byterian Hospital, and also the secretarial assistance of Geneva Jackson.

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Urea Dependence of Thiol–Disulfide Equilibria in Thioredoxin: Confirmation of the Linkage Relationship and a Sensitive Assay for Structure†

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Received January 10, 1989; Revised Manuscript Received February 16, 1989

ABSTRACT: Thioredoxin contains a single disulfide bond that can be reduced without perturbing significantly the structure of the enzyme. Upon reduction of the disulfide, protein stability decreases. We have experimentally tested the expected linkage relationship between disulfide bond formation and protein stability for thioredoxin. In order to do this, it is necessary to measure the equilibrium constant for disulfide bond formation in both the folded and unfolded states of the protein. Using glutathione as a reference species, we have measured the equilibrium constant for forming the disulfide bond (effective concentration) in thioredoxin as a function of urea concentration. As a control, we show that urea per se does not interfere with our measurements of thiol–disulfide equilibrium constants. Comparison of the values obtained for disulfide bond formation in the folded and unfolded states with the free energies for unfolding oxidized and reduced thioredoxin using circular dichroism confirms the expected linkage relationship. The urea dependence of thiol–disulfide equilibria provides a sensitive assay for folded structure in peptides or proteins. The method should also be useful to evaluate the stabilizing or destabilizing effect of natural or genetically engineered disulfides in proteins. In future work, the effects of amino acid substitutions on disulfide bond formation could be evaluated individually in the native and unfolded states of a protein.

A useful way to think about thermodynamic linkage relationships in protein stability is to consider the effective concentrations of specific interactions in the protein (Creighton, 1983). Effective concentrations represent a ratio of equilibrium or rate constants for otherwise identical intra- and intermolecular reactions. The concept of effective concentrations, used to explain the chelate effect in inorganic chemistry (Schwarzenbach, 1952; Adamson, 1954), is recognized as a useful concept in enzymology (Page & Jencks, 1971). Ef-

† This research was supported by grants from the NIH (GM37241) and the Biotechnology Process Engineering Center at MIT (NSF CDR-8803014).
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0006-2960/89/0428-5282$01.50/0

fective concentrations often exceed the concentrations of molecules in liquids or solids. This is understood in terms of the loss of rotational and translational entropy when unimolecular interactions are compared with their bimolecular counterparts (Page & Jencks, 1971).

Because it involves a reversible covalent change, the disulfide is the only interaction found in proteins for which an effective concentration (C_{eff}) can be measured directly and specifically. With glutathione as a reference thiol (Creighton, 1984), C_{eff} is the ratio of the intramolecular equilibrium constant for disulfide bond formation in a peptide or protein to the intermolecular equilibrium constant for forming a disulfide between two molecules of reduced glutathione (Creighton, 1983). Expressing both equilibria (eq 1a and 1b) as half-reactions:

$$P_{SH}^{SH} \overset{K_{intra}}{\rightleftharpoons} P_S^S + 2H^+ + 2e^- \quad (1a)$$

$$2GSH \overset{K_{inter}}{\rightleftharpoons} GSSG + 2H^+ + 2e^- \quad (1b)$$

$$C_{eff} = \frac{K_{intra}}{K_{inter}} = \frac{[P_S^S][GSH]^2}{[P_{SH}^{SH}][GSSG]} \quad (1c)$$

where P_{SH}^{SH} and P_S^S refer to the reduced and oxidized forms of the polypeptide and GSH and GSSG refer to reduced and oxidized glutathione. C_{eff} has units of concentration since a unimolecular reaction is being compared to a bimolecular one. In these equations, and subsequently, a concentration of thiols is meant to include the thiolate species [cf. Houk et al. (1987)]. C_{eff} is an empirical parameter, measured relative to a standard species (in this case, glutathione) at a given pH and set of conditions. Experimentally, C_{eff} can be measured in a redox equilibrium mixture of peptide and glutathione:

$$P_{SH}^{SH} + GSSG \rightleftharpoons P_S^S + 2GSH \quad (2a)$$

$$K_{eq} = \frac{[P_S^S][GSH]^2}{[P_{SH}^{SH}][GSSG]} = C_{eff} \quad (2b)$$

For convenience, the concentrations of GSH and GSSG are much larger than that of the protein or peptide so that the redox potential of the solution is fixed (Snyder, 1987). The amount of oxidized and reduced protein or peptide is quantitated by HPLC, and C_{eff} is calculated from eq 2b. The reaction between protein and glutathione proceeds through mixed disulfides. Although the mixed disulfide species do not enter into the relevant equilibrium constant (eq 2b), they must be distinguished from other species in the separation.

The ratio of C_{eff} in the folded (N) and unfolded (U) states of a polypeptide gives, by linkage, the difference in free energy for unfolding with or without the disulfide bond:

$$\begin{array}{ccc} N_{SH}^{SH} & \overset{K_1}{\rightleftharpoons} & N_S^S \\ \Big\downarrow\uparrow K_3 & & \Big\downarrow\uparrow K_4 \\ U_{SH}^{SH} & \overset{K_2}{\rightleftharpoons} & U_S^S \end{array} \quad (3a)$$

$$C_{eff}^N / C_{eff}^U = K_1/K_2 = K_3/K_4 \quad (3b)$$

where C_{eff}^N and C_{eff}^U represent C_{eff} of folded and unfolded states of a polypeptide, respectively.

In this paper, we demonstrate that C_{eff}^U can be measured in concentrated urea solutions: the principal concern is that the urea per se will affect measurements of C_{eff}. We show that the measured value of C_{eff} in a model "random-coil" peptide [Ac-Cys-$(Gly)_6$-Cys-NH_2], called $[Gly]_6$, is independent of urea concentration from 0 to 7 M urea. Changes in C_{eff} with [urea] in proteins or peptides are thus likely to represent unfolding and/or destabilization of structure.

Using *Escherichia coli* thioredoxin, we also show that the effective concentration for the single disulfide in the protein decreases in a sigmoidal fashion as urea is added. Then, we test quantitatively the linkage relationship (eq 3b) by comparing the ratio of C_{eff}^N:C_{eff}^U obtained at low and high urea concentrations, respectively, to the ratio of K_3:K_4 obtained by using traditional measurements of the unfolding equilibrium in the absence or presence of the disulfide bond.

The urea titration method used here provides a sensitive probe for folding and stability in polypeptides that can form a disulfide bond. The method can also be used to evaluate the stabilizing or destabilizing effect of a natural or genetically engineered disulfide [cf. Villafranca et al. (1983), Wells and Powers (1986), Schultz et al. (1987), and Wetzel et al. (1988)] and to evaluate the effects of amino acid substitutions on disulfide bond formation in the native and unfolded states of a protein.

Materials and Methods

The model random-coil peptide [Ac-Cys-$(Gly)_6$-Cys-NH_2] was synthesized on an Applied Biosystems Model 430A peptide synthesizer using standard reaction cycles. A *p*-methylbenzhydrylamine resin was used to give the C-terminal amide, and the amino terminus was blocked by acetylation. The peptide was cleaved from the resin with trifluoromethanesulfonic acid (Yajima & Fujii, 1983; Tam et al., 1986). The cleaved peptide was desalted in the reduced form on a Sephadex G-10 column in dilute acetic acid. It was then purified by reverse-phase HPLC on a Vydac C18 column, using a mobile phase composed of acetonitrile, water, and trifluoroacetic acid.

E. coli thioredoxin was purchased from Chemical Dynamics Corp. and purified by reverse-phase HPLC before use. GSH and GSSG were obtained from Sigma and used without further purification. The values of C_{eff} obtained did not depend, within experimental error, on the concentration of GSH over a 3.4-fold range or on the concentration of GSSG over a 2.6-fold range. The redox potential depends on the ratio of [GSSG] to $[GSH]^2$, so the independence of C_{eff} on the concentrations of the reagents used indicates that any impurities are not significant for these measurements. All other chemicals were of reagent grade or better.

The HPLC assay used to determine C_{eff} in peptides or proteins is as follows. The peptide (or protein) is incubated with a mixture of GSH and GSSG in 0.1 M Tris, 0.2 M KCl, and 1 mM EDTA, pH 8.7. For measurements of C_{eff} in urea solutions, the same conditions are used and the measured pH is adjusted to 8.7 after addition of urea. The concentrations of GSH and GSSG in the reaction mixture are at least 50-fold higher than that of the peptide, in order to fix the redox potential of the solution. A low concentration of peptide ($\sim$50 μM) is used to prevent dimer formation, and the reaction is performed under argon to prevent air oxidation. The reaction is allowed to proceed to equilibrium, as judged by lack of a time dependence for C_{eff} (generally checked after 1–2 h of incubation). After equilibration, the reaction mixture is loaded directly onto a reverse-phase column, previously equilibrated with acidic solvents. Alternatively, the reaction mixture is quenched with HCl (to pH 2) and then loaded onto the column. The reduced and oxidized forms of the peptide (or protein) are detected by absorbance at 229 nm, and the peaks are quantitated by integration. Corrections are made for differences in the relative extinction coefficient at 229 nm for the reduced and oxidized forms, which are determined by injecting the same amount of peptide (or protein) in the reduced or oxidized state.

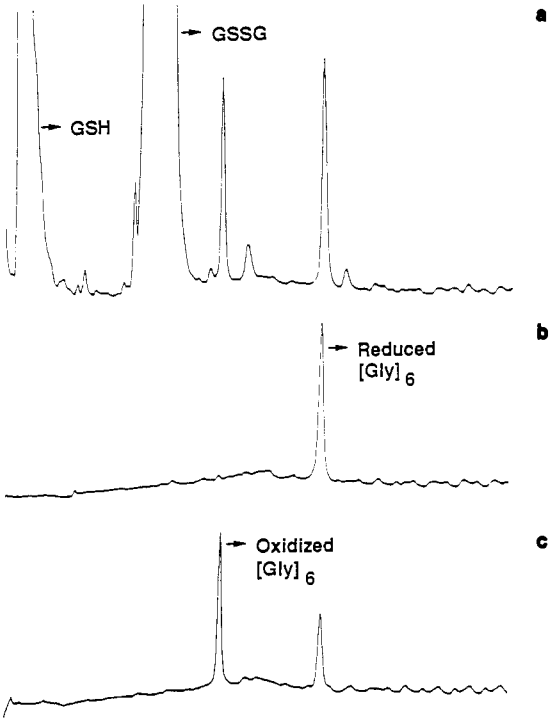

FIGURE 1: Separation of oxidized and reduced forms of a peptide [Ac-Cys-$(Gly)_6$-Cys-NH_2] by reverse-phase HPLC. Solvent A is 0.1% TFA in water, and solvent B is 70% acetonitrile, 30% water, and 0.1% TFA. The gradient is from 100% to 97% solvent A in 72 min. (a) Chromatogram of the peptide in a C_{eff} reaction mixture, which contained 0.1 M Tris, 0.2 M KCl, 1 mM EDTA, 21.6 mM GSH, and 3.6 mM GSSG, pH 8.7. (b) Chromatogram of the reduced peptide obtained by incubation with DTT. (c) Chromatogram of the peptide after air oxidation for 5.5 h at room temperature in 0.28 M Tris, pH 8.7.

As a check on the acid quench, the free thiols of reduced thioredoxin were also quenched with iodoacetamide. The final concentration was between 0.25 and 0.68 M iodoacetamide in 0.1 M Tris, 0.2 M KCl, and 1 mM EDTA, pH 8.7. After incubation at room temperature for 1–2 min, the solution pH was decreased to 2 by adding HCl and then analyzed by HPLC. HPLC experiments were carried out to ensure that the iodoacetamide quench was not modifying the protein except at thiol groups.

The stability of thioredoxin to unfolding by guanidine hydrochloride (Gdn-HCl) was measured by circular dichroism analysis at 220 nm (Kelley et al., 1987). An Aviv 60DS spectropolarimeter was used with a thermostated cell having a 1-mm path length. Spectra were measured at 23 °C in a solution of 0.1 M Tris-HCl, 0.2 M KCl, and 1 mM EDTA, containing different concentrations of Gdn-HCl (Schwarz-Mann Ultrapure). The pH of the solution was adjusted to 8.7 after addition of Gdn-HCl. Spectra were accumulated from 400 to 210 nm, and the buffer spectrum was subtracted. Solutions of reduced thioredoxin also contained a 10-fold molar excess of reduced DTT.

RESULTS

A representative HPLC chromatogram of a reaction mixture for measuring C_{eff} in the peptide $[Gly]_6$ is shown in Figure 1a. GSH and GSSG elute early in the gradient. The reduced peptide (P_{SH}^{SH}) peak was identified by reducing the peptide with 0.1 M DTT (Figure 1b). The oxidized peptide peak was identified by using a dilute ($\sim$50 μM) sample of peptide that was allowed to oxidize at pH 8.7 in the presence of air (Figure 1c). Mass spectrometry analysis confirmed assignments of these peaks.

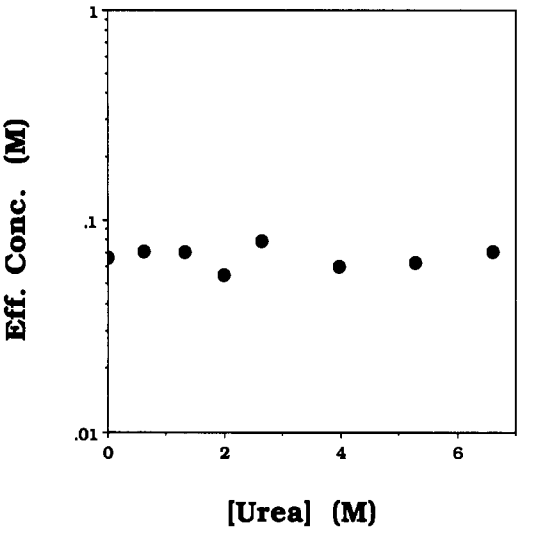

FIGURE 2: Urea dependence of C_{eff} in the peptide Ac-Cys-$(Gly)_6$-Cys-NH_2. C_{eff} was measured in a buffer of 0.1 M Tris, 0.2 M KCl, 1 mM EDTA, and different concentrations of urea at pH 8.7. The measurement was done at room temperature (23 °C). The concentrations of GSSG and GSH were 3.6 and 21.6 mM, respectively. The concentration of peptide was approximately 50 μM.

The relative amounts of P_{SH}^{SH} and P_S^S in the equilibrium mixture are determined by integration of the HPLC peak areas, after correction for differences in extinction coefficients at 229 nm. In the case of $[Gly]_6$, the ratio of the extinction coefficients of P_S^S to P_{SH}^{SH} at 229 nm is 1.5. This difference probably results from the absorbance of the disulfide bond at 229 nm. Knowing the concentrations of GSH, GSSG, P_{SH}^{SH}, and P_S^S, C_{eff} is obtained from eq 2b.

In eq 3b, the ratio between K_1 and K_2 can be obtained by measuring C_{eff} in the folded and unfolded states, which is proportional to K_3 and K_4, respectively. In order to do so, it is necessary to find conditions in which C_{eff} of the unfolded state can be measured accurately. We decided to investigate urea as a denaturant since there is little effect of urea on the pK_a of thiols (Creighton, 1977). The model random-coil peptide, $[Gly]_6$, was used. As shown in Figure 2, C_{eff} for $[Gly]_6$ is about 60 mM, and there is little dependence of C_{eff} on [urea]. This indicates that urea per se is not affecting our measurements of C_{eff}.

E. coli thioredoxin is a relatively small enzyme of 108 amino acids containing a single disulfide bond in the active site (Holmgren et al., 1975). It has been shown that there is little change in the conformation of the protein upon reduction of the disulfide bond (Stryer et al., 1967; Holmgren & Roberts, 1976; Hiraoki et al., 1988).

Figure 3 depicts C_{eff} in thioredoxin as a function of urea concentration. In the absence of urea, C_{eff} is 10 M, indicating a high propensity for the cysteines to form a disulfide in the native protein. As the concentration of urea is increased, C_{eff} decreases to a value of 26 mM, indicating that the propensity to form a disulfide in the unfolded protein is approximately 400 times lower than that in the folded protein. C_{eff} remains constant between 7.7 and 9 M urea (Figure 3), which supports our previous conclusion that urea per se is not affecting our measurements of C_{eff}. The values of C_{eff} shown in Figure 3 are independent of the ratio of GSSG and GSH used in the redox buffer, as described in the preceding section.

Because free thiols are apt to undergo oxidation and thiol–disulfide exchange reactions, a good quench is necessary for measuring C_{eff}. The commonly used quench reagents are acid, iodoacetamide, or iodoacetate. Quenching with acid is

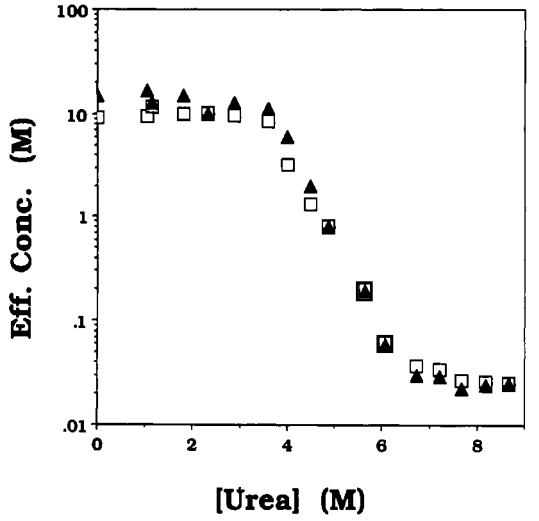

FIGURE 3: Urea dependence of C_{eff} in *E. coli* thioredoxin. C_{eff} were measured at room temperature (23 °C) in the same buffer as described in Figure 2. The concentration of GSSG was 0.56 mM and that of GSH from 3.3 to 94 mM. The concentration of protein was 5–7 μM. (□) Results obtained when the reaction mixture was quenched by acid to pH 2. (▲) Results obtained when the reaction mixture was quenched with iodoacetamide.

very fast, with a rate constant greater than 1×10^9 s^{-1} M^{-1} (Eigen, 1964). It also produces only a small change in the thiol groups as compared to other quenching reagents. The drawback of the acid quench is that it is not irreversible. Quenching with iodoacetate or iodoacetamide is an irreversible reaction. The reaction, however, is relatively slow and thiol–disulfide exchange can occur during quenching. Iodoacetamide or iodoacetate (0.1 M) has been used in studies of BPTI (Creighton, 1974a,b) and an immunoglobulin light chain fragment (Goto & Hamaguchi, 1981). Synder (1987), in studying a soybean trypsin inhibitor fragment that contains two cysteines, found that 1/7 of the thiols convert to disulfides during quenching with 0.1 M iodoacetamide or iodoacetate at pH 7. Creighton showed that acid, iodoacetate, and iodoacetamide give the same one-disulfide intermediates for BPTI but found that the two-disulfide intermediates trapped by acid rearrange intramolecularly (Creighton, 1974a,b, 1984).

We generally used an acid quench, since it is fast and easy. We have compared the results of C_{eff} measurements obtained with an iodoacetamide quench and an acid quench. Quenching with iodoacetamide at high concentrations (≥0.25 M) for 1–2 min at pH 8.7 converts reduced thioredoxin completely to the carboxamidomethylated protein. Figure 3 shows that results obtained with an iodoacetamide quench are comparable to those obtained with an acid quench.

As indicated in eq 3, the finding that C_{eff} in native thioredoxin is higher than that in unfolded thioredoxin is consistent with previous reports that oxidized thioredoxin is more stable than the reduced protein (Kelley et al., 1987). The ratio of C_{eff} for native and unfolded thioredoxin (C_{eff}^N and C_{eff}^U) should be the same as the ratio of equilibrium constants for unfolding reduced and oxidized thioredoxin (K_3 and K_4, respectively, in eq 3). This linkage relationship was tested by measuring K_3 and K_4 independently, in the same conditions used for the C_{eff} measurements. Circular dichroism was used to monitor the Gdn-HCl-induced unfolding of reduced and oxidized thioredoxin. Figure 4a shows that, as predicted, reduced thioredoxin is less stable than the oxidized form. Linear extrapolations (Schellman, 1978; Pace, 1986) of the unfolding free energies were used to estimate the free energy of unfolding reduced and oxidized thioredoxin in the absence of Gdn-HCl,

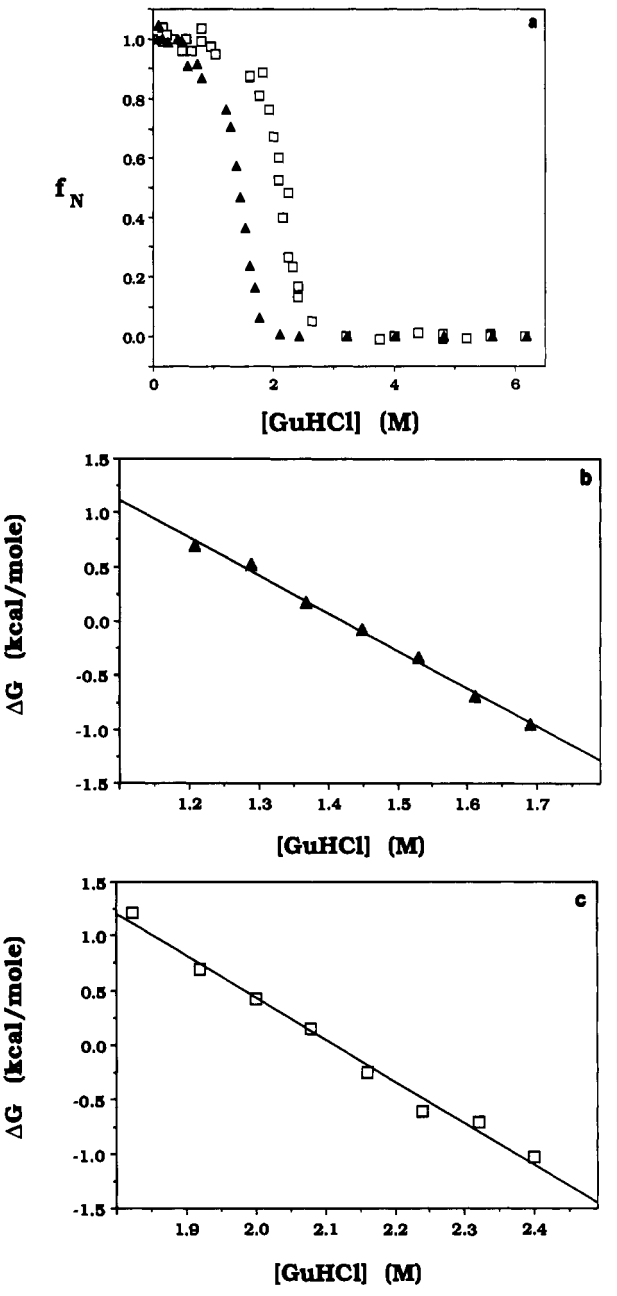

FIGURE 4: (a) Equilibrium unfolding transition for thioredoxin in the presence (□) and absence (▲) of the disulfide bond. (b) Linear extrapolation of the unfolding free energy for reduced thioredoxin showing data within the transition region. The extrapolation gives a $\Delta G°$ of 5.0 kcal/mol for unfolding reduced thioredoxin. (c) Linear extrapolation of the data for oxidized thioredoxin. The extrapolation gives a $\Delta G°$ of 8.1 kcal/mol for unfolding oxidized thioredoxin. Conditions as in Figure 2, except that Gdn-HCl was used instead of urea. Experiments with reduced thioredoxin were performed in the presence of a 10-fold molar excess of DTT.

as shown in panels b and c of Figure 4. The results give unfolding free energies of 5.0 and 8.1 kcal/mol for the reduced and oxidized species, respectively. The difference in stability ($\Delta\Delta G$) is 3.1 ± 0.6 kcal/mol, in good agreement with the predicted $\Delta\Delta G$ of 3.5 ± 0.3 kcal/mol from the ratio of C_{eff}^N and C_{eff}^U.

Discussion

Linkage between Disulfide Bond Formation and Protein Stability. Our results show that the thermodynamic cycle shown in eq 3b can be experimentally confirmed in the case of thioredoxin. This suggests that the two-state approximations

indicated in eq 3b are reasonable descriptions of the linkage between protein stability and disulfide bond formation. Because there appears to be little intrinsic effect of [urea] on disulfide bond stability, the assay described here circumvents the base-line extrapolation problems normally associated with evaluating K_3 and K_4 by traditional methods (Schellman, 1987; Pace, 1986).

Thus, measurements of the urea dependence of disulfide bond stability, similar to those used here, are intrinsically more accurate than measurements of conformational stability and involve fewer assumptions. As described below, it may also be possible to use such measurements to investigate the effects of amino acid substitutions on disulfide bond formation in the native and denatured states of a protein or peptide.

Effective Concentration Assay for Structure. In the structure assay used here, the effective concentration between two thiols in the peptide or protein is measured at varying concentrations of a denaturant (urea). Unfolding of the structure by urea is likely to change C_{eff}: the extent of change in C_{eff} is related by linkage to the effect of the disulfide bond on stability (eq 3b). The assay described here can be used as a sensitive test for structure in proteins, peptides, and protein-folding intermediates.

An assumption of the assay is that urea does not have any intrinsic effects on measurements of C_{eff}. This assumption is supported by our finding that C_{eff} in the model random-coil peptide ($[Gly]_6$) is independent of urea concentration (Figure 2). In addition, C_{eff} in thioredoxin is independent of urea concentration at high [urea] where the protein is unfolded (Figure 3).

The sensitivity of the assay depends on the difference between C_{eff} in the folded and unfolded conformations. In the case of thioredoxin this difference is approximately 400-fold. Assuming that one can detect a 2-fold change in C_{eff} with confidence (this seems conservative given the scatter in the data shown in Figures 2 and 3), a population containing 0.3% folded molecules could be detected for thioredoxin. In other proteins with a larger difference between C_{eff}^{N} and C_{eff}^{U}, the sensitivity of the assay can be orders of magnitude greater. For example, C_{eff} for the three disulfides in bovine pancreatic trypsin inhibitor has been estimated to be 200, 2×10^3, and 1×10^5 M, while C_{eff} for these disulfide bonds in unfolded BPTI has been estimated to be approximately 0.05 M (Creighton & Goldenberg, 1984; Creighton, 1988).

In addition to the high sensitivity of the assay, it is also precise over a wide range of C_{eff} values. This is because the redox potential of the solution can be varied by adjusting the ratio of [GSH]:[GSSG] so that the oxidized and reduced forms of the peptide or protein are present in approximately equal amounts (i.e., where quantitation of the relative amounts of the two species is most precise). Since the redox potential of the solution varies with the square of [GSH] but linearly with [GSSG], it is possible to accommodate a large range of C_{eff} values. Measurements of large C_{eff} values, however, will be limited by the solubility of GSH ($\sim$0.4 M at pH 8.7). In such cases, other disulfide reagents with greater reducing potential (e.g., DTT) could be used instead of GSH. C_{eff} for DTT, relative to glutathione, has been estimated to be 1200 M at pH 8.7 in the same conditions as used here (Creighton & Goldenberg, 1984; Creighton, 1986), so it should be possible to compare results obtained with DTT to those obtained with GSH.

It should be noted that the assay will also work if C_{eff} in the native state is *lower* than that in the unfolded state. In such a case, structure would be detected by an increase in C_{eff} as urea is added. The linkage relationship in eq 3 indicates that such a result would identify a disulfide bond that has a destabilizing effect on the protein or peptide.

The major limitation of the assay described here is that it is best suited for peptides or proteins that contain a single disulfide. In theory it is possible to extend the assay to include systems containing multiple disulfides, but it is simpler to work with single disulfide containing species. The assay will also not detect structure if C_{eff} in the native state is the same as that in the unfolded state. This latter situation is expected to be rare, although as pointed out earlier, it is the difference between the two C_{eff} values that determines the sensitivity of the assay.

From a practical point of view, the assay is advantageous because little material is required. A complete urea dependence study will typically require less than 1 mg of peptide or protein, which can be recovered for reuse. Air oxidation must be avoided, and we find it necessary to work in an argon atmosphere and to purge solutions with argon before use. We have found that reverse-phase HPLC gives good resolution and recovery for reduced and oxidized thioredoxin as well as many other peptides. If adequate recovery or separation is not obtained by HPLC, other methods such as electrophoresis might be used [cf. Creighton (1974a), Goto and Hamaguchi (1981), and Wells and Powers (1986)].

The reaction between protein and glutathione proceeds through mixed disulfide species. Mixed disulfides do not need to be quantitated since they are not part of the equilibrium being considered (eq 2) and because GSH and GSSG are in vast excess (i.e., the presence of mixed disulfides does not alter the redox potential of the solution). It is important, however, to separate the mixed disulfide species from the reduced and oxidized species of the peptide or protein. We check for incomplete separation using a very slow gradient (<0.2% acetonitrile increase/min).

Problems of Quenching. Although quenching is necessary to stop the thiol–disulfide reaction, it is difficult to find an ideal method. The following problems are often encountered: (i) The quenching reaction is not irreversible or the quenched product is not stable; therefore, the spectrum of species changes with time. (ii) The rate of quenching may be different for different species. Thus, some species may be trapped while others may interconvert.

Our results with thioredoxin indicate that both acid and iodoacetamide quenching give comparable results. Thus, both problems mentioned above are probably negligible, provided that the concentration of iodoacetamide used is high ($\geq$0.25 M). At high concentrations of iodoacetamide, however, side reactions can become a problem. For thioredoxin, we find that exposure to iodoacetamide gradually converts the protein to another species, indicating that there are reactive groups other than thiols that can be modified. Our quench conditions, 0.25–0.68 M iodoacetamide for 1–2 min, were chosen to ensure effective quenching of thiols with negligible side reactions.

Effects of Mutation on the Native and Denatured States of a Protein. In theory, amino acid replacements can affect the stability of proteins by changing the free energy of the native state, denatured state, or both. Traditional thermodynamic measurements determine the *difference* in free energy between the native and denatured states, so it is difficult to evaluate how an amino acid replacement is altering protein stability. Most structural studies of amino acid replacements focus on the native state; it has been difficult to study the effects of amino acid replacements on the structure of the unfolded state.

It may be possible to use disulfide bonds to probe the effects of mutations in the native and denatured states of a protein. This would involve determining values of C_{eff}^{N} and C_{eff}^{U} (eq 3) in two proteins that differ by a single amino acid. These considerations have been discussed by Goldenberg and Creighton (1984) and Alber (1988). Our finding that urea does not have intrinsic effects on measurements of C_{eff} permits determination of C_{eff}^{U} directly (eq 3). Thus, it should now be possible to determine the effects of amino acid substitutions on an interaction (i.e., formation of a specific disulfide bond) in both the native and unfolded states of proteins. A caveat is that this approach introduces two new states to be evaluated; instead of considering the native and denatured states alone, one needs to consider the reduced and oxidized forms of each state.

ACKNOWLEDGMENTS

We thank R. Rutkowski for peptide synthesis; Drs. T. Alber, R. L. Baldwin, and T. G. Oas for discussion; T. E. Creighton, J. P. Staley, and a referee for comments on the manuscript; and the MIT Mass Spectrometry Facility (supported by the NIH) for performing analyses.

Registry No. Urea, 57-13-6.

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