyll a and b to form a complex active as a light receptor. This complex, which has long been known to undergo reversible phosphorylation, is generally thought to mediate the distribution of energy between Photosystems I and II (1). The finding of a link to thioredoxin supports a role for redox in regulating this distribution of energy. Others have previously proposed such a role for thioredoxin (or an unidentified thiol) based on studies with isolated chloroplast thylakoids (24, 31), but a protein target was not identified. If confirmed, the results suggest that thioredoxin regulates not only the chlorophyll a/b binding protein substrate, but, according to recent evidence, the kinase that phosphorylates it as well (10). In such a system, light, acting via thioredoxin, could control energy distribution by via both the redox and phosphorylation state of the protein.

Electron transport chain

Two components of the photosynthetic electron transport chain linking Photosystems I and II—plastocyanin and Rieske FeS protein—were identified as potential thioredoxin targets. Plastocyanin, located on the lumenal side of the thylakoid membrane, transfers electrons from the cytochrome b6f complex to P700. It possesses one conserved cysteine that is involved in the ligation of the copper. However, oxidation is known to result in the formation of disulfide-linked dimers (30)—a change that could possibly be reversed by reduced thioredoxin or a related protein. The Rieske FeS protein is part of the cytochrome b6f complex. Three of its conserved cysteines are involved in the iron–sulfur cluster, while the remaining two form a disulfide that faces the lumen (18) that could be subject to regulation. This possibility is strengthened by recent work indicating a signaling function of the Rieske FeS protein in addition to its role in electron transport (9). Additional evidence for a regulatory role comes from experiments with yeast mitochondria showing that the disulfide loop is required for electron transport, but not for assembly of the iron–sulfur cluster (27).

ATP synthesis

The gamma subunit of the ATP synthase complex, found to be regulated by thioredoxin nearly 30 years ago (5), was the only thylakoid membrane protein known to be regulated by redox until the recent identification of oxygen evolving enhancer protein 1 (14, 20, 23). The present study suggests that the beta-subunit of the ATP synthase enzyme, containing one conserved cysteine, is also a thioredoxin target (Table 1). Thus, based on current evidence, both the gamma and beta subunits of the chloroplast ATP synthase complex appear to be linked to thioredoxin. The beta subunit of the ATP synthase was also identified as a potential thioredoxin target in plant mitochondria (3).

Hypothetical protein

One hypothetical protein containing seven conserved cysteines was detected as a candidate target in this study. Predicted to be localized in the plastid, this protein is likely to have two strong transmembrane domains (15).

Bound soluble proteins

Four stromal proteins recovered in our thylakoid preparations—rubisco large and small subunits, rubisco activase, and fructose bisphosphate aldolase—were reduced by thioredoxin in our experiments (Table 1). All were previously identified as thioredoxin target proteins (20, 28, 34). They were likely detected in our experiments as a result of a weak association with the thylakoid membranes (29).

DISCUSSION

A regulatory link to redox provides a mechanism whereby light modulates photosynthetic electron flow and the accompanying synthesis of ATP via thioredoxin or potentially other disulfide proteins. Originally proposed to be controlled by phosphorylation (1), the optimization of light utilization more recently has also been linked to redox (24, 31). Current evidence strengthens this role and suggests that the distribution of light energy is controlled by both phosphorylation (favoring Photosystem I) and thioredoxin (favoring Photosystem II). Light also appears to regulate the associated synthesis of ATP via the ATP synthase gamma-subunit. The basis for the presently identified connection between thioredoxin and the beta-subunit of the enzyme remains to be elucidated.

The importance of thioredoxin, or related redox proteins, in regulating the other candidate targets presently identified, notably those of the lumen, requires further investigation (plastocyanin, Rieske protein, oxygen enhancer protein 2,

Photosystem I reaction center subunits N, and psaK). It seems possible that, as recently proposed for the immunophilin FKBP13, redox status could be linked to the regulation or transport of these proteins into the lumen (7, 12). It remains to be seen whether the activity of these proteins is also controlled by redox, for example, diurnally as has been established for stromal enzymes, or whether regulation by redox is a one time only event. It is anticipated that the ability to study membrane-bound proteins linked to redox will provide answers to these questions as well as to the role of the transmembrane regulatory network that has surfaced in promoting communication between cells and organelles (7).

Finally, the present study with solubilized bound thioredoxin targets raises questions relating to membrane topography. It becomes of interest to examine these proteins under more physiological conditions, such as while they remain anchored to the membrane. When compared to the present study, experiments of this type are expected to give information on targets that are buried in isolated thylakoids, but become exposed under other conditions, for example, in the light. Studies of this nature promise to advance our knowledge not only of redox regulation, but also of membrane function.

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ABBREVIATIONS

mBBr, monobromobimane; NTR, NADP-thioredoxin reductase; Trx, thioredoxin.

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