Differential Scanning Calorimetric Study of the Thermal Unfolding Transitions of Yeast Iso-1 and Iso-2 Cytochromes *c* and Three Composite Isozymes†

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ABSTRACT: The effects of regional sequence differences on the thermodynamic stability of a globular protein have been investigated by scanning microcalorimetry. Thermal transitions have been measured for two isozymes of yeast cytochrome *c* (iso-1-MS and iso-2) and three composite proteins (Comp1-MS, Comp2-MS, and Comp3-MS) in which amino acid segments are exchanged between the parental isozymes. There are three main observations. (1) In the temperature range of the unfolding transitions (40–60 °C) the unfolding free energies for the composite proteins are only slightly different from those of the the parental isozymes, although in some cases there are large but compensating changes in the transitional enthalpy and entropy. At lower temperatures (0–30 °C), all the composites are significantly less stable than the two parental proteins. (2) Long-range structural effects are responsible for at least some of the observed differences in stability. For example, in the temperature range of the unfolding transitions (40–60 °C), the Comp1-MS protein which contains only a small amount of iso-2-like sequence is less stable than either of the parental isozymes, despite the fact that none of the iso-2-specific amino acid side chains impinges directly on any of the iso-1-specific amino acid side chains. (3) Changes in ionization of His 26 appear to be linked to thermal unfolding. Iso-1-MS and Comp1-MS contain a histidine residue at position 26 while iso-2 and the other two composites do not. On lowering the pH from pH 6 to 5, both iso-1-MS and Comp1-MS show a decrease in stability (lower T_m) within the unfolding transition region (40–60 °C), whereas the stabilities of iso-2, Comp2-MS, and Comp3-MS are essentially unchanged. The thermal unfolding transitions are highly reversible (>95%) but mechanistically complex. A moderate dependence of T_m on protein concentration and the ratio of the van't Hoff enthalpy to the calorimetric enthalpy suggest that thermal unfolding involves the reversible association of a significant fraction of the unfolded species, at least at elevated protein concentrations.

Comparisons of the thermodynamic parameters of the unfolding transitions of wild-type proteins with those of genetically altered mutant proteins provide information about the determinants of protein conformational stability. Cytochrome *c* is a good model protein for study of the basic principles of protein folding and stability because it is a small globular protein (13 000 daltons) of known structure with a highly reversible thermal unfolding transition.

There are two naturally occurring isozymes of cytochrome *c* in the yeast *Saccharomyces cerevisiae*. These are iso-1-cytochrome *c*, encoded by the *CYC1* gene (Sherman et al., 1966), and iso-2-cytochrome *c*, encoded by the *CYC7* gene (Downie et al., 1977). There is approximately 84% identity between the iso-1[1] and the iso-2 sequences (Borden & Margoliash, 1976; Montgomery et al., 1980). The iso-1 sequence contains 108 amino acid residues of which only 17 are different from the corresponding residues in the iso-2 sequence. The 17 different amino acid residues are distributed throughout the sequence; they are not localized in specific regions. In addition, the iso-2 sequence has an additional four amino acid residues at the amino terminus. It is known from X-ray crystallographic studies that the secondary and the tertiary structures of iso-1 and iso-2 yeast cytochromes *c* are very similar to each other with an overall average deviation of 0.3 Å for the mainchain atoms (Murphy et al., 1992). Indeed, the three-dimensional structures of both isozymes of yeast cytochrome *c* are quite similar to those of the broad family of eukaryotic cytochromes *c* (Louie et al., 1988; Murphy et al., 1992).

Several years ago, three composite forms of yeast cytochrome *c* were isolated (Ernst et al., 1982). The composite proteins from the alleles *CYC1-136-B*, *CYC1-158-B*, and *CYC1-136-C* are denoted herein as Comp1, Comp2, and Comp3, respectively (Table 1). The sequences of the amino- and the carboxy-terminal regions of the composite proteins are identical to those of the corresponding regions of the iso-1 protein, while the sequences of the central regions of the

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[1] Abbreviations: DSC, differential scanning calorimetry; iso-1, isozyme 1 of cytochrome *c* from the yeast *Saccharomyces cerevisiae*; iso-2, isozyme 2 of cytochrome *c* from the yeast *Saccharomyces cerevisiae*; Comp1, Comp2, and Comp3, cytochromes *c* which are composites of iso-1 and iso-2; -MS, S-methylated at C102, i.e., cytochrome *c* blocked with methyl methanethiosulfonate; Gdn·HCl, guanidine hydrochloride; SDS, sodium dodecyl sulfate.

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Table 1: Yeast Strains and Alleles Producing Cytochrome *c* Variants

protein	allele	strain number	differences	reference
iso-1	CYC1+	D-2023-2C	0	
iso-2	CYC7+	B-4926	21	Downie et al. (1977)
C102A iso-1	CYC1-820	B-7706	1	Hickey et al. (1991)
Comp1	CYC1-136-B	B-1904	4	Ernst et al. (1982)
Comp2	CYC1-158-B	B-2080	8	Ernst et al. (1982)
Comp3	CYC1-136-C	B-2036	10	Ernst et al. (1982)

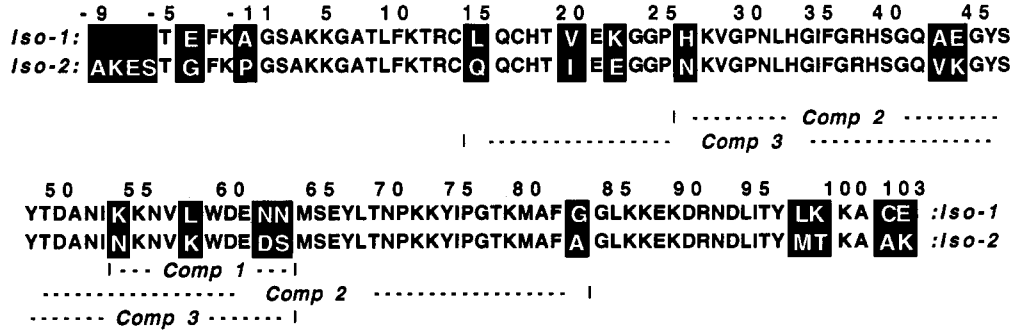

FIGURE 1: Amino acid sequences of yeast iso-1 and iso-2 cytochromes *c* (Montgomery et al., 1978, 1980). Amino acid residues which differ in the two isozymes are shown in open type. The regions of the iso-2 sequence which replace the corresponding regions of the iso-1 sequence in the three composite cytochromes *c* (Ernst et al., 1982) are indicated by the brackets.

composite proteins are identical to those of the corresponding regions of the iso-2 protein. Within the primary structure, the length and the location of the region with the iso-2-like sequence is different in each of the composite proteins (Figure 1).

These five forms of yeast cytochrome *c*, iso-1, iso-2, and the three composite proteins, present an excellent opportunity to examine the effects of regional sequence alterations on the thermodynamic stability of a globular protein. Each of the composite proteins folds into a compact structure with spectral properties essentially the same as those of iso-1 and iso-2. The three-dimensional structure of the Comp3 protein has been elucidated by X-ray crystallography. The overall structure of the Comp3 protein is similar to the structures of the iso-1 and the iso-2 proteins although there are small localized structural differences between all three proteins (Murphy et al., 1992). The three-dimensional structures of the Comp1 and the Comp2 proteins have not yet been elucidated, but it is reasonable to suppose that they also are similar to those of the iso-1 and the iso-2 proteins (Figure 2).

Differential scanning calorimetry (DSC) has emerged as a powerful tool for the investigation of protein thermostability (Privalov, 1979). In principle, differential scanning calorimetry can provide a simultaneous determination of the calorimetric enthalpy (ΔH^{cal}) and the van't Hoff enthalpy (ΔH^{vH}) of the reversible unfolding transition of a globular protein (Mabrey & Sturtevant, 1978; Privalov, 1979; Sturtevant, 1987). The ratio of the van't Hoff enthalpy to the calorimetric enthalpy ($\Delta H^{vH}/\Delta H^{cal}$) can thus be determined. This is important because it provides an accurate thermodynamic test of the mechanistic complexity of folding: a $\Delta H^{vH}/\Delta H^{cal}$ value of unity is regarded as being indicative of an idealized two-state transition, a $\Delta H^{vH}/\Delta H^{cal}$ value of less than unity indicates the presence of intermediates, and a $\Delta H^{vH}/\Delta H^{cal}$ value of greater than unity is thought to indicate the occurrence of reversible intermolecular association (Sturtevant, 1987).

DSC can also provide a measurement of the difference in the heat capacity between the folded protein and the unfolded protein (ΔC_P). ΔC_P is, perhaps, the most important thermodynamic parameter characterizing the thermal unfolding transitions of proteins because it is a measure of the hydrophobic stabilization of the protein. As such ΔC_P is believed to be related to the hydrophobic surface area buried on folding. Moreover, ΔC_P is required to fully describe the temperature dependence of the stabilty function for a protein [$\Delta G(T)$].

In this work we have used differential scanning calorimetry to investigate the thermal unfolding transitions of both iso-1 and iso-2 yeast cytochromes *c* and of the three composite cytochromes *c*. The objective is to determine how the exchange of amino acid segments between closely related proteins affects the thermodynamic parameters of the thermal unfolding transitions. Are proteins thermodynamically the sum of their parts? Is the total enthalpy of unfolding for an intact protein the sum of the unfolding enthalpies of the constituent amino acid segments? What about the entropies, heat capacity changes, and the free energies of unfolding? Are there complementary changes in some thermodynamic properties, for example, enthalpy–entropy compensation, which help in maintaining the constancy of other thermodynamic properties like the unfolding free energy?

Cytochrome *c* contains a heme which can be in either the oxidized state (Fe^{3+}) or the reduced state (Fe^{2+}). The work presented here was done with the heme in the oxidized state. Iso-1-cytochrome *c*, and each of the three composite cytochromes *c*, has a cysteine with a free sulfhydryl group at position 102.[2] In order to prevent covalent dimerization caused by the formation of intermolecular disulfide bonds, C102 of each of these proteins was blocked with methyl methanethiosulfonate (Smith et al., 1975; Ramdas et al., 1986). The blocked proteins are referred to as iso-1-MS, Comp1-MS, Comp2-MS, and Comp3-MS.

[2] The vertebrate cytochrome *c* numbering system is used to denote amino acid positions so as to permit comparison between members of the cytochrome *c* family. Iso-1 has five additional amino terminal residues and iso-2 has nine. Thus, the vertebrate numbering system of iso-1 starts at position –5 and extends to position 103 while that of iso-2 starts at position –9 and extends to position 103 (Dickerson, 1972; Hampsey et al., 1986).

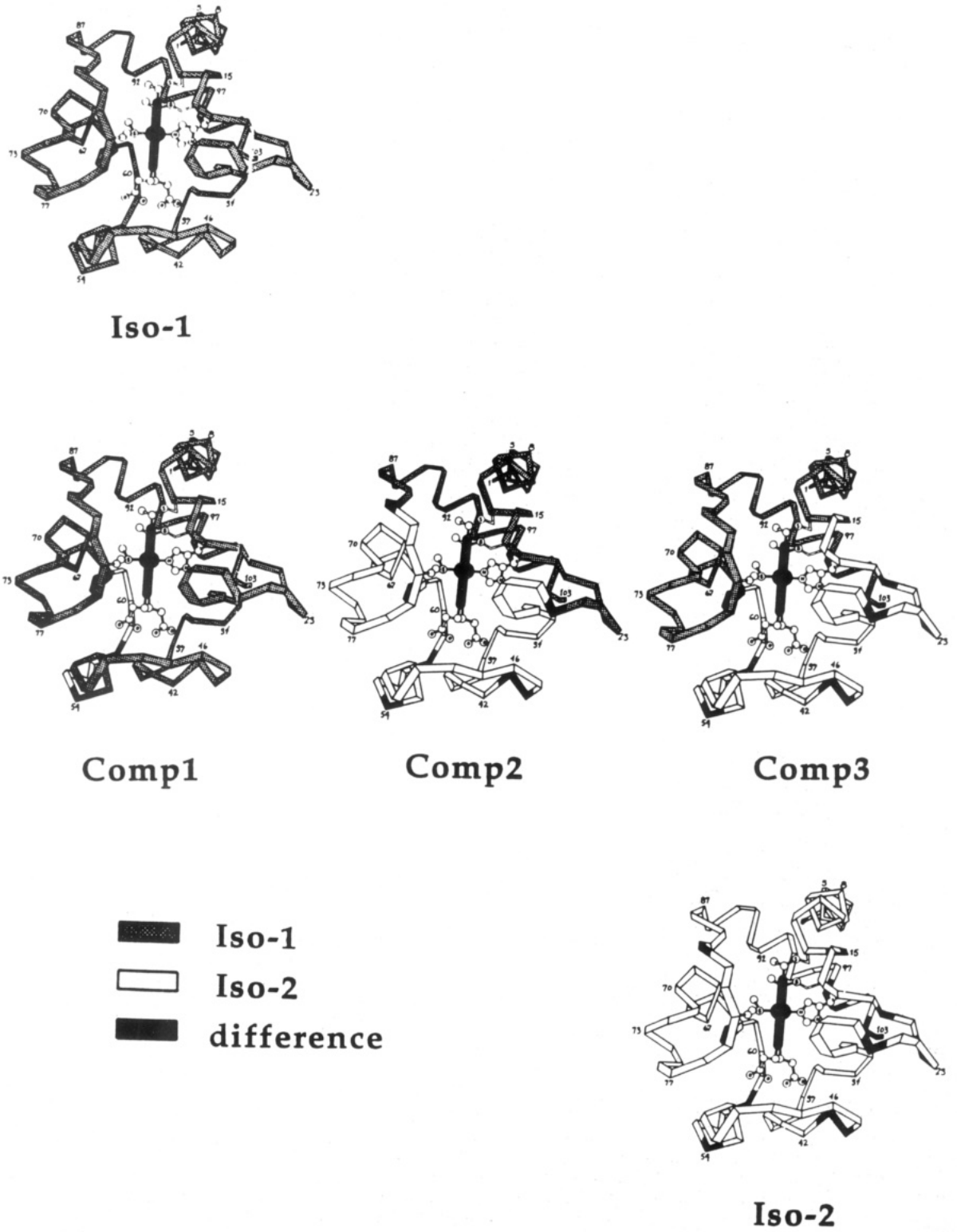

FIGURE 2: Three-dimensional structures of yeast iso-cytochromes *c*. The structures of iso-1, iso-2, and composite Comp3 have been determined by X-ray crystallography (Louie et al., 1988; Murphy et al., 1992). The structures shown for composites Comp1 and Comp2 are models based on the structures of the parental isozymes, iso-1 and iso-2. Peptide segments of the composite proteins which come from iso-1 or iso-2 are indicated by partial shading or no shading, respectively. Specific amino acid residues of iso-1 which are different in iso-2 or the composite proteins are fully shaded. In addition, iso-2 cytochrome *c* has an extra four amino acid residues at the N-terminus which are absent in iso-1 and the composite proteins.

MATERIALS AND METHODS

Growth of yeast (*S. cerevisiae*) and the isolation of cytochrome *c* were performed as described previously (Nall & Landers, 1981; Zuniga & Nall, 1983). The purified stocks of cytochrome *c* were lyophilized and then stored at −70 °C until needed.

Modification of Iso-1 and the Composite Cytochromes c. As previously described (Ramdas et al., 1986) the free sulfhydryl of C102 of iso-1 and the three composite cytochromes *c* was blocked by treatment with methyl methanethiosulfonate (Smith et al., 1975). The absence of dimers in the methyl methanethiosulfonate-treated preparations was

ascertained by running samples on polyacrylamide–SDS gels in the absence of sulfhydryl reagents. Samples were tested for the presence of dimerized material both before and after thermal unfolding.

Preparation of Cytochrome c Solutions. To prepare a cytochrome *c* solution for scanning calorimetry, lyophilized yeast cytochrome *c* was dissolved in buffer in the presence of 3 M guanidine hydrochloride (Gdn·HCl) and heated to 55 °C for 7 min. This treatment causes the cytochrome *c* to unfold, whereupon the heme is oxidized by aqueous oxygen. The Gdn·HCl was then removed by dialysis against four successive changes of buffer at 4 °C. For each of the four buffer changes, the ratio of the volume of buffer to the volume of cytochrome *c* solution was at least 100:1. After dialysis the cytochrome *c* solution was passed through a 0.2-μm filter immediately prior to addition to the sample cell of the calorimeter.

Differential Scanning Microcalorimetry. Scanning microcalorimetry was performed using an MC-2 microcalorimeter from MicroCal Inc., Northampton, MA. The calorimeter was calibrated with the temperature standards supplied by MicroCal Inc. and with solutions of bovine pancreatic ribonuclease A (Schwarz & Kirchhoff, 1988). Immediately before each cytochrome *c* solution was added to the sample cell, buffer was added to both the sample and the reference cells, and a series of scans was made in order to determine the instrumental baseline. The buffer was then removed from the sample cell, and the cytochrome *c* solution was added. Each cytochrome *c* solution was subjected to a series of upward scans from 10 °C to either 70 or 77.5 °C. After the scans of the protein solution were completed, buffer was added to both cells. Several additional baseline scans were collected to verify the baseline stability throughout the data collection process. The scanning rate was 90 °C h^{-1} for most of the experiments. However, scan rates of 45, 20, and 10 °C h^{-1} were occasionally used to check for the possibility of scan rate dependence of the transition parameters.

Protein Concentrations. Cytochrome *c* concentrations were measured spectrophotometrically in a Hewlett-Packard 8452A UV–visible spectrophotometer using a quartz cuvette with a 1-cm optical path. Concentrations were calculated using the molar extinction coefficient which was determined as described below.

Molar extinction coefficients of yeast cytochrome *c* were obtained by calibrating the UV–visible spectra of iso-2-cytochrome *c* solutions using amino acid analysis. Protein samples were hydrolyzed by HCl vapor at 110 °C for 22.5, 49.5, and 70.75 h before being analyzed in a Beckman 7300 amino acid analyzer. The quantity of each amino acid was plotted as a function of the time of hydrolysis, and zero-time extrapolations were made. The number of each type of amino acid in the iso-2-cytochrome *c* molecule is known from the nucleotide sequence of the *CYC7* gene (Montgomery et al., 1980). Therefore, an estimate of the concentration of cytochrome *c* in the calibration solution could be obtained from each amino acid by dividing the amount of a particular amino acid present by the number of residues of that amino acid in the iso-2-cytochrome *c* molecule.

Some amino acids are either degraded or completely destroyed by the hydrolysis procedure. Estimates of the amounts of these amino acids were obtained by extrapolating to zero time of hydrolysis. The cytochrome *c* concentrations obtained from quantitation of the D, T, E, L, K, and P residues were averaged. The average value was judged to be accurate to ±4%. The UV–visible spectrum was calibrated using this value.

The extinction coefficient so obtained was checked by dry weight calibration. The dry weight measurements were performed by Felix Vhjdos in the laboratory of Dr. C. N. Pace at the Department of Biochemistry at Texas A&M University, College Station, TX. The results of the amino acid analysis and the dry weight calibrations agreed within experimental error. The extinction coefficient at 410 nm is 104×10^3 M^{-1} cm^{-1} when the heme is in the oxidized state and 101×10^3 M^{-1} cm^{-1} when the heme is in the reduced state. These extinction coefficients were used to calculate the concentrations of cytochrome *c* in the solutions that were heated in the calorimeter. It was assumed that the spectra of the C102A iso-1, iso-1-MS, iso-2, and the three composite cytochromes *c* are so similar that the iso-2 extinction coefficient could be used to calculate the concentration of each of the six types of yeast cytochrome *c* in solution.

Analysis of Data. Data from the scanning calorimeter were analyzed using the Origin computer program supplied by MicroCal, Inc. The program uses the Marquardt–Levernberg algorithm (Bevington, 1969) for optimization of the thermodynamic parameters by nonlinear least-squares fitting of the data to the thermodynamic expression. Generally, 3–4 repetitive scans were performed on the same sample to test the reproducibility and reversibility of the transition. On the assumption that the calorimetric enthalpy of transition obtained from a particular scan is proportional to the amount of undegraded protein present, analysis of data obtained from successive scans of the same cytochrome *c* solution showed that the reversibility after a single scan was approximately 96%. The second scan from each series was chosen for detailed analysis. First, the instrumental baseline was subtracted. The number of millimoles of cytochrome *c* in the sample cell was calculated from the protein concentration and corrected for the slight loss of reversibility after the first scan. Plots of the molar heat capacity (C_P) of the protein as a function of temperature were obtained by making the necessary corrections for the partial specific volume of the protein (Privalov & Potekhin, 1986). The corrected data were fit to a theoretical curve, either for a two-state transition or for a modified two-state transition. Supplementary Material is available that gives detailed derivations for the fitting functions. Three types of fitting functions were used: (i) Fitting function for a two-state unfolding transition with a temperature-dependent ΔC_P; (ii) fitting function for a two-state unfolding transition with a variable $\Delta H^{vH}/\Delta H^{cal}$ value and a temperature-dependent ΔC_P (Sturtevant, 1987; Kitamura & Sturtevant, 1989; Connelly et al., 1991); (iii) fitting function for a two-state transition with reversible dimerization and a temperature-independent ΔC_P (Sturtevant, 1987; Tanaka et al., 1993).

One practical aspect of using the fitting functions (i) and (ii) should be noted. In order to maximize the reversibility of the scans, the calorimetric cells were cooled as soon as possible after the cytochrome *c* had fully unfolded. For this reason the posttransition baselines of the data sets are generally rather short. This means that the slope of the posttransition baseline is often poorly defined for data sets with the highest reversibility. For scans taken to temperatures with well-defined posttransition baselines the slopes are generally zero, or very close to zero. Therefore, for most data sets the slope of the posttransition baseline was held fixed at zero when fitting an experimental data set (by substituting $-D$ for B in eqs A4, A17, and A21 as described in the Supplementary Material).

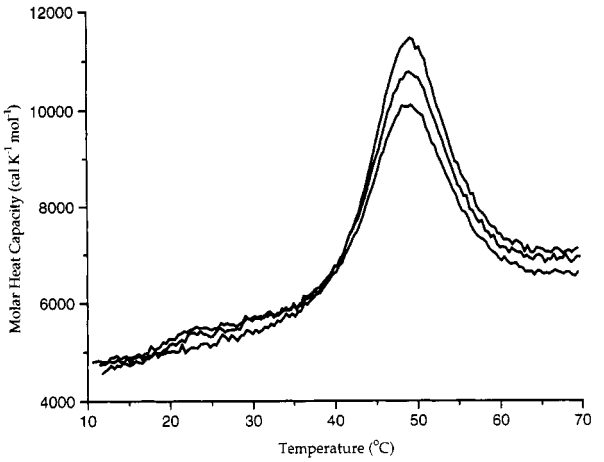

FIGURE 3: Reversibility of the thermal unfolding transition for the Comp3-MS protein. From top to bottom the first, fifth, and ninth scans are shown for the same protein sample. Each repeated scan retains ~0.96 of the transitional enthalpy of the preceding scan. The other proteins used in this study (Table 1) also exhibit about 96% reversibility for repeated scans. Conditions are 0.1 M sodium phosphate, pH 6, and a protein concentration of 144 μM.

RESULTS

Reversibility of Thermal Transitions. For a reliable reversible thermodynamic description of protein stability, it is necessary that the experimentally measured unfolding transition be reversible. Figure 3 shows repeated thermal scans for a single sample of the Comp3-MS protein at pH 6, 0.1 M sodium phosphate. Each repeated scan retains ~0.96 of the transitional enthalpy of the preceding scan, showing that thermal unfolding is highly reversible. The high thermal reversibility is retained at protein concentrations in excess of 1 mM. The other yeast cytochromes *c* described herein exhibit similar highly reversible thermal transitions. Highly reversible thermal unfolding transitions for the yeast cytochromes *c* near neutral pH contrast sharply with the irreversible thermal transitions exhibited by many other cytochromes *c* from higher eukaryotes. For example, high reversibility of the thermal transition for horse cytochrome *c* occurs only at acid pH where the folded conformation of the protein is no longer fully native.

Thermal Unfolding of Mutant and Normal Proteins. Differential scanning calorimetry was performed for the thermal unfolding transitions of iso-2, C102A iso-1, iso-1-MS, and the three composite cytochromes *c* at pH 5.0 and 6.0. A typical data set is shown in Figure 4 together with a theoretical curve for a two-state transition in which $\Delta H^{vH}/\Delta H^{cal}$ has been allowed to vary. The thermodynamic parameters obtained from the unfolding transitions for these proteins are given in Table 2. At pH 6 the values of T_m for the three composite proteins are all lower than those of the normal proteins from which they are derived. At pH 5 two of the composites (Comp2-MS, Comp3-MS) have a T_m in between those of iso-1-MS and iso-2, while one composite, Comp1-MS, has a T_m lower than either parental protein. The values of ΔH and ΔS given in Table 2 are the quantities measured by calorimetry at $T = T_m$ for each individual protein. The subscript "m" indicates that these are the thermodynamic quantities measured at T_m, the midpoint of the thermal unfolding transition (i.e., ΔH_m, ΔS_m). To compare these quantities for proteins that differ in T_m, it is necessary to measure ΔC_P so that ΔH and ΔS can be calculated for a common reference temperature.

Determination of ΔC_P. While ΔC_P can be determined, in principle, from fits of the data from a single calorimetry scan

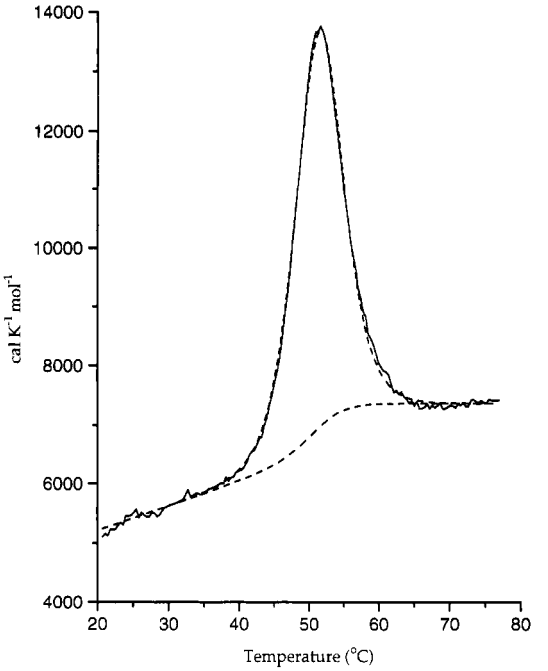

FIGURE 4: Typical DSC scan obtained with a 160 μM solution of iso-1-MS cytochrome *c*, pH 6.0, and 0.1 M sodium phosphate. Molar heat capacity as a function of temperature was obtained by correcting the data as described by Potekhin and Privalov (1986). Also shown is a theoretical curve obtained by a least-squares fit of the data to a two-state transition with an adjustable $\Delta H^{vH}/\Delta H^{cal}$ value and a temperature-dependent ΔC_P. The root mean square deviation of the data from the theoretical curve is 1.2% of the temperature maximum of the transitional heat capacity, $C_{P,tr}$ (eq A20, Supplementary Material). The baseline heat capacity for the theoretical curve, $C_{P,bs}$ (eq A5, Supplementary Material), is shown extrapolated across the transition region. The parameters for the fit to the theoretical curve (eq A21, Supplementary Material) are ΔH_m = 61.5 kcal mol^{-1}; T_m = 50.7 °C; $\Delta H^{vH}/\Delta H^{cal}$ = 1.48; ΔS_m = 190 cal mol^{-1} K^{-1}; ΔC_{Pm} = 862 cal mol^{-1} K^{-1}; pretransition baseline slope (B in eq A3, Supplementary Material) = 41.5 cal K^{-2} mol^{-1}; y-intercept (at 0 K) of the pretransition baseline (A in eq A3, Supplementary Material) = –6.96 kcal K^{-1} mol^{-1}. The slope of the posttransition baseline was fixed at zero as described in the Supplementary Material.

to a theoretical curve (i.e., Figure 4), a more accurate value is obtained by measuring the temperature dependence of the enthalpy over a wide temperature range. This can be acomplished by measuring ΔH_m [$\Delta H_m \equiv \Delta H^{cal}(T_m)$] as a function of pH where the T_m is strongly dependent on pH. A value for ΔC_P is obtained from the slope of a plot of ΔH_m vs T_m. For iso-2 the thermal unfolding transition was investigated between pH 2.5 and 5.0 (Table 3). At pH 2.5, there is no calorimetrically observable unfolding transition. As the pH decreases from 5.0 to 3.0, the melting temperature and the calorimetric enthalpy decrease. Similar behavior is observed for several other globular proteins (Privalov, 1979).

A graph of the calorimetric enthalpy of unfolding as a function of the melting temperature for all of the six proteins is shown in Figure 5. The slope of the plot of the calorimetric enthalpy of unfolding of iso-2 vs temperature gives ΔC_P of 1.24 kcal mol^{-1} K^{-1} with a correlation coefficient of 0.98. The value of ΔC_P shows that ΔH has a strong temperature dependence, but the excellent fit of the plot of ΔH_m vs T to a straight line suggests that ΔC_P is largely temperature independent, at least over the temperature range for which ΔH^{cal} has been measured. This is in contrast to the less accurate determinations of ΔC_P from fitting single calorimetric scans (e.g., Figure 4) which indicate that ΔC_P depends on temperature. The reason for the discrepancy is unknown, but values of ΔC_P obtained in plots like those in Figure 5 are

Table 2: Thermodynamic Parameters of the Unfolding Transitions of Iso-1, Iso-2, and the Three Composite Cytochromes *c*[a]

protein	pH	T_m (°C)[b]	ΔH_m (kcal mol^{-1})[b]	$\Delta H^{vH}/\Delta H^{cal}$ [b]	ΔS_m (cal mol^{-1} K^{-1})[b]
C102A iso-1	6.0	56.2	69.9 ● 3.9	1.48 ± 0.10	212 ± 12
iso-1-MS	6.0	50.7	64.5 ± 3.7	1.43 ± 0.11	199 ± 12
iso-2	6.0	54.5	67.4 ± 2.2	1.36 ± 0.06	206 ± 7
Comp1-MS	6.0	47.6	52.2 ± 4.3	1.55 ± 0.13	163 ± 13
Comp2-MS	6.0	49.8	50.8 ± 2.7	1.30 ± 0.06	157 ± 8
Comp3-MS	6.0	48.4	52.3 ● 1.6	1.37 ± 0.06	163 ± 5
C102A iso-1	5.0	55.5	68.9	1.44	210
iso-1-MS	5.0	47.4	58.3	1.41	182
iso-2	5.0	54.9	73.4	1.27	224
Comp1-MS	5.0	43.9	47.7	1.40	150
Comp2-MS	5.0	49.8	55.5	1.23	172
Comp3-MS	5.0	49.5	59.2	1.25	183

[a] The buffer used was 0.1 M sodium phosphate. Concentrations of cytochrome *c* were approximately 160 μM except for that of Comp2-MS at pH 5.0 which was 102 μM and that of Comp2-MS at pH 6.0 which was 130 μM. The heme was in the oxidized state in every experiment. T_m is defined as the temperature at the midpoint of the unfolding transition (i.e., when $K = 1$) as obtained from fits of the C_P vs T curves to a thermodynamic expression for $C_P(T)$ with a temperature dependent ΔC_P and variable $\Delta H^{vH}/\Delta H^{cal}$ (eq A21, Supplementary Material). ΔH_m is the calorimetric enthalpy of the unfolding transition evaluated at the temperature (T_m) corresponding to the midpoint of the unfolding transition [i.e., $\Delta H_m \equiv \Delta H^{cal}(T_m)$]. The quantity $\Delta H^{vH}/\Delta H^{cal}$ is the ratio of two enthalpies, where ΔH^{vH} is the van't Hoff enthalpy of unfolding (eqs A18 and A21, Supplementary Material). ΔS_m is the entropy of the unfolding transition at T_m, evaluated from ΔH_m and T_m (eq 3). [b] At pH 6 the thermodynamic parameters are averages of three or more determinations and the standard deviations are indicated. The data at pH 5.0 were obtained from single scans so errors are not listed explicitly. Errors in T_m are estimated to be ±0.1 °C.

assumed to be more reliable. The available data suggest that the values of ΔC_P for the other proteins used in this study (Table 1) are similar to that of iso-2.

Protein Concentration Dependence of the Unfolding Transition. DSC scans were performed on both iso-2 and C102A iso-1 at different protein concentrations. The data are presented in Table 4. When the concentration of C102A iso-1-cytochrome *c* is increased by a factor of 30, the T_m decreases by 3.0 °C (Table 4). This suggests reversible intermolecular association in the unfolded state. When the concentration of iso-2 increases 23-fold, the T_m decreases by 1.8 °C. This is again indicative of reversible intermolecular association in the unfolded state. It should be noted, however, that the value of $\Delta H^{vH}/\Delta H^{cal}$ remains approximately constant and greater than unity at all of the protein concentrations tested for iso-2 and for C102A iso-1.

Guanidine Hydrochloride Concentration Dependence of the Unfolding Transition. Data are presented in Table 5 for differential scanning calorimetry of C102A iso-1 at different concentrations of Gdn·HCl. As the concentration of Gdn·HCl is increased from 0 to 1 M, both T_m and ΔH_m steadily decrease. However, the value of $\Delta H^{vH}/\Delta H^{cal}$ remains remarkably constant. When the protein concentration is increased from 152 to 667 μM at 0.75 M Gdn·HCl, the T_m decreases by 2.6 °C (Table 4), suggesting that reversible intermolecular association occurs even in the presence of Gdn·HCl. The value of $\Delta H^{vH}/\Delta H^{cal}$ remains greater than unity and is independent of the concentration of Gdn·HCl.

Scan Rate Dependence of the Unfolding Transition. If the rates of folding and unfolding in the transition region are sufficiently slow, then equilibrium is not attained as the temperature of the sample cell is continually increased during a scan. The result is distortion of the shape of the curve. This could lead to errors in T_m and cause an *apparent* $\Delta H^{vH}/\Delta H^{cal}$ value of greater than unity (Lepock et al., 1992). If so, the values of both T_m and $\Delta H^{vH}/\Delta H^{cal}$ should be markedly affected by changing the scan rate of the calorimeter. Decreasing the scan rate from 90 to 10 °C h^{-1} does not cause a decrease of T_m nor does it result in the value of $\Delta H^{vH}/\Delta H^{cal}$ being closer to unity (data not shown).

Differences in Stability of Parental and Composite Proteins. At both pH 5.0 and 6.0 the differences in the stabilities of the six proteins [$\Delta\Delta G(T_{ref})$] are given with respect to iso-1-MS at a reference temperature equal to the melting temperature of iso-1-MS (Table 6). To obtain $\Delta\Delta G(T_{ref})$, we start with the expression for $\Delta G(T)$ of a protein in terms of the transitional enthalpy and entropy measured at T_{ref} (Becktel & Schellman, 1987):

$$\Delta G(T) = \Delta H(T_{ref}) - T\Delta S(T_{ref}) + \Delta C_{Pm}\left[T - T_{ref} - T\ln\left(\frac{T}{T_{ref}}\right)\right] \quad (1)$$

Using eq 1, an expression for $\Delta\Delta G(T_{ref}) = \Delta G_{mut}(T_{ref}) - \Delta G_{wt}(T_{ref})$ for unfolding of a mutant protein with respect to a normal (wild-type) protein can be derived. For $T_{ref} = T_{m;wt}$ (where $T_{m;wt}$ is the transition midpoint for the normal protein), $\Delta G_{wt}(T_{ref}) = \Delta G_{wt}(T_{m;wt}) = 0$, and the expression is

$$\Delta\Delta G(T_{ref}) = \Delta H_m^{mut} - T_{ref}\Delta S_m^{mut} + \Delta C_P\left[T_{ref} - T_{m;mut} + T_{ref}\ln\left(\frac{T_{ref}}{T_{m;mut}}\right)\right] \quad (2)$$

ΔH_m^{mut}, ΔS_m^{mut}, and $T_{m;mut}$ are the enthalpy, entropy, and thermal transition midpoint for the mutant (superscript or subscript "mut") protein. In these expressions, ΔS_m^{mut}, the calorimetric entropy of unfolding of the mutant protein at the midpoint of the thermal transition for the mutant protein, $T_{m;mut}$, is given by

$$\Delta S_m^{mut} = \frac{\Delta H_m^{mut}}{T_{m;mut}} \quad (3)$$

ΔC_P in eq 2 is the difference in the heat capacities of the fully folded and fully unfolded forms of the protein. A value for $\Delta C_P = 1.24$ kcal mol^{-1} K^{-1} is obtained from the slope of the graph of the temperature dependence of the calorimetric enthalpy of unfolding of iso-2 (Figure 5). It is assumed that ΔC_P is temperature independent and that it has the same value for the mutant and normal proteins. Any errors resulting from these assumptions are expected to be small because the term which contains ΔC_P in eq 2 is small compared to other terms in the equation.

Protonation and the pH Dependence of Unfolding. Protein unfolding often leads to changes in the protonation of a protein. Generally, protonation changes depend on pH. The proton uptake on unfolding is related to the transitional enthalpy and the pH dependence of the transition temperature [see, for example, Tanaka et al. (1993)]. Table 7 gives the proton uptake on unfolding, $\Delta\nu$, at various pH values for the composite proteins and for the parental isozymes from which they are derived. The data show that protein unfolding involves the uptake of one or more protons at acid pH, but that proton uptake decreases on approaching neutral pH. At pH 6, note that proton uptake is positive for iso-1-MS and Comp1-MS, which contain histidine at position 26. The change in protonation on unfolding at pH 6 is slightly negative or zero for proteins containing asparagine rather than histidine at position 26: iso-2, Comp2-MS, and Comp3-MS. C102A iso-1

Table 3: Thermodynamic Parameters of the Unfolding Transitions of Iso-2 Cytochrome *c* as a Function of pH[a]

pH	buffer	T_m (°C)	ΔH_m (kcal mol^{-1})	$\Delta H^{vH}/\Delta H^{cal}$	ΔS_m (cal mol^{-1} K^{-1})
6.0	0.1 M sodium phosphate	54.5	67.4	1.36	206
5.0	0.1 M sodium phosphate	54.9	73.4	1.27	224
5.0	0.1 M sodium acetate	51.2	68.9	1.34	212
4.5	0.1 M sodium acetate	48.6	65.0	1.33	202
4.0	0.1 M sodium acetate	43.7	62.7	1.21	198
3.5	0.1 M sodium acetate	37.2	50.8	1.31	164
3.5	0.05 M glycine/HCl	40.7	56.5	1.21	180
3.0	0.05 M glycine/HCl	(25.7)	(34.0)	(1.39)	(114)

[a] The quantities T_m, ΔH_m, $\Delta H^{vH}/\Delta H^{cal}$, and ΔS_m are defined in the footnote to Table 2. The concentration of cytochrome *c* in all experiments was 160 μM. The data obtained at pH 3.0 are in brackets because of the uncertainty due to both the broad transition and the lack of a measurable pretransition baseline. In analyzing the data at pH 3.0 the slope of the pretransition baseline was assumed to be approximately the same as the slopes of the pretransition baselines obtained at higher pH values.

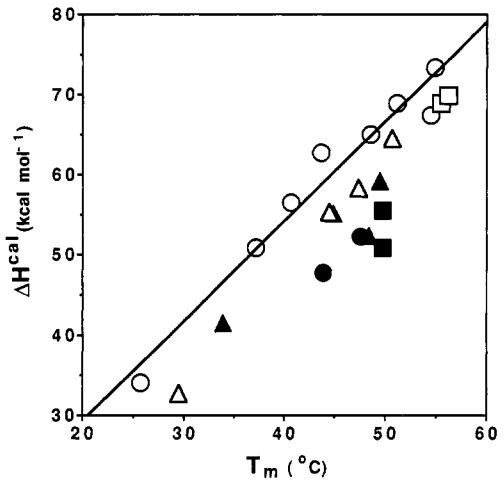

FIGURE 5: Calorimetric enthalpies (ΔH) of iso-1, iso-2, and the three composite cytochromes *c* as a function of the melting temperature (T_m). (□) C102A iso-1; (△) iso-1-MS; (○) iso-2; (●) Comp1-MS; (■) Comp2-MS; (▲) Comp3-MS. The solid line represents a fit of the data for iso-2 to the function $\Delta H(T) = \Delta C_P T + b$, where the slope, ΔC_P, is the transitional change in heat capacity, and *b* is the calorimetric enthalpy at 0 °C. Protein concentrations are ~160 μM.

has a positive change in protonation on unfolding, as do the other proteins with His 26, but the magnitude of the protonation change for unfolding of C102A iso-1 is of marginal significance.

DISCUSSION

Features of the Unfolding Transitions. Four aspects of the data are apparent: (i) At pH 6.0 and a common temperature, $T_{ref} = T_m$ for iso-1-MS, the transitional enthalpies of unfolding of the three composite proteins are significantly less than those of the two isozymes (Table 6). (ii) At pH 6 and the reference temperature, T_{ref}, all composite proteins are less stable [smaller $\Delta\Delta G(T_{ref})$] than either of the parental isozymes (Table 6; Figure 8). (iii) At pH 5 and $T = T_{ref}$ the composite proteins fall into two groups: (1) those which are less stable than either parental protein (Comp1-MS) and (2) those that are intermediate in stability between the two parental proteins (Comp2-MS, Comp3-MS) (Table 6). (iv) The ratio of the van't Hoff enthalpy to the calorimetric enthalpy ($\Delta H^{vH}/\Delta H^{cal}$) is significantly greater than unity for all six proteins (Table 2).

Measurement of ΔC_P. The difference between the heat capacities of the folded and the unfolded states of the protein (ΔC_P) can be measured in two ways: (i) by fitting the calorimetric data to thermodynamic expressions for the heat capacity in which ΔC_{Pm} [$\equiv \Delta C_P(T_m)$] appears as an adjustable parameter (see Supplementary Material, eqs A17 and A21) or (ii) by pH variation of T_m and measuring the slope of the graph of the temperature dependence of the enthalpy of unfolding (Figure 5). For reasons which are not fully understood, the values of ΔC_P obtained from the two different ways are markedly different for many protein unfolding transitions (Connelly et al., 1991; Hu et al., 1992a). Moreover, ΔC_P values obtained from curve fitting are often temperature dependent while plots of ΔH_m vs T_m usually indicate that ΔC_P is independent of temperature. Compared to the value obtained by pH variation, the value of ΔC_P from curve fitting is somewhat smaller and tends to fluctuate widely from scan to scan. The slope of the high temperature baseline may be influenced by reactions not related to unfolding which occur preferentially at higher temperatures. Also, small errors in fitting the high-temperature and the low-temperature baselines can result in large errors in ΔC_{Pm}. Thus, it is generally regarded as more reliable to measure ΔC_P from the temperature dependence of the enthalpy of unfolding (Becktel & Schellman, 1987; Privalov, 1979; Hu et al., 1992b).

Privalov and co-workers have shown that the ΔC_P for unfolding depends on temperature (Makhatadze et al., 1993; Privalov & Makhatadze, 1990, 1992) for many proteins including horse cytochrome *c*. Therefore, our assumption that ΔC_P is independent of temperature is likely to be an approximation. Comparisons of the thermodynamic parameters at a common reference temperature near the transition temperatures (e.g., Table 6) should be accurate, since contributions of terms involving ΔC_P are small. Extrapolations to temperatures far removed from the transition region (e.g., the lower temperature region of Figure 8) are, however, more suspect.

Comparison of Calorimetric and van't Hoff Enthalpies. The value of $\Delta H^{vH}/\Delta H^{cal}$ is consistently greater than unity but should be equal to one for a two-state transition. Perhaps the most straightforward interpretation is that reversible intermolecular association occurs. This can be tested by measuring the protein concentration dependence of the T_m for the transition, since the T_m will depend on protein concentration unless an association reaction among folded conformers exactly balances an association reaction among unfolded species. The results presented in Table 4 show that there is indeed a dependence of T_m on protein concentration such that T_m decreases slightly with increasing protein concentration. The sign of the T_m change with increasing protein concentration suggests preferential association among unfolded species. Notice that the other thermodynamic parameters, ΔH_m, ΔS_m, and $\Delta H^{vH}/\Delta H^{cal}$, show no significant dependence on protein concentration. Thus, with the possible exception of the T_m values, there is no need to correct the thermodynamic parameters for protein concentration, at least

Table 4: Thermodynamic Parameters of the Unfolding Transition of Cytochrome *c* as a Function of Protein Concentration[a]

protein	concentration (μM)	T_m (°C)	ΔH_m (kcal mol^{-1})	$\Delta H^{vH}/\Delta H^{cal}$	ΔS_m (cal mol^{-1} K^{-1})
iso-2	37	54.7	65.7	1.43	200
iso-2	149	54.5	67.4	1.36	206
iso-2	835	52.9	66.6	1.50	204
C102A iso-1	36	56.7	67.0	1.49	203
C102A iso-1	146	56.2	69.9	1.48	212
C102A iso-1	586	54.9	71.1	1.50	217
C102A iso-1	1096	53.7	69.3	1.54	212
C102A iso-1 0.75 M Gdn·HCl	152	40.4	44.1	1.49	141
C102A iso-1 0.75 M Gdn·HCl	667	37.8	41.5	1.49	134

[a] The quantities T_m, ΔH_m, $\Delta H^{vH}/\Delta H^{cal}$, and ΔS_m are defined in the footnote to Table 2. In all experiments the buffer used was 0.1 M sodium phosphate and the pH was 6.0.

Table 5: Thermodynamic Parameters of the Unfolding Transitions of C102A Iso-1 Cytochrome *c* as a Function of Guanidine Hydrochloride Concentration[a]

Gdn·HCl (M)	T_m (°C)	ΔH_m (kcal mol^{-1})	$\Delta H^{vH}/\Delta H^{cal}$	ΔS_m (cal mol^{-1} K^{-1})
0	56.2	69.9	1.48	212
0.25	51.6	66.6	1.36	205
0.5	46.3	57.4	1.36	180
0.75	40.4	44.1	1.49	141
1	(31.4)	(34.6)	(1.15)	(114)

[a] The quantities T_m, ΔH_m, $\Delta H^{vH}/\Delta H^{cal}$, and ΔS_m are defined in the footnote to Table 2. ΔH^{vH} at T_m is not given explicitly but may be calculated from $\Delta H^{vH}(T_m) = (\Delta H^{vH}/\Delta H^{cal})\Delta H_m$. The concentration of cytochrome *c* in all experiments was 160 μM, and the buffer was 0.1 M sodium phosphate, pH 6.0. The data obtained at 1 M Gdn·HCl are in brackets because of the uncertainty due to the broad transition and the lack of a measurable pretransition baseline. In analyzing the data for 1.0 M Gdn·HCl the slope of the pretransition baseline was assumed to be approximately the same as the slopes of the pretransition baselines obtained at lower concentrations of Gdn·HCl. The difficulty in obtaining a good pretransition baseline at 1.0 M Gdn·HCl occurs because of the Gdn·HCl-induced unfolding transition. Assuming a linear dependence of the free energy on Gdn·HCl concentration, the Gdn·HCl-induced transition midpoint is calculated to be 1.25 M Gdn·HCl at 20 °C.

Table 6: Comparison of the Thermodynamic Parameters for Unfolding at the Reference Temperature[a]

protein	pH	$\Delta H(T_{ref})$ (kcal/mol)	$\Delta S(T_{ref})$ (cal mol^{-1} K^{-1})	$\Delta\Delta G(T_{ref})$ (kcal/mol)
C102A iso-1	6.0	63.0 ± 3.9	191 ± 12	1.11 ± 0.07
iso-1-MS	6.0	64.5 ± 3.7	199 ± 12	(0)
iso-2	6.0	62.7 ± 2.2	191 ± 7	0.75 ± 0.03
Comp1-MS	6.0	56.1 ± 4.3	175 ± 13	–0.52 ± 0.04
Comp2-MS	6.0	51.9 ± 2.7	160 ± 8	–0.14 ± 0.01
Comp3-MS	6.0	55.2 ± 1.6	172 ± 5	–0.38 ± 0.01
C102A iso-1	5.0	58.8	179	1.62
iso-1-MS	5.0	58.3	182	(0)
iso-2	5.0	64.0	195	1.61
Comp1-MS	5.0	52.1	164	–0.54
Comp2-MS	5.0	52.5	163	0.41
Comp3-MS	5.0	56.6	175	0.38

[a] The thermodynamic parameters from Table 2 have been calculated for $T_{ref} = T_m$ of iso-1-MS as described by Becktel and Schellman (1987) using $\Delta C_p = 1.24$ kcal mol^{-1} K^{-1}. $\Delta\Delta G(T_{ref})$ has been obtained from eq 2. At pH 6.0, $T_{ref} = 50.7$ °C, while at pH 5.0, $T_{ref} = 47.4$ °C (Table 2). At pH 6.0 the errors have been estimated from the standard deviations of multiple measurements. The results at pH 5.0 are for single determinations, so errors have not been listed explicitly.

Table 7: Proton Uptake on Unfolding per Molecule of Cytochrome *c*[a]

protein	pH	$\Delta\nu$
iso-2	5.0	+0.2
	4.5	+1.1
	4.0	+1.5
	3.5	+2.1
	3.0	+4.2
iso-1-MS	5.0	+0.6
	3.5	+1.4
Comp3-MS	5.0	+0.3
	3.5	+1.2
C102a iso-1	6.0	+0.1
iso-1-MS	6.0	+0.5
iso-2	6.0	–0.1
Comp1-MS	6.0	+0.4
Comp2-MS	6.0	0
Comp3-MS	6.0	–0.1

[a] The change in the number of protons bound to a cytochrome *c* molecule upon unfolding was calculated using

$$\Delta\nu = [\Delta H_m/(2.303RT_m^2)](dT_m/dpH)$$

The values of the enthalpy of unfolding at the melting temperature (ΔH_m) and the melting temperature (T_m) were obtained from Tables 2 and 3 and, for Iso-1-MS and Comp 3-MS at pH 3.5, from unincluded data. The values of (dT_m/dpH) were obtained from the slopes of plots of T_m vs pH.

when comparing unfolding of proteins measured at similar protein concentrations. In many instances it is possible to decrease the degree of intermolecular association by adding small amounts of protein denaturants. The data presented in Table 5 show that the value of $\Delta H^{vH}/\Delta H^{cal}$ does not depend on the presence of low concentrations of denaturant. This is somewhat surprising. One might have expected a weak association among unfolded species to be sensitive to protein denaturants. Moreover, the T_m for C102A iso-1 still depends on protein concentration, even in the presence of 0.75 M Gdn·HCl (Table 4).

Dimerization might occur by an intermolecular heme ligation reaction. Histidine is known to be a heme ligand for unfolded cytochrome *c* (Babul & Stellwagen, 1971; Muthukrishnan & Nall, 1991), at least when the histidine side chain is in the neutral, uncharged form present above pH ~5. Furthermore, dimerization via histidine side chains would not be influenced by low concentrations of Gdn·HCl, since even high concentrations of Gdn·HCl do not disrupt heme ligation by histidines. Additional evidence favoring a role for histidine side chains in dimerization is found by comparing the values of $\Delta H^{vH}/\Delta H^{cal}$ for different variants of cytochrome *c* listed in Table 2. The cytochrome *c* variants with His 26 (four histidines total) have somewhat larger ratios of $\Delta H^{vH}/\Delta H^{cal}$ than variants with Asn 26 (three histidines total) (Figure 1 and Table 2). However, there is little if any pH dependence of the $\Delta H^{vH}/\Delta H^{cal}$ ratio (Table 3), a result which is at odds with the idea of dimerization via intermolecular ligation by histidine side chains. In contrast to the results presented in Table 3, protonation of the histidine side chains at low pH should lower the $\Delta H^{vH}/\Delta H^{cal}$ ratio by blocking both heme ligation and dimerization.

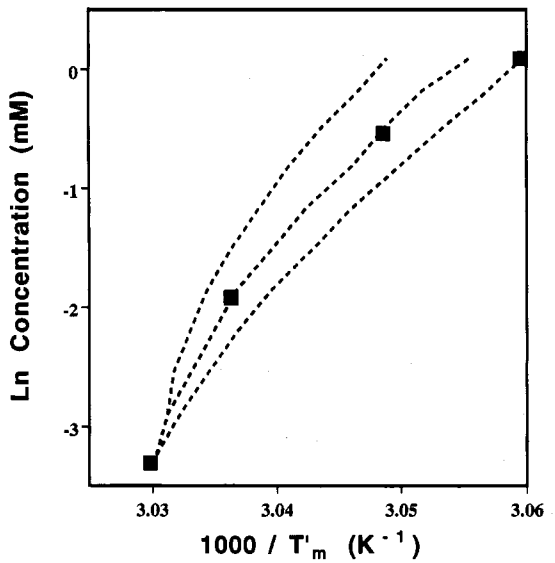

FIGURE 6: Protein concentration dependence of the transition temperature for C102A iso-1-cytochrome *c*. The theoretical protein concentration dependence of the T'_m, the temperature of maximum excursion of the transitional heat capacity, is shown by plotting ln C vs $1/T'_m$ for a two-state transition with reversible dimerization of the unfolded protein (eqs A22–A36, Supplementary Material). Experimental points for C102A iso-1 taken from the data in Table 4 are included for comparison (■). Estimated errors in the experimental points are of the order of the size of the symbols or smaller. The fixed parameters for the theoretical curves are ΔH_m = 78.9 kcal mol^{-1} and ΔC_P = 1.24 kcal mol^{-1} K^{-1}. Theoretical curves for K_u = 40 M$^{-1/2}$ (upper dashed line), K_u = 60 M$^{-1/2}$ (middle dashed line), and K_u = 80 M$^{-1/2}$ (lower dashed line) are shown. For each value of K_u, a value of T_m was chosen so that the value of T'_m at a protein concentration of 39 μM would be equal to the experimental value.

Reversible intermolecular association among unfolded species may provide a *quantitative* explanation for both properties: (1) protein concentration dependence of T_m, and (2) $\Delta H^{vH}/\Delta H^{cal} > 1$. This was tested with a thermodynamic model for a two-state transition that includes reversible dimerization of the unfolded protein (see Supplementary Material, eqs A22–A36). Using this model it is possible to calculate the dependence of ln C (where C = protein concentration) on $1/T_m$ for various trial values of K_u (the equilibrium constant for the monomer–dimer transition for the unfolded protein). A comparison of the predicted dependence of ln C on $1/T_m$ to the data for C102A iso-1 provides a sensitive means of estimating K_u (Figure 6). For the best overall value of K_u = 60 M$^{-1/2}$, the temperature at which the unfolding equilibrium constant is unity (which, according to the model, is the true T_m), is predicted to be 56.9 °C. This theoretical value of T_m = 56.9 °C would equal the apparent T_m in the absence of dimerization and, within errors, is equal to the experimental value of T_m measured at the lowest protein concentrations, C = 36 μM (Table 4). The dimerization model provides a good description of the protein concentration dependence of T_m for protein concentrations of up to ~600 μM, but there is significant deviation between the theoretical curve and the experimental result at C = 1096 μM. This may be because of higher order association of the unfolded protein, which is not adequately accounted for by the simple dimerization model.

Figure 7 compares the theoretical heat capacity curves calculated using the model for a two-state transition with reversible dimerization to the experimental thermal transitions at concentrations of C102A iso-1 of 36 and 586 μM. For these simulations the ratio of the van't Hoff enthalpy to the calorimetric enthalpy is held equal to 1, so the agreement between the experimental and theoretical curves is not as good as when this ratio is allowed to vary (as in Figure 4). The simulations in Figure 7 use a value of K_u = 60 M$^{-1/2}$. For K_u = 60 M$^{-1/2}$, the fraction of protein molecules in the dimeric state when the protein is fully unfolded ranges from 18% for a protein concentration of 36 μM to 62% for a protein concentration of 586 μM. As shown in Figure 7, the reversible dimerization model provides a plausible explanation for the observed protein concentration dependence of the transitional heat capacity curves and the fact that $\Delta H^{vH}/\Delta H^{cal} > 1$. $\Delta H^{vH}/\Delta H^{cal}$ values of greater than unity have been detected with some other globular proteins (Connelly et al., 1991; Kitamura & Sturtevant, 1989; Plaza del Pino et al., 1992; Xie et al., 1991).

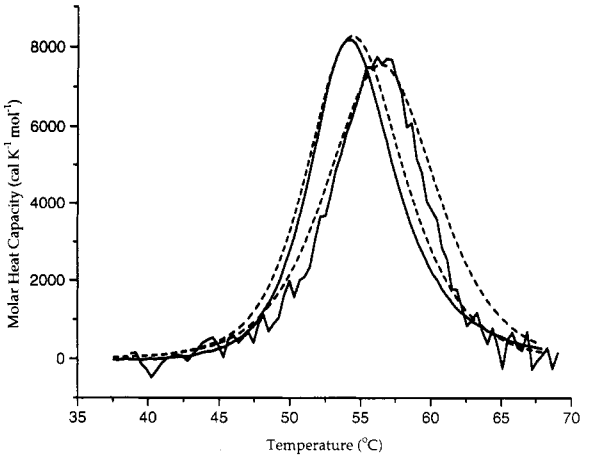

FIGURE 7: Differential scanning calorimetry of C102A iso-1 over a 16-fold concentration range of C102A iso-1 at pH 6.0, 0.1 M sodium phosphate. The solid lines give the experimental heat capacity traces for protein concentrations of 586 μM (trace with T'_m = 54.9 °C, T'_m being defined in the legend to Figure 6) and 36 μM (trace with T'_m = 56.7 °C). For comparison, theoretical curves are shown (dashed lines) for a two-state transition with reversible dimerization in the unfolded state (eq A36, Supplementary Material). For the transition with C = 586 μM, the root mean square deviation of the data from the theoretical curve is 4.6% of the temperature maximum of the transitional heat capacity. For the transition with C = 36 μM the deviation is 8%. The parameters for the theoretical curves are ΔH_m = 78.9 kcal mol^{-1}, T_m = 56.9 °C, K_u = 60 M$^{-1/2}$, and ΔC_P = 1.24 kcal mol^{-1} K^{-1}. Before the fits were made, the baseline heat capacities for the fully folded and fully unfolded protein were subtracted from the data.

Stability Differences: Is the Whole the Sum of the Parts? Inspection of the three-dimensional structures provides possible explanations for the differences in the stabilities. In the structure of iso-1 cytochrome *c* which contains a free S-H at C102 rather than the C102-MS blocked side chain, there is a small cavity buried behind the heme group (Louie et al., 1988). C102 is one of the residues whose side chains line this cavity. Iso-1 has a leucine at position 98, whereas iso-2 has a methionine at this position which causes the cavity to be eliminated in the iso-2 structure (Murphy et al., 1992). One can surmise that the attachment of the S-methyl blocking group to C102 of iso-1 cytochrome *c* causes significant local structural rearrangement. This seems the most reasonable explanation for the fact that iso-1-MS is significantly less stable than C102A iso-1 and iso-2 cytochromes *c*.

The three composite cytochromes *c* are all less stable than both the C102A iso-1 and the iso-2 cytochromes *c*. This is not surprising in view of the fact that the three composites all contain S-methylated C102 side chains. At pH 6.0, both Comp2-MS and Comp3-MS cytochromes *c* are slightly less stable than iso-1-MS (Table 6) while at pH 5.0 they are slightly

more stable. However, Compl-MS is significantly less stable than iso-1-MS at both pH 6.0 and 5.0 (Table 6). This is especially interesting in view of the fact that Compl-MS cytochrome *c* has only a small fraction of the iso-2 sequence (Figures 1 and 2). Even more interesting is the fact that in the model of the three-dimensional structure of Compl cytochrome *c* based on the X-ray structures of iso-1 and iso-2 (Louie et al., 1988; Murphy et al., 1992) none of the iso-2 specific amino acid side chains impinges directly on any of the iso-1 specific amino acid side chains (Figure 2). This strongly suggests that subtle long-range structural rearrangements are responsible for the destabilization of Compl-MS.

Both C102A iso-1 and iso-2 cytochromes *c* have an alanine residue at position 102 while iso-1-MS cytochrome *c* has a blocked cysteine residue at that position. It is interesting that there is no change in T_m for iso-2 cytochrome *c* between pH 6 and 5. Over the same pH range the cytochromes *c* with blocked C102 (the -MS forms) fall into two classes: (1) those with little or no change in T_m (Comp2-MS, Comp3-MS); (2) those with a significant decrease in T_m (iso-1-MS; Compl-MS). So why are some of these proteins more sensitive to pH changes between pH 6 and 5 than others? The sequences of iso-1-MS and Compl-MS cytochromes *c* each contain a histidine residue at position 26, while iso-2, Comp2-MS and Comp3-MS, cytochromes *c* contain an asparagine residue at this position. The imidazole side chain of free histidine has a pK_a of 6.5. The pK_a of the side chain of H26 can be expected to be approximately 6.5 in unfolded cytochrome *c*, but it may be significantly different from this value in folded cytochrome *c* (Muthukrishnan & Nall, 1991). Therefore it seems that the most likely explanation for the fact that iso-1-MS and Compl-MS cytochromes *c* are destabilized when the pH is decreased from 6.0 to 5.0 is that a change in pK_a of the H26 side chain on folding thermodynamically links H26 protonation to protein folding. Proton uptake on unfolding (Table 7) provides another way of viewing the same phenomena. Proton uptake is positive and significantly greater than zero for iso-1-MS and Compl-MS, both of which contain His 26. Proton uptake is negative, or zero, for iso-2, Comp2-MS, and Comp3-MS, all of which have asparagine at position 26.

If the above supposition is correct, one would expect C102A iso-1 cytochrome *c* to be destabilized when the pH is decreased from 6.0 to 5.0, since this protein also contains a histidine residue at position 26. This is indeed the case (Table 2) but the destabilization (0.7 °C) is considerably less than that which occurs with iso-1-MS and Compl-MS cytochromes *c*. In addition, proton uptake on unfolding for C102A iso-1, while positive, is smaller than for iso-1-MS and Compl-MS (Table 7). The three-dimensional structure of iso-1 cytochrome *c* (Louie et al., 1988) reveals that the side chain of H26 is physically adjacent to the side chain of the amino acid residue at position 102. One can surmise that the folding-induced change in the pK_a of the side chain of H26 for C102A iso-1 is slightly less than for iso forms in which C102 is blocked by the S-methyl blocking group. Presumably, this difference is brought about by the proposed structural change induced by the S-methyl blocking group (see above).

Enthalpy–Entropy Compensation. Figure 5 shows that, regardless of the temperature, the enthalpies of unfolding of the wild-type isozymes, iso-1-MS and iso-2 cytochromes *c*, are significantly greater than the enthalpies of unfolding of the three composite cytochromes *c*. Therefore, significant enthalpy–entropy compensation occurs with the unfolding transitions of the three composite cytochromes *c*. As a result of the lower enthalpies of unfolding for the composites, the free energy differences between composites and parental proteins are greater at lower temperatures (T less than ~50 °C) as shown in Figure 8. Similar enthalpy–entropy compensation has been noted for the unfolding transitions of other mutant proteins when compared to those of the respective wild types (Connelly et al., 1991; Hecht et al., 1984; Shortle et al., 1988; Sturtevant et al., 1989).

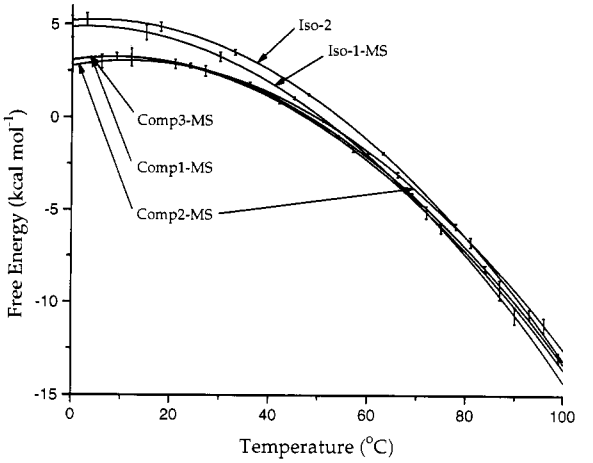

FIGURE 8: Free energy of unfolding, $\Delta G(T)$, at pH 6.0 for the two isozymes and the three composite proteins as a function of temperature as calculated from the data in Table 2 using eq 2. Error bars shown for $\Delta G(T)$ are estimated from the errors in the parameters given in Table 2.

CONCLUSIONS

Construction of composite cytochromes *c* from the two parental isozymes results in little change in thermostability. The transitional enthalpies of unfolding of the composites are all somewhat less than those of the two wild-type isozymes, but the unfolding free energies are similar because there is significant enthalpy–entropy compensation with the unfolding transitions of the three composite proteins. Particularly interesting is the role of H26 in these proteins. The subtle structural interactions between the side chain of the amino acid residue at position 102 and the side chain of H26 result in the modulation of the magnitude of the linkage between the ionization of the side chain of H26 and the stability of the folded protein conformation.

The values of $\Delta H^{vH}/\Delta H^{cal}$ of the unfolding transitions of these proteins are significantly greater than unity suggesting reversible intermolecular association in the unfolded state. This hypothesis is supported by the fact that the T_m decreases when the protein concentration is increased above ~160 μM.

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SUPPLEMENTARY MATERIAL AVAILABLE

A detailed derivation of the fitting functions and a description of how they are used to obtain the thermodynamic parameters are available. Derivations for three models are included: (i) two-state unfolding with a temperature-dependent ΔC_P, (ii) two-state unfolding with a temperature-dependent ΔC_P and variable $\Delta H^{vH}/\Delta H^{cal}$, and (iii) two-state unfolding with a temperature-independent ΔC_P, but including reversible dimerization of the protein (14 pages). Ordering information is given on any current masthead page.

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