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New Concepts in Biochemistry

The CXXC Motif: A Rheostat in the Active Site[†]

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ABSTRACT: The active-site CXXC motif of thiol:disulfide oxidoreductases is essential for their catalysis of redox reactions. Changing the XX residues can perturb the reduction potential of the active-site disulfide bond of the *Escherichia coli* enzymes thioredoxin (Trx; CGPC) and DsbA (CPHC). The reduction potential is correlated with the acidity of the N-terminal cysteine residue of the CXXC motif. As the pK_a is lowered, the disulfide bond becomes more easy to reduce. A change in pK_a can account fully for a change in reduction potential in well-characterized CXXC motifs of DsbA but not of Trx. Formal analysis of the Nernst equation reveals that reduction potential contains both *pH-dependent* and *pH-independent* components. Indeed, the difference between the reduction potentials of wild-type Trx and wild-type DsbA cannot be explained solely by differences in thiol pK_a values. Structural data for thiol:disulfide oxidoreductases reveal no single factor that determines the pH-independent component of the reduction potential. In addition, the pH-dependent component is complex when the redox state of the CXXC motif affects the titration of residues other than the thiols. These intricacies enable CXXC motifs to vary widely in their capacity to assist electron flow, and thereby engender a family of thiol:disulfide oxidoreductases that play diverse roles in biochemistry.

Thiol:disulfide oxidoreductases are found in all living cells. These widespread enzymes catalyze the oxidation of protein thiols and the reduction and isomerization of protein disulfide bonds. These reactions are important not only to the structural integrity of proteins but also to the function of other enzymes (Holmgren, 1985; Ziegler, 1985; Buchanan et al., 1994). For example, thiol:disulfide oxidoreductases serve as the physiological reducing agents for ribonucleotide reductase (Thelander & Reichard, 1979; Stubbe, 1990).

The Cys-Xaa-Xaa-Cys (CXXC[1]) motif of thiol:disulfide oxidoreductases is essential for their catalysis of redox reactions (Laboissière et al., 1995; Walker et al., 1996). This motif can be highly reducing [as in *Escherichia coli* thioredoxin, Trx; $E^{\circ\prime} = -0.27$ V (Moore et al., 1964)], quite oxidizing [*E. coli* DsbA; $E^{\circ\prime} = -0.09$ to -0.11 V (Wunderlich & Glockshuber, 1993; Grauschopf et al., 1995)], or of intermediate reduction potential [protein disulfide isomerase, PDI; $E^{\circ\prime} = -0.18$ V (Lundström & Holmgren, 1993)].[2] Altering the XX residues of Trx (Krause et al., 1991; Chivers et al., 1996), DsbA (Grauschopf et al., 1995), or T_4

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[1] Abbreviations: CXXC, Cys-Xaa-Xaa-Cys; DsbA, periplasmic protein thiol:disulfide oxidoreductase from *Escherichia coli*; EC, effective concentration; Grx, glutaredoxin from phage T_4; GSH, reduced glutathione; GSSG, oxidized glutathione; PDB, Protein Data Bank; PDI, protein disulfide isomerase; Trx, thioredoxin from *E. coli*.

[2] To calibrate these differences in $E^{\circ\prime}$, consider that a $\Delta E^{\circ\prime}$ of 0.03 V corresponds near room temperature to a ΔG° of 1.4 kcal/mol or to a 10-fold change in the equilibrium constant.

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glutaredoxin [Grx; $E^{\circ\prime} = -0.23$ V (Joelson et al., 1990)] perturbs the reduction potential of the disulfide bond of each enzyme. For the mutant Trx's and DsbA's, changes in $E^{\circ\prime}$ are manifested as changes in catalytic activity *in vivo* (Grauschopf et al., 1995; Chivers et al., 1996).

The effects of changing the XX residues in Trx and DsbA are opposite. Trx becomes a less potent reductant, but DsbA becomes a less potent oxidant. These changes in $E^{\circ\prime}$ are accompanied by a change in the pK_a of the N-terminal cysteine residue (CXXC) of the motif. (Hereafter, the first and second cysteines of the motif will be referred to as Cys_N and Cys_C, respectively.) In each mutant enzyme, a lower pK_a of Cys_N corresponds to an elevated $E^{\circ\prime}$. Analysis of the mutant DsbA's shows that pK_a can be correlated strongly with $E^{\circ\prime}$ (Grauschopf et al., 1995). Yet, analysis of the mutant Trx's shows that pK_a is not the sole determinant of $E^{\circ\prime}$ (*vide infra*). What then are the elements that determine $E^{\circ\prime}$? Here, we delineate the factors that modulate the reduction potential of the common CXXC motif. Our analysis suggests that deviations from a direct correlation between pK_a and $E^{\circ\prime}$ in the active sites of thiol:disulfide oxidoreductases are necessary for efficient catalysis in different cellular compartments.

Reduction Potential

The general equilibrium for a reduction half-reaction is ox + ne^- ⇄ red. When this equilibrium also involves protons, the reduction potential is pH-dependent. Additionally, functional groups with different pK_a's in the reduced and oxidized molecules will also contribute to this pH dependence. Thus, an enzyme that has a CXXC motif has (at least) two additional acid−base equilibria present in its reduced form. The reduced enzyme must be considered a population of dithiol, thiolate−thiol, thiol−thiolate, and dithiolate forms. The relative free energies of these forms, determined by their cysteine (Cys_N and Cys_C) pK_a's, give rise to the pH dependence of the reduction potential.

The reduction potential (E) for the half-reaction $PS_2 + 2H^+ + 2e^- \rightleftarrows P(SH)_2$ is given by a form of the Nernst equation:

$$E = E^\circ - \frac{RT}{nF}\ln\left[\frac{[P(SH)_2]}{[PS_2][H^+]^2}\right] \quad (1)$$

where E°, the standard reduction potential, is the reduction potential at the standard state of $[P(SH)_2] = [PS_2] = [H^+] = 1$ M. A pH-dependent term can be extracted from the Nernst equation by using the formal (*i.e.*, total) concentrations of the reduced [$F_{P(SH)_2}$] and oxidized (F_{PS_2}) molecules. This term accounts for the acid−base equilibria present in the reduced and oxidized molecules. For example, the fraction (α_0) of fully protonated dithiol species is

$$\alpha_0 = \frac{[P(SH)_2]}{F_{P(SH)_2}} = \frac{[H^+]^2}{[H^+]^2 + [H^+]K_1 + [H^+]K_2 + K_1K_2} \quad (2)$$

where K_1 and K_2 are the acid dissociation constants corresponding to the pK_a's of Cys_N and Cys_C, respectively. The Nernst equation then becomes

$$E = E^\circ - \frac{RT}{nF}\ln\left[\frac{\alpha_0 F_{P(SH)_2}}{[H^+]^2 F_{PS_2}}\right] \quad (3)$$

Substitution for α_0 yields

$$E = E^\circ - \underline{\frac{RT}{nF}\ln\left(\frac{1}{[H^+]^2 + [H^+]K_1 + [H^+]K_2 + K_1K_2}\right)} - \underline{\frac{RT}{nF}\ln\left[\frac{F_{P(SH)_2}}{F_{PS_2}}\right]} \quad (4)$$

The two underlined terms in eq 4 describe the formal reduction potential of the reaction (Swift, 1939), which we define here as E_F°:

$$E_F^\circ = E^\circ - \frac{RT}{nF}\ln\left(\frac{1}{[H^+]^2 + [H^+]K_1 + [H^+]K_2 + K_1K_2}\right) \quad (5)$$

If $F_{P(SH)_2} = F_{PS_2}$, then $E = E_F^\circ$. This condition is met by both PDI in its natural environment [which is the endoplasmic reticulum of eukaryotic cells (Hwang et al., 1992)] and Trx in its natural environment [which is the cytosol of *E. coli* (Gilbert, 1990)]. At pH 7, E_F° is defined as $E^{\circ\prime}$, the quantity listed commonly in biochemistry textbooks.

Equation 5 has two terms. The first term is simply E°, which reports on the intrinsic tendency of a disulfide bond to suffer reduction. The second term depends on the values of K_1, K_2, and pH. This term reveals that E_F° depends on pK_a and pH, and that $E^{\circ\prime}$ depends on pK_a. The pH dependence of E_F°, though absent from biochemistry textbooks, is treated in textbooks on biophysical chemistry (Wyman & Gill, 1990) or analytical chemistry (Harris, 1995).

The effect of thiol pK_a and pH on E_F° for three thiol:disulfide oxidoreductases is shown explicitly in Figure 1. To highlight the pH dependency of E_F°, the value of E° in Figure 1 is assumed to be zero for each enzyme. The breaks in the curves in Figure 1 correspond to pK_1 and pK_2. Once the pH is above pK_2, the value of E_F° is no longer pH-dependent.

According to the analysis used to derive Figure 1, the E_F° values of Trx and PDI are nearly identical at pH 7.0. Yet, experiments show that $E^{\circ\prime}$ for Trx and PDI differ by 0.090 V (Holmgren, 1984; Lundström & Holmgren, 1993). Although the difference in Figure 1 between DsbA and PDI is close to the value expected from experiment (~0.090 V) (Lundström & Holmgren, 1993; Wunderlich & Glockshuber, 1993; Grauschopf et al., 1995), Trx is again in discord. Thus, the assumption used to derive Figure 1 must be incorrect; the value of E° for Trx must differ from that for PDI and DsbA.[3]

Changing pK_a

A correlation between E_F° and thiol pK_a's is provided by eq 5. This correlation should be evident from experimental

[3] The relationship between $E^{\circ\prime}$ and thiol pK_a can be thought of in another way. The values of $E^{\circ\prime}$ for DsbA and Trx differ by ~0.18 V. Near room temperature, this difference corresponds to a ΔG° of ~8.4 kcal/mol. The values of pK_1 for DsbA and Trx differ by ~4. Near room temperature, this difference corresponds to a ΔG° of ~5.6 kcal/mol. An aspect of protein structure other than pK_1 must therefore provide an additional ~2.8 kcal/mol of stability to the reduced form of DsbA or the oxidized form of Trx. Likewise, glutaredoxins have a pK_1 of <5 but an $E^{\circ\prime}$ of <−0.20 V (Wells et al., 1993).

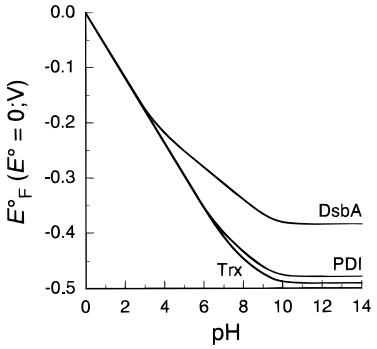


FIGURE 1: Formal potential vs pH for three thiol:disulfide oxidoreductases, each with two titrating groups (Cys_N and Cys_C) in their reduced forms. Curves were calculated by using eq 5 with $E°$ = 0 for each enzyme. DsbA data are from Grauschopf et al. (1995). PDI data are from Lundström and Holmgren (1993) and Freedman et al. (1988). Trx data are from Moore et al. (1964) and Jeng et al. (1995).

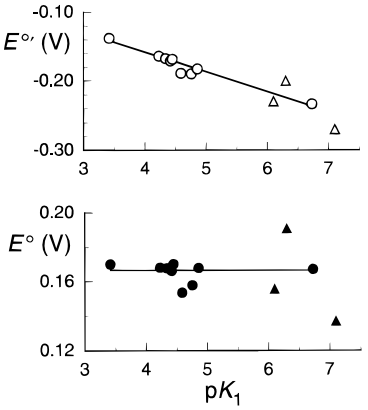


FIGURE 2: Reduction potential (open symbols, $E°'$; closed symbols, $E°$) vs pK_1 for DsbA (circles) and Trx (triangles). Lines are linear least-squares fits of the DsbA data from Grauschopf et al. (1995). Trx data are from Chivers et al. (1996).

data whenever $E°$ is constant, and it has been observed in structurally related small molecules (Szajewski & Whitesides, 1980; Burns & Whitesides, 1990). This correlation should in theory also appear in wild-type and mutant CXXC-containing enzymes. At a minimum, the size (14 atoms) and composition ($C_9N_3S_2$) of the ring containing the disulfide bond remains constant within the CXXC motif.

A plot of pK_1 vs $E°$ for both wild-type and mutants of DsbA and Trx is shown in Figure 2. The value of $E°$ (=0.17 V) for wild-type DsbA is unaffected by mutations to the CXXC motif. Thus, the change in the $E_F°$ of DsbA imposed by mutating its CXXC motif is due solely to a change in pK_1. The relationship between pK_1 and $E°'$ for DsbA is $\Delta E°' = -0.0285\ \text{V} \times \Delta\text{p}K_1$.[2]

The same plot for wild-type and mutant Trx's reveals a breakdown in this correlation. Although the pool of mutant Trx's is smaller, the change in $E°$ is greater. Thus, some property other than pK_1 is changing $E_F°$ in the mutant Trx's.

In these analyses, the pK_a of Cys_C in DsbA and Trx is assumed to be constant (pK_2 = 9.5). This assumption has little effect on the conclusions. A plot of $E°' - E°$ vs pK_1 vs pK_2 can be derived from eq 5 and is shown in Figure 3. This plot reveals that, if a thiol has a pK_a of $\gg$7, then that pK_a has a negligible effect on $E°' - E°$ (and therefore on $E°'$ if $E°$ is constant). In other words, a thiol pK_a will have no significant effect on the formal potential when its value

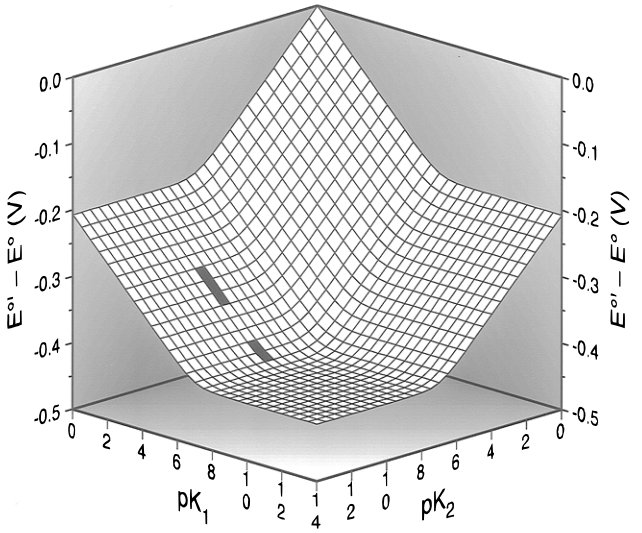


FIGURE 3: $E°' - E°$ vs pK_1 vs pK_2 for CXXC motifs. The surface is derived from eq 5 at pH 7, where $E_F° = E°'$. The shaded squares indicate the values of pK_1 and pK_2 observed for mutants of DsbA (Grauschopf et al., 1995) and Trx (Chivers et al., 1996).

is at least 1 unit higher than the pH at which the formal potential is measured. Because the pK_a of Cys_C is changed little in the mutant Trx's (Chivers et al., 1997b), this thiol almost certainly does not produce the observed effect on $E°$.

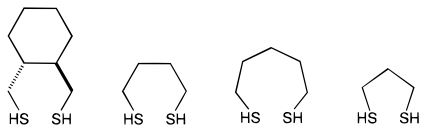
Changing E°

A quantity related to reduction potential is the effective concentration (EC) of one thiol relative to the other. This term describes the tendency of a dithiol to form an intramolecular disulfide bond relative to that of a reference monothiol to form an intermolecular (*i.e.*, mixed) disulfide bond. In biochemical thiol—disulfide interchange reactions, glutathione (GSH) is used as the reference monothiol (Creighton, 1984; Lin & Kim, 1989; Gilbert, 1990). The relevant equilibria are $PS_2 + 2H^+ + 2e^- \overset{K_{intra}}{\rightleftarrows} P(SH)_2$ and $GSSG + 2H^+ + 2e^- \overset{K_{inter}}{\rightleftarrows} 2GSH$, with EC being defined as

$$\text{EC} = \frac{K_{\text{intra}}}{K_{\text{inter}}} = \frac{[\text{PS}_2][\text{GSH}]^2}{[\text{P(SH)}_2][\text{GSSG}]} \qquad (6)$$

Dithiols with a stronger tendency to form an intramolecular disulfide than that of two molecules of GSH to form an intermolecular disulfide have an EC of >1. For the equilibria described by eq 6, EC = K_{ox} (Gilbert, 1990). The value of K_{ox} can be substituted into the Nernst equation to determine $E°'$ for a dithiol when it is in equilibrium with GSH, with an $E°'$ for GSH of −0.252 V (Lees & Whitesides, 1993).

As part of an extensive effort to design strong dithiol-reducing agents, Whitesides and co-workers used molecular mechanics calculations to reveal the factors that affect the EC of two thiols in molecules with a similar structure and thiol pK_a (Burns & Whitesides, 1990). The three parameters found to be important were the dihedral angle of the disulfide bond (θ_{CSSC}), the two thiol bond angles ($\angle_{CCS}$), and van der Waals forces in the vicinity of the sulfur atoms. The importance of each of these factors varied from molecule to molecule, as did the chemical structure and disulfide ring size. Therefore, comparisons based on these parameters are valid only on related dithiols. Such a series of related thiols



EC (M)	1500	77	3.6	2.3
$E^{\circ\prime}$ (V)	− 0.354	− 0.316	− 0.277	− 0.271

FIGURE 4: EC of related small-molecule dithiols and $E^{\circ\prime}$ of their corresponding disulfide bonds. Data are from Lees and Whitesides (1993).

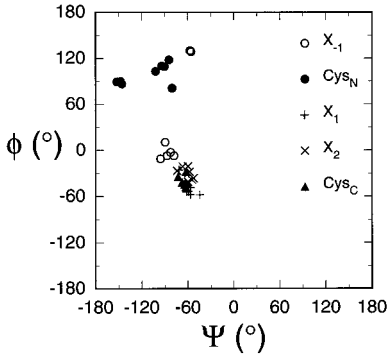


FIGURE 5: Main chain dihedral angles for amino acid residues in CXXC motifs. Values were determined with the program MIDAS PLUS (Ferrin et al., 1988) and PDB files 1AAZ (Eklund et al., 1992), 1ABA (Eklund et al., 1992), 1DSB (Martin et al., 1993), 1THX (Saarinen et al., 1995), 2TRX (Katti et al., 1990), and 1XOA (Jeng et al., 1994). In PDB files containing more than one structure, bond angles were determined for each molecule.

is shown in Figure 4. Because thiol pK_a's of the molecules in this series are equivalent (*i.e.*, the value of the pH-dependent term in eq 5 is the same for each molecule), conformational factors must change EC by altering E°.

In the crystalline and solution structures of the oxidized (Katti et al., 1990) and reduced (Jeng et al., 1994) forms of Trx, oxidized DsbA (Martin et al., 1993), and oxidized wild-type and mutant Grx's (Eklund et al., 1992), the disulfide dihedral angles (θ_{CSSC}) fall between 60 and 90°. This data set includes a mutant Grx in which $E^{\circ\prime}$ but not θ_{CSSC} is affected significantly (Joelson et al., 1990). Although differing dramatically in $E^{\circ\prime}$, oxidized DsbA and oxidized Trx have almost identical values of θ_{CSSC} (≈80°). Molecular mechanics calculations have shown that there is little change in the relative energy of H_3CSSCH_3 for 60° < θ_{CSSC} < 90° (Burns & Whitesides, 1990). Thus, the dihedral angle of these disulfide bonds is not a reliable indicator of $E^{\circ\prime}$. Additionally, virtually all structural protein disulfide bonds have values of θ_{CSSC} near 90° (Richardson, 1981; Thornton, 1981). These bonds have a wide range of reduction potentials (Gilbert, 1990), providing further evidence that θ_{CSSC} does not correlate with $E^{\circ\prime}$. Similarly, the values of $\angle_{CCS}$ for Cys_N and Cys_C are almost identical for all three enzymes, with 105° < $\angle_{CCS}$ < 115°.

The main chain dihedral angles for the residues in the active-site region of these enzymes show little correlation with $E^{\circ\prime}$ (Figure 5). The ϕ and ψ angles for X_1, X_2, and Cys_C are in the range expected for residues in an α-helical conformation. The ϕ and ψ angles for Cys_N and X_{-1} (which precedes Cys_N) are more dispersed, but do not correlate with $E^{\circ\prime}$. As with θ_{CSSC} and $\angle_{CCS}$, the ϕ and ψ angles of the CXXC motifs in Trx and DsbA are almost identical.

The value of $E^{\circ\prime}$ for a CXXC motif is not determined by the sequence of amino acid residues in the active site. Octapeptides corresponding to the active sites of Trx, PDI, Grx, and thioredoxin reductase have an $E^{\circ\prime}$ of −0.20 ± 0.01 V (Siedler et al., 1993, 1994). Interactions arising from the three-dimensional fold of the enzyme must modulate E° of each CXXC motif.

The value of $E^{\circ\prime}$ is likely to result from noncovalent interactions between the CXXC motif and surrounding residues. Each atom of the 14-membered disulfide-containing rings of oxidized Trx, DsbA, and Grx makes numerous contacts within a 3.25 Å radius. Differences in these interactions could alter E°, and hence $E^{\circ\prime}$. Thus, knowledge of the structures of both the reduced and the oxidized forms is required for a molecular rationalization of $E^{\circ\prime}$. Even with both structures, an analysis would be limited to the pH at which the structures were determined.

Experimental Determination of E°

The data presented above show that E° cannot be the same for all CXXC motifs. Can the value of E° be determined by experiments on a single enzyme? Equation 5 provides the pH dependence of the formal potential for a reduction half-reaction. The pH dependence of a complete reduction−oxidation reaction can be derived from eq 5. In this redox reaction, glutathione is used as a reference thiol because its reduction potential and pH titration behavior are well characterized (Jung et al., 1972; Rabenstein, 1973; Reuben & Bruice, 1976; Burns & Whitesides, 1990). The Nernst equation for a dithiol in equilibrium with glutathione is

$$E^{\circ\prime}_{P(SH)_2} = E^{\circ\prime}_{GSH} - \frac{RT}{nF}\ln K_{ox} \qquad (7)$$

In terms of formal concentrations, eq 7 is

$$\ln K_{ox} = \frac{nF}{RT}(E^\circ_{GSH} - E^\circ_{P(SH)_2}) + \ln\left(\frac{[H^+]^2 + 2[H^+]K_{GSH} + K_{GSH}{}^2}{[H^+]^2 + [H^+]K_1 + [H^+]K_2 + K_1K_2}\right) \qquad (8)$$

where $K_{ox} = F_{PS_2}F_{GSH}{}^2/[F_{P(SH)_2}F_{GSSG}]$. The value of K_{ox} can be determined as a function of pH, and when $E^{\circ\prime}$ and all relevant pK_a values are known, a value of E° can be extracted from the equation. Creighton and co-workers have determined experimental values for K_{ox} over a wide range of pH's for both Trx and DsbA (Nelson & Creighton, 1994; Takahashi & Creighton, 1996). The results for Trx fit eq 8 only when pK_1 and pK_2 are close to 9. This value for pK_1 is greater by 2 units than that determined by direct measurement (Dyson et al., 1991; Li et al., 1993; Jeng et al., 1995; Wilson et al., 1995; Chivers et al., 1997b). Use of eq 8 assumes that the only additional equilibria in the reduced enzyme arise from the titration of the thiols of the CXXC motif. For example, the amino group of glutathione has a different pK_a in GSH and GSSG (Jung et al., 1972). Such a pK_a, which differs in the reduced and oxidized species, requires the addition of terms to eq 8. There is much debate about the pK_a of Asp26 in the reduced and oxidized forms of *E. coli* Trx (Wilson et al., 1995; Jeng & Dyson, 1996). A change in the pK_a of Asp26 has indeed been observed upon oxidation of human thioredoxin (Qin et al., 1996). Consideration of additional titrating groups is also important for DsbA and PDI because both contain histidine residues within the CXXC

motif. The experimental determination of K_{ox} therefore requires an accurate and thorough analysis of the titration of the reduced and oxidized species of the enzyme. Extending eq 8 to CXXC motifs is difficult because enzymes are complex molecules with many acid−base equilibria (Chivers et al., 1997b).

Relevance to Biological Function

Enzymes containing CXXC motifs appear to have evolved both divergently (PDI and Trx) and convergently (DsbA and Trx). The versatility of the CXXC motif has resulted in a family of enzymes that catalyze the same reactions, but with different efficiencies. Each enzyme has evolved in an environment (*e.g.*, the cytosol, periplasm, or endoplasmic reticulum) that imposes constraints for its optimization (Burbaum et al., 1989; Chivers et al., 1997a). In addition, each enzyme has different substrates (*e.g.*, unfolded, misfolded, or folded proteins), and some of the enzymes are themselves substrates for different oxidases or reductases. Each enzyme is a solution to a particular energetic problem.

The biological screen used to identify mutant DsbA's did not demand any correlations (Grauschopf et al., 1995). Still, $E^{\circ\prime}$ is well-correlated to pK_1 in these mutant enzymes. Yet in a plot of $E^{\circ\prime}$ vs pK_1 (Figure 2), most of the mutant DsbA's are clustered within a narrow range. This grouping suggests that the structure of DsbA favors a limited range of $E^{\circ\prime}$ and pK_1 values. Further, both the reduced and the oxidized forms of the mutant DsbA's are more stable than are the two forms of wild-type DsbA (Grauschopf et al., 1995). CXXC sequences of DsbA that lie outside the narrow pK_1 and $E^{\circ\prime}$ range [*i.e.*, CPHC (wild-type) and CPPC] must have some unique properties that destabilize either the oxidized or reduced species. This destabilization has apparently endowed wild-type DsbA with optimal biological activity.

In contrast, a biological selection was used to identify mutant Trx's that could replace PDI in a eukaryotic cell (Chivers et al., 1996). The selection required a narrow range of pK_1 and $E^{\circ\prime}$ values, which were similar to those of PDI. Kinetic constraints could also have been important because the mutant Trx's must be reduced (or oxidized) by glutathione, which reacts slowly with wild-type Trx (Nikkola et al., 1991). In addition, interactions with protein substrates may have required the production of favorable binding energies. Thus, the mutant Trx's may represent the few enzymes that meet all the challenges imposed by the *in vivo* selection.

The determinants of the reduction potential of a CXXC motif are complex, involving the titration of thiol and other groups as well as noncovalent interactions imposed by three-dimensional structure. The reduction potential is also malleable, as it can be changed by pH or mutation. The ability of CXXC motifs to vary widely in their capacity to assist electron flow makes the motif a molecular rheostat, which can be set to a particular reduction potential to fit a particular need. The result is a family of thiol:disulfide oxidoreductases that play diverse roles in biochemistry.

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