Partitioning the Effects of Changes in a Protein to the Folded or Unfolded Forms by Using a Thermodynamic Cycle: A Change in *Escherichia coli* Thioredoxin Does Not Affect the Unfolded State†

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ABSTRACT: Previously, we have introduced a method whereby novel disulfide side chains can be produced in the interior of a protein by modifying a cysteine residue after denaturant-induced unfolding [Wynn, R., & Richards, F. M. (1993) *Proteins*: *Struct., Funct., Genet. 2*, 395–403]. Here the disulfide exchange equilibrium constant, K_{red}, between the protein C32S,C35S-L78C thioredoxin and 2-hydroxyethyl disulfide is studied as a function of urea concentration. Since the disulfide exchange reaction and the stability of the proteins are thermodynamically linked, independent knowledge of the stabilities of the modified and unmodified proteins along with measurements of K_{red} allows us to assign K_{red} values for the folded and urea-unfolded forms of the protein. We find that the disulfide exchange reaction is more favorable for the folded protein, in agreement with the increased stability of the modified protein, and that the K_{red} values for both states are independent of the urea concentration. Finally, K_{red} values for the unfolded protein are the same, within experimental error, as that for *N*-acetylcysteine methylamide, an analog of cysteine in a peptide chain without the possibility of intramolecular interactions. Thus, we conclude that modification of position 78 of thioredoxin does not affect the unfolded state. The relevance of these results toward protein stability studies is discussed.

Thioredoxin is a small protein of molecular weight approximately 12K with a single active-site disulfide which can exist in either the oxidized or the reduced form in the native protein. It serves as a reducing agent for a plethora of biological partners and also has activities not dependent upon a redox cycle [for a review, see Holmgren (1989)]. The structure of the oxidized protein has been determined by crystallography (Katti et al., 1990) (see Figure 1) while the reduced-form structure has been determined by NMR methods (Dyson et al., 1990). The two forms show only minor differences in the neighborhood of the disulfide bond. Previously, we have made changes at position 78 of thioredoxin by a combined mutagenesis/chemical modification technique (Wynn & Richards, 1993). After unfolding the protein C32S,C35S,L78C thioredoxin (the active-site disulfide has been removed in order to have a single thiol-containing protein) in guanidine hydrochloride (Gdn-HCl),[1] the lone cysteine residue was converted to a mixed disulfide with a variety of aliphatic thiols. Although completely buried in the wild-type structure (see Figure 1), this position, surprisingly, will accept almost any amino acid regardless of size and polarity (Hellinga et al., 1992; Wynn & Richards, 1993).

Here we form the mixed disulfide between the same protein and BME. The modified protein is produced from the unmodified protein by a simple disulfide exchange reaction:

$$\mathrm{PSH} + \mathrm{RSSR} \leftrightarrow \mathrm{PSSR} + \mathrm{RSH} \qquad (1)$$

where PSH and PSSR designate the protein with a free thiol and mixed disulfide, respectively. RSSR and RSH are the small-molecule disulfide and thiol involved in the reaction.

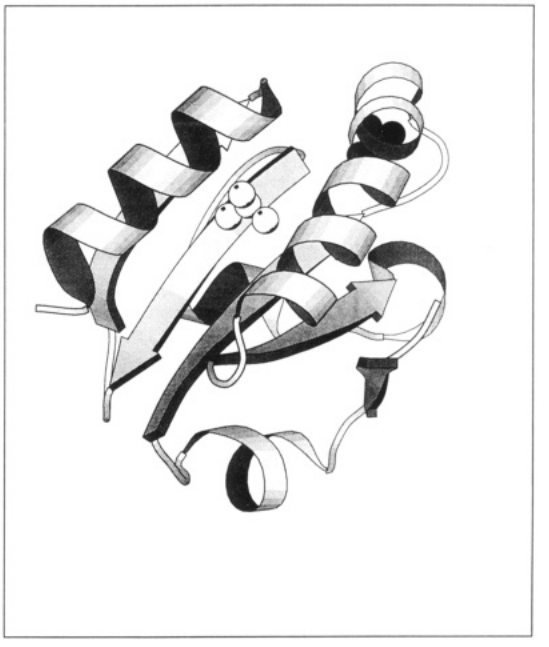

FIGURE 1: Ribbon diagram of thioredoxin with the wild-type leucine at position 78 in white. The active-site disulfide, which has been removed in this study, is shown in black. Coordinates are from Katti et al. (1990).

This process can be described by an equilibrium constant:

$$K_{red,app} = [\mathrm{PSSR}]/[\mathrm{PSH}]r \qquad (2)$$

where r is equal to [RSH]/[RSSR]. If no protein–protein disulfides are formed, then the following relationship between the fraction of protein containing a free thiol, F_{SH}, and $K_{red,app}$ holds:

$$F_{SH} = [\mathrm{PSH}]/[\mathrm{P_t}] = [\mathrm{PSH}]/([\mathrm{PSH}] + [\mathrm{PSSR}]) = r/(r + K_{red,app}) \qquad (3)$$

$[\mathrm{P_t}]$ is equal to the total concentration of protein in all forms.

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[1] Abbreviations: Trx, thioredoxin; BME, β-mercaptoethanol; BMEDS, 2-hydroxyethyl disulfide; ACM, *N*-acetylcysteine methylamide; CBME, *N*-acetylcysteine methylamide 2-hydroxyethyl disulfide; Gdn-HCl, guanidine hydrochloride.

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Determination of F_{SH} as a function of r under conditions that do not allow protein–protein disulfide formation should yield a hyperbolic plot and determination of $K_{red,app}$.

The disulfide exchange reaction is thermodynamically linked to the unfolding equilibrium of the protein:

$$\begin{array}{ccc} RSSR + P_NSH & \xrightarrow{K_{PSH}} & P_USH + RSSR \\ K_{red,N}\uparrow\downarrow & & \uparrow\downarrow K_{red,U} \\ RSH + P_NSSR & \xrightarrow[K_{PSSR}]{} & P_USSR + RSH \end{array} \quad (4)$$

where P_NSH and P_USH designate the unmodified protein in the native and unfolded forms, respectively. P_NSSR and P_USSR designate the modified protein in the native and unfolded forms, respectively. The small-molecule thiol and disulfide, RSH and RSSR, are spectator molecules in the horizontal unfolding reactions. The associated equilibrium constants are

$$K_{PSH} = [P_USH]/[P_NSH] \quad (5)$$

$$K_{PSSR} = [P_USSR]/[P_NSSR] \quad (6)$$

$$K_{red,N} = [RSH][P_NSSR]/[RSSR][P_NSH] = \{[P_NSSR]/[P_NSH]\}r \quad (7)$$

$$K_{red,U} = [RSH][P_USSR]/[RSSR][P_USH] = \{[P_USSR]/[P_USH]\}r \quad (8)$$

Since these equilibria are thermodynamically linked, the following relationship holds:

$$K_{PSH}/K_{PSSR} = K_{red,N}/K_{red,U} \quad (9)$$

Thus, under the above thermodynamic cycle, determination of any three of the above constants necessarily specifies the fourth. When there is more than one protein species present, $K_{red,app}$, as defined in eq 2, should be written so as to include the sum of both the oxidized and reduced species:

$$K_{red,app} = \{([P_NSSR] + [P_USSR])/([P_NSH] + [P_USH])\}r \quad (10)$$

which would be equivalent to

$$K_{red,app} = K_{red,N}[1/(1 + K_{PSH})] + K_{red,U}[K_{PSH}/(1 + K_{PSH})] \quad (11)$$

Finally, using eq 9 and 11, $K_{red,U}$ can be described in terms of three experimentally determinable parameters:

$$K_{red,U} = K_{red,app}/\{(K_{PSH}/K_{PSSR})(1 + K_{PSSR})/(1 + K_{PSH})\} \quad (12)$$

Examination of the individual equilibrium constants in the above thermodynamic cycle can help partition the effects of a mutation (in this case, also a chemical modification) on the stability of a globular protein as judged by the unfolding equilibrium constants, K_{PSH} and K_{PSSR}, only if the appropriate controls are accounted for. For instance, if a mutation causes K_{PSSR} to be smaller than K_{PSH} (the modified protein has a larger fraction of molecules in the folded state), then $K_{red,N}$ is necessarily greater than $K_{red,U}$ due to the constraints imposed by the thermodynamic cycle and eq 6. However, due to the fact that a covalent bond change is also involved in this scheme, it is not possible to determine whether the intramolecular interactions in either the folded or the unfolded state are favorable because there is also a free energy associated with the covalent bond change. The mutation may be favorable in both states but less so in the unfolded state or unfavorable in both states but more so in the unfolded state. Either scenario would give an increase in stability for the mutant protein. By accounting for the covalent bond change in the absence of intermolecular interactions, one can determine the effect of doing the reaction in the context of the protein environment. In this case, that means accounting for the differences in the redox potentials of cysteine in a peptide chain and BME. We have done this by conducting measurements of the disulfide exchange reaction between *N*-acetylcysteine methylamide and BMEDS. The work described in this study was underway before the appearance of the paper by Lu et al. (1992). The basic approach and analyses are similar in the two papers. Lu et al. measured an apparent stability constant for a single cysteine mutant of T4 lysozyme at approximately the T_m of the unmodified protein as a function of r. The disulfide exchange equilibrium constants for the folded and unfolded states were inferred from this profile without direct experimental corroboration. Here we present a method for measuring $K_{red,app}$ and extend this type of analysis to urea-induced denaturation. In addition, by following the redox behavior through most of the unfolding transition, we are able to verify the constant values of $K_{red,N}$ or $K_{red,U}$ with different denaturing conditions. The fact that these values do not change with urea concentration indicates little or no structural alteration in either the folded or the unfolded form with differing amounts of denaturant. This was untested in the Lu et al. work.

MATERIALS AND METHODS

The production of C32S,C35S,L78C Trx has been described elsewhere (Wynn & Richards, 1993). Proton NMR spectra were recorded on a Bruker WM-250 spectrometer. Fluorescence measurements were carried out on a SPEX FluoroMax 3000 fluorometer. A CARY 219 spectrophotometer was used for all absorbance measurements. Molecular weights were determined by fast atom bombardment mass spectrometry at the mass spectrometry facility of the Yale Medical School. The mass spectra for 2-hydroxyethyl methanethiosulfonate were determined by tandem gas chromatography–mass spectrometry as previously described (Wynn & Richards, 1993). BME, BMEDS, *S*-tritylcysteine, methylamine hydrochloride, *N*-hydroxysuccinimide, and dicyclohexylcarbodiimide were purchased from Aldrich. Sodium methanesulfinate was purchased from Lancaster Synthesis Inc. Silver nitrate was purchased from Brand-Nu Laboratories, Inc. (CT).

Organic Synthesis. 2-Hydroxyethyl methanethiosulfonate was synthesized following a published procedure (Bentley et al., 1972): 5.18 g of BMEDS (0.034 mol) was dissolved in 100 mL of 50% acetone/H_2O; 100 mL of 50% acetone containing 6.28 g of $AgNO_3$ 0.037 mol) and 3.96 g of CH_3SO_2Na 0.037 mol) was added with stirring. After 90 min, the reaction mixture was filtered. The filtrate was extracted with 4–50-mL portions of ethyl acetate which were combined and dried with $MgSO_4$. The solvent was removed under reduced pressure, and the remaining residue was purified by silica gel chromatography in 3:1 chloroform/ethyl acetate (v/v): yield 4.80 g (91%); ^1H NMR ($CDCl_3$) δ 3.94 (2H, t), 3.43 (3 H, 3), 3.36 (2 H, t), 2.76 (1 H, s). Mass spectral analysis gave a molecular weight of 156.0.

N-Acetyl-*S*-tritylcysteine: 8.99 g of *S*-tritylcysteine (0.025 mol) was suspended in 225 mL of chloroform/acetone/ethanol (1:1:1), and 11.5 mL of acetic anhydride was added. After 30 min, the reaction mixture, which had become clear, was filtered, and the solvent was removed under reduced pressure. The resultant white powder weighed 9.02 g (90%): ^1H NMR ($CDCl_3$) δ 7.1–7.5 (15 H, m), 6.12 (1 H, d), 4.50 (1 H, q), 2.75 (2 H, m), 1.92 (3 H, s).

N-Acetyl-*S*-tritylcysteine methylamide: 8.65 g of *N*-acetyl-*S*-tritylcysteine (0.021 mol), 1.51 g of methylamine hydrochloride (0.022 mol), 2.58 g of *N*-hydroxysuccinimide (0.022 mol), and 2.27 g of triethylamine (0.022 mol) were dissolved in 400 mL of 50:50 dimethylformamide/acetonitrile. After being cooled to 10 °C, 4.67 g of dicyclohexylcarbodiimide was added, and the reaction mixture was stirred overnight. The sample was filtered, and the solvent was removed under reduced pressure. The remaining residue was purified by silica gel chromatography in chloroform/acetone (4:1 v/v): yield 6.43 g (72%); ^{1}H NMR ($CDCl_3$) δ 7.1–7.5 (15 H, m), 6.32 (1 H, q), 6.16 (1 H, d), 4.14 (1 H, q), 2.70 (3 H, d), 2.60 (2 H, m), 1.88 (3 H, s).

N-Acetylcysteine methylamide: 4.01 g of *N*-acetyl-*S*-tritylcysteine methylamide (9.59 mmol) was dissolved in 15 mL of TFA and 15 mL of ethanethiol. After 30 min, 50 mL of water was added, and the mixture was washed with 3–50-mL portions of chloroform. The water layer was dried under reduced pressure, and the remaining residue was purified by silica gel chromatography in ethyl acetate/acetone (2:1): yield 1.05 g (63%); ^{1}H NMR (D_2O) δ 4.37 (1 H, t), 2.84 (2 H, d), 2.69 (3 H, s), 2.00 (3 H, s). Mass spectral analysis gave an m/z of 177.2.

N-Acetylcysteine methylamide 2-hydroxyethyl disulfide: 0.20 g of *N*-acetylcysteine methylamide (1.14 mmol) was dissolved in 2 mL of methanol and 2 mL of H_2O; 0.18 g of 2-hydroxyethyl methanethiosulfonate (1.15 mmol) was added. After 5 min, the solvent was removed under reduced pressure, and the remaining residue was purified by silica gel chromatography in acetone/ethyl acetate (2:1): yield 0.25 g (87%); ^{1}H NMR (D_2O) δ 4.50 (1 H, q), 3.75 (2 H, t), 2.98 (2 H, m), 2.78 (2 H, m), 2.66 (3 H, s), 1.97 (3 H, s). Mass spectral analysis gave an m/z of 253.2.

Protein Modification. The modified protein was produced by dissolving the unmodified protein in 50 mM potassium phosphate/3.0 M Gdn-HCl, pH 7.0, and adding BMEDS (2000-fold excess) or 2-hydroxyethyl methanethiosulfonate (1.1-fold excess). After a 3-h incubation, the protein was separated from small molecules on a Sephadex G-25 column. The modified protein was checked for unreacted thiol and protein dimers as described elsewhere (Wynn & Richards, 1993). The two protocols gave proteins with indistinguishable stabilities to urea denaturation.

$K_{red,app}$ and K_u Measurements. All measurements were carried out in 50 mM Tris/1 mM EDTA, pH 8.0, plus the indicated amount of urea at 25 °C. Urea concentrations were determined by index of refraction measurements (Nozaki, 1972). All solutions were purged with N_2 prior to usage, and all urea solutions were made up fresh and used within 12 h.

The measurement of urea unfolding equilibrium was determined as described previously (Wynn & Richards, 1993). K_u (=K_{PSH} or K_{PSSR}) values were obtained from the fluorescence data on the fully reduced (PSH) or fully oxidized (PSSR) form of the protein. Data treatment involves the assumption that the free energy of unfolding varies linearly with the urea concentration such that $\Delta G_u = \Delta G_{u(H_2O)} - m$[urea] where $\Delta G_{u(H_2O)}$ is the estimated unfolding free energy at zero urea concentration and m is the dependence of the free energy on the urea concentration. While there is debate as to whether this assumption holds to zero denaturant concentration, it is generally agreed that it is suitable in the transition region where these measurements are being made (Pace, 1986). The unfolding equilibrium constant at any particular concentration of urea can be determined from

$$K_u = e^{-\Delta G_{u(H_2O)}/RT + m[\text{urea}]/RT} \quad (13)$$

For measurement of $K_{red,app}$ between protein and BMEDS, reaction mixtures contained approximately 10 μM protein (modified or unmodified) and a mixture of BME and BMEDS where both concentrations were at least 200 times higher than the protein concentration and served to poise the redox potential of the solution. Equilibration was judged to be complete when reaction mixtures started with modified or unmodified protein at the same [BME]:[BMEDS] ratio gave the same fraction of protein containing thiol. Typically, a 4-h incubation was sufficient. Measurements near 2.0 M urea were incubated for longer periods of time. No incubation was longer than 12 h. This was necessary to avoid modification of the protein by cyanate ions formed by the decomposition of urea (Stark, 1965). Measurements below a urea concentration of approximately 2.0 M were not practical due to the long incubation times required. Guanidine hydrochloride was not used because it has been shown to have an effect on disulfide exchange reactions (Creighton, 1977).

In order to assay the amount of protein modified, the reaction mixture was quenched to pH 3.0 with 1 N HCl, applied to a Sep-pak C18 cartridge, and rinsed with 35% ACN/0.01% TFA (buffer A) until the eluant showed no detectable thiol with Ellman's reagent. The protein was eluted in 65% ACN/0.01% TFA (buffer B). The protein concentration was determined by the absorbance at 280 nm. The modified and unmodified proteins had the same extinction coefficient at 280 nm ($\epsilon_{280} = 1.52 \times 10^4$ M^{-1} cm^{-1}). Thiol concentrations were determined by mixing protein solution, buffer B, and 0.5 M Tris/6.0 M Gdn-HCl/0.2 mM Ellman's reagent, pH 8.0, such that the resulting solution was always 50% buffer B (the volume of protein solution plus the volume of buffer B was equal to half of the assay solution volume). The extinction coefficient at 412 nm for reaction of Ellman's reagent with a thiol was determined to be 1.34×10^4 cm^{-1} M^{-1} under these conditions. Plots of the absorbance at 412 nm versus the protein concentration gave straight lines (Figure 2A), the slopes of which divided by 1.34×10^4 are equal to the fraction of protein containing free thiol. At least eight different reaction mixtures were analyzed covering the range of fraction of thiol-containing protein from less than 0.2 to at least 0.8. The data were then fit by nonlinear least squares to eq 3. Protein samples separated from small molecules were also analyzed by SDS–PAGE, and no protein dimers were detectable. Reactions started with unmodified or modified protein gave indistinguishable results.

For measurements on small molecules, *N*-acetylcysteine methylamide or *N*-acetylcysteine methylamide 2-hydroxyethyl disulfide was mixed with BME and BMEDS such that the concentrations of BME and BMEDS were in at least 5-fold excess. After incubation, the reaction mixture was quenched to approximately pH 2.0 with 1 M HCl and injected on a Rainin Dynamax-300A C18 reverse-phase column previously equilibrated in 0.01% TFA. After 5 min of this buffer, a gradient to 20% acetonitrile/0.01% TFA over 30 min was run to elute all compounds while the effluent was monitored at 219 nm. The relative extinctions at 219 nm of BME and BMEDS or ACM and CBME were determined by injecting known mixtures at acidic pH and integrating the appropriate peak areas. Equilibrium constants reported are the average of at least five separate determinations.

Table I: Urea Unfolding Data

	PSH[a]	PSSR[b]
$\Delta G_{u(H_2O)}$ (kcal/mol)	5.14	5.95
m [(kcal/mol·M)]	1.72	1.71
$[urea]_{1/2}$ (M)	3.00	3.49

[a] PSH ≡ protein in reduced form. [b] PSSR ≡ protein in modified form.

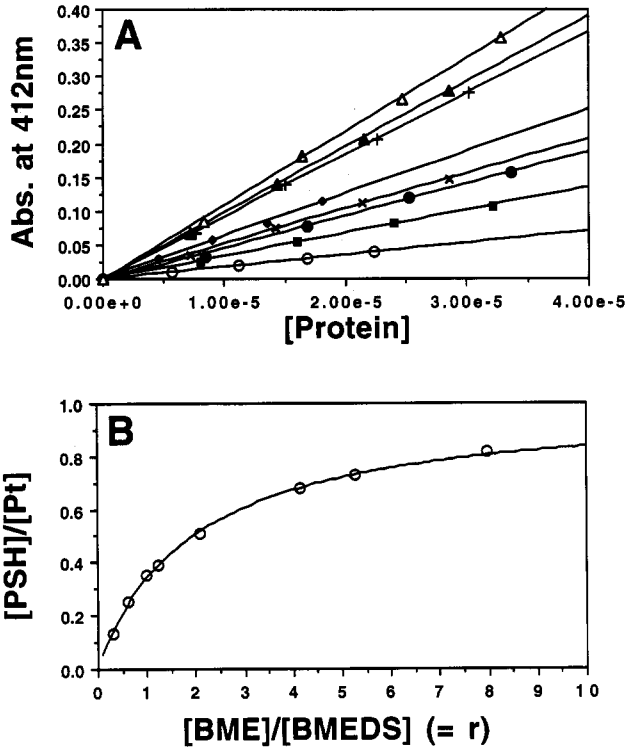

FIGURE 2: Example of measurement of $K_{red,app}$. The urea concentration for this measurement was 8.00 M. Panel A shows the absorbance at 412 nm after reacting the protein with Ellman's reagent as a function of the total protein concentration, $[P_t]$, at different [BME]/[BMEDS] [r values of 8.00 (△), 5.29 (▲), 4.15 (+), 2.07 (♦), 1.25 (×), 1.00 (●), 0.627 (■), and 0.309 (○)]. Lines drawn are the least-squares fit of the experimental data. F_{SH} is estimated from the derived slopes divided by 1.34×10^4. Panel B shows F_{SH} versus [BME]/[BMEDS] derived from the data in panel A. The solid line is the theoretical curve determined from a nonlinear least-squares fit to eq 3. The value of $K_{red,app}$ for this curve is 1.97.

RESULTS

The formation of a mixed disulfide between the protein and BME results in a protein that is 0.8 kcal/mol more stable than the thiol-containing protein (see Table I). This is manifested solely in a change in the denaturation midpoint, $[urea]_{1/2}$. The dependence of the free energy on the urea concentration, m, is essentially the same for the modified and unmodified proteins.

Figure 2 shows data from a typical $K_{red,app}$ measurement. Panel A shows that protein separated from small molecules gives linear plots when reacted with Ellman's reagent and that the slopes of these plots increase as [BME]/[BMEDS] increases. A plot of F_{SH} versus [BME]/[BMEDS] (=r; see eq 3) is given in Figure 2, panel B. The solid line indicates a nonlinear least-squares fit to eq 3. The good fit indicates that a simple one-step exchange reaction is taking place and that no protein–protein disulfides are being formed. SDS–PAGE analysis also supports this conclusion. Further proof of the postulated mechanism lies in the fact that experiments starting with either modified or unmodified protein give superimposable curves.

Table II: K_{red} Data for the Protein–BMEDS Exchange Reaction

[urea] (M)	$K_{red,app}$[a]	K_{PSH}[b]	K_{PSSR}[b]	$K_{red,N}$[c]	$K_{red,1}$[d]
2.26	7.82	0.119	0.0294	8.50	2.11
2.75	6.08	0.490	0.121	8.08	1.99
3.51	3.37	4.43	1.07	8.82	2.14
4.50	2.04	77.9	18.6	8.23	1.96
6.24	1.93	1.20×10^4	2.78×10^3	8.35	1.93
8.00	1.97	1.97×10^6	4.40×10^5	8.80	1.97
				8.46	2.02

[a] Determined as described under Materials and Methods. [b] Calculated from eq 13 using the parameters in Table I. [c] Calculated from eq 9. [d] Calculated from eq 12.

Table III: $K_{red,app}$ Data for the Acetylcysteine Methylamide–BMEDS Disulfide Exchange Reaction

[urea] (M)	$K_{red,ACM}$	[urea] (M)	$K_{red,ACM}$
0.00	2.05	6.00	2.06
2.01	2.00	7.99	2.08
4.01	2.02		2.04

The formation of a protein–protein disulfide bond is probably prevented for steric reasons, especially under weakly denaturing conditions where a significant amount of the protein assumes the folded structure since the thiol in the native state would be inaccessible. The fact that BME and BMEDS are in large excess relative to protein also inhibits the formation of protein–protein dimers due to a mass action effect. When a protein–BME mixed-disulfide is produced, one molecule of BMEDS is used, and one molecule of BME is produced. Hence, the level of protein being modified is controlled by [BME]/[BMEDS]. When a protein–protein disulfide is produced, one unmodified protein molecule must react with one modified protein molecule to produce the protein–protein disulfide and BME. Since only BME is in large excess relative to the rest of the reactants and products in this second step, it is inhibited by the already large chemical potential of BME.

The data for $K_{red,app}$ measurements are given in Table II. $K_{red,app}$ stays constant at approximately 2.0 until the urea concentration is low enough so that some protein molecules begin to assume the native state. $K_{red,U}$ could be calculated from eq 12 using values for K_{PSH} and K_{PSSR} given in Table II. $K_{red,N}$ could then be calculated from eq 9. Table II shows that both $K_{red,N}$ and $K_{red,U}$ are constant with changes in urea concentration. This is expected since the two proteins have identical m values. Therefore, K_{PSH}/K_{PSSR} has no urea concentration dependence. By eq 9, $K_{red,N}/K_{red,U}$ must have the same dependence on urea concentration as K_{PSH}/K_{PSSR}. The simplest way to satisfy this requirement is for $K_{red,N}$ and $K_{red,U}$ to have no dependence on the urea concentration. $K_{red,N}$ is approximately 4 times larger than $K_{red,U}$ as is necessary to account for the stability difference of the two proteins.

The data for the disulfide exchange reaction between *N*-acetylcysteine methylamide and BMEDS are presented in Table III. Once again, the value of $K_{red,app}$ is independent of the urea concentration. The values here are strikingly close to the average value of $K_{red,U}$.

DISCUSSION

Validity of the Thermodynamic Cycle. The thermodynamic cycle postulated here is dependent upon knowledge of each process involved. First, a two-state approximation is assumed for the unfolding of each protein species. This is a common assumption in the study of reversible protein folding in general and has been studied extensively for thioredoxin in particular. Although kinetic intermediates do seem to occur along the folding pathway of thioredoxin, only two states are significantly

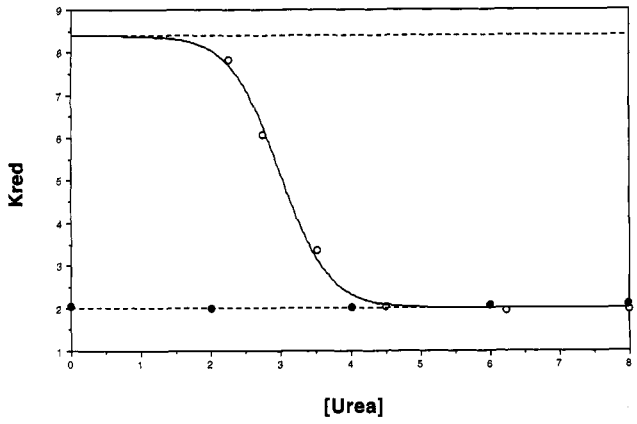

FIGURE 3: Plot of $K_{red,app}$ versus urea concentration. The open circles denote the equilibrium between protein and BMEDS while the filled circles denote the equilibrium between acetylcysteine methylamide and BMEDS. The solid line is derived from a nonlinear least-squares fit of eq 11 to the protein–BMEDS data. The dashed lines represent the fitted values of $K_{red,N}$ (higher line) and $K_{red,U}$ (lower line).

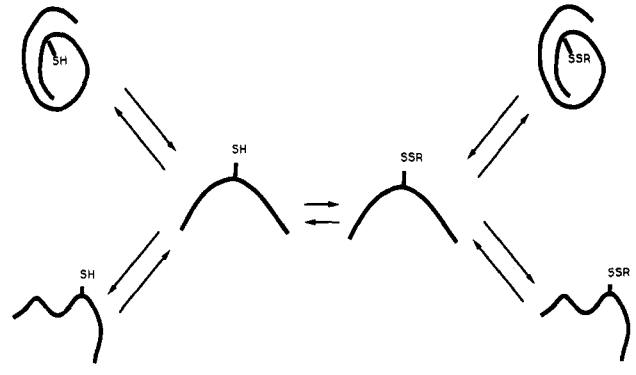

FIGURE 4: Hypothetical breakdown of the disulfide exchange reaction between the protein and a small-molecule disulfide.

populated at equilibrium (Kelley & Stellwagen, 1984; Wilson et al., 1986). Additionally, the disulfide exchange mechanism has been firmly established (Rosenfeld et al., 1977; Snyder & Carlsen, 1977), and the kinetic and equilibrium aspects for a large variety of disulfide/thiol pairs have been studied [for example, see Szalewski and Whitesides (1980)]. Nucleophilic displacement of a thiolate anion from the disulfide occurs via a single concerted step. Since no intermediates are observed, the equilibrium can be described in a two-state fashion.

Figure 3 shows a plot of $K_{red,app}$ (open circles) versus urea concentration. The solid line in the figure is a nonlinear least-squares fit of $K_{red,app}$ to K_{PSH}, which are experimentally determined independently, according to eq 11 which is based only on the equilibria 5–8 and not on eq 9. The values determined for $K_{red,N}$ and $K_{red,U}$ (shown as dashed lines) by this method are 8.39 and 1.99, respectively, well within experimental error of the average values determined assuming eq 9 as described under Materials and Methods. Note that no knowledge of K_{PSSR} is involved in the above treatment. The similar values of $K_{red,N}$ and $K_{red,U}$ determined whether eq 9 is assumed or not imply the validity of the thermodynamic cycle postulated here.

Using Small-Molecule Analogs To Account for Intrinsic Reactivity. A hypothetical breakdown of the disulfide exchange reaction is presented in Figure 4. The first step is the relaxation of the protein structure from the populated conformation (folded or unfolded) to a hyperextended conformation in which no intramolecular interactions occur. While this state may be physically unattainable, it serves as a reference point from which intramolecular interactions can be gauged. This is analogous to setting the interaction energy of two atoms at infinite distance to zero as is standard for a Lennard–Jones-type potential energy function.

The next step involves the disulfide exchange reaction while the protein is maintained in the hyperextended conformation. It is our assumption that this step can be modeled by small molecules. We have used *N*-acetylcysteine methylamide as a suitable analog for cysteine in a peptide chain. Both ends are in peptide bonds with sp3 carbons attached. All amino acids except proline at the carboxyl end would give identical covalent configurations up to the α-carbons of the surrounding amino acids. It is highly unlikely that the redox character of the γ-sulfur atom would be changed by effects propagated through five covalent bonds (from the terminal methyl groups/α-carbons to the sulfur atom). Since the inductive effects in the cysteine analog and a peptide chain should be equal, any difference in the redox behavior of the cysteine residue in a protein must be due to interactions with protein, either directly mediated or indirectly mediated through solvent. Therefore, we judge this to act equivalently to cysteine in the supposed hyperextended peptide chain.

The final step is relaxation of the peptide chain to the conformation(s) appropriate to the given solution conditions and the new side chain at the position of modification. The free energy for this step would be determined by interactions of the protein with the new side chain at the site of disulfide exchange.

The free energy of the entire process can be given as the sum of two terms: $\Delta G_{modification} = \Delta G_{cb} + \Delta\Delta G_{ii}$ where the free energy of modification is given by the free energy of covalent bond change, ΔG_{cb}, and the difference in the free energy of intramolecular relaxation for the modified and unmodified proteins, $\Delta\Delta G_{ii}$. If one is interested in protein structural changes, then the term accounting for the covalent bond change is of secondary interest. The sign of $\Delta\Delta G_{ii}$ will tell which side chain (modified or unmodified) is favored under a set of given conditions. A zero value for this term would indicate no preference between two side chains and, presumably, a lack of interactions under these conditions. A nonzero value would indicate some preference for one fo the side chains.

By assuming the ΔG_{cb} term can be accounted for with the data for *N*-acetylcysteine methylamide [$\Delta G_{cb} = -RT \ln(K_{red,app}) \approx -0.4$ kcal/mol; Table III], the $\Delta\Delta G_{ii}$ term may be determined for the folded and unfolded states [$\Delta\Delta G_{ii} = \Delta G_{modification} - \Delta G_{cb}$ where $\Delta G_{modification} = -RT \ln(K_{red,N})$ or $-RT \ln(K_{red,U})$]. In the case of the folded state, it appears that the modified side chain is preferred by 0.8 kcal/mol. This seems reasonable considering that the wild-type residue is leucine at this position. In the unfolded state, the $\Delta\Delta G_{ii}$ term is nil. This presumably indicates an absence of intramolecular interactions in the unfolded state and equivalence of side-chain hydration in the protein and the cysteine analog (or the hyperextended peptide chain). Isoenergetic interactions in the two forms of the protein cannot be ruled out formally. However, any clustering in the unfolded form of a protein is thought to occur mainly by nonspecific hydrophobic interactions. The cysteine–mercaptoethanol side chain is likely to be significantly more hydrophobic than an unmodified cysteine side chain based on the relative transfer free energies of thiols and disulfides (Saunders et al., 1993) and group transfer free energies (Eisenberg & McLachlan, 1986). Coupled to the greater size and much greater flexibility of the modified side chain, it is highly unlikely that the two forms would be

considered equivalent by the ensemble of conformations in the unfolded state if interactions occur.

Protein Stability. The stability of a protein is determined by the free energy of both the folded and unfolded forms. Experiments on any single protein can only determine a difference between the two forms. Studies of mutant proteins produced by conventional mutagenesis techniques can provide relative stabilities for a series of proteins, but once again, the effect a mutation has on each conformational state will state be indeterminate. Early studies seem to indicate that unfolded proteins were true random coils (Tanford, 1968) and this has often been assumed in protein stability studies. However, contradictory evidence from a structural (Evans et al., 1991; Neri et al., 1992), thermodynamic (Green et al., 1992; Shortle & Meeker, 1989), and theoretical (Shortle et al., 1992) point of view is mounting. For the present, the magnitude of the thermodynamic consequence of interactions in the unfolded state with regard to protein stability is unknown.

Thermodynamic cycles with proteins containing disulfide bonds have been used to help partition effects of disulfide bond formation between conformational states. This was an interesting approach because cross-links were thought to decrease the conformational entropy of the unfolded state while having little effect on the folded state. Using such a cycle to explore the effect of amino acid changes is not as straightforward and is useful only in certain circumstances. A particularly relevant example is that of Lin and Kim (1989, 1991). In these studies, the authors used the thermodynamic linkage between protein unfolding and the redox state of the active-site disulfide bond of thioredoxin (which has been removed in our study). Upon mutation of a proline residue that lies in between the two cysteine residues that make up the active-site disulfide to a serine, they find changes in the disulfide exchange equilibrium constant with glutathione in both the native and denatured states. In order to connect the two cycles (the wild type and mutant protein each make up a complete cycle), they estimate the free energy difference between the two unfolded reduced forms to be equal to the difference in the hydration free energy of the side chains plus a conformational free energy term. While this estimation is reasonable, one would like to have a more direct method. We think the cycle presented in this paper is useful for examining amino acid mutations because the disulfide exchange reaction *is* the mutation being investigated and both proteins are contained within a single cycle.

A second difference lies in the fact that the mutation must be interacting in some manner with the disulfide bond. The substitution above was in the reactive-site loop which contains the disulfide bond. It is not surprising that a change there would affect the redox potential of the active-site disulfide. On the contrary, preliminary studies show that the active-site disulfide redox equilibrium is not affected by changes at position 78 of thioredoxin (unpublished results). This is not surprising in view of the fact that the oxidized and reduced structures show very little change in the hydrophobic core of the protein (Dyson et al., 1990). NMR studies indicate that no change in the dynamics of residue 78 occurs upon reduction either (Stone et al., 1993). The cycle which utilizes the active-site disulfide is likely to be useful only in specific regions of the protein where communication occurs between the active-site disulfide and the site of mutation. While this may be a good tool to judge whether two parts of a protein are interacting, it cannot be expected to be generally useful in analyzing amino acid changes. Once again, this problem is circumvented when the disulfide exchange equilibrium is the mutation being studied.

The work by Lu et al. (1992) on a cysteine-146 T4 lysozyme mutant, using temperature as a denaturant and cysteamine/cystamine as the thiol/disulfide pair, shows similar results. The $\Delta\Delta G_{ii}$ term in the folded state is 0.9 kcal/mol while that for the unfolded state is 0.2 kcal/mol. The authors claim that 0.2 kcal/mol is close to experimental error in their system. Once again, interactions in the unfolded state must be slight or of very little thermodynamic consequence.

In summary, formation of a disulfide between a cysteine residue at position 78 of thioredoxin and BME produces a protein that is 0.8 kcal/mol more stable than the unmodified protein. For this particular location and modification, the entire effect seems to stem from interactions in the native state. It is not presently known whether this will hold true for other changes at this position, other positions in thioredoxin, other proteins, and in unfolded forms produced by other methods of denaturation. Future work using the thermodynamic cycle presented here should help answer these questions.

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