Electrostatic Interactions in the Active Site of the N-Terminal Thioredoxin-like Domain of Protein Disulfide Isomerase

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ABSTRACT: Proteins with the thioredoxin fold have widely differing stabilities of the disulfide bond that can be formed between the two cysteines at their active site sequence motif $Cys_1-Xaa_2-Yaa_3-Cys_4$. This is believed to be regulated not by varying the disulfide bond itself, but by modulating the stability of the dithiol form of the protein through interactions with the ionized form of the Cys_1 thiol group. A consistent relationship between disulfide bond stability and Cys_1 thiol pK_a value is found here for DsbA, thioredoxin, and the N-terminal thioredoxin-like domain of protein disulfide isomerase (PDI *a*), which has a very low thiol pK_a value of 4.5. This thiolate anion is stabilized by 5.7 kcal/mol in the dithiol form, giving rise to the corresponding instability of the disulfide bond and the oxidizing properties of PDI *a*. Electrostatic interactions in the active site of the PDI *a*-domain have been characterized in order to understand the physical basis of this stabilization. Linkage with the ionization of the imidazole group of His_3 in the active site demonstrates that this charge−charge interaction contributes 1.1 kcal/mol. The remainder of the stabilization is believed to be due primarily to interactions with the partial positive charges at the N-terminus of an α-helix, which are exceedingly sensitive to changes of surrounding residues.

Electrostatic effects in proteins are believed to be important for the structure, stability, and biological function of many proteins (Allewell & Oberoi, 1991; Nakamura, 1996). The magnitudes of electrostatic interactions of charged groups are apparent from the extent to which their pK_a values are shifted from the intrinsic values. Electrostatic interactions are most significant in the folded state, where pK_a values are perturbed to the greatest extent, whereas those in unfolded proteins tend to be more normal. Consequently, electrostatic effects modulate the stability of the folded state, either increasing or decreasing it. Most electrostatic effects in folded proteins cause pK_a shifts of less than 1 pH unit, although effects of greater magnitude are known (e.g., Polgár & Halász, 1978; Anderson et al., 1990).

Substantial, but variable, electrostatic effects are apparent in the active sites of proteins of the thioredoxin family: thioredoxin, glutaredoxin, DsbA, DsbC, and two domains of protein disulfide isomerase (PDI[1]). All of these proteins have very similar folded conformations (Martin, 1995), with active sites containing two cysteine residues, separated by two other residues in the sequence $-Cys_1-Xaa_2-Yaa_3-Cys_4-$, that reversibly form a disulfide bond. These residues in all members of the thioredoxin family will be referred to here using the indicated local numbering scheme. The identities of the two central residues vary between, but normally not within, the subfamilies, being Gly−Pro in thioredoxins, mostly Pro−Tyr in glutaredoxins, Gly−His for

eukaryotic PDI, and Pro−His for DsbA. Only Cys_1 has a sulfur atom that is accessible and reactive with external thiol and disulfide reagents; that of Cys_4 is buried and unreactive, forming a disulfide bond only with Cys_1. The disulfide bonds formed between the two cysteine residues are extremely similar structurally in the various members of the thioredoxin family (Martin et al., 1993; Katti et al., 1995), and the proteins undergo only minimal changes in structure upon breaking the disulfide bond (Dyson et al., 1990; Forman-Kay et al., 1991; Qin et al., 1994; Jeng et al., 1994; Weichsel et al., 1996). Nevertheless, these disulfide bonds differ greatly in their stabilities; the equilibrium constant for forming that of thioredoxin is 10^5 times greater than that of DsbA at neutral pH (Zapun et al., 1993; Wunderlich & Glockshuber, 1993). The varying stabilities of these disulfide bonds correlate with the functions of the proteins: thioredoxin is a reductant of protein disulfide bonds and forms a stable active site disulfide bond, whereas DsbA, DsbC, and PDI are oxidants and form unstable active site disulfide bonds (Zapun et al., 1995; Darby & Creighton, 1995b). In each case, the equilibrium constant for the physiological reaction is favorable.

The electrostatic effects in these proteins are most apparent in the ionization of their accessible Cys_1 thiol groups; those of DsbA and glutaredoxin have very low pK_a values of about 3.5 (Gan et al., 1990; Yang & Wells, 1991; Mieyal et al., 1991; Nelson & Creighton, 1994), more than 5 pH units lower than that of a normal thiol group (usually about 8.7). There must be a very substantial stabilizing interaction between the thiolate anion and the folded conformation, which stabilizes both by about 7 kcal/mol. A positive charge in very close proximity to the sulfur atom would seem the most likely source of this interaction, as occurs in the active site of papain (Pólgar & Halász, 1978). In the case of the crystal structure of the disulfide form of DsbA (Martin et al., 1993), however, the closest positive charges are the side

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[1] Abbreviations: CD, circular dichroism; DTT, dithiothreitol; EDTA, ethylenediaminetetraacetic acid; GSH and GSSG, reduced and oxidized glutathione, respectively; ϵ_x, molar absorbance at wavelength x nm; HPLC, high-pressure liquid chromatography; NMR, nuclear magnetic resonance; PDI, protein disulfide isomerase; PDI *a*-domain, residues 1−120 of mature human PDI; TOCSY, total correlation spectroscopy; Tris, tris(hydroxymethyl)aminomethane; TSP, sodium 3-(trimethylsilyl)-(2,2,3,3-$^2H_4$)propionate; 1D, one-dimensional; 2D, two-dimensional.

chain nitrogen atoms of His$_3$, which are 6 Å away. Other likely candidates for interacting groups in the thioredoxin fold are the microdipoles at the N-terminus of the α-helix that initiates with Cys$_1$ (Hol, 1985; Katti et al., 1990; Åquist et al., 1991). In qualitative confirmation, cysteine residues at the corresponding position in designed model α-helical peptides have been shown to have pK_a values decreased by up to 1.6 pH units (Kortemme & Creighton, 1995).

The greatly decreased pK_a value of the active site thiol group of DsbA has important functional consequences, for it seems to account for the instability of any disulfide bond that it forms (Nelson & Creighton, 1994). The negative charge of the thiolate anion disappears when the sulfur atom forms a disulfide bond, so the stabilizing interaction with the native conformation should also disappear. The stabilization of the thiol form of the protein in effect destabilizes any disulfide form involving that thiol group (Zapun et al., 1993, 1994).

There would then be expected to be a correlation in the thioredoxin motif between the pK_a of the thiol group and the stability of the disulfide bond formed between residues Cys$_1$ and Cys$_4$, and this has been observed in DsbA by changing residues Xaa$_2$ and Yaa$_3$ (Grauschopf et al., 1995). An increase in the cysteine thiol group pK_a value of 1 pH unit resulted in about a 10-fold increase in the equilibrium constant for forming the disulfide bond at neutral pH. An important question is whether this correlation also holds for other members of the thioredoxin family. Thioredoxin forms a very stable disulfide bond, with a 10^5-fold greater equilibrium constant for disulfide bond formation than that of DsbA, and its exposed cysteine thiol group has a much greater pK_a value. It had long been considered to be in the region 6.3−7.5 (Kallis & Holmgren, 1980; Dyson et al., 1991; Forman-Kay et al., 1992; Li et al., 1993; Wilson et al., 1995; Jeng et al., 1995; Jeng & Dyson, 1996; Qin et al., 1996), which is significantly lower than that of a normal thiol group. More direct methods, however, indicate that it actually has a somewhat elevated pK_a value in the region 9−9.5 (Takahashi & Creighton, 1996).

Further data on these parameters are required in other members of the thioredoxin family, and the possible importance of other aspects of the thioredoxin fold need to be examined. For example, the side chain of the His$_3$ residue that occurs in some members of the thioredoxin family, especially those with unstable disulfide bonds, has been suggested on the basis of electrostatic calculations to interact favorably with the thiolate anion of Cys$_1$, with an energy of about 0.7−1.0 kcal/mol (Gane et al., 1995). To broaden the data to other members of the thioredoxin family, and to address the role of residue His$_3$, we present data on the thioredoxin-like N-terminal domain of PDI (designated as PDI *a*) (Edman et al., 1985). The isolated domain has been produced by protein-engineering methods and characterized functionally (Darby & Creighton, 1995a,b) and structurally using multidimensional NMR techniques (Kemmink et al., 1995, 1996). This facilitates the direct analysis of electrostatic effects through the determination of pK_a values of ionizable groups. The isolated domain has about the same stability as in intact PDI, so it seems to represent an independent structural module of that protein. It forms a disulfide bond between Cys$_1$ and Cys$_4$ with an equilibrium constant 9-fold greater than does DsbA, so the pK_a of the PDI *a*-domain Cys$_1$ thiol group would be expected to be

nearly 1 pH unit greater; it also has a His$_3$ residue. The pK_a of Cys$_1$ has been measured, as has its linkage with the ionization of His$_3$ and the effect of replacing His$_3$. The expected relationship between the cysteine thiol ionization and disulfide stability has been found to be valid in this protein also. The role of the charge on His$_3$ in stabilizing the negatively charged thiolate is established, and this, plus the interaction with the NH microdipoles at the helix terminus, probably accounts for much of the reduction of the cysteine thiol pK_a value.

MATERIALS AND METHODS

Materials. The PDI *a*-domain consists of residues 1−120 of human PDI, plus the initiating methionine residue; its expression, purification, and properties have been described previously (Darby & Creighton, 1995a). The His38 residue, which is designated His$_3$ here, was changed to a proline residue by oligonucleotide-directed mutagenesis using the "megaprimer" method (Sarker & Sommer, 1990). The sequence of this His$_3$/Pro mutant was confirmed by direct sequencing of the gene in the expression vector, and the protein was expressed and purified by the same procedures as the wild-type protein. The concentrations of the wild-type PDI *a*-domain and of the His$_3$/Pro mutant were determined from their absorbance at 280 nm, using the molar absorbance coefficient of 19 060 M^{-1} cm^{-1} calculated by the method of Gill and von Hippel (1989).

GSH and GSSG from Sigma were used without further purification; only freshly prepared solutions were used. All other chemicals were of reagent grade or better.

Ultraviolet Absorption Spectroscopy. The presence of the thiolate anion was monitored by its absorbance at 240 nm, using a Uvikon 930 UV−vis spectrometer (Kontron). All absorbance measurements were carried out at 25 °C in 1 mM each of potassium citrate, borate, and phosphate, with 10 mM, 200 mM, or 2 M KCl. Reduced PDI *a*-domain was prepared by adding DTT to a final concentration of 5 mM to the protein in 0.1 M Tris/HCl, pH 7.4. After 10−30 min incubation, the DTT was removed by gel filtration on a NAP-5 column (Pharmacia) equilibrated in the citrate/borate/phosphate buffer at the appropriate pH value. UV−absorbance measurements at 240 and 280 nm in a 1-cm pathlength cuvette were taken immediately after gel filtration to minimize air oxidation. Protein concentrations were usually between 5 and 15 μM. The absorption of the protein sample was measured against air and then corrected for the buffer absorbance by subtracting the absorbance of a reference buffer solution treated in the same manner. The pH of the protein and reference solutions was checked using a GK2402C Radiometer pH electrode with a Radiometer PHM 93 pH meter. The amount of disulfide form of the protein after the UV measurements was measured by reverse-phase HPLC and was found to be negligible.

The disulfide form of the PDI *a*-domain was prepared by adding GSSG to a final concentration of 0.1 mM to the protein in 0.1 M Tris/HCl, pH 7.4 and otherwise treated as described above.

Circular Dichroism Spectroscopy. Circular dichroism (CD) measurements were recorded on a Jasco-710 spectropolarimeter at 25 °C, in 1 mM each of phosphate, borate, and citrate, 0.1 mM EDTA, 200 mM KCl, at pH 4 or 7, using a 1-mm quartz cell. The protein concentrations were 10−15 μM.

^{1}H-NMR Spectroscopy. NMR measurements were performed at 25 °C in 99.8% ^2H_2O, with protein concentrations between 0.5 and 2 mM. Samples contained sodium 3-(trimethylsilyl)(2,2,3,3-$^2H_4$) propionate (TSP) as internal reference; in addition, reduced protein samples contained 5 mM deuterated DTT. The disulfide form of the PDI *a*-domain was prepared by adding GSSG to a final concentration of 0.1 mM to the dithiol protein in 0.1 M Tris/HCl, pH 7.4, incubating at 25 °C for about 30 min, and then dialyzing against H_2O. The protein samples were lyophilized and dissolved in 99.8% ^2H_2O. Spectra were recorded on a Bruker AMX-600 spectrometer operating at 600.141 MHz, using a spectral width of 8196 Hz. 1D NMR spectra were collected with 8192 points accumulating 512 or 1024 scans. TOCSY spectra (Braunschweiler & Ernst, 1983) were recorded at an uncorrected pH meter reading of 5.1 with a mixing time of 60 ms. Free induction decays were acquired with 2048 data points accumulating 64 scans in 512 t_1 increments. Water suppression was achieved by selective presaturation (1 s) during the relaxation delay.

Data were processed on a Silicon Graphics Indy workstation using the program UXNMR. Data points were weighted by a Lorentz−Gauss transformation in the t_2 dimension and a shifted squared sine-bell in the t_1 dimension. After zero-filling and Fourier transformation, the final data matrix contained 1024 × 1024 real data points. Polynomial baseline corrections were applied in both dimensions.

For titration experiments, the pH of the protein sample was adjusted using small amounts of NaO^2H or ^2HCl solutions and measured directly before and after recording of each 1D NMR spectrum, usually with good agreement. Measurements of the pH were not corrected for deuterium isotope effects and are therefore designated as pH*. The chemical shifts are given relative to the TSP standard, corrected for the pH-dependence of the chemical shift of TSP (De Marco, 1977).

Determination of pK$_a$ Values. pH titration curves obtained from monitoring UV-absorbance or chemical-shift changes were analyzed using the nonlinear regression routine included in the program Kaleidagraph (Abelbeck software). Data were fitted to a simple model derived from the Henderson−Hasselbach equation for one apparent pK_a value (pK_{app}). The errors given for the pK_{app} values are those obtained from the least-squares fitting routine.

Determination of the Equilibrium between His$_3$/Pro PDI a-Domain and Glutathione. Reduced His$_3$/Pro PDI *a*-domain at 4−6 μM was equilibrated at 25 °C in 0.1 M Tris, pH 7.4, 200 mM KCl, and 0.1 mM EDTA with different ratios of freshly prepared, excess GSH and GSSG for 30−40 min before quenching the reaction by addition of HCl to a final concentration of 0.1 M. Varying incubation times demonstrated that equilibrium had been reached under these conditions. Separation of the trapped thiol and disulfide species was performed by HPLC on a Vydac 218TP54 C18 reverse-phase column heated to 25 °C, eluting with a linear gradient of 30−36% acetonitrile in 0.1% TFA over 50 min. The absorbance was monitored at 220 nm, and the peaks were integrated using the Gilson HPLC software. Equal amounts of reduced and oxidized PDI *a*-domain His$_3$/Pro gave peaks of identical areas, showing that correction for different extinction coefficients or recoveries was not necessary. The extinction coefficients and recoveries of the mixed-disulfide species were assumed to be the same as those

of the reduced and oxidized proteins. A small portion of the reaction mixture was used to determine its total thiol content at the time of acid quenching by the method of Ellman (1959) in the absence of guanidinium chloride, using an ϵ_{412} of 14 150 M^{-1} cm^{-1} for the thionitrobenzoate anion. Since the concentration of GSH was in all cases in large excess over the protein, the total thiol content was taken as a measure of [GSH]. Air-oxidation of thiol groups was negligible with the short incubation times used. GSSG concentrations of stock solutions were measured by the ϵ_{248} of 381 M^{-1} cm^{-1} (Chau & Nelson, 1991).

RESULTS

pK$_a$ Determination of the Accessible Active Site Cysteine Thiol Group in the Wild-Type PDI a-Domain. The first objective in analyzing the electrostatic interactions in the active site of the thioredoxin-like PDI *a*-domain was to determine the ionization constant of the accessible active site Cys$_1$ thiol group (which is Cys36 of the protein). Previous data on the pH-dependence of the reactivity of the dithiol form of the PDI *a*-domain with GSSG suggested that the Cys$_1$ thiol group was substantially ionized at pH values as low as 5 (Darby & Creighton, 1995b). This did not, however, explicitly determine the thiol pK_a value, and such kinetic measurements may be very misleading with proteins of the thioredoxin family (Takahashi & Creighton, 1996). A direct way to follow the ionization of thiol groups is to measure the absorbance at 240 nm of the thiolate anion (Benesch & Benesch, 1955; Polgár, 1974; Graminski et al., 1989; LoBello et al., 1993). This requires comparison with a very similar control protein, differing only in the absence of the thiol group. The disulfide form of thioredoxin-like proteins suffices within the pH range where neither form of the protein unfolds. The buried thiol group of Cys$_4$ generally does not ionize in the accessible pH range and does not affect the measurements.

The pH-dependence of ϵ_{240} was measured for the dithiol form of the PDI *a*-domain at 25 °C and our standard conditions of 200 mM KCl (Figure 1). The data between pH 4 and 7.5 could be fitted to a single transition with an apparent pK_a value (pK_{app}) of about 4.5 (Figure 1). The best fits obtained in all cases yielded an amplitude of the absorbance change at 240 nm of about 5000 M^{-1} cm^{-1}, which is that expected for the titration of a single thiol group (Benesch & Benesch, 1955). The absorbance of the disulfide form of the PDI *a*-domain did not exhibit any pH-dependence between pH 4 and 7.5 (data not shown), confirming that the transition depicted in Figure 1 is due to thiol ionization. Above pH 7.5, the absorbance behavior of both the disulfide and dithiol forms of PDI *a*-domain was complicated by additional contributions of other ionizing residues and eventual unfolding.

Thermodynamic Linkage between the Thiol and Histidine Ionizations in the Wild-Type PDI a-Domain. For a detailed investigation of the energetic contribution of the electrostatic interaction between the thiolate anion of Cys$_1$ and His$_3$, the thermodynamic linkage between their ionizations must be considered. Any group stabilizing the thiolate anion and therefore lowering its pK_a value should be stabilized by the negative charge of the thiolate to the same extent. In quantitative terms, this is described by the thermodynamic cycle:
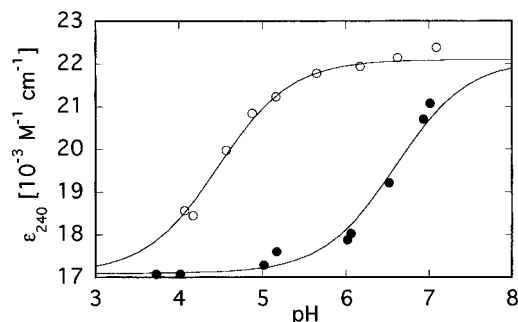
FIGURE 1: Cysteine thiol ionization of the dithiol forms of the wild-type and His$_3$/Pro PDI a-domains monitored by the absorbance at 240 nm at 25 °C and 200 mM KCl as a function of pH. The lines represent the fit using the Henderson−Hasselbalch equation to one apparent pK_a value. Open circles: wild-type PDI a-domain, pK_{app} = 4.50 ± 0.04. Filled circles: His$_3$/Pro PDI a-domain, pK_{app} = 6.57 ± 0.05. Data below pH 3.5 and above pH 7.5 reflect absorbance changes due to unfolding and ionization of titratable groups other than the accessible cysteine and were not included in the fit. Therefore, it was assumed that the two proteins had the same UV absorbance properties, except for that of the Cys$_1$ thiol group, as would be expected (Gill & von Hippel, 1989). Best fits were obtained using a low-pH base line of about 17 100 M^{-1} cm^{-1} and a high-pH base line of about 22 000 M^{-1} cm^{-1}, obtained from the His$_3$/Pro and wild-type proteins, respectively.

$$\begin{array}{c} & & P_{S^-}^{His+} & & \\ pK^{Cys}\nearrow & & & \searrow pK^{His} & \\ P_{SH}^{His+} & & & & P_{S^-}^{His} \\ pK^{\#His}\searrow & & & \nearrow pK^{\#Cys} & \\ & & P_{SH}^{His} & & \end{array} \qquad (1)$$

pK^{Cys} and pK^{His} are the pK_a values of the cysteine thiol group and the histidine imidazole group ionizing in the presence of a charge on the other side chain, while p$K^{\#Cys}$ and p$K^{\#His}$ correspond to when the other group is not ionized. The four ionization constants are linked by:

$$pK^{Cys} - pK^{\#Cys} = pK^{\#His} - pK^{His} \qquad (2)$$

$$\Delta pK^{Cys} = -\Delta pK^{His} \qquad (3)$$

In other words, if the ionization of Cys$_1$ increases the pK_a of His$_3$, ionization of His$_3$ must decrease the pK_a of Cys$_1$ to the same extent. The electrostatic stabilization of the thiolate provided by the charge−charge interaction with the histidine imidazole ($\Delta G_{His-Cys}$) is given by

$$\Delta G_{His-Cys} = 2.3RT\Delta pK^{Cys} = -2.3RT\Delta pK^{His} \qquad (4)$$

As Cys$_1$ ionizes with a pK_a lower than that of His$_3$, which will be raised by interaction with a thiolate anion (see below), the thiol pK_{app} value of 4.5 measured in Figure 1 should reflect mainly the value of pK^{Cys}, and the pK_{app} of His$_3$ in the dithiol form of PDI a will then correspond closely to the value of pK^{His}. Measurement of the value of p$K^{\#His}$ requires the absence of the charge on Cys$_1$, which can be approximated by the disulfide form of PDI a. Consequently, if the titration of the histidine imidazole is linked to the ionization of the cysteine thiol group, it should display different pK_{app} values in the disulfide and dithiol states of the protein.
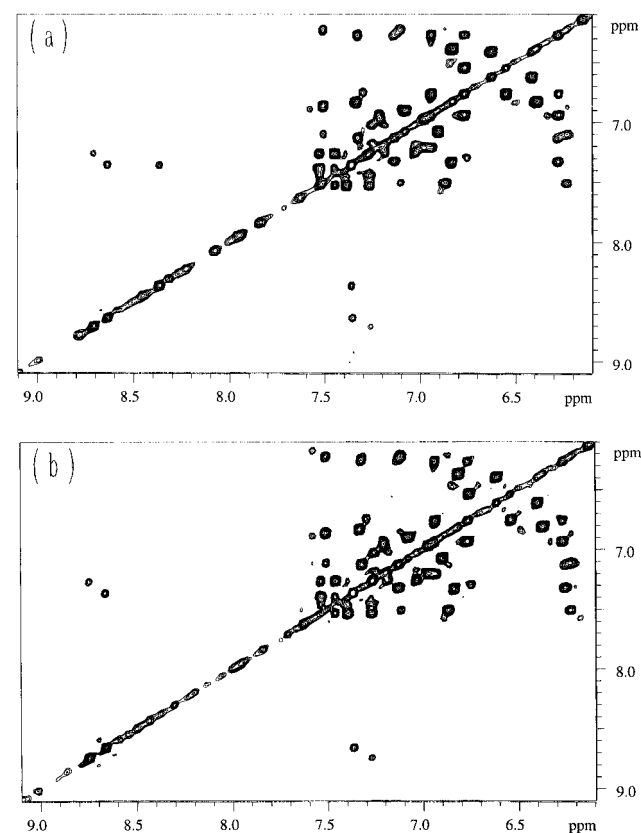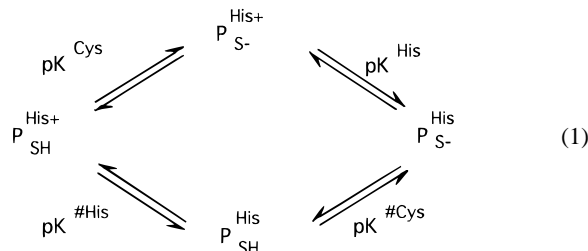

FIGURE 2: Assignment of the aromatic resonances of His$_3$. The TOCSY spectra were recorded with a mixing time of about 60 ms in ^{2}H$_2$O at 25 °C and pH* 5.1, the uncorrected pH meter reading: (a) wild-type PDI a-domain; (b) His$_3$/Pro PDI a-domain.

Assignment of the Active Site Histidine Residue in the Wild-Type PDI a-Domain. The resonance assignments and the solution structure of the PDI a-domain are known (Kemmink et al., 1995, 1996), but the side chain assignments for the three histidine residues in the protein were not complete, so it was necessary to assign the aromatic proton resonances corresponding to His$_3$. One prominent sequence difference in the active sites of the PDI a-domain and thioredoxin is the exchange of the active site His$_3$ residue to proline in thioredoxin. The high three-dimensional similarity of these two proteins (Kemmink et al., 1996) indicated that a histidine to proline exchange at this position of the PDI a-domain should cause minimal structural perturbations. This was observed, for the far-UV CD spectra of the wild-type PDI a-domain and the His$_3$/Pro mutant protein were indistinguishable (data not shown). Figure 2 shows the aromatic−aromatic TOCSY correlations for the disulfide forms of the wild-type and His$_3$/Pro PDI a-domains, obtained under identical conditions in 99.8% ^{2}H$_2$O. The spectra were extremely similar, except for the cross peak at 8.37 and 7.36 ppm observed only in the wild-type spectrum; this could be immediately assigned to the 2H−4H cross peak of His$_3$ of normal PDI a.

pK_a *Determination of the His$_3$ Imidazole Group in the Dithiol and Disulfide Forms of the Wild-Type PDI a-Domain.* The pH-dependence of the chemical shifts of the 2H histidine resonances was used to measure the pK_a values of the three histidine residues. The chemical shifts measured by 1D ^{1}H NMR in 99.8% ^{2}H$_2$O are depicted in Figure 3 for the dithiol and disulfide forms of the wild-type PDI a-domain. In the dithiol state, all the data points from all three histidine
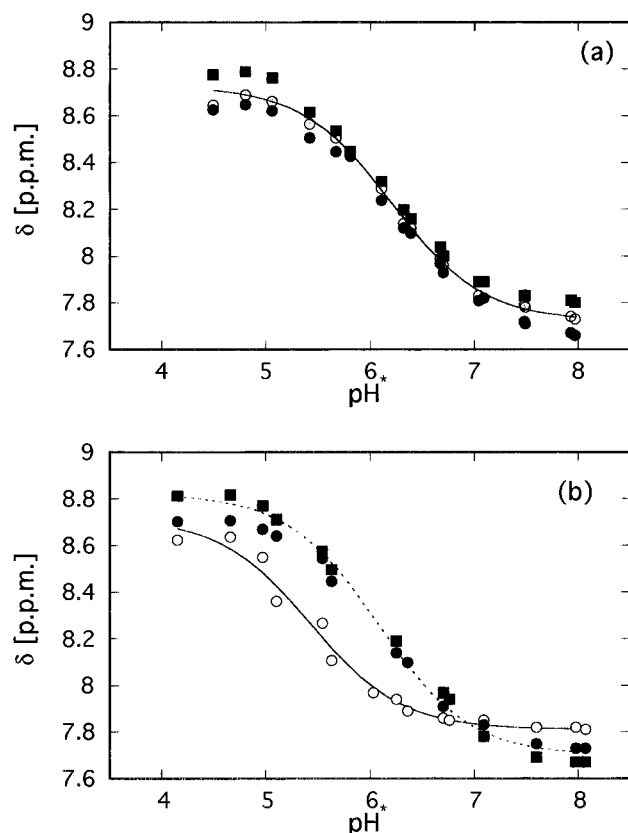
FIGURE 3: Titration of the histidine imidazole groups of PDI *a*-domain in the dithiol and disulfide forms. The chemical shifts of the 2H aromatic protons of the three histidine residues were measured at 25 °C in ^2H_2O as a function of pH*, the uncorrected pH meter reading. The curves give the fit using the Henderson−Hasselbalch equation to one apparent pK^*_{app} value. (a) Dithiol PDI *a*: all three histidine signals could be fitted within ± 0.1 pH unit to the same pK^*_{app} of 6.2. (b) Disulfide PDI *a*-domain: two histidine ionizations could be fitted to within ±0.1 pH unit to a single pK^*_{app} of 6.1. The third transition reflects the ionization of the active site His₃, with pK^*_{app} of 5.4 ± 0.1.

residues could be fitted with the same single ionization transition, with pK^*_{app} of 6.2 ± 0.1 (the asterisk indicates that the pH meter reading was uncorrected for deuterium isotope effects). In contrast, only two transitions overlapped in the disulfide form, displaying within experimental error the same pK^*_{app} of 6.1 ± 0.1. The third resonance, that assigned to the side chain of His₃, displayed the significantly altered pK^*_{app} of 5.4 ± 0.1. These results are consistent with expectation, in that only His₃ is close to the active site of PDI *a*. The other two histidine residues are on the surface of the protein, distant from the active site; consequently, they have normal pK_a values that are not affected by the changes at the active site. A similarly altered, but slightly greater histidine imidazole pK^*_{app} value of about 5.7 was found in a mutant of PDI *a* in which Cys₁ was replaced by serine (data not shown).

pKₐ Determination of the Cys₁ Thiol Group in the His₃/Pro Mutant PDI a-Domain. From the linkage between the thiolate and histidine titrations demonstrated above, removal of the charge on the His₃ side chain would be expected to increase the thiolate pK_{app}, to $pK^{\#Cys}$, and to increase the stability of the disulfide bond in the His₃/Pro protein, if the structures of the wild-type and mutant proteins are otherwise comparable. Their almost identical CD spectra and patterns of aromatic side-chain correlations in the two proteins (Figure

Table 1: Equilibrium Constants for the Thiol−Disulfide Exchange Reactions between the PDI *a*-Domain and Glutathione To Form the Protein Disulfide Bond[a]

| PDI *a*-domain	K_{GSSG} (mM)	K_1	K_2 (mM)
His₃/Pro	142 ± 10	3.7 ± 0.3	40 ± 5
wild-type[b]	0.7 ± 0.13	0.01 ± 0.002	69 ± 10

[a] The equilibrium constants K_1, K_2, and K_{GSSG} are defined in eqs 5 and 6. The values are the mean ± standard deviation of 11 or 12 experiments for the His3/Pro and wild-type PDI *a*-domains, respectively. [b] From Darby and Creighton (1995a), under the same conditions as used in this study.

2) indicate that they are structurally very similar. Therefore, the ionization properties of the His₃/Pro mutant of the PDI *a*-domain were investigated in more detail.

Figure 1 shows the thiolate absorbance at 240 nm of the dithiol form of the reduced His₃/Pro mutant as a function of pH. With this protein also, the control experiment performed with the disulfide form of the mutant protein did not reveal any pH-dependent changes of ϵ_{240} in the respective pH range (data not shown). Comparison of the ionization transitions of the wild-type and His₃/Pro proteins in Figure 1 shows clearly that the latter has its thiol pK_{app} increased by about 2.1 pH units, to 6.57 ± 0.06. This increase in pK_a is consistent qualitatively with the linkage measured using the $pK_{app}*$ of His₃ (Figure 3), but is considerably greater in magnitude.

Stability of the Disulfide Bond Measured in the His₃/Pro PDI a-Domain. The inverse relationship between the pK_a value of the accessible cysteine thiol group and the stability of the disulfide bond (Grauschopf et al., 1995) suggests that the increased pK_a of the Cys₁ thiol group in the His₃/Pro mutant should cause its disulfide bond to be more stable than in the wild-type protein. Therefore, the equilibrium constants for the reaction between the His₃/Pro mutant PDI *a*-domain and glutathione were measured.

$$ (5) $$
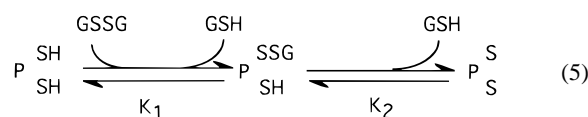
K_1 describes the equilibrium for forming a mixed disulfide, P^{SSG}_{SH}, between Cys₁ of the dithiol protein, P^{SH}_{SH}, and glutathione; K_2 is the equilibrium constant for the formation of the protein disulfide bond from the mixed disulfide. The overall equilibrium constant for the oxidation of the dithiol species, K_{GSSG}, is given by

$$ K_{GSSG} = K_1K_2 \qquad (6) $$

The value of K_{GSSG} for the PDI *a*-domain His₃/Pro mutant was determined to be 142 mM, about a factor of 200 greater than that determined for wild-type PDI *a* (Table 1). The two microscopic equilibrium constants could be determined by quantifying the amount of the mixed-disulfide intermediate. The accuracy of these values depends on the validity of the trapping procedure (Darby & Creighton, 1995b). The results indicated that most of the difference in the overall equilibrium constants was reflected in the value of K_1, which is the equilibrium constant that should be affected if the factor responsible is ionization of Cys₁ in P^{SH}_{SH}. The alternative possibility, of changes in the energy of the protein disulfide bond, should affect the value of K_2. That primarily K_1 is

Table 2: Effects of Salt on the Thiol pK_{app} Values in the Wild-Type and His₃/Pro PDI *a*-Domains[a]

PDI *a*-domain	pK_{app}			
	0.01 M KCl	0.2 M KCl	2 M KCl	ΔpK_{app}[b]
wild-type	3.95 (0.05)	4.50 (0.04)	5.25 (0.04)	1.30
His₃/Pro	6.45 (0.07)	6.57 (0.05)	7.04 (0.10)	0.59

[a] Apparent pK_a values were obtained from the pH dependence of the UV absorbance at 240 nm at 25 °C, as in Figure 1. The errors given in parentheses are those obtained from the least-squares fitting routine. [b] Difference between the pK_{app} values at 2 and 0.01 M KCl.

altered indicates that the difference in the disulfide bond stabilities is caused mainly by different thiolate stabilization in the dithiol protein, which is reflected in the altered thiol pK_{app} value.

Salt-Screening Effects on the Thiol Ionization in the Wild-Type and His₃/Pro PDI a-Domains. A charge—charge interaction between the thiolate and the protonated histidine imidazole should be screened by salt, whereas an interaction like a hydrogen bond should be resistant to screening effects (Huyghues-Despointes et al., 1993). Table 2 summarizes the thiol pK_{app} values obtained for the normal and His₃/Pro PDI *a*-domains at 10 mM, 200 mM, and 2 M KCl. Both proteins displayed an increase in the apparent thiol pK_a values with increasing ionic strengths, consistent with the screening of electrostatic interactions in both cases. The magnitude of the effect was greater, however, with the wild-type protein, where the His₃ residue is present. Even at a high ionic strength of 2 M KCl, the Cys₁ thiol pK_a value was significantly perturbed from normal in both proteins.

DISCUSSION

Linkage of Disulfide Bond Stability and Thiol Ionization in the Thioredoxin Motif. The disulfide bond that can be formed at the active site of the thioredoxin fold, between the residues −Cys₁−Xaa₂−Yaa₃−Cys₄−, can vary in stability by at least 7 kcal/mol in the different family members, a difference in equilibrium constant of 10^5-fold. The structural identity of the active site disulfide bonds makes it unlikely that their stability differences are due to different extents of strain in the disulfide form of the thioredoxin-like proteins. Instead, the variation in stability appears to arise primarily from differing degrees of stabilization of the dithiol forms of each protein. This variable stabilization involves the thiolate anion of Cys₁, which interacts to varying extents with the folded conformation and is reflected in the pK_a value of the thiol group. Strong interaction and stabilization of the thiolate anion cause it to have a low pK_a value and the disulfide bond to have a low stability. This relationship between the pK_a value of the Cys₁ thiol group and the stability of the disulfide bond it forms makes it possible to predict one parameter from the other.

This relationship was first demonstrated in DsbA (Nelson & Creighton, 1994; Grauschopf et al., 1995), but the present results show that the very same relationship seems to hold in the PDI *a*-domain (Figure 4). It also applies to thioredoxin if its Cys₁ thiol group has a pK_a of about 9, as determined by Takahashi and Creighton (1996). The same relationship need not necessarily apply to all members of the thioredoxin fold. Any mutations altering interactions of the nonionized thiol groups of Cys₁ or Cys₄ with the folded conformation, or the stability of the disulfide bond directly, would be
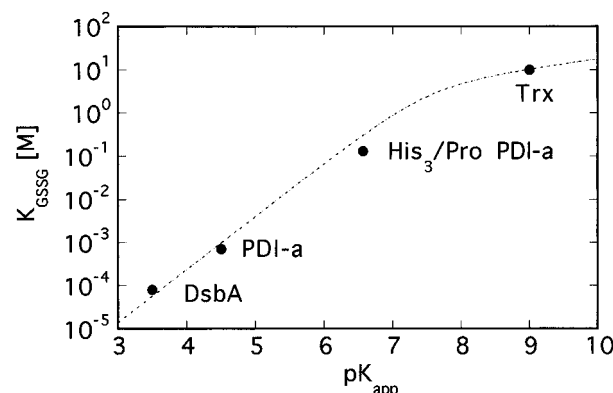

FIGURE 4: Observed relationship between the stability of the disulfide bond of the thioredoxin fold, $\log(K_{GSSG})$, and the pK_{app} of the Cys₁ thiol group. The data are for DsbA (Zapun et al., 1993; Nelson & Creighton, 1994), the wild-type and His₃/Pro mutant PDI *a*-domains (Figure 1 and Table 1), and *E. coli* thioredoxin, designated Trx (Takahashi & Creighton, 1996). The curve represents the expected pH-dependence of the equilibrium constant for reaction with glutathione, K_{GSSG}, at pH 7.5 calculated for the case where only the Cys₁ thiol pK_a differs, no other groups differ in ionization in the thiol and disulfide forms when K_{GSSG} is measured, and with the thiol pK_a of GSH taken as 8.7. All the measurements were made under the same conditions; the values of K_{GSSG} were measured in 0.1 M Tris HCl, pH 7.4 or 7.5, 200 mM KCl at 25 °C. A very similar relationship was demonstrated over the pK_a range 3.4−6.7 for mutant forms of DsbA by Grauschopf et al. (1995), but their measurements were performed under somewhat different conditions.

expected to alter independently either the ionization of the thiol group or the net stability of the disulfide bond. The remarkable relationship between these two parameters in different proteins (Figure 4) suggests that the nonionized dithiol and the disulfide forms of the various thioredoxin folds have the same relative free energies. They will then have the same stability of the disulfide bond at low pH, where the thiol groups are not ionized. The stabilities of the disulfide bonds will diverge at higher pH values, simply due to mass-action effects of differential ionization of the thiol groups. Measurements with model peptides (Siedler et al., 1993) indicate that there are likely to be small, but significant, differences in conformational strain in the various −Cys₁−Xaa₂−Yaa₃−Cys₄− disulfide loops, relative to the unstructured polypeptide chain, but the same strain will be present in the dithiol form of the protein if its conformation prevents these residues from relaxing when the disulfide bond is broken, as appears to be the case in thioredoxin (Dyson et al., 1990; Forman-Kay et al., 1991; Qin et al., 1994; Jeng et al., 1994; Holmgren, 1995).

Any protein disulfide bond that is more stable in the folded conformation than when the protein is unfolded must, for thermodynamic reasons, stabilize the folded conformation, as has been observed for thioredoxin (Kelley et al., 1987). In contrast, any disulfide bond that is less stable in the folded state, as are those of DsbA, DsbC, and PDI *a*, must destabilize the folded conformation, as has also been observed (Wunderlich et al., 1993; Zapun et al., 1993, 1995; Darby & Creighton, 1995b). When the native disulfide bond is destabilized indirectly, by stabilization of the dithiol protein, as in these cases, the physical basis of the destabilization is clear: the favorable interaction with the thiolate anion in the reduced protein must stabilize the folded conformation, and this stabilization is absent in the disulfide form of the protein. Disulfide stability, thiol ionization, and folded conformation stability are all linked functions.

Electrostatic Factors Contributing to Thiolate Stabilization. As the low Cys_1 thiol pK_a value appears to be a crucial factor for the oxidizing properties of the PDI *a*-domain and DsbA, the question remains how this substantial thiolate stabilization is achieved in the thioredoxin motif. One major factor is believed to be the location of Cys_1 at the N-terminus of an α-helix, with its positive NH microdipoles (Hol, 1985; Katti et al., 1990; Aquist et al., 1991; Nelson & Creighton, 1994). In confirmation, cysteine thiol groups at the N-termini of model α-helical peptides also have decreased pK_a values, whereas those at the C-terminus are increased (Kortemme & Creighton, 1995). Yet, all proteins of the family have Cys_1 residues in the same position, but their Cys_1 thiol pK_a values and disulfide stabilities vary widely. Additional charge−charge interactions important for thiolate stability can be unraveled by considering the linkage of thiol ionization to that of other titratable groups (eq 1). The present study demonstrates such a linkage of the ionizations of the Cys_1 thiol group and the imidazole group of His_3 present in the thioredoxin-like domains of the oxidizing proteins DsbA and PDI, but absent in thioredoxin. The His_3 imidazole pK_a value was altered in the presence and absence of the thiolate. The latter state is considered to be best approximated by the disulfide form of the protein, with the assumption that forming the disulfide bond in the PDI *a*-domain only removes the charge on the thiolate, with otherwise only minimal perturbations, as was observed with the highly similar structures of oxidized and reduced thioredoxin (Holmgren, 1995). This comparison gives a His_3 pK_a value difference of about 0.8 pH units in the presence and the absence of the thiolate and an energy of the interaction (eq 4) of about 1.1 kcal/mol. A very similar energy of interaction was obtained from electrostatic calculations in DsbA (Gane et al., 1995).

The analysis carried out here is further strengthened by the results of the salt screening on the wild-type and the His_3/Pro mutant PDI *a*-domains. Both proteins had increased apparent thiol pK_a values at greater ionic strengths (Table 2), as expected, since both proteins should possess a charge−helix interaction that was screenable by salt to some extent in model α-helical peptides (Kortemme & Creighton, 1995). The magnitude of the salt-screening effect was, however, greater in the wild-type protein, where the thiol pK_{app} was increased by about 1.3 pH units in 2 M salt, compared to only 0.6 pH units in the His_3/Pro protein. If one assumes that the charge−dipole interactions in both forms of the protein are screened to the same extent by 2 M salt, the difference of 0.7 pH units can be attributed to screening of the charge−charge interaction between the thiolate and the histidine side chain present in the wild-type protein. This interaction should nearly be abolished in high salt, and the magnitude of the screening effect is very similar to the 0.8 pH units found from the thermodynamic linkage to the ionization of the His_3 side chain.

In both the wild-type and His_3/Pro PDI *a*-domains, the Cys_1 thiol pK_a value was significantly perturbed from normal even at the extremely high ionic strength of 2 M KCl. Such a resistance to salt screening is commonly taken as an indication of hydrogen bonding (Huyghues-Despointes et al., 1993). The presumed interaction between the thiolate anion and the NH microdipoles at the end of the helix can be considered as a combination of simple electrostatics and more specific hydrogen bonding "capping" interactions (Baker &

Hubbard, 1984). With the present data, however, it is not possible to exclude a role of other electrostatic interactions sufficiently shielded from the solvent to be unaffected by the ionic strength of the medium.

The ionization of the His_3 side chain also seems to be affected by the α-helix dipole, as was previously observed for histidine residues at α-helix termini in barnase (Sancho et al., 1992). Otherwise, one would expect the histidine imidazole pK_a value to be elevated in the dithiol state by the interaction with the thiolate anion, but normal in its absence in the disulfide form. The pK_a was, however, depressed in the disulfide form of the protein, where there is no charged group in the active site (Figure 3), other than the partial charges of the dipoles of the α-helix. In contrast, the pK_a of His_3 was normal, when the Cys_1 thiolate anion was present.

The Importance of the Central Residues in the $−Cys_1−$ $Xaa_2−Yaa_3−Cys_4−$ *Motif for Thiol Ionization and Disulfide Bond Stability.* The identities of the two central residues $Xaa_2−Yaa_3$ vary between, but normally not within, the subfamilies of thioredoxin-like proteins, and have been shown to have substantial effects on the stability of the disulfide bond formed between Cys_1 and Cys_4 (Joelson et al., 1990; Krause et al., 1991; Lundström et al., 1992; Gleason, 1992; Wunderlich, 1994) and on the pK_{app} of the Cys_1 thiol group (Grauschopf et al., 1995). The importance of these residues is confirmed here by the large change in both parameters observed upon replacing His_3 of PDI *a* with Pro, to generate the active site sequence of thioredoxin, $−Cys−Gly−Pro−Cys−$. If this change were simply due to the removal of the charge on the His_3 side chain, the thiol pK_a value should be increased by about 0.8 pH. The observed increase was, however, much greater; it varied from 1.8 to 2.5 pH units, depending on the salt concentration (Table 2), corresponding to an interaction energy of up to about 2.9 kcal/mol. The difference cannot be solely explained by the absence of the charge−charge interaction, which should be nearly fully screened at high salt concentrations, and indicates the subtlety of the effects of changing residues Xaa_2 and Yaa_3.

Substantial changes occur in the pK_a value of the Cys_1 thiol groups, and in their tendencies to form disulfide bonds, upon altering residues Xaa_2, Yaa_3, and Cys_4, even when the residues altered are nonpolar (Wunderlich, 1994; Zapun et al., 1994, 1995; Grauschopf et al., 1995). Such effects upon altering the residues adjacent to the cysteine can also be observed in model peptides, although diminished in magnitude (T. Kortemme and T. E. Creighton, unpublished observations); they may reflect subtle differences in the accessibility to solvent of the various polar groups (Warwicker & Gane, 1996).

Implications for the Differences in Redox Potential in the Thioredoxin Family. In all proteins with the thioredoxin motif, the Cys_1 residue is located at the N-terminus of an α-helix, which should decrease the thiol pK_a through an interaction with the positive NH microdipoles (Hol, 1985; Åquist et al., 1991; Kortemme & Creighton, 1995). Yet, in thioredoxin the Cys_1 pK_a value is certainly not decreased to the same extent as in DsbA or the PDI *a*-domain (Holmgren, 1995), and is probably increased (Takahashi & Creighton, 1996). The solution to this apparent paradox is most likely due to a combination of factors. Firstly, the interaction between the Cys_1 thiolate anion and the α-helix microdipoles

appears to be exceedingly sensitive to the microenvironment at the helix terminus. Secondly, the charge−charge interaction of the Cys_1 thiolate with His_3 appears to provide additional stabilization in the oxidizing proteins only, consistent with modeling studies (Gane et al., 1995). The linkage of thiol ionization to ionization of another group that ionizes in another pH region might be physiologically important. The intrinsic reactivity of a sulfur atom is related to its ionization when a thiol group (Szajewski & Whitesides, 1980); thiol groups with low pK_a values are more ionized, which is usually necessary for thiol reactivity, but such sulfur atoms have a lower intrinsic reactivity (as a thiolate anion or as part of a disulfide bond). The linkage of the thiol ionization of the PDI *a*-domain Cys_1 residue to that of His_3, with a pK_{app} of 6.1, results in the Cys_1 sulfur atom having a pK_a of 4.5 at low pH but an effective pK_a of 5.3 at higher pH, and an increased reactivity.

Finally, factors other than just residues Xaa_2 and Yaa_3 must also be important for the functional properties of the thioredoxin motif. Changing these two residues of the PDI *a*-domain to those of thioredoxin, in the His_3/Pro mutant protein, produced changes in the thiol pK_a and disulfide bond stability only about $^2/_3$ of those observed in authentic thioredoxin (Figure 4), in spite of the high structural and topological similarity of the PDI *a*-domain and thioredoxin (Kemmink et al., 1996).

Finite difference electrostatic calculations (Gane et al., 1995; Warwicker & Gane, 1996) attributed a large proportion of the redox potential difference between DsbA and thioredoxin to the altered contributions of protein backbone dipoles to thiolate stabilization. Part of this effect was proposed to be influenced by the insertion of one residue into the active site α-helix of DsbA and by the presence of an additional α-helical domain, inserted into the thioredoxin fold of DsbA, extending the electric field range in this protein. In this context, it is significant that the PDI *a*-domain accommodates a thiol pK_a value almost as low as that measured for DsbA, and correspondingly a very unstable disulfide bond, in the absence of both of the above mentioned regions adjacent to the active site. These considerations pose the question whether a modulation of main-chain dipolar interactions, similar to that proposed for DsbA, is provided by a different region of the protein in the PDI *a*-domain or whether other mechanisms are involved. Due to the high structural similarity of the PDI *a*-domain to thioredoxin and the identical topology of the two proteins, PDI *a* represents an ideal model to investigate these effects.

CONCLUSIONS

The ionization properties of active site residues are crucial determinants in modulating the redox potentials in oxidoreductases of the thioredoxin superfamily and therefore are important for an understanding of the various reactivities exhibited by members of the family. The current study confirms that the accessible cysteine thiol group in the thioredoxin-like PDI *a*-domain possesses an unusually low pK_a value, which is comparable to that measured previously in DsbA, another very oxidizing member of the thioredoxin family, and consistent with the high oxidizing potential of these two proteins.

In order to have its pK_a value lowered from a normal value of about 8.7 to the value of 4.5 measured here, the cysteine thiolate has to be stabilized by about 5.7 kcal/mol in the reduced protein. The histidine residue present in the active sites of DsbA and the PDI *a*-domain is shown to provide a charge−charge stabilization of the thiolate in the reduced form of the PDI *a*-domain of about 1.1 kcal/mol. This interaction can be added to the list of factors contributing to the unusual cysteine thiol pK_a in the protein. A large proportion of the remaining stabilization energy is thought to arise from an interaction with the peptide dipoles at the N-terminus of an α-helix pointing toward the active site. The strength of this interaction appears to be modulated through subtle conformational effects. The thermodynamic linkage of ionization properties should prove to be a useful method to identify other factors influencing the redox potential and to estimate their contributions.

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