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Reactivity of Thioredoxin as a Protein Thiol-Disulfide Oxidoreductase

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Supporting Information

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1. INTRODUCTION

Thiol-disulfide reactions are crucial for redox homeostasis in the cell.[1,2] As a disulfide oxidoreductase, thioredoxin (Trx, ~12 kDa) regulates a wide variety of biological molecules in both eukaryotic and prokaryotic species, including ribonucleotide reductase, peroxiredoxin, methionine sulfoxide reductase, phosphatase and tensin homologue (PTEN), transcription factors such as nuclear factor-κB (NF-κB), redox factor-1 (Ref-1), and activator protein-1 (AP-1).[3] Thus, Trx has been implicated in such diverse processes as antioxidant defense, DNA repair and synthesis, redox regulation, and apoptosis.[4−9]

In the evolution of Trx catalysis important physical factors (e.g., a hydrophobic binding groove) have appeared to make Trx bind the disulfide substrate in a specific fashion and generate stabilizing interactions.[10,11] The interactions and substrate binding were shown to regulate the geometry and orientation of the target disulfide in the catalytic site of the enzyme, and account for Michaelis−Menten−type kinetics of disulfide reduction by Trx.[10,11] Trx molecules contain two cysteines in the active site in a CXXC motif. The chemistry of Trx-catalyzed protein disulfide reduction following substrate-binding includes a nucleophilic attack on the disulfide of target proteins by the N-terminal cysteine thiolate of Trx (which is Cys32 in the *Escherichia coli* numbering) to form an intermolecular mixed disulfide, and a subsequent attack on the disulfide intermediate by the thiolate of the C-terminal cysteine of Trx (Cys35), producing the reduced target protein and oxidized Trx (Figure 1).[12,13]

The 3-D structures of Trx proteins are highly conserved, with the five central β-strands surrounded by four α-helices (Figure 2, A and B). Part of the redox active center (-CGPC- motif) protrudes at the surface of the molecule at the very N-terminus of helix α_2, with Cys35 largely covered by the N-terminal portion of the helix α_2. Only one side of the Cys32-SH is accessible to solvent for the transfer of reducing equivalents in Trx_{ox} but the side chain of Cys32 turns into the solvent upon reduction.[14−16] The Trx fold is also found in several other classes of enzymes that interact with substrates containing either disulfides or dithiols. Protein families that have a Trx-fold include the thioredoxin, Dsb (disulfide bond formation protein) proteins, glutaredoxin (Grx), glutathione S-transferase, and protein disulfide isomerase (PDI) families.[2,16,17] We performed a structural bioinformatics study on 515 sequences of the Trx family, 495 sequences of the DsbA family, and 382 sequences of the PDI family (from the Conserved Domain Database (CDD),[18] http://www.ncbi.nlm.nih.gov/sites/entrez?db=cdd). This search confirmed a recent observation that the two components that are most conserved across these 3 families of proteins include an active site CXXC motif, and a conserved proline that is distant in sequence from this active site but immediately adjacent in 3-dimensional space.[19] In the Trx family, the CXXC motif has the sequence CGPC and is present at position 32−35, while the proline residue is at position 76 in its best-studied member, *E. coli* thioredoxin 1 (*Ec*Trx; PDB 2TRX_A) (Figure 2C). The highly conserved proline is at position 151 in *E. coli* DsbA (PDB 1DSB_A) and at position 83 in human PDI (PDB 1MEK) (Figure 2, D and E). In *Ec*Trx, the distances between the N-atom of Pro76 and the two sulfur atoms of the cysteine pair are 4.07 (Cys32) and 3.62 Å (Cys35) (Figure 2F). The next closest residue is Asp26, and the shortest distance between an O-atom of Asp26 and the sulfur atom of Cys35 is 5.59 Å (Figure 2G). Pro76 and Asp26 have been shown to play important roles in the redox reactions due to their close contact with the Cys pair.[20,21]

Conserved structural features such as the Trx-fold and secondary structure in Trx family members, nevertheless, allow diverse reactivities in catalyzing protein disulfide interchange reactions. Quantum mechanical calculations suggest that the relative stability of thiolates in the CXXC motif determines whether these enzymes catalyze oxidation, reduction, or isomerization.[2,22,23] Because the cysteine thiolates in Trx are both poorly stabilized, Trx is a good reducing agent. In contrast, DsbA stabilizes both cysteine thiolates and thus is a good oxidizing agent; isomerases such as PDI have one thiolate relatively solvent exposed and poorly stabilized while the other (relatively buried) thiolate is highly stabilized.[22,23] Static calculations, such as the quantum mechanical calculations, provide insights into the thermodynamics of thiol-disulfide reactions, but as

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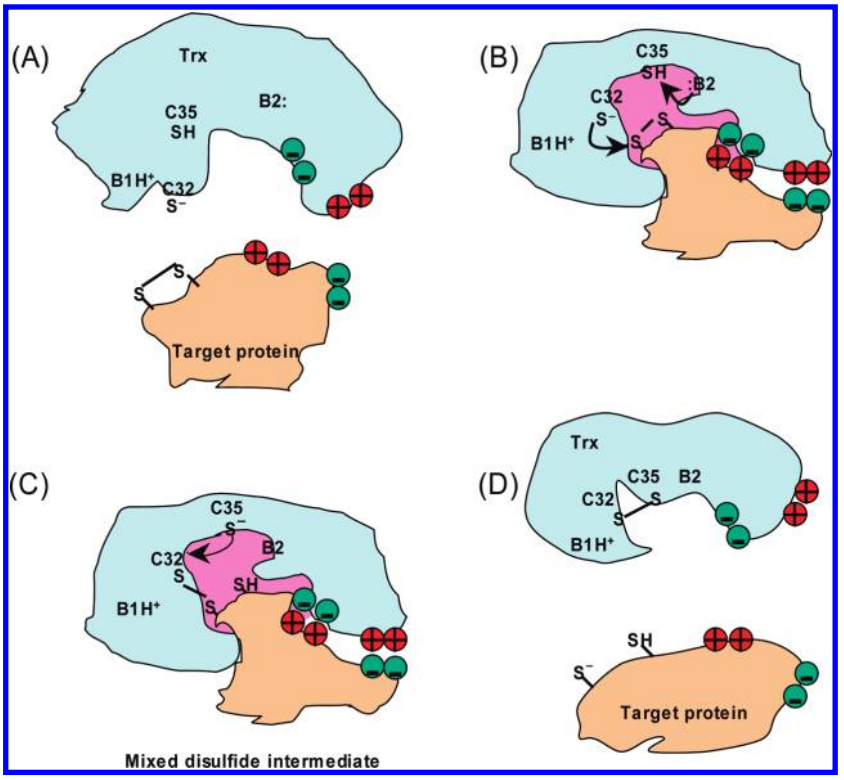

Figure 1. Schematic cartoon view of a Trx-catalyzed disulfide reduction. A number of factors are involved in the regulation of Trx activity, including the amino acid residues spanning the redox active CXXC motif, molecular interactions (e.g., electrostatic force), acid/base catalysts (B1 and B2), conformational adjustment to orientate the reactive groups to facilitate reaction, and hydrophobic microenvironments formed after substrate binding (indicated in pink). Taking *Ec*Trx as an example, the reaction of Trx with target disulfide proteins includes binding (panel A to B), the first nucleophilic attack of Cys32-S^- (somewhat exposed to the solvent, panel A) on the target disulfide to form an intermolecular mixed disulfide intermediate (panel B to panel C), followed by a second nucleophilic attack of Cys35-S^- (buried in *Ec*Trx) on the mixed disulfide (panel C), with the generation of Trx-S_2 and the reduced protein (panel D). Binding of the protein substrate to Trx could create a more apolar environment for Cys32 and place it in proximity to acid/base catalysts. However, the identity of acid/base catalysts still remains to be established, although they are critical for the ionization of thiol groups in the CXXC motif for the nucleophilic attacks.

previously noted and as recently further investigated,[1,24] the reactivity in an actual reaction is more complex due to dynamics and conformational changes that occur during substrate binding. For instance, studies with site-specific mutagenesis indicate that the relative stability of the oxidized versus the reduced form determines the difference in the redox potentials of Trx from *Staphylococcus aureus* (*Sa*Trx).[25−27] Replacement of the conserved proline in the CXXC motif by threonine or serine greatly reduced the relative stability of *Sa*Trx and made it less reducing[25] Moreover, the activation energy barrier for forming a transition state complex must be overcome by dynamics or other properties to accomplish a thermodynamically favorable thiol-disulfide reaction.[1] However, DsbA from *Staphylococcus aureus* shows identical stabilities in oxidized and reduced forms, suggesting that alternative mechanisms beyond thermodynamic stability underlie the activity of *Sa*DsbA in thiol-disulfide reactions.[28,29]

To date a number of factors, including the identity of the amino acid residues spanning the CXXC motif, the pK_a of the redox active thiols, the redox potential of the disulfide/dithiol couple, the acid/base catalysts, the molecular interactions and the local conformational changes after substrate binding, have been implicated in the regulation of Trx activity as a protein disulfide oxidoreductase, albeit with controversies and debates. In this work, we review the emerging evidence that support or dispute the regulating roles of these factors, and propose the likely prime determinants of Trx activity that deserve more attention in future research, with the hope to promote a better understanding of the mechanistic factors of Trx reactivity.

2. pK_a Values of the Active Thiols in the CXXC Motif

The pK_a of the thiol of a reactive cysteine residue determines what fraction of the cysteine will be in the thiolate form (RCH_2S^-), which is required for nucleophilic attack on a disulfide (Figure 1). Cysteines with low pK_a values are ionized and can be highly reactive in buffers of physiological pH, but the lower pK_a values also are associated with lower intrinsic nucleophilicity and higher redox potentials.[1] Thus, the pK_a values of cysteine residues in thioredoxin family members have multiple effects on their reactivity.[1] It has been found that mutations of the dipeptide between the two active site cysteine residues in the thioredoxin family of proteins alter the redox potential of the resulting variants in ways that can at least in part be explained by the effects of these alterations on the thiol pK_a values.[25,30−32] For example, when the intervening dipeptide of the CXXC motif in *Ec*Trx was replaced by the dipeptide from glutaredoxin, PDI, or DsbA, all the variants had lower Cys32-SH pK_a values and increased redox potentials relative to wild type Trx.[31] The glutaredoxin-like variant had the lowest pK_a value (5.9) and the highest redox potential (ΔE_m = 75 mV), as reflected by the 420-fold decrease in the equilibrium constant ($K_{GSH/GSSG}$ or K_{ox}) for

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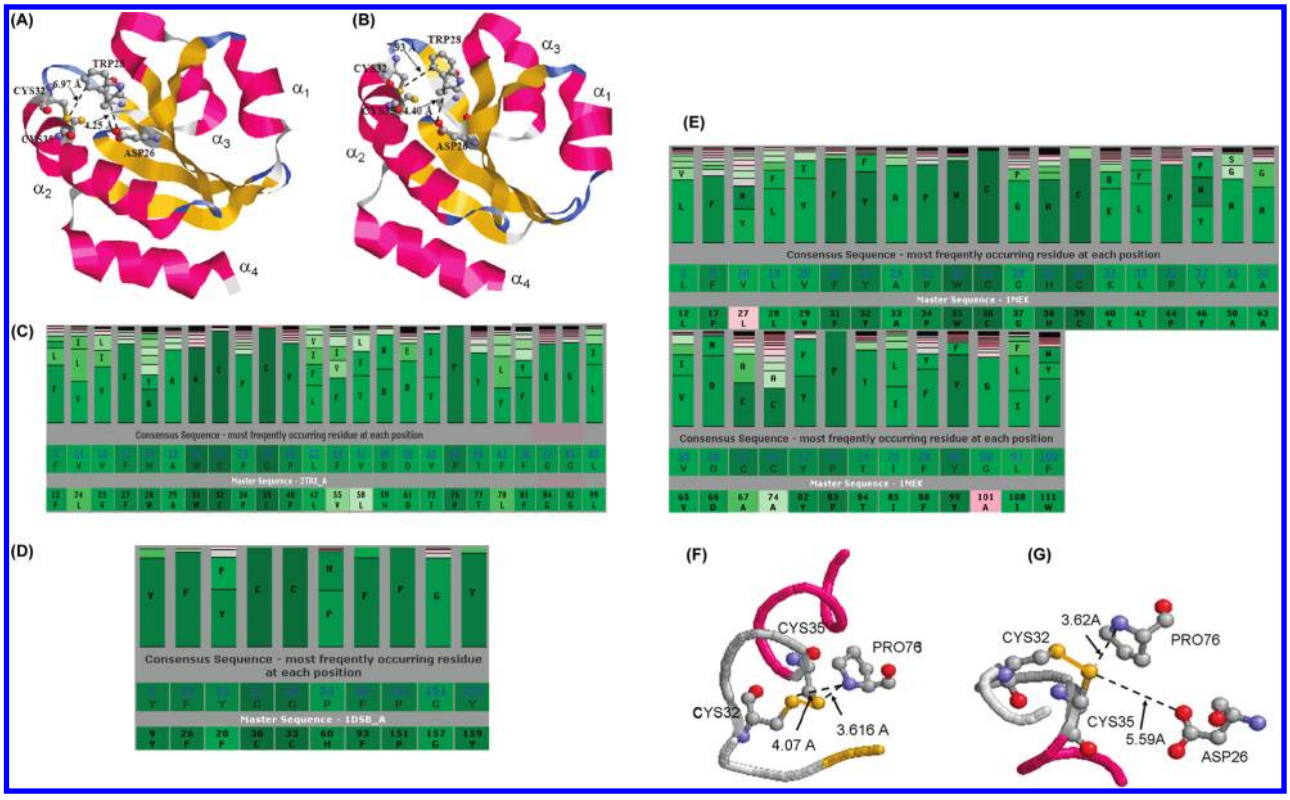

Figure 2. The structure of *Ec*Trx and conserved amino acid residues in the Trx family. (A) and (B) are the ribbon diagrams of oxidized and reduced *Ec*Trx, respectively. They show the spatial distances between Trp28 and Cys32, and between Trp28 and Asp26. The color of the backbone labels the secondary structures, where red indicates alpha-helices, yellow indicates beta-sheets, and gray indicates turns or coils. The atom coordinates of proteins are obtained from the PDB,[141] and the figures of protein structures were generated using RasMol.[142] (C-E) show the conserved residues with sequence alignments of 515 Trx sequences (C), 495 DsbA sequences (D), and 382 PDI-α protein sequences (E) from the Conserved Domain Database of NCBI (note that only the more conserved residues are shown).[18] For each panel (C−E), the top portion shows the relative conservation of residues at each conserved position, the middle shows the consensus sequence, and the bottom portion shows the Master Sequence of each class (2TRX_A, 1DSB_A, and 1MEK, respectively). The Master Sequence is that of a real protein, and is the sequence to which all other sequences in the Conserved Domain (CD) alignment are related pairwise. The Consensus Sequence for a CD contains, at each position, the most frequently occurring amino acid at that position in the alignment of the CD. The relative heights of the green boxes show the residue (in black) having the highest fraction among all the aligned sequences. The color gradation from green to red indicates a decreased conservation of the corresponding residue. From the alignment of the Master Sequences, we can see that the two Cys residues of the three representative proteins (32 and 35 of 2TrxA, 30 and 33 of 1DsbA, and 36 and 39 of 1MEK) are highly conserved in their corresponding families. The alignment also suggests a proximal proline is almost completely conserved in these families (Pro76 in *Ec*Trx, PDB 2TrxA; Pro151 in *Ec*DsbA, PDB 1DsbA; and Pro83 in human PDI, PDB 1MEK). (F) Spatial distance of active site residues Cys32 and Cys35 from Pro76 in *Ec*Trx. (G) Spatial distance between Asp26 and Cys35, and between Pro76 and Cys35 of *Ec*Trx.

its reaction with GSSG compared to wild type (eqs 1 and 2).

$$\mathrm{Trx(SH)_2} + \mathrm{GSSG} \underset{k_2}{\overset{k_1}{\rightleftharpoons}} \mathrm{GSS-Trx-SH} \underset{k_4}{\overset{k_3}{\rightleftharpoons}} \mathrm{Trx(S)_2} + 2\mathrm{GSH} \quad (1)$$

$$K_{\mathrm{GSH/GSSG}} = \frac{[\mathrm{Trx(S_2)}]\times[\mathrm{GSH}]^2}{\mathrm{Trx(SH)_2}\times[\mathrm{GSSG}]} = \frac{k_1\times k_3}{k_2\times k_4} \quad (2)$$

For at least some thioredoxin-related proteins, such as DsbA, it has been reported that there is a clear and simple relationship between cysteine pK_a values and redox potentials, that is, the lower the pK_a of the resultant thiols, the easier it is to reduce the disulfide bond.[30,33] However, the situation appears to be more complicated for thioredoxin.[33] On the basis of the substitution effects on rate constants, Mossner et al. tried to predict redox potentials and Cys32 pK_a values, but *wt*Trx was always an outlier in simulations. For example, the calculated K_{ox} value of *wt*Trx based on this correlation was 22-fold lower than the experimental one.[32] Moreover, DsbA- and PDI-like variants of Trx showed 3-fold changes in the maximal reactivity of the Cys residue thiolate (analogous to Cys32 in *Ec*Trx) with iodoacetamide, and this could be expected to reflect the changes in their pK_a values; however, all of the pK_a values of Cys32-SH were virtually identical.[31] Additionally, Trx from the hyperthermophilic archaeon *Methanococcus jannaschii* (*Mj*Trx) was a stronger reducing catalyst than *Ec*Trx despite an intrinsically lower pK_a of 6.28 versus the p*K*a of 7.1 for *Ec*Trx (see Table 1), arguing against the pK_a being the sole determinant of the reactivity and the redox potential of Trx.[34] Therefore, Trx reactivity is associated with the pK_a value, but the relationship is not straightforward. Consistent with this, theoretical studies on cysteine-containing small molecules and proteins indicated that features causing decreased thiol pK_a values also caused decreased nucleophilicity of the nascent thiolate, hence making it less reactive.[1] Particularly, the presence or introduction of electron-withdrawing groups or

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Table 1. pK_a Values of Active Thiols in *Ec*Trx Measured by Different Methods

thiols	pK_a values	methods/ assays	references
Cys32-SH 6.7	6.7	alkylation by IAA or IAM	46, 136
	6.28	Trp28 fluorescence	39
	9−10	UV absorbance (A_{240})	
		GSSG/GSH equilibria	45
	7.1	Raman spectroscopy	42
	7.4	^{1}H−^{13}C NMR	101
	7.0−7.1	^{1}H−^{13}C NMR	58
	7.5 or 9.2	^{13}C NMR	97
Cys35-SH			
	9.0	alkylation by IAA or IAM	
		coupled to HPLC analysis	46
	9−10	UV absorbance (A_{240})	
		GSSG/GSH equilibria	45
	7.9	Raman spectroscopy	42
	9.4	^{1}H−^{13}C NMR	101
	7.0−7.1	^{1}H−^{13}C NMR	58
	>11	^{13}C NMR	97
	13.5−14.0	density functional theory	37

positive charges in proximity to the thiol decreased the pK_a by stabilizing the negative charge of the thiolate anion;[20,31,35] however, both decreased and increased values of the equilibrium constants for the thiol disulfide reactions were observed despite a general increase in the rate of disulfide bond formation.[36] The effects of thiolate stability on the reactivities of Trx family members were investigated recently using quantum mechanical calculations.[22,23,37] These studies suggested that the degree of overall stabilization of thiolates in the active site determines whether the thiol-disulfide reaction equilibrium shifted toward reduction, oxidation, or isomerization in reactions associated with Trx, DsbA, or PDI, respectively.[22,23] In particular, the presence of proline in the CXXC motif was found to decrease the overall stability of the attacking thiolate in Trx and render the thiol prone to oxidation.[22,23,25] Of note, the conserved central proline also critically mediates the thermodynamic stabilities of Trx and its driving force to reduce substrate proteins.[25] The oxidized form of wild-type *Sa*Trx is far more stable than the reduced form, with a relative stability $\Delta(T_{1/2})^{ox/red}$ of 15.5 °C. However, mutation of the central proline (Pro31) to threonine markedly reduced $\Delta(T_{1/2})^{ox/red}$ to 5.8 °C, concomitant with a 7-fold decrease in the reducing activity relative to wild-type *Sa*Trx.[25] Further study suggested the P31T mutation did not introduce extra hydrogen bonds with the sulfur of the nucleophilic cysteine, but induced a more hydrophilic milieu surrounding the nucleophilic cysteine that makes the thiol more reactive (or less stable). As might be expected, the pK_a value of the nucleophilic cysteine in the mutant decreased by one pH unit and redox potential increases by 30 mV.[25]

Because thiolate is the reacting species in the thiol-disulfide exchange reactions (Figure 1), a number of methodologies have been developed to measure the pK_a values of reactive thiols in Trx. These methods include measurements of changes in fluorescence[38,39] or UV absorption at 240 nm (A_{240}),[31,34,40] Raman spectroscopy,[41,42] NMR analysis,[39,43,44] and measurements of the pH dependence of the rate of alkylation.[31] Different techniques give pK_a values for the Cys32 that vary from 6.3 to 10.0. The pK_a value for the Cys35 is even less well-defined, lying somewhere between 7 and 14 (Table 1). The large variation in the values obtained for this important parameter is surprising and somewhat disconcerting, and it has drawn attention to some of the shortcomings of the methods used to measure the pK_a values.[20,45] For example, because it was thought that the Cys32 thiolate quenched Trp28 fluorescence, the fluorescence changes as a function of pH were plotted to calculate the pK_a of Cys32-SH as 6.28.[46−48] However, the 3D structure of *Ec*Trx shows that the Cys32-SH is almost 8 Å from the tryptophan ring in Trx-$(SH)_2$, even at its closest approach, while the Asp26 side chain is much closer to the Trp28 ring (average distance between Trp28 C^{γ} and Asp26 $O^{\delta 1}$ was 4.4 Å) (Figure 2A and 2B).[49−51] Given the knowledge that the deprotonation of Asp26 occurs in the range of pH 5−7.7[47,52]and that the Trp28 fluorescence of the D26A variant was less pH-dependent than wild type enzyme within the pH 5−7 range,[20] it is more likely that the Trp28 fluorescence changes are due to quenching of the tryptophan emission by the Asp26 side chain rather than the Cys32-S^-. Given these types of concerns it is clear that at a minimum it is necessary to use several methods to determine pK_a values.

We have therefore attempted to re-examine these data with the notion that consideration of all the data together, coupled with the advantages of hindsight, might help to resolve this important question. The data in Table 1 show that most determinations of the pK_a of Cys32-SH of Trx are below the 8.5−9.0 value for cysteine that is free in solution or is in unfolded proteins.[39,53,54] The likely physical basis for the apparently anomalously low pK_a of Trx-Cys32 is its proximity to the N-terminus of an α-helix where the positive dipole of the helix can stabilize the negative thiolate form of Cys32.[55−57] The pK_a of Trx-Cys35 proved more difficult to measure reliably than the pK_a of Trx-Cys32, partly because it is buried in the interior of Trx. Some attempts to measure the pK_a of the Cys35 thiol have been made with Cys32 in the free thiol or thiolate form.[42,45,58] However, the more relevant pK_a of Cys35 is the one that applies to the dithiol−disulfide interchange between Trx$(SH)_2$ and target proteins; this is when Cys32 is in the form of a mixed disulfide with the target protein (Figure 1).

It should also be kept in mind that the pK_a values of Cys32-SH and Cys35-SH in Table 1 are based on static measurements in the absence of protein ligands (e.g., A_{240}, NMR, and Trp28 fluorescence); unfortunately, static measurements are not necessarily informative about how dynamic processes affect the relevant pK_a values during the docking of a target protein onto Trx.[24] Szajewski and Whitesides examined Brønsted coefficients for the dithiol−disulfide reaction between dithiothreitol and GSSG, and have developed an equation that relates the rate constant for steps of the interchange reaction to the pK_a values of the nucleophilic, central, and leaving group thiols.[53]

$$\log k^{obs} = (6.3 + 0.59pK_a^{nuc} - 0.40pK_a^{c} - 0.59pK_a^{lg}) - \log(1 + 10^{pK_a,\,nuc\,-\,pH}) \quad (3)$$

where pK_a^{nuc}, pK_a^{c}, and pK_a^{lg} are the pK_a values of the nucleophilic, central, and leaving group sulfurs, respectively; 6.3 is the log of the pH-independent rate constant. The Brønsted coefficients are explained in the Discussion of reference.[53]

As discussed more fully in section 5, conformational changes occur in Trx through strong molecular interactions during substrate recognition and binding, and these can generate a local

hydrophobic milieu and a juxtaposition of titratable groups that affect the pK_a values of the Trx thiols and their net reactivity.[59,60] Some attempts were made to use the kinetics of the reaction of Trx with GSSG (or IAM) to estimate the pK_a values,[45,46] but these processes are somewhat different than reactions of Trx with proteins. For instance, thiol-containing proteins react with $TrxS_2$ orders of magnitude faster than with small molecule thiols, presumably because conformational changes and reorientation of reactive groups occur during protein−protein binding that act to accelerate the reactions.[5,39,40,45,53,61−63]

Therefore, to account, in part, for the contribution of dynamic structural factors, a correction factor (F_c) has been introduced into the empirical eq 3 to yield eq 4 for calculating rate constants of steps in the reaction of $Trx(SH)_2$ with proteins.[31,32]

$$log\ k^{obs} = (6.3 + 0.59pK_a^{nuc} - 0.40pK_a^{c} - 0.59pK_a^{lg}) - log(1 + 10^{pK_a,\,nuc\,-\,pH}) + F_c \quad (4)$$

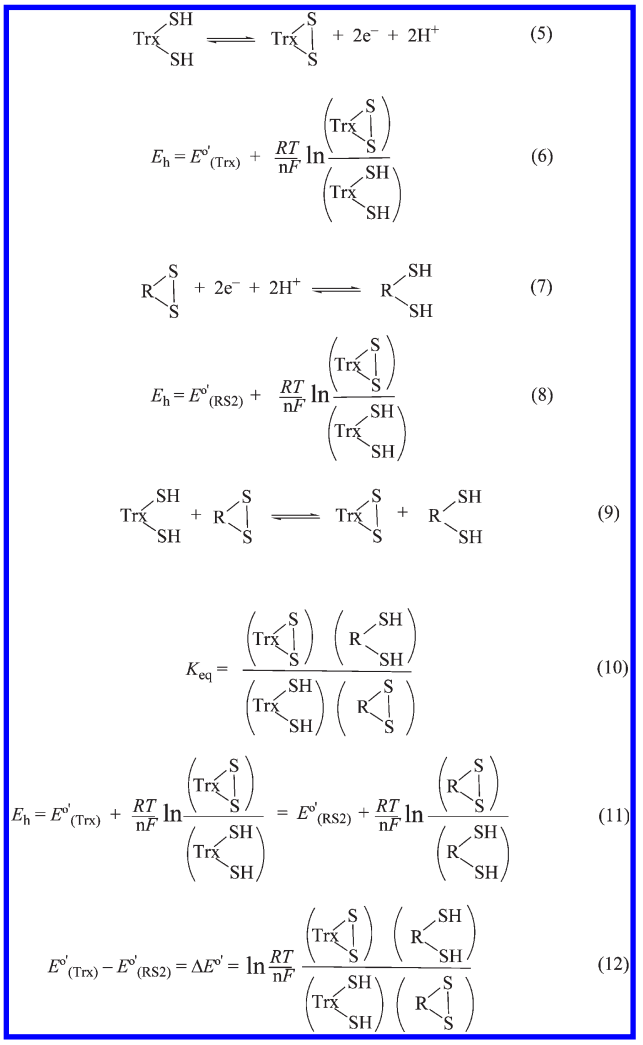
These equations show that in addition to the pK_a values of Cys32-SH and Cys35-SH in Trx, the thiol pK_a of the target (GSSG or client protein) also contributes to the dependence of the observed rate constant and the overall equilibrium constant of the reaction on the pH of the buffer. For the second nucleophilic attack that occurs on the mixed disulfide by the Cys35 thiolate, the pK_a value of GS-S-Cys32-Trx-Cys35-SH or RS-S-Cys32-Trx-Cys35-SH, instead of Trx-Cys35-SH, should be used to calculate the rate constant k_{obs} (k_3, the rate constant for the third step of the interchange reaction). However, the pK_a of GSS-Trx-SH or the relevant pK_a values of Cys35 thiols that contribute to Trx reactivity have not been measured because the rate of mixed disulfide intermediate formation is slow relative to its rate of resolution.[64] It is not sufficient to use static pK_a values for evaluation of reactivity because (1) in addition to not being reproducible by the different methods of measurement, the static pK_a also fails to reflect the dynamic effects in the reaction, (2) reliable pK_a values of protein-Trx-SH mixed disulfides are unavailable, and (3) there is no consensus for the pK_a values of Trx-Cys35-SH. Recent studies with computational methods, such as molecular dynamics (MD) simulations, have revealed that structural factors, particularly conformational changes during the reaction, could critically regulate the relevant pK_a values of active thiols and dissociation of the mixed disulfide intermediate complex between Trx family members and target proteins.[24,37,65]

3. REDOX POTENTIALS OF THE ACTIVE THIOLS IN THE CXXC MOTIF

Oxidized and reduced Trx form a conjugate redox pair. The redox potential is related to the ratio of the oxidized to the reduced component at equilibrium and can be calculated with the Nernst equation (see eqs 5 and 6).[1,66] This also applies to target disulfide reagents (RS_2) that potentially react with $Trx(SH)_2$ (eqs 7 and 8). Equations 5 and 7 are the oxidative and reductive half reactions, respectively, of the thiol-disulfide reaction between $Trx(SH)_2$ and RS_2 (eq 9). $E^{o\prime}_{(Trx)}$ and $E^{o\prime}_{(RS2)}$ represent the standard state redox potentials at pH 7 (E_m or $E_{1/2}$) of Trx and the disulfide reagent, respectively; n refers to the number of electrons transferred and F is the Faraday constant. Under the standard state conditions common in biochemistry, the redox potential is standardized as a reduction potential at pH 7, 25 °C ($E^{o\prime}$ or E_{m7}). All other chemical participants are standardized at 1 M.

Equation 10 defines the equilibrium constant, K_{eq}, of the reaction between $Trx(SH)_2$ and RS_2. E_h refers to the redox potential of either reacting pair of thiol-disulfide reactions at the experimental concentrations of oxidized and reduced forms. When the system is at equilibrium, E_h is identical for both conjugated redox pairs (eq 11).

Therefore, if the $E^{o\prime}$ of the $RS_2/R(SH)_2$ couple is known, the redox potential of Trx can be calculated using eq 12 (ref 66 and references therein). The more negative the redox potential of the active thiols in the CXXC motif, the more reducing is the Trx. Using these relationships, the redox potentials of a number of thioredoxins have been determined.[34,67−71]



On the basis of the E_m values available in the literature for the Trx superfamily, the thioredoxin family is the most reducing group (with E_m values −260 to −285 mV at pH 7, 25 °C for most thioredoxins).[66−70] Glutaredoxins (Grx) are somewhat less reducing (−198 mV to −233 mV),[67] protein disulfide isomerases (PDI) have intermediate redox values (−147 to −175 mV),[71,72] and DsbA(s) are the most oxidizing (−90 mV to −110 mV).[33,73] The −XX− dipeptides that intervene between the two cysteines have been shown to influence the redox potential, with G−P, P−T, P−H, and G−H found in Trx, Grx, DsbA, and PDI, respectively. Thus, upon replacing CGPC of *Ec*Trx with CGHC (found in PDI), the redox potential increased modestly toward that of PDI (ΔE_m = 35 mV),[74] and the inverse mutation (G−H→G−P) in

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Table 2. Activity of *Ec*Trx, *Dm*Trx, *Pf*Trx, *Tb*Trx, and *Ec*Trx Variants in Reacting with Thiol Compounds

Trx and mutants	E_m (mV)	k (DTT^{red})[a] (M^{-1} s^{-1})	k (DTT^{ox})[a] (M^{-1} s^{-1})	k (lipoic acid)[a] (M^{-1} s^{-1})	k (sulfate)[b] (mm)	k (methionine sulfoxide)[b] (mm)	k(GSSG)[c] (M^{-1} s^{-1})
wild-type *Dm*Trx (CGPC)	−287[d]						170
wild-type *Pf*Trx (CGPC)	−284[d]						650
wild-type *Tb*Trx (CGPC)	−282[d]						23
wild-type *Ec*Trx (CGPC)	−296[d]						100
wild-type *Ec*Trx (CGPC)	−270[e]	1.1	8.8	313	2.3	1.8	
PDI-like *Ec*Trx (CGHC)	−221[e]	24	5.8	55.8	1.3	0.83	
DsbA-like *Ec*Trx (CPHC)	−204[e]	46	2.4	11.6	0.4	0.55	
Grx-like *Ec*Trx (CPYC)	−195[e]	47	3.3	9.7	0.12	0.17	

[a] The preliminary data were from ref 78. [b] The reduction of sulfate and methionine sulfoxide was measured with *trxA, grxA* strain FÅ47 and *trxA, metE* strain A313, respectively. The *trxA, grxA* strain FÅ47 was transformed with the pBAD33 derivatives containing the genes of the corresponding thioredoxin variants and then grown at 37 °C on agar plates with M63 minimal medium (containing 10 g/L ammonium sulfate as sole source of sulfur). Similarly, the *trxA, metE* strain A313 was transformed with the pBAD33 derivatives containing the genes of the corresponding thioredoxin variants and grown at 37 °C on agar plates with M63 minimal medium supplemented with methionine sulfoxide as sole methionine source. The preliminary data were from ref 78. [c] The preliminary data were from refs 66, 70, 79, and 80. [d] The redox potentials of *Ec*Trx, *Dm*Trx and *Pf*Trx determined at pH 7.0, 25 °C as reported [66] were recalculated for pH 7.4 at which the GSSG reduction rates were measured.[79,80] The redox potential of *Tb*Trx determined at pH 7.0, 25 °C [70] was recalculated for pH 7.5 at which the GSSG reduction rate was measured in the same work. [e] The redox potentials of wild type *Ec*Trx and variants are from ref 78.

PDI led to a variant that was more Trx-like ($\Delta E_m = -66$ mV);[75,76] similarly, the replacement of PH in DsbA by GP resulted in a Trx-like DsbA and a 1200-fold decrease in K_{eq} for the reaction with GSH.[73,77] These interesting studies have revealed a reasonable quantitative correlation between the E_m values and the activities in reacting with small molecule disulfide reagents (Table 2).[78] The plots of E_m versus reactivity of thioredoxin and the Grx-, DsbA-, and PDI-like variants yielded an average linear correlation coefficient of $r \geq 0.90$ (Supporting Information Figure 1, A−E). Studies extended to Trx-protein reactions showed a similar linear pattern for the E_m-reactivity relationship ($r = 0.99$) (Table 3, Supporting Information Figure 2A).[78] These correlations strongly indicate that redox potential plays an important role in the thiol-disulfide interchange reactions.

We note, however, that the modified Trx proteins involving residues between the redox active cysteine residues must be distinguished from the variants involving other residues. Specifically, wild type thioredoxins from different species, as measured by reactions with glutathione disulfide (Table 2 and Supporting Information Figure 1F), as well as the variants D26A, P34S, and P76A, reacting with NADPH catalyzed by TrxR or RNR (Table 3 and Supporting Information Figure 2C and D), show poor correlation between the E_m values and thiol-disulfide interchange activity.[66,79,80] Of the four wild-type thioredoxins (*Ec*Trx, *Dm*Trx, *Pf*Trx, and *Tb*Trx), *Pf*Trx reduced GSSG much more rapidly than did *Ec*Trx (650 vs 100 M^{-1} s^{-1}), although its redox potential appears less reducing than that of *Ec*Trx (−284 vs −296 mV, pH 7.4, 25 °C) (Supporting Information Figure 1F). This result shows that factors in addition to redox potential play a role in determining Trx reactivity. This is not surprising given our discussion of the influence of thiol pK_a values on reactivity (section 2). A recent report indicates that molecular binding interactions that link the two reactant molecules and position the reactive thiols optimally for the reaction increase the effective thiolate concentration and reduce spatial degrees of freedom much like intramolecular reactions.[1] Such effects are common for enzymatic processes, whereby the favorable enthalpy of binding is used to overcome the unfavorable entropic effects inherent in bimolecular reactions, namely, the loss of translational and rotational entropy. Thus, the optimal binding of substrates to Trx provides some of the driving force for catalysis; it lowers the effective transition state energy for the reaction.

Sequence alignment of *Ec*Trx, *Dm*Trx, *Pf*Trx, and *Tb*Trx reveals that the amino acid interposed between the two highly conserved residues (Lys-36 and Ile-38 in *Pf*Trx) is not conserved among the Trx family. In *Pf*Trx, residue 37 is the basic arginine, while the analogous residue in *Ec*Trx and *Dm*Trx is the apolar methionine, and a threonine replaces the arginine residue in *Tb*Trx.[81] The positively charged arginine at physiological pH may increase the electrostatic interactions between *Pf*Trx and the negatively charged GSSG, and this additional binding parameter could facilitate overcoming the entropic barrier of bringing the reacting groups together in a confined volume and in the optimal orientation for reaction.[1,66,79,80] In contrast, in *Tb*Trx the hydroxyl group might diminish electrostatic interactions, which together with its more oxidizing E_m could account for the poorer reactivity of *Tb*Trx in reducing GSSG (Table 2, Supporting Information Figure 1F).

Consistent with the hypothesis that molecular interactions, in addition to the E_m effects, accelerate thiol-disulfide reactions by diminishing the entropic effects of bimolecular reactions, two lines of evidence were obtained from both types of Trx variants, those that involve the residues intervening the active site cysteines and those involving other sites.[78,82] Thioredoxin and variants such as D26A, P34S, and P76A did not show high correlations between their enzymatic efficiency (k_{cat}/K_m) and their E_m values; the correlation coefficients were only 0.71 in thioredoxin reductase activity (TrxR) and 0.85 in ribonucleotide diphosphate reductase (RDR) activity (Table 3, Supporting Information Figure 2C and D). In contrast, a plot of TrxR or RDR efficiency (k_{cat}/K_m) versus $1/K_m$, a parameter that indicates the enzymatic affinity toward Trx and its variants D26A, P34S, and P76A, exhibited a nearly perfect linear relationship ($r = 0.999$ and 0.997, respectively) (Table 3, Supporting Information Figure 2E and F). Thus, we find that molecular interactions in this case can play a dominant role in driving the reaction, although E_m also contributes. A second example is the linear increase of enzymatic activity with respect to $1/K_m$ that can be related to the affinity of

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Table 3. Catalytic Efficiency (k_{cat}/K_m) of *Ec*Trx and *Ec*Trx Variants in Reacting with Thioredoxin Reductase (TrxR) or Ribonucleotide Diphosphate Reductase (RDR)

Trx variants	E_m (*mV*)	K_m (μM)		k_{cat}/K_m ($M^{-1}s^{-1}$)	
		TrxR	RDR	TrxR	RDR
wild-type (CGPC) [a]	−284				
wild-type (CGPC) [b]	−270	2.7		1.1×10^7	
PDI-type (CGHC) [b]	−221	1.4		1.6×10^7	
DsbA-type (CPHC) [b]	−204	1.2		1.9×10^7	
Grx-type (CPYC) [b]	−195	1.2		1.9×10^7	
wild-type (CGPC) [c]	−260 (−282)	1.8	2.0	1.4×10^7	1.5×10^6
Trx D26A (CGPC) [c]	−258 (−280)	10.8	33.3	2.4×10^6	8.5×10^4
Trx P34S (CGPC) [c]	−269 (−291)	1.8	1.2	1.4×10^7	2.3×10^6
Trx P76A (CGPC) [c]	−245 (−267)	8.5	18.5	3.7×10^6	2.3×10^4

[a] As noted in the text, we prefer the value of −284 mV (at pH 7.0, 25 °C, I = 0.28) determined by an improved methodology as reported[88] with precise corrections with regard to pH, ionic strength, temperature, and reference potential, per ref 89. [b] All the parameters were determined at pH 8.0 and 25 °C for variants involving residues between the redox active cysteine residues (ref 78). These values were not corrected because a different buffer system was used and the correction factors were unavailable. [c] The redox potentials for wild type Trx and the three Trx variants involving residues other than those between the redox active cysteine residues, were determined at pH 7.0 and 20 °C, based on a reference potential of −305 mV for the $NADP^+$/NADPH couple.[82] The corrections to 25 °C and to the correct reference potential for the $NADP^+$/NADPH couple, per ref 89, are given in parentheses. Briefly, the E_m of the redox reference $NADP^+$/NADPH was corrected to −327 mV according to ref 89; pH correction: −29.5 mV/pH; temperature correction: −1.39 mV/C.

Trx and its variants DsbA-, PDI- and Grx-like (r = 0.99) toward TrxR (Supporting Information Figure 2B). This correlation coefficient (0.99) is the same as that of activity versus E_m (0.99) (Supporting Information Figure 2A), suggesting that both E_m and molecular interactions affect Trx reactivity. Therefore, we conclude that large differences in E_m are important for the variation in reactivity (e.g., in DsbA-, PDI-, and Grx-like variants), but additional factors, such as specific binding, also participate in regulating Trx activity, especially when the differences in E_m are small (e.g., in the variants D26A, P34S, and P76A).

Several methods for measurement of E_m are based on redox titrations and the equilibrium theory shown in eqs 5−12. In addition, varying ratios of GSSG/GSH or DTT^{ox}/DTT^{red} have been used at relatively high concentrations as redox buffers that equilibrate with Trx according to the relevant K_{eq}. In these cases, after Trx has equilibrated with the redox buffers, iodoacetic acid (IAA) was used to rapidly alkylate the remaining thiols, and HPLC or native gel electrophoresis was used to quantify the proportions of oxidized and alkylated (reduced) Trx products. The equilibrium constant K_{eq} could thereby be determined using different ratios of the redox buffers, and from the K_{eq} values obtained, the E_m of various Trx family members could be calculated.[67,68,71] However, caution must be exercised in the use of these methods as has been pointed out by these authors and others.[68,75,83−85] First, IAA may shift the thiol-disulfide equilibrium of proteins;[83] therefore, it is ideal to perform the experiments with different concentrations of IAA to ensure that the concentration of IAA does not affect the results.[68] Second, incomplete or slow quenching of thiol-disulfide exchange reactions may reduce the confidence of K_{eq} and E_m values measured by this method.[75,84,85] Third, because of the relationship between the equilibrium constant and the square of the GSH concentration (eq 2), the error present in the quantification of GSH can be substantial and can be more pronounced in the presence of millimolar or lower concentrations of GSH/GSSG. In 1997, Aslund et al. developed a direct protein−protein method that allows quantification of all species present in a thiol-disulfide interchange reaction using a single HPLC analysis.[67] This method is straightforward and operates at micromolar concentrations of oxidized and reduced proteins.[34,67,69,70]

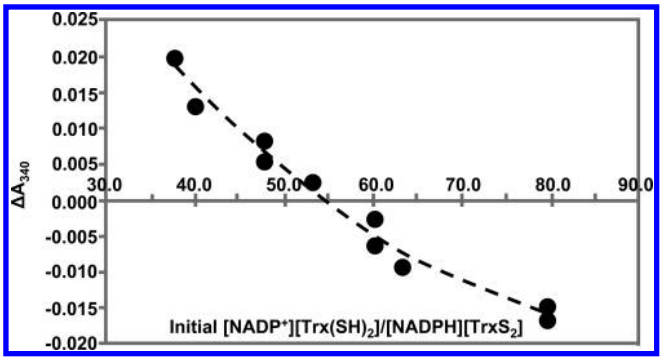

Figure 3. Plot of the change in A_{340} as the reaction comes to equilibrium vs the initial ratios of reactants. The crossover point where the change in A_{340} is zero gives the K_{eq} value. Shown are data for determining K_{eq} for *Dm*Trx2 (pH 7.0, 0.1 M potassium phosphate buffer containing 0.3 mM EDTA). The initial ratio of $[DmTrx(SH)_2]/[DmTrx(S)_2]$ was kept constant at 5. The initial concentrations of NADPH and $NADP^+$ were varied systematically to give the ratios of $NADP^+$/[NADPH] from 8 to 16, but the sum of the concentrations of $NADP^+$ and NADPH was maintained at 220 μM. The initial ratios of $[NADP^+]$ $[DmTrx(SH)_2]/[NADPH]$ $[DmTrx(S)_2]$ ranged from 40 to 80 accordingly. Further details concerning the calculation and related examples can be found in ref 66. Reprinted with permission from ref 66. Copyright 2007 American Chemical Society.

Another noteworthy method is the spectrophotometric assay of the kinetic equilibrium of the Trx/NADPH/TrxR system, which is convenient and has been widely used since 1964.[13,66,82,86−88] In this method, nonequilibrium concentrations of NADPH, $NADP^+$, $Trx(SH)_2$, and $TrxS_2$ are mixed together with TrxR, and the system is allowed to come to equilibrium. When the starting concentrations of NADPH and $TrxS_2$ are in excess of equilibrium values, the reaction will proceed toward reduction of $TrxS_2$; conversely, when starting concentrations of $NADP^+$ and $Trx(SH)_2$ are in excess of equilibrium, the reaction will proceed toward oxidation of $Trx(SH)_2$.[86] The reaction can be monitored easily and quantitatively by the changes in absorbance due to NADPH. However, if the initial ratios of reactants are far from equilibrium, it has been observed that somewhat unreliable results can be obtained, thereby reducing the confidence in E_m values that are calculated from the measured K_{eq} values.[66,86,88] To counter this problem, a systematic variation of the initial ratios of $[NADP^+][Trx(SH)_2]/[NADPH][Trx(S)_2]$ can be conducted as shown in Figure 3.[66,88] The plot of initial ratios of concentrations versus the change in absorbance that occurs to reach equilibrium (A_{340}) yields a crossing point where no change in absorbance occurs. This point is the most trustworthy equilibration point for calculating $E^{o\prime}$. The most reliable results are obtained when the initial ratio and concentration of one of the pairs (e.g., $[NADPH]/[NADP^+]$) is kept constant and the ratio of the

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other pair is varied, but the sum of concentrations of the latter pair remains constant. Using these criteria, a smooth curve can be obtained as shown in Figure 3. It should be noted that the E_m of the redox reference ($NADP^+$/NADPH) is sensitive to temperature and ionic strength; thus, the calculated E_m of Trx should be corrected according to the reaction conditions (Table 3, footnotes a and c).[66,89]

Direct protein electrochemistry has recently been shown to be a versatile tool to determine the electron transfer pathway in Trx catalysis and to measure the E_m of Trx.[90−96] To use this technique, Trx or its variants, are immobilized on a modified gold electrode and uniquely oriented relative to the electrode surface via a histidine tag; this enables the redox mechanism of the protein to be examined electrochemically. Upon scanning the applied voltage in the reducing direction followed by reoxidation in the reverse direction, two one-electron reduction waves were identified for *Ec*Trx. Such studies yielded a range of E_m values between −261.5 and −265 mV for *Ec*Trx, and −210 mV for both *m* and *f* spinach thioredoxins.[91−93] These E_m values measured by direct protein electrochemistry are comparable to those calculated from K_{eq} measurements as described above. More importantly, protein-film voltammetry revealed a fast electrochemical connection between a submonolayer of the protein being analyzed and the electrode, which has allowed the direct observation of the reversible $2e^-/2H^+$ redox couple of thioredoxins.[94]

Taken together, the redox potentials of the active site thiols play an important role in Trx reactivity. The E_m values show a linear correlation with the activities for Trx and Trx variants involving residues between the redox active cysteine residues (DsbA-, PDI- and Grx-like variants) in the Trx superfamily, but the relationship appears more complicated for Trx and Trx variants involving residues other than those between the redox active cysteine residues (D26A, P34S, and P76A). The observation of a reversible reaction with $2e^-/2H^+$ stoichiometry suggests that, in addition to electron transfer, proton transfer processes are important in Trx-catalyzed thiol-disulfide reactions.[94] In the following section, acid/base catalysts that can promote proton transfer and accelerate the reactions of the Trx superfamily will be discussed.

4. ACID/BASE CATALYSTS

Deprotonation of the redox active thiols in the CXXC motif is critical for the generation of the nucleophilic thiolate form that will attack the target disulfide in thiol-disulfide interchange reactions. Factors that affect the deprotonation of reactive thiols, and thus determine the pK_a values, influence the acid/base catalysts per se. There are two affecters of the deprotonation of the exposed thiol in forming the mixed disulfide, substrate binding, and the positive charge of helix α_2 as discussed in section 2 and below.[55,56] A number of proposed factors affecting the deprotonation of the buried thiol prior to mixed disulfide complex dissociation have been the subject of study and debate for years.[24] These include (1) a potential hydrogen bond between the peptidic N of the partially exposed cysteine and the Sγ of the buried cysteine,[50,51,24] (2) another hydrogen bond between the buried Cys-SH and the backbone NH of the proximal Trp,[24] (3) proton sharing between the two sulfur atoms of the active site cysteines,[101] and (4) a hydrogen bond between the Sγ of the buried Cys, and the conserved, proximal Asp26 (*Ec*Trx numbering) as shown in Figure 2G; this long hydrogen bond would need to be bridged by a water molecule.[13,20,46,47,97−100] The positively charged side chain of the proximal Lys36 may not participate in the deprotonation of active thiols because structural study revealed this side chain was disordered and far from the active thiols in reduced Trx.[49] In addition, the hypothesis of a hydrogen bond between the Cys35-NH and Cys32-S^- was questioned due to its incompatibility with the structures: the van der Waals radius of sulfur was such that the Cys32 thiol (in *Ec*Trx) is moved out into the solvent from the pocket, where it resides as part of the disulfide bond in Trx-S_2.[49] Moreover, if the Cys35 amide proton is hydrogen bonded to a negatively charged group, then hydrogen exchange should be significantly slowed. Contrary to the expectation, hydrogen exchange for the Cys35 amide proton is over 10^4 times faster in Trx-$(SH)_2$ than in Trx-S_2.[49]

The proposal of proton sharing between Cys32-S^- and Cys35-SH (in *Ec*Trx) was based on the NMR observations showing that titrations of the two thiol groups were intimately linked, and two pK_a values are ∼1 pH unit above or below 8.4 (the pK_a of free cysteine thiol), respectively.[101] A close interaction between the two cysteine thiols, possibly proton sharing, could account for the opposite shift of the pK_a of the Cys32 thiol and Cys 35 thiol, with the thiolate anion of Cys 32 stabilized through the sharing of the remaining thiol proton, nominally attached to Cys35.[101] Furthermore, distances shorter than those corresponding to the sum of the van der Waals radii of the two cysteine S atoms have also been observed by X-ray crystallography for *E. coli* and human reduced thioredoxins.[101] To test the proton-sharing hypothesis, computational methods have been used but provided contrary evidence.[37] Upon performing a potential energy surface scan of the transfer of the proton between Cys32 and Cys35 in thioredoxin, the potential energy of the system was plotted versus the distance between the thiol proton and the Sγ of the nucleophilic cysteine.[37] If the proton was shared by the two active site cysteines, a curve with a minimal potential energy should appear in the position where the proton was equally shared (with a distance of 1.41 Å); however, the curve reached a maximum around 1.41 Å. Thus, this study argues against the notion of proton sharing.

A possible physical basis for deprotonation of active thiols with an anomalously low pK_a (e.g., Cys32 in *Ec*Trx) is the positive dipole of the α-helix that can stabilize the negative thiolate of Cys32.[55−57] The Cys32 thiol is positioned directly on the axis of the long helix.[55−57] Being in the positive electrostatic field of the N-terminus of an α-helix, Cys32 possibly loses a proton during binding to the substrate protein. The somewhat more apolar environment that is created around Cys32 when substrate binds may intensify the electrostatic effects of the α-helix. Although the Cys35 thiol is also situated close to the N-terminus of the α-helix, the local environment around the Cys35-SH is sufficiently different from that of Cys32, as indicated by the distance from the N-terminus of the α-helix and alignment with the helix axis, as well as its more apolar environment and proximity to the side chain of the buried Asp26.[55−57] This could account at least in part for the different pK_a values and reactivities of the N- and C-terminal reactive cysteines.

Deprotonation of the buried cysteine thiol is critical for the second nucleophilic attack to complete the thiol-disulfide interchange reaction. However, the factors that mediate the deprotonation are challenging to identify experimentally because the mixed disulfide intermediate forms only transiently.[24,102] It was hypothesized that a solvent-mediated proton transfer might take place between Asp26 and Cys35 (in *Ec*Trx) even at a distance of

5.6 Å.[13,103] To pursue evidence in this respect, theoretical studies have been conducted on the reduction of dimethyl disulfide by CGPC, which contains the active-site of Trx; the study made use of both DFT (density functional theory) calculation and MD simulation. The computations suggested that deprotonation of Cys35-SH occurred directly after the first S_N2@S step through proton abstraction by the leaving group, which was released in the vicinity of or in steric contact with the thiol group of the buried Cys35.[102] However, a caveat is needed; in this case it was not prudent to apply studies involving small molecules to the mechanism of the deprotonation of a buried thiol. Combining theoretical calculations and biochemical experiments, a recent study addressed the mechanisms responsible for the second nucleophilic attack to resolve the mixed disulfide intermediate.[24] The formation and resolution of a mixed disulfide in the reaction between *Sa*Trx (with a Cys29-Gly-Pro-Cys32 motif) and the target protein, arsenate reductase (ArsC, with Cys82 and Cys89 in the redox active center), were investigated using DFT calculations and MD simulations of the distance between Asp23$O_{\delta 1}$ or Asp23$O_{\delta 2}$, (analogue of Asp26 of *Ec*Trx) and Cys32Sγ (an analogue of Cys35γ in *Ec*Trx). The distance increased to 10 Å (out of the hydrogen bonding distance of 6 Å with a single water molecule) when the leaving thiol Cys82-ArsC was ionized. Instead of solvent-mediated proton transfer, the authors suggested that two hydrogen bonds, Cys32TrxSγ—Cys29TrxN and Cys32TrxSγ—Trp28TrxN, were introduced during the local conformational changes; these could activate Cys32Sγ without intervention of Asp23.[24] Moreover, mutation of Asp23 in *Sa*Trx (D23A) or Cys82 in ArsC (C82A) did not prevent the mixed disulfide from dissociation in the biochemical complex formation experiments, suggesting Asp23 and Cys82 might not be required for the mixed disulfide formation and resolution.[24] These findings strongly suggest that experimental validation is required for theoretical simulation and prediction, although it is a challenging task. Moreover, differences in the mechanism may exist between Trx isolated from several species.

Before the proximal aspartate (Asp26 in *Ec*Trx and Asp23 in *Sa*Trx) is dismissed as a potential acid/base catalyst, however, a few critical issues must be addressed. First, it has been established that factors lowering thiol pK_a values can also decrease the nucleophilicity of the nascent thiolate and make it less reactive.[1,20,31,35,36] The MD simulation suggested additional hydrogen bonds that were particularly strong based on geometric criteria.[24] How such strong hydrogen bonds might lower the pK_a of the Cys32-SH in *Sa*Trx but not deactivate the buried thiol(ate) must be addressed to make this hypothesis attractive. Second, the observation, in which mutation of Asp23 to Ala did not prevent the mixed disulfide formation and dissociation, does not necessarily exclude Asp23 as a potential acid/base catalyst in the reaction. Asp23 could be one of the acid/base catalysts and function in parallel with others such as the proximal tryptophan (in a catalytic tetrad, to be discussed below), proline (even closer to the reactive thiols than the aspartate, see below), small molecule bases in the buffer, and so-far-unidentified structural factors (particularly those formed during substrate binding and conformational changes). These factors, which may overlap in their function as acid/base catalysts, might adequately compensate for the effect of the mutation of Asp23 on Trx activity. This is particularly true when 5,5′-dithiobis-(2-nitrobenzoic acid) (DTNB) was used as a disulfide to react with *Sa*ArsC C10S/C15A/C82A triple mutant (*Sa*ArsC^{Trip}); the resulting mixed disulfide was reacted with reduced *Sa*Trx or *Sa*Trx D23A in the presence of small molecule bases in the buffer. The absence of a mixed disulfide, detectable on a nonreducing SDS-PAGE, between Sa_ArsC^{Trip} and either *Sa*Trx or *Sa*Trx D23A suggests that such a mixed disulfide formed and was resolved as Cys32 attacked Cys29 to form oxidized *Sa*Trx or SaTrx D23A and *Sa*ArsC^{Trip}.[24]

Establishing chemical mechanisms is always difficult, requiring very strict validation of working hypotheses by experimental data.[24] Nevertheless, considerable experimental data have emerged to support the potential role of Asp26 as an acid/base catalyst in *Ec*Trx.[13,20,47,97−100] This finding is not surprising given that Asp26 in *Ec*Trx is located close to the CXXC motif and has been shown to affect the microscopic pK_a values of the active thiols.[97]

The D26A variant was found to have only 10% of the activity of wild-type Trx-$(SH)_2$ toward reducing insulin disulfides. In the reaction of Trx-S_2 with thioredoxin reductase at pH 8.0, the k_{cat}/K_m for the D26A variant was only ∼10% that of wild type (7.5×10^5 M^{-1} s^{-1} compared to 8×10^6 M^{-1} s^{-1}).[20] In oxidizing DTT, wild-type Trx exhibited substantially greater reactivity than did D26N-Trx at low pH values, but the activities converged to nearly the same value at ∼ pH 9.0.[98] If the results are normalized to the thiolate concentrations of DTT that are present, the rates of the reactions follow a normal pH titration curve with a pK_a value of 7.23, close to that of Asp26.[47,97,98,100] Thus, when the buffer had a pH that was greater than the pK_a value, the effects of Asp26 were greatly diminished.[99]

To further understand the role of Asp26 in acid/base catalysis, the effects of exogenous bases on the activities of Trx variants at Asp26 were studied.[13] In the reduction of DTNB, the inclusion of exogenous bases substantially increased the activities of D26N-Trx and D26L-Trx.[13] Interestingly, the rates of D26A-Trx and D26N-Trx in the first step of DTNB reduction, that is, the formation of the mixed disulfide, were indistinguishable from those of wild-type Trx. However, the rate of the second phase, which corresponds to the cleavage of the Trx/TNB mixed disulfide, differs significantly.[13,98] This difference suggests that Asp26 is more likely to react with Cys35 rather than Cys32, consistent with Asp26 being closer to Cys35 than to Cys32 (Figure 2G). The addition of imidazole, acetate, or Tris-base facilitated, in a concentration-dependent manner, the cleavage of the TrxSH/S-TNB mixed disulfide in the Asp26 variants, but had no effect with wild-type Trx. This constitutes strong evidence that Asp26 serves as a base in the reaction of reduced wild type Trx with DTNB, admittedly, an atypical substrate.[13] In contrast to these results with DTNB, the buffers had less effect on the Asp26 variants in the cleavage of Trx/TrxR mixed disulfide (even with 0.10 M Tris in the buffer), presumably because the interface of the Trx-TrxR complex limits access to small molecule bases.

A catalytic tetrad was proposed to form among five highly conserved amino acids, in *Chlamydomonas reinhardtii* thioredoxin *h* (*Cr*Trx*h*); Asp30 (analogous to Asp26 in *Ec*Trx), Trp35, the redox-active disulfide, Cys36-Cys39, and Asp65 (Figure 4).[44] In the catalytic tetrad, Trp35 is proposed to be involved in a hydrogen bond between its N1−H and the carboxylate of Asp65. This hypothesis seems to extend the H-bonding (or sharing) proposal,[49,101] regardless of the controversy.[37] This suggests the conserved tryptophan near the CXXC motif is indirectly involved in the deprotonation of Cys36, and that Asp30 acts as the acid catalyst.[44] Experimentally, the W35A mutation in *Cr*Trx*h* diminished its ability to activate the thiol-regulated NADPH-dependent malate dehydrogenase, although the reduction potential was unchanged. The lesser activity in W35A was attributed to the changes

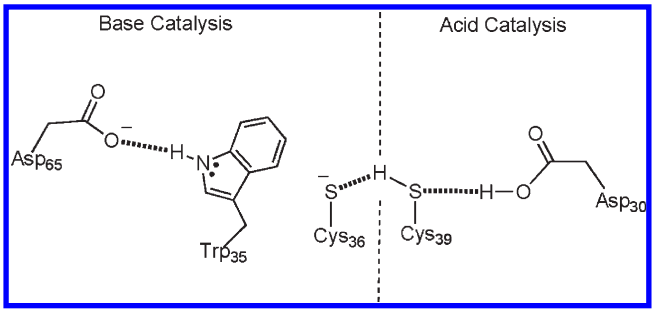

Figure 4. Schematic view of a proposed catalytic tetrad composed of Asp65, Trp35, the redox-active disulfide (Cys36-Cys39), and the protonated buried Asp30 in *Cr*Trx*h*. The catalytic tetrad provides both *base* catalysis toward Cys36-SH, provided by an aromatic side chain of Trp35, and *acid* catalysis toward Cys39-SH, provided by Asp30. Adapted with permission from ref 44. Copyright 1998 Wiley-Blackwell.

in pH sensitivity of the redox-active thiols. Wild-type *Cr*Trx*h* showed two apparent pK_a values, ~7.0 and 9.5, but the W35A variant had a single pK_a of ~ 8.3. Conceivably, the W35A mutation changed the ionization behavior of the redox active thiols, although it cannot be ruled out that the function of Trp35 in wild-type *Cr*Trxh was primarily structural, to facilitate the optimal orientation of appropriate residues. Consistent with the contribution of tryptophan to acid−base catalysis, a mutation that substituted Ala for Trp31 (W31A) in *Ec*Trx (Trp31 is an analogue to Trp35 in *Cr*Trx*h*) diminished the activity in reducing insulin.[104] A structural role may nevertheless be important because Trx-W31F and Trx-W31H exhibited substantial activity, and Trx-W31Y had an activity similar to that of wild-type enzyme; these three mutations are far less drastic than Trp to Ala.[104] A nearby lysine residue contributes little to the catalysis, as shown by a single mutation of this residue (Lys57Met in *Ec*Trx) that had no effect on the reactivity.[20] It may be noteworthy, that the conserved tryptophan in *Sa*Trx (Trp28) was recently found to be important for the thermodynamic stability of this protein.[26] The W28A mutation results in a swapped dimer that is so kinetically stable as to suggest that this mutation might occur and favor other mutations until an evolutionary advantage is obtained.[26]

Proline is traditionally thought to serve a structural role in proteins. For example, using the potential cis and trans isomers of the proline peptide bond, Pro76 in *Ec*Trx is thought to control the known partition between two folded forms that have different stabilities.[21,105] However, Pro76 is even closer to the CXXC motif and is more highly conserved than is Asp26 in the Trx superfamily (Figure 2, C and F). It is therefore tempting to speculate that Pro76 facilitates the thiol-disulfide exchange reaction via interactions with the thiols. Consistent with this notion, it was found that the P76A variant of *Ec*Trx had lower activity,[21] and mutation of the analogous residue in DsbA (Pro151) resulted in a significantly slower thiol-disulfide exchange reaction.[106] Thus, P151T or P151S mutations in DsbA resulted in detectable accumulation of intermolecular mixed disulfide intermediates, which are generally difficult to detect because they resolve very quickly.[106] Although it remains unclear how the proline residue facilitates the thiol-disulfide exchange reaction, the proximity of the proline N-atom to the CXXC motif (3.62 Å) suggests hydrogen bonding to the thiol of the substrate could promote the shuttling of the proton from one sulfur atom to the other without involving solvent (Figure 5). Note that the conserved proline (Pro76 in *Ec*Trx or Pro151 in DsbA) is located

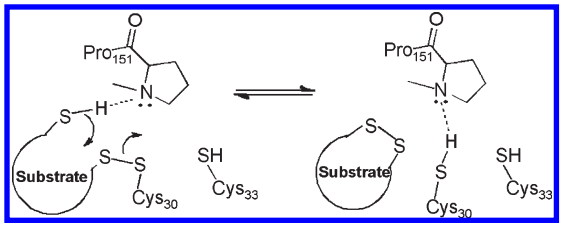

Figure 5. Schematic hypothesis of Pro151 in DsbA in aiding the shuttling of a proton in the cleavage of DsbA-substrate mixed disulfide.

at the surface where the family members bind their target proteins and form complexes stabilized within the nascent hydrophobic milieu.[107] Without this proton-transfer mechanism, the energy barrier for this reaction could be quite high because it is unlikely that a proton-donating solvent molecule would be trapped inside such a hydrophobic interface to facilitate the proton transfer. We suggest that the hypothesis of Pro76 or Pro151 being involved in a proton shuttle deserves further investigation.

5. MOLECULAR INTERACTIONS AND CONFORMATIONAL FACTORS

Trx binds to a variety of protein substrates and modulates their structures and activities.[108] The intermolecular interactions between *Ec*Trx and its substrate proteins leads to high affinity and substrate specificity, resulting in ~10^4-fold enhanced rates for both the oxidation[109,110] and reduction[111] reactions at neutral pH, as compared to similar reactions with small molecule thiols and unstrained disulfides. As already discussed in section 3, such effects are common for enzymatic processes, whereby the favorable enthalpy of binding is used to overcome the unfavorable entropic effects inherent in bimolecular reactions.

Because of the crucial role of molecular interactions in accelerating the reaction, Trx derivatives that lack redox active thiol groups, such as *S*,*S*′-dicarboxymethyl-dicarboxamidomethyl-thioredoxin or Cys → Ser variants, were capable of activating target proteins using only suboptimal amounts of native thioredoxin that acted as a catalyst.[110,112−114] When the affinity of *Ec*Trx toward its targets was changed, for example, by replacing the neutral Gly31 with charged residues (Lys or Asp), the reactivities with both insulin and TrxR were lessened, although those variants showed redox potentials that were almost identical to that of wild-type Trx.[69,87]

Chloroplasts contain two well-studied thioredoxins, Trx-*f* and Trx-*m*. Trx-*f* is a potent activator of fructose-1,6-bisphosphatase (CFBPase),[115−118] which is important in regulating phosphofructokinase and the balance between glycolysis and gluconeogenesis. The redox-active cysteines of CFBPase that are responsible for its activation are clustered in a solvent-exposed structure formed by the highly negatively charged 170s loop.[119−121] In the chloroplast ferredoxin-Trx system that consists of ferredoxin, ferredoxin−thioredoxin reductase (FTR), and Trx-*f*, the function of Trx-*f* was found to depend both on molecular interactions and on reductive activation. This redox cascade proceeds most efficiently if the target and other associated proteins form complexes.[113,114] Despite their common tertiary structures, the distribution of surface charges differs among thioredoxins (Figure 6),[14,16,122,123] and these surface charges can contribute to electrostatic interactions that enhance the affinity of Trx to its targets. For example, the positively charged residues of chloroplast thioredoxin, Trx-*f*, favor its binding to CFBPase. Indeed, variants with negatively charged residues

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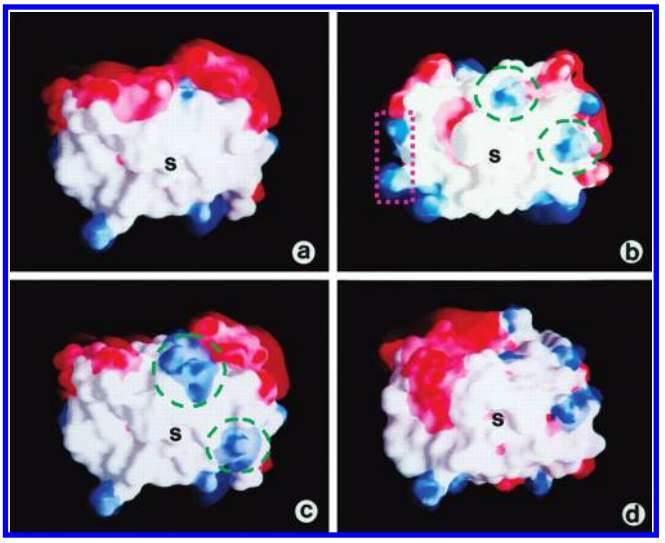

Figure 6. Electrostatic properties of thioredoxins. Charge distribution on molecular surfaces of *E. coli* wild type Trx (*a*), spinach chloroplast Trx-f (*b*), E30K/L94K *E. coli* Trx (*c*), and spinach chloroplast Trx-*m* (*d*), as calculated with GRASP. Electrostatic potentials at the protein surface and superimposed isopotential shells (calculated at ±2 *k*T) are colored as follows: *red* as negative and *blue* as positive. The active site regions of all proteins are located at the center of each image. An *S* illustrates the position of the accessible sulfur atom of Cys-32. Reprinted with permission from ref 122. Copyright 1998 American Society for Biochemistry and Molecular Biology.

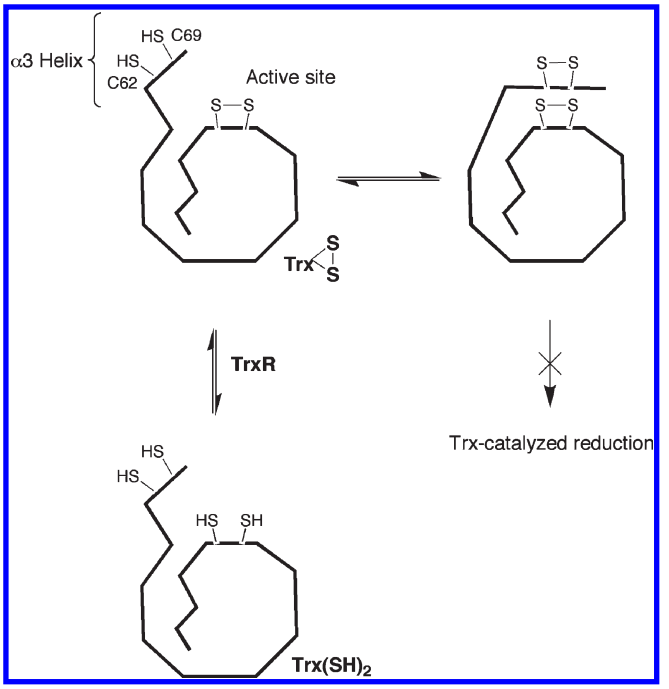

Figure 7. Scheme showing that an intramolecular disulfide formed between the catalytically inactive thiols (i.e., Cys62−Cys69 disulfide of human Trx) impaired Trx activity by disrupting Trx−target protein interactions. Due to this impairment, the half-life of the oxidized Trx becomes prolonged, for which Trx activity can be transiently inhibited under conditions of redox signaling or oxidative stress, and allows more time for the sensing and transmission of oxidative signals. Adapted with permission from ref 135. Copyright 2004 Oxford University Press.

substituting for positively charged or neutral residues (K58E, Q75D, and N74D), and deletion mutants (ΔAsn74 and ΔAsn77) lacked much of the ability of Trx-*f* to activate CFBPase.[118] Moreover, the presence of the four basic residues, Arg37, Lys70, Arg74, and Lys97, in pea Trx-*m* conferred greater efficiency of activation of CFBPase than that of spinach Trx-*m,* which lacks the positively charged residue corresponding to Lys70 of pea Trx-*m*. The K70E variant of pea Trx-*m* exhibited 50% less efficient activation of FBPase than wild type.[124] Conversely, replacement of the negatively charged Asp with the uncharged Asn in *Ec*Trx promoted its activation of spinach CFBPase,[125] while the increased abundance of Lys in *Ec*Trx variants impaired its ability to reduce a positively charged target protein.[126]

Computational analysis indicated that the surface of *Ec*Trx (Figure 6a) and of chloroplast Trx-*m* (Figure 6d) had large negatively charged areas that were absent in Trx-*f* (Figure 6b). In Trx-*f* there is an additional positively charged area (marked by the pink rectangle) (Figure 6b) that was not present in either Trx-*m* (Figure 6d) or *Ec*Trx (Figure 6a).[122] Upon introducing two positively charged residues (E30K/L94K) into *Ec*Trx (Figure 6c), the surface charge distribution of wild type *Ec*Trx became more like that of chloroplast Trx-*f* (marked by the green circles) (Figure 6b).[122] The increased number of positive charges was found to substantially enhance the affinity of the *Ec*Trx variants for CFBPase and increase the maximum specific activity.[93,122] Specifically, wild type *Ec*Trx and its three variants (E30K, L94K, E30K/L94K) have nearly identical redox potentials, but wild type *Ec*Trx is less active than these three variants toward CFBPase.[93,122] Moreover, in spite of Trx-*f* having the most oxidizing redox potential among five thioredoxins, it nevertheless exhibited the highest activity in activating CFBPase via reductive activation.[93,122] This apparent contradiction can be explained at least in part by the affinity differences among *Ec*Trx and its variants, because wild type *Ec*Trx had the lowest affinity whereas Trx-*f* had the highest affinity for CFBPase.[93,122] It is noteworthy that high concentrations of KCl impaired the affinity of otherwise highly efficient modulators like the *Ec*Trx variant E30K/L94K and chloroplast Trx-*f*.[122] Consistent with this, a quantitative relationship has been developed that links protein interactions with the ionic composition of the solution.[127]

Conceivably, long-range electrostatic interactions can contribute to the formation of a noncovalent Trx-CFBPase complex in which the noncharged patch surrounding the nucleophilic Trx-Cys32 would provide proper complementarities between the interfaces of modulator (Trx) and the target enzyme (CFBPase).[113,114,122] Such electrostatic interactions would allow for the recognition and optimal orientation of paired proteins to promote proton transfer, as well as for channeling of relevant substrates.[128] Correct docking permits the reactive thiolate anion of Trx to cleave the disulfide bond of CFBPase with the formation of a transient mixed disulfide between the two proteins.

The contribution of electrostatic interactions may also account in part for the differential reactivities of *Ec*Trx, *Dm*Trx, and *Pf*Trx (see sections 2 and 3) in reducing the negatively charged GSSG, as shown by the substantially higher reactivity of those proteins having positively charged amino acids in proximity of the active thiols (Supporting Information Figure 1F).[14,35,36,66,79,80,129]

Recently, on the basis of dynamic simulation, a hydrophobic binding groove was proposed, which allows Trx to bind the substrate specifically through stabilizing interactions that results in Michaelis−Menten−type kinetics of disulfide reduction.[10,11]

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This binding groove appears to be deepened across the evolution of Trx catalysis, from bacterial to eukaryotic Trxs. Trx catalysis has been studied using single-molecule force spectroscopy where mechanical force is applied to the disulfide bond of a substrate protein. If the bond is stretched with low force, all Trxs exhibit a Michaelis−Menten mechanism while at high force eukaryotic Trxs reduce disulfide bonds through a single-electron transfer reaction (SET), whereas bacterial Trxs show both nucleophilic substitution (S_N2) and SET reactions.[10,11]

Substrate binding that modulates the structures of the two proteins can enable them to achieve an optimal conformation to facilitate dithiol−disulfide interchange.[10,11,24,50,51,61−63,128,130,131] Although the overall molecular architecture of the Trx family is similar in both redox states, subtle conformational changes have been identified, particularly in the space surrounding the redox active cysteine residues during dithiol−disulfide interchange.[24,50,51,132,133] For instance, the catalytic site of *Ec*Trx was shown to participate in the reorientation or elongation of the substrate disulfide bond, by adjusting the local geometry of the participating sulfur atoms with subangstrom (Å) precision to achieve efficient catalysis.[11,131] Moreover, the backbone and side-chain of Cys35 in *Ec*Trx undergoes a rotation around the Cα-Cβ bond upon change of the redox state, while Cys32 remains largely fixed (cf. Figure 2A and B);[14] in contrast, formation of the Cys32−Cys35 disulfide causes a rotation of the side-chain of Cys32 away from Cys35 in *Dm*Trx, as does human Trx and spinach chloroplast Trx-*m*. The movements of Gly33 and Pro34 were smaller in *Dm*Trx than in *Ec*Trx, whereas the movement of the side chain of Phe28 that occurs when *Dm*Trx becomes oxidized was not discernible in *Ec*Trx. The stereo representations in reference[14] are particularly instructive, showing that in the fly enzyme Cys35 is not buried.

These differences in dynamics during changes in the redox states have been attributed to the mechanisms underlying the two distinct modes of catalysis that have evolved in prokaryotic (low molecular weight of 35 kDa) and eukaryotic (high molecular weight of 55 kDa) TrxRs,[13,134] and suggests that the evolutionary optimization of Trx activity has been a complex process. Given the structural and mechanistic differences between prokaryotic and eukaryotic TrxRs across the evolutionary tree, it is reasonable to consider that the evolution of the chemical mechanisms found in Trx has been tightly associated with the evolution of TrxR.[10,11,13,134] Particularly, an interconversion between two conformations occurs twice in each catalytic cycle of the prokaryotic TrxR; after reduction of the disulfide by the flavin, the pyridine nucleotide domain rotates with respect to the flavin domain to expose the nascent dithiol for reaction with thioredoxin, This motion also repositions the pyridine ring adjacent to the flavin ring to enable rereduction of the flavin for another catalytic cycle. In the eukaryotic enzyme, however, the rotation of two domains is not necessary, because a third redox active site (disulfide or selenenylsulfide) shuttles the reducing equivalent from the apolar active site thiols to the protein surface where reduction of Trx occurs.[134]

Under some conditions, dimerization of Trx may occur and cause loss of reactivity due to protein precipitation and aggregation; this may underlie the putative autoregulation of Trx availability and activity in vivo.[5,68,81,129,132] For instance, noncovalent interactions, such as hydrophobic interactions arising from abundant apolar residues on the Trx surface, can substantially contribute to its dimerization.[129,132] Although not yet discovered in nature, a single mutation of the active-site tryptophan to alanine in *Sa*Trx converts the oxidized protein into a biologically inactive dimer that is extremely stable (see above).[26] Thioredoxins that contain additional cysteine residues, such as *Pf*Trx (Cys43), *Tb*Trx (Cys67), and human Trx (Cys62, Cys69, and Cys73), are especially prone to form intermolecular covalent dimers.[68,81,129] In addition to the intermolecular disulfide/dimerization that reduces Trx activity through aggregation, an intramolecular disulfide formed between the catalytically inactive thiols (i.e., Cys62−Cys69 disulfide of human Trx) impairs Trx activity by disrupting Trx−target protein interactions as indicated in the cartoon in Figure 7.[68,135] While it is easy to understand the formation of an intermolecular disulfide between cysteines located on the protein surface (e.g., Cys73 and Cys73′ of two human Trx molecules), the structural factors facilitating the intra- and intermolecular disulfide bonds between buried cysteines (e.g., Cys62 and Cys69 in human Trx) were only delineated recently.[136,137] It was shown that human thioredoxin undergoes a conformational change that unravels helix α_3 and exposes residues Cys62 and Cys69 (both buried beneath and located at either end of helix α_3) in a more open conformation.[136,137] As such, the buried cysteine residue becomes exposed in the open conformation and generates a structural basis for the cysteine to participate in the inter- and intramolecular disulfide formation.[136,137] The impaired activity caused by the extremely stable dimerization of oxidized Trx provides a mechanism by which Trx activity is transiently inhibited under conditions of redox signaling or oxidative stress, and allows more time for the sensing and transmission of oxidative signals.[68]

6. CONCLUSIONS

Trx catalyzes disulfide oxidoreduction in a wide variety of biological processes that are controlled by dithiol−disulfide interchange reactions. Because the reversible dithiol−disulfide reaction has $2e^-/2H^+$ stoichiometry, factors that regulate electron and proton transfer affect the reactivity of Trx family members. Consistent with this notion, the redox potential and pK_a values of the active thiols in the CXXC motif show a strong association with Trx reactivity. Moreover, emerging evidence shows that Trx activity can be regulated by the relative stability of oxidized and reduced forms of Trx, molecular interactions (e.g., electrostatic force), acid/base catalysts, conformational adjustments to orientate the reactive groups to facilitate reaction, and hydrophobic microenvironments formed after substrate binding. However, several outstanding questions must be addressed before we fully understand the mechanisms that underlie Trx reactivity as a protein disulfide oxidoreductase.

First, considerable controversy exists in the reported pK_a values of the active site thiols. Thus, the use of multiple methods and the development of new, more reliable methodologies are desirable for more accurate measurement of the pK_a values for the active thiols in the CXXC motif. In particular, the pK_a value of the buried Cys35-SH (in *Ec*Trx), which is important for resolving the mixed disulfide, should be measured in Trx in which the Cys32 thiol is actually in the form of a mixed disulfide (RS-S-Cys32-Trx-Cys35-SH) rather than the form of a free thiol (HS-Cys32-Trx-Cys35-SH). Second, complex rather than simply linear correlations between redox potentials (or pK_a values) and reactivity of Trx suggests that other regulating factors as discussed above must be taken into consideration for a reliable simulation of Trx activity. Third, considerable evidence has revealed the critical role of acid/base catalysts in the regulation

of Trx activity. Asp26 (in *Ec*Trx) can be one of the acid/base catalysts, but further corroborating evidence is required, as some recent studies have questioned its role in modulating Trx reactivity. The highly conserved residue, Pro76 (in *Ec*Trx) that is uncharged and even closer to the CXXC motif, deserves future attention with respect to its possible role as an aid in shuttling protons during dithiol disulfide catalysis. Fourth, the nature of the nascent hydrophobic milieu, which forms at the surface when thioredoxins bind their target proteins and form complexes, requires further study to understand the residues and the particular interactions involved. Finally, with the realization that its subcellular localization is crucial to defining Trx as a reductant or an oxidant,[138−140] more in vivo evidence is needed to establish the nature of Trx reactivity under physiological conditions.

To address these questions, use of new techniques seems to be a promising strategy. For instance, direct protein electrochemistry as a versatile tool has been to employed to determine the electron transfer pathway in Trx catalysis and produce reliable measurement of the E_m of Trx comparable to classic methods; this has allowed the direct observation of the reversible $2e^-/2H^+$ redox couple of thioredoxins. Single molecule force spectroscopy has emerged as a powerful tool to probe the signature of dynamic subangstrom molecular rearrangements within the Trx active site during catalysis. By coupling new techniques with traditional methods (e.g., protein biochemistry, X-ray crystallography, and NMR), future studies on Trx will substantially advance our understanding of the fundamental mechanisms underlying Trx activity and evolution as a protein disulfide oxidoreductase.

ASSOCIATED CONTENT

Supporting Information

Figures S1 and S2. This information is available free of charge via the Internet at http://pubs.acs.org/.

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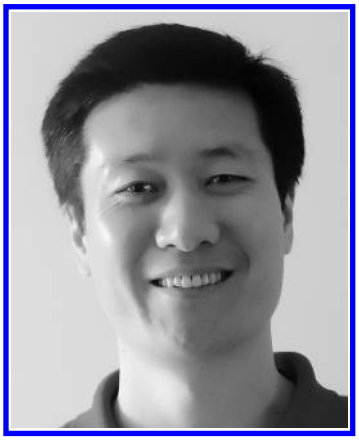
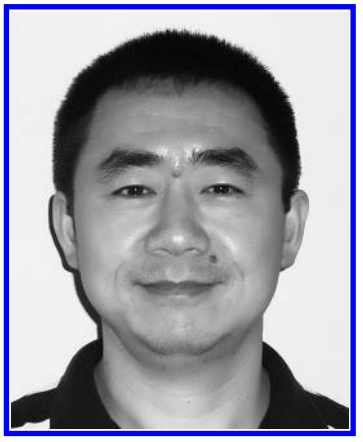
BIOGRAPHIES



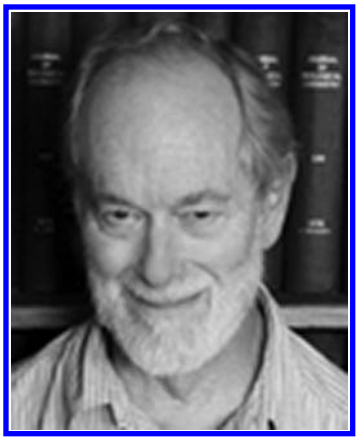
Zhiyong Cheng received his Ph.D. degree from Peking University (China) in 2003, and his thesis was focused on the study of antioxidant functions and mechanisms of phenolic compounds in in vitro oxidation systems, under the supervision of late Prof. Yuanzong Li. In 2004−2006, Dr. Cheng conducted his postdoctoral research with Profs. Charles H. Williams and David P. Ballou, at University of Michigan Medical School (Ann Arbor) where he studied the redox properties and catalysis mechanism of *Drosophila Melanogaster* thioredoxin system. Currently Dr. Cheng is working with Prof. Morris F. White (Howard Hughes Medical Institute, Harvard University Medical School) on redox regulation of insulin signaling and mitochondrial dysfunction metabolic diseases such as diabetes, obesity, and nonalcoholic fatty liver diseases.



Jinfeng Zhang got his B.S. in chemistry from Peking University (PKU) of China in 1997. He obtained his Master's degree in chemistry (2000), Master's degree in computer science (2002), and Ph.D. in Bioinformatics (2004 with Prof. Jie Liang) at University of Illinois at Chicago. The focus of his Ph.D. thesis is on studying protein structures using Monte Carlo method. In 2004, he joined the Department of Statistics, Harvard University, as a post-doc fellow continuing his research in computational study of protein structures. Currently, Dr. Zhang is an assistant professor at Department of Statistics, Florida State University. His current research lies in several areas including protein structure analysis and simulation, biological data mining, and biological networks.

David P. Ballou grew up in Connecticut. He received a B.S. in Chemistry with a minor in music at Antioch College in 1965. He received his M.S. (1967) and Ph.D. (1971) from the University

of Michigan under the direction of Graham Palmer. From 1971−1972 he did postdoctoral work with Vincent Massey and M.J. Coon at Michigan. He served as an instructor (1972−1974), assistant professor (1974−1981), associate professor (1981−1987) and full professor (1987−present) at the University of Michigan. He did one sabbatical with J. Groves at Michigan. Dr. Ballou has studied a wide variety of enzymes, especially those carrying out oxygenations and redox reactions. These mostly involve flavins, non-heme iron centers, heme iron (cytochrome P450), cobalamin, and pyridoxal phosphate. He has specialized in developing rapid reaction techniques including stopped-flow spectroscopy, rapid freeze-quench EPR methods, and anaerobic methods. Dr. Ballou has had the opportunity to collaborate with many wonderful scientists in carrying out these studies. He has taught in a variety of didactic biochemistry courses, including a laboratory course for which he co-authored a text, "*Fundamental Laboratory Approaches to Biochemistry and Biotechnology*". He is co-author of more than 170 publications, 75 chapters in books, two textbooks, and he edited a volume on Metalloproteins for Essays in Biochemistry.



Charles H. Williams, Jr., grew up in Washington, DC. He received a B.S. in Chemistry from the University of Maryland in 1956 and a Ph.D. in Biochemistry from Duke University in 1961 under the direction of Dr. Henry Kamin. From 1961 to 1963 he held an American Cancer Society Postdoctoral Fellowship in the Department of Biochemistry at the University of Sheffield; Dr. Vincent Massey was his mentor. Dr. Williams married Angela Small in 1962 and they have two sons. He joined the Department of Veterans Affairs Medical Center in 1963 and was promoted to Research Career Scientist in 1978; he had a joint appointment in the Department of Biological Chemistry at The University of Michigan from 1963 and was promoted to Professor in 1979, becoming Professor Emeritus in 2005. He taught in the biochemistry course for medical students for thirty years. Following his retirement from the VA in 2001, he joined the laboratory of Dr. Massey, until Dr. Massey's death in 2002. Since then he has enjoyed research in the laboratory of Prof. David P. Ballou. His sabbatical leaves included four venues in Italy, Germany, and the UK. Charles owes much to his research associates, many of whom have distinguished themselves in various ways.

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ABBREVIATIONS

Trx	thioredoxin
*wt*Trx	wild-type Trx
*Ec*Trx	Trx from *Escherichia coli*
*Pf*Trx	Trx from *Plasmodium falciparum*
*Dm*Trx	Trx from *Drosophila melanogaster*
*Tb*Trx	thioredoxin from *Trypanosoma brucei*
*Cr*Trxh	thioredoxin h from *Chlamydomonas reinhardtii*
*Sa*Trx	thioredoxin from *Staphylococcus aureus*
Trx-m	chloroplast thioredoxin m
Trx-f	chloroplast thioredoxin f
TrxR	thioredoxin reductase
Trx-S_2	oxidized thioredoxin
Trx-$(SH)_2$	reduced thioredoxin
PDI	protein disulfide isomerase
IAM	iodoacetamide
E_m	the midpoint redox potential
K_{eq}	equilibrium constant
GR	glutathione reductase
GSH	glutathione
GSSG	glutathione disulfide
Grx	glutaredoxin
Dsb	disulfide bond formation protein

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