# Protein Folding: Assignment of the Energetic Changes of Reversible Chemical Modifications to the Folded or Unfolded States†

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ABSTRACT: Reversible chemical modifications of a series of single cysteine-containing variants of T4 lysozyme combined with thermal denaturation studies have been used to study the effects of these modifications on the stability of the protein. This allows dissection of the energetic effects of the modification on both the native and denatured states of this protein. At some sites modifications with various chemical reagents have essentially no effect on the stability of the protein, while at others, substantial changes in stability are observed. For example, chemical modification of cysteine at site 146 by cystamine ($^+NH_3CH_2CH_2SSCH_2$-$CH_2NH_3^+$) to form the mixed disulfide lowers the stability of the protein by about 1.1 kcal/mol. The reduction in the free energy of folding caused by the chemical modification is attributed to the destabilization of native state (0.9 kcal/mol), with only a relatively small effect from stabilization of the denatured state (0.2 kcal/mol). Chemical modifications of T4 lysozyme at site 146 with various chemical reagents show that the stability of the protein is lowered by a positively charged group and is relatively independent of the size of the side chains. This approach allows the investigation of the thermodynamic consequences of the reversible insertion of a wide variety of chemical entities at specific sites in proteins and, most importantly, allows dissection of the contribution of the chemical modifications to both the folded and unfolding states. It can be applied to almost any suitable macromolecular system.

The use of single amino acid replacements is one of the most powerful methods available to dissect the contributions of individual residues to the stability of the folded state of a protein. Amino acid substitutions can change the free energy difference between the folded and unfolded states of a protein by changing the free energy of either the native state, the denatured state, or both. There are several examples of modifications that are thought to change the relative stability of the folded state by acting primarily on the free energy of the unfolded state. For example, it has been suggested that replacement of residues by glycine or proline can increase or decrease the configurational entropy of the unfolded state, resulting in net destabilization or stabilization of the folded state (Matthews et al., 1987). Similarly the introduction of disulfide bonds is thought to stabilize the folded state largely as a result of an increase in the free energy of the unfolded state (Johnson et al., 1978; Lin et al., 1984; Schellman et al., 1955; Flory et al., 1956). Studies on the temperature and solvent denaturation of mutants of staphylococcal nuclease have been interpreted in terms of large changes in the energetics of the denatured state (Shortle & Meeker, 1986; Shortle et al., 1988).

Thermodynamic and crystallographic studies of bacteriophage T4 lysozyme, λ cro, and λ cI repressor mutants have shown that most temperature-sensitive mutations occur at sites with low crystallographic thermal factors and are generally inaccessible to solvent (Alber et al., 1987; Pakula et al., 1986; Hecht et al., 1983). These observations have been used to argue that the mutations change the stability of proteins by changing the free energy of their folded states (Alber et al., 1987; Goldenberg et al., 1988; Alber, 1989). In addition, these results are consistent with the view that the interactions contributing most to the stability of the native state are those that are most rigidly fixed by the other cooperative interactions in the native conformation (Crieghton, 1983; Goldenberg, 1985).

Clearly, it is possible that both the energy of folded state and unfolded state can be changed due to amino acid substitutions. To determine the energetic effect on both folded state and unfolded state, it is necessary to measure not only the difference in free energy between the folded and unfolded states of a protein but also the "free energy of mutation" (Schellman, 1987). This requires a means of equilibrating the wild-type and mutant proteins. Such equilibrations are almost impossible to achieve for natural amino acid substitutions and for irreversible covalent modifications.

We were interested in developing a thermodynamic method to assign energetic differences to the folded or unfolded states. Reversible chemical modifications provide a powerful way to change the relative stabilities of the folded and unfolded states. For example, Lindorfer and Becktel (1990) have measured the effect of reversible oxidation and reduction of a disulfide bridge on the folded and unfolded states of T4 lysozyme. A similar approach was taken by Lin and Kim (1991) to monitor thioredoxin folding energetics.

In this work, we exploit reversible chemical reactions that involve thiol–disulfide exchange of small disulfide molecules with a protein containing only one cysteine residue. By measuring the equilibrium constants for the thiol–disulfide exchange reaction and comparing these values to that of the same disulfide with a suitable model compound, we can calculate the free energy change for interconversion of denatured and native states of the unmodified protein and modified protein. Consequently the energetic effect of the chemical modification on native state as well as denatured state can be directly inferred.

We have investigated the effects of chemical modifications on the stability of the protein at eight locations (55, 79, 96, 119, 135, 142, 146, and 159) in T4 lysozyme. A detailed analysis of the modifications at position 146 using the method

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described above is presented. This site had previously been identified as one that is strongly coupled to the stability of the protein. Modification of T4 lysozyme at site 146 by the formation of a mixed disulfide with cystamine decreases the stability of the protein by about a factor of 5, or 1.1 kcal/mol at 56 °C and pH 5.3. Approximately 80% of this energy change is due to destabilization of the native, folded state. Chemical modifications with several other reagents have little effect at this site.

## EXPERIMENTAL PROCEDURES

(*1*) *T4 Lysozymes.* Wild-type T4 lysozyme contains two cysteine residues at position 54 and 97. Using site-directed mutagenesis (Alber & Matthews, 1987), the gene for a pseudo-wild-type protein with the substitutions of cysteines 54 and 97 to serine was constructed. The eight new cysteine substitutions, summarized in Table I, were introduced into this pseudo-wild-type background. The genes for these T4 lysozyme variants were incorporated into our standard bacterial expression system, and the corresponding proteins were prepared as described previously (Muchmore et al., 1989). The purity of the proteins was monitored by cation-exchange HPLC. The proteins were denatured in 4.0 M guanidinium hydrochloride and 0.1 M sodium phosphate at pH 7 and were titrated with dithionitrobenzoic acid (Ellman, 1959), confirming that there was only 1 mol of cysteine per mole of each protein.

(*2*) *Reagents.* Chemicals used for the modification of the protein were from commercial sources, except for *N*-acetylcysteinamide, which was synthesized from L-cystine dimethyl ester dihydrochloride following the procedure published by Martin et al. (1967). The pH values for stock solutions and the thermal denaturation buffer were measured using a PHM 84 research pH meter at room temperature. All the solutions were made from deionized water.

(*3*) *Chemical Modifications of the Proteins.* The chemical modifications of the single thiol-containing T4 lysozyme variants by cystamine, and the oxidized forms of mercaptoacetic acid, mercarptoethanol, and dithionitrobenzoic acid, were performed by adding a 1000-fold excess amount of reagents to an approximate 50 $\mu$M protein sample. The protein samples were previously dialyzed against pH 7.0, 100 mM sodium phosphate and 1.0 mM DTT solution under $N_2$.

Cation-exchange HPLC with a CX-300 column was used to monitor the conversion of proteins with charged reagents. The proteins were eluted with a linear salt gradient from 50 mM sodium phosphate to 300 mM sodium phosphate, pH 7.0. The absorbance at 229 nm was monitored with time. More than 95% of the proteins were converted to mixed disulfide of proteins as indicated by HPLC after 1-h reaction at room temperature. After 3 h, the modified protein samples were dialyzed against 0.2 M KCl, pH 2.0, to quench the thiol–disulfide exchange and to remove the excess reagents.

(*4*) *Stability and Equilibrium Constant Measurements.* (*a*) *Reversible Thermal Denaturation of the T4 Lysozymes.* The thermal denaturation of the lysozymes was monitored by the change in the circular dichroism signal at 223 nm (Becktel & Baase, 1987).

Both the unmodified and modified proteins were dialyzed against thermal denaturation buffer (25 mM KCl and 20 mM potassium phosphate at pH 2.44) in the same container to ensure identical solution conditions. 1.0 M KOH was added directly to the cuvette to adjust pH for measurements at higher pH values. Protein concentrations for thermal denaturation experiments were about 20 $\mu$g/mL.

(*b*) *Measurement of Equilibrium Constants for the Reaction of Cystamine with T4 Lysozyme.* Apparent equilibrium constants of unfolding ($K_{app}$) for A146C in different concentration ratios of cystamine and cysteamine were measured in 100 mM potassium acetate, pH 5.13, at 56 °C. The change in CD signal with time was monitored during the following process. The cuvette with 25 $\mu$g/mL A146C in 100 mM potassium phosphate, pH 5.13, was allowed to thermally equilibrate at 0 °C for about 10 min. The temperature was then raised to 56 °C within 2 min. These conditions are near the midpoint of the denaturation transition of A146C and the modified A146C by cystamine. The system was allowed to reach equilibrium. Into the cuvette were added 5.0 $\mu$L of 0.459 M cystamine and 5.0 $\mu$L of various concentrations of cysteamine. After about 2 h, the reaction due to chemical modification of A146C and folding–unfolding transition of the modified protein reached equilibrium, and the CD signal reflected the new equilibrium. Finally, the temperature was raised to 70 °C to completely unfold the protein and the system was allowed to reach equilibrium again.

The equilibrium constant of *N*-acetylcysteinamide, a model compound, with cystamine was measured by CD spectroscopy under the same conditions.

The equilibrium constants for reaction of native L79C, R96C, and R119C with cystamine, respectively, were measured by HPLC at 20 °C, pH 7.0. The protein samples, about 3 mg/mL, were dialyzed against redox buffer (20 mM cysteamine and 15 mM cystamine) for 2 h. A 100-$\mu$L aliquot of a 1:5 dilution of each sample was injected into a HPLC with CX-300 column. The proteins were eluted with same salt gradient as described previously.

## RESULTS

A number of variants of T4 lysozyme containing single cysteine residues were constructed for this study. We began with the replacement by serine of the two naturally occurring cysteine residues at positions 54 and 97. We refer to this pseudo-wild-type version of the protein as the thiol-free protein. The stability of T4 lysozyme is descreased by these two substitutions, with its $T_m$ decreasing from 42 to 36 °C at pH 2.44. We then constructed eight variants of the thiol-free protein which contained a single cysteine residue at each of eight positions. For example, the asparagine residue at position 55 was replaced by cysteine; we refer to this protein as N55C. Other cysteine substitutions were made at Leu79 (L79C), Arg96 (R96C), Arg119 (R119C), Lys135 (K135C), Thr142 (T142C), Ala146 (A146C), and Asp159 (D159C).

(*1*) *Thermal Denaturation Studies of the Eight Single Cys-Containing T4 Lysozymes.* The reversible thermal unfolding of each of these eight T4 lysozymes, as well as the pseudo-wild-type parent, was monitored by circular dichroism (CD) at 223 nm. Under the conditions used for study, all proteins displayed reversible two-state transitions.

Figure 1 shows the CD signal of a solution of A146C in 100 mM potassium acetate buffer at pH 5.32 as a function of temperature. There is a gradual decrease in the CD signal at temperatures below 40 °C. This is followed by the cooperative two-state transition with a midpoint ($T_m$) at 58.3 °C. At temperatures above 70 °C, the protein is fully unfolded and the weak temperature dependence of the CD signal of the unfolded state is observed. The observed temperature dependences of the folded and unfolded states are approximately linear over these temperature ranges with slopes of $8.0 \times 10^{-4}$/°C and $1.2 \times 10^{-3}$/°C, respectively.

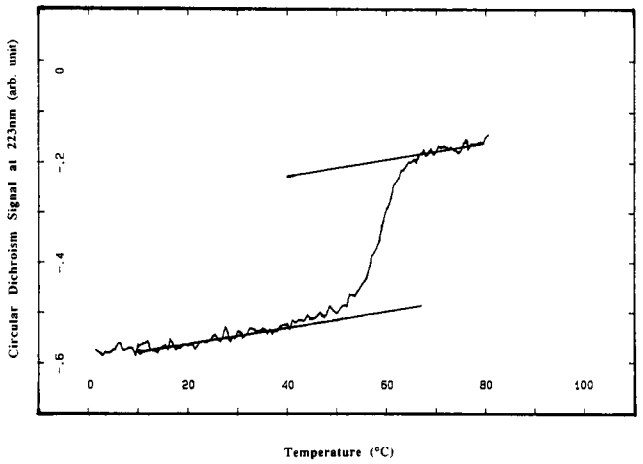


FIGURE 1: Temperature dependence of the circular dichroism signal at 223 nm of unmodified A146C at pH 5.32 in 100 mM potassium acetate. The folded and unfolded base lines, determined by using a least squares fit, are indicated by straight lines. Data from the transition zone were transformed to the fraction of folded protein using a simple two-state model with correction for these base lines.

Table I: Transiton Temperatures of T4 Lysozyme Variants and Derivatives[a]

| variant | native $T_m$ (°C) | $\Delta T_m$ (°C) | mixed disulfide $T_m$ (°C) | $\Delta\Delta T_m$ (°C) |
|---|---|---|---|---|
| pH 2.4 | | | | |
| parent (C54S,C97S) | 36.4 | | | |
| A146C | 32.3 | −4.1 | 24.5 | −7.8 |
| T142C | 35.9 | −0.5 | 31.7 | −4.2 |
| D159C | 37.1 | +0.7 | 33.9 | −3.2 |
| N55C | 32.8 | −3.6 | 31.4 | −1.4 |
| R96C | 27.7 | −8.7 | 25.6 | −1.1 |
| L79C | 31.3 | −5.1 | 30.6 | −0.7 |
| R119C | 33.4 | −3.0 | 33.9 | +0.5 |
| K135C | 33.8 | −2.6 | 34.1 | +0.7 |
| pH 5.3 | | | | |
| A146C | 58.3 | | 54.9 | −3.4 |
| R96C | 58.4 | | 58.1 | −0.3 |

[a] $T_m$ is the thermal denaturation midpoint transition temperature. Reproducibility of $T_m$ measurements is ±0.2 °C. $\Delta\Delta T_m$ is the difference in $T_m$ between the modified protein and the unmodified protein. $\Delta T_m$ is the difference in $T_m$ between the cysteine-containing protein and the parent.

Table I is a summary of the denaturation studies of the various mutant proteins. The single cysteine substitutions have varying effects on stability of T4 lysozyme as compared to their pseudo-wild-type parent. The lysozymes with cysteine at positions 142 and 159 have similar stabilities as the parent protein while those with substitutions at the other positions are somewhat less stable. The most extreme case is R96C for which replacement of arginine by cysteine reduces the $T_m$ of lysozyme by about 9 °C at pH 2.44.

*(2) Thermal Denaturation Studies of the Cys-Containing T4 Lysozymes Modified with Cystamine.* Each of the lysozyme variants was treated with an excess of cystamine to completely form the mixed disulfide of the protein and cystamine according to the reaction:

$$PSH + RSSR = PSSR + RSH$$

where PSH is the reduced form of the protein, RSSR is the cystamine, PSSR is the mixed disulfide of the protein and cystamine, and RSH is cysteamine. The midpoints of the thermal unfolding curves of the modified protein were also determined from the temperature dependence of the CD signal at 223 nm (Table I). In all cases the unfolding transitions appeared as well-behaved, two-state equilibria.

Table II: Change in $T_m$ of A146C Due to Mixed Disulfide Formation with Several Reagents[a]

| A146C derivatives | $\Delta T_m$ (°C) pH = 2.44 | pH = 2.85 | pH = 5.32 |
|---|---|---|---|
| $PSSCH_2CH_2NH_3^+$ | −7.8 | −7.1 | −3.4 |
| $PSSCH_2CH_2OH$ | 0.5 | nd | 0.1 |
| $PSSCH_2COOH$ | −1.1 | −0.1 | −0.3 |
| PSSTNB | 0.1 | nd | −1.2 |

[a] nd = not determined. $\Delta T_m$ is the difference in the midpoint transition ($T_m$) between the modified and unmodified A146C ($T_{m(mod)} - T_{m(unmod)}$). $T_m$'s of A146C are 32.3 °C at pH 2.44, 40.6 °C at pH and 58.3 °C at pH 5.32. PSS refers to the oxidized A146C, and TNB is thionitrobenzoic acid.

Table III: Thermodynamic Analysis of the Denaturation of A146-$SSCH_2CH_2NH_3^+$ [a]

| pH | $T_m$ (°C) | $\Delta T_m$ (°C) | $\Delta H$ (kcal/mol) | $\Delta S$ [cal/(mol·deg)] | $\Delta\Delta G$ (kcal/mol) |
|---|---|---|---|---|---|
| 2.44 | 24.5 | −7.8 | 47.0 | 158 | 1.2 |
| 2.85 | 33.5 | −7.1 | 67.9 | 221 | 1.6 |
| 5.32 | 54.9 | −3.4 | 103.2 | 315 | 1.1 |
| 5.13* | 55.7 | −3.3 | 113.9 | 346 | 1.1 |

[a] $T_m$ is the melting temperature of the chemically modified derivatives, and $\Delta T_m$ is the change in melting temperature relative to pseudo-wild-type A146C. $\Delta H$ and $\Delta S$ are respectively the enthalpy and entropy of unfolding of the modified protein, and $\Delta\Delta G$ (=$\Delta S\Delta T$) is the difference in free energy between the free energies of the chemically modified protein and A146C estimated from the change in melting temperature. "pH 5.13*" is in 100 mM potassium acetate. Otherwise 25 mM KCl and 20 mM potassium phosphate were used.

The formation of the mixed disulfides with the single cysteine residues at positions 79, 119, and 135 had essentially no effect on the thermal unfolding of the proteins at low pH. Four of the variants (N55C, R96C, D159C, and T142C) had intermediate properties upon mixed disulfide formation, with decreases in $T_m$ ranging from 1 to 4 °C. The most dramatic effect on stability was observed when the mixed disulfide was formed with the single cysteine substitution at position 146. This resulted in a decrease of 8 °C in $T_m$.

We chose position 146 for further study since it was the most strongly affected by cystamine modification. We also chose three proteins, L79C, R96C, and R119C, as examples of mutant proteins in which there was little change in stability upon disulfide formation.

*(3) Modification of A146C Lysozyme with Thiol Reagents.* The variant A146C was modified with cystamine, the oxidized forms of mercaptoacetic acid, mercaptoethanol, and dithionitrobenzoic acid. The thermal denaturations of A146C and the chemically modified forms of the protein were monitored at pH 2.44, 2.85, and 5.32. In all cases, the conformational transitions were apparently two state. The melting temperature differences between the chemically modified proteins and the unmodified A146C are listed in Table II. In contrast to cystamine, the chemical modification of A146C with the oxidized forms of mercaptoethanol, mercaptoacetic acid, and dithionitrobenzoic acid had only small effects on the stability of the lysozyme.

The thermodynamic parameters for the thermal unfolding of A146-$SSCH_2CH_2NH_3^+$ at two pH values are listed in Table III. Chemical modification by positively charged cystamine lowers the protein stability of 7.8 °C at pH 2.4. This corresponds to a decrease in free energy of unfolding, $\Delta G_{mod}$, by about 1.2 ± 0.3 kcal/mol using the formula $\Delta G_{mod} = (\Delta T_{mod})(\Delta S)$, where $\Delta T_{mod}$ is the change in $T_m$ due to chemical modification and $\Delta S$ is the entropy change of unfolding of the modified A146C at its transition midpoint temperature (Beck-

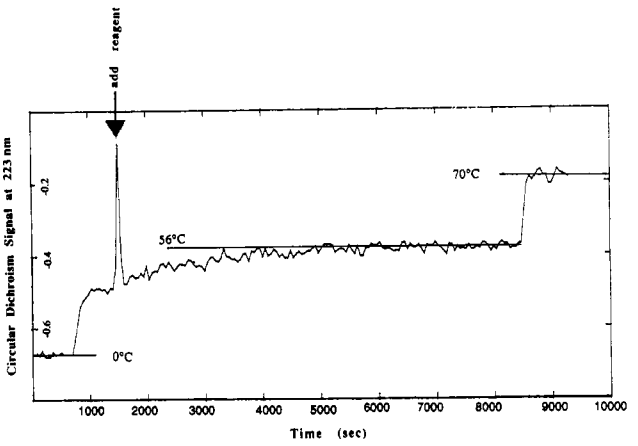


FIGURE 2: Measurement of $K_{app}$ for the reaction of A146C with cystamine. A146C (25 $\mu$g/mL A146C in 100 mM potassium acetate, pH 5.13) in a cuvette was equilibrated at 0 °C for 12 min. Then the temperature was raised to 56 °C, near the midpoint of the unfolding transition of the protein, and the system was allowed to reach equilibrium. Into the cuvette, 5.0 $\mu$L of 0.459 M cystamine and 5.0 $\mu$L of 0.251 M cysteamine were quickly added and very well mixed. After the reaction reached equilibrium, the temperature was then raised to 70 °C.

tel & Schellman, 1987). At pH 5.3, the $T_m$ is lowered by only about 3.4 °C. However, the entropy of unfolding the protein at this pH and temperature is larger, and the change in stability also corresponds to 1.1 ± 0.3 kcal at pH 5.3. This suggests that there is very little pH and temperature dependence to the change in stability caused by mixed disulfide formation in the acidic range of pH.

*(4) Measurement of Overall Unfolding Equilibrium Constant of A146C in Different Redox Buffers of Cystamine and Cysteamine.* The thiol–disulfide reaction of A146C and cystamine at 56 °C can proceed only at pH values above 5. The ionic strength and pH dependence of the denaturation of lysozyme between pH 5 and 6 are slight (Anderson et al., 1990). In order to maintain the exact pH for these studies, we used buffer containing 100 mM potassium acetate, pH 5.13, instead of usual 25 mM KCl and 20 mM potassium phosphate buffer. This new buffer system did not have any specific effects on the stability of the modified and unmodified protein.

Since the formation of the mixed disulfide at position 146 with cystamine changes the stability of the lysozyme, one can monitor the increased amount of unfolded protein caused by adduct formation at pH 5.13 and constant temperature with a CD spectrophotometer. Such an experiment is shown in Figure 2 as a plot of the CD signal intensity at 223 nm as a function of time. After an initial period of thermal equilibration at 0 °C the sample in the cuvette is heated to 56 °C. This temperature is near the midpoint of the thermal unfolding transition of the unmodified protein. After allowing the unmodified protein to reach equilibrium between its native and unfolded states, a redox buffer is added to the cuvette. For the example shown, this buffer contains both cystamine and cysteamine at a molar ratio of 1.8 mol of cystamine/mol of cysteamine. Both are present in at least a 100-fold excess over the protein. In this redox buffer, the protein is partially modified. The oxidized protein is less stable than the reduced protein under these conditions, and the CD signal decreases as further unfolding proceeds. The system is allowed to come to equilibrium, as evident by no further change in CD signal (at least 5 half-lives). Finally, the cuvette is heated to 70 °C to complete the unfolding process.

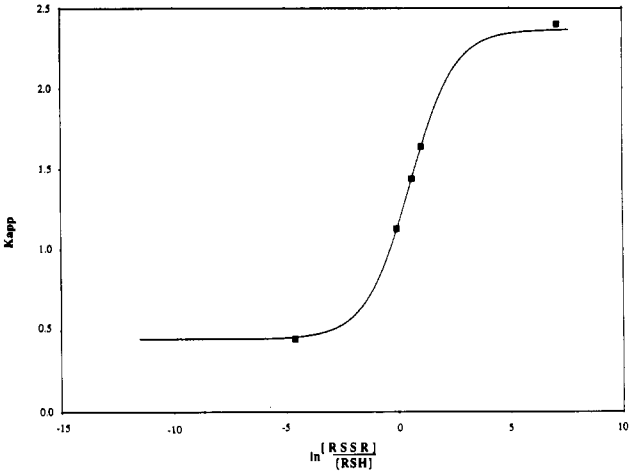


FIGURE 3: $K_{app}$ vs redox buffer potential [RSSR]/[RSH]. The solid squares are the experimental values, and the calculated values from average $K_n$ = 0.59, $K_u$ = 3.1, and $K_f$ = 0.45 by eq 2 are shown as the smooth curve.

The CD signal of the unfolded state at 56 °C, $\theta_u$, is determined from the signal of the protein at 70 °C by a linear-extrapolation using the previously measured value of the temperature dependence of the CD of the unfolded protein (Figure 1). The corresponding value for the folded state, $\theta_n$, is obtained by the linear temperature correction of the signal due to the folded state measured at 0 °C. The value of $K_{app}$, the overall equilibrium constant for the unfolding reaction, is then calculated from the CD signal observed at 56 °C, $\theta$, after the addition of redox buffer using the relationship:

$$K_{app} = (\theta - \theta_n)/(\theta_u - \theta) \quad (1)$$

This assumes that the native protein and the disulfide adduct have the same CD signal at 223 nm in the folded and the unfolded states and the same temperature dependence of CD signal outside the transition region. This assumption appears to be valid since we observe no change in the CD signal when redox buffers are added to protein solutions at temperatures where adduct formation does not significantly change the fraction of the total protein in the folded or unfolded states. Analysis of the data shown in Figure 2 gives a value of $K_{app}$ of 1.13 for this experimental condition.

Figure 3 shows a plot of the dependence of $K_{app}$ as a function of the logarithm of the ratio of cystamine to cysteamine present in the redox buffer used in a series of experiments similar to that shown in Figure 2. The measured values of $K_{app}$ vary from about 0.4 to 2.4. With low relative concentration of cystamine, only a very small fraction of the protein is modified and $K_{app}$ is approximately the same as the unfolding equilibrium constant for the unmodified protein. With higher values of cystamine to cysteamine concentration ratio, nearly all the protein is present as the mixed disulfide and $K_{app}$ approaches the unfolding equilibrium constant for the fully derivatized protein.

The thermodynamic coupling of the unfolding equilibria to the thiol–disulfide equilibria is shown in Figure 4. Here NSH is the unmodified folded protein, NSSR is the folded form of the derivatized protein, USH and USSR denote the respective unfolded protein species, RSSR is cystamine, and RSH is cysteamine. The equilibrium constants $K_n$ and $K_u$ describe the formation of the mixed disulfide of the protein with the small disulfide molecule in the folded and unfolded states, respectively. The equilibrium constants $K_f$ and $K_f'$ define the

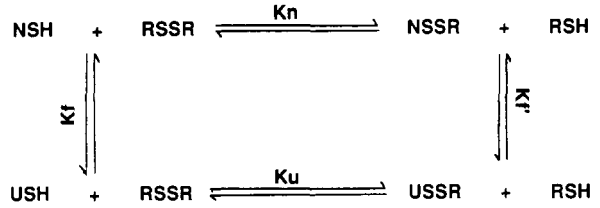


FIGURE 4: Thermodynamic cycle linking the reversible thermal denaturation of a protein and the reversible chemical modification of side-chain groups. $K_n$ and $K_u$ are the equilibrium constants of the native protein and denatured protein with small disulfide, respectively. $K_f$ and $K_f'$ are the equilibrium constants of unfolding of the modified protein and the unmodified protein, respectively.

unfolding equilibria of the native and derivatized forms of the protein. Using these definitions

$$K_{app} = \frac{[USH] + [USSR]}{[NSH] + [NSSR]} = \frac{[USH](1 + [USSR]/[USH])}{[NSH](1 + [NSSR]/[NSH])} = K_f\left(\frac{1 + K_uP}{1 + K_nP}\right) \quad (2)$$

where $P$ is the redox buffer potential, $P = [RSSR]/[RSH]$. The solid line in Figure 3 is the theoretical curve using $K_f = 0.45$, $K_n = 0.59$, and $K_u = 3.1$. As can be seen, there is an excellent fit to the theoretical curve using the experimental values of $K_{app}$ determined at several values of $P$. The thermodynamic coupling shown in Figure 4 requires that the ratio of the stabilities of the native and derivatized proteins be the same as the ratio of the equilibrium constants for disulfide formation in the folded and unfolded states, or

$$K_f/K_f' = K_n/K_u \quad (3)$$

Thus the formation of the disulfide adduct results in a 5-fold reduction in the thermodynamic stability of the folded as compared to the unfolded state. This corresponds to a difference in stability of about 1.0 kcal/mol under these conditions, agreeing well with the results reported in Table III for the unfolding of unmodified and fully modified A146C proteins.

*(5) Determination of the Reference Equilibrium Constant for Mixed Disulfide Formation.* In order to assign the source of the 5-fold change in stability to the folded or unfolded states, it is necessary to compare the values of $K_n$ and $K_u$ to a suitable noninteracting model thiol. The difference in $K_n$ and $K_u$ from that of model compound will reflect the effects of folded or unfolded state on the thiol–disulfide exchange reaction. This will be further explored in the Discussion section. It is essential that this thiol has the same electronic properties as the protein-associated thiol. We chose *N*-acetylcysteinamide (NACA) as the model compound.

The equilibrium constant for the reaction of NACA with cystamine was measured under the same conditions as those used to measure $K_{app}$ by following the CD signal change at wavelength 223 nm. Figure 5 shows the CD spectrum of the reaction mixture in different conditions. Curve A is the CD spectrum of NACA, and curve E is essentially the spectrum of the resulting product of NACA with cystamine. Curves B–D result when a constant concentration of NACA is incubated in various ratios of oxidized cystamine to reduced cysteamine. The presence of the isosbestic point at 213 nm is consistent with equilibration between two chiral, CD-active species, NACA and the mixed disulfide of NACA and cystamine. Cystamine and cysteamine alone do not have a CD

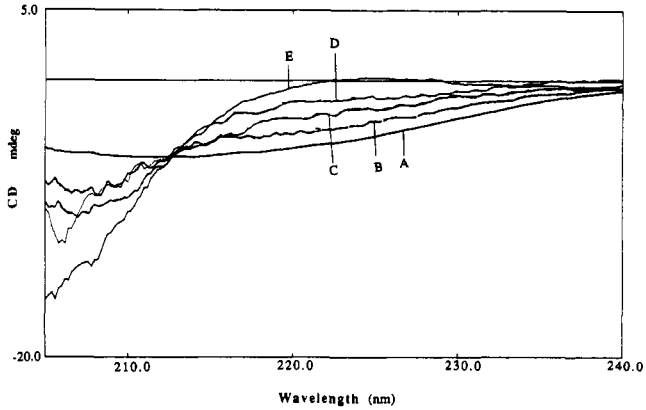


FIGURE 5: Determination of equilibrium constant for cystamine with *N*-acetylcysteinamide at pH 5.13 and 56 °C. The starting reaction mixtures were as follows: (A) 9.5 mM *N*-acetylcysteinamide, (B) 9.5 mM *N*-acetylcysteinamide, 41.6 mM RSSR, and 178.3 mM RSH, (C) 9.5 mM *N*-acetylcysteinamide, 59.7 mM RSSR, and 170.4 mM RSH, (D) 9.5 mM *N*-acetylcysteinamide, 62.5 mM RSSR, and 133.6 mM RSH, and (E) 9.5 mM *N*-acetylcysteinamide and 229.0 mM RSSR. RSSR is $(^+H_3NCH_2CH_2)_2S_2$, RSH is $^+H_3NCH_2CH_2SH$.

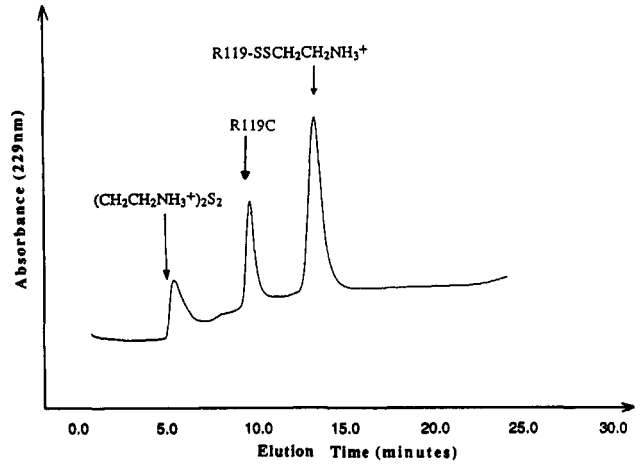


FIGURE 6: HPLC separation of unmodified R119C and the mixed disulfide of R119C and cystamine. A 3 mg/mL solution of R119C was dialyzed against 20 mM cysteamine and 15 mM cystamine in pH 7.0, 100 mM sodium phosphate buffer at 20 °C for 2 h. 100 μL of a 1:5 dilution of the sample was injected into an ion-exchange HPLC column. The proteins were eluted with a salt gradient as described in the text.

spectrum. As we can see from the figure, the NACA has a CD signal at wavelength 223 nm while the resulting product of NACA with cystamine essentially has no CD signal at this wavelength. The amount of NACA and of the mixed disulfide at equilibrium can be calculated from the CD signal. From several independent determinations, the equilibrium constant of the model reaction is 2.2 ± 0.3. Within the errors of measurement, this value was found to be independent of both temperature between 20 and 56 °C and pH between 5.0 and 7.0.

To further explore the validity of NACA as a model for the mixed disulfide equilibrium, we also measured the equilibrium constant of the reaction of the protein with cystamine for L79C, R96C, and R119C, respectively. Modification results in little change in stability for these variants, and thus $K_n$ approximately equals $K_u$ for each of these proteins. Because of the lack of the change in stability, we could not monitor the modification equilibrium using CD spectroscopy. The modified and native proteins can be separated using cation-exchange HPLC, however. Figure 6 shows the separation of the oxidized and reduced R119C. The chemical modification of cystamine resulted in an additional positive charge on the

protein, and thus the modified lysozyme elutes later than the unmodified species. The equilibrium constant can be determined by quantitating the area of the peaks for modified protein and unmodified protein when equilibrium is reached. The equilibrium constant measured for the reaction of L79C with cystamine was 2.2, for R96C with cystamine the value was 2.6, and for the reaction of R119C with cystamine the value was 2.8.

## DISCUSSION

The thiol–disulfide exchange reactions described in this report provide a convenient means to incorporate a variety of side-chain modifications into proteins. These are reversible reactions which can be studied under equilibrium conditions. By linking the equilibria of a redox reaction and the folding–unfolding reaction, it is possible to dissect the effects of chemical changes on the stability of the folded and unfolded states. The use of disulfide chemistry is advantageous since it is both highly specific and reversible and allows a wide variety of modifications. There are some technical features of the approach which must be considered. First, since thiol–disulfide exchange requires the participation of the thiolate anion (Creighton, 1978), the range of pH values which can be used is limited. Below pH 5, the exchange reactions become rather slow, and reactions carried out below pH 4 will not likely to be useful for these studies. Second, the experimental range of free energy difference between unmodified protein and modified protein is limited by the analytical methods used to quantitate the extent of modification. Third, there must be a defined number of sites at which thiol–disulfide exchange can occur. The simplest case is the one illustrated in this work in which a single thiol is available for reaction. While multiple sites can be used, the thermodynamic scheme needed for quantitative analysis becomes complicated.

(*1*) *Effects of Cysteine Substitutions in T4 Lysozyme.* We began this study with a survey of potentially interesting sites for the incorporation of a single cysteine residue into T4 lysozyme which could then be reversibly derivatized. We chose a total of eight sites involving positions 55, 79, 96, 119, 135, 142, 146, and 159. As summarized in Table I, the effects of the cysteine substitutions are generally to reduce the transition temperature for the unfolding of the proteins. These changes range from a small increase of about 0.7 °C for the variant D159C to a large 8.7 °C decrease for the R96C substitution. In each case, except for A146C, these substitutions insert a cysteine residue into a relatively solvent-exposed region of the protein.

Positions 96 and 146 had previously been identified as potentially important sites for determining stability by random mutagenesis of T4 phage, followed by screening and selection for temperature-sensitive halo formation (Grütter et al., 1979). The other sites were chosen arbitrarily with the proviso that the side chain appeared to be at least partially solvent accessible in the structure of the wild-type protein determined by X-ray crystallography. While we have not determined the structures of the variants, the cysteine of each protein in the native state was found to be reasonably reactive to the bulky thiol reagent DTNB (data not shown). A detailed structural explanation of the stability changes caused by the substitutions awaits X-ray structural analysis. However, they most likely reflect rather small energetic changes as a result of the details of the side-chain interactions in the folded or unfolded states or both. This is exemplified by previous studies of T4 lysozyme mutants with the substitution R96H and A146T (Gray, 1985).

(*2*) *Cystamine as an Amino Acid Analogue.* We were especially interested in the effects of formation of the mixed disulfide with cystamine on the stability of single-cysteine containing T4 lysozymes. One might imagine that the resulting cysteinyl aminoethyl disulfide residue could mimic the long positively charged side chain of arginine or lysine residues. For example, we had hoped that the mixed disulfide might resemble the original arginine at position 96 and restore at least some of the nearly 8 °C decrease in stability caused by the arginine to cysteine substitution. As shown in Table I, the formation of the mixed disulfide did not increase stability, but rather resulted in a further slight decrease at low pH and had virtually no effect at pH 5.6. This argues that arginine 96 has a precise steric role in its contributions to the folding energetics of wild-type T4 lysozyme. While it is possible that these contributions lie in the unfolded state, we believe that these precise steric interactions are more likely to be associated with interactions of the folded state. A detailed structural and thermodynamic analysis of the mutant R96H has been published (Weaver et al., 1989). In general, this supports the view that specific interactions of arginine residue 96 in the folded state determine its energetic contributions to the folding thermodynamics.

Similarly the formation of the mixed disulfide with cystamine did not restore stability to R119C or K135C. While the destabilization caused by the cysteine substitutions is 3.0 and 2.5 °C, only about 0.5 °C of this decrease in stability was regained by mixed disulfide formation. This is probably not significant since we estimate the error in the measurement of $T_m$ to be about 0.2 °C.

Overall, we conclude that the cysteinyl aminoethyl disulfide residue should not be considered as a mimic for lysine or arginine side chains but rather has its own properties. It is likely that the unique bond lengths and geometry of the disulfide moiety itself play an important role in distinguishing the properties of the mixed disulfide side chain from those of lysine or arginine.

(*3*) *Effects of Chemical Modification on the Folded or Unfolded States of T4 Lysozyme.* We are particularly concerned with the assignment of changes in stability to changes in the energetics of the folded or of the unfolded states of T4 lysozyme. It may be useful to consider the conceptually simpler acid–base equilibria for the reversible interconversion of chemically distinct forms of the folded and unfolded states of a protein. In this case the $K_a$ values for proton dissociation from particular residues have the same information as the thiol–disulfide equilibrium constants. If a given p$K_a$ value of side chain in the native or denatured state is different from that of the side-chain acidic residue in a noninteracting peptide model, we infer an interaction which accounts for the difference observed. The size of the p$K_a$ shift reflects the strength of the differential interaction between the protonated and unprotonated states which, in turn, reflects structural events in the folded or the unfolded states of the protein (Anderson et al., 1990). The thiol–disulfide exchange reaction is somewhat more complicated than simple proton binding since steric considerations are more likely to be important for the thiol–disulfide exchange equilibria, although electrostatic interactions may be less relevant.

This energetic analysis consists of two steps. First, the equilibrium constant for the formation of the mixed disulfide with the folded and unfolded protein must be determined. As a result of the thermodynamic cycle shown in Figure 4, the change in stability of the folded state due to mixed disulfide formation is mirrored in the change in the equilibrium constants for mixed disulfide formation in the folded and unfolded states, respectively. Thus the 5-fold decrease in the

equilibrium constants for mixed disulfide formation of cystamine with A146C in the unfolded versus folded states reflects the 5-fold decrease in stability of the folded state of the modified protein relative to the reduced parent as a result of mixed disulfide formation. As can be seen from the data in Table III and in Figure 3, the thermodynamic changes introduced can be monitored either by the thermal unfolding equilibria or by the thiol–disulfide exchange equilibria, or, most ideally, by both.

The second step in the assignment of energetic changes to the folded or unfolded state requires comparison of the values of the equilibrium constants for mixed disulfide formation of the folded and unfolded states to the corresponding equilibrium constant for mixed disulfide formation with a noninteracting model thiol. We chose *N*-acetylcysteinamide as this model. This compound provides a thiol with nearly identical electronic properties as a cysteine residue of a protein and should display no steric restriction, electrostatic attraction or repulsion, or other environmental effects which might alter the reactivity of the thiol to the formation of the mixed disulfide. The measured value of 2.2 ± 0.3 for the equilibrium constant of the model compound with cystamine demonstrates that mixed disulfide formation is slightly favored as compared to the formation of the symmetric disulfide or cystamine in the presence of the model thiol.

We tested the validity of the choice of this model by measuring the equilibria for three of the lysozyme variants with cystamine under conditions where mixed disulfide formation had no effect on protein stability. For these variants and conditions, the folded and unfolded states should have the same values for the equilibrium constants describing the reaction with cystamine. While it is possible that a given variant may have no change in stability of folding because the folded and unfolded states are equally perturbed, it is more likely that mixed disulfide formation is simply not perturbed by the protein for these positions. Our measured values of 2.2, 2.6, and 2.8 for the formation of the mixed disulfide of cystamine with the single thiols of L79C, R96C, and R119C, respectively, agree very closely with the value of 2.2 ± 0.3 measured for the model compound. These observations support the validity of the model and argue that for these variants there is essentially no interaction of the rest of the protein with the cysteinyl aminoethyl disulfide side chain.

Modification of A146C with cystamine lowers the stability of the protein by about 1.1 kcal/mol at pH 5.3. The contributions of the folded and unfolded states to this change in stability were estimated as follows. The analysis of mixed disulfide formation of cystamine with A146C (56 °C, pH 5.14) demonstrated a value for the equilibrium constant of 3.1 in the unfolded state as compared to 0.6 in the folded state. Thus it appears that mixed disulfide formation may be slightly favored in the unfolded state as compared to the model. Factors contributing to errors in $K_{app}$ measurements include the base line of the CD of the native and denatured protein, concentration of the redox solution, and resolution of the CD spectrum. We estimate that these factors can lead to about 10% error in $K_{app}$. Given 10% of the error associated with $K_{app}$ values, this stabilization seen in the unfolded state is on the borderline of statistical significance. Nevertheless, if we assume the differences seen are real, the unfolded state appears to be stabilized upon modification by $\Delta G^\circ = -RT \ln (K_u/K_{model})$ of about 0.2 kcal/mol. The 5-fold decrease in the equilibrium constant for mixed disulfide formation observed in the folded state is clearly significant and represents a destabilization of the folded state by a factor of 5 upon the formation of the mixed disulfide. The free energy of the destabilization is given by $\Delta G^\circ = -RT \ln (K_n/K_{model})$ of about 0.9 kcal/mol.

Previous crystallographic and thermodynamic studies of various T4 lysozyme mutants have shown that temperature-sensitive mutants occur at sites with low crystallographic thermal factors and low solvent accessibility in the X-ray crystal structure. These observations suggest that the observed reductions in thermodynamic stability generally involve significant effects on the folded conformation (Alber et al., 1987). Studies reported here are consistent with these observations. Side-chain modifications at sites relatively exposed on the surface have little effect on the stability of the protein. In contrast, alanine 146 is buried in a hydrophobic interior of the protein and is important in determining the stability of this protein.

Modifications with various chemical reagents also allow us to determine the role of different forces in stability of the protein. Previous work has shown that substitutions of Ala at this site by Val, Ile, Thr, Cys, and Phe lower protein stability by about the same amount (6–7 °C) at pH 2 (unpublished data). As shown in this work, although replacement of alanine by cysteine lowered the stability of the protein by about 5 °C at pH 2.44 compared to pseudo-wild-type T4 lysozyme, further chemical modification of the cysteine side chain with a neutral group and a relatively large negatively charged group such as DTNB did not affect the stability of the protein. These results suggest that the stability of the protein is relatively independent of the size of the side chain at this site and the specific interactions of alanine with groups surrounding it in wild type rather than hydrophobic forces at this site are important to the stability of the protein.

The stability of A146C protein is further reduced by modifying the cysteine with the positively charged aminoethyl disulfide. This decreased stability appears to be the result of the positive charge since the neutral mixed disulfide formed with mercaptoethanol and the negatively charged mixed disulfide formed by reaction with DTNB and oxidized mercaptoacetic acid do not have nearly as large a destabilizing effect. The decrease in the stability of the protein is due mainly to the destabilization of the folded state.

Although detailed analysis of the energetic effects in the folded state requires comparison of the structures of A146C and A146-$SSCH_2CH_2NH_3^+$, some qualitative arguments can be made based on the structure of the wild-type T4 lysozyme. The side chain of 146 resides in the hydrophobic interior of the protein. Therefore, replacement of an alanine side chain by a charged group may cause unfavorable interactions. Furthermore, there are three positively charged groups, Arg145, Lys147, and Arg148, nearby in the sequence, and Lys147 is nearby in the three-dimensional structure. Replacement of the neutral cysteine by a positively charged group may be more unfavorable due to repulsive charge–charge interactions. This repulsive force is likely to be more pronounced in the native structure where the charged group is fixed in space. Modification of a side chain with small moieties such as cystamine is not likely to introduce a large increment of conformational flexibility to the unfolded state. However, the slight stabilization of the unfolded state we observed could be due to the increase in configurational entropy of the unfolded state caused by the modification.

Chemical modifications provide a convenient way to make variant proteins with variable side-chain sizes and different charges. This permits us to examine the contribution of different forces in determining the stability of the protein.

Reversible chemical modification of cysteine side chains provides us an opportunity to dissect the energetic contribution of the side chain modification to both the native state and the denatured state. The approach described here can be applied to almost any suitable macromolecular system.

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