Destabilizing Effects of Replacing a Surface Lysine of Cytochrome *c* with Aromatic Amino Acids: Implications for the Denatured State†

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Received August 6, 1992; Revised Manuscript Received October 21, 1992

ABSTRACT: A series of mutations at the highly solvent-exposed lysine 73 of iso-1-cytochrome *c* have been prepared by site-directed mutagenesis. These mutations were designed to probe denatured-state effects on the unfolding equilibrium of this protein. The hydrophilic amino acid Lys was replaced with the hydrophobic amino acids Met, Tyr, Phe, and Trp. The idea was to induce stabilizing hydrophobic interactions in the unfolded state, while having little effect on the folded-state energy due to the high solvent exposure of this site. Fourier transform infrared spectral analyses indicate that none of these mutations significantly affect the native fold of the protein. The stability of each protein to guanidine hydrochloride denaturation was monitored at 25 °C by circular dichroism spectroscopy. All four hydrophobic mutants decreased the value of $\Delta G°_{u}{}^{H_2O}$, the free energy of unfolding of the protein in the absence of denaturant, by 1.0–1.5 kcal/mol. The $\Delta G°_{u}{}^{H_2O}$ values for these proteins correlate linearly (correlation coefficient of 0.98) with the hydrophobicity of the amino acid at position 73 of the sequence. These data are consistent with the idea that the position-73 mutants are more buried in the denatured state than in the native state, suggestive of a compact denatured state where such interactions would be possible.

We have begun a program of research to investigate the effect of solvent-exposed amino acids on the stability and folding properties of a protein. Much of the research on protein stability and folding has emphasized amino acids buried in the hydrophobic core of a protein [for a recent review, see Dill (1990)]. Indeed, random mutagenesis studies have demonstrated that destabilizing amino acid substitutions occur almost exclusively in the hydrophobic core of a protein (Hecht et al., 1984; Shortle & Meeker, 1986; Matthews, 1987; Alber et al., 1987; Shortle et al., 1988; Bowie et al., 1990; Reinhaar-Olson & Sauer, 1991). Hydrophobic forces, core volume, and shape complementarity appear to interact in a complex manner to mediate the large stabilization due to the hydrophobic core of a protein (Shortle et al., 1990; Lim et al., 1992). Surface mutations have mainly been studied to probe the importance of electrostatic interactions to protein stability. Generally, only small effects have been observed in these investigations (Dao-pin et al., 1991a,b; Serrano et al., 1990).

Our interest in surface mutations stems from recent theoretical and experimental investigations which suggest that the denatured state of a protein is not a random coil and hence can be expected to have a significant role in controlling the equilibrium between the native and denatured state of a protein (Dill & Shortle, 1991; Alonso & Dill, 1991; Shortle et al., 1990, 1992; Shortle & Meeker, 1986). Surface mutations are an ideal means by which to investigate denatured-state effects on protein stability. Surface-exposed amino acids often have relatively little steric interaction with adjacent parts of the folded state of a protein. Hence, mutations at such sites are less likely to affect the structure and interactions that lead to stabilization of the native state of a protein. Surface mutations thus provide a way to probe mutational effects on denatured-state energy with less complication from native-state energy effects than would be expected for mutations at buried sites.

In this initial report, we have chosen to replace lysine 73 (tuna numbering, for yeast numbering add 5) of iso-1-cytochrome *c* with the hydrophobic amino acids methionine, tyrosine, phenylalanine, and tryptophan. This site was chosen because it is evolutionally-conserved (Hampsey et al., 1988) and has a relatively high degree of solvent exposure in the native state (Louie & Brayer, 1990; Berghuis & Brayer, 1992). In the folded state, the hydrophobic position 73 cytochrome *c* mutants should be highly solvated due to the steric constraints of the main chain conformation. Upon unfolding, these main chain steric constraints will be relaxed, and if the denatured state is not a random coil we might expect nonpolar position-73 mutants to stabilize the denatured state relative to the native state due to an increase in the hydrophobic interactions in this state. One previous report has demonstrated such effects on protein stability for a hyperexposed amino acid on the surface of the λ Cro protein (Pakula & Sauer, 1990). Our results show similar effects on protein stability for a surface amino acid that is not hyperexposed.

EXPERIMENTAL PROCEDURES

Materials. Oligonucleotides used in mutagenesis were synthesized commercially by Operon Technologies, Inc. or at the Biotechnology Core Facility of the University of Colorado Health Sciences Center. Guanidine hydrochloride[1] was ultrapure grade (U.S. Biochemical) and was used without

† This work was supported in part by NSF REU Grant CHE 9000781 (K.M.), NIH minority High School Grant 2S03RR03329-05 (T.Z.), NIH BRSG Grant 2S07RR07138-20 (B.E.B.), Colorado Agricultural Experimental Station Project 643 (W.S.C.), and a gift from Strohtech, Inc. (W.S.C.).

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[1] Abbreviations: gdnHCl, guanidine hydrochloride; FT-IR, Fourier transform infrared; CD, circular dichroism; SDS, sodium dodecyl sulfate; EDTA, ethylenediaminetetraacetic acid; $\Delta G°_{u}$, free energy of unfolding; $\Delta G°_{u}{}^{H_2O}$, free energy of unfolding in the absence of denaturant; A_d, solvent-exposed surface area of the denatured state; A_n, solvent-exposed surface area of the native state.

0006-2960/93/0432-183$04.00/0

further purification. All solutions were made with glass-distilled water from a Corning MP-6A Mega-Pure System running off a deionized water source. All chemicals used in buffers were reagent grade. Restriction enzymes were obtained from Boehringer Mannheim Biochemical or U.S. Biochemical. T4 DNA ligase was obtained from IBI/Kodak or U.S. Biochemical. R408 helper phage was obtained from Promega. DNA isolations were carried out with columns and buffers from Qiagen, Inc. DNA was sequenced using the Sequenase version 2.0 kit (U.S. Biochemical). Site-directed mutagenesis was performed with the oligonucleotide-directed in vitro mutagenesis kit version 2.1 (Amersham Corp.). All materials for growth media were from Difco. YPD, YPG, SD Leu$^-$, and lactate media were prepared from standard recipes (Sherman et al., 1974, 1986).

Site-Directed Mutagenesis. All mutants were prepared by the Eckstein phosphorothioate method (Nakamaye & Eckstein, 1986). A 2.5-kb fragment containing the CYC1 gene (Montgomery et al., 1978; Smith et al., 1979) cloned between the *Bam*HI and *Hin*dIII sites of M13mp8 (Pielak et al., 1985) was obtained from Michael Smith at the University of British Columbia. Single-stranded DNA used as a template for site-directed mutagenesis was prepared by standard methods (Messing, 1983). All mutations were made to a CYC1 gene containing a single mutation of Cys 102 to Ser which we will refer to as the wild-type gene (Mayo, 1988). The oligonucleotide 5′-d(CCAGGAATATA,CAT,CTTTGGGTT)-3′ was used to prepare the Met 73 mutant and the oligonucleotide mixture 5′-d(CCAGGAATATA,(A, G or C)NA,CTTT-GGGTTAGTC)-3′ was used to prepare the Tyr 73, Phe 73, and Trp 73 mutants (mutation site delineated by commas). Single-stranded DNAs derived from viral plaques produced by transformation of the mutagenesis reaction mixtures into *Escherichia coli* TG-1 cells (genotype: K12, Δ(*lac–pro*), *supE*, *thi*, *hsdD5*/F′*traD36*, *proA*$^+$*B*$^+$, *lacI*^{q}, *lacZ*Δ*M15* from Amersham Corp.) were sequenced by the dideoxy method (Biggin et al., 1983). Mutants with amino acid codons corresponding to the known codon preference of *Saccharomyces cerevisiae* were chosen (Ikemura, 1982; Bennetzen & Hall, 1982). Mutant CYC1 genes were then ligated into plasmid YEp213 (Hicks et al., 1982) or phagemid pRS425 (Christianson et al., 1992) to produce YEp213/CYC1 and pRS425/CYC1 DNAs. Transformation into the *S. cerevisiae* cell line GM-3C-2 (α, *leu2–3*, *leu2–112*, *trp1–1*, *his4–519*, *cyc1–1*, *cyp3–1*; Faye et al., 1981) was carried out by the LiCl method (Ito et al., 1983).

Analysis of Mutants. Transformed yeast colonies were subjected to a curing prcedure (Pielak et al., 1985) to test that the cytochrome *c* phenotype was plasmid-based and not due to chromosomal reversion. Briefly, a transformed yeast colony was grown for 2 days on YPD media. The cells were diluted with sterile distilled water. One hundred microliters of 10^{-4} and 10^{-5} dilutions was spread onto YPD plates. Single colonies were then tested for growth on YPD, YPG, and SD Leu$^-$ plates to monitor the loss of plasmid-based phenotypes.

Plasmids were also reisolated from GM-3C-2 cells by a literature method (Hoffman & Winston, 1987) and used to transform *E. coli* TG-1 cells. For pRS425/CYC1 plasmids, ssDNA was isolated using the helper phage R408 (Russel et al., 1986; Titus, 1991). The CYC1 fragment from YEp213/CYC1 was ligated into M13mp8 and ssDNA produced as above. All ssDNA was purified using Qiagen tip-20 columns. The ssDNAs were then sequenced as above.

Isolation of Iso-1-cytochrome c. Yeast cultures were grown on YPG media in a New Brunswick Scientific BioFlo IIC 5-L fermenter. The media was supplemented with 40 mg/L ampicillin to inhibit bacterial infections and with 1 mL/L antifoam C (Sigma). Cells were generally grown to an optical density (600 nm) of 8–10 producing 200–250 g wet weight of cells. The purification procedure of Cutler et al. (1987) was followed. The cytochrome *c* was then HPLC purified using a Pharmacia Mono S HR5/5 column. HPLC buffers were (A) 50 mM sodium phosphate, pH 7.0, and (B) buffer A + 1.0 M NaCl. The following gradient (1 mL/min flow rate) was used: 5 mL at 0% B, 0–30% B over 20 mL, 7 mL at 30% B. Purified yields average 85% of crude yields giving typical overall yields of 12.5–25 mg of cytochrome *c*/5-L fermentation. The estimated purity of our cytochrome *c* samples is 98% (integration of an HPLC chromatogram of purified protein). Only a single band is detectable on Coomassie blue stained SDS–polyacrylamide gels. Prior to HPLC purification, significant impurities are detectable by SDS–PAGE.

FT-IR Spectroscopy. Iso-1-cytochrome *c* mutants were oxidized with a large excess of $K_3Fe(CN)_6$ (5 mg/1 mg of protein) for a minimum of 2 h. The protein was then isolated from the iron hexacyanide by G-25 chromatography using 50 mM sodium phosphate, pH 7.2, 1 mM EDTA as the running buffer. The eluant was then concentrated in a Centricon-3 ultrafiltration device (Amicon), rediluted with 2 mL of the buffer, and reconcentrated to approximately 50 μL. The final concentration of protein ranged from 10 to 18 mg/mL in samples prepared for infrared measurement in a Beckman FH-01 cell with CaF_2 windows and a 6-μm path length. Infrared spectra were recorded at 20 °C with a Perkin-Elmer Model 1800 FT-IR spectrophotometer equipped with a Hg/Cd/Te detector and interfaced with a Perkin-Elmer 7700 computer. Spectral measurement and analysis followed the criteria and double-subtraction procedures of Dong et al. (1990, 1992) with slight modification. The factoring of water vapor subtraction was based on the elimination of water vapor bands outside the amide I region between 1850 and 1720 cm^{-1} which resulted in a featureless spectrum in this "window" region of the second-derivative spectrum.

Guanidine Hydrochloride Denaturation. All experiments were performed with ferricytochrome *c*. Preparation of oxidized protein was as described for FT-IR samples except that the running buffer for G-25 chromatography was 20 mM Tris, pH 7.5, 40 mM NaCl. Unfolding as a function of [gdnHCl] was monitored by CD spectroscopy at 220 nm. Measurements were made with a JASCO 500C spectropolarimeter using a time constant of 2 s, a sensitivity of 2 millidegrees/cm, and a slit width of 1800 μm. Samples were prepared from concentrated stocks of protein, gdnHCl, and buffer and adjusted to a final volume of 1.0 mL with distilled water using gravimetrically-calibrated Gilson microliter pipetmen. The concentration of oxidized iso-1-cytochrome *c* stocks was determined spectrophotometrically at the isosbestic points 339, 526.5, and 541.75 nm of the cytochrome *c* spectrum as well as with the oxidized-state extinction coefficients at 360 and 550 nm (Margoliash & Frohwirt, 1959). The guanidine hydrochloride stock used was 6.0 M. Its concentration was evaluated by refractive index measurements versus distilled water (25 °C) prior to each experiment (Nozaki, 1972). Cytochrome *c* concentrations in measured samples were 1.5–2.1 μM. For each experiment, the [gdnHCl] was varied from 0 to 1.6 M in intervals of 0.1 M. Samples with [gdnHCl] of 1.8, 2.0, 2.3, 2.5, and 3.0 M as well as additional samples in the transition region were measured. Samples were measured within approximately 10 min of preparation. The

signal at 220 nm was measured for a minimum of 2 min and signal averaged manually. All measurements were made at 25 ± 0.1 °C using a jacketed cell attached to a Neslab Model RTE 5 circulating bath. All stock solutions were maintained at 25 °C in a Napco Model 220 water bath, as was each sample prior to measurement. To test for reversibility of gdnHCl denaturation, we prepared 100-μL samples of each iso-1-cytochrome c in approximately 3.0 M gdnHCl. The sample was kept at 25 °C for 5–10 min and then diluted to a final volume of 1.0 mL and a gdnHCl concentration of 0.3 or 0.4 M. The sample was allowed to equilibrate for 5–10 min, and then the CD signal at 220 nm was measured as above.

Analysis of Guanidine Hydrochloride Denaturation Data. Analysis of denaturation curves followed standard procedures (Pace, 1986). The native state baseline was generated by a linear least-squares fit of the data from 0 to 0.6 M gdnHCl. In some instances, the zero concentration point was not included in the fit since the ellipticity of cytochrome c can increase in the presence of low concentrations of gdnHCl (Hickey et al., 1988). The denatured state baseline was derived from a linear least-squares fit to data from 1.8 to 3.0 M gdnHCl. The unfolding equilibrium constant at each [gdnHCl] in the unfolding region was calculated as $K_u = f_u/(1 - f_u)$, where the fraction unfolded, $f_u = (\theta - \theta_N)/(\theta_D - \theta_N)$, θ is the ellipticity of the sample, and θ_N and θ_D are the ellipticities of the native and denatured state, respectively, at the [gdnHCl] of the sample determined from the baseline curves. Ellipticity at 220 nm is measured at $(\theta_{220} - \theta_{250})$ to compensate for slight instrumental baseline drift during the course of an experiment.

Free energy of unfolding is calculated from the expression, $\Delta G°_u = -RT \ln K_u$. For each iso-1-cytochrome c mutant, data from three separate experiments were then merged. Average values and standard deviations of $\Delta G°_u$ were calculated at each [gdnHCl] in the transition region. Only data for 10–90% unfolded protein was included since data closer to the native or denatured state baseline tends to be unreliable. Data were then fit to the equation $\Delta G°_u = \Delta G°_u{}^{H_2O} - m[\text{gdnHCl}]$ (Pace, 1986), using a weighted least-squares fit (Taylor, 1982) where the weighting factor was taken as $1/\lambda(95\%)$ (the 95% confidence limit; Shoemaker et al., 1974) to account for gdnHCl concentrations where only two (as opposed to three) data points were available. The standard deviations (SD) in slopes and intercepts were calculated as $SD = \{\Sigma[(\delta F/\delta \Delta G_i)(SD(\Delta G_i))]^2\}^{1/2}$, where F is the function for the weighted least-squares slope or intercept and $SD(\Delta G_i)$ is the standard deviation of $\Delta G°_u$ at each [gdnHCl] (Shoemaker et al., 1974).

Calculation of Solvent Surfaces and Intraprotein Distances. The solvent-exposed surface area of the lysine 73 side chain was calculated using the program Biograf (Molecular Simulations, Inc.) and the method of Lee and Richards (1971) of rolling a 1.4-Å sphere over the surface of interest. The 1.23 Å X-ray coordinates of reduced yeast iso-1-cytochrome c (Louie & Brayer, 1990; Bernstein et al., 1977; Abola et al., 1987) were used. Intraprotein distances were measured using the Tripos graphics program Sybil.

RESULTS

Characterization of Mutants. All mutant iso-1-cytochromes c also contain the mutation C102S (Mayo, 1988). This mutation is present to prevent formation of intermolecular disulfide dimers which would complicate interpretation of our physical studies. This mutation does not significantly alter the properties of iso-1-cytochrome c (Mayo, 1988; Cutler et al., 1987).

We have tested the Met 73, Tyr 73, Phe 73, and Trp 73 iso-1-cytochrome c mutants for growth on the nonfermentable carbon sources glycerol (YPG) and the more stringent lactate (Sherman et al., 1974). Qualitatively, all four mutants display growth rates similar to that of the wild-type (C102S) iso-1-cytochrome c. In general, the evolutionally-conserved lysine 73 is readily replaced, 12 of 13 mutants at this site are fully functional (Bowler et al., unpublished results).

We have carried out several tests to ensure that our amino acid 73 mutants are genuine. A curing procedure was used to test that the iso-1-cytochrome c phenotype was not due to chromosomal reversion. In all cases the loss of the cytochrome c phenotype was linked to the loss of the leucine biosynthesis phenotype. The Leu2+ gene is carried on the yeast expression vectors used and complements a genetic deficiency of the GM-3C-2 cell line. Linkage of loss of the Leu2+ and CYC1 phenotypes is expected for a plasmid-based CYC1 gene. Since we are mutating at an evolutionally-conserved site, we have reisolated the plasmids from the transformed *S. cerevisiae* GM-3C-2 cells and resequenced the entire coding region of the CYC1 gene. In each case, only the expected mutations (C102S and amino acid 73) were observed.

An important supposition of the experiments presented here is that mutation of lysine 73 to other amino acids does not affect the native structure of the protein. To test this supposition, we have carried out FT-IR spectroscopic analysis of protein amide I spectra. The protein amide I band (1700–1620 cm^{-1}) arises almost entirely from the C=O stretch of amide groups in the peptide linkages (Krimm & Bandekar, 1986; Susi & Byler, 1986; Surewicz & Mantsch, 1988) and, therefore, is very sensitive to protein conformation. Each type of secondary structure gives a different C=O stretch frequency as a result of differences in molecular geometry and hydrogen bonding involving the C=O and NH moieties of amide groups. Using second-derivative analysis, band components ascribed to α-helix (band at 1656 ± 2 cm^{-1}), β-sheets (bands between 1698–1689 cm^{-1} and 1642–1623 cm^{-1}), turns (multiple bands between 1688 and 1666 cm^{-1}), and random structure (band at 1648 ± 2 cm^{-1}) under the amide I contour can be clearly resolved (Dong et al., 1990, 1992; Dong et al., unpublished data). The extraordinary sensitivity of the spectrum to subtle changes in protein conformation is demonstrated by an overlay of both the primary and second-derivative spectra of oxidized and reduced species of iso-1-cytochromes c in Figure 1. While only minor structural differences between the two oxidation states were found in structures derived from X-ray diffraction data (Louie & Brayer, 1990; Berghuis & Brayer, 1992), significant spectral differences, especially at the bands assigned to β-sheet and turn structures, in the amide I region are observed in infrared spectra; these differences are highly reproducible (Dong et al., 1992). Figure 2 shows overlays of the second-derivative spectrum of wild type (C102S) and the spectra of Met 73, Phe 73, Tyr 73, and Trp 73. The differences between wild-type and position 73 mutant spectra are in each case very small with the greatest difference occurring with Met 73. In all mutants, the spectral changes are much less than observed upon reduction of heme, which is consistent with our supposition that only very minor structural changes result from our mutations to Lys 73.

Guanidine Hydrochloride Denaturation. Two important conditions must be met when using solvent denaturation to characterize the unfolding thermodynamics of a protein. It must be demonstrated that the process of unfolding has reached

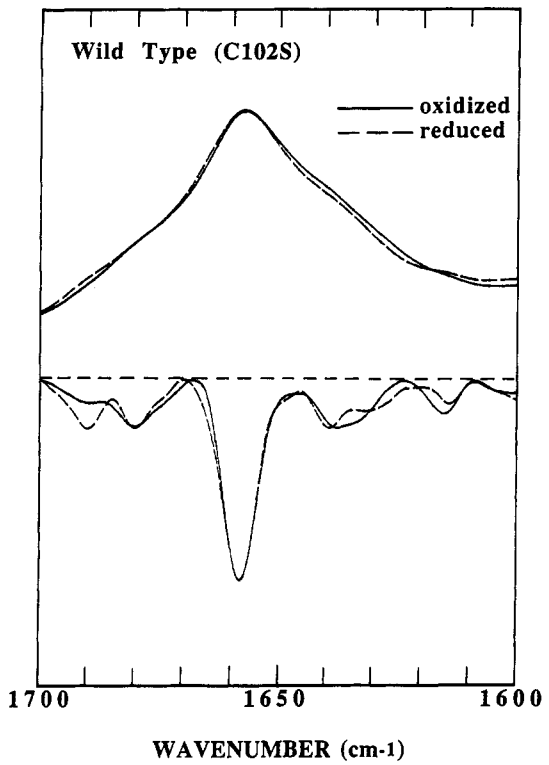

FIGURE 1: Amide I infrared spectra of oxidized and reduced species of wild-type iso-1-cytochrome *c*. (Top) Primary spectra. (Bottom) Second-derivative spectra.

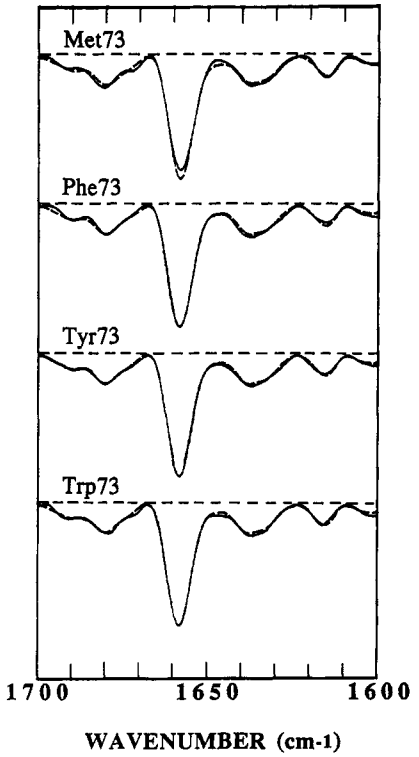

FIGURE 2: Comparisons of the second-derivative amide I infrared spectra of oxidized wild-type (C102S) and position-73 mutant iso-1-cytochromes *c*. The second-derivative spectrum of each mutant is compared directly with the spectrum of wild type (- - -).

equilibrium and that it is reversible. Studies on the kinetics of unfolding of both yeast iso-1- and iso-2- cytochromes *c* indicate that over a wide range of gdnHCl concentrations even the slow phases of folding and unfolding are complete within 5 min (Ramdas & Nall, 1986; Nall, 1990). Our measurements are made approximately 10 min after sample preparation, so equilibrium should have been reached. In general, we monitored the CD signal of each sample at 220 nm for 2–3 minutes. However, for each iso-1-cytochrome *c* we measured some samples for longer periods of 5–9 min. Over this period of time, no noticeable change in the CD signal occurred. For the Tyr 73 and Phe 73 mutants, we investigated the time dependence of the CD signal more extensively. For each protein, we monitored one sample near the midpoint of the transition and one at 0.4 M gdnHCl. Over the course of 1 h (measurements at 15-min intervals), no significant loss of signal was observed for the Phe 73 protein. For both proteins about a 5% loss in CD signal was observed over the course of 4 h. After approximately 24 h, a 15–30% loss in signal was observed (samples were maintained at room temperature between measurements). We measured out to 95 h for the Tyr 73 sample and CD signal loss continued. For the Tyr 73 sample we also monitored protein with no gdnHCl added. This sample showed similar loss of signal with time, losing 60% of the original CD signal after 95 h. For iso-1-cytochrome *c*, it appears that there is a slow irreversible loss of native structure at 25 °C irrespective of the presence of gdnHCl.

Tests for reversibility of the gdnHCl denaturation of the iso-1-cytochrome *c* mutants were carried out as described in the Experimental Procedures section. Samples orginally at about 3.0 M in gdnHCl were diluted so the final gdnHCl concentration was 0.3 or 0.4 M. These samples gave CD signals that were 94–100.6% of the intensity of samples with the same final gdnHCl concentration prepared from native iso-1-cytochrome *c*. These experiments indicate that the gdnHCl-induced unfolding process we are observing is reversible. The long-term irreversible loss of native structure in both the presence and absence of gdnHCl suggests a very slow aggregation process proceeding from the denatured state as shown in eq 1. From these data, we believe that for short

$$N \rightleftharpoons D \overset{\text{slow}}{\rightarrow} A \qquad (1)$$

times the CD signal we are measuring is due almost exclusively to a reversible process that has reached equilibrium. Previous unfolding studies of yeast cytochrome *c* (Hickey et al., 1988, 1991; Ramdas et al., 1986) have not commented on the existence of a slow irreversibility; however, these studies may not have looked at the unfolding process on such long time scales. Our data and previous kinetic studies (Ramdas & Nall, 1986; Nall, 1990) suggest that the most accurate thermodynamic values for reversible unfolding of iso-1-cytochrome *c* will be obtained if samples are measured within 15 min to an hour of preparation.

In Figure 3, a typical denaturation curve is shown with the native- and denatured-state baselines drawn in. The data were analyzed as described in the Experimental Procedures section. Plots of ΔG°_u versus [gdnHCl] for the four position 73 mutants are given in Figure 4. Data for the wild-type protein are plotted on each graph for comparison. The values of $\Delta G^\circ_u{}^{H_2O}$, the slope, *m*, and the midpoint of the transition, $[\text{gdnHCl}]_{1/2}$, are given in Table I. It is evident both from Figure 4 and Table I that both the $\Delta G^\circ_u{}^{H_2O}$ and *m* values decrease when lysine 73 is replaced with a hydrophobic amino acid. The Phe 73 mutant is somewhat unusual. Unlike the other mutants, its free energy plot deviates from linearity, in particular near the end of the unfolding transition (Figure 4). To fit the Phe 73 data, we have included only the central data points (0.9–1.15 M gdnHCl). If the data points at 0.8 and

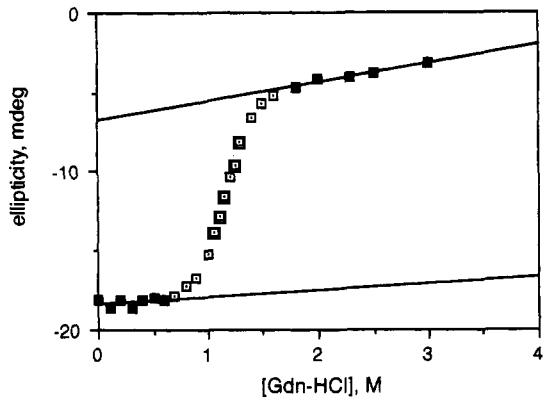

FIGURE 3: Guanidine hydrochloride induced unfolding of wild-type (C102S) iso-1-cytochrome c. The unfolding transition is presented as a graph of the ellipticity at 220 nm in millidegrees as a function of guanidine hydrochloride concentration. The two lines in the graph represent the native (lower) and denatured (upper) state baselines used to analyze the free energy of unfolding as a function of guanidine hydrochloride concentration in the region of the unfolding transition.

Table I: Thermodynamic Parameters for the Denaturation of Wild-Type and Mutant Cytochromes c

cytochrome c	$\Delta G°_u{}^{H_2O}$ (kcal/mol) (25 °C)	$[gdnHCl]_{1/2}$ (M)	m (kcal/(mol·M))
wild type (C102S)	5.66 ± 0.41	1.16 ± 0.07	4.87 ± 0.32
Met 73 (C102S)	4.66 ± 0.16	1.05 ± 0.03	4.43 ± 0.16
Tyr 73 (C102S)	4.61 ± 0.29	1.00 ± 0.05	4.62 ± 0.29
Phe 73 (C102S)	4.51 ± 0.31	1.02 ± 0.05	4.44 ± 0.30
Trp 73 (C102S)	4.23 ± 0.35	1.02 ± 0.06	4.16 ± 0.34

1.2 are included in the fit, they do not fall within one standard deviation of the least-squares line. On this basis, they were omitted from the final fit. The curvature in this plot compromises the ability to derive completely reliable parameters from the least-squares fit. Hence, the precision of the values of $\Delta G°_u{}^{H_2O}$ and m for the Phe 73 mutant is probably less than indicated by the standard deviations reported in Table I. The curvature we observe for the Phe 73 free energy plot is in the opposite direction to what one might expect if the denatured state was expanding as a function of [gdnHCl] (Shortle & Meeker, 1986). We do not have a good explanation for this result at the current time.

Our gdnHCl denaturation data for the wild-type (C102S) iso-1-cytochrome c agree reasonably well with other data in the literature for wild type (Cys 102 or Ala 102) iso-1-cytochrome c (Hickey et al., 1991). Our $\Delta G°_u{}^{H_2O}$ and m values are somewhat higher and our $[gdnHCl]_{1/2}$ value is somewhat lower. These differences may be due to the fact that our buffer and pH conditions were different from those used previously. The identity of the amino acid at position 102 may also be of some significance.

DISCUSSION

The data presented here are consistent with the folded state structure of wild-type (C102S) iso-1-cytochrome c being largely unaffected by our mutations to position 73. All mutants qualitatively support normal growth of GM-3C-2 yeast cells under conditions requiring oxidative metabolism, indicating a functional cytochrome c fold has been produced. DNA sequence data show that mutant functionality is not due to second site reversion. FT-IR data are consistent with only very minor structural perturbations resulting from these mutations. Structural rearrangement of these mutant cytochromes c to bury the position 73 side chain in the hydrophobic core would require significant native-state structural perturbations. Such rearrangements would be readily observed by FT-IR spectroscopy if they occurred. The X-ray diffraction structure of iso-1-cytochrome c (Louie & Brayer, 1990; Berghuis & Brayer, 1992) shows that lysine 73 is in a very isolated position, projecting well out into the solvent. It makes no close contacts with the rest of the protein beyond the β-carbon and hence solvation of the side chain is quite complete. Similarly, we expect our mutants to be highly solvated at this position. If indeed the denatured state is a random coil, then our native-state structural data would lead us to predict minimal or no change in the $\Delta G°_u{}^{H_2O}$ as a function of these mutations. The position-73 side chains would be highly solvated in both the native and the denatured state and thus have minimal effect on the free energy change of the unfolding transition. This is not what we observe.

We must consider possible effects on the energy of the folded state due to our mutations. Since we are removing a charged residue we could either be relieving charge repulsions or removing charge attractions on the surface of iso-1-cytochrome c. Although, lysine 73 is in a relatively isolated position, inspection of nearby charges is necessary. Lysine 73 is immediately adjacent to trimethyllysine 72. Crystallographic data show that these two side chains are part of a short stretch of α-helix and are oriented at nearly 90° with respect to each other. This orientation should minimize charge–charge repulsion. The ϵN–ϵN distance between Lys 73 and Lys 72 is 13.83 Å. Lys 86 is slightly closer to Lys 73 at a ϵN–ϵN distance of 11.39 Å. These two distances represent the closest approach of a positive charge center to Lys 73. We note that removal of a charge in this case would be expected to lower the energy of the folded state, producing an effect opposite to what we actually observe for our mutations. The nearest negatively charged residue is Glu 66. No direct interaction is observed between Glu 66 and Lys 73 and in fact the Asn 70 side chain would sterically block such an interaction. The crystallographic distance from ϵN to the nearest carboxylate oxygen of Glu 66 is 12.81 Å. Besides Asn 70, several crystallographic water molecules occupy the space between Glu 66 and Lys 73. Although we cannot entirely rule out electrostatic effects on the energy of the native state on the basis of this analysis, the distance involved and the presence of intervening water molecules should minimize them. The only specific contact made to lysine 73 is a hydrogen bond from the side chain of Asn 70 to the main chain amide of lysine 73 (Louie & Brayer, 1990; Berghuis & Brayer, 1992), which should be side-chain independent.

Given the above discussion, we feel the most reasonable explanation for our data is that the denatured state of iso-1-cytochrome c is not a random coil and that our mutations affect primarily the structure and energy of the denatured state. Recent theoretical studies (Shortle et al., 1992) predict that mutation of a hydrophobic amino acid at the surface of a protein to a polar amino acid will stabilize that protein toward unfolding. This stabilization is linked to a loss of possible hydrophobic interactions in the ensemble of conformations that make up the denatured state, thus destabilizing the denatured state. In our experiments, we have mutated in the opposite direction (polar to hydrophobic) and hence expect to observe the converse effect. The relatively low concentrations of gdnHCl required to denature our proteins are consistent with a compact denatured state favoring hydrophobic clustering since gdnHCl is not a good solvent for hydrophobic amino acids at these concentrations (Dill & Shortle, 1991).

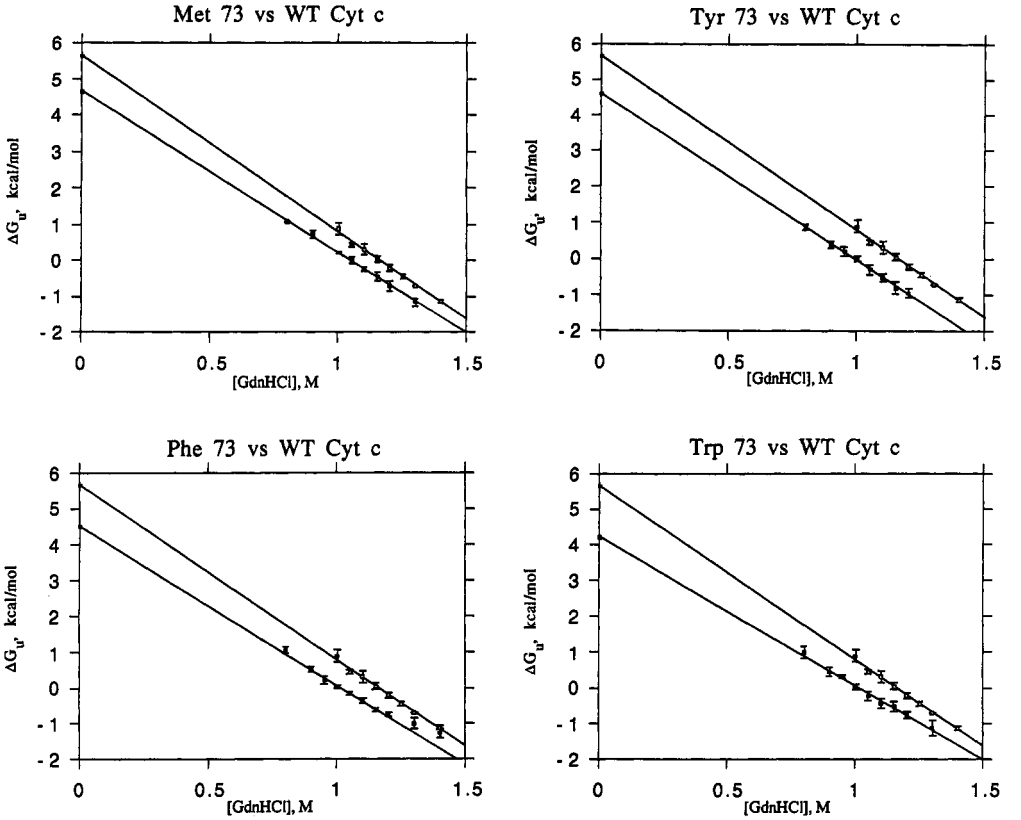

FIGURE 4: Free energy plots, $\Delta G°_u$ versus guanidine concentration, shown for the Met 73, Tyr 73, Phe 73, and Trp 73 mutants. The upper plot on each graph (open squares) is that of the wild-type (C102S) protein. The lower plot (closed squares) on each graph is that of the mutant protein. The error bars represent one standard deviation. The lines are weighted least-squares fits to each set of data points as described in the Experimental Procedures section. The intercepts on the $\Delta G°_u$ axes are the $\Delta G°_u{}^{H_2O}$ values for each mutant. All data are at 25 °C in 20 mM Tris, pH 7.5, 40 mM NaCl.

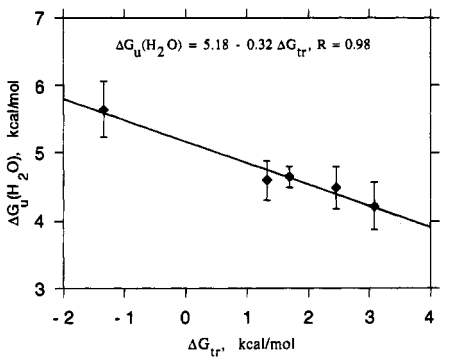

FIGURE 5: Plot of $\Delta G°_u{}^{H_2O}$ for each mutant versus the hydrophobicity of the amino acid at position 73. Hydrophobicity is taken to be the free energy of transfer of the amino acid from octanol to water, ΔG_{tr}, (Eisenberg & MacLachlan, 1986; Fauchere & Pliska, 1983). The equation for the linear least-squares fit to this data is given on the graph along with the correlation coefficient, R. Error bars for one standard deviation are included for each $\Delta G°_u{}^{H_2O}$ value.

If hydrophobic interactions are important in controlling the denatured-state energy, then we might expect a correlation between the stability of a protein and the hydrophobicity of a surface-exposed amino acid residue. A plot of our $\Delta G°_u{}^{H_2O}$ values against the hydrophobicity (*n*-octanol to water transfer energies; Fauchere & Pliska, 1983; Eisenberg & McLachlan, 1986) of the amino acid at position 73 shows a very good linear correlation (Figure 5). The correlation coefficient, R, for this fit is 0.98. The probability that five uncorrelated data points would produce a correlation coefficient of 0.98 or higher is 0.24% (Taylor, 1982). The $\Delta G°_u{}^{H_2O}$ decreases with increasing hydrophobicity as would be expected from increased hydrophobic interactions stabilizing the denatured state. The fractional solvent accessibility of the lysine 73 side chain in iso-1-ferrocytochrome *c* is 0.63 (solvent-exposed surface area of lysine 73 divided by the solvent-exposed surface area in an Ala-X-Ala model peptide; Lee & Richards, 1971). Hence, hydrophobic interactions at amino acid 73 should be of little importance to the stability of the native state of iso-1-cytochrome *c*. Due to the complexity of protein-folding energetics, we cannot entirely rule out folded-state effects; however, given the location and environment of Lys 73 described above, we feel this correlation is due primarily to a denatured state stabilized by increased hydrophobic interactions.

We also note that the slope, m, of our free energy versus [gdnHCl] plots decreases for all of our mutants. From theoretical analysis (Schellman, 1978), the slope, m, is proportional to ΔA, where $\Delta A = A_d - A_n$, the difference in the solvent-exposed surface areas of the denatured and native states. Since FT-IR analysis indicates that the folded-state structures of our mutants are unchanged relative to the wild-type protein and since the random coil solvent-exposed surface areas of the amino acid side chains we have substituted for lysine are similar to that of lysine (Lee & Richards, 1971), the decreased values of m imply that our mutants have more compact denatured states than the wild-type protein (Shortle & Meeker, 1986). Such a result is consistent with mutation of hydrophilic lysine to the hydrophobic side chains, Met, Tyr, Phe, and Trp for which hydrophobic clustering in the denatured state should be more pronounced. It is noteworthy that the slope of the unfolding transition, m, correlates with the hydrophobicity of the amino acid at position 73 (slope = 0.15, R = 0.94, probability of an uncorrelated set of five data points producing this R value is 1.63%). According to the

theory of Shortle et al. (1992), the parameter m is related to the relative populations of the two most compact conformations of the denatured state. A decrease in m implies that the relative population of the most compact denatured state increases due to the mutation. Without specific knowledge of these two denatured-state conformations and how they are affected by each mutation, it is not possible to determine whether such a correlation should exist. This correlation is however consistent with the correlation of the absolute value of m with $\Delta\Delta G^{\circ}_{u}$ observed by Shortle et al. (1990).

A similar correlation between the hydrophobicity of an amino acid at a solvent-exposed site and the stability of a protein has been observed for the thermal unfolding of the λ Cro protein (Pakula & Sauer, 1990). In this case, the residue being mutated was hyperexposed to solvent, having a fractional solvent accessibility of 1.4. It appears that mediation of protein stability by solvent-accessible amino acids may be more prevalent than originally expected—a hyperexposed side chain or one with a fractional solvent accessibility near 1.0 not being required (Pakula & Sauer, 1990). Interestingly, our data would require the fractional solvent accessibility of the position 73 mutants to be less than 0.63, suggesting a fairly compact denatured state for the wild-type and mutant forms of iso-1-cytochrome c. This conclusion is consistent with Trp 59 to heme fluorescence energy transfer data for horse heart cytochrome c as a function of [gdnHCl] (Tsong, 1974, 1975), which show that the distance between Trp 73 and the heme is not that of a random coil after the main unfolding transition.

CONCLUSIONS

We have demonstrated that mutation of a hydrophilic solvent-exposed amino acid into hydrophobic amino acids can significantly impact the folding equilibrium of a protein. These observations are consistent with the denatured state of a protein having a nonrandom structure. The correlation of $\Delta G^{\circ}_{u}{}^{H_2O}$ with the hydrophobicity of the amino acid at position 73 of iso-1-cytochrome c strengthens this interpretation of the data. The potential of a mutation strategy involving substitution of solvent-exposed amino acid side chains to either stabilize or destabilize a protein is evident as surface mutations are much less likely to interfere with the normal function of a protein. Since these kinds of mutations seem to affect primarily the denatured state of the protein whereas mutations to the hydrophobic core have more complex effects (Lim et al., 1992) which may act on both the native and denatured state of a protein (Shortle et al., 1990), the effects of surface mutations on protein stability may prove to be more predictable. Efforts to further explore the effects of surface mutations on protein stability and the generality of our current results are ongoing in our laboratory.

ADDED IN PROOF

Reevaluation of the solvent accessibility (ACCESS computer program of M. D. Handschumacher and F. M. Richards, obtained from C. N. Pace) of the Lys 73 side chain indicates that its fractional accessibility is 0.85 (oxidized) to 0.90 (reduced), significantly higher than our original determination.

ACKNOWLEDGMENT

We thank Dani Casimiro of the California Institute of Technology for solvent-exposed surface area calculations on Lys 73 and Karl Hahn of the University of Colorado Health Sciences Center for help in determining intraprotein distances.

REFERENCES

Abola, E. E., Bernstein, F. C., Bryant, S. H., Koetzle, T. F., & Weng, J. (1987) in *Crystallographic Databases—Information Content, Software Systems, Scientific Applications* (Allen, F. H., Bergerhoff, G., & Siever, R., Eds.) pp 107–132, Data Commission of the International Union of Crystallography, Bonn, Cambridge, Chester.

Alber, T., Dao-pin, S., Nye, J. A., Muchmore, D. C., & Matthews, B. W. (1987) *Biochemistry 26*, 3754–3758.

Alonso, D. V. O., & Dill, K. A. (1991) *Biochemistry 30*, 5974–5985.

Berghuis, A. M., & Brayer, G. D. (1992) *J. Mol. Biol. 223*, 959–976.

Bernstein, F. C., Koetzle, T. F., Williams, G. J. B., Meyer, E. F., Jr., Brice, M. D., Rodgers, J. R., Kennard, O., Shimanouchi, T., & Tasumi, M. (1977) *J. Mol. Biol. 112*, 535–542.

Bennetzen, J. L., & Hall, B. D. (1982) *J. Biol. Chem. 257*, 3026–3031.

Biggin, M. D., Gibson, T. J., & Hong, G. F. (1983) *Proc. Natl. Acad. Sci. U.S.A. 80*, 3963–3965.

Bowie, J. U., Reidhaar-Olson, J.-F., Lim, W. A., & Sauer, R. T. (1990) *Science 247*, 1306–1310.

Christianson, T. W., Sikorski, R. S., Dante, M., Shero, J. H., & Hieter, P. (1992) *Gene 110*, 119–122.

Cutler, R. L., Pielak, G. J., Mauk, A. G., & Smith, M. (1987) *Protein Eng. 1*, 95–99.

Dao-pin, S., Sauer, U., Nicholson, H., & Matthews, B. W. (1991a) *Biochemistry 30*, 7142–7153.

Dao-pin, S., Soderlind, E., Baase, W. A., Wozniak, J. A., Sauer, U., & Matthews, B. W. (1991b) *J. Mol. Biol. 221*, 873–887.

Dill, K. A. (1990) *Biochemistry 29*, 7133–7155.

Dill, K. A., & Shortle, D. (1991) *Annu. Rev. Biochem. 60*, 795–825.

Dong, A., Huang, P., & Caughey, W. S. (1990) *Biochemistry 29*, 3303–3308.

Dong, A., Huang, P., & Caughey, W. S. (1992) *Biochemistry 31*, 182–188.

Eisenberg, D., & McLachlan, A. D. (1986) *Nature 319*, 199–203.

Fauchere, J.-L., & Pliska, V. (1983) *Eur. J. Med. Chem. 18*, 369–375.

Faye, G., Leung, D. W., Tatchell, K., Hall, B. D., & Smith, M. (1981) *Proc. Natl. Acad. Sci. U.S.A. 78*, 2258–2262.

Hampsey, D. M., Das, G., & Sherman, F. (1988) *FEBS Lett. 231*, 275–283.

Hecht, M. H., Sturtevant, J. M., & Sauer, R. T. (1984) *Proc. Natl. Acad. Sci. U.S.A. 81*, 5685–5689.

Hickey, D. R., McLendon, G., & Sherman, F. (1988) *J. Biol. Chem. 263*, 18298–18305.

Hickey, D. R., Berghuis, A. M., Lafond, G., Jaeger, J. A., Cardillo, T. S., McLendon, D., Das, G., Sherman, F., Brayer, G. D., & McLendon, G. (1991) *J. Biol. Chem. 266*, 11686–11694.

Hicks, J. B., Strathern, J. N., Klar, A. J. S., & Dellaporta, S. L. (1982) in *Genetic Engineering Vol. 4* (Setlow, J. K., & Hollaender, A., Eds.) pp 219–248, Plenum Press, New York.

Hoffman, C. S., & Winston, F. (1987) *Gene 57*, 267–272.

Ikemura, T. (1982) *J. Mol. Biol. 158*, 573–577.

Ito, H., Fukada, Y., Murata, K., & Kimura, K. (1983) *J. Bacteriol. 153*, 163–168.

Krimm, S., & Bandekar, J. (1982) *Adv. Protein Chem. 38*, 181–364.

Lee, B. K., & Richards, F. M. (1971) *J. Mol. Biol. 55*, 379–400.

Lim, W. A., Farruggio, D. C., & Sauer, R. T. (1992) *Biochemistry 31*, 4324–4333.

Louie, G. V., & Brayer, G. D. (1990) *J. Mol. Biol. 214*, 537–555.

Margoliash, E., & Frohwirt, N. (1959) *Biochem. J. 71*, 570–572.

Matthews, B. W. (1987) *Biochemistry 26*, 6885–6887.

Mayo, S. L. (1988) Ph.D. Thesis, California Institute of Technology.
Messing, J. (1983) *Methods Enzymol. 101*, 20–78.
Montgomery, D. L., Hall, B. D., Gillam, S., & Smith, M. (1978) *Cell 14*, 673–680.
Nall, B. T. (1990) in *Protein Folding* (Gierasch, L. M., & King, J., Eds.) pp 198–207, AAAS, Washington, D.C.
Nakamaye, K., & Eckstein, F. (1986) *Nucleic Acids Res. 14*, 9679–9698.
Nozaki, Y. (1972) *Methods Enzymol. 26*, 43–50.
Pace, C. N. (1986) *Methods Enzymol. 131*, 266–280.
Pakula, A. A., & Sauer, R. T. (1990) *Nature 244*, 363–364.
Pielak, G. J., Mauk, A. G., & Smith, M. (1985) *Nature 313*, 152–154.
Ramdas, L., & Nall, B. T. (1986) *Biochemistry 25*, 6959–6964.
Ramdas, L., Sherman, F., & Nall, B. T. (1986) *Biochemistry 25*, 6952–6958.
Reidhaar-Olson, J.-F., & Sauer, R. T. (1990) *Proteins: Struct., Funct., Genet. 7*, 306–316.
Russel, M., Kidd, S., & Kelley, M. R. (1986) *Gene 45*, 333–338.
Schellman, J. A. (1978) *Biopolymers 17*, 1305–1322.
Serrano, L., Horovitz, A., Avron, B., Bycroft, M., & Fersht, A. R. (1990) *Biochemistry 29*, 9343–9352.
Sherman, F., Stewart, J. W., Jackson, M., Gilmore, R. A., & Parker, J. H. (1974) *Genetics 77*, 255–284.
Sherman, F., Fink, G. R., & Hicks, J. B. (1986) *Methods in Yeast Genetics*, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.
Shoemaker, D. P., Garland, C. W., & Steinfeld, J. I. (1974) *Experiments in Physical Chemistry*, 3rd ed. pp 32–34, McGraw-Hill Book Co., New York.
Shortle, D., & Meeker, A. K. (1986) *Proteins: Struct., Funct., Genet. 1*, 81–89.
Shortle, D., Meeker, A. K., & Freire, E. (1988) *Biochemistry 27*, 4761–4768.
Shortle, D., Stites, W. A., & Meeker, A. K. (1990) *Biochemistry 29*, 8033–8041.
Shortle, D., Chan, H. S., & Dill, K. A. (1992) *Protein Sci. 1*, 210–215.
Smith, M., Leung, D. W., Gillam, S., Astell, C. R., Montgomery, D. C., & Hall, B. D. (1979) *Cell 16*, 753–761.
Susi, H., & Byler, D. M. (1986) *Methods Enzymol. 130*, 290–311.
Surewicz, W. K., & Mantsch, H. H. (1988) *Biochim. Biophys. Acta 952*, 115–130.
Taylor, J. R. (1982) *An Introduction to Error Analysis*, p 169 University Science Books, Mill Valley, CA.
Titus, D. E. (1991) *Promega Protocols and Application Guide*, 2nd ed., Promega Corp., Madison, WI.
Tsong, T. Y. (1974) *J. Biol. Chem. 249*, 1988–1990.
Tsong, T. Y. (1975) *Biochemistry 14*, 1542–1547.