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′) 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°′ = −0.27 V (Moore et al., 1964)], quite oxidizing [E. coli DsbA; E°′ = −0.09 to −0.11 V (Wunderlich & Glockshuber, 1993; Grauschopf et al., 1995)], or of intermediate reduction potential [protein disulfide isomerase, PDI; E°′ = −0.18 V (Lundström & Holmgren, 1993)]. Altering the XX residues of Trx (Krause et al., 1991; Chivers et al., 1996), DsbA (Grauschopf et al., 1995), or T_a

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glutaredoxin [Grx; \( E^\circ = -0.23 \text{ V} \) (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 \( E^\circ \) 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 \( E^\circ \) 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 \( E^\circ \). Analysis of the mutant DsbA’s shows that pK_a can be correlated strongly with \( E^\circ \) (Grauschopf et al., 1995). Yet, analysis of the mutant Trx’s shows that pK_a is not the sole determinant of \( E^\circ \) (vide infra). What then are the elements that determine \( E^\circ \)? 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 \( E^\circ \) 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^{-} \rightleftharpoons \text{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^- \rightleftharpoons P(SH)_2 \[1\] is given by a form of the Nernst equation:

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

where \( E^\circ \), the standard reduction potential, is the reduction potential at the standard state of \([P(SH)_2]\) and oxidized \([PS_2]\). This term accounts for the acid-base equilibria present in the reduced and oxidized molecules. For example, the fraction \((\alpha_0)\) of fully protonated dithiol species is

\[
\alpha_0 = \frac{[P(SH)_2]}{F_{PSH2}} = \frac{[H^+]^2}{[H^+]^2 + [H^+][K_1] + [H^+][K_2] + K_1K_2}
\]

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

\[
E = E^\circ - \frac{RT}{nF} \ln \left[ \frac{\alpha_0 F_{PSH2}}{[H^+]^2 F_{PS_2}} \right]
\]

Substitution for \( \alpha_0 \) yields

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

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

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

If \( F_{PSH2} = F_{PS_2} \), 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, \( E_F^\circ \) is defined as \( E^\circ \), the quantity listed commonly in biochemistry textbooks.

Equation 5 has two terms. The first term is simply \( E^\circ \), 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 \( E_F^\circ \) depends on pK_a and pH, and that \( E_F^\circ \) depends on pK_a. The pH dependence of \( E_F^\circ \), 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 \( E_F^\circ \) for three thiol: disulfide oxidoreductases is shown explicitly in Figure 1. To highlight the pH dependency of \( E_F^\circ \), the value of \( E_F^\circ \) in Figure 1 is assumed to be zero for each enzyme. The breaks in the curves in Figure 1 correspond to \( K_1 \) and \( K_2 \). Once the pH is above \( \alpha_0 \), the value of \( E_F^\circ \) is no longer pH-dependent.

According to the analysis used to derive Figure 1, the \( E_F^\circ \) values of Trx and PDI are nearly identical at pH 7.0. Yet, experiments show that \( E^\circ \) 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 \( E_F^\circ \) for Trx must differ from that for PDI and DsbA.

**Changing pK_a**

A correlation between \( E_F^\circ \) and thiol pK_a’s is provided by eq 5. This correlation should be evident from experimental

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1 The relationship between \( E^\circ \) and thiol pK_a can be thought of in another way. The values of \( E^\circ \) for DsbA and Trx differ by ~0.18 V. Near room temperature, this difference corresponds to a \( \Delta G^\circ \) of ~8.4 kcal/mol. The values of pK_a for DsbA and Trx differ by ~4. Near room temperature, this difference corresponds to a \( \Delta G^\circ \) of ~5.0 kcal/mol. An aspect of protein structure other than pH, and that

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data whenever \( E^\circ \) 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 (\( C_9 N_3 S_2 \)) of the ring containing the disulfide bond remains constant within the CXXC motif.

A plot of \( pK^a \) vs \( E^\circ \) for both wild-type and mutants of DsbA and Trx is shown in Figure 2. The value of \( E^\circ \) (=0.17 V) for wild-type DsbA is unaffected by mutations to the CXXC motif. Thus, the change in the \( E^\circ \) of DsbA imposed by mutating its CXXC motif is due solely to a change in the \( pK^a \) of Cys C in DsbA and Trx is shown in Figure 2. The value of \( E^\circ \) for wild-type DsbA is 0.17 V 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).

Changing \( E^\circ \)

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 \( PS_2 + 2H^+ + 2e^- \rightleftharpoons P(SH)_2 \) and GSSG + \( 2H^+ + 2e^- \rightleftharpoons 2GSH \), with EC being defined as

\[
EC = \frac{K_{\text{intra}}}{K_{\text{inter}}} = \frac{[PS_2][GSH]^2}{[P(SH)_2][GSSG]} \tag{6}
\]

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 = \( K_{\text{ox}} \) (Gilbert, 1990). The value of \( K_{\text{ox}} \) can be substituted into the Nernst equation to determine \( E^{\circ \prime} \) for a dithiol when it is in equilibrium with GSH, with an \( E^{\circ \prime} \) 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 (\( \theta_{\text{CSSC}} \)), the two thiol bond angles (\( \angle_{\text{CCS}} \)), 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 dithiols...
mechanics calculations have shown that there is little change in CXXC motifs in Trx and DsbA are almost identical. CCS for Cys N and Cys C are almost identical for all three dihedral angles (φ, θ, ψ) 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\textsubscript{a} of Asp26 in the reduced and oxidized forms of Trx, and the value of E\textsuperscript{o} of thioredoxin reductase have an E\textsuperscript{o} of −0.20 ± 0.01 V (Stiedler et al., 1993, 1994). Interactions arising from the three-dimensional fold of the enzyme must modulate E\textsuperscript{o} of each CXXC motif.

The value of E\textsuperscript{o} 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\textsuperscript{o}, and hence E\textsuperscript{o}. Thus, knowledge of the structures of both the reduced and the oxidized forms is required for a molecular rationalization of E\textsuperscript{o}. Even with both structures, an analysis would be limited to the pH at which the structures were determined.

Experimental Determination of E\textsuperscript{o}

The data presented above show that E\textsuperscript{o} cannot be the same for all CXXC motifs. Can the value of E\textsuperscript{o} 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 & Bruce, 1976; Burns & Whitesides, 1990). The Nernst equation for a dithiol in equilibrium with glutathione is

$$E_{\text{ox}} - E_{\text{red}} = \frac{RT}{nF} \ln K_\text{ox}$$

(7)

In terms of formal concentrations, eq 7 is

$$K_\text{ox} = \frac{F_{\text{red}}}{F_{\text{ox}}} \left( \frac{E_{\text{red}}^o - E_{\text{ox}}^o}{F_{\text{ox}}^2} \right) + \ln \left( \frac{[H^+]^2 + 2[H^+]K_{\text{GSH}} + K_{\text{GSSG}}^2}{[H^+]^2 + [H^+]K_1 + [H^+]K_2} \right)$$

(8)

where \(K_\text{ox} = F_{\text{PS}}F_{\text{GSH}}^2/[F_{\text{SOSH}}F_{\text{GSSG}}]\). The value of \(K_\text{ox}\) can be determined as a function of pH, and when E\textsuperscript{o} and all relevant pK\textsubscript{a} values are known, a value of E\textsuperscript{o} can be extracted from the equation. Creighton and co-workers have determined experimental values for K\textsubscript{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\textsubscript{1} and pK\textsubscript{2} are close to 9. This value for pK\textsubscript{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\textsubscript{a} in GSH and GSSG (Jung et al., 1972). Such a pK\textsubscript{a}, which differs in the reduced and oxidized species, requires the addition of terms to eq 8. There is much debate about the pK\textsubscript{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\textsubscript{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.

Octapeptides corresponding to the active sites of Trx, PDI, Grx, and thioredoxin reductase have an E\textsuperscript{o} of −0.20 ± 0.01 V (Stiedler et al., 1993, 1994). Interactions arising from the three-dimensional fold of the enzyme must modulate E\textsuperscript{o} of each CXXC motif.
motif. The experimental determination of $K_{\text{on}}$ 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, $E^0$ is well-correlated to $pK_1$ in these mutant enzymes. Yet in a plot of $E^0$ 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 $E^0$ 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 $E^0$ 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 $E^0$ 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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