ANTIOXIDANTS & REDOX SIGNALING
Volume 5, Number 4, 2003

Forum Review

Functions of Thiol-Disulfide Oxidoreductases in *E. coli*: Redox Myths, Realities, and Practicalities

RON ORTENBERG and JON BECKWITH

ABSTRACT

A large family of enzymes contributes to the thiol-disulfide redox environment of the cells of most organisms. These proteins belong to pathways that carry out a variety of reactions, including the promotion of disulfide bond formation in extracytoplasmic proteins, the isomerization of proteins with incorrect disulfide bonds, and the reduction of disulfide bonds in the active sites of cytoplasmic proteins. Although the redox activities of these proteins measured *in vitro* often is consistent with the role (oxidant or reductant) these proteins perform *in vivo*, this is not always the case. The measured redox potentials can even suggest a function for a protein opposite of that which it carries out in the cell. Structural features of such proteins can contribute to a direction of electron transfer inconsistent with the redox potential. Furthermore, the environment in which such proteins are found may determine the protein's physiological role. Detailed analysis of these proteins in *Escherichia coli* provides strains that are useful for biotechnological purposes. Increasing the activity of certain of these proteins in the cell envelope or altering the thiol-disulfide redox environment of the cytoplasm to make it more oxidizing enhances the yield of useful disulfide bond-containing proteins such as tissue plasminogen activator and immunoglobulins. *Antioxid. Redox Signal.* 5, 403–411.

INTRODUCTION

COVALENT LINKAGES OF AMINO ACIDS IN PROTEINS are largely limited to the peptide bond. The most common exception to this rule is the disulfide bond, a sulfur–sulfur chemical bond that results from an oxidative process that links nonadjacent (in most cases) cysteines of a protein. Proteins that contain disulfide bonds can be divided into two classes: those in which the cysteine–cysteine linkage is a stable part of their final folded structure and those in which pairs of cysteines alternate between the reduced and oxidized states. For the first class, the disulfide bond may contribute to the folding pathway of the protein and to the stability of its native state. For the second, the oxidative–reductive cycling of the disulfide bond may be central to a protein's activity as an enzyme [see, *e.g.,* certain ribonucleotide reductases (29)] or may be involved in a protein's activation and deactivation [see, *e.g.,* OxyR (4, 70)].

It has long been noted that proteins containing stable disulfide bonds are rarely found in the cytoplasmic compartments of any organism. Instead, in bacteria, they are usually located in extracytoplasmic compartments or the external milieu and, in eukaryotic cells, in compartments such as the endoplasmic reticulum and the plasma membrane or secreted into the external milieu. The restriction of stably disulfide-bonded proteins to noncytoplasmic environments has been attributed to the contrasting reductive nature of the cytoplasm and the oxidative nature of certain other compartments and the external milieu. A recent exception to this rule is the discovery of large numbers of disulfide-bonded proteins in certain Archaea (42, 63).

Proteins that are capable of catalyzing protein disulfide bond formation or reduction are members of a large collection of thiol-disulfide oxidoreductases found in all living cells. Many of these enzymes belong to the thioredoxin superfamily, which is defined by an active site containing a

Department of Microbiology and Molecular Genetics, Harvard Medical School, Boston, MA 02115.

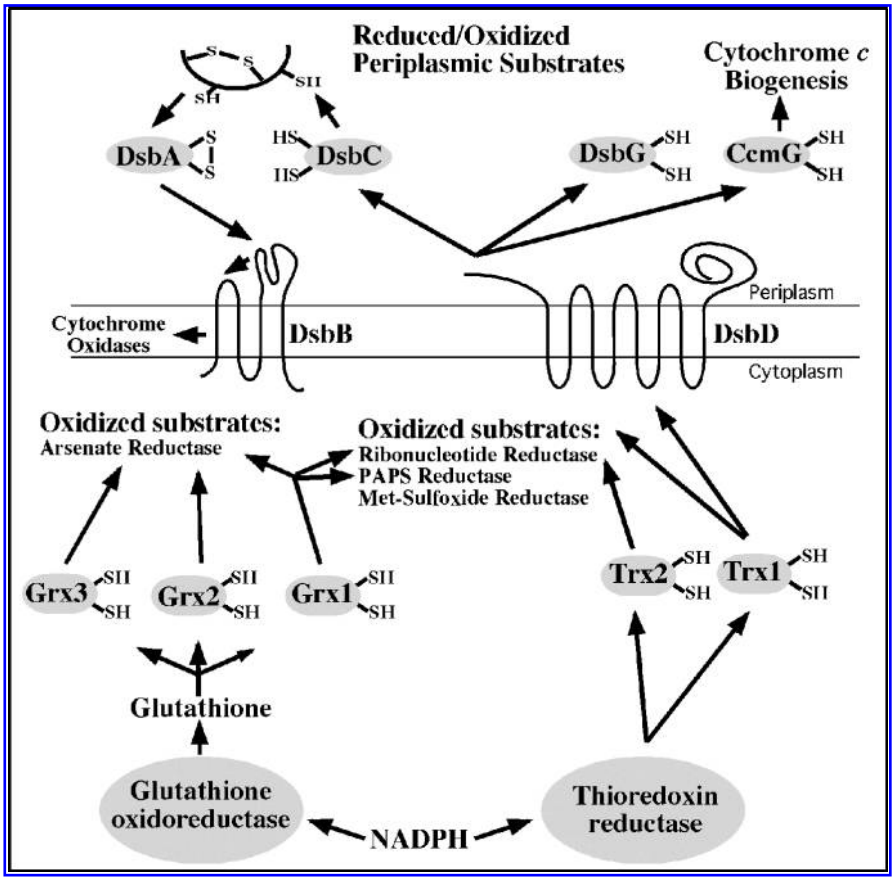

FIG. 1. Disulfide reducing and oxidizing pathways in *Escherichia coli*. The cytoplasmic NADPH-dependent reductases thioredoxin reductase and glutathione reductase reduce thioredoxins (Trx1 and Trx2) and glutaredoxins (Grx1, Grx2, and Grx3), respectively. The abilities of thioredoxins and glutaredoxins to reduce different cytoplasmic substates are indicated. In the periplasm, DsbA/DsbaB is responsible for thiol oxidation, whereas DsbD is involved in disulfide bond isomerization (via DsbC), cytochrome *c* maturation (via CcmG), and reduction of DsbG. The arrows indicate the direction of the electron flow.

Cys-Xaa-Xaa-Cys (CXXC) motif, and by a "thioredoxin fold" seen in the three-dimensional structure of the prototypical thioredoxin 1 of *E. coli*. Whereas in extracytoplasmic compartments these proteins can act as oxidants, those located in the cytoplasm perform mainly reductive steps. One of the most important of the cytoplasmic activities for many bacteria is the reduction of ribonucleotide reductase by thioredoxins and glutaredoxins (Fig. 1).

Increasingly, thiol oxidoreductases are being identified that do not belong to the thioredoxin superfamily. Typically, these show entirely different three-dimensional structures from thioredoxin and use redox-active cysteine pairs that are separated by more than two amino acids. In addition, these enzymes may use small-molecule electron donors and receptor cofactors, such as FAD, NADPH, NADH, quinones, and lipoic acid (for reviews, see 13, 53). Unlike the thioredoxin family, the structures of this latter group of proteins do not fall into a single or even a few classes. Many of these "non–thioredoxin-like" enzymes themselves receive electrons from or donate electrons to proteins belonging to the "thioredoxin-like" class. So far, those enzymes whose major role is to form disulfide bonds in extracytoplasmic proteins are members of the thioredoxin superfamily and not of this "other" class. Similarly, most of those cytoplasmic reductants that carry out the last step in a pathway of electron transfer, such as the reduction of ribonucleotide reductase, are also members of the thioredoxin superfamily.

The wide variety of thiol-disulfide oxidoreductases and their multitude of functions raise in stark fashion one of the problems confronting functional genomics. Given that it is often possible to identify the family these proteins belong to by bioinformatic analysis of their amino acid sequence, how does one determine their biological function? More specifically, how does one deduce whether their main role is to act as oxidants or reductants or what substrate they act on? In this review, we highlight the factors that have to be taken into account in considering the role and substrate specificity of these proteins.

THE CXXC SEQUENCE AS A DETERMINANT OF THIOL-DISULFIDE OXIDOREDUCTASE FUNCTION

The physiological function of a thiol-disulfide oxidoreductase is usually correlated with the redox properties of its

active-site disulfide. In the case of members of the thioredoxin superfamily, a major determinant of the redox potential of the active-site disulfide bond is the nature of the amino acid residues between the two active-site cysteines (24, 45, 46). In *E. coli*, two of the major disulfide bond reductases, thioredoxin 1, with a CGPC sequence, and glutaredoxin 1, with a CPYC sequence, have redox potentials of −270 mV (35) and −233 mV (3), respectively. In contrast, the main catalyst of disulfide bond formation (an oxidative process) in the periplasm of *E. coli*, DsbA, has a CPHC active site and a redox potential of −120 mV (24, 66, 68). Replacement of the two amino acids between the cysteines of a reducing enzyme with those from a more oxidizing one causes the redox potential of the enzyme to become more oxidizing, and vice versa. For example, changing the Gly-Pro dipeptide of the thioredoxin active site to Pro-His (from the active site of DsbA) or to Pro-Tyr (from the glutaredoxin active site) causes a shift of redox potential of thioredoxin 1 from −270 mV to −204 mV and −195 mV, respectively (46). These changes are sufficient to abolish almost entirely the ability of these variants of thioredoxin 1 to function *in vivo* as electron donors to 3′-phosphoadenylylsulfate (PAPS) reductase and methionine sulfoxide reductase (MsrA). Both of these latter enzymes ordinarily utilize thioredoxin as a source of electrons to regenerate their activities after carrying out their reductive reactions (40, 41, 44, 61, 64). In addition, experiments in which thioredoxin 1 was exported to the periplasm of an *E. coli dsbA* mutant revealed that wild-type thioredoxin 1 in the periplasm could only weakly substitute for the oxidative function of DsbA, whereas the thioredoxin variant containing the DsbA CPHC sequence could fully complement the DsbA deficiency (15, 16, 28). These thioredoxin mutants function as oxidants in the periplasm of *E. coli*.

Analogous studies have been carried out with thioredoxin 1 in the yeast *Saccharomyces cerevisiae*. Thioredoxin 1, when exported to the endoplasmic reticulum, cannot complement a protein disulfide isomerase (PDI) null mutation in yeast. However, a mutant thioredoxin, in which the redox potential of the active-site disulfide was changed to one more closely resembling the redox potential of the active-site disulfide in PDI, does complement the PDI null mutant yeast strain for growth (11).

Should these studies be taken to indicate that the redox potential of the active-site disulfide bond is the major factor determining the function of the thioredoxin superfamily members? That this is not the case follows from an examination of a series of DsbA variants in which the two amino acids between the cysteines of the active site were extensively randomized (10). The results indicated that there was no simple correlation between the redox potentials of these variants and the *in vivo* efficiency of oxidative folding of protein substrates of DsbA. For example, the steady-state level of activity of β-lactamase (having one disulfide bond) was indistinguishable among strains carrying a range of *dsbA* alleles, including ones in which the DsbA protein exhibited a redox potential as low as −220 mV. In contrast, the formation of active murine urokinase cloned into *E. coli* was markedly affected by the sequence of the active-site dipeptide. Two of the less-oxidizing mutants, CSFC (−152 mV) and CPSC (−173 mV), displayed levels of urokinase activity that were three- to fourfold higher than those displayed by the wild-type strain. Mutants with similar redox potentials resulted in widely different levels of active urokinase. Most notably, the CQLC mutant, which has an *in vitro* redox potential similar to that of CPSC, gave very low activity. The least-oxidizing mutant CPPC (−220 mV) fell in the middle of the range in terms of the urokinase yield. Thus, it is clear that although the CPHC motif of DsbA strongly affects the ability of the cell to fold urokinase, it does so in ways that are not entirely related to its role in determining the redox potential. One possible explanation for these results is that redox potentials are usually measured by determiming equilibrium constants with mixtures of reduced and oxidized glutathione (GSH/GSSG). Thus, this value may reflect only reactivity with glutathione, and may be different from that with thiols present in the substrate proteins.

REDOX ENVIRONMENT AS A DETERMINANT OF THIOL-DISULFIDE OXIDOREDUCTASE FUNCTION

Further indication that the redox potential of the active-site disulfide of oxidoreductases is not necessarily determinant of the function of these proteins as oxidants or reductants is the finding that these enzymes can be forced, by manipulating the redox environment *in vivo*, to efficiently perform the reverse of their normal enzymatic reactions. What is the redox environment, and how is it possible to change it?

The redox environment of a linked set of redox couples as found in a biological fluid, organelle, cell, or tissue is the summation of the products of the reduction potential and reducing capacity of the linked redox couples present (57). Thus, the redox environment of a cell, or particular compartment of the cell, is a reflection of the state of these couples. The reduction potential of a particular redox couple is measured as a voltage. Reducing capacity is estimated by determining the concentration of the reduced species in a redox couple. Thus, the definition of cell cytoplasm as a reducing environment with respect to thiols and disulfides means that the active-site cysteines of thiol oxidoreductases, for example, are kept in the reduced form, allowing these enzymes to function as reductants. On the other hand, the periplasmic environment of gram-negative bacteria with regard to the thiol-disulfide balance is highly oxidizing, a state established primarily by the powerful oxidant DsbA, which is kept in the oxidized form by the membrane protein DsbB (13). An important clarification that touches on general discussions of cellular redox environments is that here we have been discussing the components of the redox environment relevant to disulfide bond reduction and oxidation. Effects on these components do not necessarily affect other contributors to the redox environment or even significantly alter overall redox potential of a cellular compartment.

It is possible to readily alter the thiol-disulfide redox environment of subcellular compartments of *E. coli* by introducing mutations that eliminate the enzymes responsible for those environments. For example, a strain lacking the enzyme

thioredoxin reductase (TrxB) would no longer be able to regenerate two of the major disulfide reductants in the *E. coli* cytoplasm, thioredoxins 1 and 2. Instead, the cells accumulate the oxidized forms of both (61). Thus, the thiol-redox environment of the cytoplasm has become more oxidizing. As a consequence of this alteration, and in contrast to the wild-type situation, it becomes possible to accumulate proteins with stable disulfide bonds in the cytoplasm.

For example, when disulfide-bonded proteins such as *E. coli* alkaline phosphatase and mouse urokinase are expressed without their signal sequences in wild-type *E. coli*, they are retained in the cytoplasm where they are enzymatically inactive (17, 49). Now, when the same proteins are expressed in the cytoplasm of the *trxB* mutant, enzymatically active forms of these proteins accumulate (18, 49). Importantly, the accumulation of disulfide bonds in cytoplasmic proteins is not due simply to an "alteration of the redox environment"; rather, in the *trxB* mutant, oxidized thioredoxins actively promote disulfide bond formation in these proteins (61). This finding is expected from a thermodynamic point of view. The redox equilibrium reaction involving thioredoxin can be represented as

$$\text{Trx (red)} + \text{Protein (S-S)} = \text{Trx (oxi)} + \text{Protein (-SH -SH)}$$

If thioredoxin is maintained in the oxidized form, the equilibrium can go only backwards. Thus, in the absence of thioredoxin reductase, thioredoxins are actually performing the opposite function from their usual physiological role of reducing disulfide bonds. Interestingly, comparable disruption of the glutaredoxin reduction pathway by deletion of the *gor* gene (glutathione oxidoreductase), which results in the accumulation of oxidized glutaredoxins, did not give any appreciable accumulation of disulfide-bonded proteins in a cytoplasm. Apparently, oxidized glutaredoxins are not as effective as thioredoxins at promoting disulfide bond formation.

The efficiency of disulfide bond formation in the *E. coli* cytoplasm can be further enhanced by construction of a strain defective in both the glutathione/glutaredoxin and thioredoxin pathways (*trxB*$^-$,*gor*$^-$). However, this double mutant grows very poorly under aerobic conditions, presumably due to the failure to reduce ribonucleotide reductase. Selection for derivatives of the *trxB,gor* mutant that grow at normal rates yielded strains with suppressor mutations. In all cases, the mutations had altered the gene, *ahpC*, encoding a component of the enzyme alkylhydroperoxidase, converting it from a peroxidase to a disulfide reductase (55). The alterations in this enzyme, which itself uses disulfide bond chemistry in its reaction mechanism, caused it to direct electron flow to the glutathione pathway, thus restoring reduction of ribonucleotide reductase.

Remarkably, the suppressor mutations (*ahpC**) were all the result of the same event, the expansion of a triplet nucleotide repeat sequence. The expansion causes the addition of a single amino acid to alkylhydroperoxidase, dramatically altering the physiological function of this protein. Despite this restored viability and electron flow, the *trxB,gor,ahpC** strain still is able to efficiently catalyze cytoplasmic disulfide bond formation in the *E. coli* protein alkaline phosphatase and even in the complex eukaryotic protein, tissue plasminogen activator, which contains multiple disulfide bonds (8). Disulfide bond formation in this strain still depends on the oxidized thioredoxins.

Another example of the influence of the redox environment of a compartment on the physiological role of a protein comes from the above-mentioned studies in which a signal sequence was fused to the N-terminus of thioredoxin 1, causing it to be exported to the *E. coli* periplasm (15, 16). In the oxidative milieu of the periplasm, some of the thioredoxin is oxidized by DsbB, allowing it to partially substitute for DsbA in the formation of disulfide bonds. DsbB is a membrane protein that is normally responsible for keeping DsbA in the oxidized state.

A similar reversal of function of a protein, dependent on the redox environment, is observed with the periplasmic disulfide bond isomerase, DsbC. DsbC reduces and promotes isomerization of incorrect disulfide bonds in the periplasm. To maintain this activity, the CXXC motif must be kept in the reduced state by the membrane protein, DsbD. In a strain lacking DsbD, DsbC is found in the fully oxidized state. Oxidized DsbC is capable of partially restoring disulfide bond formation to a *dsbA* mutant (51). (See below for further discussion of DsbC as an oxidant and reductant.)

PROTEIN STRUCTURAL FEATURES AS DETERMINANTS OF THIOL-DISULFIDE OXIDOREDUCTASE FUNCTION

We have pointed out the importance of the redox potential in defining the *in vivo* role of a thiol-disulfide oxidoreductase. At the same time, we have shown that redox potential considered by itself can be misleading in assigning such roles. In particular, the nature of the environment in which the protein is functioning can reverse predictions deduced from measurement of redox potentials. Here we describe a striking case of a native protein in its native environment that contradicts simplistic expectations based on either redox potential or redox environment.

Purified periplasmic disulfide bond isomerase, DsbC, exhibits a redox potential of −130 mV (69), almost as high as that of the most oxidizing of this family of proteins, DsbA. From a simple analysis based on redox potential *and* redox environment, one would have predicted this protein to be an oxidant. It is not. As mentioned, the membrane protein DsbD maintains DsbC in the reduced state. But that is not the whole story, for DsbC can be converted by a single amino acid alteration to a protein that acts as an efficient oxidant. The explanation is as follows: DsbC is a homodimeric protein made up of monomers that contain a thioredoxin-like domain and a dimerization domain (43). The two active sites of DsbC are sequestered within a cleft of the dimer that prevents them from being oxidized by a protein such as DsbB, but allows substrates and a soluble domain of DsbD to access them.

A single mutation in the dimerization domain of DsbC results in monomerization of the protein and conversion of the

protein to an oxidant (7). In the monomeric form, the active-site CXXC motif is now accessible to DsbB, becomes oxidized, and acts as an efficient oxidant capable of restoring disulfide bond formation in the periplasm to a *dsbA* mutant. This is a remarkable cautionary finding. It means that deducing the direction of electron flow merely from the redox potential of a protein (or of a domain of a protein) can be terribly misleading in assessing the function of the protein and its mechanism of action.

The findings with DsbC may provide a partial explanation for a "redox potential paradox" seen with the protein, DsbB. DsbB is a cytoplasmic membrane protein with four transmembrane segments and two pairs of essential cysteines. The first pair of cysteines is located in the first small periplasmic loop in a CXXC arrangement (but not in a thioredoxin fold). The other pair of cysteines is in the second larger periplasmic loop. DsbB restores disulfide bonds to DsbA by transferring electrons from the reduced DsbA to membrane-embedded quinones. The reduced quinones are then oxidized aerobically by terminal oxidases using molecular oxygen or anaerobically using other electron acceptors such as nitrate or fumarate (5, 6, 34). In spite of the remarkable recent progress in studying the electron transfer reaction mediated by DsbB, puzzling questions still remain. Specifically, the redox potential of DsbB is −210 mV for its first N-terminal cysteine pair and −250 mV for the pair of cysteines in the C-terminal periplasmic domain, which interacts with DsbA (25, 50). Taken by themselves, these data suggest that DsbB should function as a reductant of DsbA, which has a higher redox potential (−122 mV). However, the DsbA oxidation reaction is carried out efficiently, despite the unfavorable redox potential differences. Several explanations for this "paradox" come to mind. First, DsbB has high affinity for quinones, a high redox potential compound, which may affect anomalously the apparent redox properties of DsbB (6, 25, 32). (The redox potentials of each of the two domains were measured with purified mutant proteins that were lacking the cysteines of the other domain, using equilibrium constants with a cysteine/cystine mixture.) Second, DsbB appears to adopt an unusual mechanism of electron transfer within the protein using two pairs of cysteines in a coordinated reaction to accept electrons from the active cysteines of DsbA (31). This mechanism may depend on structural rearrangements of the protein, which contribute to the facilitation of electron transfer in the unexpected direction. Furthermore, the multi-disulfide-bonded intermediates suggested for the quinone-DsbB-DsbA reaction (31) may exhibit redox potentials themselves that do not reflect the *in vitro* measurements. In sum, the redox potentials of these separate domains measured *in vitro* may not be the same inside the cell acting on a substrate different from glutathione (DsbA) and forming intermediates with their own redox properties and structures that favor the direction of electron flow observed *in vivo*. Finally, it is possible that DsbB and DsbA are maintained in an oxidizing state because of kinetic factors. If electrons are transferred from DsbB to quinone much faster than that from DsbA to DsbB, and the reaction network (DsbA-DsbB-quinone) is kinetically controlled, then the redox potential constants become irrelevant.

DETERMINANTS OF SUBSTRATE SPECIFICITY OF THIOL-DISULFIDE OXIDOREDUCTASES

Examples we have cited above indicate that the basic design of many thiol oxidoreductases is such that they can carry out thiol exchange reactions well in either direction and can substitute for one another in such reactions. Witness the ability of thioredoxin exported to the periplasm to partially replace the function of DsbA (15, 16). In general, a host of both *in vitro* and *in vivo* genetic studies indicate that indeed there is significant functional overlap among members of this protein family. Consider the reduction of the enzyme ribonucleotide reductase, which is essential for growth and whose active-site cysteines form a disulfide bond as result of the catalytic activity of the enzyme. Under aerobic conditions, this reduction is dependent on thiol reductants. Both thioredoxins 1 and 2 and glutaredoxin 1 can act as electron donors to ribonucleotide reductase. Mutational elimination of any one or two of these reductants does not interfere with growth of *E. coli*. However, a *trxA,trxC,grxA* triple mutant is incapable of growth (49). Similar functional overlap is seen with the reduction of the membrane protein DsbD, which acts in the periplasm to reduce DsbD and other substrates. Ordinarily, the source of electrons for DsbD is cytoplasmic thioredoxin (52). However, overexpression of the normally poorly expressed thioredoxin 2 complements the defect of a *trxA* mutant for DsbD reduction, indicating that the thioredoxins are functionally equivalent in this process when expressed at similar levels (54).

Despite these examples of functional overlap, there is also extensive evidence of significant substrate specificity among thiol-disulfide oxidoreductases. Only thioredoxin 1 and glutaredoxin 1 serve as hydrogen donors to PAPS reductase (40, 61, 64), although thioredoxin 2 can perform this function when overexpressed (54). The enzyme methionine sulfoxide reductase, which utilizes the sulfhydryls of cysteines in its reduction of methionine sulfoxide to methionine, allows *E. coli* to use methionine sulfoxide as the sole source of methionine. However, cells cannot utilize methionine sulfoxide if they lack the gene for thioredoxin 1, indicating that thioredoxin 1 is the only member of the thiol redox family that can reduce methionine sulfoxide reductase (56). (Even overexpressed thioredoxin 2 will not substitute for thioredoxin 1 in this case.) Finally, the physiological substrates of glutaredoxins 2 and 3 remain unknown. Although glutaredoxin 3 can reduce ribonucleotide reductase *in vitro* (1), it fails to do so *in vivo*; a plasmid expressing high levels of glutaredoxin 3 does not complement a *trxA,trxC,grxA* triple mutant of *E. coli* for growth. A possible function for glutaredoxin 2 is suggested by the demonstration that it is the most effective hydrogen donor *in vitro* for the reduction of arsenate by arsenate reductase (58).

The features of the thiol-disulfide oxidoreductases that are responsible for differing substrate specificities are not well studied. On the one hand, there may be a matching of the redox potentials between the oxidoreductase and its substrate that, in some manner not yet understood, results in effective

electron transfer between particular pairs of proteins. Alternatively, as these thiol exchange reactions are catalyzed processes that involve protein–protein interactions, portions of the enzyme other than the CXXC active site may determine substrate specificity. These questions appear particularly relevant when comparing enzymes that are very similar, such as the large number of members of the thioredoxin superfamily. For example, thioredoxins 1 and 2 have identical active sites and show ~36% identity in amino acid sequence (thioredoxin 2 has a 32-amino acids long N-terminal extension that is not included in estimating homology), yet only thioredoxin 1 is able to reduce methionine sulfoxide reductase (61). Similarly, glutaredoxins 1 and 3 have identical active sites and a high level of homology (39% identity), and their three-dimensional structures virtually overlap (2, 47, 48, 59, 67). But only glutaredoxin 1 can serve as electron donor to ribonucleotide reductase *in vivo*. Currently, there is no significant information available to explain these differences. We have recently begun to construct chimeras between thioredoxins 1 and 2 and between glutaredoxins 1 and 3. Our preliminary results indicate that this approach allows definition of the regions of these proteins that determine substrate specificity (Ortenberg and Beckwith, unpublished observations).

A combination of structural and genetic studies has allowed some definition of enzyme substrate interactions in the case of the reductase, DsbD. DsbD not only maintains DsbC in the reduced state, it is also required for the reduction of DsbG, a DsbC homologue, and CcmG, a protein that itself is involved in maintaining periplasmic cytochrome *c*'s in the reduced state (9, 14, 20, 33, 60). DsbD is organized into three functional domains: the N-terminal (α) hydrophilic periplasmic domain interacts with substrates; the central hydrophobic (β) core composed of eight transmembrane segments receives electrons from cytoplasmic thioredoxin; and the C-terminal periplasmic domain (γ) with an apparent thioredoxin-like fold transfers electrons between the β and α domains (12, 19, 62). Each domain has pair of redox-active cysteines (33). Recent structural studies of the DsbD α domain revealed that this domain has an immunoglobin-like fold (21, 22). Furthermore, the structure of a captured disulfide-bonded intermediate between α and DsbC reveals the regions of both proteins that interact during the reaction. Studies on the phenotypic effects of mutants of α are consistent with the importance of the regions defined by the structural analysis.

BIOTECHNOLOGICAL ASPECTS OF REDOX ENVIRONMENT MODULATION

The well developed genetic tools and detailed understanding of *E. coli* physiology make it an organism favored for the production of many recombinant proteins, including complex eukaryotic proteins. Many such proteins contain multiple disulfide bonds, which are required for their folding, stability, and/or activity. The high level of expression of this class of proteins in active form is quite challenging because (a) the ability to form disulfide bonds in such proteins in the wild-type *E. coli* periplasm is not highly efficient and (b) it is often difficult to obtain effective secretion of the proteins into the periplasmic space. The extensive research into disulfide bond reduction/oxidation/isomerization pathways in *E.coli* and into the factors that determine the function of thiol oxidoreductases has resulted in a number of diverse approaches improving dramatically the expression of proteins with multiple disulfide bonds.

When recombinant proteins with multiple disulfide bonds are expressed at high levels in the *E. coli* periplasmic space, the efficiency of the oxidation and isomerization systems is not sufficient to give good yields of active proteins. However, overexpression of protein members of the Dsb family along with the eukaryotic proteins has proved successful in improving these systems. Human nerve growth factor contains three disulfide bonds formed between pairs of cysteine residues that do not occur consecutively in the amino acid sequence. Ordinarily, it forms insoluble aggregates when expressed in *E. coli*. The nonconsecutive nature of the cysteine pairs likely leads to the formation of incorrect disulfide bonds and should require an active disulfide isomerization system to repair any incorrect disulfide bonds formed (26, 27). Co-overexpression of the components of the protein disulfide bond isomerization pathway, DsbC and DsbD, yielded up to 80% active protein of the total nerve growth factor produced (37). Very similar results were obtained with expression of horseradish peroxidase and brain-derived neurotrophic factor (23, 36). These proteins contain four and three disulfide bonds, respectively, formed between nonconsecutive cysteine residues.

A major disadvantage in attempting to express foreign disulfide bond-containing proteins in *E. coli* is the limited capacity (or specificity) of its secretion machinery. One way to overcome this problem is to remove the portion of the genes encoding the signal sequences of these foreign proteins and express them in cells whose cytoplasm now favors disulfide bond formation. A strain now widely used for such studies is the *trxB,gor,ahpC** strain described above. We have already mentioned the effectiveness of this strain in permitting expression of active tissue plasminogen activator in the cytoplasm (8). The human hemofiltrate peptide HF6478, a putative serine proteinase inhibitor, was also successfully expressed in the cytoplasm of this oxidizing strain, yielding protein that was soluble and properly folded, and accurately formed disulfide bonds (38). The same strain background was used to express two different Fab fragments (Fab 2C5 and Fab 5H4m) that recognize the integral membrane protein NhaA, a bacterial Na^+/H^+ antiporter (65). The yield of the active Fab fragments in this case was 50–250 times higher than any other reported expression strategy for Fab fragments.

Although there have been clear successes in using the *trxB,gor,ahpC** strain for expression of disulfide bond-containing proteins in the cytoplasm, there are still hurdles to overcome for obtaining even higher yields. First, the correct folding of many eukaryotic proteins requires chaperones. Second, in the cases of proteins with multiple cysteine residues, aberrant disulfide bonds are produced that require disulfide bond isomerase activity in order to be corrected. Co-overexpression of chaperones has been successful in some cases. For example, the production of Fab antibody was significantly improved by coexpression of a number of cytoplasmic chaperones, such as GroEL/ES, DnaK, and DnaJ (39). The coexpression of a signal sequenceless version of the periplasmic

chaperone Skp had the largest effect (five- to sixfold increase) on the yield of the correctly folded Fab (39). Another approach to deal with the problem of correct folding and correct disulfide bond formation is to coexpress a signal sequenceless version of DsbC, which exhibits both chaperone and isomerase activities. The resultant expression of DsbC in the cytoplasm of the *trxB,gor,ahpC** strain caused a dramatic increase in the production of fully functional single-chain Fv antibodies (30).

SUMMARY

In this review, we have highlighted the various factors that contribute to the activity of the family of proteins termed thiol-disulfide oxidoreductases. We questioned many of the assumptions that inform discussions of the function of these proteins in living cells. First, the assumption that the general redox environment of a compartment in which such proteins are found determines whether they will act as oxidants or reductants is shown to be flawed. Second, the assumption that the relative redox potential of pairs of such proteins, as determined *in vitro*, determines the direction of electron flow between them inside a living cell does not always follow. Finally, the assumption that these proteins can fulfill only one role, reductant or oxidant (or isomerase), is contradicted by experimental evidence. Although the findings that led to these new insights in recent years have sharpened our understanding of the mechanism of action of these proteins, many questions still remain, particularly concerning the substrate specificity or lack thereof of various members of this very large family of proteins. Recent research has already provided biotechnological benefits by permitting the construction of *E. coli* strains that have vastly improved efficiency in producing eukaryotic proteins with disulfide bonds. Deeper understanding of the remaining questions should contribute to this effort in the future.

ACKNOWLEDGMENTS

We thank Dr. G. Georgiou, Dr. F. Katzen, and Dr. H. Kadokura for critical readings of the manuscript. J. Beckwith is American Cancer Society Professor. Dr. R. Ortenberg was supported by a Dorot Foundation Fellowship. This work was supported by grant no. 41883 from the National Institute of General Medical Sciences.

ABBREVIATIONS

gor, glutathione oxidoreductase gene; *grx*, glutaredoxin gene; PAPS, 3′-phosphoadenylylsulfate; PDI, protein disulfide isomerase; *trx*, thioredoxin gene; TrxB, thioredoxin reductase.

REFERENCES

1. Åslund F, Ehn B, Miranda-Vizuete A, Pueyo C, and Holmgren A. Two additional glutaredoxins exist in *Escherichia coli*: glutaredoxin 3 is a hydrogen donor for ribonucleotide reductase in a thioredoxin/glutaredoxin 1 double mutant. *Proc Natl Acad Sci U S A* 91: 9813–9817, 1994.
2. Åslund F, Nordstrand K, Berndt KD, Nikkola M, Bergman T, Ponstingl H, Jornvall H, Otting G, and Holmgren A. Glutaredoxin-3 from *Escherichia coli*. Amino acid sequence, ^{1}H AND ^{15}N NMR assignments, and structural analysis. *J Biol Chem* 271: 6736–6745, 1996.
3. Åslund F, Berndt KD, and Holmgren A. Redox potentials of glutaredoxins and other thiol-disulfide oxidoreductases of the thioredoxin superfamily determined by direct protein–protein redox equilibria. *J Biol Chem* 272: 30780–30786, 1997.
4. Åslund F, Zheng M, Beckwith J, and Storz G. Regulation of the OxyR transcription factor by hydrogen peroxide and the cellular thiol-disulfide status. *Proc Natl Acad Sci U S A* 96: 6161–6165, 1999.
5. Bader M, Muse W, Zander T, and Bardwell J. Reconstitution of a protein disulfide catalytic system. *J Biol Chem* 273: 10302–10307, 1998.
6. Bader MW, Xie T, Yu CA, and Bardwell JC. Disulfide bonds are generated by quinone reduction. *J Biol Chem* 275: 26082–26088, 2000.
7. Bader MW, Hiniker A, Regeimbal J, Goldstone D, Haebel PW, Riemer J, Metcalf P, and Bardwell JC. Turning a disulfide isomerase into an oxidase: DsbC mutants that imitate DsbA. *EMBO J* 20: 1555–1562, 2001.
8. Bessette PH, Åslund F, Beckwith J, and Georgiou G. Efficient folding of proteins with multiple disulfide bonds in the *Escherichia coli* cytoplasm. *Proc Natl Acad Sci U S A* 96: 13703–13708, 1999.
9. Bessette PH, Cotto JJ, Gilbert HF, and Georgiou G. *In vivo* and *in vitro* function of the *Escherichia coli* periplasmic cysteine oxidoreductase DsbG. *J Biol Chem* 274: 7784–7792, 1999.
10. Bessette PH, Qiu J, Bardwell JC, Swartz JR, and Georgiou G. Effect of sequences of the active-site dipeptides of DsbA and DsbC on *in vivo* folding of multidisulfide proteins in *Escherichia coli*. *J Bacteriol* 183: 980–988, 2001.
11. Chivers PT, Laboissiere MC, and Raines RT. The CXXC motif: imperatives for the formation of native disulfide bonds in the cell. *EMBO J* 15: 2659–2667, 1996.
12. Chung J, Chen T, and Missiakas D. Transfer of electrons across the cytoplasmic membrane by DsbD, a membrane protein involved in thiol-disulphide exchange and protein folding in the bacterial periplasm. *Mol Microbiol* 35: 1099–1109, 2000.
13. Collet JF and Bardwell JC. Oxidative protein folding in bacteria. *Mol Microbiol* 44: 1–8, 2002.
14. Crooke H and Cole J. The biogenesis of *c*-type cytochromes in *Escherichia coli* requires a membrane-bound protein, DipZ, with a protein disulphide isomerase-like domain. *Mol Microbiol* 15: 1139–1150, 1995.
15. Debarbieux L and Beckwith J. The reductive enzyme thioredoxin 1 acts as an oxidant when it is exported to the *Escherichia coli* periplasm. *Proc Natl Acad Sci U S A* 95: 10751–10756, 1998.
16. Debarbieux L and Beckwith J. On the functional interchangeability, oxidant versus reductant, of members of the thioredoxin superfamily. *J Bacteriol* 182: 723–727, 2000.

17. Derman AI and Beckwith J. *Escherichia coli* alkaline phosphatase fails to acquire disulfide bonds when retained in the cytoplasm. *J Bacteriol* 173: 7719–7722, 1991.
18. Derman AI, Prinz WA, Belin D, and Beckwith J. Mutations that allow disulfide bond formation in the cytoplasm of *Escherichia coli*. *Science* 262: 1744–1747, 1993.
19. Goldstone D, Haebel PW, Katzen F, Bader MW, Bardwell JC, Beckwith J, and Metcalf P. DsbC activation by the N-terminal domain of DsbD. *Proc Natl Acad Sci U S A* 98: 9551–9556, 2001.
20. Gordon EH, Page MD, Willis AC, and Ferguson SJ. *Escherichia coli* DipZ: anatomy of a transmembrane protein disulphide reductase in which three pairs of cysteine residues, one in each of three domains, contribute differentially to function. *Mol Microbiol* 35: 1360–1374, 2000.
21. Goulding CW, Sawaya MR, Parseghian A, Lim V, Eisenberg D, and Missiakas D. Thiol-disulfide exchange in an immunoglobulin-like fold: structure of the N-terminal domain of DsbD. *Biochemistry* 41: 6920–6927, 2002.
22. Haebel PW, Goldstone D, Katzen F, Beckwith J, and Metcalf P. The disulfide bond isomerase DsbC is activated by an immunoglobulin-fold thiol oxidoreductase: crystal structure of the DsbC–DsbDalpha complex. *EMBO J* 21: 4774–4784, 2002.
23. Hoshino K, Eda A, Kurokawa Y, and Shimizu N. Production of brain-derived neurotrophic factor in *Escherichia coli* by coexpression of Dsb proteins. *Biosci Biotechnol Biochem* 66: 344–350, 2002.
24. Huber-Wunderlich M and Glockshuber R. A single dipeptide sequence modulates the redox properties of a whole enzyme family. *Fold Des* 3: 161–171, 1998.
25. Inaba K and Ito K. Paradoxical redox properties of DsbB and DsbA in the protein disulfide-introducing reaction cascade. *EMBO J* 21: 2646–2654, 2002.
26. Joly JC and Swartz JR. *In vitro* and *in vivo* redox states of the *Escherichia coli* periplasmic oxidoreductases DsbA and DsbC. *Biochemistry* 36: 10067–10072, 1997.
27. Joly JC, Leung WS, and Swartz JR. Overexpression of *Escherichia coli* oxidoreductases increases recombinant insulin-like growth factor-I accumulation. *Proc Natl Acad Sci U S A* 95: 2773–2777, 1998.
28. Jonda S, Huber-Wunderlich M, Glockshuber R, and Mossner E. Complementation of DsbA deficiency with secreted thioredoxin variants reveals the crucial role of an efficient dithiol oxidant for catalyzed protein folding in the bacterial periplasm. *EMBO J* 18: 3271–3281, 1999.
29. Jordan A and Reichard P. Ribonucleotide reductases. *Annu Rev Biochem* 67: 71–98, 1998.
30. Jurado P, Ritz D, Beckwith J, de Lorenzo V, and Fernandez LA. Production of functional single-chain Fv antibodies in the cytoplasm of *Escherichia coli*. *J Mol Biol* 320: 1–10, 2002.
31. Kadokura H and Beckwith J. Four cysteines of the membrane protein DsbB act in concert to oxidize its substrate DsbA. *EMBO J* 21: 2354–2363, 2002.
32. Kadokura H, Bader M, Tian H, Bardwell JC, and Beckwith J. Roles of a conserved arginine residue of DsbB in linking protein disulfide-bond-formation pathway to the respiratory chain of *Escherichia coli*. *Proc Natl Acad Sci U S A* 97: 10884–10889, 2000.
33. Katzen F and Beckwith J. Transmembrane electron transfer by the membrane protein DsbD occurs via a disulfide bond cascade. *Cell* 103: 769–779, 2000.
34. Kobayashi T, Kishigami S, Sone M, Inokuchi H, Mogi T, and Ito K. Respiratory chain is required to maintain oxidized states of the DsbA–DsbB disulfide bond formation system in aerobically growing *Escherichia coli* cells. *Proc Natl Acad Sci U S A* 94: 11857–11862, 1997.
35. Krause G, Lundstrom J, Barea JL, Pueyo de la Cuesta C, and Holmgren A. Mimicking the active site of protein disulfide-isomerase by substitution of proline 34 in *Escherichia coli* thioredoxin. *J Biol Chem* 266: 9494–9500, 1991.
36. Kurokawa Y, Yanagi H, and Yura T. Overexpression of protein disulfide isomerase DsbC stabilizes multiple-disulfide-bonded recombinant protein produced and transported to the periplasm in *Escherichia coli*. *Appl Environ Microbiol* 66: 3960–3965, 2000.
37. Kurokawa Y, Yanagi H, and Yura T. Overproduction of bacterial protein disulfide isomerase (DsbC) and its modulator (DsbD) markedly enhances periplasmic production of human nerve growth factor in *Escherichia coli*. *J Biol Chem* 276: 14393–14399, 2001.
38. Lauber T, Marx UC, Schulz A, Kreutzmann P, Rosch P, and Hoffmann S. Accurate disulfide formation in *Escherichia coli*: overexpression and characterization of the first domain (HF6478) of the multiple Kazal-type inhibitor LEKTI. *Protein Expr Purif* 22: 108–112, 2001.
39. Levy R, Weiss R, Chen G, Iverson BL, and Georgiou G. Production of correctly folded Fab antibody fragment in the cytoplasm of *Escherichia coli trxB gor* mutants via the coexpression of molecular chaperones. *Protein Expr Purif* 23: 338–347, 2001.
40. 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.
41. Lowther WT, Brot N, Weissbach H, and Matthews BW. Structure and mechanism of peptide methionine sulfoxide reductase, an "anti-oxidation" enzyme. *Biochemistry* 39: 13307–13312, 2000.
42. 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 U S A* 99: 9679–9684, 2002.
43. McCarthy AA, Haebel PW, Torronen A, Rybin V, Baker EN, and Metcalf P. Crystal structure of the protein disulfide bond isomerase, DsbC, from *Escherichia coli*. *Nat Struct Biol* 7: 196–199, 2000.
44. Moskovitz J, Rahman MA, Strassman J, Yancey SO, Kushner SR, Brot N, and Weissbach H. *Escherichia coli* peptide methionine sulfoxide reductase gene: regulation of expression and role in protecting against oxidative damage. *J Bacteriol* 177: 502–507, 1995.
45. Mössner E, Huber-Wunderlich M, and Glockshuber R. Characterization of *Escherichia coli* thioredoxin variants mimicking the active-sites of other thiol/disulfide oxidoreductases. *Protein Sci* 7: 1233–1244, 1998.
46. Mössner E, Huber-Wunderlich M, Rietsch A, Beckwith J, Glockshuber R, and Åslund F. Importance of redox poten-

tial for the *in vivo* function of the cytoplasmic disulfide reductant thioredoxin from *Escherichia coli*. *J Biol Chem* 274: 25254–25259, 1999.
47. Nordstrand K, Åslund F, Holmgren A, Otting G, and Berndt KD. NMR structure of *Escherichia coli* glutaredoxin 3-glutathione mixed disulfide complex: implications for the enzymatic mechanism. *J Mol Biol* 286: 541–552, 1999.
48. Nordstrand K, Sandstrom A, Aslund F, Holmgren A, Otting G, and Berndt KD. NMR structure of oxidized glutaredoxin 3 from *Escherichia coli*. *J Mol Biol* 303: 423–432, 2000.
49. Prinz WA, Åslund F, Holmgren A, and Beckwith J. The role of the thioredoxin and glutaredoxin pathways in reducing protein disulfide bonds in the *Escherichia coli* cytoplasm. *J Biol Chem* 272: 15661–15667, 1997.
50. Regeimbal J and Bardwell JC. DsbB catalyzes disulfide bond formation *de novo*. *J Biol Chem* 277: 32706–32713, 2002.
51. Rietsch A, Belin D, Martin N, and Beckwith J. An *in vivo* pathway for disulfide bond isomerization in *Escherichia coli*. *Proc Natl Acad Sci U S A* 93: 13048–13053, 1996.
52. Rietsch A, Bessette P, Georgiou G, and Beckwith J. Reduction of the periplasmic disulfide bond isomerase, DsbC, occurs by passage of electrons from cytoplasmic thioredoxin. *J Bacteriol* 179: 6602–6608, 1997.
53. Ritz D and Beckwith J. Roles of thiol-redox pathways in bacteria. *Annu Rev Microbiol* 55: 21–48, 2001.
54. Ritz D, Patel H, Doan B, Zheng M, Åslund F, Storz G, and Beckwith J. Thioredoxin 2 is involved in the oxidative stress response in *Escherichia coli*. *J Biol Chem* 275: 2505–2512, 2000.
55. Ritz D, Lim J, Reynolds CM, Poole LB, and Beckwith J. Conversion of a peroxiredoxin into a disulfide reductase by a triplet repeat expansion. *Science* 294: 158–160, 2001.
56. Russel M, Model P, and Holmgren A. Thioredoxin or glutaredoxin in *Escherichia coli* is essential for sulfate reduction but not for deoxyribonucleotide synthesis. *J Bacteriol* 172: 1923–1929, 1990.
57. Schafer FQ and Buettner GR. Redox environment of the cell as viewed through the redox state of the glutathione disulfide/glutathione couple. *Free Radic Biol Med* 30: 1191–1212, 2001.
58. 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.
59. Sodano P, Xia TH, Bushweller JH, Bjornberg O, Holmgren A, Billeter M, and Wuthrich K. Sequence-specific ^{1}H n.m.r. assignments and determination of the three-dimensional structure of reduced *Escherichia coli* glutaredoxin. *J Mol Biol* 221: 1311–1324, 1991.
60. Sone M, Akiyama Y, and Ito K. Differential *in vivo* roles played by DsbA and DsbC in the formation of protein disulfide bonds. *J Biol Chem* 272: 10349–10352, 1997.
61. Stewart EJ, Åslund F, and Beckwith J. Disulfide bond formation in the *Escherichia coli* cytoplasm: an *in vivo* role reversal for the thioredoxins. *EMBO J* 17: 5543–5550, 1998.
62. Stewart EJ, Katzen F, and Beckwith J. Six conserved cysteines of the membrane protein DsbD are required for the transfer of electrons from the cytoplasm to the periplasm of *Escherichia coli*. *EMBO J* 18: 5963–5971, 1999.
63. Toth EA, Worby C, Dixon JE, Goedken ER, Marqusee S, and Yeates TO. The crystal structure of adenylosuccinate lyase from *Pyrobaculum aerophilum* reveals an intracellular protein with three disulfide bonds. *J Mol Biol* 301: 433–450, 2000.
64. Tsang ML. Assimilatory sulfate reduction in *Escherichia coli*: identification of the alternate cofactor for adenosine 3′-phosphate 5′-phosphosulfate reductase as glutaredoxin. *J Bacteriol* 146: 1059–1066, 1981.
65. Venturi M, Seifert C, and Hunte C. High level production of functional antibody Fab fragments in an oxidizing bacterial cytoplasm. *J Mol Biol* 315: 1–8, 2002.
66. Wunderlich M and Glockshuber R. Redox properties of protein disulfide isomerase (DsbA) from *Escherichia coli*. *Protein Sci* 2: 717–726, 1993.
67. Xia TH, Bushweller JH, Sodano P, Billeter M, Bjørnberg O, Holmgren A, and Wuthrich K. NMR structure of oxidized *Escherichia coli* glutaredoxin: comparison with reduced *E. coli* glutaredoxin and functionally related proteins. *Protein Sci* 1: 310–321, 1992.
68. Zapun A, Bardwell JC, and Creighton TE. The reactive and destabilizing disulfide bond of DsbA, a protein required for protein disulfide bond formation *in vivo*. *Biochemistry* 32: 5083–5092, 1993.
69. Zapun A, Missiakas D, Raina S, and Creighton TE. Structural and functional characterization of DsbC, a protein involved in disulfide bond formation in *Escherichia coli*. *Biochemistry* 34: 5075–5089, 1995.
70. Zheng M, Åslund F, and Storz G. Activation of the OxyR transcription factor by reversible disulfide bond formation. *Science* 279: 1718–1721, 1998.

Address reprint requests to:
Dr. Jon Beckwith
Department of Microbiology and Molecular Genetics, HMS
200 Longwood Avenue
Armenise Building/Room 212
Boston, MA 02115, U.S.A.

E-mail: jbeckwith@hms.harvard.edu

Received for publication September 18, 2002; accepted May 9, 2003.

This article has been cited by:

1. Elke Ströher, A. Harvey Millar. 2012. The biological roles of glutaredoxins. *Biochemical Journal* **446**:3, 333-348. [CrossRef]
2. Chengyan Wu, Jing Hong, Xinli Liao, Chenyun Guo, Xueji Wu, Hongyu Hu, Donghai Lin. 2012. 1H, 13C and 15N backbone and side-chain resonance assignments of reduced CcmG from Escherichia coli. *Biomolecular NMR Assignments* . [CrossRef]
3. Thijs R.H.M. Kouwen, Jan Maarten van Dijl. 2009. Interchangeable modules in bacterial thiol-disulfide exchange pathways. *Trends in Microbiology* **17**:1, 6-12. [CrossRef]
4. Kurtis G. Knapp, James R. Swartz. 2007. Evidence for an additional disulfide reduction pathway in Escherichia coli. *Journal of Bioscience and Bioengineering* **103**:4, 373-376. [CrossRef]
5. Jimin Zheng, Chunhua He, Vinay Kumar Singh, Nancy L. Martin, Zongchao Jia. 2007. Crystal structure of a novel prokaryotic Ser/Thr kinase and its implication in the Cpx stress response pathway. *Molecular Microbiology* **63**:5, 1360-1371. [CrossRef]
6. Dr. Stéphanie Gon , Melinda J. Faulkner , Jon Beckwith . 2006. In Vivo Requirement for Glutaredoxins and Thioredoxins in the Reduction of the Ribonucleotide Reductases of Escherichia coli. *Antioxidants & Redox Signaling* **8**:5-6, 735-742. [Abstract] [Full Text PDF] [Full Text PDF with Links]
7. Adam M. Benham . 2005. Oxidative Protein Folding: An Update. *Antioxidants & Redox Signaling* **7**:5-6, 835-838. [Citation] [Full Text PDF] [Full Text PDF with Links]
8. Dmitri E. Fomenko , Vadim N. Gladyshev . 2003. Genomics Perspective on Disulfide Bond Formation. *Antioxidants & Redox Signaling* **5**:4, 397-402. [Abstract] [Full Text PDF] [Full Text PDF with Links]
9. Elizabeth A. Kersteen , Ronald T. Raines . 2003. Catalysis of Protein Folding by Protein Disulfide Isomerase and Small-Molecule Mimics. *Antioxidants & Redox Signaling* **5**:4, 413-424. [Abstract] [Full Text PDF] [Full Text PDF with Links]
10. Katrin Linke , Ursula Jakob . 2003. Not Every Disulfide Lasts Forever: Disulfide Bond Formation as a Redox Switch. *Antioxidants & Redox Signaling* **5**:4, 425-434. [Abstract] [Full Text PDF] [Full Text PDF with Links]
11. Adam Benham . 2003. Oxidative Protein Folding: Recent Advances and Some Remaining Challenges. *Antioxidants & Redox Signaling* **5**:4, 355-357. [Citation] [Full Text PDF] [Full Text PDF with Links]