Introduction of a Disulfide Bond into Cytochrome *c* Stabilizes a Compact Denatured State†

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Received November 27, 1991; Revised Manuscript Received October 5, 1992

ABSTRACT: We introduced a novel disulfide bond, modeled on that of bullfrog cytochrome *c*, into yeast iso-1-cytochrome *c*. The disulfide spontaneously forms upon purification. A variety of techniques were used to examine the denaturation of this variant and several non-cross-linked controls. Denaturation is reversible and, with the exception of the protein in which the two cysteines are blocked, consistent with a two-state process. Comparison of the calorimetric and van't Hoff enthalpy changes indicates that denaturation is two-state at pH 4.6. Calorimetric and fluorescence-monitored guanidine hydrochloride (GdnHCl) denaturation data indicate that the free energy of denaturation for the cross-linked protein (ΔG_d at 300 K) is decreased relative to non-cross-linked controls. The dependence of ΔG_d on GdnHCl concentration, the GdnHCl concentration that denatures half the protein, as well as the enthalpy, entropy, and heat capacity changes (m_{GdnHCl}, C_m, ΔH_d, ΔS_d, and ΔC_p, respectively), all decrease in magnitude upon introduction of the cross-link. The decrease in ΔH_d and ΔS_d were confirmed by monitoring absorbance at several wavelengths as a function of temperature. The cross-link also decreases the pH dependence of these observables. Circular dichroism studies indicate the denatured state of the cross-linked protein possesses more structure than non-cross-linked proteins, and this structure is refractory to increases in temperature and chemical denaturant. We conclude that the diminished values of ΔG_d, ΔH_d, ΔS_d, ΔC_p, and m_{GdnHCl} result from the denatured state of the cross-linked variant being more compact and possessing more structure than non-cross-linked controls.

An important endeavor in biological chemistry is the understanding of protein stability. In this paper, protein stability is defined by the free energy of denaturation (ΔG_d).[1,2] Of particular interest are novel disulfide bonds (Wetzel, 1987). Naturally occurring, disulfide-containing proteins possess a more positive ΔG_d than their non-cross-linked equivalents under physiological conditions (Pace et al., 1988). Disulfides were originally predicted to increase the free energy of the denatured state of a protein by decreasing its chain entropy (Flory, 1956). The thermodynamic details of this increased stability, however, appear to be more complex and involve both enthalpic and entropic effects. As opposed to Flory (1956), Doig and Williams (1991) suggest that the dominant effect of disulfide bonds is to increase ΔH for denaturation (ΔH_d), and although there *is* a decrease in chain entropy, the overall entropy change for denaturation (ΔS_d) is increased due to diminution of the hydrophobic effect.

The change in free energy for protein denaturation as a function of temperature (ΔG_T) can be calculated using the Gibbs–Helmholtz equation:

$$\Delta G_T = \Delta H_d(1 - T/T_m) - \Delta C_p[(T_m - T) + T \ln (T/T_m)] \quad (1)$$

where T_m is the temperature at which half of the protein is denatured and ΔC_p is the difference in heat capacity between the denatured and native states. ΔG_d at a particular temperature can also be calculated from chemical denaturation data [guanidine hydrochloride (GdnHCl) is used here] using

$$\Delta G_{H_2O} = C_m m_{GdnHCl} \quad (2)$$

where ΔG_{H_2O} is the free energy of denaturation in the absence of denaturant, C_m is the concentration of GdnHCl at which half of the protein is denatured, and m_{GdnHCl} is the slope of the line of $-\Delta G_d$ versus GdnHCl concentration (Pace et al., 1990). These two relationships are valid provided denaturation is reversible and the concentration of intermediates is small.

In some studies of proteins with novel disulfides, ΔG_d cannot be determined because denaturation is irreversible. Instead, increased stability relative to a wild-type protein is usually defined as an increase in either C_m or T_m or in the time required for enzymatic activity to decrease 50% ($t_{1/2}$). Even if denaturation is reversible, and the concentration of intermediates is small, determination of T_m (eq 1), C_m (eq 2), or $t_{1/2}$ (Lumry et al., 1966) alone are insufficient to determine ΔG_d.

† This work was presented in part at the 201st American Chemical Society National Meeting, April 14–19, 1991, in Atlanta, GA, and at the ASBMB/Biophysical Society Meeting, Feb 9–13, 1992 in Houston, TX. S.F.B. was supported by a U.S. Department of Education Fellowship and a North Carolina Board of Governor's Fellowship in Science and Technology. This work was supported by donors of the Petroleum Research Fund, administered by the American Chemical Society (21597-G).

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[1] Abbreviations: CD, circular dichroism; C_m, the concentration of GdnHCl at which half of the protein is denatured; C_{me}, cysteines in which the sulfhydryl group is S-methylated; GdnHCl, guanidine hydrochloride; m_{GdnHCl}, the slope of the line of $-\Delta G_d$ versus GdnHCl concentration; m_{urea}, the slope of the line of $-\Delta G_d$ versus urea concentration; NMR, ^{1}H nuclear magnetic resonance; RNase, ribonuclease; SDS–PAG, sodium dodecyl sulfate polyacrylamide gel; T_m, temperature at which half of the protein is denatured; $t_{1/2}$, time required for enzymatic activity to decrease 50%; WT, S-methylated wild-type iso-1-cytochrome *c*; ΔC_p, the difference in heat capacity between the denatured and native states; ΔG_d, ΔG for protein denaturation; ΔG_{H_2O}, ΔG_d determined from chemical denaturation data; ΔG_T, ΔG_d determined from calorimetric data; ΔH_{cal}, the calorimetric ΔH_d; ΔH_d, ΔH for protein denaturation; ΔH_m, the van't Hoff ΔH near T_m calculated from thermal denaturation data; ΔH_{vh}, the van't Hoff ΔH calculated from calorimetric data; ΔS_d, ΔS for protein denaturation; ΔS_m, ΔS_d near T_m.

[2] ΔG_d is used in place of the more common ΔG_u (for unfolding) because, as discussed by Dill and Shortle (1991), a denatured protein is not necessarily fully unfolded.

0006-2960/92/0431-12337$03.00/0

Attempts to increase the stability of monomeric proteins by introduction of novel disulfides have met with mixed success. Reversible denaturation is observed for the cross-linked and non-cross-linked forms of T4 lysozyme, dihydrofolate reductase, and ribonuclease H (RNase H). Certain single or multiple disulfides introduced into T4 lysozyme increase T_m, ΔG_d, and $t_{1/2}$ (Perry & Wetzel, 1984; Wetzel et al., 1988; Matsumura & Matthews, 1989; Matsumura et al., 1989). On the other hand, introduction of a cross-link into dihydrofolate reductase increases C_m but decreases T_m and ΔG_{H_2O} (Villafranca et al., 1987; Shortle, 1989). Introduction of a novel disulfide into RNase H increases C_m, T_m, and ΔG_{H_2O} (Kanaya et al., 1991), but there are complications in that controls that consider the effects of substituted wild-type residues were not reported. Other proteins have had novel disulfides introduced, but denaturation of these proteins is irreversible and so will not be discussed here. An important consideration when introducing novel disulfides is their effect(s) on the native state. Substantial information is available concerning spatial requirements for disulfide bonds (Richardson, 1981; Thornton, 1981). Katz and Kossiakoff (1986) suggest that the destabilization of certain subtilisin BPN′ variants is caused by highly strained torsional angles adopted by the disulfides.

The sequences of nearly 100 mitochondrial cytochromes *c* are known (Moore & Pettigrew, 1990), and only bullfrog cytochrome *c* possesses a disulfide bond (Brems et al., 1982). The disulfide is formed between cysteines at positions 20 and 102 [throughout this work the numbering scheme of higher eukaryotic cytochromes *c* is used (Cutler et al, 1987; Moore & Pettigrew, 1990)]. In their comparison of several eukaryotic ferricytochromes *c* (all of which exhibit reversible denaturation), Brems et al. (1982) showed that the bullfrog protein exhibits the highest C_m, but there are several points that complicate interpretation of this result. First, the sequences of the proteins compared differ by more than just the residues at positions 20 and 102. Second, although comparison of structural and sequence data indicate that the cytochrome *c* family possesses a conserved fold (Mathews, 1985), their stability varies greatly (Pace, 1975). Third, as eq 2 indicates, an increased C_m does not necessarily lead to an increased ΔG_d.

To overcome these complications, we undertook a study of iso-1-cytochrome *c* from the yeast *Saccharomyces cerevisiae*. The Fe^{3+} form was examined so our results can be compared directly with those of Brems et al. (1982). Iso-1-cytochrome *c* is ideally suited for examining the effect of disulfides on protein stability for three reasons. First, the protein possesses a cysteine at position 102, and only substitution of valine 20 is required to introduce the cross-link found in bullfrog cytochrome *c*. Second, the crystal structure of iso-1-cytochrome *c* has been determined to high resolution (Louie & Brayer, 1990; Berghuis & Brayer, 1992), and its solution structure has been studied extensively by ^1H nuclear magnetic resonance (NMR) spectroscopy (Pielak et al., 1988a,b; Gao et al., 1990, 1991). Third, as demonstrated here, the denaturation of both the cross-linked variant and non-cross-linked control are reversible and two-state.

MATERIALS AND METHODS

Nomenclature, Strains, and Molecular Biology Techniques. The term "variant" is reserved for discussion of proteins, and the term "mutant" refers to specific alleles. Variants are denoted by their one-letter codes (IUPAC–IUB Joint Commission on Biochemical Nomenclature, 1985) with the wild-type residue listed first, followed by the primary sequence position, and the variant residue. Multiple changes are separated by a semicolon (e.g., V20C;C102T). Cysteines in which the sulfhydryl group is S-methylated are denoted C_{me}. Except for the yeast strain used in this study, all manipulations were performed as described by Hilgen and Peilak (1991). The yeast strain used here is GM-3C-2 [*MATα leu2-3 leu2-112 trp1-1 his4-519 cyc1-1 cyp3-1*] (Faye et al., 1981). For putative mutants, the sequence of the entire cytochrome *c* gene was determined. Yeast were transformed by electroporation (Rech et al., 1990). Prior to the isolation of variant cytochromes *c*, the phagemids were rescued from yeast (Rose, 1987) and the cytochrome *c* genes subjected to DNA sequence analysis.

Protein Purification and Modification. Horse cytochrome *c* (type VI) was purchased from Sigma. Iso-1-cytochrome *c* were isolated using an FPLC-modified version of the method described by Sherman et al. (1968). The purity of the variant proteins was determined by sodium dodecylsulfate polyacrylamide gel (SDS–PAG) electrophoresis and HPLC. Amino acid analysis was performed as described by Pielak et al. (1985). Free sulfhydryls were blocked with methyl methanethiosulfonate (Ramdas et al., 1986). Ferricytochrome *c* were produced by binding the protein to Amberlyte CG-50 (Sigma) equilibrated with 50 mM potassium phosphate, pH 7.2. A 10–20-fold excess of $NH_4[Co(dipicolinate)_2]$ (Mauk et al., 1979) was then passed through the column. After all traces of $Co(dipicolinate)_2^-$ were removed, ferricytochrome *c* was eluted with 50 mM potassium phosphate and 1 M KCl, pH 7.2. Examination of the NMR spectrum indicates that this regimen removes all traces of the oxidant (G. J. Pielak, unpublished observation). The concentration of cytochromes *c* was determined using an extinction coefficient of 106 100 M^{-1} cm^{-1} at 410 nm (Margoliash & Frohwirt, 1959). The number of free sulfhydryls was determined by titration with 5,5′-dithiobis(2-nitrobenzoic) acid (Habeeb, 1972). Prior to an experiment, the ferricytochromes *c* were exchanged into the appropriate buffer system by repeated concentration with Centricon-10 microconcentrators (Amicon).

Thermal Denaturation. Absorbance was determined using a Shimadzu UV-160 spectrophotometer fitted with thermostated cuvette holders and connected to a Fisher Scientific model 900 constant temperature bath. Cuvettes (4 mL, Hellma) were stoppered with a teflon cap containing a 0.25-mm bore. Temperature was monitored in the sample using a teflon-coated 0.25-mm tissue implant probe (Physitemp). Absorbencies of the ferricytochromes *c* were monitored at 278, 287, and 399 nm. These wavelengths were chosen by comparing difference spectra of the variant proteins taken at 10 °C intervals between 20 and 80 °C (data not shown). The protein concentration for thermal denaturation was 10–15 μM. T_m, ΔH_m, and ΔS_m (ΔH_d and ΔS_d near T_m) were calculated as described by Pace et al. (1990).

Chemical Denaturation. Denaturation by GdnHCl was performed by diluting stock solutions of ferricytochromes *c* into various combinations of buffered 5 M GdnHCl and buffer to a final cytochrome *c* concentration of 2–3 μM. The buffer systems were 50 mM potassium phosphate, pH 7.2, or 40 mM glycine, pH 4.6. Ferricytochromes *c* were equilibrated in denaturant at 300 K for a minimum of 4 h. Fluorescence was measured using a SLM 8000 (Aminco) fluorimeter with an excitation wavelength of 292 nm and emission wavelength of 352 nm (Ramdas et al., 1986). ΔG_{H_2O} and m_{GdnHCl} were calculated using nonlinear least-squares analysis (Santoro & Bolen, 1988; Hughson & Baldwin, 1989). Except for $V20C_{me}$, all the data are consistent with the two-state model, and regress

Table I: Thermal Denaturation Data[a]

variant	pos 20	pos 102	pH	T_m (°C)	ΔH_m (kcal mol^{-1})	ΔS_m (cal mol^{-1} K^{-1})
WT	Val	Cys_{me}	6.0	51.7	84.5	262
$V20C_{me}$;C102T	Cys_{me}	Thr	6.0	55.9	92.3	280
$V20C_{me}$	Cys_{me}	Cys_{me}	6.0	49.7	*b*	*b*
C102T	Val	Thr	4.6	54.7	89.4	272
			6.0	59.6	108.8	327
V20C	Cys	Cys	4.6	53.4	64.5	197
			6.0	58.5	63.8	192

[a] The estimated errors in T_m, ΔH_m, and ΔS_m are ±0.1 °C, ±4 kcal mol^{-1}, and ±15 cal mol^{-1} K^{-1}, respectively. [b] Does not fit the two-state model.

to well below the X^2 1% confidence limit (Havlicek & Crain, 1988).

Calorimetry. Differential scanning calorimetry was performed with a Microcal MC-2 microcalorimeter. Ferricytochromes *c* were diluted with 50 mM sodium acetate, pH 4.6, or 50 mM sodium phosphate, pH 6.0, to a concentration of 2 mg mL^{-1}. The scan rate was 1 K min^{-1} from 15 to 75 °C for the yeast proteins. The range for horse ferricytochrome *c* was from 20 to 95 °C. Return scans (from high to low temperature) were performed to demonstrate reversibility. Data were analyzed with CpCALC software (version 2.1, Microcal). The van't Hoff enthalpy for denaturation (ΔH_{vh}) was calculated from excess heat capacity plots as described by Sturtevant (1987).

Circular Dichroism. Measurement of circular dichroism (CD) was performed with a Jasco J-600 spectropolarimeter. Protein concentration was 30 μM. In the ultraviolet, spectra were acquired using a 1-mm cell, and visible spectra were acquired using a 1-cm cell. To investigate the circular dichroism of the native state, spectra were acquired at 25 °C, pH 4.6, in water. To investigate the circular dichroism of denatured states, spectra were acquired at 63 °C, pH 4.6, in water, or at 25 °C in 1.84 M GdnHCl, pH 4.6. Independently, these conditions result in 95% denaturation of both the C102T and V20C variants as determined from calorimetric and spectroscopic data. Spectra were also acquired at 63 °C, in 1.84 M GdnHCl, pH 4.6. Molar ellipticities, Θ, were calculated as described by Pielak et al. (1986).

RESULTS

The proteins investigated are listed in Table I. The C102T variant contains only two cysteines, at positions 14 and 17, that are posttranslationally modified to form the thioether linkages to the heme. Two of the proteins in Table I contain an additional cysteine: the wild-type protein (cysteine 102) and the V20C;C102T variant. The V20C variant contains cysteines at both position 20 and 102. Changes in deduced amino acid sequence were confirmed by determining the DNA sequence of the entire coding region of the cytochrome *c* genes isolated from yeast transformants and by amino acid analysis of the variant proteins. Each of the four alleles yields a fully functional cytochrome *c* in vivo as judged by the ability of the transformants to grow in glycerol-containing media (Hampsey et al., 1986). Prior to modification, the number of free sulfhydryls in the wild-type protein and the V20C;C102T variants is 1.0 ± 0.1 per protein. The proteins listed in Table I exhibit less than 0.1 free sulfhydryls per protein as determined by Ellman titration. The presence of the disulfide in the V20C variant was verified by comparing its electrophoretic mobility in SDS–polyacrylamide gels in the presence and absence of β-mercaptoethanol. The relative mobility of non-disulfide-bonded variants is unaffected by this change in conditions, whereas the V20C variant exhibits increased mobility under nonreducing conditions. Thermal and GdnHCl denaturation is reversible for the C102T and disulfide-containing variants. Proteins with free sulfhydryls exhibit irreversible denaturation, but after S-methylation these variants exhibit reversible behavior. SDS–PAG electrophoresis under nonreducing conditions indicates that the S-methylated proteins are monomeric both before and after thermal denaturation. This and the observation that these proteins no longer react with 5,5′-dithiobis(2-nitrobenzoic) acid shows that blocking is complete. It did not prove possible to examine the form of the V20C variant in which the disulfide was reduced. First, reduction of the iron with sodium dithionite causes reduction of the disulfide. Second, using the procedures described under Materials and Methods, denaturation of ferro-C102T as well as ferro-V20C is irreversible.

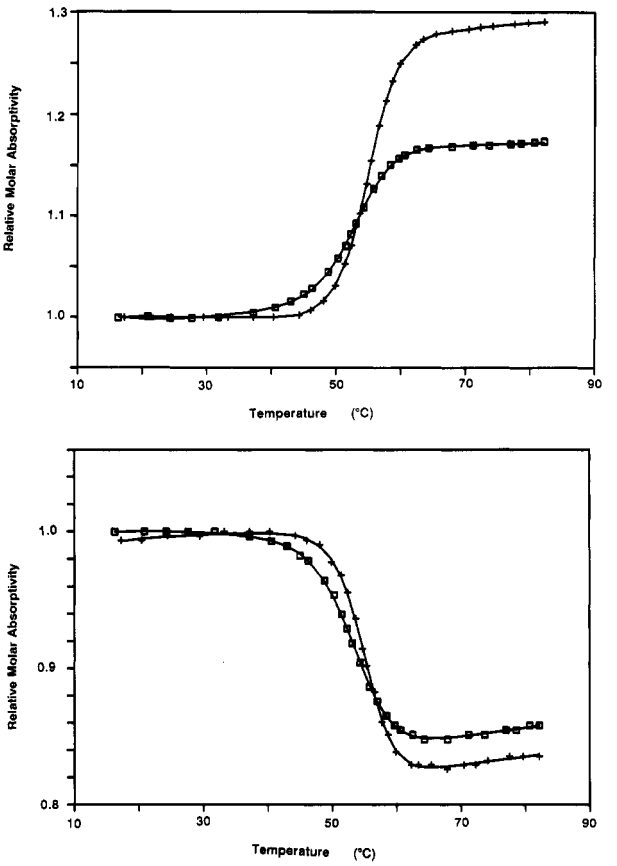

FIGURE 1: (Upper panel) Relative molar absorptivity at 399 nm versus temperature for the iron(III) form of the (+) C102T and (□) V20C variants at pH 4.6. (Lower panel) Relative molar absorptivity at 287 nm versus temperature for the iron(III) forms of the C102T and V20C variants at pH 4.6. Theoretical fits of the data to eq 1 (using ΔC_p values from calorimetry) have been drawn through the points. Relative molar absorptivity is determined by setting the molar absorptivity at 20 °C and a given wavelength equal to 1.0 and calculating the molar absorptivity at other temperatures relative to this value. The molar absorptivities of the V20C and C102T variants at 20 °C are the same for the wavelengths examined.

Representative examples of thermal denaturation data are shown in Figure 1. T_m, ΔH_m, and ΔS_m were estimated as described by Pace et al. (1990) and are presented in Table I. The T_m of the S-methylated wild-type iso-1-cytochrome *c* (WT) is 51.7 °C, in good agreement with the study by Dumont et al. (1990). These data, however, do not agree as well with other studies (Hickey et al., 1988; Das et al., 1989) that place the T_m of this protein near 46 °C. The lower T_m may be due to the presence of dimerized protein (Bryant et al., 1985).

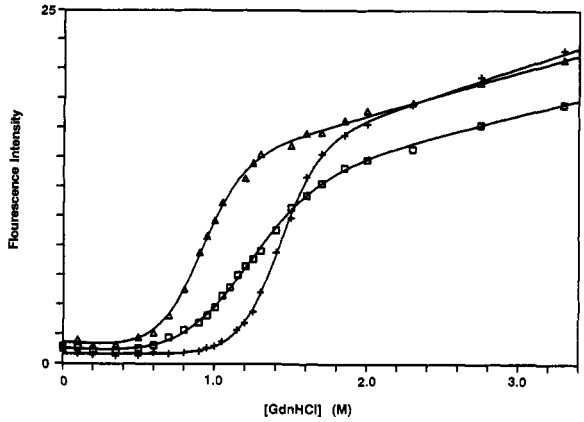

FIGURE 2: Fluorescence versus GdnHCl concentration for the iron-(III) form of the (△) WT protein, the (+) C102T variant, and the (□) V20C variant at pH 7.2 and 300 K. The curves represent the nonlinear least-squares fits of the data to the two-state hypothesis (Santoro & Bolen, 1988).

Table II: GdnHCl Denaturation at 300 K[a]

variant	pH	C_m (M)	m_{GdnHCl} (kcal mol^{-1} M^{-1})	ΔG_{H_2O} (kcal mol^{-1})
WT	7.2	0.9	4.6	4.1
V20C$_{me}$;C102T	7.2	1.1	3.7	4.1
V20C$_{me}$	7.2	0.9	*b*	*b*
C102T	4.6	1.0	6.2	6.4
	7.2	1.4	4.6	6.4
V20C	4.6	1.1	2.7	2.9
	7.2	1.2	2.6	3.0
tuna[c]	7.0	2.8	3.2	9.0
horse[c]	7.0	2.5	2.9	7.4
bullfrog[c]	7.0	3.9	1.8	7.2

[a] The estimated errors in C_m, m_{GdnHCl}, and ΔG_{H_2O} are ±0.1 M, ±0.2 kcal mol^{-1} M^{-1}, and ±0.3 kcal mol^{-1}, respectively. [b] Does not fit the two-state nonlinear regression model. [c] Calculated by linear regression from Figure 4 of Brems et al. (1982).

The proteins examined here were checked by nonreducing SDS-PAG electrophoresis, both before and after thermal denaturation, and no dimerized material was detected. The V20C$_{me}$ and the V20C$_{me}$;C102T variants exhibit T_m's close to that of the WT protein. With the exception of V20C$_{me}$, all of the proteins yield data consistent with a two-state transition. The V20C variant exhibits the smallest ΔH_m, and C102T the largest. (The V20C$_{me}$ variant appears to have a large ΔH_m, but the values for this derivative are suspect because denaturation data for this variant cannot be fit to a two-state model.) Although the calculation of ΔH_m is approximate, and only valid near T_m, the entropy change near T_m (ΔS_m) can be estimated by dividing ΔH_m by T_m. Accordingly, V20C exhibits the smallest ΔS_m. The data in Table I also indicate that ΔH_m and ΔS_m for the V20C variant are independent of pH, whereas those for C102T increase with increasing pH.

Representative examples of chemical denaturation data are shown in Figure 2. C_m, m_{GdnHCl}, and ΔG_{H_2O} were calculated by nonlinear least-squares analysis (Santoro & Bolen, 1988; Hughson & Baldwin, 1989) and are presented in Table II. GdnHCl was chosen because the variation of ΔG_d with GdnHCl concentration is linear over a wide range of concentrations (Shortle et al., 1989), and deviations from linear behavior at low concentrations are small (Alonso & Dill, 1991; Santoro & Bolen, 1992). With the exception of the V20C$_{me}$ variant, each protein yields data consistent with the two-state model.

Examination of the GdnHCl denaturation data (Table II) demonstrates that C102T is the most stable variant. At pH 7.2, both variants with single blocked cysteines are of intermediate stability, and the disulfide-bonded variant is the least stable. (Although V20C$_{me}$ appears to be poorly stabilized, the uncertainty concerning its two-state behavior prohibits detailed analysis.) At the heart of this large difference in ΔG_{H_2O} is the variation of m_{GdnHCl}. The m_{GdnHCl} value for V20C is considerably smaller than that of either C102T or WT. When the pH is lowered to 4.6, the C_m of C102T is lowered by 0.4 M and is the same or less than that of V20C, whose C_m is not significantly changed. This would seem to indicate that V20C is more stable at acidic pH, yet the values of ΔG_{H_2O} for the two proteins at these two pH's do not change. For C102T, this is because of compensatory changes in C_m and m_{GdnHCl}, but for V20C it is because C_m and m_{GdnHCl} are unaltered. There is no difference in the absorption and little change in the CD spectra of either protein between pH 7.2 and 4.6 (data not shown). The pH dependence of denaturation was monitored by absorbance below pH 4, and no changes for either the C102T or V20C variant were detected near pH 4.6. This suggests that the native states of the proteins are of similar stability, and hence the difference in free energy between the native states of both V20C and C102T on going from pH 7.2 to 4.6 is small.

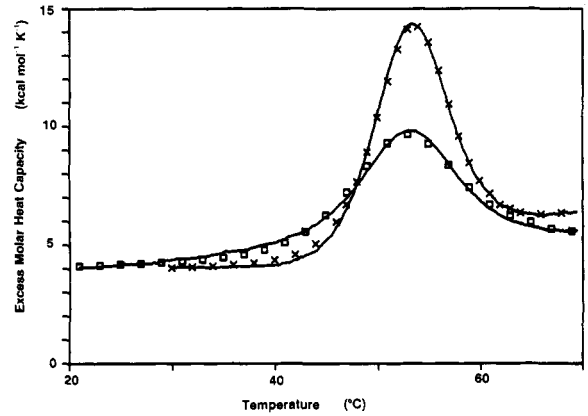

FIGURE 3: Excess molar heat capacity versus temperature for the iron(III) form of the (×) C102T and (□) V20C variants at pH 4.6. Data are shown as solid lines, and points from theoretical fits to eq 1 are shown as symbols.

If introduction of the cross-link creates, or increases the concentration of, intermediate species, the stability of the V20C variant will be underestimated (Cantor & Schimmel, 1980). Therefore, calorimetric studies were undertaken because differential scanning calorimetry allows simultaneous determination of the van't Hoff (ΔH_{vh}) and calorimetric (ΔH_{cal}) enthalpies. A value of unity for $\Delta H_{cal}/\Delta H_{vh}$ is proof that a protein undergoes a two-state transition (Privalov, 1979; Pace et al., 1990). As previously observed by Santucci et al. (1989), horse cytochrome *c* at pH 7.2 exhibits calorimetric data of poor quality, and ΔC_p could not be estimated. Calorimetry of cytochromes *c* at pH 4.6, however, provides data of good quality (Privalov & Khechinashvili, 1974). As a standard, horse ferricytochrome *c* was examined. ΔH_{cal} and ΔC_p for horse cytochrome *c* (91.3 kcal mol^{-1} and 1510 cal mol^{-1} K^{-1}) are in close agreement with the values reported for bovine cytochrome *c* (Privalov & Khechinashvili, 1974).

Plots of excess heat capacity as a function of temperature for the V20C and C102T variants at pH 4.6 along with fits of the data to a two-state process are shown in Figure 3. Calorimetry performed at pH 6.0 also provided data of good quality. Thermodynamic parameters derived from the calorimetric data are presented in Table III. The values of $\Delta H_{cal}/\Delta H_{vh}$ for C102T and V20C at pH 4.6 and 6.0 range from 0.89

Table III: Differential Scanning Calorimetry Data

variant	pH	T_m (°C)	ΔH_{vh} (kcal mol^{-1})	ΔH_{cal} (kcal mol^{-1})	ΔS_d[a] (cal mol^{-1} K^{-1})	ΔC_p (cal mol^{-1} K^{-1})	ΔG_T at 300 K (kcal mol^{-1})
C102T	4.6	53.5	95.0	88.8	272	1380	5.7
	6.0	58.5	109.5	99.4	300	2230	5.9
V20C	4.6	52.8	71.6	63.5	195	925	4.1
	6.0	56.7	66.0	62.7	190	880	4.4

[a] $\Delta S_d = \Delta H_{cal}/T_m$ (T_m in K).

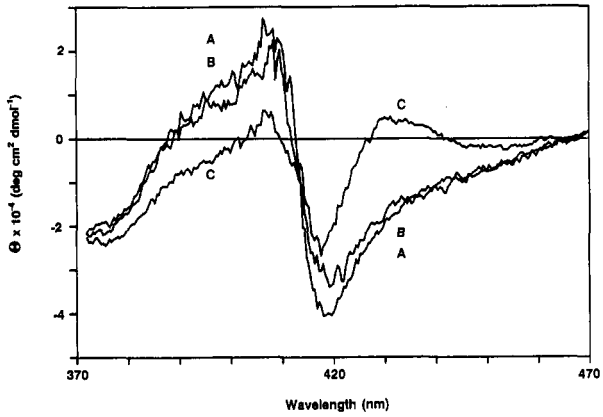

FIGURE 4: Soret CD of the iron(III) native state for the (A) C102T, (B) V20C, and (C) $V20C_{me}$ variants in H_2O at pH 4.6, 25 °C.

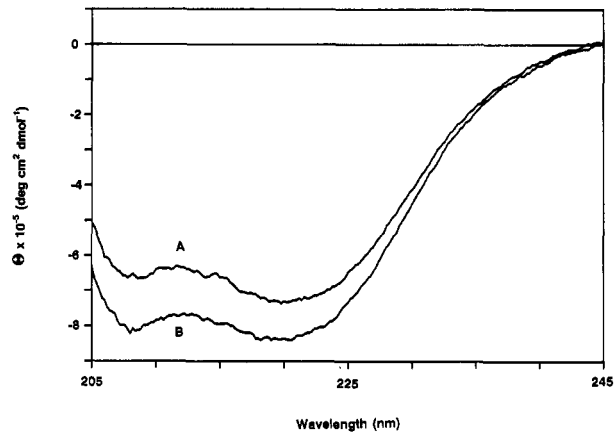

FIGURE 5: Ultraviolet CD of the iron(III) native state for the (A) C102T and (B) V20C variants in H_2O at pH 4.6, 25 °C.

to 0.95. These observations demonstrate that denaturation of these proteins is essentially two-state. The van't Hoff enthalpies determined by monitoring absorbance as a function of temperature (Table I) and calorimetry (Table III) can be compared. ΔH_{vh} is consistently slightly greater than ΔH_m, with the ratio of $\Delta H_m/\Delta H_{vh}$ varying from 0.91 to 0.99. Additionally, the differential scanning calorimetry experiment provides the variables needed to calculate ΔG_T (eq 1). A comparison of ΔG_T to ΔG_{H_2O} (both at 300 K; Tables II and III) indicates that the cross-linked protein is less stable than the non-cross-linked control, although there are differences between the chemical and calorimetric ΔG_d's.

For the C102T variant, ΔH_{cal} at pH 4.6 is approximately 25 kcal mol^{-1} greater than that of the disulfide-bonded protein (Table III). This trend is also observed for ΔH_m (Table I). The C102T variant possesses a greater ΔC_p than the V20C variant.[3] The ΔC_p values for both variants at pH 4.6 are smaller than that of horse ferricytochrome *c* under identical conditions, and the T_m for the horse protein is more than 25 °C higher than the most stable iso-1-cytochrome *c* variants. Plant and fungal cytochromes *c* possess an unstructured N-terminal extension (Cutler et al., 1987; Louie & Brayer, 1990). This region may contribute to the lower T_m of iso-1-cytochrome *c* compared to horse cytochrome *c* (Straume et al., 1992).

The negative Soret Cotton effect of cytochrome *c* is a sensitive indicator of the integrity of the heme environment (Pielak et al., 1986). As shown in Figure 4, the Soret CD of the V20C and C102T variants are nearly identical. The Soret CD of the singly S-methylated variants are also highly similar to that of C102T (data not shown). On the other hand, the Soret CD spectrum of the $V20C_{me}$ variant is attenuated (Figure 4). The ultraviolet CD of the C102T and V20C variants is shown in Figure 5. The C102T variant exhibits strong negative Cotton effects at 208 and 222 nm, as expected for a protein whose main secondary structure is α-helix (Figure 5). Inspection of the ultraviolet CD of the V20C variant indicates that the cross-link increases helical content. These observations suggest that neither the singly S-methylated cysteines nor the cross-link significantly alters the native state but the presence of both blocking groups results in more drastic changes.

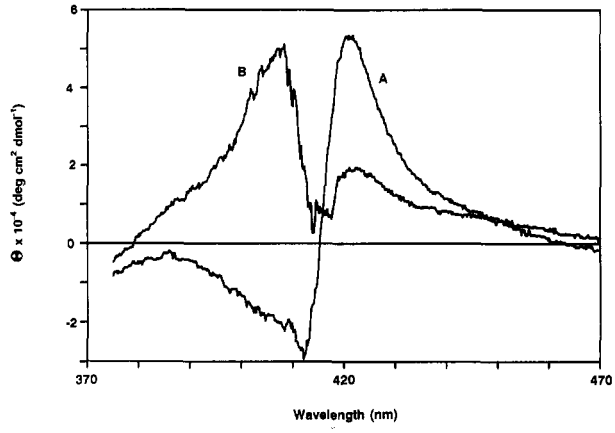

FIGURE 6: Soret CD of iron(III) denatured state for the (A) C102T and (B) V20C variants in 1.84 M GdnHCl, pH 4.6, 25 °C. Note change in scale from Figure 4.

The CD of the C102T and disulfide-bonded variants was also examined under denaturing conditions (Figures 6 and 7). Both proteins exhibit decreases in the α-helical band at 222 nm upon thermal or chemical denaturation (compare Figures 5 and 7). Inspection of the CD of the thermally denatured proteins shows that the denatured state of V20C has a greater negative ellipticity at 222 nm than does C102T. This difference persists even after the difference between the native proteins is subtracted. This same trend is observed for the GdnHCl-denatured proteins. These observations indicate that the thermally denatured state of the cross-linked protein contains more structure than that of C102T. Without further evidence, it is premature to conclude that this residual structure is α-helical (Robertson & Baldwin, 1991), although for a

[3] Values of ΔC_p were estimated from several experiments. ΔC_p for the C102T variant has also been examined by pH dependent ΔH versus T_m plots, and is indistinguishable from the value reported here (D. Cohen and G. Pielak, manuscript in preparation).

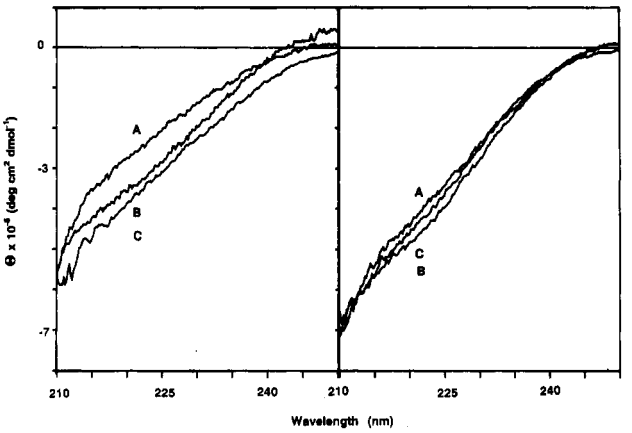

FIGURE 7: Ultraviolet CD of iron(III) denatured state for the (left panel) C102T and (right panel) V20C variants at pH 4.6. Conditions: (A) GdnHCl, 1.84 M, 25 °C; (B) 63 °C, in water; (C) GdnHCl, 1.84 M, 63 °C.

protein with little other secondary structure than α-helix, this assertion is not unreasonable. Additionally, as shown in Figure 6, at 25 °C upon the addition of GdnHCl, the Soret CD maxima of C102T changes extensively (from 407 to 422 nm), whereas the change for the V20C variant is minimal (from 407 to 409 nm), suggesting a more native-like heme environment for the denatured cross-linked variant.

Denaturation conditions were made more drastic by raising the temperature of the GdnHCl-containing sample to 63 °C (Figure 7). Compared to the same sample at 25 °C, the C102T variant continues to unfold in a manner consistent with observations for other proteins at high temperature in the presence of GdnHCl (Privalov et al., 1989). On the other hand, for the V20C variant little change is observed when both effects are combined.

The CD data are summarized as follows. The cross-link's effects on the native state of the protein are small compared to its effects on the denatured state(s). Denaturation of both C102T and V20C by either temperature or GdnHCl does not result in a random coil. For both the V20C and C102T variants, the thermally denatured forms seem to possess more structure than the chemically denatured forms. The denatured forms of the cross-linked variant, however, possess significantly more structure than those of the C102T variant. The structure present in the C102T variant after thermal denaturation is reduced by addition of GdnHCl. However, the structure present within the V20C variant after thermal denaturation is nearly refractory to the addition of GdnHCl.

DISCUSSION

There are three reasons to suppose that the novel disulfide bond described here should increase ΔG_d. First, the disulfide links the "ends" of the protein. Theory suggests that the reduction in entropy of the unfolded protein is a function of the number of residues between the cross-link (Flory, 1956; Poland & Scheraga, 1965), and, although not directly related, experiments show that a large loop size increases T_m (Matsumura et al., 1989). The cross-link between positions 20 and 102 of cytochrome *c* encompasses over 75% of the chain length. Second, the disulfide links two units of secondary structure, namely, the N- and C-terminal helices. The interaction of these helices is the most conserved structural feature of this class of proteins (Mathews, 1985). The disulfide connects the end of the C-terminal helix with the region following the N-terminal helix. This linking motif is common to many disulfide-containing proteins (Richardson, 1981). Furthermore, the interaction of the N- and C-termini appears to be an early event in the folding of cytochromes *c* (Roder et al., 1988). Third, molecular modeling (data not shown) of the crystal structures (Takano & Dickerson, 1981; Louie et al., 1988) of tuna and iso-1-cytochrome *c*, and the fact that the disulfide forms spontaneously upon purification, suggest that the disulfide is unstrained. Nevertheless, the disulfide-bonded protein possesses a smaller ΔG_d at 300 K compared to that of the C102T variant (Tables II and III) despite the fact that it possesses a similar T_m and has a greater C_m than the C102T variant at pH 4.6 (Table II).

In an attempt to determine whether the decreases in m_{GdnHCl} and ΔG_{H_2O} upon introduction of the cross-link are specific to iso-1-cytochrome *c*, we calculated ΔG_{H_2O} and m_{GdnHCl} (Table II) from the data on bullfrog cytochrome *c* presented in Figure 4 of the paper by Brems et al. (1982). Even though the C_m of the bullfrog protein is considerably greater than the C_m's of the other two proteins, the bullfrog protein exhibits the smallest ΔG_{H_2O}. The source of this behavior is the same as for the V20C variant: a decreased m_{GdnHCl}. Because the data are for proteins that differ by more residues than those in the cross-link, it cannot be interpreted with the same rigor as that for the iso-1-cytochromes *c*. Furthermore, the data from Brems et al. (1982) cannot be subjected to nonlinear regression analysis.

In comparing ΔG_d values for variant proteins, it is often difficult to gauge whether changes are caused by alteration of the native or the denatured state. That is, the diminished ΔG_d observed for the cross-linked protein compared to the C102T or WT proteins could be caused by destabilization of V20C's native state and/or by stabilization of its denatured state.

Examination of the spectroscopic data for the V20C variant suggests that its native state is similar to that of the C102T variant. The absorption spectra of the native C102T and V20C variants are indistinguishable. Inspection of the CD spectra indicates there are very small changes between the native forms of V20C and C102T. Additional evidence for the stability of the disulfide bond in the native state comes from the observation that no free sulfhydryls are detected for V20C by Ellman titration (and no dimers are detected by SDS–PAG electrophoresis). Given the low sensitivity of the Ellman titration, the smallest ratio of cross-linked to non-cross-linked protein is 10, indicating that the disulfide is *at least* 1.5 kcal mol^{-1} more stable than the free cysteines.

If the V20C variant is unable to unfold to the same extent at the C102T variant, C_m and T_m would still be expected to be high, but ΔG_d would decrease. We conclude that the cross-link between positions 20 and 102 affects the denatured state of cytochrome *c* by locking it into a more compact structure compared to non-cross-linked controls. Evidence leading to this conclusion is summarized below. Comparing the electrophoretic mobility of the variants, both before and after thermal denaturation, under nonreducing denaturing conditions, all of the variants in Table I migrate as monomers, and all except V20C have identical mobilities. The disulfide-bonded protein has a greater mobility indicating a smaller hydrodynamic volume in the denatured state. With β-mercaptoethanol present, however, all five proteins exhibit the same mobility. Although the visible spectra of the native states of V20C and C102T are identical, the change in molar absorptivity upon thermal denaturation is greater for the non-cross-linked protein (Figure 1). This observation is consistent with the idea that the denatured state is affected by introduction of the cross-link and that the change between the

denatured and native states is smaller for the cross-linked protein. The GdnHCl denaturation data demonstrate that the major effect of the cross-link on the denaturation of cytochrome c is to decrease m_{GdnHCl} (Table II). The value of m_{GdnHCl} is related to the difference in solvent-exposed, nonpolar surface area between the denatured and native states (Schellman, 1978; Shortle, 1989). The m_{GdnHCl} value for V20C at neutral pH is 55% that of WT or C102T, indicating that V20C exposes less surface area upon denaturation. When the pH of the experiment is lowered to 4.6, the value of m_{GdnHCl} for C102T increases by 1.6 kcal mol^{-1} M^{-1}. This is in agreement with the chemical denaturation data from Pace et al. (1992), who find that for RNase T1 and barnase the changes in ΔG_d versus urea concentration (m_{urea}) increases with decreasing pH. This effect is proposed to be due to increasing the overall positive charge of the protein upon lowering the pH. This increases the charge repulsion, causing an expansion of the denatured state, thus increasing m_{urea}. However, there is no change in m_{GdnHCl} for the V20C variant upon changing the pH. Calorimetric data indicate that ΔC_p for the disulfide-bonded protein is smaller than for C102T. This decrease in ΔC_p as well as the decrease in m_{GdnHCl} upon introduction of the disulfide bond both support the idea of a more compact denatured state and are in agreement with theoretical work concerning the thermodynamic effects of disulfide bonds (Doig & Williams, 1991).

Both the thermal denaturation and the calorimetric data indicate that ΔH_d and ΔS_d are smaller for the cross-linked protein than for the C102T variant. This behavior is at odds with the theoretical study of Doig and Williams (1991), who contend that both ΔH_d and ΔS_d for a disulfide-bonded protein should be greater than that of the isostructural non-cross-linked protein. Part of this discrepancy comes from the use of the C102T variant as the "isostructural" non-cross-linked control. The C102T variant is used because cysteine 102 of the wild-type protein must be blocked for denaturation to be reversible. If left unblocked, the wild-type protein forms disulfide-bonded dimers (Cutler et al., 1987). Although the structural consequences of blocking cysteine 102 are unknown, two possibilities are presented. First, examination of the evolutionary record reveals that amino acids with heavy atoms at the δ position are never found at position 20 and rarely found at position 102 (Cutler et al., 1987; Moore & Pettigrew, 1990). Therefore, the decreased stability of the WT protein compared to the C102T variant is consistent with a deleterious steric interaction resulting from the S-methylation of cysteine 102. Alternatively, this differential stability may result from a stabilizing interaction involving threonine 102. The replacement of the native cysteine 102 by threonine does not adversely affect the function of iso-1-cytochrome c in vivo (Cutler et al., 1987) and eliminates the possibility of dimer formation in vitro. Over 85% of the cytochrome c whose sequences are known possess a threonine at position 102. The amino acid residue at position 102 is the first after the C-terminal helix. Threonine 102 provides a hydrogen-bond donor with the ability to form a "helix cap" (Presta & Rose, 1988) for the unsatisfied carbonyl of leucine 98 in the C-terminal helix. Inspection of the primary sequences of cytochromes c (Moore & Pettigrew, 1990) between positions 102 and 104 shows a preponderance of amino acids with side chains that are potential hydrogen-bond donors. Tuna cytochrome c possesses a hydrogen bond between the hydroxyl of threonine 102 and the carbonyl of leucine 98 (Takano & Dickerson, 1981), and rice cytochrome c contains the homologous hydrogen bond as well as one involving the hydroxyl of serine 103 and the carbonyl of lysine 99 (Ochi et al., 1983). The S-methylated sulfhydryl has *no* hydrogen-bond donating capacity. Therefore, the loss of this potential helix cap could be a source of the instability of the WT protein relative to the C102T variant. In summary, although the use of the C102T variant may overestimate the differences in ΔG_d between a variant and wild-type iso-1-cytochrome c, it is the best control for investigating such differences.

The difference in ΔH_d between V20C and C102T is at least 25 kcal mol^{-1}. ΔH_d is related to the number of hydrogen bonds and van der Waals interactions lost on going from the native to the denatured state. This difference cannot be entirely accounted for by steric interaction or the loss of one helix-capping hydrogen bond. The smaller ΔH_d, as well as the smaller ΔS_d, ΔC_p, and ΔG_d, probably results from a similarity between the compact denatured state of V20C and the native state. These state functions are expected to be smaller for the cross-linked variant than for the non-cross-linked control because the denatured state of the cross-linked variant is more compact.

CONCLUSIONS

The introduction of a novel disulfide into ferricytochrome c does not significantly alter the native state of the protein but results in a more compact denatured state. The greater similarity between its native and denatured states, compared to the non-cross-linked proteins, results in diminished values of ΔG_d, ΔH_d, ΔS_d, ΔC_p and m_{GdnHCl} for the cross-linked variant. The observations that lead to these conclusions are summarized below.

(1) Formation of this disulfide is concomitant with protein isolation.

(2) The absorbance, fluorescence, and CD of the native states of the cross-linked and non-cross-linked control show only small differences.

(3) The disulfide increases electrophoretic mobility under nonreducing denaturing conditions.

(4) Denaturation of the cross-linked variant as well as its non-cross-linked controls is reversible and consistent (for all but one control) with a two-state transition.

(5) For the cross-linked variant and non-cross-linked control, differential scanning calorimetry studies show that denaturation is a two-state process.

(6) The cross-linked variant has a high T_m and the greatest C_m at pH 4.6, despite the fact that ΔG_d for the cross-linked variant is less than that of the non-cross-linked controls.

(7) ΔH_d and m_{GdnHCl} are pH-independent for the cross-linked protein.

(8) CD studies of the denatured states of the two proteins indicate that the cross-linked variant possesses significantly more structure than the non-cross-linked control and is more resistant to denaturation conditions (pH, GdnHCl, or temperature).

ACKNOWLEDGMENT

We thank N. Thompson for use of the fluorimeter, C. Hardin for use of the spectropolarimeter, B. Lentz for use of the microcalorimeter, and J. Wu, K. Pearce, D. Cohen, and A. Saunders for helpful discussions.

SUPPLEMENTARY MATERIAL AVAILABLE

Thermal denaturation data for other proteins at pH 6.0, chemical denaturation data for other proteins, and thermo-

grams at pH 6.0 (10 pages). Ordering information is given on any current masthead page.

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Registry No. Fe, 7439-89-6; heme, 14875-96-8; cytochrome *c*, 9007-43-6.