Conformational properties of the bis-μ-(thiolato) dicopper center in cytochrome c oxidase

Jitumani Rajbongshi a,b, Manas Kumar Ghosh a, Nusrat J M Sanghamitra b, Sayan Gupta a & Shyamalava Mazumdar b, *

aDepartment of Chemistry, Gauhati University, Guwahati 781 014, India
bDepartment of Chemical Sciences, Tata Institute of Fundamental Research, Homi Bhabha Road, Mumbai 400 005, India
Email: shyamal@tifr.res.in

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The novel bis-μ(thiolato) dicopper center (CuA) forms the electron entry site in the respiratory enzyme, cytochrome c oxidase. While most of the electron transfer copper proteins consist of a mono-nuclear copper center, the presence of a dinuclear copper in cytochrome c oxidase has attracted immense interest. The CuA center from the mesophilic organism, P. denitrificans (PdCuA) and from the thermophilic organism T. thermophilus (TtCuA) have very similar spectroscopic and electronic properties, albeit the stability of the two proteins are significantly different from each other. This dinuclear copper center undergoes interesting conformational change induced by change in the pH of the solution, which involves equilibrium conversion of the purple ‘charge-delocalized’ form to a ‘valance-trapped’ form of the metal center. The pH dependent conformational changes in the PdCuA and TtCuA show different pKₐ values indicating involvement of different amino acids in the process. The conformation change near the dinuclear center in the mesophilic protein PdCuA is extremely fast while that in the thermophilic protein TtCuA is very slow. The results of recent studies on the conformational properties of this novel metal center in the protein have been outlined in this mini-review in the light of understanding their implications in biology.

Keywords: Bioinorganic chemistry, Electron transfer proteins, Cytochrome c oxidase, Time-resolved fluorescence, Protein electrochemistry, Conformational changes, Dinuclear copper, Copper

The heme copper oxidases are terminal respiratory enzymes involved in catalyzing the reduction of molecular oxygen to water with electrons from NADH or succinate in the electron transfer chain. Mitochondrial cytochrome oxidases (CcO) and most oxidases from aerobic bacteria utilize cytochrome c as the first electron donating substrate, while several bacteria express the homologous enzyme called cytochrome bo ubiquinol oxidase (QO) that uses ubiquinol as the electron donor. The overall mechanism of biochemical function of these enzymes has been reviewed earlier. The basic functions of these oxidases involve transmembrane electron transfer leading to reduction of molecular oxygen to water at the binuclear heme-copper catalytic site (heme a₃-CuB), and active proton translocation across the membrane in the direction opposite to the electron transfer. The electron transfer and the proton translocation activities are coupled to each other. The protons from inside the membrane enter into the enzyme through certain specific channels in the enzyme and the overall reaction is:

O₂ + 8H⁺ (inside) + 4e⁻ (cytc) → H₂O + 4H⁺ (outside).

The protons are required for reduction of molecular oxygen to water formation (‘chemical’ protons) and for translocation from inside to outside of membrane (‘pumped’ protons). The bis-μ(thiolato) dicopper center, a type IIIA purple dinuclear copper complex (CuA) located at the solvent exposed part of membrane bound subunit II of cytochrome c oxidase, acts as the primary acceptor of electron from cytochrome c. The architecture of the CuA is such that unlike other copper containing electron transfer proteins, the electron entry and exit paths for the CuA are distinctly different. The protein structure, electronic properties, redox potentials, etc., of the bis-μ(thiolato) dicopper core have been studied by several groups. The crystal structure of the enzyme have been reported from various sources, which suggests that the basic topology of the solvent exposed part of the subunit II of the oxidases from all sources are almost the same with a beta-barrel structure holding the copper ions binding site in a ‘greek key’ cupredoxin fold. The structure shows that
the metal active site is slightly exposed towards the surface adjacent to the binding site of cytochrome c \(^{3,21}\) (Fig. 1). The coordination geometry of the metal center is formed by the specific conformation of the protein bringing the coordinating residues close to each other to conserve the biological electron transfer process \(^{29}\). The conserved residues (Fig. 2) for the bis-\(\mu\)-(thiolato) dicopper core from all sources are two cysteines, two histidines, and a methionine \(^{28,30,31}\). Spectroscopic and biochemical studies have shown that this center is ‘charge delocalized’ binuclear center with a Cu-Cu distance of 2.5Å, having two bridging cysteine ligands and a histidine ligand on each copper \(^{23,30,32}\).

The biological function of cytochrome c oxidase (CcO) involves electron transfer across the membrane and as mentioned above, the CuA center in the subunit II forms the electron entry site. Electrons from cytochrome c are transferred sequentially to the \(\text{O}_2\) reduction site of CcO (heme \(a_3\)-CuB) via the CuA and six-coordinated heme \(a\), which is coupled to the vectorial proton transfer across the membrane. This redox linked proton pumping by CcO is a subject of immense interest and forms one of the main themes of bioenergetics. Most models for redox-linked proton pumping by cytochrome c oxidase are based on consideration of the redox activity at a single metal center, either heme \(a_3\) or CuA, to provide the driving force for proton translocation \(^{33}\). However, the redox activity at both the metal centers, heme \(a_3\) and CuA, are now believed to be linked to the electron coupled proton translocation \(^{34}\). It has been proposed that part of the redox energy between the low-potential centers and the dioxygen-activated binuclear heme \(a_3\)-CuB center is transferred to the protein, changing its conformation reversibly in a manner that gates the electron flow as well as the protons to be pumped \(^{35}\). This gating of the electron flow allows the enzyme to switch on a coupled pathway, accelerating the downhill electron transfer as well as the coupled proton transfer across the membrane. Several experimental results support the role of CuA in the proton pumping mechanism \(^{36-38}\). Recent results indicate that the unique structure of CuA may have crucial role in the proton pumping machinery of...
cytochrome c oxidase\textsuperscript{36} and support a role of CuA in the proton pumping mechanism\textsuperscript{36-38} of the intact enzyme.

The electronic and redox properties of the CuA have been shown to depend on the pH, indicating that there is definite role of the concentration of protons on the bis-μ(2-thiolato) dicopper core\textsuperscript{25,39}. The pH dependent conformational equilibrium in the protein is often linked to the binding of protons\textsuperscript{25} and depends on the protonation/ deprotonation of certain amino acid residues around the active site of the protein\textsuperscript{25-26,40-43}. The two histidine residues ligated to the copper centre (Fig. 2) at the active site are also susceptible towards protonation/deprotonation. Moreover, a little change in the coordination geometry can have drastic effects on the stability and conformational properties of the CuA center of the protein along with its biological function. The conformational changes in the CuA protein as a function of pH and temperature have been investigated by several groups\textsuperscript{24-26}. The present article outlines our recent work in this area in the light of the current status towards the understanding the correlation of the biochemical function with the structural, redox and conformational properties of the dinuclear copper center of the cytochrome c oxidase from the various sources.

The CuA Center of CcO from Mesophilic and Thermophilic Sources

The mesophilic organisms, i. e., organisms grown under ambient conditions, consist of proteins that are generally unstable at elevated temperatures. On the other hand, the extreme environmental conditions of the hyperthermal habitat enable the thermophilic organisms to thrive at high temperatures where mesophilic organisms often lose their functions and structures. The proteins from thermophilic organisms, though often very similar in structures to their mesophilic homologues, show high intrinsic resistance to thermal as well as chemical denaturation\textsuperscript{44,45}. Several factors such as increased hydrophobic and electrostatic interactions, extensive H-bonds, higher compactness, enhanced polar and non-polar contribution, enriched salt bridges, etc., were shown to be responsible for the thermostability\textsuperscript{46-51}.

The soluble dinuclear copper protein (PdCuA) obtained from the solvent exposed domain of the subunit II of CcO from Paracoccus denitrificans is a mesophilic protein, while the analogous dinuclear copper protein (TtCuA) from Thermus thermophilus is highly thermostable. The coordinating residues and the structure of the metal center (Fig. 2) in the PdCuA and the TtCuA are very similar, though there are several interesting factors that remain distinctly different in each other.

The pH-induced Conformational Changes in CuA

The copper center in all these cases shows deep purple color at ambient pH, which is characteristic of the metal complex present in the protein. The UV-visible absorption spectrum of the CuA from mesophilic bacteria Paracoccus denitrificans (PdCuA) shows absorption bands at 360, 480, 535 and 810 nm corresponding to metal ligand and metal-metal charge transfer transitions\textsuperscript{22}. The absorption bands at ~480 nm and ~530 nm of the CuA have been assigned to extensive S(Cys)→Cu charge transfer (LMCT) transitions in the dinuclear Cu₂(Cys)₂ active site, while the band at ~800 nm has been assigned to the transition associated with the ‘valance delocalized’ CuA center and the ~360 nm band is attributed to the N(His)→Cu coordination\textsuperscript{25}.

The UV-visible absorption and visible CD spectra of the CuA site from Paracoccus denitrificans (PdCuA) show significant change with pH. Figure 3 shows the UV-visible spectra of PdCuA\textsuperscript{21,22,24} at pH 6 and pH 10. The pH change was associated with gradual decrease in the absorbance of the 480 nm, 535 nm and 810 nm bands with subsequent increase in the 360 nm band. The plot of the extinction coefficient at the absorption maxima against pH (Fig. 3) was analysed using a one-site equilibrium model (Eq. 1) and the associated pK\textsubscript{s} with the process for PdCuA was found\textsuperscript{21,24} to be ~8.2.

\[
A_{\lambda obs} = \frac{C_0^* e^\lambda_A e^\lambda_B \left( pH - pK_{app}^\lambda \right)}{1 + 10^{\left( pH - pK_{app}^\lambda \right)}} \quad \ldots(1)
\]

The CD spectra of PdCuA in the visible region show increase in ellipticity of 365 nm and 522 nm bands at the expense of 333 nm and 443 nm bands with increase in pH from 6.0 to 10.0, which was also associated with a pK\textsubscript{s} of ~8.2, indicating that the
observed pH dependence in the spectrum indeed corresponds to changes in the conformation of the protein. This pH has been proposed to correspond to deprotonation of a tyrosine residue lying close to the copper center. Saraste et al. proposed that at high pH, the deprotonated tyrosine might co-ordinate with a copper through oxygen ligation. Site directed mutagenesis and NMR studies also indicated breaking of the coordinate bond between a copper and the coordinated H224 at high pH (Fig. 2).

The UV visible spectrum as well as the CD spectrum of the CuA from Thermus thermophilus (TtCuA) shows slightly shifted bands in the visible region at 365, 477, 530, 790 nm compared to those in the case of PdCuA. Unlike the mesophilic analogue, increase in pH does not immediately show any change of the spectra of TtCuA, which was assigned to be due to rigidity of the structure of the protein backbone. Increase in pH of the TtCuA solution caused a slow but distinct change in the spectra, indicating changes in the environment around the metal ion at high pH. Analogous to the mesophilic protein, increase in pH cause decrease in the absorbance of the visible bands of TtCuA with consecutive increase in absorbance at ~ 320 nm with an isosbestic point at ~ 355 nm (Fig. 4). The protein solution however requires incubation for 48 hours under nitrogen atmosphere at each pH ranging from 6.5 to 12 to ensure equilibrium. The variation in the absorbance and CD at different wavelength as a function of pH was fit to a one-site protonation/deprotonation equilibrium model to get an apparent pH value of 9.7. The pH induced conformational transition was also shown to be reversible in nature and decreasing the pH from 12 to 6.5 slowly restore the original spectrum of the protein observed at pH 6.5 on incubation for ~48 hrs.

A new absorption band at 510 nm and blue shifted band at very high pH (pH 12) possibly indicate a significant distortion in the dinuclear copper active site environment at higher pH. Artificial CuA center produced by genetic engineering of the single copper protein, azurin, by inserting the amino acid sequence appropriate for formation of bis-μ(thiolato) dicopper complex in a chimeric CuA protein also showed pH induced conformation change in the metal center analogous to that discussed above. (Fig. 4)
Unlike the visible CD, the far UV-CD of the protein remains almost independent of pH in all CuA proteins indicating that the secondary structure, i.e., the beta-barrel topology remains unaffected by the subtle conformation change at the metal binding site due to the change in pH.

**Fluorescence Studies of CuA Protein**

Steady state fluorescence of PdCuA that consists of five tryptophan residues, shows an emission maximum\(^{21,24}\) at 345 nm when excited at 295 nm. It is interesting that the fluorescence emission spectrum of the protein did not show any significant change in the emission maximum with pH. This indicated that the environment around the tryptophan residues in the protein was not significantly affected by the change in pH implying that the overall structure of the protein remains intact. However, the change in pH was shown to be associated with subtle changes in the conformation of the active site of the protein, detected by the steady state fluorescence quenching at pH 6.0 and 10.0 using neutral quencher acrylamide and ionic quencher cesium chloride\(^{21,24}\). The fluorescence quenching data were analysed by Lehrer\(^{56,57}\) plots (Eq. 2) for quenching by acrylamide and CsCl (Fig. 5), where \(\Delta F\) is the change in the fluorescence intensity due to quenching, \(K_Q\) is the quenching constant, \(f_a\) is the fraction of the total fluorophore accessible to the quencher and \(F_o\) is the fluorescence intensity in absence of the quencher of concentration \([Q]\).

\[
\frac{F_o}{\Delta F} = \frac{1}{(K_Q f_a [Q])} + \frac{1}{f_a} \quad \ldots (2)
\]

Acrylamide, a neutral and hydrophobic quencher can access all the tryptophan residues in the protein, hence the fraction of the fluorophore (\(f_a\)) exposed to the quencher obtained from the Lehrer plot is equal to 1 in such cases. The ionic quencher, cesium chloride, on the other hand cannot access the tryptophan residues which are buried inside the hydrophobic core of the protein. Thus, the fraction of tryptophan exposed to the surface is equal to the fraction (\(f_a\)) of the fluorophore exposed to quenching by CsCl. The intercept of the Lehrer plots gave the values of \(f_a\) to be 0.4 for the ‘low pH’ form, i.e., at pH 6 and 0.27 for the ‘high pH’ form at pH 10 of the PdCuA, which suggested that there is indeed distinct change in the surface of the CuA protein associated with the pH induced conformation change at the dinuclear copper core.

Time resolved decay studies of tryptophan fluorescence of the protein at different pHs provided important information on the specific tryptophan residues that may be affected by the pH induced conformational change of the metal centre. The observed fluorescence decay data \(F(t)\), is a convolution of the instrument response function \(R(t)\), and the intensity of the decay fluorescence function of the sample \(I(t)\) (Eq. 3),

\[
F(t) = \int R(s + \delta)I(t - s)ds \quad \ldots (3)
\]

where \(\delta\) is the shift parameter that was optimized in the analysis. Data analysis involves the determination of the best values for the unknown parameters in \(I(t)\). The fluorescence decay curves deconvoluted with the excitation function and the fluorescence intensity can be analyzed as a sum of discrete exponentials given by Eq. (3a)\(^{58,59}\),

\[
I(t) = \sum_k A_k \exp(-t / \tau_k) \quad (3a)
\]

where \(k\) is the number of discrete exponentials required to fit the emission profile (\(k = 3\)). The amplitudes (\(A_k\)) and lifetimes (\(\tau_k\)) were determined by iterative reconvolution using non-linear regression and Marquardt's algorithm for parameter optimization\(^{58,59}\). Stability of the multi-exponential fits are determined from the randomness of the weighted residual distribution\(^{58,59}\) \(W(t)\) and \(\chi^2\). Typical time resolved fluorescence decay profile of PdCuA is shown in Fig. 6. The weighted residuals distribution for the three- and two-exponential fits (Fig. 6) showed that the data were best fitted to the sum of three exponentials.
pH dependent lifetime of tryptophan fluorescence

Figure 7 shows the plot of lifetime components of the tryptophan fluorescence decay of PdCuA at different pHs. The fastest component ($\tau_1 = 0.8$ ns) constitutes $\sim 10\%$ of the fluorescence decay. The magnitude of this lifetime ($\tau_1$) was found to decrease with increase in pH with a $pK_a$ of $\sim 8.2$. The other two lifetime components ($\tau_2 \sim 2$ ns, $\tau_3 \sim 5$ ns) remain almost constant over the pH range 6.0 to 10.0.

The contributions from each lifetime component to the steady state fluorescence spectrum were used to construct the decay-associated spectra (DAS)\textsuperscript{59-62} from the time-resolved fluorescence decay of PdCuA (Fig. 8). The three lifetime components ($\tau_1$, $\tau_2$ and $\tau_3$) obtained by single curve analysis at different emission wavelengths were found to show only a small variation with wavelength\textsuperscript{59,62}. The decay associated spectra show that the fastest lifetime $\tau_1$ contributes least to the steady state fluorescence with the emission maximum at $\sim 330$ nm, while steady state fluorescence is dominated by contributions from $\tau_3$ with emission maximum at $\sim 350$ nm. The steady state contribution of $\tau_2$ is associated with an emission maximum at $\sim 345$ nm. Increase in pH from 6 to 10 was found to have no significant effect on the emission maxima of the steady state spectra associated with the lifetime components, but the...
relative intensity of the contribution of \(\tau_1\) was found to be slightly increased at pH 10 compared to that at pH 6 suggesting that the environment around the tryptophan residue residing close to the metal center indeed undergoes change on change in the pH or the protein solution. The crystal structure of PdCuA showed that the coordinated residues C216, E218, C220, H224 and M227 (Fig. 2) belong to the loop joining \(\beta 9\) and \(\beta 10\) while H181 belongs to the loop joining \(\beta 5\) and \(\beta 6\). The H224 residue is exposed to the solvent. The protein has \(\beta\)-barrel (Greek key type) topology, which is stabilized by hydrophobic interactions of the nonpolar side chains. The sheets are also connected through inter-sheet hydrogen bonding interactions. The inter-sheet hydrogen-bonding network may be formed by polar aromatic amino acid residues like tyrosines and tryptophans directed towards the core of the \(\beta\)-barrel. Change in pH was proposed to perturb one or more of these interactions leading to change in the tertiary structure and hence, the geometry of the metal center in PdCuA.

The conformation change associated with the conversion of the ‘low-pH’ form to the ‘high-pH’ form in the TtCuA was also investigated by steady-state and time-resolved tryptophan fluorescence of the thermostable protein at different pHs. TtCuA consists of a single tryptophan residue (Trp 64) and the fluorescence quantum yield as well as the lifetime of the time-resolved fluorescence decay of the tryptophan residue are affected due to quenching by the metal center. The intensity of the tryptophan emission band (\(\lambda_{ex}=295\) nm, \(\lambda_{em}(\text{max})=325\) nm) was shown to decrease with increase in pH of the protein solution. The emission band was slightly broad at high pH indicating possible increase in polarity of the environment around the tryptophan residue at high pH. The decrease in Trp fluorescence intensity at high pH was proposed to arise due to the decrease in distance or change in the relative orientation between Trp residue and copper center. The Trp fluorescence lifetime decay profiles (Fig. 9) for the TtCuA was best fit to a triple exponential decay function at each pH. Unlike in case of the PdCuA, all the three lifetime components (\(\tau_1, \tau_2\) and \(\tau_3\)) decreased with increase in pH. Moreover, the amplitude of the fastest lifetime component (\(\tau_1\)) increases from \(~34\%\) at pH 8 to \(~87\%\) at pH 12. The discrete analysis of the fluorescence decay (Eq. 3) for a heterogeneous solution containing both the ‘low-pH’ and ‘high-pH’ forms of the protein gave a weighted average of the lifetime values at each pH. This decrease in lifetime thus could be due to the changes in the relative position of the tryptophan residue (Trp 64) from the copper center (CuA) due to the conformational change occurring during the conversion of ‘low-pH’ form to ‘high-pH’ form. This further confirmed that there is indeed a conformational change in the protein on increase in pH and this change in the tertiary structure possibly involves movement of the strand-1 towards the strand-9 due to distortion in the \(\beta\)-barrel of the protein (see Fig. 1) bringing the Trp64 closer to the metal centers.

### EPR Spectra of TtCuA at Different pHs

EPR spectra provide direct information on the nature of the metal center in a copper complex. The EPR of PdCuA as well as of TtCuA at ambient pH 6.5 show an axial signal, \(g\perp \sim 0.0\) and \(g\parallel \sim 2.18\).
The $g_{||}$ of the EPR spectrum shows seven line splitting at ambient pH indicating delocalization of one unpaired electron over two equivalent copper atoms (class III); the hyperfine coupling constant is generally very small ($A_z \sim 2.5$ mT) in the CuA proteins from all sources including in the chimeric CuA protein. Increase in $pH$ of the solution leads to breaking of the symmetry of the EPR signal and also shows change in the $g_{||}$ to a four line spectra characteristic of a ‘valence-trapped’ [Cu(2+), Cu(+1)] form of the dinuclear center. Figure 9 shows the EPR spectra of TtCuA at 4 K at ambient pH (6.5) and at higher $pH$ (10.5 and 12). As $pH$ was increased from 6.5 to 10.5, the EPR signal in the $g_{||}$ region changed significantly with increased copper hyperfine splitting signals. Simulation of the EPR spectrum of TtCuA at $pH$ 10.5 gave the values of $g_{||} \sim 2.00$, $g_{\perp} \sim 2.01$ and $g_{\perp} \sim 2.30$, with $A_z \sim A_y = 2.5$ mT and $A_x = 7.5$ mT, which are also similar to those of the ‘high-$pH$’ form of the PdCuA. The EPR spectrum of the protein at $pH$ 12 was very broad and indicated presence of a mixture of at least two isolated Cu$^{2+}$ species with $A_z$ values of 10 mT and 8.5 mT. Similar $pH$ dependent changes in the EPR spectra were also observed in the H252N mutant of PdCuA. The EPR spectra at different $pH$s thus supports that the metal center was converted from the native ‘charge-delocalized’ form to ‘valence-trapped’ [Cu(+2), Cu(+1)] form(s) at high $pH$.

The EPR along with the fluorescence and absorption spectral results demonstrate that increase in $pH$ not only leads to the breaking of the ‘charge-delocalised’ dinuclear copper center, but also causes drastic change in the tertiary organization of the strands in the protein. The $pK_a$ of this change in the case of TtCuA ($pK_a = 9.7$) is close to the $pK_a$ for deprotonation of the OH- of a tyrosine or of the NH- of a histidine or of the SH- of a cysteine or of the NH$_3^+$ of a lysine residue. The last two possibilities were ruled out as both the cysteines in the TtCuA are deprotonated to coordinate to the copper centers, and all the lysine residues in the protein are solvent exposed and far away from the metal centers. The change in the conformation at the metal binding site in TtCuA was proposed to be initiated by deprotonation of a tyrosine or a histidine residue in the protein. The $pK_a$ of the NH- proton of histidine varies over a large range depending upon the environment and presence of prosthetic group in a protein. The $N^6$ of His114 is coordinated to a Cu center and the $pK_a$ of the imidazole is known to change drastically upon complexation with a metal ion. Based on this argument, deprotonation of the $N^6$- of His114 was proposed to increase the electron density at the $N^6$- of the imidazole ring strengthening the Cu$^{2+}$-His114 bond at high $pH$ giving rise to the observation of the new absorption band at 320 nm in the ‘high $pH$’ form of the protein. The analysis of crystal structure of TtCuA suggested that $N^6$- of His114 is hydrogen bonded to Asp111 in the protein at ambient $pH$, and thus the observed $pK_a$ ($9.7$) in TtCuA could also correspond to deprotonation of the $N^6$H- of the coordinated histidine residue in the protein in the [Cu(2+), Cu(1+)] of the ‘valence-trapped’ form. The mixture of species observed in the EPR spectrum of the protein at $pH$ 12 was proposed to correspond to heterogeneous nature of ‘valence-trapped’ forms of [Cu(1+), Cu(2+)] configuration in the solution with both the cysteine residues coordinated to the Cu$^{2+}$ center at high $pH$ as indicated by the observation of weak absorption band at 512 nm at $pH$ 12 (Fig. 4). Crystal structure further indicated that Tyr90 in TtCuA is hydrogen bonded to Gly115 connecting beta strands 3 to 6. Thus, analogous to the observation in the case of PdCuA, deprotonation of the Tyr90 could also cause a distortion at the cupredoxin fold leading to a conformation change at the metal center.

**Redox Properties of CuA**

Equilibrium intermolecular electron transfer reaction between reduced horseheart cytochrome c and PdCuA was studied by UV visible spectroscopy at different $pH$s to determine the redox potentials of PdCuA. The equilibrium constant for the reaction was determined from decrease in intensity of the absorption of the 550 nm band of reduced cytochrome c in presence of increasing concentrations of PdCuA at a given $pH$. The redox potential of PdCuA determined from the equilibrium constant ($K_{eq}$) was reported to be −240 mV at $pH$ 7. The redox potential of the protein however increases to −270 mV at $pH$ 8.5, indicating that the redox potential of the metal center is $pH$ dependent and that the redox potential of the ‘high $pH$’ form might be higher than that of the ‘low $pH$’ form.

Direct (unmediated) electrochemical studies often give an unbiased estimate of the redox properties of the metal center. However, such studies require careful design of the electrode surface appropriate for the electrochemistry of the redox protein. The cyclic
voltammetry was achieved for CuA protein from *Thermus thermophilus*, which shows a well defined quasi-reversible Faradic response with a peak-to-peak separation ($\Delta E_p$) of 65 mV at a neomycin modified glassy carbon electrode at pH 7.0 (Fig. 10). TiCuA yields good quasi-reversible responses on neomycin modified glassy carbon electrode in the pH range 3-12. The reduction potentials were also measured from the square wave voltammetry for TtCuA protein in the broad pH range from 2-12. The reduction potential at pH 7.0 is 255 ± 5mV, consistent with that estimated by other methods. As the pH value increases, the reduction potential gradually decreases and a value of -150 ± 4 mV is obtained at pH 12. This is contrary to that observed in the case of PdCuA where the redox potential was proposed to increase with pH. As the pH is lowered from neutral pH, the reduction potential of the TiCuA increases and a value of 460 ± 8 mV is obtained at pH 3. The variation of the reduction potential of TtCuA protein as a function of pH is shown in Fig. 11. Non-linear least squares fit of the data gave the $pK_a$ values of 3.5 and 9.6 for the acidic and alkaline transitions respectively.

The heterogeneous electron transfer between the modified GC electrode and the TtCuA was determined at different pH. The heterogeneous electron transfer rate constant ($k_s$) was calculated by Nicholson’s method assuming $\alpha = 0.5$ in the derivation of Eq. (4),

$$k = \frac{\psi}{(RT / \pi n FD)} \quad \ldots(4)$$

where $\psi$ is a current function (dimensionless rate parameter in CV), $k_s$ is the heterogeneous rate constant, $R$ is ideal gas constant, $T$ is the temperature, $F$ is the Faraday constant and $n$ is the number of electron.

Values of $k_s$ were found to be 0.003 ± 0.001 cm/s, 0.61 ± 0.01 cm/s and 0.008 ± 0.001 cm/s respectively at pH 3, 7 and 12 at the scan rate of 5 mV/s. The results show that at high and low pH, the electron transfer rates between the electrode and the protein decrease drastically compared to that at ambient pH. This means that the electron transfer rates are very slow at acidic and alkaline pH as compared to that at physiological pH.

The thermodynamic parameters for the electrode reaction of the [Cu$_2$(Cys)$_2$] complex were determined by variable-temperature electrochemistry. Such studies were feasible only in the case of TtCuA as it is extremely thermostable. Using a non isothermal cell configuration, potentials for the TtCuA reduction were measured between 25 °C and 60 °C (Fig. 12). For a reversible cell, the change in the Gibbs energy of reaction, $\Delta G$, is proportional to the cell potential, $\Delta G = -nFE$. Since the standard emf of a cell is related to the Gibbs energy, electrochemical measurements can be used to obtain other thermodynamic functions using the Eq. (5).

$$\left(\frac{\partial G}{\partial T}\right) = -S \quad \ldots(5a)$$
The standard entropy change for the complete cell reaction $\Delta S$ at pH 8, was shown to be $-76.23$ J mol$^{-1}$ K$^{-1}$, giving $\Delta H$ as $-49.21$ kJ mol$^{-1}$ and $\Delta G$ as $-25.63$ kJ mol$^{-1}$, which agree with results obtained by other methods$^{71}$. The thermodynamic parameters for TtCuA protein at different pHs show that there was no major change in the thermodynamic parameters for TtCuA protein at pH 6-8, but significant changes occurred in the parameters for CuA at pH 12. The transition of the native conformer to alkaline form is therefore clearly a thermodynamically distinct process.

**Kinetic Properties of CuA**

The kinetics of conversion from the ‘low pH’ form to the ‘high pH’ form of the CuA protein from *P. denitrificans* (PdCuA) was investigated by stopped flow pH jump technique$^{24}$. The variations in the absorbances at 360 nm, 480 nm and 810 nm on change in the pH of the solution were monitored. The time evolution of the absorbance ($y$) was fitted to a single exponential model (Eq. 6),

$$y = A \times \exp(-t \times k_{app}) + B$$

where $t$ is time, $k_{app}$ is the apparent rate constant and $A$ and $B$ are constants.

The change in the apparent extinction coefficient (Fig. 13) in each of these wavelengths was fitted to a single exponential function (Eq. 6) and the apparent rate constant ($k_{app}$) was calculated at each pH. The magnitude of the apparent rate constant ($k_{app}$) was shown to be independent of the initial pH ($pH_i$) in the pH jump experiment indicating that the change in pH
of the solution on mixing of two buffers takes place within the mixing time of the instrument and the rate of the reaction depends only on the final pH \( (pH_f) \). The conversion from the ‘low pH’ \( (P_A H) \) to the ‘high pH’ \( (P_B) \) form of PdCuA was investigated in detail to determine the mechanism of the pH induced change in the metal center\(^3\). It was shown to involve two main steps in case of PdCuA\(^2\): (1) the first involving deprotonation of an amino acid leading to the formation of the deprotonated species \( (P_A^-) \) may still maintain the conformation of the ‘low pH’ form, and, (2) the second involving conformational rearrangement in which the tertiary structure around the metal site is changed leading to formation of the ‘high pH’ form. Two reaction schemes have been proposed\(^2\). The first model (Scheme 1) consists of a slow (rate determining) proton transfer step (Step 1) followed by a fast (equilibrium) conformational change \( (K_{iso}) \) (Step 2). The apparent rate constant \( (k_{app}) \) in this model linearly increases with proton concentration \( ([H^+]_f) \).

\[
\text{Step 1: } \begin{align*}
\text{Low pH form (P}_AH\text{)} & \xrightleftharpoons[k_2]{k_1} \text{Low pH form (P}_A^-\text{)} + \text{H}^+ \\
\text{Step 2: } & \xrightleftharpoons[K_{iso}]{k_3} \text{High pH form (P}_B\text{)}
\end{align*}
\]

\[\text{Scheme 1}\]

The time evolution of the concentration of the low pH form \( (P_AH) \) in Scheme 1 would be given as Eq. (7b),

\[
[P_AH] = [P_AH]_f - \left( [P_AH]_f - [P_AH]_i \right) \times \exp \left[ - \frac{k_2 [H^+]_f}{1 + K_{iso}} t \right]
\]

where \([P_AH]_i\) and \([P_AH]_f\) are the concentrations of the ‘low pH’ form at initial \( (pH_i) \) and final \( (pH_f) \). The apparent rate constant would thus be as given in Eq. (7c).

\[
k_{app} = \left( \frac{k_1 + k_2 [H^+]_f}{1 + K_{iso}} \right)
\]

\[\text{Scheme 2}\]

The second model (Scheme 2) consists of a fast equilibrium \( (K_a) \) in the Step 1 (proton transfer), which is followed by a slow conformational change in the Step 2 (rate determining step). The apparent rate constant \( (k_{app}) \) in this model would increase with decrease in the proton concentration.

\[
\text{Step 1: } \begin{align*}
\text{Low pH form (P}_AH\text{)} & \xrightleftharpoons[K_a]{k_3} \text{Low pH form (P}_A^-\text{)} + \text{H}^+ \\
\text{Step 2: } & \xrightleftharpoons[k_4]{K_{iso}} \text{High pH form (P}_B\text{)}
\end{align*}
\]

The experimental \( k_{app} \) values for PdCuA could be fitted to Eq. 7 suggesting that the protonation induced conformation change in the mesophilic CuA center follows Scheme 1. The values of the parameters \( k_1 \) and \( k_2/(1+K_{iso}) \) calculated from the fit to the experimental \( k_{app} \) against pH were found to be: \( k_1 = 0.029 \pm 0.005 \) s\(^{-1}\) and \( k_2/(1+K_{iso}) = 1.7 \pm 0.05 \times 10^6 \) M\(^{-1}\) s\(^{-1}\). The rate of deprotonation of amino acids in aqueous medium is generally diffusion controlled process while that inside the protein matrix could become very slow depending on the nature of the surrounding environment.

Unlike in the case of the \textit{Paracoccus} protein\(^2\), the conversion of the ‘low pH’ form to the ‘high-pH’ form in the case of the thermostable TtCuA was found to be extremely slow\(^2\). The thermostable protein takes almost 48 hours to equilibrate at each pH.
whereas in the mesophilic counterpart the whole process occurs in millisecond timescale. The possible two mechanistic schemes (Schemes 1 and 2) were evaluated in the pH dependence kinetics of TtCuA. The time evolution of the absorbance at 530 nm subsequent to a pH jump was fitted to single exponential decay function and the apparent rate constant \( k_{\text{app}} \) was determined (Fig. 14) at each pH (pH). The magnitude of \( k_{\text{app}} \) in the case of TtCuA was shown to increase with the increase in the value of the final pH (pHf) in the pH jump experiment and followed Scheme 2 (Eq. 8). The values of the rate constants (\( k_1 \) and \( k_2 \)) associated with the conformation change in the protein are very small (\( k_1 = 0.002 \text{ s}^{-1} \), \( k_2 = 1.3\times10^{-4} \text{ s}^{-1} \) at 25 °C) and the value of the \( K_a \) for the deprotonation (Step 1, Eq. 8) equilibrium determined from the apparent \( pK_a \) (Eq. 6) obtained from the equilibrium experiment was \( K_a = 4.06 \times 10^{-11} \text{ M}^{-1} \) (\( pK_a = 10.4 \)) at room temperature, suggesting that the conformational change in the protein subsequent to the deprotonation of the residue (with \( pK_a = 10.4 \)) occurs quite slowly.

The reverse conversion of the ‘high pH’ form to the ‘low pH’ form in the TtCuA was also determined by decreasing the pH of the ‘high pH’ species, which was found to be much slower (Fig. 14) compared to the forward reaction. Moreover, unlike in the case of the forward reaction (Scheme 2), the rate of the reverse reaction in TtCuA was shown to obey Scheme 1 (Eq. 7). The apparent rate constant for the reverse reaction, \( k_{\text{app}}^r \), was extremely small (\( \sim 5\times10^{-4} \text{ s}^{-1} \) at 25 °C for pHf = 6.5) and it decreased with increase in the value of the final pH (pHf) in the pH jump experiment. The value of the equilibrium constant, \( K_a^r \), was \( 1.1 \times 10^{-9} \text{ M}^{-1} \) for the deprotonation of TtCuA.

The kinetics of the pH-induced conformational change in TtCuA was investigated at different temperatures to estimate the activation parameters for the pH induced reactions in the protein. Analysis of the temperature dependence of \( K_a \) using the van’t Hoff equation in TtCuA gave the estimate of the enthalpy change as \( \Delta H_a = 164 \pm 21 \text{ kJ/mol} \) and the entropy change, \( \Delta S_a = 347 \pm 66 \text{ J/mol/K} \) associated with the overall process.

The activation parameters for the energy barrier of this conformational transition in TtCuA were determined from the temperature dependence of the \( k_1 \) and \( k_2 \) values using the Arrhenius equation. The activation energy for the forward reaction, i.e., the conversion of the ‘low pH’ form to the ‘high pH’ form was shown to be 51 (±5) kJ mol\(^{-1}\) whereas that for the reverse process was 82 (±6) kJ mol\(^{-1}\) (ref. 25). Duy et al. reported that the irreversible unfolding process of α-amylases from different sources were in the range of 260-360 kJ mol\(^{-1}\), which is larger than that observed in the pH induced conformational change in the TtCuA. However, the pH induced conformation change in the CuA protein is mainly a distortion around the dinuclear copper center, which is expected to have a relatively smaller energy barrier compared to the more drastic changes involved in overall unfolding of the protein.

The fast deprotonation/protonation equilibria that was proposed in the case of TtCuA indicate that the rate constants for proton transfer in the Thermus...
protein\textsuperscript{25} can be fast as shown by H/D exchange studies on hyperthermophile rebredoxin\textsuperscript{54}. The hydrogen exchange kinetics in TtCuA was further probed by ESI-mass spectrometry (Fig. 15). The native TtCuA in water shows 8+, 9+ and 10+ charge states at ambient pH corresponding to the dinuclear copper bound folded holoprotein\textsuperscript{25, 53} as estimated on the basis of the crystal structure of the folded protein\textsuperscript{79}. The aqueous solution of TtCuA at ambient pH injected into the mass spectrometer with 50% D\textsubscript{2}O in a continuous-flow setup resulted in a distinct shift in the positions of all the three charge state peaks towards larger m/z values owing to deuteration of the protons in the protein. The results show that \textasciitilde73\% of all exchangeable protons (i.e., amide and other exchangeable protons) were exchanged within the mixing time of D\textsubscript{2}O in TtCuA indicating that the thermostable CuA can undergo fast protonation/deprotonation equilibrium on change in the pH of the solution\textsuperscript{25}. Based on these results a model was proposed to rationalize the slow conformational transition that follows the protonation/deprotonation of the protein\textsuperscript{25}. The conformation of the protein is stabilized by large number of hydrogen bonds between the residues. Any conformational change of the protein backbone would require these hydrogen bonds to be opened at the same time. This model proposed that although the rate of exchange of individual hydrogen bonds can be quite fast, the opening of the hydrogen bonds may not be coherent and thus the effective rate of simultaneous exchange of several hydrogen bonds associated with a particular segment of the protein may be very small in the case of the thermostable protein\textsuperscript{25}. The complex nature of interactions in stabilization of the structure of thermostable proteins could thus result in fast exchange rates for individual exchangeable hydrogens in a protein while the conformational change remains effectively very slow in a thermostable protein.

Discussion

The pH-induced alkaline conformational change in the CuA is indicated by the hypochromism in the visible absorption bands at different wavelengths\textsuperscript{24, 25}, which reflects the distortion in the coordination of the dinuclear active site of the protein. The decrease in absorbance of the visible bands for the CuA protein at higher pH indicates the weakening of the Cu\textright\textleft\textharpoonright\textleft S(Cys) bridge\textsuperscript{25}, suggesting that the native dinuclear active site of CuA may be disrupted at high pH as a result of deprotonation of the coordinated amino acid residues like histidine, methionine and the glutamic acid. On the other hand, the hypochromism in the band intensities of CuA at lower pH was attributed to the protonation of the coordinated amino acid residues in the active site of the CuA domain. This type of feature has also been found in other cupredoxins that exhibit an active site protonation at their C-terminal Histidine residue\textsuperscript{40}. The bis-\mu(thiolato) dicopper core thus was suggested to exist in two conformational states, viz., a ‘low pH’ form that is present at ambient pH with purple color of the metal center corresponding to a ‘charge delocalised’ [Cu(1.5)-Cu(1.5)] species and a ‘high pH’ form stabilized at high pH that corresponds to a ‘valence trapped’ [Cu(I)-Cu(II)] species\textsuperscript{24, 25}. The spectroscopic studies demonstrate that increase in pH not only leads to the breaking of the ‘charge-delocalised’ dinuclear copper center leading to formation of a ‘valence-trapped’ metal center, but also causes drastic change in the tertiary organization of the strands in the protein.

Analyses of the crystal structure of the PdCuA protein suggested that the polar side chains of Tyr125 and Tyr127 are directed towards the core of the \beta-barrel and a water molecule inside the core of the \beta-barrel was proposed to be close to Tyr125\textsuperscript{24}. This showed that the core of the \beta-barrel possibly contains hydrogen-bonding network involving the Tyr125 and water. Deprotonation of the Tyr125 residue at high pH would decrease the stability of the \beta barrel and
lead to conformational change in the PdCuA. The ‘high pH’ species of PdCuA may thus be formed by deprotonation of the Tyr125 residue leading to a small conformational change in the β barrel causing changes in the geometry of the dicopper center. These results thus propose that the tyrosine residue in the hydrophobic pocket in PdCuA might be deprotonated with \( pK_a \) 8.2, which would lead to breaking of the associated hydrogen bonding network of PdCuA at high pH. This subsequently may cause a change in the conformation of the β barrel leading to breaking or weakening of the H224 coordination to copper giving rise to the formation of the ‘high pH’ form\(^{24}\). Deprotonation of the tyrosine residue in the hydrophobic region was associated with a slower rate constant possibly because of slow diffusion of protons into the hydrophobic pocket of the protein. The subsequent conformational change in the β barrel is triggered by breaking of the hydrogen-bonding network at high pH and this process would be very fast in the mesophilic protein, PdCuA.

These temperature dependent kinetics studies of the pH induced conformational change in the thermostable, TtCuA show that it not only has exceptional thermodynamic stability but also it is kinetically very inert\(^7\). The slow conformational change was also noted in several other thermostable proteins\(^{23,75-78}\). However, deuteration exchange kinetics by NMR on the thermostable rubredoxin from \( P. furiosus \) showed that the amide H/D exchange rates for different residues are very fast suggesting high flexibility of the protein backbone\(^5\). Similar H/D exchange studies using electrospray mass spectrometry in the TtCuA also showed very fast amide exchange rates. The slow conformational transition albeit presence of fast proton exchange\(^25\) thus may be characteristic of thermostable proteins. Although the rate of exchange of individual hydrogen bonds associated with the amides can be quite fast, the opening of the hydrogen bonds in a thermostable protein were proposed to be unsynchronized with each other and thus the effective rate of simultaneous exchange of several hydrogen bonds associated with a particular segment of the protein may be very slow in the case of the thermostable protein\(^{25}\). The thermostable proteins have been proposed to be optimized for their function at high temperature so that the polypeptide backbone could maintain the integrity of the active site structure at high temperature\(^5\). The dynamics of the metal active center in the TtCuA is also proposed to be optimized for the function of the protein at elevated temperatures\(^{25}\).

**Concluding Remarks**

The electron entry site in the ubiquitous multifunction respiratory enzyme, cytochrome c oxidase comprises a novel bis-μ(thiolato) dicopper center in the solvent exposed part of the subunit II of the enzyme. The dinuclear copper center undergoes interesting conformational change induced by change in the pH of the solution. This pH induced change involves equilibrium conversion of the purple ‘charge-delocalized’ form to a ‘valence-trapped’ form of the metal center. The pH dependent conformational change in the CuA from the mesophilic organism \( P. denitrificans \) and from the thermophilic organism \( T. thermophilus \) show distinct differences albeit having very similar structure and function of the metal center. Variation of redox potential with pH in TtCuA gives two \( pK_a \) values (3.5 and 9.7), while UV-visible absorbance and other studies for the protein could detect only the high pH form. The \( pK_a \) in the case of the mesophilic analogue PdCuA was 8.2. Both the acidic and alkaline conformational changes for TtCuA may be necessary for the regulation of the proton translocation coupled with the electron transfer process by cytochrome c oxidase in the mitochondrial respiratory chain.

Kinetic studies on the pH induced conformation change show two distinct steps, proton transfer and conformation change at the metal center. The conformation change near the dinuclear center in the thermostable PdCuA is extremely fast while the thermophilic protein TtCuA shows very slow conformational change that is ascribed to the intrinsic structural rigidity of thermostable protein. The H/D exchange studies further propose that the proton exchange in thermostable protein could be analogous to the mesophilic analogues in spite of having slow backbone dynamics due to structural rigidity in the thermostable protein.

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