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Structure, Function, and Mechanism of Thioredoxin Proteins

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Abstract

Thioredoxins are ubiquitous antioxidant enzymes that play important roles in many health-related cellular processes. As such, the fundamental knowledge of how these enzymes work is of prime importance for understanding cellular redox mechanisms and for laying the ground for the development of future therapeutic approaches. Over the past 40 years, a really impressive amount of data has been published on thioredoxins. Here, we review the most significant results that have contributed to our knowledge regarding the structure, the function, and the mechanism of these crucial enzymes. *Antioxid. Redox Signal.* 13, 1205–1216.

Introduction

In extracellular proteins, most cysteine residues are involved in disulfide bonds. The inside of the cell, on the other hand, is a reducing environment (35) in which most cysteines are kept reduced. Although cells have millimolar concentrations of glutathione and alternatives that serve as redox buffers preventing the formation of unwanted disulfides, they need more efficient reducing systems to catalyze the reduction of disulfide bonds. One of the major reducing pathways involves thioredoxin (Trx).

Trx is a ubiquitous antioxidant enzyme that is found in organisms ranging from archae to mammals. Trx has a long evolutionary history. The first Trx was originally discovered in 1964 in *Escherichia coli* as an electron donor for ribonucleotide reductase, an enzyme required for DNA synthesis (64, 90). From then onwards, it has become clear that thioredoxins (Trxs) play multivalent cellular roles. They act as reductases in redox control (48), protect proteins from oxidative aggregation and inactivation (48–51), help the cells cope with various environmental stresses (reactive oxygen species (ROS), peroxynitrite, arsenate) (63, 83), and regulate programmed cell death (100) via denitrosylation (5). Some Trxs also act as growth factor (98), modulate the inflammatory response (94), promote protein folding (56), or play important roles in the lifecycles of viruses and phages (49).

Organisms have developed their specialized subset of Trxs, which are located in various cellular compartments. For instance, some Trxs are abundant in the cytosol (2), while others are translocated to the nucleus (46, 47) or mitochondria, associated with the cell membrane (74) or secreted to the extracellular environment (1, 122).

All Trx proteins have a canonical CGPC catalytic motif located on a highly conserved fold. The cysteine residues of the CGPC motif are the key players used by Trxs to break disulfide bonds in oxidized substrate proteins (Fig. 1). Upon completion of a catalytic cycle, these two cysteine residues are oxidized and form a disulfide. They are converted back to the reduced state by thioredoxin reductase (TrxR) at the expense of the reduced form of nicotinamide adenine dinucleotide phosphate (NADPH) (Fig. 1).

The Trx literature is immense. Here, we will focus first on the structural properties of these proteins. Then, we will show how the structural properties of Trxs dictate their catalytic mechanism. Finally, we will highlight the diversity of the Trx proteins by describing the characteristics of the most important members of this ubiquitous family.

The Structure of Thioredoxins

The first structural architecture of a Trx protein was described by Holmgren *et al.* (52) in 1975. Today, the crystal and solution structures of many oxidized and reduced Trxs are known. The fold of Trxs consists of five β-strands surrounded by four α-helices (52) (Fig. 2A). The β-sheets and α-helices can be divided in a N-terminal $\beta_1\alpha_1\beta_2\alpha_2\beta_3$ and a C-terminal $\beta_4\beta_5\alpha_4$ motif connected by the α_3-helix. The β-strands of the N-terminal motif run parallel, and the β-strands of the C-terminal motif run anti-parallel. The α_2 and α_4 helices are located on one side of the central β-sheet while the α_3-helix is located on the opposite side (Fig. 2A). The α_3-helix is oriented perpendicularly to helices α_2 and α_4. The catalytic CGPC motif is located on the surface of the protein in a short segment at the amino-end of the α_2-helix.

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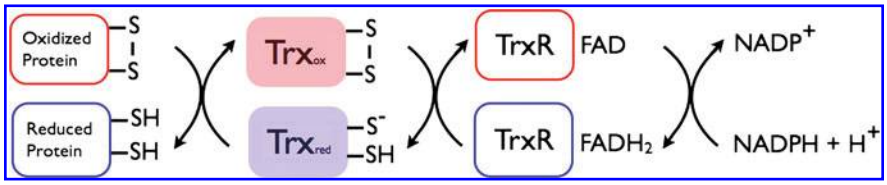

FIG. 1. Electron flow from NADPH to oxidized substrate proteins. Schematic representation of the electron flow in which thioredoxin (Trx) reduces oxidized proteins with electrons coming from NADPH via the NADPH-dependent flavo-enzyme thioredoxin reductase (TrxR). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article at www.liebertonline.com/ars).

A minimal version of this fold is known as the Trx-fold (75). It lacks the ß$_1$-strand and the α_1-helix. The Trx-fold is the complete structural fold of glutaredoxins (119) (Fig. 2B), and is also observed in protein disulfide isomerases (44, 79, 115), in disulfide oxidases such as DsbA (76), as part of the inner membrane electron transport protein DsbD (38, 55), glutathione transferases (101), glutathione peroxidases (102), chloride intracellular channels (CLIC) (43), or in the recently characterized protein DsbG (21, 44) that controls the level of sulfenylation in the periplasm. So, Nature has used the Trx-fold as a conserved protein fold to engineer proteins for particular tasks (26, 96). Most of the

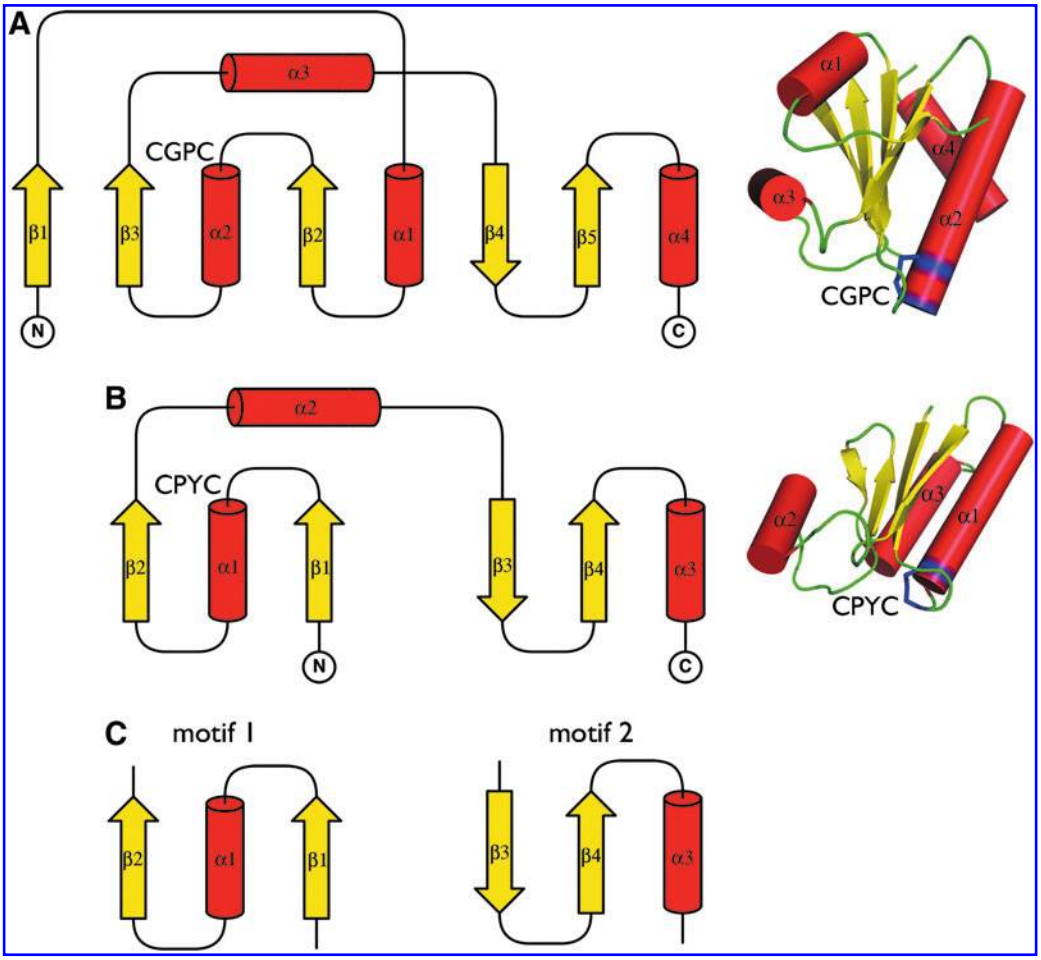

FIG. 2. The fold of thioredoxin and the Trx-fold. (**A**) The secondary and three-dimensional structures (PDB code: 2O7K)(106) of Trx are shown. The structure consists of 4 α-helices and of a central 5- stranded β-sheet. The conserved CXXC motif is located at the N-terminus of the α_2-helix. The α-helices are in *red*, the β-strands in *yellow*, and the disulfide bond in *blue*. (**B**) The secondary and three-dimensional structures of glutaredoxin are shown (PDB code: 1EGO)(120). Glutaredoxins have a Trx-fold consisting of 2 motifs connected by the α_2-helix. The conserved active site CXXC motif is always located at the same position in space, at the N-terminal site of an α-helix. (**C**) The two motifs of the Trx-fold. The figure was generated using TopDraw from CCP4 suite and MacPyMol (Delano Scientific LLC 2006, San Francisco CA). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article at www.liebertonline.com/ars).

proteins with a Trx-fold present a conserved CXXC catalytic motif.

Conserved Residues on a Scaffold

Several residues that play important structural and catalytic roles are conserved among Trx proteins (Fig. 3A). Two of them, the cysteine residues found in the catalytic site, are essential for the activity of Trxs. The other conserved residues are not strictly required for activity but dictate the thermodynamic and redox properties of the protein.

Trxs are characterized by the presence of three conserved prolines, with one located between the catalytic cysteine residues of the CGPC motif. This proline is the key residue that determines the reducing power of Trxs; replacing it by a serine or a threonine has a dramatic effect on the redox and stability properties of the protein (17, 37, 59, 69, 91, 106, 111). The second conserved proline is located five residues beyond the catalytic motif (Fig. 3B). This proline introduces a kink in the α_2-helix that separates the CGPC motif, located at the amino end of the α_2-helix, from the rest of the helix. Mutating this residue destabilizes the Trx structure but has no effect on the redox properties of the protein (14, 19).

The third important proline in the sequence of Trx is positioned on the opposite side of the CGPC active site motif. This proline, which is always found in cis-conformation, is important for maintaining both the conformation of the active site and the redox potential of the protein. Replacing it by an alanine has an effect on the efficiency of catalysis (36).

Next to this cis-proline, there is a conserved threonine. This residue is involved in structuring the region opposite to the CGPC active site motif (Fig. 3). The side chain oxygen ($O\gamma1$) of this threonine makes hydrogen bonds with the main chain oxygens of the cis-proline and of the preceding residue (26).

Three glycine residues are conserved among Trxs. The first one, which is in the CGPC motif, maintains the conformation of the active site and influences the redox potential. The other two flank the β_5-strand and determine its length. They are structurally important as they are found at equivalent positions for this type of turns in other structures (61, 108).

Several aromatic residues are also conserved in Trxs. First, there are two phenylalanine residues, which are located in the N-terminal α_1-helix and at the end of the β_2-strand, respectively. These residues are both part of the same internal hydrophobic cluster (green oval shape in Fig. 3B) in which several isoleucine and valine residues of the central β-sheet

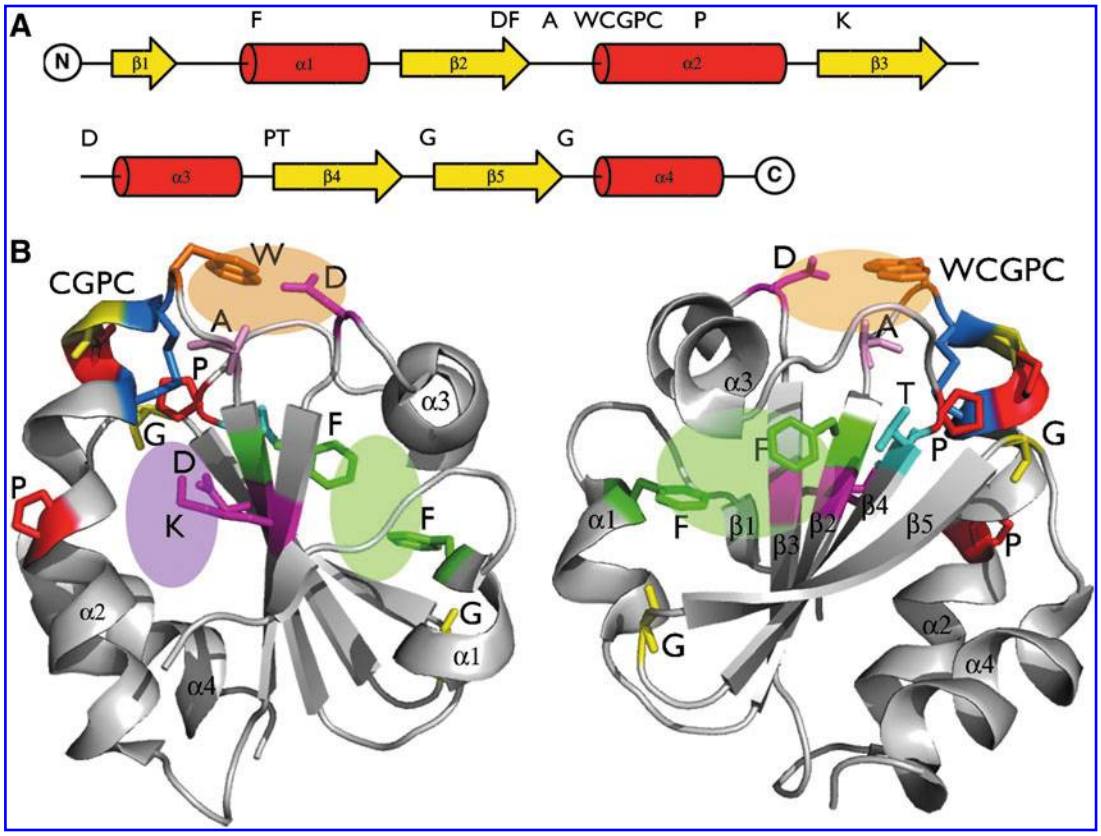

FIG. 3. Conserved residues in thioredoxins. (**A**). The conserved sequence of the proteins of the Trx family is shown in one letter code on top of the secondary structure. The conserved sequence was computed with the program BLASTP (BLOSUM62 as matrix) using the SWISSPROT database (3). (**B**) The conserved regions are located in the center and around the active site of Trx. The conserved residues are shown in stick presentation: tryptophan (*orange*), charged residues (*magenta*), cysteines (*blue*), glycines (*yellow*), phenylalanines (*green*), and prolines (*red*). The hydrophobic region (*green*), the charged region (*magenta*), and the exposed structural cluster (*orange*) are indicated with a semitransparent *oval shape*. The ribbon diagram of the overall structure of Trx is visualized from two different positions. The figures were generated using MacPyMol (Delano Scientific LLC 2006). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article at www.liebertonline.com/ars).

are also involved (26). This cluster is structurally important. For instance, the N-terminal phenylalanine positions the α_1-helix correctly.

There is also a conserved tryptophan, located immediately before the CGPC sequence motif. This residue is important for the thermodynamic stability of Trx (31). The conserved alanine located in the turn before the tryptophan is in van der Waals contact with this residue. It is important to have a small residue as an alanine in this position, because a larger residue would shift the position of the indole side chain. Both the tryptophan and the alanine form a structural cluster in which there is also a conserved aspartate located in the turn between β_3 and α_3 (Fig. 3B, orange oval).

Noteworthy, a mutant of Trx in which this tryptophan is replaced by an alanine has the tendency to form a domain-swapped dimer that is devoid of any of the biochemical activities known for Trx-fold proteins (31). This domain-swapped dimer is kinetically stable with a half-life of more than 150,000 years and might be an example of how multimeric proteins evolved from their monomeric ancestors by domain swapping (6, 81, 121).

Finally, Trxs contain two conserved charged residues (*i.e.*, an aspartate and a lysine located in the β_2-strand and the β_3-strand, respectively) (25). They are part of a charged region present between the β-sheet and the kinked α_2-helix (Fig. 3B, purple oval shape). This region is shielded from the environment by the disulfide present in the oxidized state of the protein. This conserved aspartate has long been considered to be the key residue activating the C-terminal cysteine of the CGPC motif as a nucleophile (17, 80). However, results reported recently by Roos *et al.* (105) suggest that it is not the case. Their results showed that the C-terminal thiol can be activated even in the absence of the aspartate and that the distance between the oxygens ($O_{\delta 1}$ and $O_{\delta 2}$) of the aspartate and the C-terminal thiol (S_γ) increases up to 10 Å in the mixed disulfide complex, preventing them to interact. They proposed that the C-terminal thiol is activated by hydrogen bonds to backbone amides (see next section).

Catalytic Mechanism

The reaction catalyzed by Trx is a bimolecular nucleophilic substitution reaction (SN_2) (Fig. 4). The reaction can be seen as a transfer of the disulfide bond from the substrate protein to Trx (Fig. 1). In other words, the electrons coming from Trx are shuttled to the substrate protein. Thus, in spite of the reducing environment of the compartment in which they are usually located, Trx forms a disulfide bond after a single catalytic cycle. This disulfide bond is not a structural disulfide, but functions as a redox switch (118). It is surface exposed and only accessible from one side of the protein (Fig. 3B).

Oxidized Trx is more stable than reduced Trx (Fig. 5). The difference in stability between the oxidized and reduced states of Trx provides the necessary driving force for the reaction and predicts the way the reaction is thermodynamically favorable (Fig. 5). Factors that affect the rate of the thiol–disulfide exchange are the pK_a of the nucleophilic cysteine (113), the local electrostatic environment of nearby

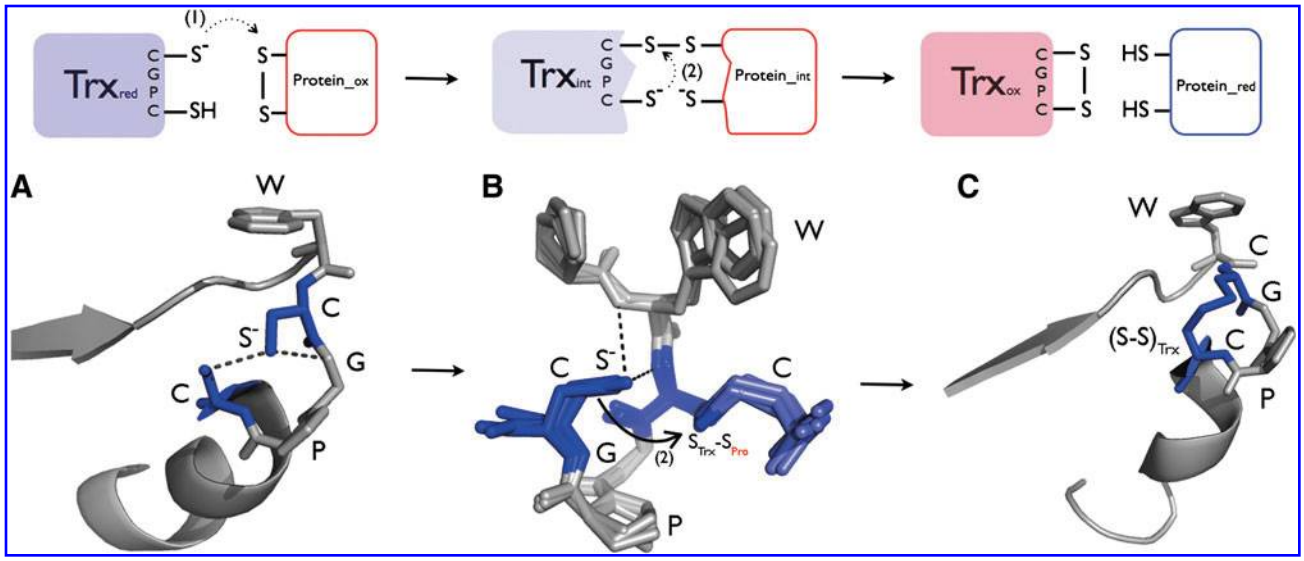

FIG. 4. Reaction mechanism of disulfide reduction by thioredoxin. A schematic representation of the reaction mechanism is shown on *top of the structures* of Trx at each step of the reaction. The reaction takes off with a nucleophilic attack of the N-terminal cysteine of the conserved CGPC motif targeting the disulfide (1). The thiolate of the nucleophilic cysteine is stabilized by two hydrogen bonds with the NH of the glycine and the SH of the C-terminal cysteine (PDB code: 1TRV(99)) (**A**) As a result, an intermediate mixed disulfide complex is formed between Trx and the substrate protein, which in turn is reduced by a nucleophilic attack of the C-terminal cysteine of the CGPC motif (2). The C-terminal cysteine is primed for nucleophilic attack in the Trx–protein mixed disulfide complex. Selected snapshots from an MD simulation of the *B. subtilis* Trx and arsenate reductase complex show that the thiolate on the C-terminal cysteine is stabilized with two backbone amide hydrogen bonds, which lowers its pK_a to 7.4 (**B**)(105). Further, the N-terminal cysteine of Trx has been found to be more susceptible for the nucleophilic attack of the C-terminal cysteine and is also sterically closer to the C-terminal cysteine (105). One single catalytic reduction cycle stops with the release of a reduced substrate protein and oxidized Trx (PDB code: 1TRU (99)) (**C**). The figures were generated using MacPyMol (Delano Scientific LLC 2006). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article at www.liebertonline.com/ars).

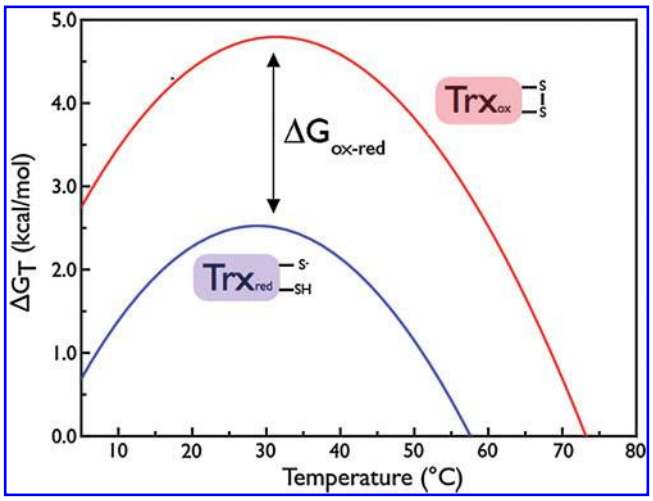

FIG. 5. Thermodynamic stability of Trx as function of temperature. The stability of oxidized Trx (*red curve*) is higher compared to the stability of reduced Trx (*blue curve*). This $\Delta G_{ox\text{-}red}$ drives Trx to reduce oxidized proteins. Curves are calculated with the Gibbs–Helmholtz equation based on urea-unfolding data of wild-type *S. aureus* Trx (106). Curves were generated with pro Fit 6.1.9. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article at www.liebertonline.com/ars).

amino acids (10, 42), the geometry to form a linear transition-state (27), the molecular strain (95), and entropy (9). Moreover, the pH of the surrounding solvent (112) and the pK_a values of the leaving thiol groups of the oxidized substrate (113) also determine the rate of the thiol–disulfide reaction. For all the details of the kinetics of this reaction, we refer to the article by Jensen *et al.* and to its references (53).

The reaction starts with a nucleophilic attack of the N-terminal thiol of the CGPC motif on the disulfide of the target protein, releasing a free thiol and forming a mixed disulfide between Trx and the target protein (Fig. 4). This first step of the mechanism depends on the low pK_a value of the N-terminal cysteine of the CGPC motif ($pK_a \sim 7$) (22, 24), which is significantly lower than the pK_a of free cysteine residues in solution ($pK_a \sim 9$). Under physiological conditions, a large fraction of the sulfur of the N-terminal cysteine is therefore present as a thiolate, which enables this residue to act as a nucleophile, attacking disulfides in proteins. The low pK_a value results from the stabilization of the negative charge of the thiolate anion via the formation of hydrogen bonds between the sulfur of the cysteine and its neighboring residues (Fig. 4, first step of the reaction) (29). In contrast, the sulfur of the C-terminal cysteine has a high pK_a (~ 9) and is present as a thiol (10, 72). This contributes in stabilizing the N-terminal thiolate, therefore increasing the reaction rate.

Once a mixed disulfide has been formed between Trx and its substrate, the C-terminal thiol has to be activated as a thiolate to allow the dissociation of the complex (Fig. 4, second step of the reaction). The dissociation mechanism of a Trx mixed disulfide complex has been a matter of controversy for more than 20 years (11, 12, 17, 23, 36, 54, 80), and opposing arguments for the activation of the C-terminal cysteine as a thiolate have been put forward. Based on the solution structure of a mixed disulfide complex between Trx and *Bacillus subtilis* arsenate reductase (ArsC) (70), fresh insights into the dissociation mechanism have been provided (105). As mentioned in a previous section, it now appears that the C-terminal cysteine is stabilized as a thiolate by hydrogen bonds between this residue and the backbone amides of the active site tryptophan and of the N-terminal cysteine. As a consequence, the pK_a of the C-terminal cysteine in the mixed disulfide complex drops (105) (Fig. 4, second step of the reaction). Noteworthy, even a transient stabilization of the thiolate is sufficient to allow the dissociation of the mixed-disulfide complex. From a mechanistic point of view, the pK_a of the C-terminal cysteine needs to be low only for a split second for a nucleophilic attack to take place (105). As such, instantaneous low pK_a values are more relevant than an average value. Similar essential short timescale events were also observed in the active site of glutaredoxin 3 from *Escherichia coli* (28, 29).

The last step of the catalytic cycle (Fig. 4, second step of the reaction) needs also to be completed with the correct selectivity. Based on quantum chemical calculations (107), Roos *et al.* (105) rationalized the mechanism behind the favored reaction during complex dissociation: the C-terminal cysteine of Trx attacks the mixed disulfide complex on the N-terminal cysteine of Trx and not on the cysteine from the substrate protein, releasing a reduced substrate protein and oxidized Trx. Trx is then reduced by TrxR at the expense of NADPH (68) (Fig. 1), enabling the protein to start another reaction cycle.

Trapping Trx Substrates

Trxs play a central role in living cells where they are often at the crossroads between various processes. Several techniques and methods have been developed in order to identify the proteins that interact with Trxs. These approaches, which are often complementary, have led to the identification of several dozens of potential Trx substrates. Here, we present three of the most commonly used techniques to trap Trx substrates.

In one of the favored approaches, the C-terminal cysteine of the catalytic site of Trx is mutated to an alanine (4) (Fig. 6A). As explained above, the C-terminal cysteine is essential for the dissociation of the mixed disulfide complex. Mutation of the C-terminal cysteine prevents the dissociation, allowing the formation of stable complexes between Trx and its substrates. The mutant protein can be expressed *in vivo* and the complexes are then purified by affinity chromatography (21). Alternatively, the single cysteine mutant of Trx can be immobilized on a resin and used to capture potential target proteins in cellular extracts (67, 88, 92).

A second approach consists in the differential labeling of reduced and oxidized cysteines. Although various molecules and protocols can be used, the rationale is often the same and consists in modifying the reduced cysteine residues with a molecule such as iodoacetamide. The oxidized cysteines are subsequently reduced and modified with a different molecule, such as 4-vinylpyridine (73) or N-ethylmaleimide (65, 116). The proteins or the peptides are then analyzed by mass spectrometry. The difference in mass is used to determine whether the cysteines were either reduced or involved in disulfide bonds in the cell. Alternatively, the cysteines can be modified by two different isotopes of the same molecule, such as "light" (^{12}C) and "heavy" (^{13}C or ^{14}C) iodoacetamide-based Isotope Coded Affinity Tag (ICAT) reagents (30, 39). We will

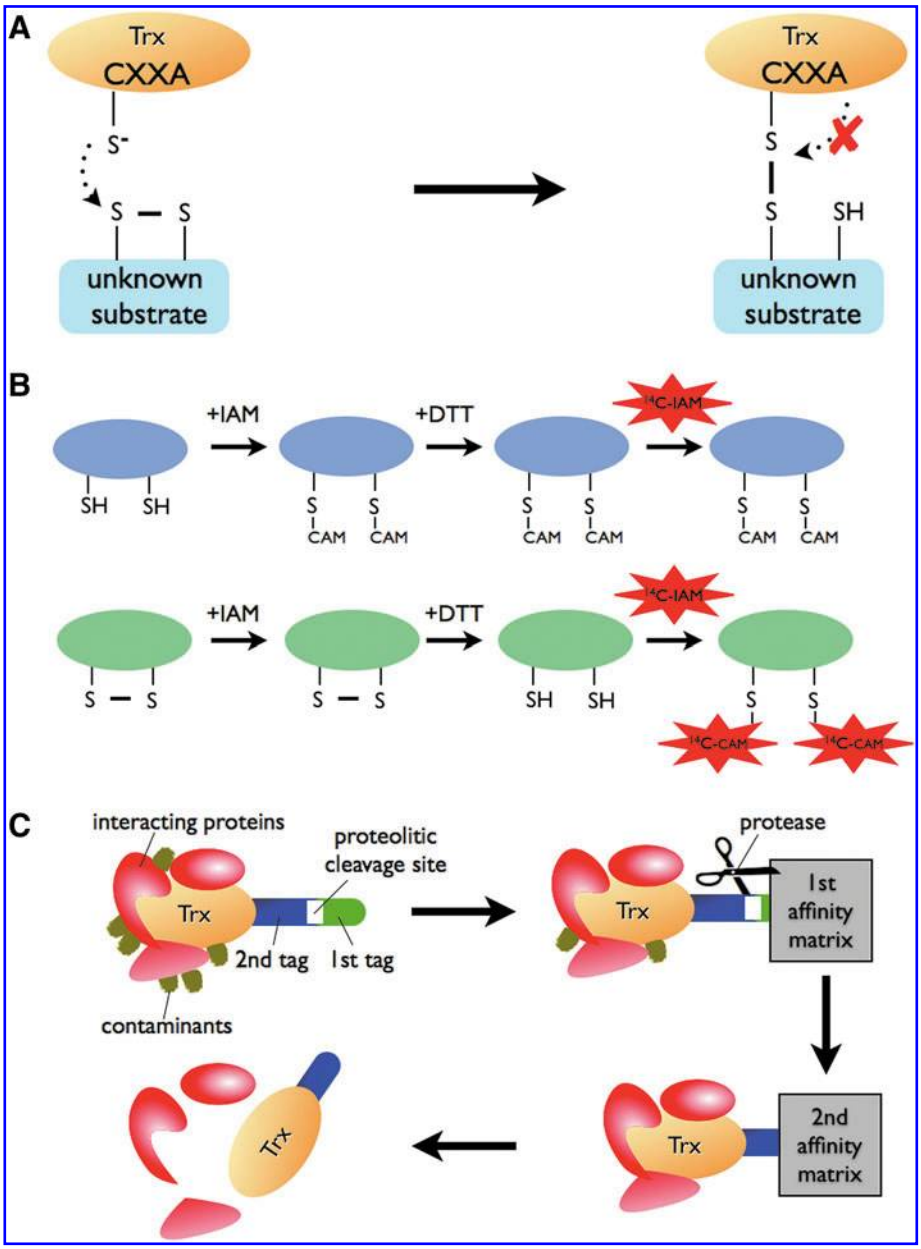

FIG. 6. Trapping thioredoxin substrates. (**A**) Mutation of the second cysteine of the CGPC motif to an alanine allows the trapping of Trx with its substrates. The mixed disulfide complexes formed upon the nucleophilic attack of the first cysteine residue on the target proteins cannot be resolved. (**B**) Thiol differential labeling is often used to identify the proteins that are kept reduced by Trxs. In the approach illustrated here, the accessible thiol groups are carbamidomethylated (CAM) with iodoacetamide (IAM) and blocked for the subsequent reduction and alkylation steps. The disulfide bonds are then reduced using dithiothreitol (DTT), and the newly accessible thiols are then labeled with radioactive iodoacetamide (^{14}C-IAM). Radioactivity is therefore incorporated into the proteins that were originally in an oxidized form. Comparison of cellular extracts prepared from wild-type and Trx knockout strains allows the identification of the proteins that depend on thioredoxin for reduction. (**C**) Addition of a TAP-tag at the C-terminus of Trx allows purification of the complexes involving Trx and interacting proteins by using two different affinity columns. A cleavage site for a specific protease is present between the tags, allowing the removal of the most C-terminally located tag before starting the second affinity purification. The Trx partners are then identified using mass spectrometry. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article at www.liebertonline.com/ars).

use the *in vivo* differential thiol-trapping technique developed by Leichert *et al*. in 2004 as an example to illustrate the approach (66) (Fig. 6B). Jakob and co-workers prepared extracts from both a wild-type and a Trx-deficient strain of *E. coli* (66). After quenching the extracts under acid conditions, cysteines in their thiol form were first alkylated using cold, unlabeled iodoacetamide (IAM). As such, accessible thiol groups are quickly blocked, preventing further exchange reactions. In the next step, the disulfide bonds were reduced with dithiothreitol and the newly accessible thiol groups were modified with [^{14}C]-labeled IAM. Radioactivity was therefore incorporated into the proteins that originally contained disulfide bonds. The differentially trapped proteins were then analyzed on a two-dimensional gel, the protein spots stained and the level of incorporated [^{14}C]-radioactivity determined. This method allowed the identification of proteins that were oxidized in the Trx-deficient strain, indicating that these proteins depend on Trx for reduction.

In a third approach, Trx substrates have also been identified by purifying proteins bound to Trx using Tandem Affinity Purification (TAP) followed by ms/ms mass spectrometrical analysis (60). A TAP-tag was appended to the C-terminus of *E. coli* Trx1 (Ec_Trx1) (see below) and the Trx-substrate complexes were sequentially purified on two different affinity columns under mild conditions to preserve the protein–protein interactions (Fig. 6C). More than 80 *E. coli* proteins co-purifying with Ec_Trx1 have been identified this way, implicating Ec_Trx1 in at least 26 distinct cellular processes.

Diversity Within a Ubiquitous Family

Bacterial thioredoxins

Thioredoxin 1 (Ec_Trx1) and thioredoxin 2 (Ec_Trx2) are two redundant reductases present in the cytoplasm of *E. coli*. Ec_Trx1, which contains 108 amino acids, has become the paradigm of the Trx superfamily. In addition to ribonucleo-

tide reductase, Ec_Trx1 catalyzes the reduction of other cytoplasmic proteins such as methionine sulfoxide reductases and 3-phosphoadenosine 5′-phosphosulfate (PAPS) reductase (51). It also provides reducing equivalents to the inner membrane protein DsbD, which transfers electrons to the periplasm (103). Moreover, Ec_Trx1 is required for the growth of several bacteriophages including T7, M13, and f1 (109). For this latter function, Ec_Trx1 binds to a DNA polymerase encoded by the viral genome and increases its processivity by remodeling the protein to favor interaction with DNA and other replication proteins (34, 41). The redox activity of Trx is not required for this function, as active site cysteine mutants of Ec_Trx1 support nearly normal viral growth (71, 110).

Ec_Trx1 is the most reducing protein present in the cytoplasm of *E. coli*. Its redox potential value has been estimated to be −270 mV (58), but a more recent study based on an improved protocol designed to minimize experimental errors suggests that it might be even lower ($E^{o'} = -284$ mV) (16).

In 1974, Chamberlin *et al.* (15) isolated a deletion mutant strain that completely lacked Ec_Trx1. This indicated that other cytoplasmic proteins were able to reduce ribonucleotide reductase. The search for alternative hydrogen donors led to the discovery of several glutaredoxins and of a second thioredoxin, Ec_Trx2 (84).

Ec_Trx2, which is encoded by the gene *trxC* (84), shares 28% sequence identity with Ec_Trx1. Like Trx1, Ec_Trx2 is able to reduce ribonucleotide reductase, DsbD, and PAPS reductase. Ec_Trx2 has two striking characteristics that differentiate it from Ec_Trx1 and suggest that this protein may have a specific cellular function. First, the expression of Ec_Trx2 is controlled by OxyR, a transcription factor that controls a response to oxidative stress (104). Second, Ec_Trx2 presents an additional N-terminal domain of 32 amino acids that contains two CXXC motifs (Fig. 7). We found that these additional cysteine residues bind Zn^{2+} with an extremely high affinity ($10^{18}\ M^{-1}$) (18). These zinc-binding CXXC motifs are conserved in all Ec_Trx2 homologues that have been identified so far, making Ec_Trx2 the prototype of a new zinc-binding Trx family. Ec_Trx2 has a redox potential of −221 mV (40), and is therefore a significantly less reducing enzyme than Ec_Trx1. Further, we recently showed that the zinc center of Ec_Trx2 fine tunes its redox and thermodynamical properties (40).

Remarkably, the function of Trxs seems to depend on the cellular environment. Under the more oxidizing conditions of the periplasm (82), Ec_Trx1 becomes an oxidase that promotes disulfide bond formation (20). Similarly, both Ec_Trx1 and Ec_Trx2 promote disulfide bond formation in the more oxidizing cytoplasm of strains lacking TrxR (7, 114). Moreover, Masip *et al.* (78) have shown that an Ec_Trx1 mutant with a CXCC active site motif can mediate oxidative protein folding in the periplasm independently of the Dsb-oxidative folding system (82). This extra active site cysteine leads Trx to dimerize via the formation of an iron sulfur cluster (78).

Yeast and mammalian thioredoxins

In the yeast *Saccharomyces cerevisiae*, there are two cytosolic (Sc_Trx1, Sc_Trx2) and one mitochondrial (Sc_Trx3) Trxs (45). Yeast mutants lacking both cytosolic Trxs are viable. They have a longer S phase in their cell cycle and are auxotrophic for sulfur amino acids (93). Mutants lacking Sc_Trx3 are hypersensitive to hydroperoxide. So, Sc_Trx3 seems to function as an antioxidant against ROS generated in mitochondria (97).

Mammalian cells possess two Trx isoforms, ma_Trx1 (∼12 kDa), which is present in the cytosol, and ma_Trx2 (∼18 kDa), which contains a 60 residue N-terminal sequence for targeting to the mitochondria. Although ma_Trx1 has no nuclear localization sequence, it has also been detected in the nucleus of certain normal and tumor cells (46, 77). ma_Trx1 was first identified in humans in 1967 (hu_Trx) (89), and ma_Trx2 in pig heart mitochondria in 1991 (8).

In addition to the catalytic cysteines present in the CGPC motif, ma_Trx1 contains 3 extra cysteine residues (99). In the human protein, two of these cysteines (C62 and C69) flank the α_3-helix that links both motifs of the Trx-fold (Fig. 2A). The third extra cysteine (C73) is located in a turn close to the active site CGPC motif. Several reports have shown that these extra cysteines are involved in regulating the function of Trx via post-translational modifications, such as glutathionylation and S-nitrosylation (13, 62). For instance, C73 has been shown to be *S*-nitrosylated after treating the human protein with S-nitrosoglutathione (85). This S-nitrosothiol can be transferred from C73 to caspase 3 *in vitro* (86) and *in vivo* (87). Moreover, under oxidizing conditions, a disulfide can be formed between C69 and C72 (117). The formation of this disulfide is predicted to have a profound effect on the structure of Trx and decreases the rate by which the active site is regenerated by TrxR.

Plant thioredoxins

The Trx family is particularly important in plants. Plant Trxs are involved in multiple processes, such as photorespi-

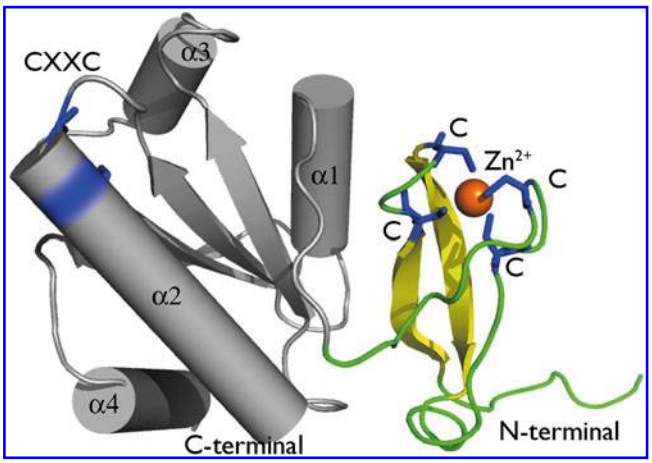

FIG. 7. The structure of a thioredoxin protein with a Zn^{2+}-binding domain. The structure of Trx2 from *Rhodobacter capsulatus* is shown (PDB code: 2PPT)(123). The Trx domain is *gray*, the N-terminal Zn^{2+}-binding domain is *colored*, and the cysteine residues are in *blue*. The N-terminal zinc-binding domain is composed of short *β*-strands (*yellow*) connected by short loops. The four zinc-binding cysteine residues form a tetragonal binding site, which is located about 30 Å away from the catalytic CXXC motif. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article at www.liebertonline.com/ars).

ration, lipid metabolism, membrane transport, hormone metabolism, and ATP synthesis (4). They also play an important role in sustaining early seedling growth of germinating cereal seeds (4).

Plants possess the greatest group of Trxs found in all organisms. For instance, at least 20 Trx genes have been detected in the genome of *Arabidospis thaliana* and it is likely that the diversity found in *A. thaliana* is representative of higher plants, such as poplar, pinus and tomato (33). Plant Trxs can be divided in six major groups: Trx f, h, m, o, x, and y on the basis of their sequence. Whereas Trxs m, x, and y are related to prokaryotic Trxs, Trxs f, h, and o are specific to eukaryotic organisms. Plant Trxs are found in the cytoplasm, in mitochondria, and in chloroplasts. Whereas TrxR reduce mitochondrial and cytoplasmic Trxs, Trxs present in chloroplasts are recycled by a ferredoxin-thioredoxin reductase (FTR) [reviewed in Gelhaye *et al*. (33)]. FTR is only found in photosynthetic organisms and uses the reducing power provided by light to reduce Trxs. Although plant Trxs possess the classical structural features of Trxs, some of them have specific characteristics. For instance, Trx *h* from poplar has an additional cysteine residue playing a role in an atypical catalytic mechanism (57). Here, electrons are transferred via an intramolecular disulfide cascade in which three cysteines and glutathione are involved. Moreover, some Trxs *h* harbor an unusual CXXS active site motif and are recycled by glutathione, whereas others have a canonical CGPC motif and are reduced by glutaredoxins [reviewed in Gelhaye *et al.* (32)].

Conclusion

Since its discovery less than 50 years ago, Trx has been promoted from a protein functioning as an electron donor for an *E. coli* enzyme to a protein that is at the heart of numerous fundamental processes in all sorts of living organisms. The story is not over and we believe that Trxs, as well as the proteins presenting a Trx-fold, have only offered us a first glimpse at their important roles. There is no doubt that in the next few years, several Trxs for which a specific function has not been assigned yet will be characterized, broadening our knowledge and opening new fields of investigation.

The Trx-fold has proven its solidity over the last billion years. This scaffold has been used for a number of different purposes, revealing its high adaptability. We can therefore assume that the Trx-fold will continue to evolve in order to catalyze new reactions and to deal with new substrates, helping the cell to cope with various stresses coming from the environment. In such a way, it will help the cell to adapt and to survive.

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Abbreviations Used

ArsC = arsenate reductase
CAM = carbamidomethylated
CGPC = Cysteine-Glycine-Proline-Cysteine
CLIC = chloride intracellular channels
DTT = dithiothreitol
Ec_Trx1/2 = *Escherichia coli* thioredoxin 1 and 2
FTR = ferredoxin-thioredoxin reductase
hu_Trx = human thioredoxin
IAM = iodoacetamide
ma_Trx = mammalian thioredoxin
OxyR = a transcription factor that controls a response to oxidative stress
PAPS = 3-phosphoadenosine 5′-phosphosulfate
PDB = Protein Data Bank
ROS = reactive oxygen species
Sc_Trx1/2/3 = *Saccharomyces cerevisiae* thioredoxin 1, 2 and 3
TAP = tandem affinity purification
Trx = thioredoxin
TrxR = thioredoxin reductase
Trxs = thioredoxins

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