Direct Detection of Heat and Cold Denaturation for Partial Unfolding of a Protein

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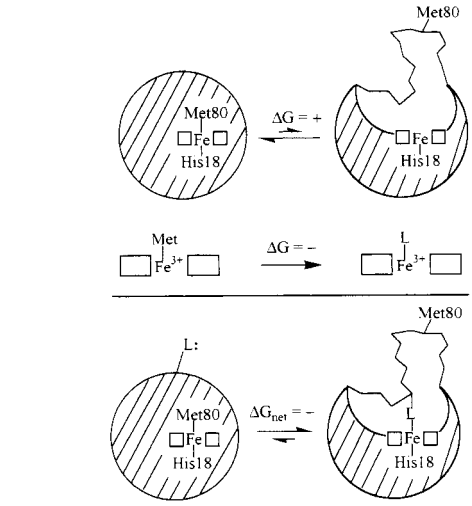
Partially unfolded states of proteins are an important focus of research in the investigation of protein folding due to their presumed role as intermediates in the folding of proteins from the denatured state to the native state.[1] Interest in such states has intensified of late because of the apparent role of partially unfolded proteins in aggregation in protein misfolding diseases.[2] It is difficult to significantly populate partially unfolded states of proteins near physiological pH. Normally, to stabilize such states, extremes of pH must be used, as for the molten globule state which is stabilized at low pH and high salt[3] or the alkaline conformer of cytochrome *c*.[4] Partially unfolded states of proteins have also been observed indirectly using NMR-detected hydrogen/deuterium (H/D) exchange experiments.[5] Here, we describe direct detection of heat and cold denaturation for partial unfolding of yeast iso-1-ferricytochrome *c*, at pH 7.5.

Recently, we have been developing methods to couple a favorable equilibrium to the unfavorable partial unfolding of a protein to allow substantial population of partially unfolded states of proteins.[6] In particular, we have been using the free energy provided by heme ligand exchange reactions to drive partial unfolding, as outlined below (Scheme 1) for cytochrome *c*. When ligand L: is histidine, the methionine → histidine ligand exchange for Fe(III) heme can provide ∼5 kcal/mol of stabilization energy based on data from heme−peptide model systems.[7] In previous work, we have demonstrated that mutation of lysine 73 to histidine in the least stable substructure of cytochrome *c*[5a] stabilizes an equilibrium folding intermediate during guanidine hydrochloride (gdnHCl) unfolding of iso-1-cytochrome *c* at pH 7.5.[6,8] In the present investigation, we determine the temperature dependence of the population of this partially unfolded state.

Since this partial unfolding involves loss of the Met 80 heme ligand (Scheme 1), the conformational transition can be monitored using the absorbance band at 695 nm, which is characteristic of heme-Met 80 ligation.[9] In Figure 1, the absorbance at 695 nm, A_{695}, is highest near 15 °C. The native (Met 80 ligated heme) state is lost in favor of the partially unfolded (His 73 ligated heme)

Scheme 1. Stabilization of Partially Unfolded Cytochrome *c*



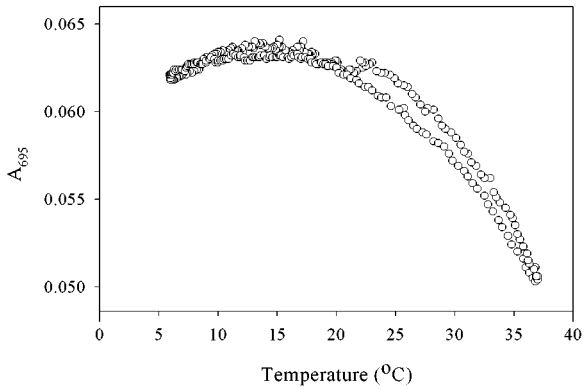


Figure 1. Temperature dependence of partial unfolding of His 73 iso-1-cytochrome *c* monitored at 695 nm in 20 mM Tris, pH 7.5 (25 °C), 40 mM NaCl. Temperature is scanned at 1 °C/min from 20 °C to 5 °C, then from 5 °C to 37 °C and back down to 20 °C.

state[10] either by increasing or decreasing the temperature, indicating heat and cold denaturation. The behavior of the A_{695} band as a function of temperature is similar in the presence of 0.1 to 0.5 M gdnHCl, except that the magnitude of A_{695} becomes progressively smaller as the [gdnHCl] increases (data not shown).

The A_{695} data as a function of temperature and [gdnHCl] were converted into free energy of partial unfolding, ΔG_{obs}, using standard methods (Figure 2).[11] The A_{695} data used to generate the ΔG_{obs} versus T data in Figure 2 were collected with cyclic temperature scans as in Figure 1. Inspection of Figure 2 shows that the partial unfolding reaction is reversible. Since ΔG_{obs} is ∼0 for partial unfolding of His 73 iso-1-cytochrome *c*, ΔG_{obs} can be obtained over a broad temperature range, allowing the curvature due to the heat capacity increment, ΔC_p, to be observed directly. Normally, long extrapolations of temperature-dependent gdnHCl or urea denaturation data are required to obtain ΔC_p from

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(10) Previous studies on the alkaline conformational transition of the His 73 variant of iso-1-cytochrome *c* (ref 13) have confirmed that the heme ligand in the partially unfolded state is a histidine.

(11) The observed equilibrium constant at each temperature for partial unfolding was calculated as $K_{obs} = (\epsilon_N - \epsilon_{695})/(\epsilon_{695} - \epsilon_P)$, where ϵ_N is the extinction coefficient at 695 nm for the native Met 80 ligated state at temperature T, ϵ_P is the extinction coefficient for the partially unfolded state at 695 nm at temperature T, and ϵ_{695} is the observed extinction coefficient at temperature T. Evaluation of ϵ_N and ϵ_P as a function of temperature and [gdnHCl] is described in the Supporting Information. Free energy of partial unfolding is evaluated as $\Delta G_{obs} = -RT \ln K_{obs}$.

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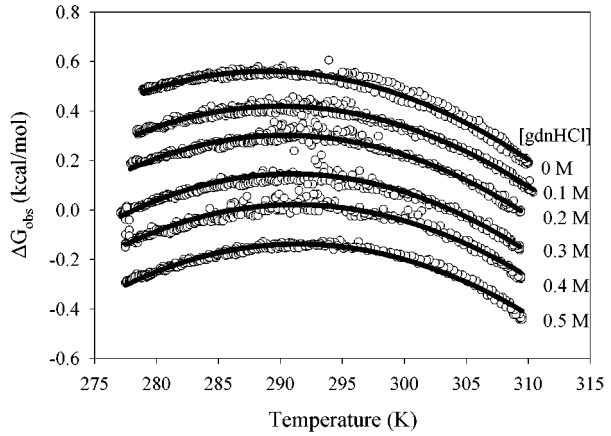

Figure 2. Observed free energy of partial unfolding, ΔG_{obs} (kcal/mol), as a function of temperature (Kelvin) at different [gdnHCl]. The solid lines are fits to eq 1.

Table 1. Thermodynamic Parameters for Partial Unfolding of His 73 Iso-1-cytochrome *c*[a]

[gdnHCl], M	ΔG_{max}, kcal/mol	T_{max}, K	ΔC_p, kcal/(mol·K)
0	0.45 ± 0.09	287 ± 1	0.46 ± 0.03
0.1	0.30 ± 0.08	289 ± 1	0.46 ± 0.03
0.2	0.15 ± 0.09	289 ± 1	0.47 ± 0.04
0.3	-0.01 ± 0.09	290 ± 1	0.48 ± 0.05
0.4	-0.11 ± 0.11	290 ± 1	0.48 ± 0.02
0.5	-0.25 ± 0.11	290 ± 1	0.51 ± 0.04

[a] The parameters are the average of three separate experiments. The error indicated is the standard deviation of the reported value.

ΔG versus T plots for protein conformational transitions.[12] The data in Figure 2 are fit to eq 1,[13] where ΔG_c is the conformational free energy and the second term[14]

$$\Delta G_{obs} = \Delta G_c + RT \ln(1 + 10^{(pK_a - pH)}) \quad (1)$$

accounts for the effect of the histidine protonation equilibrium on ΔG_{obs}. The temperature dependence of eq 1 is predominately in the ΔG_c term (eq 2[15]),[14] and is treated with a modified Gibbs−Helmholtz equation.[16] The parameters from fitting the data to eq

$$\Delta G_c = \Delta G_{max} + \Delta C_p\{T - T_{max} - T \ln(T/T_{max})\} \quad (2)$$

1 are given in Table 1. The maximal value of ΔG_c, ΔG_{max}, decreases monotonically with [gdnHCl], as expected. The values of ΔC_p and T_{max} are, within error, invariant as a function of [gdnHCl].

The magnitude of ΔC_p is expected to reflect the extent of buried surface area exposed to solvent when a protein unfolds.[17] Thus, our experimental ΔC_p can be used to evaluate the degree of unfolding of this partially unfolded form of iso-1-cytochrome *c*. The ΔC_p for full unfolding of wild-type iso-1-cytochrome *c* is 1.40 ± 0.06 kcal/(mol·K),[18] suggesting that about one-third of the buried hydrophobic surface area of iso-1-cytochrome *c* is exposed in the partially unfolded state of His 73 iso-1-cytochrome *c*. In previous work on the pH and gdnHCl dependence of the formation of this partially unfolded form of His 73 iso-1-cytochrome *c*, the gdnHCl *m* value was about one-third the value for full unfolding of this protein.[8,13] GdnHCl *m* values also correlate with the exposure of buried surface area coincident with a protein conformational transition.[17b,19] Thus, the partially unfolded state of iso-1-cytochrome *c* stabilized by the His 73 variant appears to be the same whether generated by heat or gdnHCl denaturation methods. The invariance of ΔC_p with [gdnHCl] is also consistent with this conclusion. It has been argued that fully unfolded states of proteins generated by heat or solvent denaturation methods are thermodynamically equivalent.[20] Our data show that partially unfolded states of proteins behave similarly with respect to solvent and heat denaturation.

Unfolding of the least stable substructure of cytochrome *c*, which is presumably stabilized by the His 73 mutation, only encompasses ~15% of the amino acid sequence of cytochrome *c*.[5a] It is perhaps surprising that both heat and gdnHCl denaturation methods are consistent with exposure of one-third of the buried surface of iso-1-cytochrome *c*. The gdnHCl *m* value for the least stable substructure of cytochrome *c* obtained by H/D exchange methods is also high relative to the number of amino acid residues in the substructure.[5a] However, the partial unfolding of a substructure exposes not only the residues in the substructure to water, but also the buried surfaces of adjacent substructures that are packed against the portion of the protein which has become unstructured (see Scheme 1). Thus, fractional surface exposure significantly greater that the fractional number of amino acid residues in the unfolded substructure is not unreasonable.

In conclusion, coupling ligand exchange free energy to partial unfolding of cytochrome *c* produces a stabilized partially unfolded state that is amenable to careful thermodynamic analysis over a broad temperature range. This system will permit careful studies of cooperativity between protein substructures and provides a well-defined model system to study the aggregation behavior of partially unfolded states of proteins near physiological pH, potentially affording new insight into aggregation in protein misfolding diseases.

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Supporting Information Available: Parameters for deriving native and partially unfolded state baselines at 695 nm as a function of temperature and [gdnHCl] and methodology for assessing the temperature dependence of the (pK_a−pH) term in eq 1 (PDF). This material is available free of charge via the Internet at http://pubs.acs.org.

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(14) The pK_a of the group causing partial unfolding of His 73 iso-1-cytochrome *c* has been shown to be 6.7 ± 0.1 in previous work, ref 13. This value is used for the pK_a in eq 1. The pK_a of histidine and Tris buffer are both temperature dependent. An equation for the expected temperature dependence of the (pK_a − pH) term in eq 1 is derived in the Supporting Information.
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