Proteomic analysis of thioredoxin-targeted proteins in *Escherichia coli*

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Thioredoxin, a ubiquitous and evolutionarily conserved protein, modulates the structure and activity of proteins involved in a spectrum of processes, such as gene expression, apoptosis, and the oxidative stress response. Here, we present a comprehensive analysis of the thioredoxin-linked *Escherichia coli* proteome by using tandem affinity purification and nanospray microcapillary tandem mass spectrometry. We have identified a total of 80 proteins associated with thioredoxin, implicating the involvement of thioredoxin in at least 26 distinct cellular processes that include transcription regulation, cell division, energy transduction, and several biosynthetic pathways. We also found a number of proteins associated with thioredoxin that either participate directly (SodA, HPI, and AhpC) or have key regulatory functions (Fur and AcnB) in the detoxification of the cell. Transcription factors NusG, OmpR, and RcsB, not considered to be under redox control, are also associated with thioredoxin.

The role of redox regulatory pathways in signal transduction is well established (1). Thiol-disulfide exchange reactions control the structure and activity of proteins that contain regulatory cysteines (2). This reversible disulfide bond formation is mediated by thiol-dependent proteins, such as thioredoxin and glutaredoxin, that exchange reducing equivalents between their active site cysteines and the cysteines of target proteins (3). The precise molecular mechanisms underlying redox regulation continue to be elucidated. To this end, identification of the cellular targets of thiol-disulfide exchange proteins is an important goal.

Thioredoxins are members of a class of small (≈12-kDa) redox active proteins that maintain the reductive intracellular redox potential (4). The thioredoxin fold comprised of five β-strands surrounded by four short α-helices and the active site cysteines (CXXC) are evolutionarily conserved in all organisms (Fig. 1) (5). Thioredoxin participates in redox reactions by oxidation of its active-site thiols and is then reduced by NADPH in a reaction catalyzed by thioredoxin reductase (4).

Originally isolated from *Escherichia coli* in 1964 as an electron donor for ribonucleotide reductase (6), thioredoxin is now known to play a role in a multitude of processes (7). Apart from its oxidoreductase activity, thioredoxin exerts control over the activity of its target proteins via reversible thiol-disulfide exchange reactions (Fig. 2*a*). In plant chloroplasts, thioredoxin regulates the light-activated Calvin cycle by reducing specific regulatory disulfides (8). In eukaryotes, thioredoxin regulates the activity of transcription factors such as NF-κB and AP-1 (9, 10). Thioredoxin also plays a critical role in the oxidative stress response; peroxiredoxins that catalyze the reduction of H_2O_2 are activated in turn by reduction by thioredoxin (11). In *E. coli*, transient disulfide bonds formed in the presence of reactive oxygen species in proteins such as Hsp33 (12) and the transcriptional regulator OxyR (13) are reduced by the thioredoxin and glutaredoxin systems.

A second regulatory mechanism independent of thiol redox activity depends on the ability of thioredoxin to interact with other proteins to form functional protein complexes (Fig. 2*b*). *E. coli* thioredoxin is an essential component of a protein complex required for filamentous phage assembly (14). Thioredoxin is also an essential processivity factor for bacteriophage T7 DNA polymerase (15). Only the reduced form of thioredoxin binds T7 DNA polymerase (16, 17). In eukaryotes, reduced thioredoxin inactivates the apoptosis signaling kinase-1 (ASK-1) (18). This mode of regulation is incumbent on stringent protein interactions, because these thioredoxin-linked proteins do not contain regulatory cysteines.

To identify the regulatory pathways in which thioredoxin participates, we have characterized the thioredoxin-associated *E. coli* proteome. A genomic tandem affinity purification (TAP) tag (19) was appended to thioredoxin, and proteins associated with TAP-tagged thioredoxin were identified by MS.

Methods

TAP Tagging of *trxA*. The DNA sequence encoding the TAP cassette from plasmid pFA6a-CTAP (20) was fused to the C terminus of the sequence encoding thioredoxin in plasmid pTrx-3 (21) by using two sequential PCR to yield plasmid pTrx-TAP.

Formation of Thioredoxin-Associated Complexes. *E. coli* HMS 262 cells harboring pTrx-TAP (1.25 liters) were grown at 37°C to an A_{580} = 0.6, harvested, and resuspended (5 ml) in either buffer A [50 mM Tris·HCl (pH 7.4)/25 mM EDTA/10% sucrose] or buffer B [50 mM Tris·HCl (pH 7.4)/50 mM NaCl/10 mM $MgCl_2$/1 mM EDTA/0.5 mM 2-mercaptoethanol/10% glycerol/0.01% Nonidet P-40]. A protease inhibition mixture (Complete Mini, EDTA-Free, Roche Molecular Biochemicals) was added in each case.

Isolation of Thioredoxin-Associated Complexes. Cells resuspended in buffer A were subjected to three freeze–thaw cycles to isolate thioredoxin-associated proteins localized at the inner membrane (22). Cells resuspended in buffer B were subjected to three freeze–thaw cycles to enrich membrane-associated proteins. In a third experiment, cells resuspended in buffer B were incubated with lysozyme (0.2 mg/ml), and Benzonase (0.5 units/ml) (Invitrogen) to degrade DNA and RNA at 4°C for 1 h. The entire thioredoxin-associated *E. coli* proteome was isolated from this total cell lysate. In all cases, the cell debris was removed by centrifugation (100,000 × *g*) at 4°C for 45 min. Thioredoxin-associated complexes were isolated from the cell extracts as described by Rigaut *et al.* (19).

Control Experiments. *E. coli* HMS 262 proteins that associate nonspecifically with the chromatographic media constitute the background of this experiment. To subtract this background, exponentially growing *E. coli* HMS 262 cells (1.25 liters) were subjected to the same treatment and analysis as that outlined above. These proteins have been excluded from this paper (see the supporting information, which is published on the PNAS web site).

Results

Tandem Affinity Tagging of Thioredoxin. A TAP tag was appended to the C terminus of thioredoxin. *E. coli* HMS 262 (*trxA*$^-$) cells were used for the expression and the formation of TAP-tagged thiore-

Abbreviation: TAP, tandem affinity purification.

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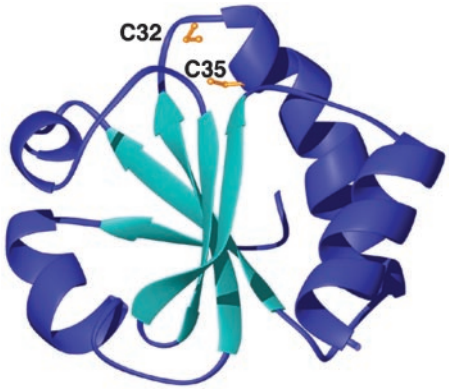

Fig. 1. NMR structure of *E. coli* thioredoxin (5). The active site cysteines (C32 and C35) are indicated in yellow.

doxin associated protein complexes to eliminate endogenous thioredoxin.

Protein complexes associated *in vivo* with TAP-tagged thioredoxin are purified from extracts by using two affinity chromatography steps (Fig. 3). The sequential use of two affinity tags reduces the nonspecific protein background, and the native conditions used for purification preserve protein–protein interactions.

Expression and Cellular Localization of TAP-Tagged Thioredoxin. The expression level of wild-type thioredoxin from the bacterial chromosome was estimated from *E. coli* C600 cells, and that of TAP-tagged thioredoxin from *E. coli* HMS 262 (*trxA*$^-$) cells harboring pTrx-TAP. *E. coli* C600 is the parent strain of HMS 262 (*trxA*$^-$) that produces a normal level of thioredoxin.

Exponentially growing *E. coli* C600 cells or *E. coli* of HMS 262 (*trxA*$^-$) cells harboring pTrx-TAP were harvested and subjected to freeze–thaw cycles in a Tris·EDTA buffer. Thioredoxin associates peripherally with the inner cell membrane and is quantitatively released by this procedure (22). Immunoblot analysis of the supernatant from *E. coli* C600 cells shows a single band of 12 kDa that

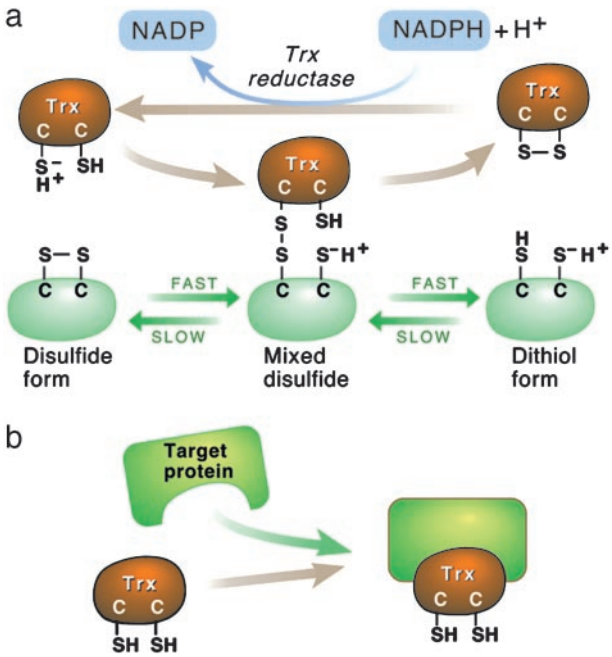

Fig. 2. Regulation of protein activity by thioredoxin. (*a*) Thioredoxin as an oxidoreductase. (*b*) Thioredoxin as a structural component.

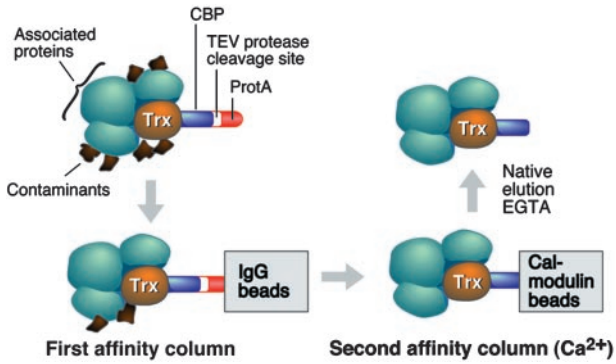

Fig. 3. Overview of the TAP procedure. The first step utilizes the binding of the ProtA moiety in the tag to matrix-bound IgG. The complex is released from the beads by cleavage of a seven-amino acid residue recognition sequence located between the two tags by TEV protease. The complex is then immobilized via the calcium-dependent binding of the calmodulin-binding peptide in the tag to calmodulin-coated beads and released by the addition of EGTA (19).

corresponds to thioredoxin (Fig. 4, lane 1). With *E. coli* HMS 262 (*trxA*$^-$) cells harboring pTrx-TAP, two bands of 32 and ≈13 kDa are visible (Fig. 4, lane 2). The 32-kDa protein corresponds to TAP-tagged thioredoxin. The second band that migrates slower than wild-type thioredoxin corresponds to untagged thioredoxin formed by proteolytic cleavage. This proteolytic cleavage takes place once the protein is released by the freeze–thaw procedure; only the 32-kDa protein is observed when *E. coli* HMS 262 (*trxA*$^-$) cells harboring pTrx-TAP were lysed directly without the freeze–thaw cycles (Fig. 4, lane 3). This cleavage may be effected by the outer membrane protease OmpT, which cleaves other overproduced proteins only after cell lysis (23). The absence of any band from *E. coli* HMS 262 cells that do not harbor a plasmid (Fig. 4, lane 4) confirms that *E. coli* HMS 262 cells do not have endogenous thioredoxin.

A comparison of the amount of wild-type thioredoxin produced from the *E. coli* chromosome (Fig. 4, lane 1) with the amount of TAP-tagged thioredoxin produced in *E. coli* HMS 262 (*trxA*$^-$) cells (Fig. 4) indicates that tagged thioredoxin is not overproduced, reducing the likelihood of formation of nonphysiological complexes. TAP-tagged thioredoxin (Fig. 4, lane 2) and wild-type thioredoxin (Fig. 4, lane 1) are both released on freeze–thaw treatment, indicating that the TAP tag does not interfere with the intracellular localization of tagged thioredoxin. TAP-tagged thi-

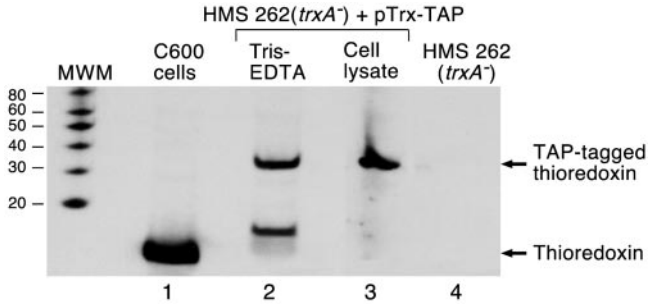

Fig. 4. Western blot analysis of thioredoxin expressed in *E. coli*. Extracts of *E. coli* were subjected to SDS/PAGE and Western blotting with an antibody to thioredoxin. Lane 1, wild-type thioredoxin produced in *E. coli* C600 cells, and released by Tris·EDTA treatment. Lane 2, TAP-tagged thioredoxin produced from pTrx-TAP in *E. coli* (*trxA*$^-$) cells, and released by Tris·EDTA treatment. Lane 3, TAP-tagged thioredoxin produced from pTrx-TAP in *E. coli* (*trxA*$^-$) cells, and released by cell lysis by using SDS. Lane 4, a total cell lysate of *E. coli* HMS 262 (*trxA*$^-$) cells which do not produce thioredoxin.

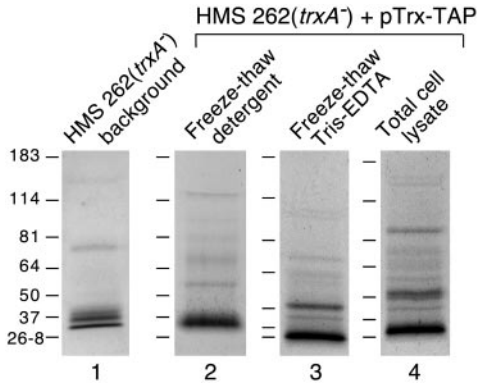

Fig. 5. SDS/PAGE analysis of TAP-tagged thioredoxin-associated proteins from *E. coli*. The position of molecular weight markers are indicated to the left of each lane. Lane 1, proteins purified from an *E. coli* (*trxA*⁻) cell lysate; lane 2, proteins from *E. coli* cells producing TAP-tagged thioredoxin that were released from the cells by freeze–thaw treatment in the presence of a nonionic detergent; lane 3, proteins from *E. coli* cells producing TAP-tagged thioredoxin that were released on treatment with Tris·EDTA; lane 4, proteins from a total cell lysate of *E. coli* cells producing TAP-tagged thioredoxin.

oredoxin is stable in unlysed *E. coli* HMS 262 based on the single band observed on immunoblot analysis (Fig. 4, lane 3).

Isolation of Thioredoxin-Associated Complexes. A crude homogenate of *E. coli* HMS 262 cells harboring pTrx-TAP was subjected to the TAP procedure. Proteins associated with TAP-tagged thioredoxin were separated by SDS/PAGE and visualized by Coomassie blue staining (Fig. 5, lane 4). The entire gel slice that corresponds to lane 4 was excised and the proteins subjected to tryptic digestion. The tryptic peptides were subjected to microcapillary reverse-phase HPLC nanoelectrospray tandem MS (MS/MS). The fragmentation mass spectra were correlated with theoretical spectra using the Sequest algorithm along with the method of Chittum *et al.* (24). The MS/MS peptide sequences were then reviewed for consensus with known proteins. This experiment was repeated three times. Sixty-three thioredoxin-binding proteins were identified from total cell lysates (Table 1, column 3).

Given the complexity of the crude extract, it is possible that other thioredoxin-associated complexes that are only minor components in the total cell lysate have escaped detection. To reduce the sample complexity, we made use of the fact that thioredoxin associates with the inner membrane. Two different fractionation techniques that do not disrupt the cell wall were used to isolate thioredoxin-associated complexes localized to the inner membrane.

In one experiment, *E. coli* HMS 262 cells harboring pTrx-TAP were subjected to freeze–thaw cycles in Tris·EDTA to release thioredoxin without extensive leakage of other cytoplasmic proteins (22). The proteins associated with thioredoxin were purified by using the TAP procedure and visualized by Coomassie blue staining (Fig. 5, lane 3). Thirty-one proteins were identified (Table 1, column 2), 10 of which had escaped detection in the total cell homogenate.

In a second experiment, *E. coli* HMS 262 cells harboring pTrx-TAP were subjected to freeze–thaw cycles in the presence of a nonionic detergent to release periplasmic proteins (25). Thioredoxin-associated proteins purified from this fractionation method were visualized after SDS/PAGE (Fig. 5, lane 2). Proteomic analysis of this sample identified 10 proteins (Table 1, column 1), six of which had escaped detection in a total cell homogenate.

Discussion

In this study, thioredoxin-associated complexes formed *in vivo* were purified by means of a TAP tag and identified by MS. A total of 80 proteins belonging to 26 distinct functional classes were identified. The proteins have been classified into functional categories in Table 1 according to the definitions of Riley and Labedan (26). The results are qualitative, and the stoichiometry of the components was not determined.

Our study expands the number of proteins that are potential targets of thioredoxin. Previous analyses of thioredoxin-associated proteins from spinach chloroplasts (27) and from *Chlamydomonas reinhardtii* (28) identified 35 and one proteins, respectively. These studies used immobilized thioredoxins to capture target proteins from extracts. Thioredoxin reduces proteins by forming a mixed disulfide intermediate, which is resolved by the vicinal cysteine in the active site (Fig. 2*a*). A genetically altered thioredoxin with one of the active site cysteines replaced with alanine was used to trap mixed disulfide-linked complexes with the target proteins. Thus, the studies selected proteins that interact with thioredoxin primarily via dithiol/disulfide exchange.

Our approach differs from these previous studies in four ways. First, our strategy is to characterize complexes formed *in vivo*. Second, there is no competition with endogenous thioredoxin. Third, neither of the active-site cysteines in thioredoxin is altered. Fourth, the purification is carried out in the presence of reducing agents. Under these conditions, we are not limited to isolating proteins that interact with thioredoxin primarily via dithiol/disulfide exchange. Many of the proteins identified in this study do in fact interact with thioredoxin independent of mixed-disulfide formation; 20 of the 80 proteins do not contain cysteines.

Established Functions of Thioredoxin in *E. coli*. In *E. coli*, thioredoxin participates in reactions catalyzed by thioredoxin reductase, ribonucleotide reductase, PAPS reductase, methionine sulfoxide reductase (MrsA), and DsbD (4). We detected an interaction only with ribonucleotide reductase. DsbD, MrsA, thioredoxin reductase, and PAPS reductase interact transiently with thioredoxin via mixed disulfide intermediates that are unstable in the presence of reducing agents (29, 30). This suggests that the proteins identified in this study interact with thioredoxin by additional specific contacts.

We found that both class I and III ribonucleotide reductases associate with thioredoxin. Although thioredoxin is the electron donor required by the class I ribonucleotide reductase (6), it is not required for catalytic activity of the class III anaerobic enzyme. Instead, thioredoxin plays a regulatory role; it reduces conserved cysteines in the anaerobic reductase, thereby activating the protein (31). Our study suggests a stable complex between thioredoxin and the class III ribonucleotide reductase.

Thioredoxin-Associated Pathways

Cell Division. Two bacterial cytoskeletal proteins, FtsZ and MreB, were identified in a thioredoxin-associated complex localized to the inner membrane. FtsZ, the prokaryotic tubulin homolog essential for cell division, localizes to the midcell position and remains at the invaginating septum throughout cell division (32). The MinCDE proteins position FtsZ. MreB, the prokaryotic actin homolog, is postulated to serve as the scaffolding for the MinCDE proteins (33). Our finding of a similar subcellular localization of otherwise cytosolic proteins implicates thioredoxin in cell division. Interestingly, yeast cells lacking thioredoxin have an extended S phase (34), and chloroplast thioredoxins associate with FtsZ (27).

Detoxification/Oxidative Stress Response. *E. coli* thioredoxin-deficient mutants are sensitive to H_2O_2 (35). Thioredoxin participates in protection against H_2O_2 by scavenging reactive oxygen species and by regulating the activity of detoxification proteins. A number of detoxification proteins were found to bind thioredoxin.

Two peroxiredoxins, alkyl hydroperoxide reductase C22 (AhpC) (36), and thiol peroxidase associate with thioredoxin (37). The peroxide scavenging properties of peroxiredoxins are regulated via

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Table 1. Thioredoxin-associated proteins in *E. coli*

Swiss-Prot ID	Protein	No. of peptides identified I*	II†	III‡
Energy metabolism				
Anaerobic respiration				
P32176	Formate dehydrogenase-O major subunit	(0)	(0)	(2, 0, 0)
ATP-proton motive force interconversion				
P00822	ATP synthase, F_1 sector, α-chain	(0)	(0)	(2, 0, 0)
P00824	ATP synthase, F_1 sector, β-chain	(0)	(2)	(0, 0, 2)
Glycolysis				
P08324	Enolase	(0)	(5)	(1, 0, 0)
P11604	Fructose-1,6-bisphosphate aldolase	(0)	(1)	(0, 0, 0)
P06977	GAPDH	(0)	(2)	(4, 0, 0)
P21599	Pyruvate kinase II	(0)	(0)	(6, 0, 2)
P04790	Triose phosphate isomerase	(0)	(0)	(2, 0, 0)
Pentose phosphate pathway				
P30148	Transaldolase B	(0)	(0)	(1, 0, 0)
TCA				
P36683	Aconitate hydrase B	(8)	(3)	(6, 2, 0)
P08200	Isocitrate dehydrogenase	(0)	(3)	(7, 0, 2)
P10444	Succinate dehydrogenase, flavoprotein subunit	(11)	(0)	(0, 0, 0)
P07459	Succinyl-CoA synthetase, α-subunit	(0)	(2)	(3, 0, 0)
P07460	Succinyl-CoA synthetase, β-subunit	(0)	(1)	(2, 3, 0)
Processes				
Cell division/cellular structure				
P06138§	FtsZ	(0)	(11)	(0, 0, 0)
P13519	Rod shape-determining protein mreB	(0)	(4)	(0, 0, 0)
Chaperones				
P05380§	GroES	(0)	(0)	(4, 4, 0)
P10413§	Hsp 90	(2)	(0)	(1, 0, 0)
P18274	dksA (DnaK suppressor)	(0)	(3)	(0, 0, 0)
P30856	FKBP-type peptidyl prolyl cis trans isomerase	(7)	(0)	(1, 1, 0)
P39311§	FKBP-type 22-kDa peptidyl prolyl cis trans isomerase	(0)	(0)	(1, 0, 0)
P32168	hslVU, ATPase subunit	(0)	(2)	(6, 13, 0)
Detoxification/oxidative stress response				
P18391	Alkyl hydroperoxide reductase, C22 subunit	(0)	(0)	(7, 2, 2)
P13029	Catalase, hydroperoxidase HP(I)	(15)	(0)	(0, 0, 0)
P00448§	Superoxide dismutase [Mn]	(0)	(0)	(3, 0, 0)
P37901	Thiol peroxidase	(0)	(0)	(3, 0, 0)
Regulatory functions				
P06975	Fur, negative regulatory protein	(0)	(0)	(2, 0, 0)
P03025	OmpR	(0)	(0)	(4, 3, 0)
P16921§	NusG	(0)	(4)	(3, 4, 5)
P14374§	RcsB	(0)	(5)	(2, 3, 0)
P09546	Proline dehydrogenase	(0)	(0)	(1, 0, 1)
P15277§	Cold shock protein cspA, transcriptional activator	(0)	(0)	(0, 2, 0)
Transport/binding proteins				
P08837§	Glucose specific II A component of the PTS system	(0)	(1)	(4, 1, 2)
P02928§	Maltose-binding periplasmic protein	(0)	(0)	(1, 1, 0)
P02943	Lambda receptor protein (LamB)	(3)	(0)	(0, 0, 0)
P02925§	D-ribose-binding periplasmic protein [Precursor]	(0)	(0)	(2, 0, 0)
P07019	Signal recognition particle protein	(0)	(2)	(0, 0, 2)
Small molecule biosynthesis and degradation				
Amino acid biosynthesis				
P03948	THP succinyltransferase	(0)	(1)	(1, 1, 0)
Amino acid degradation				
P00913	Tryptophanase	(5)	(0)	(0, 0, 0)
P30744	L-serine deaminase	(0)	(2)	(0, 0, 0)
Carbon degradation				
P00722	β-Galactosidase	(0)	(1)	(3, 3, 1)
2′-Deoxyribonucleotide metabolism				
P28903	Anaerobic ribonucleoside triphosphate reductase	(0)	(0)	(12, 5, 3)
P00452	Ribonucleoside diphosphate reductase, B1 subunit	(0)	(0)	(0, 1, 1)
Fatty acid biosynthesis				
P29132	Enoyl-[ACP] reductase	(0)	(0)	(1, 0, 0)
P21177	Fatty oxidation complex α-subunit	(0)	(0)	(0, 2, 0)
P18391	β-hydroxydecanoyl-[ACP] dehydrase	(0)	(0)	(6, 4, 3)
P21774	(3R)-hydroxymyristol [ACP] dehydratase	(0)	(0)	(0, 0, 5)
P25716‡	3-oxoacyl-[ACP] reductase	(0)	(0)	(3, 2, 2)
Purine/pyrimidine ribonucleotide biosynthesis				
P24233	NDK, nucleoside diphosphate kinase	(0)	(0)	(1, 0, 1)
P29464	Uridylate kinase	(0)	(0)	(2, 2, 0)

Table 1. (continued)

Swiss-Prot ID	Protein	No. of peptides identified I*	II†	III‡
Salvage of nucleosides and nucleotides				
P22333	Adenosine deaminase	(0)	(2)	(0, 0, 0)
P23331	Thymidine kinase	(0)	(0)	(0, 0, 2)
P12758	Uridine phosphorylase	(0)	(0)	(1, 1, 0)
Sugar-nucleotide metabolism				
P17114	*N*-acetyl glucosamine-1-phosphate uridyltransferase	(0)	(0)	(0, 2, 1)
P27306	Soluble pyridine nucleotide transhydrogenase	(0)	(1)	(0, 2, 0)
Ubiquinone/menaquinone biosynthesis				
P27851	Ubiquinone/menaquinone methyltransferase	(0)	(0)	(2, 3, 0)
Macromolecules				
Cell envelope/porins				
P02931§	OmpF	(5)	(0)	(0, 0, 0)
P06996§	OmpC	(0)	(1)	(0, 0, 0)
P02934	OmpA (precursor)	(2)	(0)	(0, 0, 0)
DNA replication and Recombination				
P03017	RecA	(0)	(3)	(1, 3, 0)
P02341§	HU-1 (post transcriptional regulator)	(0)	(0)	(2, 3, 0)
Protein translation and modification				
P33398	EF-P	(0)	(0)	(0, 1, 0)
P02997	EF-Ts	(0)	(0)	(7, 0, 0)
P02996	EF-G	(0)	(3)	(7, 3, 0)
RNA synthesis				
P08374§	RNA polymerase, ω subunit	(0)	(3)	(5, 5, 0)
Protein degradation/proteases				
P19245	ATP dependent specificity component of clpP protease	(0)	(0)	(3, 0, 2)
P23865§	C-terminal protease for penicillin-binding protein 3	(0)	(0)	(0, 3, 0)
Peptidoglycan biosynthesis				
P31120	Protein mrsA	(0)	(0)	(4, 0, 0)
Phage-related proteins				
P39180	Flu (outer membrane fluffing protein, sim to adhesin)	(3)	(0)	(0, 0, 0)
Unknown proteins				
P30977§	ybeD (uncharacterized conserved protein, COG2921)	(0)	(2)	(2, 1, 0)
P31220	yhbG (ABC transporter, COG1137)	(0)	(1)	(0, 0, 0)
P24203	yjiA (putative GTPase, COG0523)	(0)	(1)	(0, 0, 0)
P77475	yqaB (putative phosphatase, COG0637)	(0)	(1)	(0, 0, 0)
P77482§	yajQ (uncharacterized conserved protein, COG1666)	(0)	(1)	(2, 0, 0)
P24250	ydjA (NADPH-flavin oxidoreductase, COG0778)	(0)	(0)	(2, 0, 0)
P25538	ybaD (predicted translational regulator, COG1327)	(0)	(0)	(1, 0, 0)
P33940§	yojH, (malate: quinone oxidoreductase, COG0579)	(0)	(2)	(0, 1, 2)
P33218	yebE	(0)	(0)	(1, 0, 1)
P42641	yhbZ (hypothetical GTP-binding protein)	(0)	(0)	(1, 1, 2)
P23199	hypothetical protein mglA Galactose ABC transporter	(0)	(1)	(1, 0, 0)

Numbers in parentheses correspond to the number of peptides detected that match the identified protein.
*I, Thioredoxin-associated *E. coli* proteins isolated by freeze–thaw treatment in presence of a detergent.
†II, Thioredoxin-associated proteins isolated by Tris·EDTA.
‡III, Thioredoxin-associated proteins isolated from a total cell lysate.
§Proteins do not contain cysteines.

reduction of specific regulatory thiols (38). During their catalytic cycle, the active disulfide formed is recycled back to the thiol form by an oxidoreductase. Although thiol peroxidase (Tpx, p20, scavengase) requires thioredoxin, the activity of AhpC is restored by its dedicated reductase, AhpF (36). Our finding points to a role of thioredoxin in the catalytic cycle of the *E. coli* AhpC. The observation that Tpx and AhpC are thioredoxin-associated suggests that a stable complex exists *in vivo*. The only other report of a peroxiredoxin copurifying with its reductase that did not use a single cysteine mutant is from *Thermus aquaticus* (39).

Both manganese-dependent superoxide dismutase (SodA) and catalase (hydroperoxidase I, HPI) associate with thioredoxin. As SodA does not contain cysteines, its interaction may be akin to the interaction of thioredoxin with T7 DNA polymerase (16) and with ASK-1 (18). HPI can dismutate H_2O_2 either as a catalase or as a peroxiredoxin (40). In the peroxidase pathway, the cognate reductase of HPI has not been identified. Our finding implicates thioredoxin as the partner of HPI. An alternative explanation is that thioredoxin associates with SodA and HPI via ribonucleotide reductase, because both are required for generation of the ribonucleotide reductase radical (41).

Energy Transduction. Proteins involved in glycolysis, the citric acid cycle, and the pentose phosphate cycle were found to bind thioredoxin. In plants, thioredoxins regulate these processes via reductive activation of enzymes such as fructose1,6-bisphosphatase and GAPDH (8). We find thioredoxin associated with GAPDH and fructose 1,6-bisphosphate aldolase.

Outer Membrane Proteins. Outer membrane proteins OmpA, OmpC, OmpF, and LamB were identified in fractions enriched for periplasmic and membrane-associated proteins. It is not surprising to find outer membrane proteins because they are exported into the periplasm after synthesis, before transport to the outer membrane (42). Immature conformers of OmpA fold in the periplasm (43). Thioredoxin has a chaperone activity (44), and the colocalization of

thioredoxin could be indicative of a role in protein folding in the periplasm. This association may also indicate a role for thioredoxin as a receptor for bacteriophages. OmpA is a receptor for T2 phage, and LamB is both the phage λ receptor protein and a porin for transport of maltose and maltodextrins (45).

Protein Folding and Degradation. Hsp90 and GroES, neither of which contains cysteines, and FKBP-type peptidyl prolyl cis trans isomerase were found associated with thioredoxin. The FKBP-type peptidyl prolyl cis trans isomerase is a molecular chaperone that is also required for lysis of ϕX174-infected cells (46). The ATP-binding regulatory subunit of the clp serine protease and the ATPase subunit of the ATP-dependent protease, HslVU (47), were found to bind thioredoxin. The ATP-binding subunit of the clp protease was also trapped with chloroplast thioredoxin (27). These results suggest a role for thioredoxin in protein folding and degradation.

Transcription Regulation. The transcription antitermination factor, NusG, and RcsB were repeatedly isolated in complexes with thioredoxin, even though they do not contain cysteines. It is of interest to examine whether NusG and RcsB are regulated by thioredoxin, as is ASK-1, which likewise does not contain regulatory cysteines (18).

Three proteins that respond to changes in the environment were identified: the ferric uptake regulator (Fur), aconitate hydrase B (AcnB), and OmpR, which responds to changes in osmolarity. Fur is a global regulator that uses ferrous ion as a cofactor to regulate the expression of >90 genes involved in the oxidative stress response, glycolysis, the citric acid cycle, purine metabolism, and virulence factors (48). It is one of four global regulators (ArcA, SoxQ, SoxS, and Fur) that control the transcription of the gene for superoxide dismutase (SodA) in *E. coli*. It also acts posttranscriptionally to activate SodA (48). Thioredoxin regulates the expression of SodA in mammalian cells (49), most likely via its modulation of DNA binding of transcription factor NF-κB (10). A model whereby thioredoxin regulates the DNA-binding activity of Fur would link thioredoxin to iron homeostasis and the oxidative stress response.

AcnB contains a [4Fe-4S] cluster and catalyzes the conversion of citrate into isocitrate. Under conditions of iron depletion and oxidative stress, AcnB is demetallated, and the apo-protein functions as an RNA-binding protein that regulates genes encoding proteins of iron metabolism and the oxidative stress response such as SodA (50). Thioredoxin activates the apo-protein form of aconitase in macrophages for RNA binding (51). We speculate that thioredoxin plays a similar role in the activation of AcnB.

Translation. Chloroplast thioredoxins associate with elongation factors Tu and G, implying a role of thioredoxin in translation (27). We detected elongation factors G, P, and Ts.

Unknown Function. Eleven proteins that do not have an annotated function associate with thioredoxin. Functional predictions based on phylogenetic analysis were made by using the clusters of orthologous groups database (52) and are indicated in Table 1.

Summary

The observation that thioredoxin is associated with numerous proteins that function in various regulatory processes provides compelling evidence for an extensively coupled network of redox regulation. It also demonstrates that a single redox active protein can have pleiotropic roles. This role is particularly significant in redox signaling where proteins often have different functions depending on the intracellular environment. Thioredoxin adopts different conformations in its reduced or oxidized forms and may use protein–protein interactions that depend on a specific conformation as a mechanism for signaling. The identification of 20 thioredoxin-associated proteins that do not contain cysteines reinforces such a role of thioredoxin as a redox sensor in the cell. Finally, the large representation of proteins involved either directly or through regulation of the oxidative stress response in *E. coli* reinforces the central role of thioredoxin in redox signaling.

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