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Linkage of Thioredoxin Stability to Titration of Ionizable Groups with Perturbed pK_a†

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ABSTRACT: The highly conserved, buried, Asp 26 in *Escherichia coli* thioredoxin has a pK_a = 7.5, and its titration is associated with a sizable destabilization of the protein [Langsetmo, K., Fuchs, J., & Woodward, C. (1991) *Biochemistry* (preceding paper in this issue)]. A fit of the experimental pH dependence of thioredoxin stability to a theoretical expression for the pH/stability relation in proteins agrees closely with a pK_a value of 7.5 for Asp 26. The agreement between the experimental and theoretical changes in protein stability due to substitution of Asp 26 by alanine is also good. The local structure in the vicinity of Asp 26 in the low-pH crystal structure (with uncharged Asp 26) is hydrophobic, indicating that the aspartate would be highly destabilized. In theoretical calculations, the desolvation penalty for deprotonating Asp 26 in this environment is similar to the total protein folding energy. As a consequence, the Asp 26 pK_a would be much greater than 7.5, and/or the protein might not fold. This suggests that a compensating process partially stabilizes the Asp 26 carboxyl group when it is charged. A simple model for this is proposed, whereby the Lys 57 side chain rotates to form a salt bridge with Asp 26 when it is deprotonated.

Thioredoxin contains a buried aspartic acid at residue 26, with a pK_a of 7.5 (Langsetmo et al., 1991). Deprotonation of Asp 26 significantly destabilizes the protein. Conversely, replacement of Asp 26 with alanine enhances the stability of the "mutant" D26A[1] relative to WT (Langsetmo et al., 1990). The evolutionary conservation of Asp 26 in various species of thioredoxin (Gleason & Holmgren, 1988) implies an important biological role for its destabilizing effect. Its function may be to exert a critical influence on thioredoxin catalytic activity by pH-dependent modulation of the redox potential of the active site disulfide bond.

Expressions have been developed for the pH dependence of the stability of a protein when stability is linked to titration of an ionizable group with perturbed pK_a (Yang et al., 1991). We show here that the pH dependence of $\Delta G°$ for thioredoxin unfolding in the vicinity of Asp 26 pK_a, pH 6-9, is in excellent agreement with the predictions of this analysis. Likewise, the

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[1] D26A, thioredoxin with Asp 26 replaced by alanine; WT, wild-type thioredoxin.

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pH dependence of the difference in free energy of WT thioredoxin and D26A, $\Delta\Delta G^{\circ}_{(WT \rightarrow D26A)}$, fit very well with this analysis in the range pH 6–9.

To understand the structural basis for the abnormal pK_a of Asp 26, we have examined the electrostatics of thioredoxin using calculations that explicitly include the effects of solvation. The destabilization arising solely from desolvation (burial) of unprotonated Asp 26 is substantial, around 12 kcal/mol for the charged form, and comparable to the net stabilization energy of the folded protein, 8.6 kcal/mol at pH 8.5 (Langsetmo et al., 1991). Unless a compensating stabilization is provided by other protein interactions, the desolvation penalty alone would prevent folding of the protein. The low-pH crystal structure, in which Asp 26 is presumably uncharged, provides no direct indication of a mechanism for stabilization of the charged aspartate. No differences between thioredoxin with charged versus uncharged Asp 26 are indicated by circular dichroism, suggesting that no substantial conformational changes increase the Asp 26 exposure to solvent. However, modeling with the combined use of molecular mechanics and electrostatic calculations predicts a small but critical conformational change in thioredoxin as it goes from unprotonated to protonated Asp 26. This involves a rotation of the Lys 57 side chain that leads to formation of a salt bridge between Asp 26 and Lys 57. This stabilizing interaction would offset the desolvation penalty of charging Asp 26.

The model for thioredoxin containing the Lys 57–Asp 26 interaction was developed with a simple procedure where the key parameter is the salt-bridge distance. The procedure combines use of molecular mechanics to generate conformations with use of electrostatic calculations to assess the effect of conformational changes on solvation energies. The model of thioredoxin with the salt bridge predicts a pK_a value for Asp 26 of 7.5 ± 1 in good agreement with the experimental value of 7.5. An expression for the multiple equilibria describing the effect on thioredoxin stability of the putative Lys 57–Asp 26 salt bridge is derived from the treatment of Yang et al. (1991). A pK_a for Lys 57 of 10.25 is obtained from fits of this relationship to thioredoxin stability data over the range pH 6–10.5, which includes the titration range of lysine.

Materials and Methods

Determination of pK_a. The average degree of protonation of an ionizable group, f, in a protein is given by

$$\ln [f/(1-f)] = (\mathrm{pH} - pK_a)/2.303 \quad (1)$$

where the right-hand side is the energy of proton dissociation. The pK_a can be written as

$$pK_a = pK_a^{int} + \Delta G^{ttr}/(2.303kT) = pK_a^0 + (\Delta\Delta G^{env} + \Delta G^{ttr})/2.303kT \quad (2)$$

where pK_a^{int} is the intrinsic pK_a of the group in the protein, i.e., the pK_a it would have in the absence of all other formally charged groups on the protein (Sharp & Honig, 1990). The term ΔG^{ttr} accounts for the pK_a shift due to the potential at the group arising from other ionizable groups. The intrinsic pK_a is comprised of two terms, the reference state pK_a^0 (typically the pK_a of the isolated group in water, which is known) and $\Delta\Delta G^{env}$, the extra electrostatic energy of dissociating the proton in the protein environment relative to its reference state. The latter in turn may be split into two contributions:

$$\Delta\Delta G^{env} = \Delta\Delta G^{slv} + \Delta\Delta G^{dip} \quad (3)$$

where $\Delta\Delta G^{slv}$ is the difference in solvation energy of the group in the reference state and in the protein and $\Delta\Delta G^{dip}$ is the energy due to the potential from the protein dipoles acting upon the group. $\Delta\Delta G^{slv}$ also includes any difference in ionic strength effects upon the protonation energy between the reference and folded states.

Calculation of Electrostatic Potentials. The program DelPhi [Gilson et al., 1988; Nicholls & Honig, 1990; Sharp & Nicholls, 1990; for a review see Sharp and Honig (1990)] was used to calculate electrostatic potentials. This program solves the Poisson–Boltzmann (PB) equation

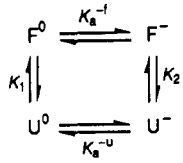
$$\nabla(\epsilon(r)\ \nabla\phi(r)) - \epsilon\kappa^2 \sinh(\phi(r)) = -4\pi\rho^f(r) \quad (4)$$

which relates the potential ϕ at any position r to the protein charge density distribution ρ^f, the dielectric constant ϵ in both the protein and the solvent, and the ionic strength of the solvent (through the Debye length $\lambda = 1/\kappa$). Physiological ionic strength (0.15 M, $\lambda = 8$ Å) and a dielectric of 80 were used for the solvent. A Stern layer of 2 Å was placed around the protein, which limits the closest approach of solvent ions. A dielectric of 4 was used for the protein, on the basis of a number of theoretical and experimental studies (Gilson & Honig, 1986; Nakamura et al., 1988; Soman et al., 1989). DelPhi uses a finite difference method to solve the PB equation. The protein is mapped onto a 65 × 65 × 65 grid, so that the molecule fills about 70% of the grid, the remainder being filled by solvent. Charges were assigned to atoms with use of the DISCOVER all-atom potential function (Hagler et al., 1973). The charge assigned to each atom is distributed onto the lattice as described previously (Gilson et al., 1988). Lattice points that fall inside the molecular surface of the molecule are assigned a dielectric of 4, those outside a dielectric of 80. The molecular surface defines the region inaccessible to any part of a 1.4-Å-radius solvent probe sphere (Lee & Richards, 1971). Debye–Huckel boundary conditions were applied to the edge of the grid (Klapper et al., 1986). After assignment of the parameters, the finite difference equations are solved by optimized successive overrelaxation (Nicholls & Honig, 1990) to obtain the potential at all the grid points. A typical calculation takes about 15 s of computer time on a Convex C2 computer.

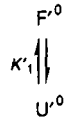
Solvation Energy Calculations. The contribution of the change in solvation energy to the pK_a of a ionizable group, $\Delta\Delta G^{slv}$, is determined by four DelPhi calculations. Charges are assigned only to the ionized form of the group in question, and (i) the potentials ϕ^W are determined for the isolated group in water (i.e., all other protein atoms are omitted) and (ii) The potentials ϕp are determined for the group as part of the uncharged protein (i.e., all other protein atoms are included, but they are not assigned a charge). Then, the sum $\Delta G^{rxn} = \sum q_i(\phi_i^P - \phi_i^W)/2$ is computed over all charges q_i of the group, where $\Delta\phi_i^x = \phi_i^P - \phi_i^W$ is the change in reaction potential due to the desolvation of the group by the low dielectric environment of the protein and ΔG^{rxn} is the change in the solvation energy of the group (Sharp & Honig, 1990; Nicholls & Honig, 1990; Sharp, 1990). The same pair of calculations is repeated for the neutral form of the group, and $\Delta G^{slv} = \Delta G^{rxn}(\text{charged}) - \Delta G^{rxn}(\text{neutral})$.

Charge–Charge Interactions. The charge–charge interaction term arising from the ionizable group–protein dipole interaction, $\Delta\Delta G^{dip}$ is obtained from DelPhi run (ii) above, by summing the potential from the group at all the dipolar (partially charged) atoms of the protein, q_j, excluding the ionizable group itself and any formally charged groups: $\Delta\Delta G^{dip} = \sum_{j \neq i} q_j(\phi_j^P(\text{charged}) - \phi_j^P(\text{neutral}))$. The contribution from the other charged groups, $\Delta\Delta G^{ttr}$, is given by the comparable sum over all the formally charged groups. The Asp 26–Lys

Scheme I



Scheme II



57 interaction was evaluated separately as $\Delta G^{Asp/Lys} = [\Delta G(Asp^-/Lys^+) - \Delta G(Asp^-/Lys^0)] - [\Delta G(Asp^0/Lys^+) - \Delta G(Asp/Lys)]$, where the superscripts +, −, and 0 refer to the charged and neutral forms.

Model Building. Various models for the Asp 26–Lys 57 salt bridge were generated with use of DISCOVER (Hagler et al., 1973). Initial coordinates were taken from Katti et al. (1990) and kindly provided by Dr. Hans Eklund. The coordinates of all residues except Lys 57 were fixed, and a series of distance constraints applied to the Lys 57 NZ–Asp 26 OD1 distance. For each constraint the system was minimized with use of 500 steps of conjugate gradient minimization. Minimization was performed without any electrostatic interactions. The result is a series of 16 stereochemically and van der Waals unstrained conformations of thioredoxin, closely resembling the crystal structure, but with varying salt-bridge distances. The pK_a's of Asp 26 and Lys 57 and the Asp 26–Lys 57 interaction energy were evaluated for the original crystal structure and each of the conformations with use of DelPhi.

Stability Calculations. The shift in pK_a of a titratable group upon incorporation into a protein is related to protein stability (Tanford & Kirkwood, 1957; Matthew & Gurd, 1986). For the case of Asp 26 in thioredoxin, the thermodynamic cycle representing this linkage is given in Scheme I of Langsetmo et al. (1991). This cycle may be written in the more general notation of Yang et al. (1991) (Scheme I) where F is the folded form of the protein, U the unfolded form, with the superscripts 0 and − referring to neutral (protonated) and acidic (unprotonated) Asp 26; $K_a = 10^{-pK_a}$, with the superscripts f and u referring to the folded and unfolded forms of the protein; K_1 and K_2 are the equilibrium constants for cooperative unfolding of thioredoxin with protonated and unprotonated Asp 26: $K_1 = [F^0]/[U^0]$ and $K_2 = [F^-]/[U^-]$.

The net protein unfolding equilibrium constant for the wild-type (WT) protein is $K_{WT} = \sum[F]/\sum[U]$. The variation of K_{WT} with pH for the equilibria in Scheme I is given by

$$K_{WT} = \frac{[F^0](1 + K_a^{-f}/[H^+])}{[U^0](1 + K_a^{-u}/[H^+])} = K_1\frac{(1 + K_a^{-f}/[H^+])}{(1 + K_a^{-u}/[H^+])} \quad (5)$$

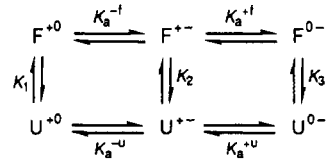
If we now consider the mutant D26A, with aspartate replaced by alanine at position 26, the equilibrium corresponding to Scheme I is given in Scheme II, where the prime indicates the mutant and $K'_1 = [F'^0]/[U'^0]$. The superscript 0 stands for the neutral alanine at position 26 in both folded and unfolded protein. For D26A, the net protein unfolding equilibrium constant, K_{mut}, is simply K'_1.

Then, for thioredoxin over the pH range of Asp 26 titration

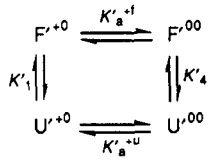
$$K_{WT}/K_{mut} = \frac{K1}{K'1}\frac{(1 + K_a^{-f}/[H^+])}{(1 + K_a^{-u}/[H^+])} \quad (6)$$

If $K_1 \cong K'_1$, the first term on the right is one. In the case of Asp 26, K_1 and K'_1 are close (see below).

Scheme III



Scheme IV



If, in addition to Asp 26 titration, the ionization state of Lys 57 also affects thioredoxin stability, the unfolding equilibria for the three relevant protonation states may be written as Scheme III, where now the superscripts −, +, and 0 refer to the acidic, basic and neutral forms of Asp 26 and Lys 57. The predominant species are +− at neutral pH's, +0 at low pH's (where the acidic group is protonated, below pK_a^{-f} and pK_a^{-u}), and 0− at high pH's (where the basic group is unprotonated, above pK_a^{+f} and pK_a^{+u}). Note that technically there is a fourth protonation state, 00, but this will be present in extremely small amounts and may be ignored for the present analysis. K_1, K_2, and K_3 are equilibrium constants between the folded and unfolded forms of these three protonation species. pK_a^{-f} and pK_a^{+f} are the pK_a's of the acidic and basic groups in the folded protein in the presence of the other charged partner of the salt bridge and are obtained from the pK_a calculations above. pK_a^{-u} and pK_a^{+u} are the corresponding pK_a's in the unfolded form and are assumed to be the same as for the isolated amino acids, i.e., 3.86 for aspartate and 10.79 for lysine. This is reasonable if the unfolded state of the protein has little structure and these groups are well solvated. For scheme III, the net protein unfolding equilibrium constant for WT protein is $K_{Wt} = ([F^{+0}] + [F^{+-}] + [F^{0-}])/([U^{+0}] + [U^{+-}] + [U^{0-}])$, and summing over all the protonation states gives

$$K_{WT} = \frac{[F^{+0}](1 + K_a^{-f}/[H^+] + K_a^{-f}K_a^{+f}/[H^+]^2)}{[U^{+0}](1 + K_a^{-u}/[H^+] + K_a^{-u}K_a^{+u}/[H^+]^2)} = K_1\frac{(1 + K_a^{-f}/[H^+] + K_a^{-f}K_a^{+f}/[H^+]^2)}{(1 + K_a^{-u}/[H^+] + K_a^{-u}K_a^{+u}/[H^+]^2)} \quad (7)$$

For the mutant D26A, the equilibria corresponding to Scheme III is given in Scheme IV, where $K'_a{}^{+f}$ is the pK_a of the basic group in the folded mutant protein (which has no salt bridge since Asp 26 is replaced by alanine). This gives for the mutant net unfolding constant:

$$K_{mut} = \frac{[F'^{+0}](1 + K'_a{}^{+f}/[H^+])}{[U'^{+0}](1 + K'_a{}^{+u}/[H^+])} = K'_1\frac{(1 + K'_a{}^{+f}/[H^+])}{(1 + K'_a{}^{+u}/[H^+])} \quad (8)$$

Assuming $K_1 \cong K'_1$, K_1 and K'_1 can be eliminated from eqs 7 and 8 to give

$$K_{WT}/K_{mut} = \frac{(1 + K_a^{-f}/[H^+] + K_a^{-f}K_a^{+f}/[H^+]^2)(1 + K'_a{}^{+u}/[H^+])}{(1 + K_a^{-u}/[H^+] + K_a^{-u}K_a^{+u}/[H^+]^2)(1 + K'_a{}^{+f}/[H^+])} \quad (9)$$

With the assumption that replacement of Asp 26 by alanine leaves the pK_a of Lys 57 in D26A unperturbed ($pK'_a{}^{+f}$ = 10.79), and given that the Asp 26 pK_a = 7.5, eq 9 can be used

Table I: Energies and pK_a's of the Asp 26-Lys 57 Salt Bridge for the Crystal, Minimum Energy and Average Structures

distance (Å)	$\Delta\Delta G^{slv}$ Asp 26	$\Delta\Delta G^{slv}$ Lys 57	$\Delta\Delta G^{ttr}$ Asp⇄Lys	ΔG^{vdw}	ΔG^{total}	pK_a Asp 26	pK_a Lys 57
4.79	11.82	5.08	−4.31	0.00	0.00	9.78	10.72
2.83	11.80	8.52	−8.82	−1.02	−2.12	6.46	11.51
2.90	11.80	8.29	−8.30	−1.16	−1.96	6.84	11.30
2.98	11.80	7.97	−7.97	−1.20	−1.99	7.08	11.29
3.06	11.82	7.39	−7.40	−1.17	−1.96	7.51	11.30
3.17[b]	11.79	7.31	−8.50	−0.80	−1.88	7.36	11.49

[a] Distance is between Asp 26 OD1 and Lys 57 NZ atoms. Energies are in kilocalories per mole. $\Delta\Delta G^{vdw}$ is the van der Waals energy relative to the crystal structure (top row). $\Delta\Delta G^{total}$ is the sum of the van der Waals energy, the desolvation energies, $\Delta\Delta G^{slv}$, and the Lys–Asp interaction energy, $\Delta\Delta G^{ttr}$ relative to the crystal structure. pK_a's are calculated from eq 2. [b] Values are for the Boltzmann-weighted average over all conformations.

to solve for the pK_a of Lys 57 from fits to the data for pH versus $\Delta\Delta G^{\circ}_{(WT\rightarrow D26A)}$.

Results and Discussion

Thermodynamic Linkage of Thioredoxin Stability to Asp 26 Titration. The highly conserved carboxyl side chain at position 26 in *Escherichia coli* thioredoxin titrates with an anomolous pK_a of 7.5, and its titration is linked to global stability of the protein (Langsetmo et al., 1991). The thermodynamic cycle describing the linkage is shown in Scheme I. The expected variation with pH of the overall folding/unfolding equilibrium constant, K_{WT}, is given by eq 5. From this, the expression for the pH dependence of the overall free energy, ΔG°_{WT}, is obtained from the standard relationship. The experimental variation of ΔG°_{WT} with pH is given by the data points in Figure 1a. The curve is the best fit to eq 5, with use of a pK_a of 7.5 for Asp 26, with K_1 in eq 5 as the variable parameter. The good agreement supports the value of 7.5 for Asp 26 obtained by the electrophoresis method.

The fitted value of K_1 determines the limiting value of $\Delta G^{\circ}_{WT}(=-RT \ln K_1)$ at low pH, where protonated Asp 26 is the only species present. As can be seen in Figure 1a, the fitted curve gives a limiting value of ΔG°_{WT} at the y intercept of 13.5 kcal/mol, close to, but not identical with, the experimental value of 13.2 kcal/mol for ΔG°_{D26A} (Langsetmo et al., 1991). That is, $K_1 \cong K'_1$ (see eq 6). This means that the unfolding energy of WT with neutral Asp 26 is similar to the unfolding energy of the mutant with a methyl group at the same position. The small difference of 0.3 kcal/mol in free energy is likely to be due to the difference in hydrophobicity between the side chains of protonated aspartic acid and alanine. In Figure 1b the solid curve is calculated from eq 6 with use of the known pK_a of Asp 26 (7.5), the pK_a of fully solvated aspartate, the value of K_1 from the fit in Figure 1a, and the measured $K'_1 = \exp(\Delta G^{\circ}_{D26A}/-RT)$. Our calculations assume pK_a values of 3.86 and 10.79 respectively for the aspartic acid and lysine groups in the unfolded (unperturbed) state. The sensitivity of our calculations to this assumption is small since the uncertainty is only of the order of tenths of pK units, while the shifts observed here are several pK units. Further, in the relevant pH range, 5–9, the change in stability depends rather weakly on the absolute pK_a values used for the unfolded form (see eq 9).

Lys 57 Conformation Change. An essential question remains, however. What structural feature accounts for the anomalous pK_a of Asp 26? The crystal structure of thioredoxin is solved at pH 3.8 (Katti et al., 1990) where Asp 26 is in the –COOH form. In this structure, Asp 26 is deeply buried in a hydrophobic cleft. Lysine 57 sits at the opening of the cleft. The only other ionizable group in the vicinity is His 6, >9 Å away from the carboxyl of Asp 26 and the amino group of Lys 57. One Asp 26 carboxyl oxygen, OD2, is completely buried;

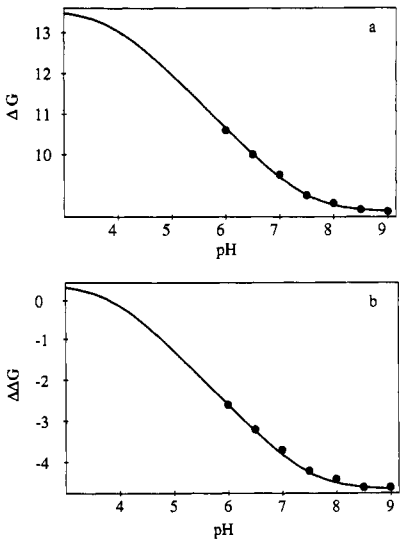


FIGURE 1: The pH dependence of the unfolding free energy of thioredoxin. The data are taken from Table II in Langsetmo et al. (1991). The points in (a) give the experimental values of the wild-type unfolding free energy, ΔG°_{WT}, as a function of pH. The curve in (a) represents the nonlinear least-squares fit to eq 5, with $K_a^{-f} = 7.5$, $K_a^{-u} = 3.9$, and, at each pH, $K_{WT} = \exp(\Delta G^{\circ}_{WT}/-RT)$ computed from ΔG°_{WT} values in Table II of Langsetmo et al. (1991). In the fit, the value of $K_1 = \exp(y \text{ intercept}/-RT)$ is allowed to vary. The points in (b) give the experimental values of the difference in free energy of WT and D26A, $\Delta\Delta G^{\circ}_{(WT\rightarrow D26A)}$, as a function of pH. The solid curve in (b) is calculated from eq 6 with K_1 obtained from (a) above, $K_a^{-f} = 7.5$, $K_a^{-u} = 3.9$, and $K'_1 = \exp(\Delta G^{\circ}_{D26A}/-RT)$, where $\Delta G^{\circ}_{D26A} = 13.2$ kcal/mol [from Table II in Langsetmo et al. (1991)].

OD1 has a slight exposure of its molecular surface and is H-bonded to one of the crystallographic water molecules in the cleft.

Electrostatic calculations on the pH 3.8 crystal structure, not surprisingly, give a large net destabilization of 7.51 kcal/mol for Asp 26. About 12 kcal/mol arises from its desolvation, or burial (first entry, Table I). Another 0.57 kcal/mol arises from the rest of the protein's dipoles and charged groups, excluding Lys 57. This is slightly destabilizing, though the small value indicates that Asp 26 is quite isolated electrostatically from the rest of the protein. The dominant electrostatic interaction in this region is between Lys 57 and Asp 26. Some stabilizatioon, −4.31 kcal/mol comes from this interaction, but not enough to compensate for the Asp 26 desolvation. Although the lysine itself is fairly desolvated, by 5.1 kcal/mol, the amino group points into solution away from Asp 26 and is 4.8 Å from the nearest Asp 26 oxygen. There are no dipolar groups with the requisite distance and alignment to stabilize the charged Asp 26, except those already involved in hydrogen bonds with other dipolar groups. Only the amide dipole of residue 76 could potentially provide some stabilization, were it not a proline.

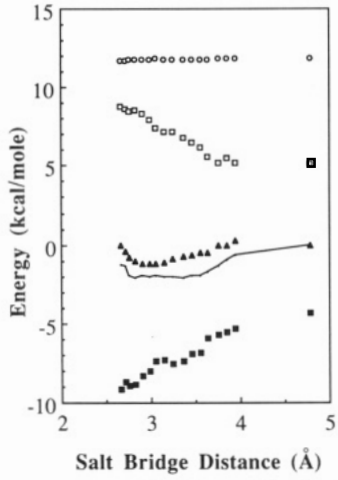


FIGURE 2: Plot of energy as a function of the Asp 26 ODl–Lys 57 NZ salt-bridge distance: (■) lysine–aspartate electrostatic interaction energy; (▲) van der Waals energy relative to crystal structure (with distance 4.79 Å); (□) Lys 57 desolvation energy; (○) Asp 26 desolvation energy. The solid line is the total energy (sum of above) relative to the crystal structure. On the far right of the plot, values for each contribution are given for Asp 26 and Lys 57 in the crystal structure.

Clearly, the charged Asp 26 side chain is stabilized by some means, or its pK_a would be much higher than 7.5 (9.8 from our calculations, Table I). A simple and reasonable source for the stabilization is a rotation of the Lys 57 side chain, which would move the amino group into the pocket to make a salt bridge with the aspartate. To test the plausibility of this, various conformations of the Lys 57 side chain were generated and the electrostatics and stability arising from an Asp 26–Lys 57 salt bridge were examined. The crucial parameter is the separation between the Asp 26 carboxyl oxygens (especially OD1) and the Lys 57 amino group. In modeling the change in Lys 57 conformation, the only degrees of freedom examined were the χ angles of that side chain. While it is possible that other groups rearrange in this region, for example, to allow greater solvent exposure of Asp 26, we examined the simplest possibility first, since in the absence of other structural data one wants to minimize changes from the crystal structure. With many degrees of freedom, and potentially large structural changes, modeling is highly dependent upon a reliable evaluation of the energies of these structures. This is difficult because electrostatics, especially the solvation interaction (which is likely to be the most important factor here), is difficult to treat accurately with current molecular mechanics methods (Harvey, 1989).

The model building was done with use of the molecular mechanics package DISCOVER (Hagler et al., 1973) without electrostatic interactions. The conformations generated were subsequently evaluated with the continuum electrostatics package DelPhi. This approach was adopted to use the strengths of both methods. Potential biasing of structures due to poor treatment of electrostatics/solvation in molecular mechanics is avoided, while generating energetically good structures by steric and van der Waals criteria. Given suitable structures, continuum electrostatic calculations can provide good estimates of solvation energy (Gilson & Honig, 1989; Jean-Charles et al., 1991). The contribution of the dipolar groups and other charged residues is assumed to be essentially unchanged from the crystal structure; Asp 26 and Lys 57 effectively form an isolated electrostatic system. The shift in pK_a of these two groups due to desolvation, their mutual interaction, and the potential from the rest of the protein is then determined with use of DelPhi.

Figure 2 shows the various energy contributions for the 16 different salt-bridge conformations as a function of the salt-bridge distance. The Asp 26 solvation remains essentially unchanged. The lysine is progressively desolvated as it moves closer to the aspartic acid and into the pocket. This is offset by an increase in the Asp–Lys interaction. When combined with the van der Waals contribution, the total energy profile shows a shallow minimum of about −2 kcal/mol in the range 2.8–3.5 Å, which covers the typical range for salt bridges in proteins (Barlow & Thornton, 1983). The energy contributions for four of the minimum energy conformations and the resulting Asp 26 and Lys 57 pK_a's are given in Table I. For the minimum energy structures, Lys 57 has a conformation similar to that of the crystal structure but the amino group is swung around to form a good salt bridge with the Asp 26 ODl atom (Figure 3). Due to the shallowness of the minimum, it is likely that the lysine has an appreciable mobility and samples several conformations. This was modeled by taking a Boltzmann-weighted average of the pK_a's over all 16 conformations with use of the total energy (last entry in Table I).

The favorable Lys 57–Asp 26 interaction of this putative salt bridge partially compensates for the large unfavorable desolvation energy of Asp 26 and brings the calculated pK_a into the range 6.5–7.5. The Boltzmann-weighted average gives a pK_a of 7.36 (Table I). This good agreement with the ex-

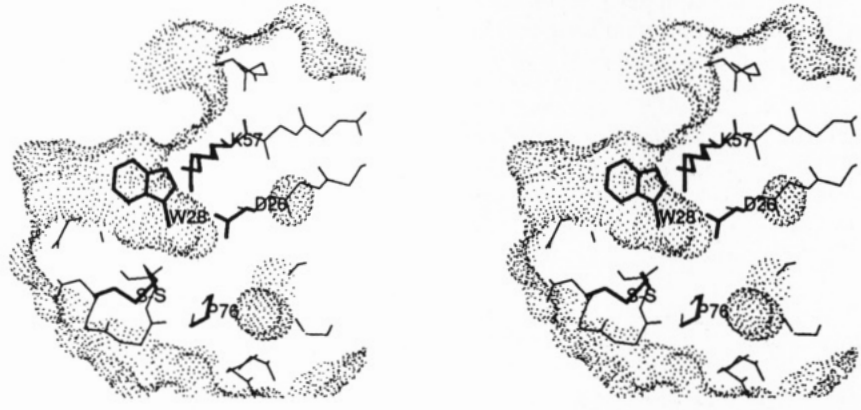


FIGURE 3: Stereo drawing of thioredoxin with a Lys-57–Asp 26 salt bridge. The view shows a cross-section through the region of the Asp 26 cleft. The thin lines represent backbone atoms, and the bold lines are the side-chain atoms of Asp 26, Lys 57, Pro 76, Cys 32, Cys 35, and Trp 28. The two configurations of the Lys 57 side chain represent the crystal coordinates (Katti et al., 1990) and the modeled position at a salt-bridge distance of 2.8 Å. The orientation of the molecule is similar to that in Figure 6 of Langsetmo et al. (1991). The molecular surface of the protein is represented by dots (Connolly, 1983). Two internal cavities are observed close to Asp 26 and Pro 76. Stereo drawings are given with the right eye image on the left side, and vice versa.

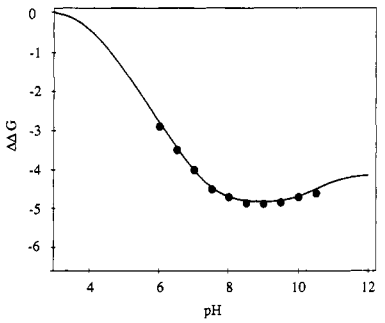


FIGURE 4: Estimation of Lys 57 pK_a from the pH dependence of $\Delta\Delta G°_{(WT\rightarrow D26A)}$. The curve is a nonlinear least-squares fit to eq 9 where $K_a^{-f} = 7.5$, $K_a^{-u} = 3.9$, $K_a^{+u} = K'^{+f}_a = K'^{+u}_a = 10.8$, $K_{WT} = \exp(\Delta G°_{WT}/-RT)$, and $K_{mut} = \exp(\Delta G°_{D26A}/-RT)$. $\Delta G°_{WT}$ and $\Delta G°_{D26A}$ at pH 6–9 are taken from Table II in Langsetmo et al. (1991) and at pH 9–10.5 are determined for GuHCl unfolding as described in the same paper. The value of K_a^{+f} is allowed to vary. The best fit corresponds to $K_a^{+f} = 10.25$, which we take to be the pK_a of Lys 57 in WT thioredoxin. The program NonLin, described in Langsetmo et al. (1991), was used for fitting.

perimental pK_a of Asp 26 supports the hypothesis that the Lys 57 side chain swings into the hydrophobic cleft to make a salt bridge with the Asp 26–COO^- and then rotates out again when Asp 26 is protonated.

pK_a of Lys 57. Assuming that Lys 57 does affect thioredoxin stability due to interactions with the aspartate (Figure 3) and given the pK_a of Asp 26, eq 9 can be used to compute the pK_a of Lys 57 from the pH dependence of thioredoxin stability when the data set is extended to include higher pH's. With the assumption that replacement of Asp 26 by alanine leaves the pK_a of Lys 57 in D26A unperturbed (pK'^{+f}_a = 10.79), and given that the Asp 26 pK_a = 7.5, eq 9 can be used to solve for the pK_a of Lys 57 (pK_a^{+f}) from fits to the data for pH versus $\Delta\Delta G°_{(WT\rightarrow D26A)}$. Figure 4 shows the experimental values of $\Delta\Delta G°_{(WT\rightarrow D26A)}$ at pH 6–10.5. A pK_a of 10.25 for Lys 57 in folded thioredoxin is obtained from the best fit of eq 9 as described.

This shift of −0.54 pK_a units indicates a slight destabilization of the charged lysine. That the lysine can greatly stabilize the aspartate, while having a relatively unperturbed pK_a itself, is explained in our model by the nearly compensating changes in lysine–aspartate interaction and lysine desolvation with salt-bridge distance (Figure 2). This is borne out by the relatively small calculated shift in lysine pK_a (+0.7) and its insensitivity to the Asp 26–Lys 57 distance (Table I). The calculated direction of the shift is reversed, but discrepancies of this order probably reflect the limits of accuracy in such a calculation, given the combination of uncertainties in partial charge distribution, modeling, and electrostatics.

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