Thermostability of Proteins: Role of Metal Binding and pH on the Stability of the Dinuclear Cu_A Site of *Thermus thermophilus*

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ABSTRACT The dinuclear copper center ($TtCu_A$) forming the electron entry site in the subunit II of the cytochrome *c* oxidase in *Thermus thermophilus* shows high stability toward thermal as well as denaturant-induced unfolding of the protein at ambient pH. We have studied the effect of pH on the stability of the holo-protein as well as of the apo-protein by UV-visible absorption, far-UV, and visible circular dichroism spectroscopy. The results show that the holo-protein both in the native mixed-valence state as well as in the reduced state of the metal ions and the apo-protein of $TtCu_A$ were extremely stable toward unfolding by guanidine hydrochloride at ambient pH. The thermal unfolding studies at different values of pH suggested that decreasing pH had almost no effect on the thermal stability of the protein in the absence of the denaturant. However, the stability of the proteins in presence of the denaturant was considerably decreased on lowering the pH. Moreover, the stability of the holo-protein in the reduced state of the metal ion was found to be lower than that in the mixed-valence state at the same pH. The denaturant-induced unfolding of the protein at different values of pH was analyzed using a two-state unfolding model. The values of the free energy of unfolding were found to increase with pH. The holo-protein showed that the variation of the unfolding free energy was associated with a pK_a of ~5.5. This is consistent with the model that the protonation of a histidine residue may be responsible for the decrease in the stability of the holo-protein at low pH. The results were interpreted in the light of the reported crystal structure of the protein.

INTRODUCTION

Proteins from thermophilic organisms, though often very similar in structures to their mesophilic homologs, are much more resistant to thermal as well as chemical denaturation (1–4). Several studies have identified a number of potential interactions that may be responsible for the observed thermostability of the protein (5,6). An increase in the hydrogen bonds (7), presence of additional electrostatic interactions and salt bridges (3,8), enhanced hydrophobic interactions (5,9), increased compactness (10), stronger binding of metal ions (11,12), etc., have been proposed to play important roles in the high thermostability of the protein.

Systematic studies on the pH dependence of chemical and thermal stability of thermostable proteins can provide us important information on the role of protonation-deprotonation of certain amino acids in imparting thermostability to the protein. We have investigated the effect of pH on the stability of the thermostable Cu_A site ($TtCu_A$) from the subunit II of the ba_3-type cytochrome *c* oxidase of *Thermus thermophilus*. The Cu_A site is a unique metalloprotein in which the redox center of the oxidized protein contains a mixed-valence [Cu(1.5)-Cu(1.5)] complex in which the unpaired electron is completely delocalized over the two copper atoms (13–15). These two copper ions are coordinated by two cysteine residues forming thiolate bridges, two histidine residues along with the weakly bound methionine (sulfur) and glutamate (peptide carbonyl) residues. The strong charge-transfer between S(Cys)-Cu and N(His)-Cu gives rise to a typical purple color of the protein and a characteristic absorption spectrum in the visible range. The Cu_A site has a β-barrel cupredoxin topology analogous to that observed in most electron transfer copper proteins such as the blue copper proteins (16,17). Unfolding studies on the β-barrel containing proteins are the subject of intensive studies in recent years (18,19). Although there are several reports on the stability of both thermal as well as denaturant-induced unfolding of various thermostable proteins (1,15,20), no systematic studies on the effect of pH on the thermodynamic stability of any thermostable β-barrel protein have been reported.

We have studied equilibrium unfolding of the $TtCu_A$ by temperature and chemical denaturant (guanidine hydrochloride, i.e., GuHCl). The unfolding of the protein was monitored by electronic absorption in the visible spectral region at different values of pH. Changes in the secondary structure as well as the tertiary structure of the protein were studied by far-UV and visible circular dichroism (CD) spectrometry at different values of pH in the presence of the denaturant or at different temperatures. The role of the metal ion on the stability of the protein was determined from the studies on the holo-protein in the native mixed-valence state and in the reduced state of the metal center as well as from the studies on the apo-protein at different values of pH. The results were analyzed using a two-state unfolding model and the effect of pH on the thermodynamics of unfolding of this thermostable β-barrel protein was determined.

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MATERIALS AND METHODS

Ampicillin sodium salt, bacteriological agar, bacto tryptone, DEAE, Sephadex column packing material, dithiothreitol (molecular biology grade), guanidine hydrochloride (GuHCl), isopropyl β-D-thiogalacto-pyranoside, L-histidine, lysozyme, phenyl methyl sulphonyl fluoride, and yeast extract were purchased from Sigma Chemicals (St. Louis, MO). DNase I was purchased from Boehringer Mannheim (Mannheim, Germany). All buffer components, solvents and other chemical reagents were of analytical grade. Deionized water (Millipore, Billerica, MA) was used in the preparation of all the buffers.

Expression and purification of $TtCu_A$ from *Thermus thermophilus*

E. coli BL-21 DE3 containing the modified pMA10 vector was used for expression of the soluble Cu_A fragment of subunit II of the cytochrome *c* oxidase (13,21,22). The protein was extracted from the harvested cells by a procedure based on the previously reported by Slutter et al. (13). The harvested cells were resuspended in 50 mM Tris-HCl, pH 8.2, 1.5 mM phenyl methyl sulphonyl fluoride was added to the sample to inhibit proteolysis and stirred for 30 min (13). The sample was lysed by sonication in ice. The lysed cells were centrifuged at 17,000 rpm, for 30 min at 4°C. Supernatant was collected and diluted with 1 volume of 50 mM NaOAc and the pH of solution adjusted to 4.8 and centrifuged at 17,000 rpm for 30 min at 4°C to remove the precipitate. Subsequently, 15 μL/ml $Cu(His)_2$ was added to the supernatant and the protein solution was heated at 65°C for 10 min to denature most of the mesophilic proteins. The sample was then centrifuged at 10,000 rpm for 30 min at 4°C to remove precipitates and the supernatant was concentrated using Vivaspin concentrator (Amicon, Houston, TX; 10 kDa cutoff). The final concentration of 100 μM protein was loaded onto preequilibrated DEAE Sepharose column, washed, and eluted with 50 mM sodium acetate buffer, pH 6.5, containing 0–1 M NaCl gradient using Gradifrac (Pharmacia, Biotech, Uppsala, Sweden). Fractions were checked by UV-visible spectroscopy (model No. UV 2100; Shimadzu, Kyoto, Japan). The purity of the protein was determined by polyacrylamide gel electrophoresis in the presence of SDS using the buffer system as described earlier (23), which resulted in single observable band. The UV-visible absorbance spectra of the protein agreed with that reported earlier (13).

Preparation of Apo-protein

The apo-protein of the $TtCu_A$ from *T. thermophilus* was prepared by the following method: 4 M GuHCl was added to freshly prepared holo-protein of $TtCu_A$ and the pH was adjusted to 3.5 with acetic acid. Protein solution was incubated for 10–12 h at 40°C until it turned colorless. The absorption spectrum of the protein solution was checked spectrometrically until a complete absence of any absorption bands in the 400–600 nm region indicated removal of the copper-ions from the protein. The apo-$TtCu_A$ sample was concentrated on Centricon concentrators (Amicon YM10) and desalted (GuHCl was removed) with 50 mM sodium acetate buffer pH 4, containing 200 μM of dithiothreitol (to avoid protein aggregation) by using Sephadex G-10 column. Samples were stored under anaerobic conditions.

Spectroscopic measurements

Absorption spectra (220–900 nm) were collected on Shimadzu 2100 UV-visible spectrophotometer equipped with a thermostated holder. Before spectroscopic measurements sample was exchanged on a Sephadex G-10 column into 50 mM sodium acetate at various pH (from pH 3.5–7) followed by concentration of the solution at 4°C in a Centricon concentrator (Amicon YM10). Protein concentration and purity was determined using the molar extinction coefficients of 3100, 3200, or 1900 M^{-1} cm^{-1} at 480, 530, or 790 nm, respectively, at pH 6.5 (13). For the pure holo-protein, the ratio of the absorbance at 276 and 530 nm was found to be 6.7, which agreed with earlier report (13).

Circular dichroism (CD) measurements were carried out on a Jasco J-810 (Jasco, Tokyo, Japan) spectropolarimeter. The CD spectra were recorded from 250 to 200 nm and from 600 to 300 nm in 0.1 and 1-cm path-length quartz cell cuvettes, respectively, with resolution of 0.1 nm, scan speed of 20 nm/min, time constant 1.0 s, 1.0 band width and sensitivity of 100 mdeg. The measured CD spectra in the far-UV region (250–200 nm) were analyzed to estimate a secondary structure of the protein at different conditions using the Jasco Secondary Structure Estimation Program with the reference CD spectra of Yang (1986). All experiments were carried out under nitrogen atmosphere and temperature of the sample was controlled using a thermostated cell holder attached to a Peltier device. All the spectroscopic measurements were done at 20 $\pm$ 1°C unless specified.

Unfolding studies of the holo-protein as well as the apo-protein were carried out by equilibration of the protein solution at different concentrations of GuHCl at different values of pH by incubating the solution for overnight under nitrogen atmosphere. The samples were degassed thoroughly and ensured that no aggregation of the unfolded protein took place during the experiment. Reversibility of the unfolding of the protein was checked by removing the denaturant from the protein solution by gel filtration through a Sephadex G-10 column with a buffer at the same pH (but no denaturant) and monitoring the spectra (UV-visible and CD) of the resulting denaturant-free protein solution. Greater than 95% recovery of the folded structure of the protein was observed at all pH on removal of the denaturant from the protein solution. Analogous result was also obtained by dialysis of the unfolded protein solution against a buffer at the same pH. Dilution of the unfolded protein solution in presence of high GuHCl with buffer of the same pH also resulted in refolding of the protein confirming reversibility of the unfolding of the protein.

Analysis of unfolding data

The equilibrium unfolding data were analyzed in terms of a two-state equilibrium model (24–26),

$$Native\ (N) \rightleftharpoons Unfolded\ (U), \tag{1}$$

where N and D represent native and denatured states of the protein. The apparent equilibrium constant of unfolding (K_{eq}) and the associated free energy change (ΔG) were estimated in the transition region by using

$$K_{eq} = \frac{(y_n - y)}{(y - y_d)}; \quad \Delta G = -RT\ln K_{eq} = \Delta G_{H_2O} + m_G[D]. \tag{2}$$

The unfolding data were fit to Eq. 3 to obtain the free energy of unfolding of the protein at different values of pH,

$$y = \frac{(y_n^0 + m_n[D]) + (y_d^0 + m_d[D]\exp(-\Delta G/RT)}{1 + \exp(-\Delta G/RT)}, \tag{3}$$

where $y_n = y_n^0 + m_n[D]; y_d = y_d^0 + m_d[D]$.

Where y is the spectral property (such as absorbance of a peak, CD intensity in visible or in far-UV regions) observed at a given GuHCl concentration ([D]). The values y_n and y_d are the spectral properties characteristic of the native (N) and the unfolded (U) protein, respectively. The values of y_n and y_d linearly change with the denaturant concentration because of the solvent effect on the properties of the native and the denatured states. The constants y_n^0 and y_d^0 represent the intercepts, and m_n and m_d the slopes of the pre- and posttransitional baselines, respectively. The GuHCl-induced unfolding curves were fitted to the nonlinear equation (Eqs. 2 and 3) relating y to the denaturant concentration withy_n^0, y_d^0, m_n, m_d, m_G, and ΔG_{H_2O} as fitting parameters. The goodness of fit was ascertained from χ-square test ($\chi^2 \approx 1$) as well as from the randomness of the residuals distribution.

RESULTS AND DISCUSSION

The visible absorption spectrum of the $TtCu_A$ protein in the native mixed-valence state of the metal center arises

primarily from ligand-to-metal charge transfer transitions between the two copper ions and the histidine as well as the bridging cysteine residues coordinated to the metal ions (14,27). Increase in temperature of the protein solution up to 95°C at ambient pH did not affect the absorption spectra indicating very high thermostability of the protein as reported earlier (15). Earlier studies (15) on GuHCl-induced unfolding of the $TtCu_A$ protein from the thermophilic bacterium showed that the native form cannot be unfolded at room temperature even in the presence of 7 M denaturant. Fig. 1 shows the temperature dependence of the visible absorption spectra of the $TtCu_A$ at pH 6.5 in the presence of 4 M GuHCl. These results indeed show that even at 90°C the protein was not completely unfolded in the presence of 4 M GuHCl, which supports the earlier report (15) that the protein could be unfolded at 75°C only in the presence of 7 M GuHCl.

To check whether the changes in the absorption spectrum of the metal center indeed correspond to unfolding of the protein in the present case, we studied the CD spectra (Fig. 2) of the protein at different values of pH in the presence of GuHCl. The far UV-CD spectrum of the protein (Fig. 2, *inset*) showed characteristic β-sheet content in the secondary structure of the protein while the visible region CD spectra (Fig. 2) correspond to the tertiary structure around the metal center in the protein. Temperature dependence of the CD spectra in the far UV as well as in the visible region was very similar to that observed in the temperature dependence of the visible absorption spectra of the protein. This indicated that the secondary and tertiary structure of the native protein unfolds simultaneously in this case, which agreed with earlier observation (15).

We have studied the thermal unfolding of the metal binding site using the temperature dependence of the UV-visible absorption spectra of the protein at different values of pH in the presence as well as in the absence of denaturant. Fig. 3 shows the typical temperature dependence of the absorbance of the protein at 530 nm at different values of pH and in the presence of different concentrations of GuHCl. The results indicate that the thermal stability of the protein did not decrease on decreasing pH from 6.5 to pH 4 in the absence of denaturant and the holo-protein remained >95% folded even at 95°C, in the pH range 6.5–4. Thus, simple change in pH does not seem to affect the thermostability of the protein.

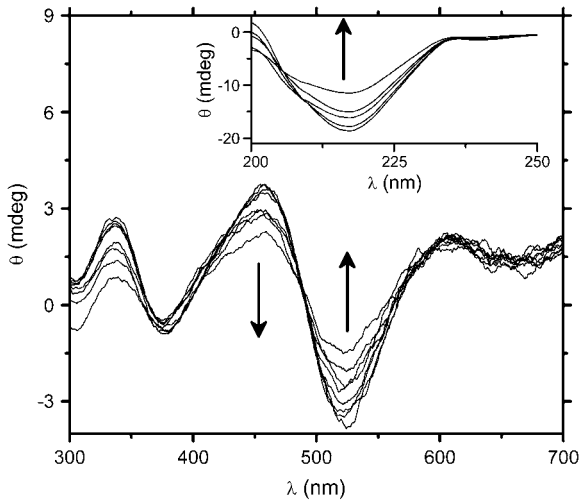

FIGURE 2 Effect of temperature on the CD spectra of *T. thermophilus* Cu_A ($TtCu_A$) in the visible region showing bands from the di-copper center at pH 6.5 in presence of 4 M GuHCl. Inset shows the far-UV CD spectra of the protein, which is characteristic of β-barrel protein. Protein concentration was 23.2 μM. Temperature range was 20–95°C and the arrows show the direction of increasing temperature.

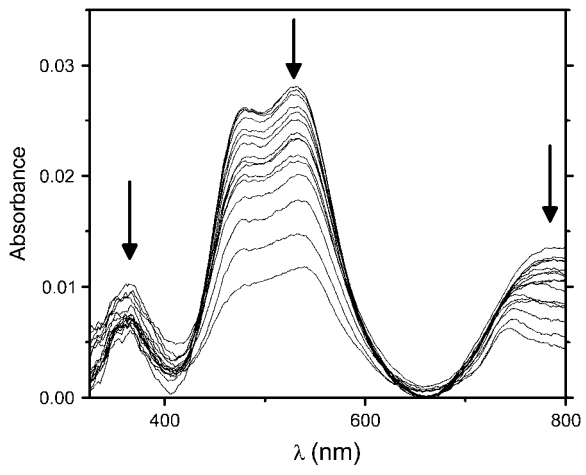

FIGURE 1 Temperature dependence of visible absorption spectra of *T. thermophilus* Cu_A ($TtCu_A$) at pH 6.5 in presence of 4 M GuHCl. Protein concentration was 8.5 μM, path-length 1 cm, and the temperature range 20–95°C (*top* to *bottom spectra*).

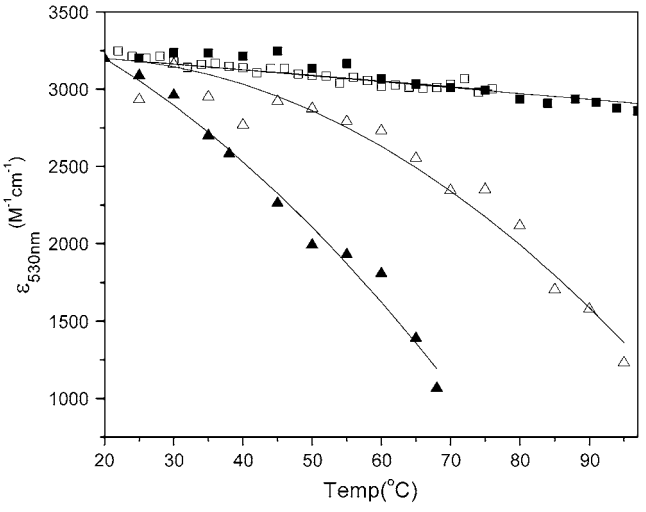

FIGURE 3 Temperature dependence of absorbance of the holo-protein of $TtCu_A$ at 530 nm (ε_{530nm}) at pH 6.5 (□) and pH 4 (■) in absence of any denaturant and in presence of 4 M (pH 6.5, △) GuHCl and 2 M (pH 4, ▲) GuHCl.

However, addition of GuHCl showed significant effect on the thermostability of the protein and the effect of denaturant on decrease in the thermostability was more significant at lower pH. Earlier studies (15) showed that the midpoint unfolding temperature (T_m) for the holo-protein of $TtCu_A$ was >100°C at pH 7.0 in absence of the denaturant while the T_m decreased to 70°C in presence of 7 M GuHCl, suggesting that the thermal stability of the protein decreases with increase in GuHCl concentration in the solution. Fig. 3 shows that 70% of the $TtCu_A$ protein was unfolded at pH 4 at 65°C in the presence of 2 M GuHCl, while the same amount of unfolding of the protein at pH 6.5 could be brought about at 95°C in the presence of 4 M GuHCl. Furthermore, the holo-protein in the native mixed-valence state lost the absorbance of the metal center by ~20% at pH 6.5 (Fig. 3) in presence of 4 M GuHCl at 75°C, while earlier report (15) showed that there was almost no unfolding of the protein under similar denaturant concentration at 75°C at pH 7.0, indicating that decrease in pH in presence of the chemical denaturant has a drastic effect on the thermostability of the protein.

The effect of pH on the thermodynamic stability of the $TtCu_A$ protein was investigated by monitoring the CD as well as visible absorption spectra of the protein at room temperature and at different values of pH in the presence of different concentrations of GuHCl. The CD as well as the visible absorption spectra of the protein were found to be almost unchanged on decreasing the pH of the solution from pH 7 to pH 3.5 in the absence of denaturant indicating that the structure of the protein was not affected by a pH change. However, the stability of the protein was found to be significantly affected by decrease in pH. Fig. 4 *A* shows the equilibrium unfolding of the holo-protein in the native mixed-valence state of the metal ion at different values of pH. The results shown in Fig. 4 *A* clearly suggest that decrease in pH significantly decreases the stability of the $TtCu_A$ protein toward unfolding by GuHCl. Thus, while the protein was not unfolded even at 7.5 M GuHCl at pH 7, the midpoint concentration (C_m) of GuHCl was ~3 M for unfolding of the protein at pH 4. The pH-dependent unfolding curves (except that at pH 7) could be analyzed by the two-state unfolding model, and the thermodynamic parameters such as ΔG_{H_2O} for unfolding of the protein were calculated at each pH by global fit to the data at different values of pH and GuHCl concentration using Eq. 3.

The stability of the redox protein has earlier been shown to depend on the oxidation state of the metal ion (14,15,28). Earlier studies by our group on subunit II of cytochrome c oxidase from *P. denitrificans* showed that the conformational stability of the protein at the native mixed-valence state is more than that of the fully reduced form at ambient pH (14). The thermostable Cu_A ($TtCu_A$) also shows analogous redox-state dependent stability at ambient pH (15). The effect of pH on the stability of the protein at different oxidation states of the dinuclear copper center was investigated by studying the unfolding of the reduced protein at different values of pH.

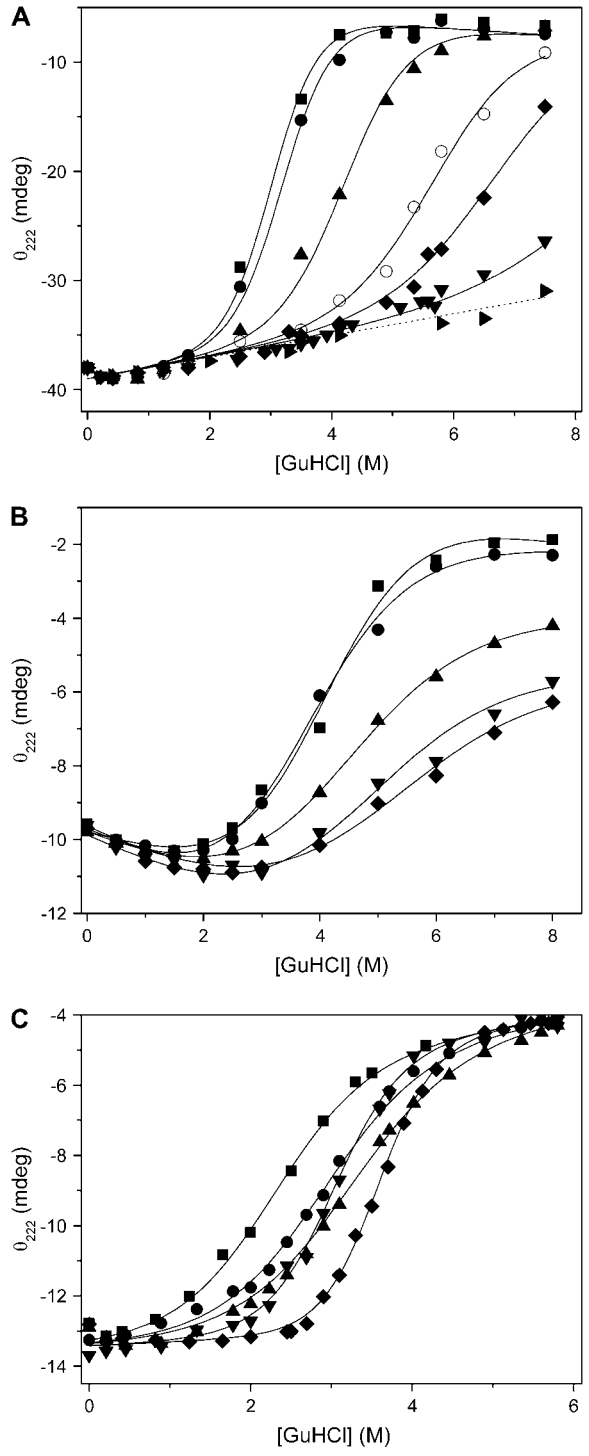

FIGURE 4 GuHCl-induced unfolding of *T. thermophilus* $TtCu_A$ at different values of pH at 20°C. (*A*) Oxidized holo-protein (46.2 μM) at pH 3.5 (■), pH 4 (●), pH 4.5 (▲), pH 5 (○), pH 5.5 (♦), pH 6 (▼), and pH 7 (►). (*B*) Reduced holo-protein (12.2 μM) at pH 4.5 (■), pH 5 (●), pH 5.5 (▲), pH 6 (▼), and pH 7 (♦). (*C*) Apo $TtCu_A$ (16 μM) at pH 3.5 (■), pH 4 (●), pH 4.5 (▼), pH 5 (▲), and pH 6 (♦).

The reduced form of the Cu_A center does not show any bands in the visible region hence the unfolding of the protein was studied by monitoring the far-UV CD (at 222 nm) with increasing concentration of GuHCl. Fig. 4 *B* shows variation of CD (222 nm) of the reduced $TtCu_A$ with GuHCl concentration at different values of pH from 4.5 to 7.0. The data could be fitted to the two-state unfolding model (Eq. 3) and the values of the thermodynamic parameters associated with the unfolding of the reduced protein were obtained. The results show that the apparent midpoint concentration (C_m) of GuHCl for unfolding of the reduced $TtCu_A$ decreases with pH. Moreover, the values of C_m for unfolding of the native form of Cu_A and that of the reduced protein are markedly different at pH values above pH ~5, indicating that the effect of reduction of the metal ion on the stability of the protein is possibly more significant at pH above ~5. The value of ΔG_{H_2O} for the reduced protein at pH 7 agreed with that reported earlier (15).

To understand the role of the metal ion bound to the protein matrix on the thermodynamic stability of the protein, we carried out a denaturant-induced unfolding of the apo-protein of the *T. thermophilus* Cu_A center ($TtCu_A$) at different values of pH. The apo-protein formed by depletion of the copper ions under anaerobic conditions showed almost identical far-UV CD spectrum to that of the holo-protein, indicating that the secondary structure of the protein was not changed on removal of the metal ions. The apo-protein, however, did not show any visible absorption or CD spectra that are characteristic of the metal center of the holo-protein. The apo-protein was found to be thermostable as observed with the holo-protein under the same experimental conditions. Analogous to the holo-protein, the apo-protein of $TtCu_A$ also showed pH dependence of the stability to denaturant-induced unfolding of the protein. The apo-protein of $TtCu_A$ was found to be unfolded by ~80% in the presence of 4 M GuHCl at pH 6, while it was almost completely unfolded at pH 4 at this denaturant concentration. The unfolding curves (Fig. 4 *C*) of the apo-protein at different values of pH were analyzed by the two-state unfolding model, as described in the case of the holo-protein, and the best fit of the data to Eq. 3 gave the values of the thermodynamic parameters of the GuHCl-induced unfolding of the protein at each pH value.

The values of free energy of unfolding ΔG_{H_2O} obtained from the fit of the data to Eq. 3 for the holo protein at the native mixed-valence state as well as in the reduced state of the metal ion and for the apo-protein were found to depend on the pH. The value of ΔG_{H_2O} could not be determined at pH 7, as there was essentially no change in the CD or visible spectra of the protein on addition of GuHCl at this pH. This result agrees with an earlier report (15) that estimated the value of ΔG_{H_2O} as 85 kJ/mol at pH 7. Fig. 5 shows the variation of the free energy of unfolding (ΔG_{H_2O}) for the holo-protein at the mixed-valence as well as at the reduced state and the apo-protein of the $TtCu_A$ at different values of pH. The values of ΔG_{H_2O} for the holo-protein were found to be

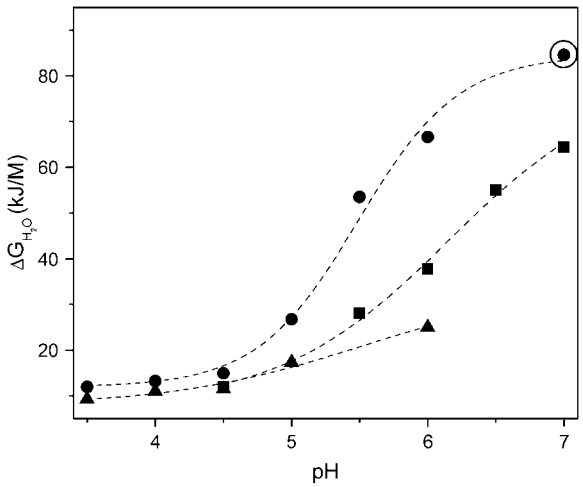

FIGURE 5 Variation of free energy parameters of unfolding with pH for holo-protein of $TtCu_A$ containing the native mixed-valence state of copper center (●), holo-protein of $TtCu_A$ containing the reduced state of copper center (■) and apo-protein of $TtCu_A$ (▲) at 20°C. The value at pH 7 for the native holo-protein was taken from Wittung-Stafshede et al. (15).

larger than the corresponding value for the apo-protein at a given pH. This indicated that the copper ions bound to the protein indeed contribute to the stabilization of the overall structure of the protein. Moreover, the holo-protein of *Thermus thermophilus* Cu_A in the native mixed-valence state of the metal centers was associated with higher value of ΔG_{H_2O}, which indicated that the native oxidation state of the metal centers makes the protein thermodynamically more stable than the one with the metal centers in the reduced form as reported earlier (15).

The metal ion in the electron-transfer copper proteins such as the type I copper proteins or the $TtCu_A$ site has been proposed to exist in an entatic state (29) in which the orientation of the ligand amino acids is primarily controlled by the β-barrel cupredoxin fold of the protein. Removal of the metal ion from the protein in these systems has hence very little effect on the structure of the protein. However, these studies as well as earlier reports (15) suggest that the metal-ligand interactions in the $TtCu_A$ protein indeed plays an important role in imparting extra stability to the metal binding site of the protein. The results also suggested that the mixed-valence native state of the metal ion stabilizes the protein structure by ~30 kJ/mol more than the reduced state of the metal ion at pH 6 (Fig. 5). Moreover, the free energy of unfolding of the mixed-valence state of the holo-protein was found to be ~40 kJ/mol larger than that obtained for the apo-protein at pH 6. However, the difference in the free energy of unfolding between the holo- and the apo-protein was found to decrease with decrease in pH and they become almost equal at pH <4. Since decrease in pH weakens the metal-ligand bonds, the overall bond strength between the metal ions and the coordinated amino acids becomes very weak at low pH, making the protein scaffold more flexible and prone

to denaturant-induced unfolding. This could also explain the extra stability of the holo-protein compared to that of the apo-protein at pH >4.

The values of free energy of unfolding were found to increase with increasing pH from pH 3.5 to 7 in the case of both the holo-protein and apo-protein, indicating that the stability of the protein increases with increasing pH. The results in Fig. 5 show that the pH dependence of the free energy for unfolding of the holo-protein showed definite inflection point with midpoint at pH ~5.5. This indicated that the decrease in the stability of the holo-protein might have an important contribution from protonation of an amino-acid residue with pK_a ~5.5. Apart from this residue, there could also be other acidic residues with pK_a in the low pH region, which might have contributions to the decreased stability of the protein at low pH. Unambiguous identification of the residue with pK_a ~5.5 that might have the major role in the decreased stability of the holo-protein of $TtCu_A$ is, however, not possible from these results. Consideration of the sequence and structure of the protein (PDB code: 2CUA) shows that there are three histidine residues (H114, H117, and H157) in the $TtCu_A$ protein from *T. thermophilus*. The histidine residues H114 and H157 are coordinated to the copper centers. The crystal structure of soluble Cu_A protein of *T. thermophilus* (PDB Code: 2CUA) also shows that the cysteine residues Cys-149 and Cys-153 act as bridging ligands to the two copper ions while Gln-151 and Met-160 are weakly coordinated to the metal ions. Fig. 6 shows the structure of the metal binding site of the $TtCu_A$ protein. Five of the coordinating residues to the metal ions belong to a single loop between sheets 8 and 9 in the β-barrel while the sixth ligand residue, H114, belongs to the loop between sheets 4 and 5 in the β-barrel. Protonation of a coordinated histidine residue (H114 or H157) would weaken or break the copper-histidine bond and thus decrease the stability of the protein so that relatively small concentration of the denaturant can unfold the protein at low pH. Earlier studies (30) on the pH dependence of azurin and several other mono nuclear copper proteins with cupredoxin fold showed that protonation of the coordinated histidine in the C-terminal side located at the center of a hydrophobic patch had a dramatic influence on the proteins. This suggests that protonation of His-157 in the $TtCu_A$ could perturb the hydrophobic patch in the loop and thus destabilize the metal ion binding site. Nevertheless, protonation of H114 in this case may also lead to destabilization of the Cu_A site of the protein. It is important to note that the protonation of the residue does not seem to affect the structure or the thermal stability of the protein in absence of any denaturant (see Fig. 3). On the other hand, protonation of the residue drastically decreases the stability of the protein toward unfolding by the denaturant. This indicates that there are other forces (such as hydrophobic interactions and hydrogen-bonding/electrostatic interactions involving other residues) in the protein that are possibly more important for the formation of the folded structure of this protein, and breaking of the metal coordination on protonation of the histidine facilitates the unfolding of the protein only in presence of the denaturant. The apo-protein does not have any extra stabilization that is characteristic for the holo-protein through bonding to the metal ion. Nevertheless, it shows high stability toward unfolding at ambient pH, while decrease in pH also decreases the stability of the apo-protein toward denaturant and the protein matrix becomes more flexible and permeable to denaturants. This suggests that the inter-residue interactions such as hydrogen bonding or electrostatic interactions that are weakened at low pH in the thermostable protein have important roles in the stabilization of the protein. Along with these interactions, strong coordination of the metal ions to the protein matrix in the holo-protein are also present, which brings two parts of the protein close together and thus imparts relatively higher stability than that in the case of the apo-protein.

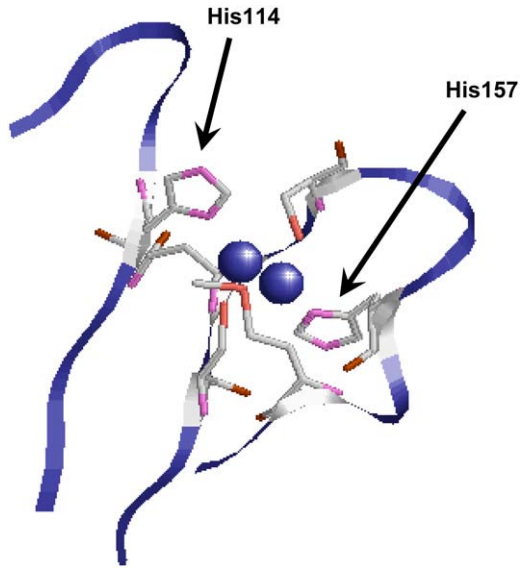

FIGURE 6 Schematic structure of the metal ion binding site in the *T. thermophilus* Cu_A ($TtCu_A$) obtained from the Protein Data Bank (PDB code: 2CuA). Arrows show the H114 and H157 residues coordinated to the dinuclear copper.

CONCLUSIONS

The thermodynamic stability of the thermostable $TtCu_A$ protein was found to decrease with lowering pH, indicating that protonation of certain residues is responsible for the decrease in stability of the protein. The denaturant-induced unfolding of the holo-protein as well as the apo-protein at different values of pH followed a two-state unfolding model and both the holo- as well as the apo-protein was found to be highly stable toward unfolding at ambient pH. The holo-protein was more stable than the apo-protein, indicating that the metal ion plays an important role in stabilization of the

protein. Moreover, the holo-protein also showed distinct redox-state dependent stability where the reduced form was less stable compared to the native state of the metal ions in the protein. The variation of the free energy of unfolding of the holo-protein at different values of pH was found to be associated with a pKa ~5.5. These results could not specifically identify the residue responsible for the protonation-dependent destabilization of the protein. Nevertheless, consideration of the crystal structure of the folded protein suggested that one of the coordinated histidines (His-114 or His-157) might be important for the higher stability of the holo-protein at ambient pH. The results also suggested that protonation of the coordinated residue(s) alone cannot cause unfolding of $TtCu_A$ in the temperature range of this study but the structure of the protein becomes weaker at low pH so that smaller concentrations of the denaturant can unfold the protein.

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