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Stability of yeast iso-1-ferricytochrome *c* as a function of pH and temperature

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Abstract

Absorbance-detected thermal denaturation studies of the C102T variant of *Saccharomyces cerevisiae* iso-1-ferricytochrome *c* were performed between pH 3 and 5. Thermal denaturation in this pH range is reversible, shows no concentration dependence, and is consistent with a 2-state model. Values for free energy (ΔG_D), enthalpy (ΔH_D), and entropy (ΔS_D) of denaturation were determined as functions of pH and temperature. The value of ΔG_D at 300 K, pH 4.6, is 5.1 ± 0.3 kcal mol^{-1}. The change in molar heat capacity upon denaturation (ΔC_p), determined by the temperature dependence of ΔH_D as a function of pH (1.37 ± 0.06 kcal mol^{-1} K^{-1}), agrees with the value determined by differential scanning calorimetry. pH-dependent changes in the Soret region indicate that a group or groups in the heme environment of the denatured protein, probably 1 or both heme propionates, ionize with a pK near 4. The C102T variant exhibits both enthalpy and entropy convergence with a ΔH_D of 1.30 kcal mol^{-1} residue^{-1} at 373.6 K and a ΔS_D of 4.24 cal mol^{-1} K^{-1} residue^{-1} at 385.2 K. These values agree with those for other single-domain, globular proteins.

Keywords: cytochrome *c*; equilibrium thermodynamics; protein denaturation; protein stability

A goal of protein chemistry is to understand how interactions between amino acid residues control stability, structure, and function. Anfinsen and coworkers showed that the amino acid sequence of a protein determines its higher-order structure and hence its function (Anfinsen, 1973). Given this knowledge, it should be possible to predict the 3-dimensional structure of a protein from its amino acid sequence and design proteins with novel structures and functions. Although progress has been made (Betz et al., 1993; Bowie & Eisenberg, 1993), these goals have yet to be realized fully.

With the advent of site-directed mutagenesis (Hutchinson et al., 1978), the protein chemist can alter specific interactions and thereby study their role in stability, structure, and function. The fundamental principles underlying the determination of protein stability, however, should be understood before variant proteins are examined. Specifically, a series of thermal denaturations was performed between pH 3 and 5 on the oxidized (Fe^{3+}) form of the C102T variant of yeast iso-1-cytochrome *c* as a prelude to experiments involving variants with substitutions at the interface between the N- and C-terminal helices (Auld & Pielak, 1991; Fredericks & Pielak, 1993).

Protein stability

Protein stability, ΔG_D, is defined as the Gibbs free energy of the denatured state minus that of the native state. Globular proteins possess thousands of interactions that stabilize N relative to D under physiological conditions. These interactions include hydrogen bonds, weakly polar interactions, van der Waals

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Abbreviations: $A_{\lambda,T}$, absorbance at wavelength λ (nm) and temperature T (K); b_D, absorbance of the denatured-state baseline linearly extrapolated to 0 K; b_N, absorbance of the native-state baseline linearly extrapolated to 0 K; C102T, the variant of yeast iso-1-cytochrome *c* in which the cysteine at position 102 in the wild-type protein is replaced by a threonine. The numbering system of higher eukaryotic cytochromes *c* is used (Cutler et al., 1987; Moore & Pettigrew, 1990); D, the denatured state; $K_{D,T}$, the apparent equilibrium constant for denaturation at temperature T; m_D, the slope of the D baseline; m_N, the slope of the native-state baseline; N, the native state; T_m, the temperature at which half the protein molecules are denatured; $y_{D,T}$, the absorbance due to the D baseline at temperature T; $y_{N,T}$, the absorbance due to the N baseline at temperature T; $\alpha_{D,T}$, the apparent fraction of D at temperature T; ΔC_p, the heat capacity of D minus N; ΔG_D, the apparent Gibbs free energy of denaturation; $\Delta G_{D,T}$, ΔG_D at temperature T; ΔH_{cal}, the calorimetric enthalpy of denaturation; ΔH_D, the apparent (i.e., van't Hoff) enthalpy of denaturation; $\Delta H_{D,T}$, ΔH_D at temperature T; ΔH_m, $\Delta H_{D,T_m}$; ΔS_D, the apparent entropy of denaturation; $\Delta S_{D,T}$, ΔS_D at temperature T; ΔS_m, $\Delta S_{D,T_m}$ (equal to $\Delta H_m/T_m$); $\Delta\nu$, the number of protons bound to D minus the number of protons bound to N; σ_x, the estimated error in parameter x.

forces, and salt bridges (Dill, 1990). Despite the cumulative stabilization these interactions impart, the average protein is only marginally stable with a ΔG_D of 5–10 kcal mol^{-1} at physiological temperatures.

The Gibbs free energy can be separated into its enthalpic and entropic components:

$$\Delta G_{D,T} = \Delta H_{D,T} - \Delta S_{D,T}. \quad (1)$$

The interactions listed above can stabilize the protein by contributing to the enthalpy of denaturation, $\Delta H_{D,T}$, whose value is on the order of 0.5–1.0 kcal mol^{-1} residue^{-1} (Murphy & Gill, 1991) near room temperature. Because most proteins are only marginally stable, the entropic component, $T\Delta S_{D,T}$, must be nearly equal to $\Delta H_{D,T}$. This large value for $T\Delta S_{D,T}$ results because the highly ordered structure of N has few degrees of freedom compared to the nearly random conformation of D.

Denaturation of small, globular proteins is highly cooperative, with the transition from N to D occurring over a small range of denaturing conditions (e.g., heat or chemical denaturants such as acid, urea, or guanidinium chloride). To simplify understanding of the denaturation reaction, sparsely populated conformations other than N or D are ignored. This simplification, known as the 2-state assumption (Lumry et al., 1966), implies that the denaturation reaction may be written as

$$N \rightleftharpoons D. \quad (2)$$

The 2-state model also implies that the reaction is reversible.

Given a probe that detects N and/or D, application of the 2-state model allows van't Hoff analysis, where $\Delta H_{D,T}$ is deduced from the dependence of an equilibrium constant on inverse temperature (Hermans & Scheraga, 1961). The observation that different probes yield nearly identical values of $\Delta H_{D,T}$ constitutes evidence for 2-state behavior (Lumry et al., 1966; Dill & Shortle, 1991). The strongest evidence for 2-state behavior comes from comparison of denaturation enthalpies from differential scanning calorimetry to values from van't Hoff analysis, because the determination of ΔH_{cal} requires no assumptions about the number states. The calorimetric and van't Hoff enthalpies are equal if the protein denatures via a 2-state mechanism. Two-state behavior allows simple calculation of thermodynamic parameters—$\Delta G_{D,T}$, $\Delta H_{D,T}$, $\Delta S_{D,T}$, and the change in heat capacity for denaturation, ΔC_p. These values are essential tools for quantifying the effects of amino acid substitutions.

Cytochrome c as a model for stability studies

Yeast iso-1-cytochrome *c* is a 108-residue globular protein that has a covalently bound heme prosthetic group (for reviews see Pettigrew & Moore, 1987; Moore & Pettigrew, 1990). The heme iron exists in either an oxidized or reduced (Fe^{2+}) form. Normally, the reduced form of the protein auto-oxidizes upon denaturation, rendering the process irreversible (Bixler et al., 1992; Hilgen-Willis et al., 1993), thus only the oxidized form is studied here. Wild-type yeast iso-1-cytochrome *c* has a free cysteine at position 102 (Smith et al., 1979) that causes dimerization upon denaturation. This covalent association makes denaturation irreversible. Eighty-seven percent of cytochromes *c* whose sequence is known have a threonine at this position. Therefore, the C102T variant is used because it is more amenable to biophysical studies (Auld & Pielak, 1991; Betz & Pielak, 1992; Auld et al., 1993; Greene et al., 1993; Hilgen-Willis et al., 1993; Marmorino et al., 1993; Hawkins et al., 1994) and is structurally and functionally identical to the wild-type protein (Cutler et al., 1987; Gao et al., 1990, 1991; Berghuis & Brayer, 1992; Willie et al., 1993).

Extracting equilibrium thermodynamic information from studies of absorbance versus temperature

Assuming the denaturation reaction is 2-state (Equation 2), the fraction of denatured protein at temperature T, $\alpha_{D,T}$, is given by

$$\alpha_{D,T} = \frac{A_{\lambda,T} - y_{N,T}}{y_{D,T} - y_{N,T}}, \quad (3)$$

where the portions of the absorbance, $A_{\lambda,T}$, due to the N and D baselines (y_N and y_D, respectively) are linear functions of temperature:

$$y_{N,T} = m_N T + b_N \quad (4)$$

$$y_{D,T} = m_D T + b_D. \quad (5)$$

In Equations 4 and 5, m_N and m_D are the changes in absorbance with respect to temperature of N and D, respectively. Likewise, b_N and b_D are the absorbances of N and D extrapolated to 0 K, respectively.

The equilibrium constant for denaturation, $K_{D,T}$, for Equation 2 is given by

$$K_{D,T} = \frac{[D]}{[N]} = \frac{\alpha_{D,T}}{1 - \alpha_{D,T}}. \quad (6)$$

The dependence of $K_{D,T}$ on temperature is obtained by fitting plots of absorbance versus temperature to

$$A_{\lambda,T} = \frac{y_{N,T} + K_{D,T}(y_{D,T})}{1 + K_{D,T}}. \quad (7)$$

To obtain additional equilibrium thermodynamic information, $K_{D,T}$ in Equation 7 is replaced by the right-hand side of Equation 8,

$$K_{D,T} = -\exp\left\{\left(\Delta H_m\left(1 - \frac{T}{T_m}\right) - \Delta C_p\left[(T_m - T) + T\ln\left(\frac{T}{T_m}\right)\right]\right)\Big/RT\right\}, \quad (8)$$

which is a modified form of the integrated Gibbs–Helmholtz equation (Equation 9; Elwell & Schellman, 1977). In Equation 8, ΔH_m is the van't Hoff enthalpy of denaturation evaluated at T_m (the temperature where $K_{D,T} = 1$), R is the gas constant, and T is absolute temperature.

$$\Delta G_{D,T} = -RT\ln(K_{D,T}) = \Delta H_m\left(1 - \frac{T}{T_m}\right) - \Delta C_p\left[(T_m - T) + T\ln\left(\frac{T}{T_m}\right)\right]. \quad (9)$$

Equation 9 is easily obtained from the Gibbs equation (Equation 1), the equation for the temperature dependence of $\Delta H_{D,T}$:

$$\Delta H_{D,T} = \Delta H_m + \Delta C_p(T - T_m), \quad (10)$$

and the equation for the temperature dependence of $\Delta S_{D,T}$:

$$\Delta S_{D,T} = \Delta S_m + \Delta C_p \ln\left(\frac{T}{T_m}\right), \quad (11)$$

where ΔS_m is defined as $\Delta H_m/T_m$, and ΔC_p is assumed to be temperature independent in the temperature range studied.

It is possible to determine ΔC_p by measuring ΔH as a function of T using Kirchoff's law,

$$\Delta C_p = \frac{\delta \Delta H}{\delta T}. \quad (12)$$

As shown below, ΔH in Equation 12 can be replaced by ΔH_m, and T by T_m. The experimentally determined values of $\alpha_{D,T}$, $K_{D,T}$, $\Delta G_{D,T}$, $\Delta H_{D,T}$, $\Delta S_{D,T}$, and ΔC_p are all apparent values because denaturation is assumed to be a 2-state process.

Results

Evidence for thermodynamic reversibility and 2-state behavior

To utilize the 2-state model, protein denaturation must be reversible and concentration independent. Reversibility was confirmed by fully heat-denaturing the protein, quickly returning the temperature to the initial value, and repeating the thermal denaturation experiment. Denaturation is deemed to be reversible if the values of absorbance for the second experiment are within 5% of the values for the initial experiment. This criterion was met over the entire pH range studied.

Thermal denaturation of the C102T variant is concentration independent. The concentration was 5–10 μM for absorbance-detected experiments, $\approx$30 μM for circular dichroism studies (D.S. Cohen, A.J. Saunders, & G.J. Pielak, unpubl.), and $\approx$5 mM for differential scanning calorimetry experiments (Betz & Pielak, 1992). These 3 methods give identical values for ΔH_m and T_m.

We examined wavelengths that monitor the heme environment (415, 399, and 360 nm) and the environment of the sole tryptophan, Trp 59 (287 and 280 nm). Plots of $\alpha_{D,T}$ versus T were overlaid for these wavelengths (Fig. 1). The fact that each probe yields the same denaturation curve implies that the probes monitor a single process. Furthermore, ΔH_m at pH 4.6 (82.5 $\pm$ 3.9 kcal mol^{-1}, see below) is within error of ΔH_{cal} (88.8 $\pm$ 4.0 kcal mol^{-1}; Betz & Pielak, 1992). This agreement strongly suggests that denaturation is well approximated by a 2-state process. Taken together, the observations that denaturation is reversible, concentration independent, and that $\Delta H_{cal}/\Delta H_m \approx 1.0$ suggest that reversible aggregation does not occur.

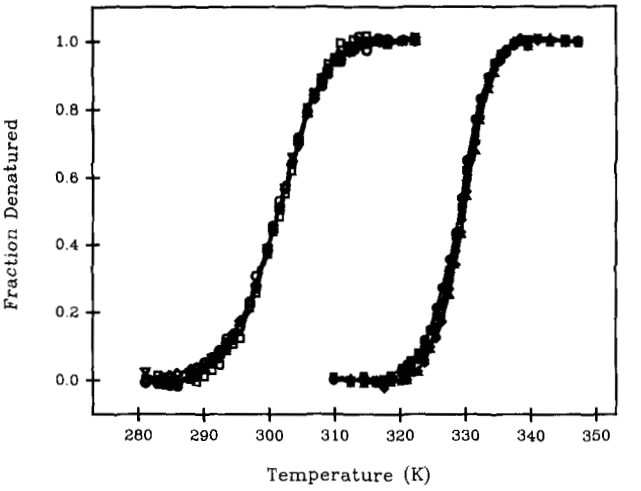

Fig. 1. Fraction denatured versus temperature for thermal denaturation experiments at pH 3.25 (open symbols) and pH 5.5 (filled symbols). Data are from 415 nm (○), 399 nm (△), 360 nm (□), 287 nm (▽), and 280 nm (◇). The curves are nonlinear least-squares fits to Equations 7 and 8.

Determination of ΔH_m and T_m

Values of ΔH_m and T_m were obtained by first substituting the right-hand side of Equation 8 into Equation 7. Each absorbance versus temperature data set was then fit to the resulting expression using a nonlinear least-squares method. Values of T_m and ΔH_m for 8 different pH values are given in Table 1. Typical standard deviations of T_m and ΔH_m from least-squares fitting of 1 data set are $\pm$0.05 K and $\pm$1.1 kcal mol^{-1}. Assuming a standard deviation of 0.003 absorbance units, the value of χ^2 is 2.2 for the data collected at pH 4.6, 399 nm. This value is less than the number of degrees of freedom (24, the number of observations in a typical data set [31] minus the number of constraints [7] in Equations 4, 5, and 8), suggesting that the results

Table 1. *Average values of ΔH_m and T_m from absorbance data at various pH values*[a]

pH (±0.02)	Number of experiments	T_m (K)	ΔH_m (kcal mol^{-1})
3.00	2	302.1 (300.7–303.5)	49.1 (46.0–52.1)
3.25	2	305.4 (305.2–305.6)	55.1 (54.2–56.0)
3.50	2	311.4 (310.2–312.7)	62.7 (61.0–64.4)
3.75	2	316.0 (315.3–316.7)	68.2 (67.4–69.0)
4.00	2	317.2 (315.5–319.1)	74.0 (72.2–75.8)
4.30	2	322.9 (322.9–322.9)	77.4 (77.0–77.4)
4.60	10	325.8 (±1.1)	82.5 (±3.9)
5.00	2	328.5 (328.4–328.6)	86.1 (85.8–86.4)

[a] Numbers in parentheses correspond to the range of experimental measurements, except at pH 4.6, where the sample standard deviation is shown.

are compatible with the model. The values of ΔH_m and T_m from least-squares fitting agree with values determined by manual selection of baselines using van't Hoff analysis as described by Pace et al. (1990). Examination of several data sets, however, revealed that nonlinear least-squares fitting reduces the standard deviations of ΔH_m and T_m, probably because the fitting routine selects baselines more consistently.

Ionization of N and D as functions of pH

To characterize the pH dependence of thermal denaturation, it is necessary to consider the ionization of N and D. From inspection of Figure 1 and Table 1 it is evident that pH affects T_m. As pH decreases, groups on the protein become protonated, hence the positive charge of both N and D increases. There is also a change in $\Delta\nu$, which is the number of protons bound to D minus the number of protons bound to N. The value of $\Delta\nu$ at each pH was determined by applying Equation 13 (Privalov & Ptitsyn, 1969; Ptitsyn & Birshtein, 1969) to the data in Table 1:

$$\Delta\nu = \frac{\Delta H_m}{2.3RT_m^2} \cdot \frac{\delta T_m}{\delta \mathrm{pH}}. \qquad (13)$$

The $\delta T_m/\delta$pH term was calculated by fitting a second-order polynomial to plots of T_m versus pH (Fig. 2) and evaluating the first derivative at each T_m. The buffer concentration (100 mM) is much larger than the protein concentration (5–10 μM), ensuring sufficient buffer capacity at all pH values studied. Figure 3 shows that $\Delta\nu$ is greatest at pH 3.5 and decreases with increasing pH, passing through 0 near pH 6.5 (data not shown). Because $\Delta\nu$ is positive and greater than 1 over the pH range studied, at least 2 groups take up protons upon denaturation. Likely candidates for these are the side chains of His 26 and His 18, whose pK_a's in N are $\leq$3.6 and $\leq$3.0, respectively (Cohen & Hayes, 1974), and one of the heme propionates, whose pK_a in N is <4.5 (Hartshorn & Moore 1988; see below).

pK of a heme-associated group

Absorbance-detected thermal denaturation experiments at varying pH can be used to measure the pK of groups that affect a chromophore. We examined the difference in the extinction coefficients between N and D as a function of pH by dividing the difference in absorbance between the N and D baselines at T_m by the protein concentration. Figure 4 shows the magnitude of the denaturation transition at 5 wavelengths as a function of pH. Wavelengths that probe the environment of Trp 59 show no change with respect to pH, whereas probes of the heme environment show marked changes. Furthermore, the absorbance of N at these wavelengths does not change between pH 3 and 5. Therefore, the transition represents a heme-associated group or groups in D that ionize with a pK of approximately 4. Likely candidates are the heme propionates whose pK_a's are <4.5 in free heme (Phillips, 1963) and <4.5 and >9.0 in N (Hartshorn & Moore, 1988).

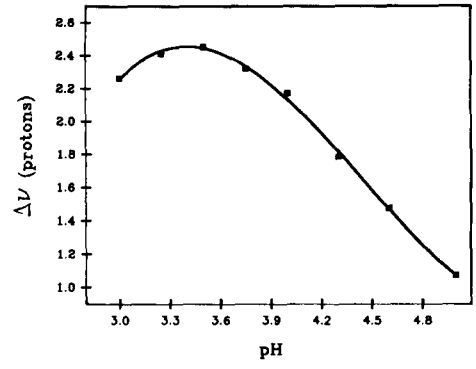

Fig. 3. Number of protons taken up by the protein upon denaturation ($\Delta\nu$) versus pH.

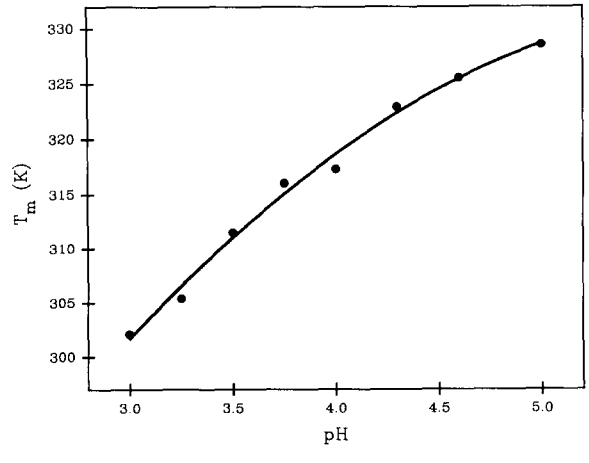

Fig. 2. Dependence of T_m on pH.

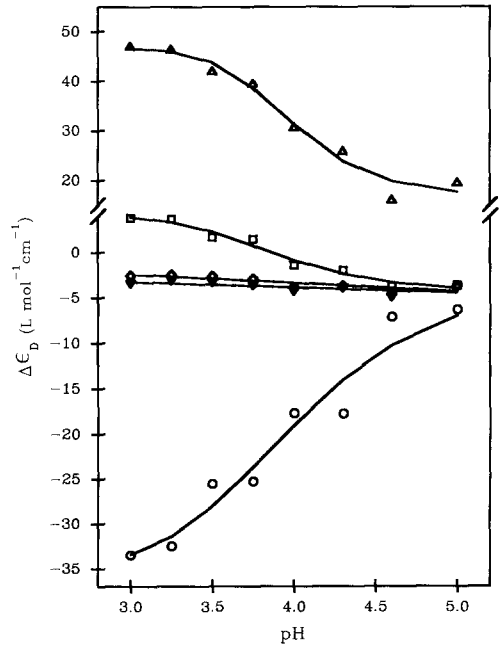

Fig. 4. Magnitude of the absorbance-detected transition, $\Delta\epsilon_D$, as a function of pH. Symbols as in Figure 1. The curves drawn through the data are of no theoretical significance.

pH dependence of ΔH_m and T_m

Figure 5 shows plots of $\alpha_{D,T}$ versus T upon varying pH. A shift toward lower T_m is observed concomitant with a decrease in pH. More subtly, the steepness of the transition, which is a measure of ΔH_m, decreases as T_m decreases. Values of ΔH_m and T_m from analysis of these curves are presented in Table 1. To produce Figure 6, the data in Table 1 were fitted to Equation 14 (Becktel & Schellman, 1987):

$$\Delta H_m = m(T_m) + b, \tag{14}$$

providing values for m and b of 1.37 ± 0.06 kcal mol^{-1} K^{-1} and −364.9 ± 1.5 kcal mol^{-1}, respectively.

Six different investigators in this laboratory have repeated the thermal denaturation experiment at pH 4.6 a total of 10 times using 2 different batches of protein. These data are shown in the inset to Figure 6. The values and sample standard deviations of ΔH_m and T_m for the 10 experiments are 82.5 ± 3.9 kcal mol^{-1} and 325.8 ± 1.0 K. The fact that the sample standard deviation from repetition of experiment is greater than the sample standard deviation from nonlinear least-squares fitting (±1.1 kcal mol^{-1} and ±0.5 K for ΔH_m and T_m; see above) of individual data sets suggests that there is an additional variable that changes from sample to sample. The observation that many of the points in the inset lie near the line suggests that the variable is pH.

Discussion

Determination of ΔC_p

The increase in the heat capacity of an aqueous protein solution upon denaturation is the hallmark of the hydrophobic interaction (Kauzmann, 1959). This increase is thought to represent the energy required to disrupt the ordered shell of water that surrounds nonpolar regions that are exposed to solvent upon denaturation. The value of ΔC_p can be directly measured from calorimetric data by subtracting the heat capacity of N from that of D, if it is assumed that ΔC_p is temperature independent.

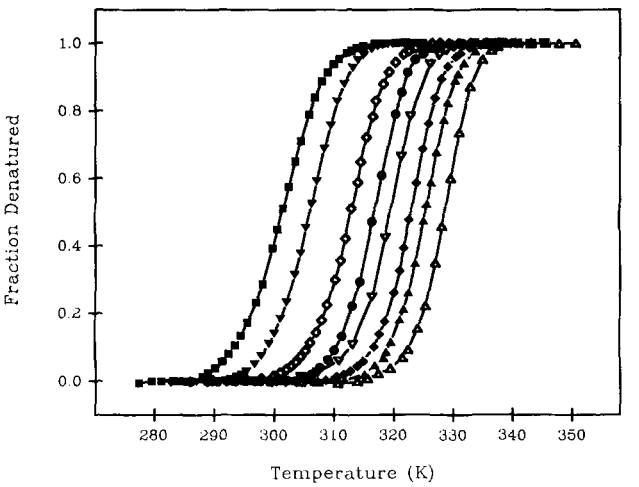

Fig. 5. Fraction denatured versus temperature as a function of pH at 399 nm. ■, pH 3.0; ▼, pH 3.25; ◇, pH 3.5; ●, pH 3.75; ▽, pH 4.0; ◆, pH 4.3; ▲, pH 4.6; △, pH 5.0.

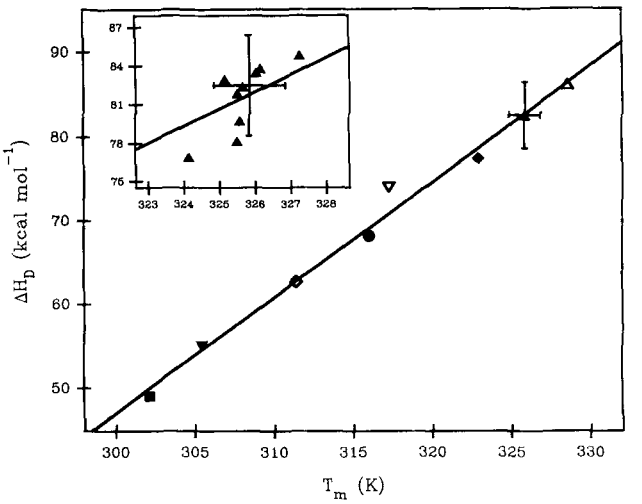

Fig. 6. ΔH_D versus T_m from the data in Table 1 (symbols as per Fig. 5). The **inset** shows the region around pH 4.6. Error bars on the inset and the main graph are of the same magnitude and represent the sample standard deviation of the data shown in the inset. Only 9 of the 10 points are visible on the inset because 2 points have nearly identical values.

For a 2-state process, ΔC_p can, in principle, be determined by fitting absorbance versus temperature data to Equation 9 (Myer, 1993). The quality of the fits shown in Figure 1, however, is insensitive to ΔC_p. This phenomenon is shown in Figure 7, where ΔH_m and T_m were fixed, and ΔC_p values of 0, 2, and 6 kcal mol^{-1} K^{-1} were used to derive plots of fraction denatured and $\Delta G_{D,T}$ versus T using Equations 7, 8, and 9. The slight curvature of the trace described by the circles in Figure 7B suggests that ΔC_p is positive, but the temperature range of the transition region is too narrow to allow quantification. The curvature in Figure 7A at low T for the largest ΔC_p represents notional cold denaturation.

If a perturbant such as pH changes T_m without changing $\Delta H_{D,T}$, ΔC_p can be obtained by measuring ΔH_m at different pH values (Fig. 6) and applying Equation 14 where m equals ΔC_p. The value of m (1.37 ± 0.06 kcal mol^{-1} K^{-1}, see above) equals the ΔC_p determined by subtraction of calorimetric baselines (Betz & Pielak, 1992), indicating that pH changes can be used to obtain ΔC_p. The agreement between the 2 values also suggests that heats of ionization are small.

The effect of pH on ΔS_D

As shown in Figure 8, $\Delta G_{D,300K}$ (calculated using Equation 9, the data in Table 1, and a ΔC_p of 1.37 kcal mol^{-1} K^{-1}) is pH dependent. Because $\Delta H_{D,T}$ appears to be pH independent (i.e., $\Delta H_m \approx \Delta H_{D,T_m}$), inspection of Equation 1 indicates that changes in $\Delta G_{D,300K}$ with pH are due to the pH dependence of $\Delta S_{D,300K}$. Values of $\Delta S_{D,300K}$ were calculated using Equation 11. These data, along with values of $\Delta H_{D,300K}$ calculated using Equation 10 and $\Delta G_{D,300K}$ calculated using Equation 9 are shown in Figure 8. Each carboxylate group that ionizes upon denaturation can contribute as much as ≈6 kcal mol^{-1} to $T\Delta S_{D,300K}$ (Edsall & Wyman, 1958). Additionally, $\Delta\nu$ and possibly the protein ionization entropy are pH dependent. Therefore, the dependence of $\Delta S_{D,300K}$ on pH results from the large ionization component

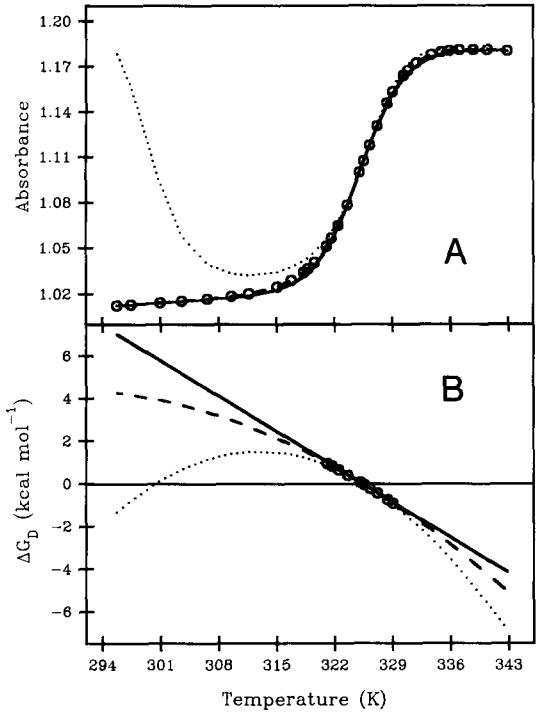

Fig. 7. A: Absorbance at 399 nm versus temperature for several values of ΔC_p calculated using Equations 7 and 8. The circles are experimental values acquired at pH 4.6. The 3 curves correspond to ΔC_p values of 6 kcal mol^{-1} K^{-1} (dotted line), 2 kcal mol^{-1} K^{-1} (dashed line), and 0 kcal mol^{-1} K^{-1} (solid line). **B:** ΔG versus temperature determined from curves in A using Equation 9. The circles were calculated from the experimental data in A.

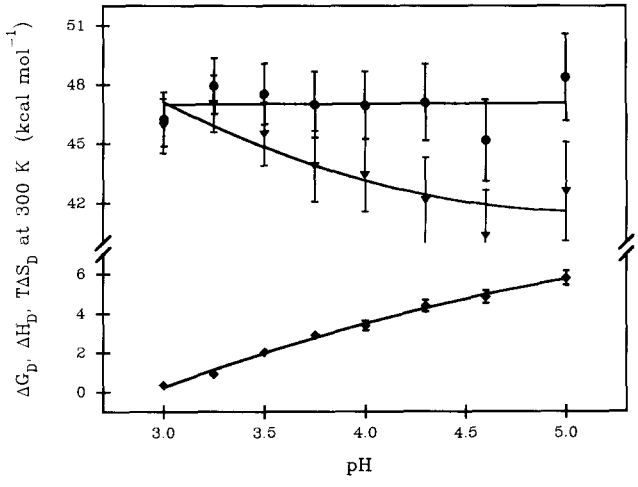

Fig. 8. $\Delta G_{D,300K}$ (◆), $\Delta H_{D,300K}$ (●), and $T\Delta S_{D,300K}$ (▼) versus pH. The line for $\Delta H_{D,300K}$ comes from Equation 10. The curves drawn through $\Delta G_{D,300K}$ and $T\Delta S_{D,300K}$ are of no theoretical significance.

of $\Delta S_{D,300K}$. Similar conclusions were reported by Pfeil and Privalov (1976) in their study of lysozyme at acid pH.

Entropy and enthalpy convergence

Many small globular proteins exhibit a common extrapolated value of ΔH_D at 373.6 K and a common extrapolated value of ΔS_D at 385.2 K when $\Delta H_{D,373.6K}$ and $\Delta S_{D,385.2K}$ are determined on a per residue (or per gram) basis and ΔC_p is assumed to be independent of temperature (Privalov & Khechinashvili, 1974; Privalov & Gill, 1988; Murphy & Gill, 1991). This assumption is not entirely correct (Baldwin, 1986; Makhatadze et al., 1993), but its application only affects the value of the convergence temperatures, not the fact that the data converge. Convergence was first discussed by Privalov and Khechinashvili (1974) upon examination of 4 different proteins, including bovine cytochrome *c*. These findings were later extended to 12 proteins by Privalov and Gill (1988).

Values of ΔH_D and ΔS_D at the convergence temperatures were calculated by dividing $\Delta H_{D,373.6K}$ and $\Delta S_{D,385.2K}$ by the effective number of residues in yeast iso-1-cytochrome *c*. The effective number of residues, 114, was obtained by dividing the molecular weight of the heme (616 g mol^{-1}) by the average residue molecular weight (111 g mol^{-1}; calculated from the sequence of Smith et al. [1979]) and adding the result to the number of residues in the protein. Using the data reported in Table 1 and Equations 10 and 11, $\Delta H_{D,373.6K}$ for the C102T variant is 1.30 kcal mol^{-1} residue^{-1} and $\Delta S_{D,385.2K}$ at pH 4.6 is 4.24 cal mol^{-1} K^{-1} residue^{-1}. Both values are close to those reported by Murphy and Gill (1991; 1.35 kcal mol^{-1} residue^{-1}) and by Privalov and Gill (1988; 4.30 cal mol^{-1} K^{-1} residue^{-1}). The origin of enthalpy and entropy convergence is discussed by Doig and Williams (1992), Fu and Friere (1992), and Privalov and Makhatadze (1993). In summary, because the C102T variant possesses the common enthalpic and entropic convergence temperatures, it can be included in the set of single-domain, globular proteins discussed by Privalov and Gill (1988). Thus, as first discussed by Privalov and Khechinashvili (1974), the covalently bound heme prosthetic group does not seem to affect the thermodynamics of denaturation.

Comparisons to the stabilities of other cytochromes c

The stability of the oxidized C102T variant at 300 K and pH 4.6 is 5.1 ± 0.3 kcal mol^{-1}, 2.2 kcal mol^{-1} less than that of equine and bovine ferricytochromes *c* (Privalov & Khechinashvili, 1974; Betz & Pielak, 1992). It is difficult to identify the sources of the stability difference because the yeast protein contains a 5-residue N-terminal extension compared to its higher eukaryotic relatives (Smith et al., 1979) and because the bovine and equine proteins differ from iso-1-cytochrome *c* at ≈37% of common positions (Moore & Pettigrew, 1990). Comparisons of chemical (White et al., 1987; Betz & Pielak, 1992) and available thermal (Dumont et al., 1990) denaturation data suggest that iso-1- is more stable than iso-2-ferricytochrome *c*, but less stable than rat ferricytochrome *c* (Koshy et al., 1994).

Conclusions

We have determined that the absorbance-detected thermal denaturation of the oxidized C102T variant of yeast iso-1-ferricytochrome *c* is reversible and is well described by a 2-state model. The value of ΔC_p determined by performing denaturation experiments at varying pH (1.37 ± 0.06 kcal mol^{-1} K^{-1}) is in agreement with the value measured calorimetrically. At 300 K and pH 4.6, ΔG_D is 5.1 ± 0.3 kcal mol^{-1}. pH-dependent

changes in the Soret region are ascribed to the ionization of 1 or both heme propionates in the denatured state. Finally, the C102T variant is one of a class of proteins whose normalized ΔH_D and ΔS_D converge at 373.6 and 385.2 K, respectively.

Materials and methods

Protein isolation, pH, spectrophotometry, and temperature control

The C102T variant of iso-1-cytochrome *c* was purified from *Saccharomyces cerevisiae* strain B6748 (Holzschu et al., 1987), harboring a plasmid carrying the gene of interest (Auld & Pielak, 1991). The cloning, transformation, and expression techniques are described in Cutler et al. (1987). Protein purification techniques are described in Willie et al. (1993), and protein oxidation methods are described in Betz and Pielak (1992).

Experiments were carried out in 100 mM sodium acetate buffer between pH 3.0 and 5.0. An Orion model SA720 pH meter equipped with an Ingold model 6030-02 electrode, calibrated with Fisher pH 4.00 and pH 7.00 standards, was used. Absorbance was monitored using a Shimadzu UV-160U spectrophotometer equipped with a constant-temperature cuvette holder. Temperature was controlled by a Fisher Scientific Isotemp refrigerated circulator (model 900). Temperature in the cuvette was measured with a Physitemp tissue thermocouple connected to a Baxter S/P digital thermometer. The quartz cuvette (Wilmad Glass Co., 1.4 mL, 1-cm pathlength) was fitted with a Teflon stopper to reduce sample evaporation. The thermocouple was inserted directly into the cuvette via a 0.75-mm-diameter hole in the stopper. Some experiments required that measurements be made between 275 and 298 K. To prevent condensation on the outside of the cuvette at low temperatures, the sample compartment was continuously purged with dry N_2.

Thermal denaturation

Initial thermal denaturation experiments were performed to determine the wavelengths to be monitored. These involved acquisition of spectra (240 nm to 600 nm) at 10 K intervals from 293 to 363 K, with subsequent subtraction of each spectrum from the first one. Wavelengths that exhibited the largest change were chosen as probes. A thermal denaturation experiment involved setting the temperature of the water bath, allowing 8 min for thermal equilibration, and recording the absorbance at the 5 wavelengths. The temperature was then increased by <2 K and the process was repeated. The temperature increments were chosen to include native and denatured baselines and a well-resolved transition region.

Propagation of errors and $\Delta G_{D,T}$

The degree to which *random* error in ΔH_m, T_m, and ΔC_p contributes to the error in $\Delta G_{D,T}$ was determined by applying propagation of error analysis to Equation 9. The general form for the combination of errors from 3 independent variables is

$$\sigma_{F(x,y,z)} \approx \sqrt{\left(\frac{\delta F}{\delta x}\,\sigma_x\right)^2 + \left(\frac{\delta F}{\delta y}\,\sigma_y\right)^2 + \left(\frac{\delta F}{\delta z}\,\sigma_z\right)^2}, \qquad (15)$$

where each term is the original function, F, differentiated with respect to a single variable, x, y, or z, multiplied by the estimated error, σ, of that variable. This type of analysis is valid when the error for the measured variable is small. Applied to Equation 9, the error formula for $\Delta G_{D,T}$ is

$$\sigma_{\Delta G_{D,T}} \approx \left\{\left[\left(1-\frac{T}{T_m}\right)\sigma_{\Delta H_m}\right]^2 + \left[\left(T-T_m-T\ln\left(\frac{T}{T_m}\right)\right)\sigma_{\Delta C_p}\right]^2 + \left[\left(\frac{\Delta C_p T}{T_m}+\frac{\Delta H_m T}{T_m^2}-\Delta C_p\right)\sigma_{T_m}\right]^2\right\}^{1/2}. \qquad (16)$$

For the C102T variant, we used values of ±3.9 kcal mol^{-1}, ±0.06 kcal mol^{-1} K^{-1}, and ±1.1 K for the σ of ΔH_m, ΔC_p, and T_m, respectively.

Inspection of Equation 16 reveals that there is a temperature dependence to the error with a minimum at T_m. At T_m, the error from the enthalpy and the heat capacity changes reduces to 0, and only the error in T_m contributes to $\sigma_{\Delta G_{D,T_m}}$ (Becktel & Schellman, 1987). The error bars for $\Delta G_{D,300K}$ in Figure 8 were calculated using Equation 16. The error bars for $\Delta H_{D,300K}$ and $\Delta S_{D,300K}$ in Figure 8 were calculated by applying Equation 15 (for 2 variables) to Equations 10 and 11, respectively. The error bars for $\Delta G_{D,300K}$ are small compared to the ones for $\Delta H_{D,300K}$ and $\Delta S_{D,300K}$ because T_m is never far from the reference temperature (300 K).

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