Effect of Redox State on the Folding Free Energy of a Thermostable Electron-Transfer Metalloprotein: The Cu_A Domain of Cytochrome Oxidase from *Thermus thermophilus*[†]

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ABSTRACT: The unfolding of the Cu_A domain of cytochrome oxidase from the thermophilic bacterium *Thermus thermophilus*, induced by guanidine hydrochloride (GuHCl)[1] at different temperatures, has been monitored by CD as well by electronic absorption (with the oxidized protein) and by fluorescence (with the reduced protein). The same unfolding curves were obtained with the different methods, providing evidence for a two-state model for the unfolding equilibrium. This was also supported by the shape of the unfolding equilibrium curves and by the observed refolding of the unfolded, oxidized protein on dilution of the denaturant. The oxidized protein cannot be unfolded by GuHCl at room temperature, and it was found to be thermally very stable as well, since, even in the presence of 7 M GuHCl, it is not fully unfolded until above 80 °C. For the reduced protein at room temperature, the unfolding equilibrium curve yielded a folding free energy of −65 kJ/mol. The corresponding value for the oxidized protein (−85 kJ/mol) could be estimated indirectly from a thermodynamic cycle connecting the folded and unfolded forms in both oxidation states and the known reduction potentials of the metal site in the folded and unfolded states; the potential is increased on unfolding, consistent with the higher folding stability of the oxidized form. The difference in folding stability between the oxidized and reduced proteins (20 kJ/mol) is exceptionally high, and this is ascribed to the unique structure of the dinuclear Cu_A site. The unfolded, reduced protein was found to refold partially on oxidation with ferricyanide.

Binding a prosthetic metal ion usually stabilizes the native conformation of a metalloprotein (*1*, *2*). If the protein is redox active, the degree of stabilization depends on the oxidation state of the metal ion. As a corollary, the reduction potential of the metal ion will be different in the folded and unfolded forms of the protein. For example, reduced cytochrome *c* has a larger folding free energy than the oxidized protein, and the heme in the unfolded protein has a lower reduction potential than in native cytochrome *c* (*3*). Similar behavior has been observed for two other heme proteins, cytochrome b_{552} (*4*) and myoglobin (*5*) in which, unlike cytochrome *c*, the porphyrin is not covalently attached to the protein. The blue copper protein, azurin, on the other hand, has a larger folding free energy in its oxidized form, and in this case the metal ion in the unfolded protein has a higher potential than in the native fold (*2*, *6*).

We have now investigated the energetics of folding of another copper protein, the soluble Cu_A domain of cytochrome oxidase from *Thermus thermophilus* (*7*). This is a unique metalloprotein in that the redox center in the oxidized protein is a mixed-valence Cu(I)−Cu(II) complex in which the unpaired electron is completely delocalized over the two copper atoms (*8*, *9*). Despite the uniqueness of the active site, Cu_A domains have the β-barrel cupredoxin topology of blue copper proteins (*10*), with some additional α-helical structure, as first suggested by sequence alignments (*11*) and far-UV CD measurements (*12*) and later established by X-ray crystallography (*13*). Since the folding of β-sheet proteins has not been investigated extensively (*2*, *14*, *15*), there is an additional reason to study a Cu_A domain. Furthermore, the *Thermus* domain, being derived from a thermophilic bacterium, is expected to exhibit unusual conformational stability.

We have unfolded the Cu_A domain by thermal denaturation and by a chemical denaturant, guanidine hydrochloride (GuHCl).[1] The kinetics and equilibrium of the process have been followed by far-UV CD. The unfolding was also monitored by electronic absorption in the visible spectral region in the case of the oxidized protein and by tryptophan fluorescence with the reduced protein. The GuHCl concentrations required to cause unfolding were found to be the

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[1] Abbreviations: GuHCl, guanidine hydrochloride; CD, circular dichroism; NHE, normal hydrogen electrode.

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same by the different methods, demonstrating that the equilibrium can be regarded as a two-state process. The oxidized protein is not unfolded by GuHCl at room temperature, even at the highest concentrations solubility allows, but it can be unfolded by GuHCl at 75 °C. At this temperature, the oxidized Cu_A domain is considerably more stable than the reduced protein (free energies of folding of −45 and −18 kJ/mol, respectively). At room temperature, the folding free energy for reduced Cu_A is −65 kJ/mol, which is much more negative than those of both oxidized and reduced azurin (−52 and −40 kJ/mol, respectively) (*2*), confirming the exceptional stability of this dinuclear copper protein. The difference in folding stability between the oxidized and the reduced Cu_A domain at 75 °C is also exceptional, being about twice that for the two redox states of azurin at room temperature, which may be related to the dinuclear nature of the Cu_A site. Our results predict that the unfolded protein will have a higher reduction potential than the native Cu_A domain, and this has been confirmed by electrochemical measurements (*6*). Finally, we have found that the unfolded, reduced protein is partially refolded upon oxidation with ferricyanide, opening up the possibility of triggering protein folding by rapid, laser-induced electron transfer (*3*, *16*).

MATERIALS AND METHODS

Protein Preparation and Chemicals. The Cu_A domain was expressed and purified as described earlier (*7*). The protein concentration was determined spectrophotometrically, using an extinction coefficient of 3240 M^{-1} cm^{-1} at 530 nm for the oxidized form. GuHCl (of highest grade), sodium dithionite, and potassium ferricyanide were obtained from Sigma.

Thermal Unfolding. Both the oxidized and the reduced forms of the Cu_A domain were thermally unfolded. The CD signal at 218 nm was followed as the temperature was raised in steps of 2 °C, from 20 to 100 °C, with an equilibration time of 2 min at each temperature. A 20 μM concentration of protein, oxidized or reduced (with a slight excess of dithionite), was used in 5 mM phosphate buffer, pH 7.0. The cell employed had a 1-mm path length, and the CD signal was measured in a Model 62A DS Aviv spectropolarimeter equipped with a programmable thermoelectric temperature controller.

Unfolding by a Chemical Denaturant. GuHCl was used in titrations to induce unfolding of the oxidized and reduced Cu_A domain, both at room temperature (20 °C) and at 75 °C. The protein concentration, buffer, and CD cell were the same as in the thermal unfolding experiments. Various GuHCl concentrations, ranging from 0 to 8 M (the solubility limit), were introduced into the protein samples, which were equilibrated for about 10 min in the room-temperature experiments and for 4 min in the 75 °C experiments. Kinetic CD experiments at 220 nm after manual mixing of the protein with GuHCl showed equilibrium to have been established within these time spans. CD was then measured between 200 and 300 nm, and each spectrum was an average of three or four scans. For the room-temperature experiments, we used a Jasco-600 spectropolarimeter, and, for the high-temperature titrations, the Aviv spectropolarimeter (see above).

The GuHCl-induced unfolding of the reduced Cu_A domain was also studied by tryptophan fluorescence at room temperature. The protein molecule contains one tryptophan residue. Samples of the reduced Cu_A domain (1 μM), under identical conditions as in the room-temperature CD experiments, were investigated in a 1-cm cell in a Hitachi F-4500 spectrofluorimeter with excitation at 280 nm, 5-nm bandpass for excitation as well as emission light, and emission detected between 300 and 400 nm. Upon unfolding of the protein, the tryptophan fluorescence is largely quenched due to exposure to a hydrophilic environment.

Since the electronic absorption of the oxidized Cu_A domain is readily observable, with distinct maxima in the 450−600 nm region, we could also follow the GuHCl-induced unfolding of the oxidized protein by absorbance changes. In these experiments, 30 μM protein, but otherwise identical conditions as in the CD experiments, was used with a 1-cm cell in a Hewlett-Packard 8452 diode array spectrophotometer. Unfolding experiments were performed both at room temperature and at 75 °C (with the use of a thermostatable cell holder). At room temperature, only a slight loss of color could be detected after several hours in 8 M GuHCl. At 75 °C, the samples were incubated for 4 min before measurements, the same time as in the corresponding CD measurements at this temperature.

Calculations of Thermodynamic Parameters. The unfolding curves (fraction folded as a function of GuHCl concentration) were analyzed in terms of a two-state model (F and U represent folded and unfolded proteins, respectively):

$$\mathrm{F} \rightleftarrows \mathrm{U} \tag{1}$$

From the standard thermodynamic relationship, $\Delta G^\circ = -RT \ln K$ (where ΔG° is the change in standard free energy, R the gas constant, T the absolute temperature, and K the equilibrium constant for the reaction in eq 1), we could calculate ΔG° as a function of GuHCl concentration. Since this is a linear function (*17*), extrapolation to zero GuHCl concentration yields the folding free energy.

From each thermal unfolding curve, the value of the standard enthalpy change [$\Delta H(T_m)$] at the temperature of the transition midpoint (T_m) was calculated from a modified van't Hoff equation. Both the enthalpy and entropy changes associated with protein unfolding depend on temperature (*18*). Thus, the experimental ln K values were corrected for the temperature dependences of both $\Delta H(T_m)$ and $\Delta S(T_m)$, assuming the change in heat capacity (ΔC_p) to have a constant value of 7.3 kJ mol^{-1} K^{-1} for the Cu_A domain; this estimate is the per residue value of 50 J mol^{-1} K^{-1} $residue^{-1}$ (*19*) multiplied by 146, the number of amino acid residues in the Cu_A domain (*7*). The corrections to the ln K values were based on the following equations:

$$\Delta H(T) = \Delta H(T_m) + \int_{T_m}^{T} \Delta C_p \, dT = \Delta H(T_m) + \Delta C_p (T - T_m) \tag{2}$$

$$\Delta S(T) = \frac{\Delta H(T_m)}{T_m} + \int_{T_m}^{T} \Delta C_p \, dT = \frac{\Delta H(T_m)}{T_m} + \Delta C_p \ln \frac{T}{T_m} \tag{3}$$

$$\ln K = \frac{\Delta H}{-RT} - \frac{T\Delta S}{-RT} = -\frac{\Delta H(T_m)}{RT} - \frac{\Delta C_p(T - T_m)}{RT} + \frac{\Delta H(T_m)}{RT_m} + \frac{\Delta C_p}{R}\ln\frac{T}{T_m} \quad (4)$$

$$\ln K^{corr} = \ln K + \frac{\Delta C_p(T - T_m)}{RT} - \frac{\Delta C_p}{R}\ln\frac{T}{T_m} = \frac{\Delta H(T_m)}{RT_m} - \frac{\Delta H(T_m)}{RT} \quad (5)$$

A plot of ln K^{corr} vs $1/T$ should give a straight line with a slope of $-\Delta H(T_m)/R$. At T_m, ln $K = 0$, so that $\Delta S(T_m)$ can be calculated. Without corrections to the ln K values, the van't Hoff plots are slightly curved, reflecting the temperature dependences of the parameters involved. After correction, on the other hand, the data points could be fitted to straight lines, suggesting that our estimated value of ΔC_p is reasonable.

RESULTS

Unfolding the Cu_A Domain at Room Temperature. The folded, oxidized Cu_A domain has intense electronic absorption in the visible and near-IR region with maxima at 480, 530, and 790 nm. Unfolding the protein leads to disappearance of these absorption peaks. By contrast, the reduced Cu_A domain exhibits no visible absorption at all, in either a folded or unfolded state. Thus, visible spectra can only be used to monitor unfolding of the oxidized protein. In attempts to unfold the oxidized domain at room temperature with a chemical denaturant, no unfolding was observed up to 8 M GuHCl, the solubility limit of this chemical.

CD in the far-UV region probes protein secondary structure and hence is an excellent tool for following protein unfolding. This technique can be applied to both the oxidized and the reduced states of the Cu_A domain. GuHCl titrations revealed again that the oxidized protein could not be unfolded by 8 M GuHCl, as shown in Figure 1A. The reduced form of the Cu_A domain could, however, be unfolded by additions of GuHCl, with a midpoint of the unfolding transition at 6.5 M GuHCl (Figure 1A).

There is one tryptophan residue in the amino acid sequence of the Cu_A domain (*7*). This amino acid has fluorescence in a hydrophobic environment, such as in the folded protein, but the emission is quenched upon exposure to a more hydrophilic environment, such as the aqueous solvent. By following the quenching of the tryptophan fluorescence, the unfolding of the reduced Cu_A domain by GuHCl at room temperature could be evaluated. The results with this technique were identical with the CD-monitored unfolding curve (Figure 1A), as shown in Figure 2.

A linear dependence of the unfolding free energy on the GuHCl concentration was found, as illustrated in Figure 1B, and from this the free energy of unfolding of the reduced Cu_A domain in water was estimated to be 65(10) kJ/mol. Using this value for unfolding of the reduced protein, and the reduction potential for the folded Cu_A domain (*20*) as well as that for the unfolded protein (*6*), a scheme connecting the folded and unfolded forms in both redox states can be drawn (Figure 3). From this scheme, the free energy of unfolding of the oxidized Cu_A domain at room temperature was calculated to be 85(10) kJ/mol in aqueous solution. The difference in folding stability between the reduced and oxidized forms of the protein is thus 20 kJ/mol (Table 1).

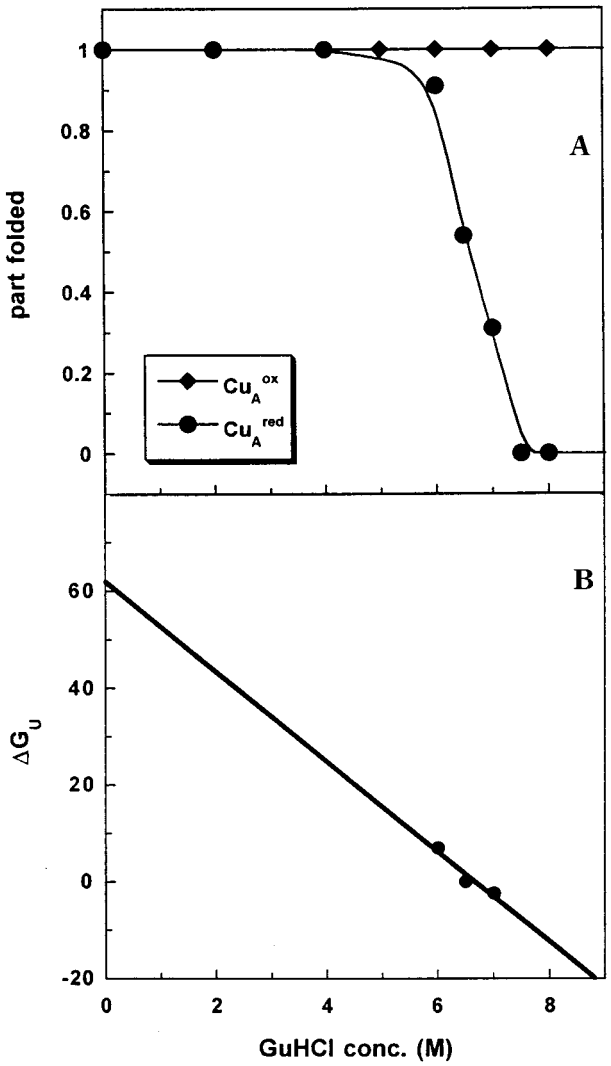

FIGURE 1: (A) GuHCl-induced unfolding of the oxidized and reduced forms of the Cu_A domain at room temperature, as monitored by CD; no unfolding of the oxidized protein could be observed, even at the highest GuHCl concentration. B. Plot of free energy (ΔG_U) versus GuHCl concentration for unfolding the reduced Cu_A domain at room temperature, extracted from the unfolding curve in panel A.

Unfolding the Cu_A Domain at Higher Temperatures. Since the oxidized Cu_A domain does not unfold at room temperature, we performed experiments at various GuHCl concentrations at higher temperatures (Table 2). We found that, in the presence of 7 M GuHCl, the oxidized protein is fully unfolded at temperatures above 80 °C. Unfolding curves obtained from CD-monitored GuHCl titrations on both oxidized and reduced states of the Cu_A domain at 75 °C are shown in Figure 4A, and the corresponding free-energy plots are shown in Figure 4B. The reduced Cu_A domain shows a midpoint of the unfolding transition at about 3 M GuHCl, whereas the oxidized protein has a midpoint of unfolding at 6.3 M GuHCl. The GuHCl-induced unfolding of the oxidized Cu_A protein was also analyzed by visible absorption at this temperature, as shown in Figure 5. The unfolding transition detected in this way (6.4 M) agreed well with that obtained by CD (Figure 4A).

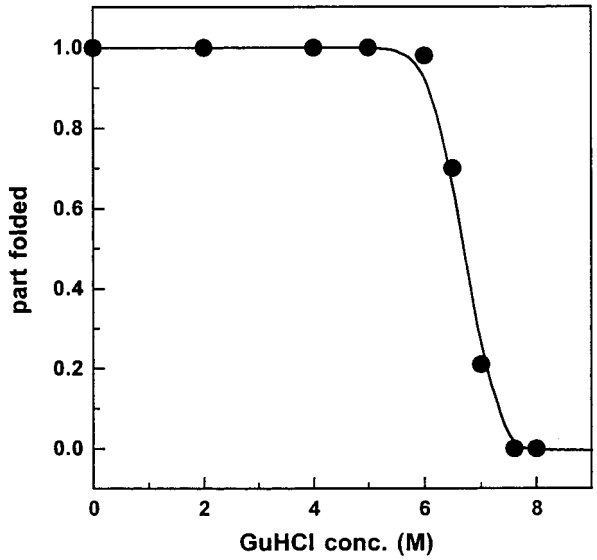

FIGURE 2: GuHCl-induced unfolding of the reduced form of the Cu_A domain at room temperature, as monitored by quenching of the tryptophan fluorescence.

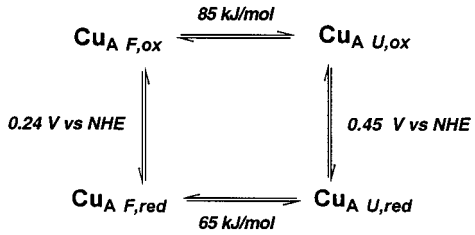

FIGURE 3: Thermodynamic cycle incorporating four states (oxidized and reduced, folded and unfolded) of the Cu_A domain. The experimental ΔG value, obtained for unfolding the reduced protein, in combination with the electrochemical potentials for the folded and unfolded forms of the protein, can be used to estimate ΔG for unfolding the oxidized protein at room temperature.

Table 1: Free Energies (kJ/mol) of Unfolding the CuA Domain in Aqueous Solution[a]

	20 °C	75 °C
ΔG Cu_A^{ox}	85(10)[b]	45(6)
ΔG Cu_A^{red}	65(10)	18(3)
$\Delta(\Delta G)$	20	27

[a] Estimated by extrapolation of the lines in Figures 1B and 4B to 0 M GuHCl concentration. [b] Calculated from the electrochemical potentials of the folded and unfolded states of the protein and the free energy of unfolding the reduced protein (Figure 3).

Table 2: Melting Temperatures for Thermal Unfolding of the Two Redox States of the Cu_A Domain at Various GuHCl Concentrations[a]

	T_m (°C)		
	0 M GuHCl	5.5 M GuHCl	7.0 M GuHCl
Cu_A^{ox}	>100	80	70
Cu_A^{red}	83	40	<20

[a] (T_m is the midpoint of the CD-detected transition).

The free-energy values for unfolding in water, extrapolated from the graph in Figure 4B, are 45(6) (oxidized) and 18(3) kJ/mol (reduced) for the two redox states of the protein. Thus, the absolute free energies are lower for both oxidation states at 75 °C compared to room temperature, which is expected. The difference in folding stability between the reduced and oxidized states of the protein is 27 kJ/mol at 75 °C (Table 1).

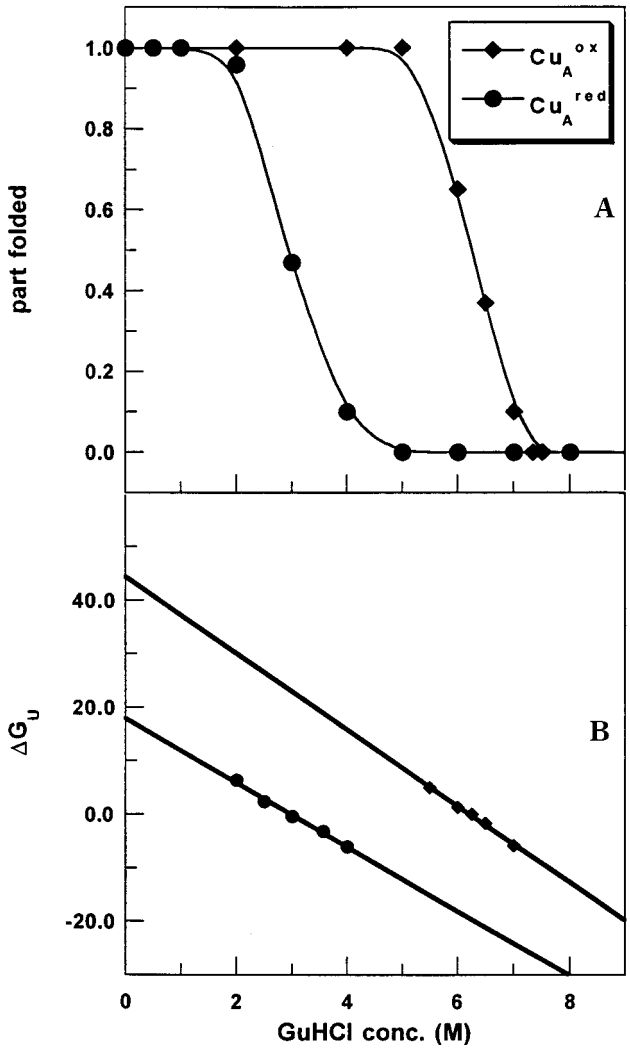

FIGURE 4: (A) Unfolding the oxidized and reduced states of the Cu_A domain at 75 °C. The data for unfolding were obtained from CD measurements. (B) Plot of the linear relation between free energy (ΔG_U) and GuHCl concentration for unfolding the oxidized and reduced states of the Cu_A domain at 75 °C. Extrapolation to 0 M GuHCl gives the free energy for protein unfolding in water.

The unfolding transitions for both the oxidized and reduced forms of the Cu_A domain are broader at 75 °C than the unfolding transition of the reduced form at room temperature. The breadth of the transition is related to the slope of the free energy plot (Figure 4B); the lower the slope, the broader the unfolding transition. The slopes are in the range of 6−7 kJ mol^{-1} M^{-1} for both oxidized and reduced states of the protein at 75 °C (Figure 4B), but 10 kJ mol^{-1} M^{-1} for the reduced protein at room temperature (Figure 1B). These broader unfolding curves at the higher temperature correspond to less cooperativity in the unfolding process at this temperature compared to room temperature.

We tried to unfold both the oxidized and reduced forms of the Cu_A domain by increasing the temperature at different constant GuHCl concentrations. We used CD in the far-UV region to monitor the amount of folded protein as the temperature was slowly raised from 20 to 100 °C in steps of 2 °C every 2 min. The midpoint temperatures of the unfolding transitions for the different samples are set out in Table 2.

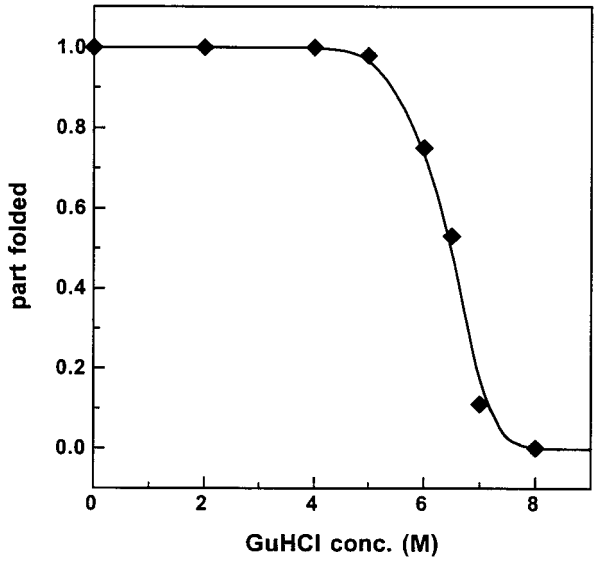

FIGURE 5: Unfolding the oxidized form of the Cu_A domain at 75 °C; the reaction was followed by changes in absorption at 530 nm.

Table 3: Standard Enthalpy [$\Delta H(T_m)$] and Entropy Changes [$\Delta S(T_m)$] at the Temperature of the Transition Midpoint (T_m) for Thermal Unfolding of the Two Redox States of the Cu_A Domain Monitored by CD at Various GuHCl Concentrations

	0 M GuHCl	5.5 M GuHCl	7.0 M GuHCl
Cu_A^{ox}			
$\Delta H(T_m)$ (kJ/mol)		225(5)	165(5)
$\Delta S(T_m)$ (J mol^{-1} K^{-1})		637(15)	481(15)
Cu_A^{red}			
$\Delta H(T_m)$ (kJ/mol)	322(5)	188(5)	
$\Delta S(T_m)$ (J mol^{-1} K^{-1})	904(15)	599(15)	

The melting temperature for both the oxidized and reduced forms of the protein decreases with increasing GuHCl concentration. The extreme folding stability of the oxidized Cu_A domain is illustrated by the fact that, in the presence of 7 M GuHCl, T_m is still as high as 70 °C; at this GuHCl concentration, the reduced protein is not folded, even at room temperature.

The thermodynamic parameters at the temperature of the transition midpoint (Table 2), derived from the thermal unfolding data at different concentrations of GuHCl, are set out in Table 3. The values have been corrected for the temperature dependences of both ΔH and ΔS, with the use of an estimated value of ΔC_p, based on the nonpolar surface area exposed by unfolding and the number of amino acid residues in the Cu_A domain, as described in detail in Materials and Methods. Both enthalpy and entropy changes associated with unfolding decrease drastically with increasing concentrations of GuHCl for the oxidized as well as the reduced Cu_A domain. The melting temperatures for the oxidized and reduced protein are different in 5.5 M GuHCl (Table 2, 80 vs 40 °C), whereas the enthalpy and entropy changes are quite similar (Table 3). By contrast, there are substantial differences in the ΔH and ΔS values for the oxidized and reduced proteins at similar melting temperatures (ca. 80 °C) but very different GuHCl concentrations (reduced at 0 M GuHCl; oxidized at 5.5 M GuHCl).

Refolding the Cu_A Domain. After unfolding by 7 M GuHCl at 75 °C, the oxidized protein refolded when diluted with buffer to lower GuHCl concentrations, as judged by electronic absorption measurements. Since the two forms of the Cu_A protein display different folding stabilities, a change in redox state should, under certain conditions, induce either folding or unfolding. To test oxidation-triggered folding, we treated a sample of the unfolded, reduced protein in 7.5 M GuHCl at room temperature with ferricyanide, whose reduction potential is about 0.5 V versus NHE at high ionic strength (*21*). Between 30 and 40% of the reduced Cu_A molecules folded to a native structure, estimated by absorption at 530 nm attributable to the oxidized, folded Cu_A domain (*7*), as shown in Figure 6; the absorption at 480 nm was masked by absorption from ferricyanide.

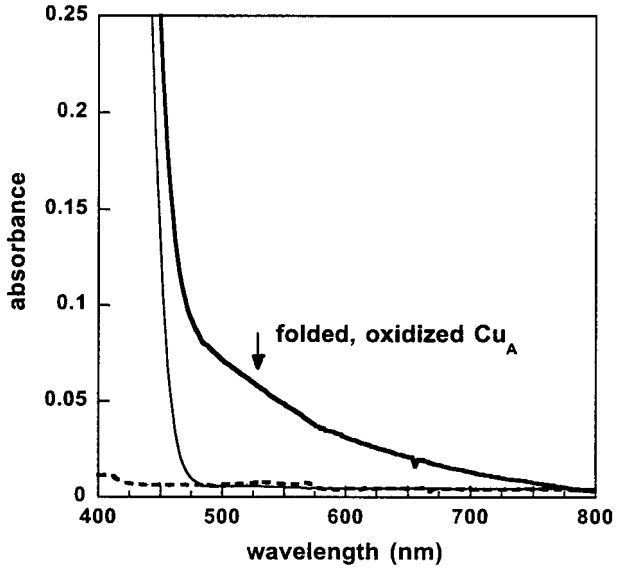

FIGURE 6: Electronic absorption spectra (dotted line, reduced unfolded Cu_A domain; thin line, ferricyanide alone; thick line, reduced unfolded Cu_A domain plus ferricyanide) show that the oxidized Cu_A domain refolds upon oxidation of the reduced, unfolded protein by ferricyanide in 7.5 M GuHCl.

DISCUSSION

Several of our observations suggest that the unfolding of both the oxidized and reduced forms of the Cu_A domain can be described as a two-state process (eq 1). First, unfolding the oxidized protein has been shown to be reversible. Second, there is a linear dependence of the unfolding free energy on the GuHCl concentration (Figures 1B and 4B). Third, the same unfolding curves are obtained by CD (Figures 1 and 4), by electronic absorption (oxidized protein) (Figure 5), and by tryptophan fluorescence quenching (reduced protein) (Figure 2).

The *Thermus* Cu_A domain possesses unusual stability against both thermal and chemical unfolding (Tables 1 and 2). The value of the unfolding free energy estimated for the oxidized protein in water at room temperature (85 kJ/mol) is almost double that for a related redox copper protein, azurin (*2*). Thus, even if binding of the metal in a metalloprotein stabilizes the native conformation (*1*), this can only contribute a small part to the extreme stability. The overall secondary structure is no different in the *Thermus* Cu_A protein compared to other Cu_A domains, as judged from far-UV CD spectra (*7*, *12*). It is likely, then, that the high folding stability of the *Thermus* domain is attributable to a combination of several small effects, such as replacement of strained conformations by glycines and an increase in the

number of hydrogen bonds, as is the case for thermostable iron−sulfur proteins (*22*, *23*). Replacement of some of these hydrogen bonds by GuH^+ interactions could be responsible for the lowered enthalpy change on unfolding in the presence high concentrations of GuHCl (Table 3). The corresponding decrease in the entropy change is probably attributable to the ordering of more GuH^+ ions around exposed hydrophobic groups in the unfolded state.

The folding stability of *Thermus* cytochrome c_{552}, which is comparable to that of the *Thermus* Cu_A domain, has been attributed to a small entropy change on unfolding, as judged from a flattened unfolding curve (*24*). It may be noted that we observe a broadened unfolding curve for the *Thermus* Cu_A domain and a corresponding low slope in the free energy plot at 75 °C (Figure 4), whereas the unfolding curve at 20 °C for the reduced protein is much steeper (Figure 1). It is thus interesting that the optimal temperature for the growth of the *T. thermophilus* bacterium is around 75 °C (*25*).

As in the case of azurin (*2*), the oxidized Cu_A domain has a higher folding free energy than the reduced protein. The difference between the two redox states at room temperature (20 kJ/mol) is almost twice that for azurin (12 kJ/mol). This can hardly be related to the high absolute folding stability of the protein but must be caused by the unique nature of the dinuclear Cu_A site. The axially ligated diamond structure of this site is expected to be less stable in the reduced form, because both the bonds to the bridging sulfurs and to the axial ligands would be weakened. Thus, it is the native fold, or protein rack, that keeps the site intact (*1*), as evidenced by the fact that there are minimal changes in bond lengths on reduction (*26*). The folded protein both excludes water from the site and provides a restricted ligand set, as discussed in detail elsewhere (*6*).

A consequence of the greater folding stability of the oxidized state of the Cu_A domain is an increase in reduction potential on going to the unfolded form (Figure 3). This increase is as large as 0.21 V compared to 0.13 V in azurin, in agreement with the larger difference in unfolding free energy between the two redox states of the Cu_A domain. As the unfolded protein has a higher potential than the native form, the effect of folding is, in this case, to decrease the potential of the redox site (cf. ref 6).

The high potential of the unfolded protein must also mean that the copper ions are still attached to the protein in the unfolded state, since the aquo Cu^{2+} ion has a relatively low reduction potential; binding of the metal to the unfolded state has been demonstrated previously for the blue-copper protein azurin (*15*). The attachment of the metal in the unfolded form of the *Thermus* domain is also supported by refolding experiments.

Finally, it should be mentioned that the different stabilities of the oxidized and reduced forms of the Cu_A domain should make it possible to trigger protein folding by laser-induced electron transfer, a new technique for studying rapid folding events (*3*, *16*). At 7.5 M GuHCl, the reduced protein is completely unfolded, whereas the oxidized form is completely folded (Figure 1A). Our experiments with ferricyanide demonstrate that refolding is induced on oxidation, and this provides additional evidence for the conclusion that the copper ions are still attached to the protein in the unfolded state (Figure 6). For rapid kinetics experiments, photochemical extraction of an electron from the reduced, unfolded form may be used to initiate folding, and such experiments are in progress.

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