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Entropic Stabilization of Cytochrome *c* upon Reduction

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Abstract: We have directly measured a 6.6 ± 0.8 kcal mol^{-1} increase in the thermal stability of *Saccharomyces cerevisiae* iso-1-ferricytochrome *c* upon reduction of the heme iron at pH 4.6 and 300 K. The stabilization is entirely entropic, contributing 13.2 ± 6.8 kcal mol^{-1} under the above conditions. The dramatic changes in the stability and entropy of denaturation cannot be rationalized in terms of redox-state-dependent changes in structure, mobility, or cross-linking but can be accounted for by redox-state-dependent heme solvation.

Introduction

Essential to the goal of rational protein design is the understanding of the structural basis of protein stability. To this end, many stability studies of proteins which differ by single amino acid residues have been reported. Two groups have shown that an even simpler protein modification, addition of an electron to the heme iron of ferricytochrome *c*, dramatically increases the free energy of denaturation, ΔG_D.[1] The stability increase, ≈10 kcal mol^{-1} at 300 K, was indirectly determined by utilizing a Born–Haber cycle relating the stability of ferricytochrome *c* (with respect to chemical denaturants) and the formal potentials of the native and chemically-denatured states. The enthalpy and entropy of denaturation for the reduced protein, however, could not be determined because the temperature dependence was not measured. The obstacle to direct determination of the thermal stability of ferrocytochrome *c* is that dissolved O_2 oxidizes the heme iron upon denaturation, making the reaction irreversible. We have circumvented this problem and report a detailed thermodynamic description of the stabilization of *Saccharomyces cerevisiae* iso-1-cytochrome *c* upon reduction.

Experimental Section

The protein utilized has its sole free cysteine, at position 102, replaced by a threonine.[2] The mutation makes the protein more amenable to biophysical studies but does not change its structure or function.[2,3] The protein was expressed in *S. cerevisiae* and purified as described in ref 4. Dissolved oxygen was removed from the buffer (100 mM sodium acetate) by vigorous stirring under vacuum. The buffers were then equilibrated with N_2(g) in a glovebox. To prevent O_2 contamination and ensure complete heme reduction, sodium hydrosulfite, which reacts with O_2 to form sulfate, was added to the buffers with subsequent storage under N_2(g). Likewise, dithiothreitol was added to 2–3 times the protein concentration. Furthermore, the methods used to probe denaturation, circular dichroism (CD) spectropolarimetry and differential scanning calorimetry (DSC), are performed under N_2(g). CD data were obtained using an Aviv 62DS spectropolarimeter equipped with a thermostated, five-position sample changer and protein concentrations of 30 or 300 μM. Denaturation was monitored at 222, 332, 416, and 424 nm. Thermodynamic parameters and their uncertainties were obtained from CD data as described in ref 5. Ferrocytochrome *c* was examined at pH 3.25, 3.50,

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Table 1. Results from Thermal Denaturations at pH 4.6

method	$\Delta H_{D,cal}$, kcal mol^{-1}	$\Delta H_{D,vH}$, kcal mol^{-1}	T_m, K
DSC scan 1	124.8	123.4	352.5
DSC scan 2	129.9	119.8	353.3
CD	na[a]	118.5 ± 1.1	352.8 ± 0.3

[a] Not applicable.

3.75, 4.00, 4.30, 4.60, 5.00, 5.50 and ferricytochrome *c* at pH 3.00, 3.25, 3.50, 3.75, 4.00, 4.30, 4.60, 5.00.[5] Values of ΔC_p (the heat capacity of denaturation) were determined by linear least-squares fitting of eq 1 to T_m (the midpoint of the transition) and $\Delta H_{D,vH}$ (the van't Hoff enthalpy at T_m) where b is the y-intercept:

$$\Delta H_{D,vH} = \Delta C_p T_m + b \quad (1)$$

Values of $\Delta H_{D,vH}$ and T_m are the mean of three to six determinations. Uncertainties are the standard deviations of the means. The enthalpy of denaturation at temperature T (ΔH_D) was calculated using eq 2:

$$\Delta H_D = \Delta H_{D,vH} + \Delta C_p(T - T_m) \quad (2)$$

The entropy of denaturation at temperature T (ΔS_D) was calculated using eq 3, where $\Delta S_{D,vH}$ equals $\Delta H_{D,vH}/T_m$:

$$\Delta S_D = \Delta S_{D,vH} + \Delta C_p \ln\left(\frac{T}{T_m}\right) \quad (3)$$

The free energy of denaturation at temperature T (ΔG_D) was determined from the CD data using the integrated form of the Gibbs–Helmholtz equation with a temperature-independent ΔC_p.

$$\Delta G_D = \Delta H_{D,vH}\left(1 - \frac{T}{T_m}\right) - \Delta C_p\left[(T_m - T) + T\ln\left(\frac{T}{T_m}\right)\right] \quad (4)$$

DSC data were obtained using a Microcal MC-2 microcalorimeter and fitted as described in ref 6. For DSC, the protein concentration was 2 mM.

Results

Thermal denaturation experiments were performed using DSC and CD under reducing conditions. Results are presented in Table 1. Two successive DSC heating-scans (1 K min^{-1}) on the same sample gave nearly identical values for T_m (the temperature of maximum excess heat capacity), $\Delta H_{D,vH}$, and $\Delta H_{D,cal}$ (the calorimetric enthalpy of denaturation). DSC yielded $\Delta H_{D,vH}$ and T_m values nearly identical to those obtained using CD. Absorbance and CD spectra (between 600 and 200 nm) collected at 300 K before and after a 10 min incubation of the protein at 20 K above T_m were the same. Not only is the $\Delta H_{D,vH}/\Delta H_{D,cal}$ ratio between 0.92 and 0.99 but also the results from each of the wavelengths used to monitor denaturation are identical, indicating two-state denaturation.[6] Denaturation is reversible because two successive DSC experiments on the same sample gave nearly identical results, and the absorption and CD spectra at 300 K were the same before and after thermal denaturation.

The value of ΔC_p was determined using CD by perturbing $\Delta H_{D,vH}$ and T_m with changes in pH and applying eq 1. The upper portion of Figure 1 shows the results of thermal denaturation at varying pH. The values of ΔC_p for both redox forms are presented in Table 2. Using these values and $\Delta H_{D,vH}$, ΔH_D at 300 K was calculated for both redox forms using eq 2. Values for $T\Delta S_D$ and ΔG_D at 300 K and pH 4.6 were calculated using eqs 3 and 4. The results are presented in Table 2 and the temperature-dependence of ΔG_D for ferro- and ferricytochrome *c* at pH 4.6 is shown in Figure 1.

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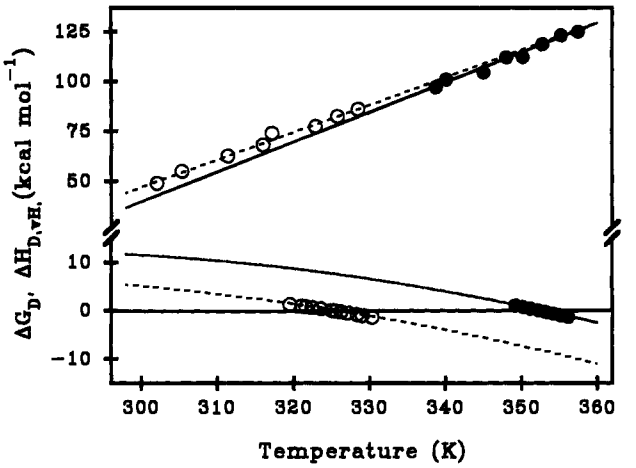

Figure 1. Plots of $\Delta H_{D,vH}$ and ΔG_D versus T for ferro- (●) and ferri- (○) cytochrome *c*. For ΔG_D, the symbols are from the transition regions of thermal denaturation experiments performed at pH 4.6. Data for ferricytochrome *c* are from ref 5.

Table 2. Thermodynamic Parameters for the Thermal Denaturation of Ferro- and Ferricytochrome *c* at 300 K and pH 4.6

parameter	ferro	ferri[a]
ΔC_p (kcal mol^{-1} K^{-1})	1.48 ± 0.08	1.37 ± 0.06
ΔH_D (kcal mol^{-1})	40.4 ± 6.0	47.1 ± 4.5
$T\Delta S_D$ (kcal mol^{-1})	28.7 ± 5.4	42.0 ± 4.2
ΔG_D (kcal mol^{-1})	11.7 ± 0.7	5.1 ± 0.4

[a] Data for ferricytochrome *c* are from ref 5.

Considering the differences in experimental approaches (i.e. chemical versus thermal denaturation), the value of ΔG_D for ferrocytochrome *c* (Table 2) is in reasonable agreement with that obtained using a Born–Haber cycle (15.8 ± 0.8 kcal mol^{-1}).[1b] In summary, upon reduction at pH 4.6 and 300 K, ΔG_D becomes 6.6 ± 0.8 kcal mol^{-1} more endergonic, ΔH_D becomes 6.7 ± 7.5 kcal mol^{-1} less endothermic, and $T\Delta S_D$ decreases by 13.2 ± 6.8 kcal mol^{-1}.[7]

Discussion

In an attempt to rationalize the changes in ΔH_D and ΔS_D upon reduction, other properties of cytochrome *c* were considered. The heme iron to Met-80 bond is stronger in the ferro form,[3a,8] yet the change in ΔH_D upon reduction is destabilizing. This means that the reduction-induced stability increase is entirely entropic. Therefore, reduction increases the entropy of the native state and/or decreases the entropy of the denatured state.

Changes in the solution structure upon reduction are small.[3b,9] In addition, analysis of the high-resolution crystal structures of ferro- and ferricytochrome *c* reveals only small redox-state-induced changes.[3a] In fact, there is only a 0.3 Å rms difference in the atomic coordinates. The mean temperature factor values for the main chain atoms are identical for both redox forms. On the other hand, the average side-chain temperature factor is 5 Å^2 in the ferro form and 12 Å^2 in the ferri form, suggesting less disorder/motion in the native state of ferrocytochrome *c*. This decrease indicates that ΔS_D should increase upon reduc-

(7) (a) The reversible thermal denaturation of the ferro and ferri forms of the non-homologous protein, cytochrome b_{562}, has been reported,[7b] but because DSC was not used and ΔC_p was not determined, only the increase in ΔG_D (4.6 kcal mol^{-1} at 300 K and pH 7.0) could be estimated. (b) Fisher, M. T. *Biochemistry* **1991**, *30*, 10012–10018.

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tion,[10] but the opposite effect is observed. In summary, changes in the native state are subtle and cannot explain the changes in ΔH_D and ΔS_D.

In light of the crystallographic results, the decrease in ΔS_D upon reduction suggests that the denatured state has changed. Two observations, however, indicate that the denatured states are similar. First, CD spectra of both redox forms are identical above their respective T_m values. Second, ΔC_p, an indirect measure of the exposure of hydrophobic surface upon denaturation, is the same for both forms. A related possibility is that, like His-18,[11] the Met-80−iron bond is intact in the denatured state of ferrocytochrome *c*. However, even using an unrealistically small value for the volume parameter,[12] such a cross-link would lower $T\Delta S_D$ by only ≈6 kcal mol^{-1} at 300 K.

The decrease in ΔS_D upon reduction can be related to the change in the number of accessible conformations using eq 5,

$$\Delta\Delta S_{ferro-ferri} = 114\, R \ln W \qquad (5)$$

where W is the number of conformations per residue and 114 is the effective number[5] of residues. According to eq 5, ferrocytochrome *c* possesses ≈1.3 fewer possible conformations per residue than the oxidized protein. This unreasonably large decrease in the number of conformations may be explained by small changes in internal entropy. For a folded protein the internal entropy is large (≈35 cal K^{-1} mol^{-1} residue^{-1}), but so is the weighted average of the configurational entropy of the individual denatured conformers.[13] If these terms are equal, they cancel, and the conformational entropy is described by eq 5. Karplus et al.[13] suggest that small perturbations (e.g. ligand binding, and amino acid substitutions) can disrupt this cancellation, invalidating the use of eq 5. In our case, the perturbation is small: the binding of an electron. In terms of ΔG_D, a change in ΔS_D is often accompanied by a compensatory change in ΔH_D.[14] Such enthalpy−entropy compensation occurs upon reduction of cytochrome *c* (Table 2) and is the hallmark of solvation changes.[14a]

In our opinion, the most reasonable explanation for the decrease in ΔS_D upon reduction is that the higher positive charge on the oxidized heme alters solvation.[15] In fact, when converted to cell potentials, the difference in ΔG_D values (−0.29 V) agrees with the change in formal potential of heme models upon transfer from water to benzene (−0.30 V).[16] As discussed by Dunitz,[17] a tightly-bound water molecule can contribute ≤7 cal mol^{-1} K^{-1} to ΔS_D. Thus, the stabilization could be caused by the binding of >6 additional water molecules to ferricytochrome *c* relative to ferrocytochrome *c*. In summary, our data, and those of others,[10] show that it is difficult to predict changes in stability from examination of even high-resolution protein structures.

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