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A Native Tertiary Interaction Stabilizes the A State of Cytochrome *c*†

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ABSTRACT: Certain kinetic intermediates in protein folding are similar to the molten globule, or A state, an equilibrium state of many proteins that is populated under high salt and low pH conditions. Many A states are nearly as compact as native proteins and have native-like secondary structure, but the extent to which nonlocal interactions stabilize the A state is unclear. In this study, thermal denaturation, monitored by circular dichroism, was used to determine the free energy of denaturation of the A state ($\Delta G_{A\rightleftharpoons D}$) for *Saccharomyces cerevisiae* iso-1-ferricytochrome *c*. Specifically, we examined the wild-type protein, seven variants with amino acid substitutions at the interface between the N- and C-terminal helices, and two variants with mutations at a position close to, but not involved in, the interface. A plot of $\Delta G_{A\rightleftharpoons D}$ versus $\Delta G_{N\rightleftharpoons D}$ (the free energy of denaturation of the native state) has a slope near unity, showing that the evolutionarily conserved helix−helix interaction stabilizes the A state to the same degree that it stabilizes the native state.

Kinetic protein folding intermediates can be detected using rapid-mixing techniques but are difficult to characterize because of their fleeting existence. Certain kinetic intermediates are similar to the molten globule (Jennings & Wright, 1993), or A state, an equilibrium state of many proteins that is populated under high salt and low pH conditions (Ptitsyn, 1992). Thus, determining what types of interactions stabilize A states may help explain protein folding. Here, we investigate the degree to which the highly conserved tertiary-structure interaction between the N- and C-terminal helices of native cytochrome *c* stabilizes the A state. The results are compared to the degree to which this interaction stabilizes the native state.

Many A states, including that of equine cytochrome *c*, are nearly as compact as native proteins (Kataoka et al., 1993) and have native-like secondary structure (Jeng & Englander, 1991). Hydrophobic core interactions stabilize the A state of staphylococcal nuclease (Carra et al., 1994), and several nonlocal interactions stabilize the molten globule-like protein, apomyoglobin, at pH 7 (Lin et al., 1994). However, the extent to which *specific* nonlocal interactions stabilize A states remains unclear.

The N- and C-terminal helices of cytochrome *c* are comprised of residues 2−14 and 87−102, respectively (Louie & Brayer, 1990). The interface between the helices is the most highly conserved feature of this protein class (Mathews, 1985), forms early in folding (Elöve et al., 1994), and involves two specific interactions: a peg-in-hole interaction between Gly 6 and Leu 94 and a weakly polar aromatic−aromatic interaction between Phe 10 and Tyr 97. Variant proteins with amino acid substitutions at this interface have been generated (Auld & Pielak, 1991; Fredericks & Pielak, 1993) and characterized (Auld et al., 1993; Pielak et al., 1995).

In this study, we use thermal denaturation experiments-monitored by circular dichroism spectropolarimetry (CD) to measure the free energy of denaturation of the A state ($\Delta G_{A\rightleftharpoons D}$) for the wild-type protein, seven interface variants, and two variants (A7L, A7Y) with amino acid substitutions in the N-terminal helix, just outside the interface. Values of $\Delta G_{A\rightleftharpoons D}$ are compared to the free energy of denaturation of the native state ($\Delta G_{N\rightleftharpoons D}$).

MATERIALS AND METHODS

Nomenclature. Variants are denoted using the one-letter code with the wild-type residue given first, followed by the position number and the new residue. The C102T variant is referred to as the wild-type protein and all variants also contain the C102T mutation. This mutation makes the protein more amenable to biophysical studies but does not change its structure or function (Cutler et al., 1987; Gao et al., 1991; Berghuis & Brayer, 1992). Values of $\Delta G_{A\rightleftharpoons D}$ are defined at 0.33 M Na_2SO_4/H_2SO_4, pH 2.1, and 308.4 K. Values of $\Delta G_{N\rightleftharpoons D}$ are defined at 0.05 or 0.1 M sodium acetate, pH 4.6, and 325.8 K.

Production of Mutants and Variants. Yeast strains harboring the A7L and A7Y variants were produced as described in Hilgen and Pielak (1991) with the following exceptions. *Escherichia coli* strain DH5αF′ [F′,ϕ80d*lac*ZΔM15, *rec*A1, *end*A1, *gyr*A96, *thi*-1, *hsd*R17(r_K^-,M_K^+), *sup*E44, *rel*A1, *deo*R, Δ(*lac*ZYA-*arg*F) U169 (Hanahan, 1983)] was used in place of JM101, all transformations were performed by electroporation (Dower et al., 1988), and a Petri plate modified (Ner et al., 1988) version of the Sequenase (USB) double-stranded DNA sequencing technique was used. Like the study of Hilgen and Pielak (1991) the sequence of the

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entire gene of each mutant was determined to confirm the absence of unwanted mutations. Variant proteins were produced as described by Willie et al. (1993). Before use, each protein was shown to be >95% oxidized.

Circular Dichroism. Data were acquired using an Aviv Model 62DS spectropolarimeter equipped with a thermostated, five-position sample changer. Spectra were acquired at 1 °C. One-millimeter quartz cells were used between 200 and 240 nm, and 10-mm cells were used between 240 and 600 nm. Thirty micromolar samples were used for both temperature and wavelength scans. Samples were prepared by adding buffer to a weighed amount of lyophilized protein. Buffers were 0.33 M Na_2SO_4/H_2SO_4, pH 2.1, for the A state; 0.01 M Na_2SO_4/H_2SO_4, pH 2.1, for the acid-denatured state; and 0.05 M potassium phosphate, pH 7.0, for the native state. To determine protein concentration, ≈300 μM samples were made in pH 2.1 buffer. An aliquot of this stock was diluted with pH 7.0 buffer and the concentration determined using an extinction coefficient of 109.4 mM^{-1} cm^{-1} at 410 nm (Hilgen-Willis, 1993).

Thermal Denaturations. For the A ⇌ D reaction, 0.33 M Na_2SO_4/H_2SO_4, pH 2.1 buffer was used. For the N ⇌ D reactions of the position 7 variants, 0.05 M acetate buffer was used instead of 0.1 M acetate buffer which was used to study the interface variants and the wild-type protein (Pielak et al., 1995; Cohen & Pielak, 1994). Changing the buffer concentration does not significantly alter the thermodynamic parameters (data not shown).

Ellipticity at 222 nm was followed from 0 to 60 °C for the A ⇌ D reaction and from ≈10 to 85 °C for the N ⇌ D reaction. The data were acquired at 1 °C intervals. The time between successive points was ≈6 min. Reversibility was checked by returning the heated samples to the initial temperature and revisiting 2−5 points. Ellipticity values for these points were compared to initial values. Data were fit to a two-state model.

RESULTS AND DISCUSSION

CD Spectra. Spectra of the native, A, and acid-denatured states of the wild-type protein are shown in Figure 1. The native and A states have nearly the same helix content, but the acid-denatured state has little regular secondary structure (Figure 1A). The peak at ≈260 nm suggests similarities between the A and acid-denatured states for the heme. The negative Soret Cotton effect at ≈415 nm in the native protein (Pielak et al., 1986) is absent from the spectra of the A and acid-denatured states (Figure 1B), also indicating that the heme environment of the A state is like that of the acid-denatured state. Finally, the spectrum of the acid-denatured state resembles that of the heat-denatured protein (data not shown).

Determination of $\Delta G_{N\rightleftharpoons D}$. The interface variants discussed here exhibit reversible, two-state denaturation (Pielak et al., 1995). Using the criteria described in this paper, the N ⇌ D transitions for the A7L and A7Y variants are >85% reversible. Fitting these data as described by Cohen and Pielak (1994) allows determination of T_m (the temperature at which half the protein molecules are denatured) and ΔH_m (the enthalpy of denaturation at T_m). The uncertainties in T_m and ΔH_m are assumed to be equal to those for the wild-type protein: ±1.1 K and ±3.9 kcal mol^{-1}. Values for the change in heat capacity upon denaturation, ΔC_p, were determined by varying pH (Cohen & Pielak, 1994).

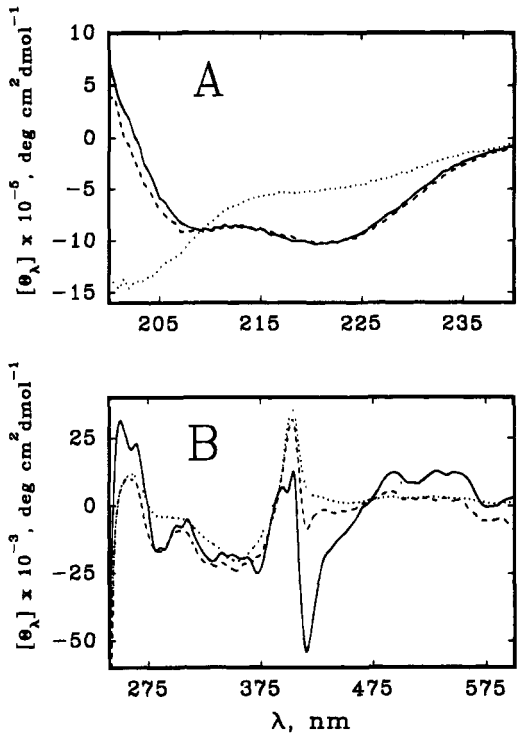

FIGURE 1: Far-UV CD (panel A) and near-UV and visible CD (panel B) spectra of the native state (—), A state (- - -), and acid-denatured state (• • •) of the wild-type protein.

Values of T_m, ΔH_m, ΔC_p, and $\Delta G_{N\rightleftharpoons D}$ for the interface variants are from Pielak et al. (1995), and values for the wild-type protein are from Cohen and Pielak (1994). Values of T_m, ΔH_m, and ΔC_p at pH 4.6 for the noninterface variants are 326.6 K, 86.3 kcal mol^{-1}, and 1.42 ± 0.27 kcal mol^{-1} K^{-1} for A7L, and 327.6 K, 81.2 kcal mol^{-1}, and 1.23 ± 0.17 kcal mol^{-1} K^{-1} for A7Y. Values for the noninterface variants are within the estimated uncertainties of the values for the wild-type protein (Cohen & Pielak, 1994). From these cardinal parameters, $\Delta G_{N\rightleftharpoons D}$ at temperature T was calculated using eq 1 (Elwell & Schellman, 1977).

$$\Delta G_{N\rightleftharpoons D} = \Delta H_m\left(1 - \frac{T}{T_m}\right) - \Delta C_p\left[(T_m - T) + T\ln\left(\frac{T}{T_m}\right)\right] \quad (1)$$

The uncertainty in $\Delta G_{N\rightleftharpoons D}$ was estimated by applying propagation of error analysis to eq 1 (Cohen & Pielak, 1994). For a variant protein, when T in eq 1 is the T_m of the wild-type protein, $\Delta G_{N\rightleftharpoons D}$ equals $\Delta\Delta G_{N\rightleftharpoons D}$ (i.e., $\Delta\Delta G_{N\rightleftharpoons D} = \Delta G_{N\rightleftharpoons D,var} - \Delta G_{N\rightleftharpoons D,wt}$). For this reason all values of $\Delta G_{N\rightleftharpoons D}$ are calculated at 325.8 K, the T_m of the wild-type protein at pH 4.6 (Cohen & Pielak, 1994). The values of $\Delta G_{N\rightleftharpoons D}$ for the A7L and A7Y variants are 0.21 ± 0.29 and 0.44 ± 0.27 kcal mol^{-1}, respectively.

Determination of $\Delta G_{A\rightleftharpoons D}$. The A ⇌ D transitions are >90% reversible for the wild-type protein and >80% reversible for all but the F10Y variant, which is >70% reversible. Wild-type protein samples placed in the A-state buffer at 60 °C for 3 h give the same bands on SDS−PAGE as protein maintained at native conditions. This indicates that little, if any, peptide bond cleavage occurs that would render the A ⇌ D transition irreversible. Unlike the equine protein (Kuroda et al., 1992), the A ⇌ D transition monitored at 282 nm for the wild-type yeast protein is not significantly

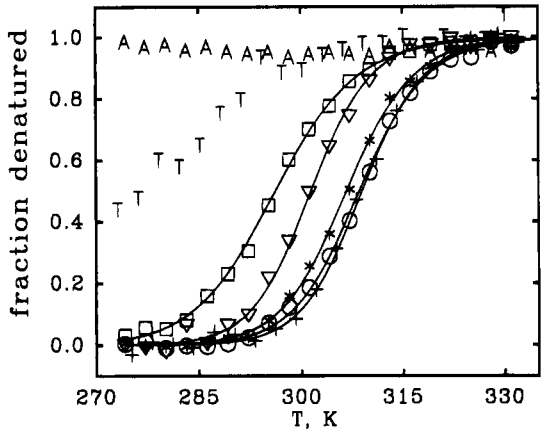

FIGURE 2: Plot of fraction denatured versus temperature for the A ⇄ D transitions of the wild-type protein (○) and the A7Y (+), A7L (*), F10Y (▽), F10W (□), L94T (T), and L94A (A) variants. Every third data point is shown. The curves are fits to a two-state model.

Table 1: Thermodynamic Parameters for the A ⇄ D Transition

protein	$T_m \pm 0.8^a$ (K)	$\Delta H_m \pm 1.2^a$ (kcal mol^{-1})	$\Delta G_{A\rightleftharpoons D,BS}{}^b$ (kcal mol^{-1})	$\Delta G_{A\rightleftharpoons D,EX}{}^c$ (kcal mol^{-1})
wild type	308.4	38.1	0.00 ± 0.14	0.00
A7L	306.7	37.9	−0.21 ± 0.14	−0.25
A7Y	309.0	41.5	0.07 ± 0.14	0.07
L94I	306.7	35.8	−0.21 ± 0.14	−0.10
Y97F	306.3	33.0	−0.26 ± 0.14	−0.25
F10Y	301.4	40.6	−0.86 ± 0.14	−0.93
F10W	296.1	28.9	−1.52 ± 0.15	−1.11
L94T	≤283	i[d]	≤−3.14	
L94A	≤273	nt[e]	≤−4.37	
Y94A	≤273	nt	≤−4.37	

[a] Uncertainties in $\Delta H_{m,A\rightleftharpoons D}$ and $T_{m,A\rightleftharpoons D}$ for the wild-type protein are the sample standard deviation from seven experiments using different protein and buffer preparations. Experiments for the variants were performed once, and standard deviations are assumed to be that of the wild-type protein. [b] Determined using eq 2. [c] Determined as described in the text. [d] Incomplete transition. [e] No transition.

different from that monitored at 222 nm (data not shown). These data are consistent with reversible, two-state denaturation. Plots of fraction denatured versus temperature are shown in Figure 2.

Values of ΔH_m and T_m for the A ⇄ D transition are shown in Table 1. The A state is stable over a narrow pH range. Therefore ΔC_p cannot be obtained by varying pH, thereby precluding the use of eq 1 to calculate $\Delta G_{A\rightleftharpoons D}$. Two methods were used to overcome this obstacle. First, $\Delta G_{A\rightleftharpoons D,EX}$ was obtained by examining plots of $-RT$ ln K_D versus temperature at the T_m of the wild-type protein, where K_D is the fraction of denatured protein divided by the fraction of protein in the A state. Values of $\Delta G_{A\rightleftharpoons D,EX}$ rely solely on the two-state assumption but can only be obtained for proteins which exhibit complete A ⇄ D reactions (see Table 1). Second, $\Delta G_{A\rightleftharpoons D,BS}$ was calculated using eq 2 (Becktel

$$\Delta G_{A\rightleftharpoons D,BS} = \frac{\Delta H_{m,wt}}{T_{m,wt}}(T_{m,var} - T_{m,wt}) \qquad (2)$$

& Schellman, 1987), where wt refers to the wild-type protein and var refers to a variant protein. Values of $\Delta G_{A\rightleftharpoons D,BS}$ rely on more assumptions (Becktel & Schellman, 1987) than do $\Delta G_{A\rightleftharpoons D,EX}$. However, $\Delta G_{A\rightleftharpoons D,BS}$ can be estimated for variant proteins with incomplete or unobservable A ⇄ D reactions.

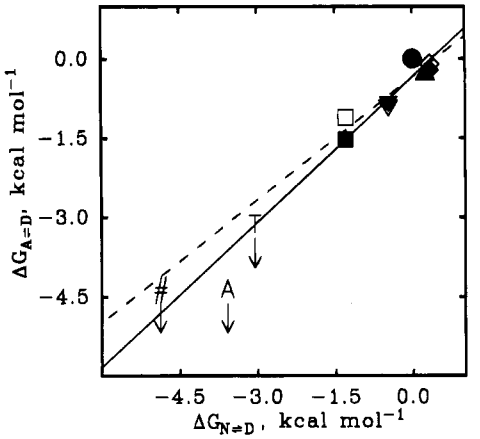

FIGURE 3: Plot of $\Delta G_{A\rightleftharpoons D,BS}$ (filled and alphanumeric symbols) and $\Delta G_{A\rightleftharpoons D,EX}$ (open symbols) versus $\Delta G_{N\rightleftharpoons D}$ for the wild-type protein and the interface variants. Symbols as per Figure 2 with the addition of the L94I (◇), Y97F (△), and Y97A (#) variants. Data are from Table 1 and Pielak et al. (1995). The size of the symbols is approximately the size of the estimated uncertainties. Arrows are shown with approximate values to indicate that these points could be lower. A best-fit line for all the data points (—) has a slope of 0.92 ± 0.06 and an intercept of −0.3 ± 0.1 kcal mol^{-1}. A best-fit line omitting data for the L94T, L94A, and Y97A variants (- - -) has a slope of 0.8 ± 0.1 and an intercept of −0.3 ± 0.1 kcal mol^{-1}.

Uncertainties in $\Delta G_{A\rightleftharpoons D,BS}$ were estimated by applying propagation of error analysis to eq 2.

A Native Tertiary Interaction Stabilizes the A State of Cytochrome c. As shown in Table 1, values of $\Delta G_{A\rightleftharpoons D,EX}$ and $\Delta G_{A\rightleftharpoons D,BS}$ are in good agreement. As shown in Figure 3, $\Delta G_{A\rightleftharpoons D,EX}$ and $\Delta G_{A\rightleftharpoons D,BS}$ follow the same trend as $\Delta G_{N\rightleftharpoons D}$. Relative to the denatured states, amino acid substitutions at the helix-helix interface affect the native state and the A state to similar degrees because the slopes of the lines in Figure 3 are near unity.[1] The A state and the native state of iso-1-cytochrome *c* possess nearly the same amount of α-helix (Figure 1). Jeng and Englander (1991) show that, in the equine protein, the helix content of the A state is present in native-like helices. Taken together, these results show that the interaction between the helices stabilizes both the A state and the native state.

As a control, we examined variants with amino acid substitutions at position 7, a solvent-exposed residue in the N-terminal helix that is close to, but not involved in, the interface. The A7L and A7Y mutations do not have a significant effect on the stability of either the native state or the A state. The analogous mutations at the interface, L94A and Y97A, are the most destabilizing. Thus, the destabilizing effect is specific for substitutions at the interface. The simplest explanation for these observations is that specific interactions between residues 10, 94, and 97 in the native state are also present in the A state. This is in accord with the classification of this A state as a native-like molten globule (Fink et al., 1994).

In summary, we provide direct evidence that an A state is stabilized by a specific and evolutionarily conserved, native tertiary interaction, providing an explanation for the observation that the A state of cytochrome *c* folds to the native state

[1] Because some $\Delta G_{A\rightleftharpoons D}$ values in Figure 3 are upper limits, it is possible that the substitutions have a larger effect on the A state than on the native state.

in one kinetic phase with a submillisecond time constant (Sosnick et al., 1994).

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