Chapter 5
Modification in the loop region of CuA protein by protein engineering: Effect on thermal stability and redox property

5.1 Introduction

Whenever nature uses copper to promote biological electron transfer (ET), the metal appears to be encased in what has come to be known as the cupredoxin fold (1). The question why this combination of metal site and fold is such a unique and successful vehicle for biological ET is addressed in this chapter. Cytochrome c oxidase is the terminal respiratory enzyme, which belongs to a heme copper oxidase superfamily (2, 3). The primary acceptor of electrons from cytochrome c is a binuclear Cu, site. This is located outside the membrane in subunit II in bacterial as well as in mitochondrial cytochrome c oxidases (2). Since subunit II is homologous also with blue copper proteins, such as azurin and plastocyanin, it is likely that its structure is the cupredoxin fold, and β-barrel motif is found in its structure like in small blue copper proteins as well as in a sub domain in multicopper oxidases (4). It is surprising that CuA, unlike type 1 Cu in blue proteins, is a binuclear site (5). The CuA site is a 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 (6, 7). The crystallographically characterized CuA protein shares a common fold of a β-barrel (or β-sandwich) and have a basic framework of copper ligation by one histidine, two cysteine, methionine and glutamine residues from the C-terminal end of the protein and a second histidine residue nearer to the N-terminal. The two cysteine residues
bridge the two Cu atoms and form a dimeric Cu$_2$S$_2$ structure (8). The strong charge-transfer between S (Cys)-Cu and N (His)-Cu gives rise to a typical purple colour of the protein and a characteristic absorption spectrum in the visible range (9).

Proteins from thermophilic organisms, though often very similar in structures to their mesophilic homologues, are much more resistant to thermal as well as chemical denaturation (10-12). Despite a series of theoretical and experimental research in this area, the molecular basis of the stability of the proteins from thermophilic organisms is not yet been unambiguously understood. Several studies have identified a number of potential interactions that may be responsible for the observed thermostability of the protein (13, 14). An increased in the hydrogen bonds, presence of additional electrostatic interactions and enriched salt bridges, enhanced hydrophobic interactions, increased compactness, stronger binding of metal ions, enhanced polar and non polar contribution and reduced ΔCp etc. have been proposed to play an important roles in the high thermo stability of the protein (15-19).

We have constructed two site-directed mutants of Cu$_A$ domain of cytochrome c oxidase from T. thermophilus in which the $\beta$ turns 14 and 17 were modified. The loop anchoring Asp111 and Leu155 in the $\beta$ turns (PDB code 2CUA) near the Cu$_A$ active site have been replaced by Alanine and Arginine respectively (Figure 5.1). We used the purified functional enzymes to investigate the effects of the mutation with various techniques. The mutant enzymes showed metal content equivalent to the wild type, which is shown by UV-visible and CD spectroscopy, but the EPR spectra were significantly changed and the redox potential ($E_0$) of the mutated site were found to be relatively high as compared to the wild type Cu$_A$ protein.
Study of thermal stability is one of the important issues for the engineered mutant proteins. Although several factors such as increased hydrophobic and electrostatic interactions, extensive H-bonds, higher compactness, enhanced polar and non-polar contribution, enriched salt bridges, reduced $\Delta C_p$ etc. have been proposed to be responsible for the thermostability, a generalized mechanism that promotes the stability or the concept that governs this phenomenon has still remained controversial (11, 20-24). Unfolding studies of proteins have helped to gain insights into the various thermodynamic aspects of stability of these proteins (25-27). The present study shows that the thermal stability of the mutant proteins decreased drastically as compared to the wild type CuA protein.

pH-dependent studies showed that increase in pH causes drastic change in the absorption spectrum of the binuclear copper site, which indicate the conformational change in the protein structure like the wild type (7, 28). Recent studies on subunit II of cytochrome c oxidase from P. denitrificans, T. thermophilus, and P. versetus indicated the protonation/deprotonation of one of the coordinated histidines due to which at high pH, valence trapped state is possibly found instead of delocalised state of the native CuA at ambient pH (7, 28, 29). But different pKa values found for the mutant proteins than those of the native CuA also support the role of Asp111 and Leu155 in the loop near the active site on the protein structure.
Figure 5.1: Modification in the loop near active site of Cu_A
5.2 Results

5.2.1 Expression and purification of mutants

The site directed mutagenesis to produce D111A and L155R mutants were carried out using QuikChange Site-Directed Mutagenesis Kit and suitable oligonucleotide primers as mentioned in chapter 2. The plasmids isolated from the XL1-blue colonies for D111A and L155R mutation were digested with EagI (Figure 5.2 (A)) and AccIII (Figure 5.2 (B)) restriction enzyme respectively.

![Agarose gel](image)

**Figure 5.2:** Agarose (0.7 %) gel of (A) EagI digested wild type CuA plasmids (Lane 5), plasmids from XL1-Blue colonies after site directed mutagenesis of C149H (Lane 2) and the BenchTop 1kb DNA Ladder marker (Lane 1), (B) AccIII digested wild type CuA plasmids (Lane 4), plasmids from XL1-Blue colonies after site directed mutagenesis of L155R (Lane 2) and the BenchTop 1kb DNA Ladder marker (Lane 1).

The plasmid shows new bands (lane 5) for wild type in Figure 5.2 (A) compared to the D111A mutant (lane 2) indicating the removal of the EagI restriction site along with the desired mutation. Similarly Figure 5.2 (B) shows plasmid bands in lane 2 and 4 migrated...
to different length suggesting the removal of Eco52I restriction site in plasmids of lane 4. The WT, D111A and L155R mutant of CuA were expressed and purified following the protocol described earlier (Chapter 2). The purified enzymes were stored at -30 °C as mentioned earlier (Chapter 2). We have constructed the site directed mutants D111A and L155R of the CuA domain of cytochrome c oxidase from Thermus thermophilus. The two bridging cysteine variants were expressed in E.coli and purified as described in chapter 2. Copper analysis was done by ICPAES (30) and Cu/protein ratios were found to be 1.75, 1.93 and 1.76 for D111A, L155R and wild type CuA respectively.

5.2.2 UV-visible Absorption Spectral studies

Figure 5.3: UV-Visible spectra of wild type CuA (—), D111A (---) and L155R (…) in 50 mM Tris buffer at pH 6.5. Concentration 7 µM

The UV-vis absorption spectra of wild type CuA and engineered mutants are shown in Figure 5.3. The absorption spectrum of wild type CuA displays a typical purple copper spectrum with strong absorption at 478 nm and 530 nm along with low intensity band at 360 nm and 790 nm. The engineered mutants of CuA center exhibit similar type
of spectra. The peak positions and the intensities are listed in Table 5.1. In the case of the mutants the spectra are similar to the wild type CuA after modification in the β turns. The ratios between the two LMCT bands are different in case of the mutants as compared to the wild type CuA protein. Nonetheless the other optical features are similar to the wild type protein.

5.2.3 CD spectra

Figure 5.4 shows the room temperature CD spectra of wild type CuA and the loop mutants of CuA.

![Graph A: Visible CD spectra of wild type CuA (---), D111A (--) and L155R (--) in 50 mM Tris buffer at pH 6.5. Concentration 30 μM.](image1)

![Graph B: Far UV CD spectra of wild type CuA, D111A and L155R with 50 mM Tris buffer at pH 6.5. Concentration 15 μM.](image2)

The visible CD spectrum of purple CuA from *Thermus thermophilus* shows a pair of characteristic features occurring at 460 nm and 525 nm, which are intense and oppositely signed. An additional weaker positive feature is also observed at around 350 nm. The
visible CD spectra for the mutants D111A and L155R depicted in Figure 5.4A are similar to the wild type Cu₄A protein. Both D111A and L155R mutants show similar positive features at 520 nm and 347 nm and negative feature at 462 nm. On the other hand there is also no change in the secondary structure of the mutants as compared to the wild type retaining the β barrel scaffold. The far UV CD spectra are shown in Figure 5.4B which shows that both the mutants contain mostly β component similar to the wild type Cu₄A protein.

5.2.4 EPR spectroscopy

The native and simulated EPR spectra for loop mutants are shown in Figure 5.5 along with the wild type Cu₄A protein. In both the mutants, the modification in the β turns anchoring the active site of Cu₄A protein has the influence in the spectra, which is highlighted by the g and A values determined from the simulations. The EPR parameters obtained from the simulation are listed in the Table 5.1. The most significant effect in the mutants is the four line spectra with higher g₂ value accompanied by a sizable increase in A₂₀ to the contrary to the seven line spectra of wild type Cu₄A protein.
Figure 5.5: X-band EPR spectra for wild type CuA (A), D111A (B) and L155R (C) were recorded at 77K with a microwave frequency 9.45 GHz. The protein samples (50 μM) were in 50 mM Tris buffer pH 6.5 plus 10% glycerol. (Solid line- experimental spectra and Dash line- simulated spectra)

5.2.5 Reduction potentials of the loop mutants

The reduction potentials of the loop mutants D111A and L155R were determined by cyclic voltammetry. The mutants yield good quasi reversible responses on glassy carbon electrode at neutral pH at a scan rate of 10mV/s shown in Figure 5.6. The peak
currents are proportional to the (scan rate)$^{1/2}$ in the range of ~5-70 mV/s. In contrast to the changes observed by UV-visible and EPR spectroscopic techniques, large variations in reduction potential were observed for the mutants from the wild type Cu$_A$. The reduction potentials obtained are 328 ± 5 mV for D111A and 294 ± 7 mV for L155R, while the reduction potential for wild type Cu$_A$ is found to be 260 ±5 mV at pH 6.5 (Table 5.1). From these values, it is seen that mutation replacing the residue in the loop region of the wild type Cu$_A$ leads to increase the reduction potentials to large extent. The reduction potential values for the mutants are within the range of normal copper proteins.

Figure 5.6: Cyclic voltammogram of D111A, L155R and wild type Cu$_A$ in 50 mM Tris buffer at pH 6.5.
Table 5.1: Properties of the mutants (D111A and L155R) as compared to the wild type CuA

<table>
<thead>
<tr>
<th>Parameter</th>
<th>WTCuA</th>
<th>D111A</th>
<th>L155R</th>
</tr>
</thead>
<tbody>
<tr>
<td>UV/Vis&lt;sup&gt;a&lt;/sup&gt;</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
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<td>0.46</td>
<td>0.48</td>
</tr>
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<td></td>
<td></td>
<td></td>
</tr>
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<td>2.05</td>
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<td>3.5 &amp; 9.5</td>
<td>3.2 &amp; 8.6</td>
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</table>

<sup>a</sup> Measured in 50 mM Tris HCl buffer at pH 6.5 (room temperature).  <sup>b</sup> Recorded at 77K in 50 mM Tris HCl buffer at pH 6.5 plus 10% glycerol. All EPR parameters are derived from simulation using the program SIMFONIA (Bruker).  <sup>c</sup> Measured at pH 6.5 (50 mM Tris HCl buffer).  <sup>d</sup> Measured from the variation of absorption and ellipticity at different pH (pH 2-12, Universal buffer).
5.2.6 Thermal stability

The visible absorption spectrum of the wild type Cu₄ 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 (7, 31). Increase in temperature of the protein solution up to 95°C at ambient pH did not affect the absorption spectra of Cu₄ protein from Thermus thermophilus indicating very high thermostability of the protein as reported earlier (32, 33). Figure 5.7 shows the temperature dependence of the visible absorption spectra of the loop mutants at pH 5.0 upto 70°C only. The absorbance of the characteristic peaks at 478 nm and 531 nm has been decreased as the temperature increases. Consequently the absorbance at 360 nm peak increased with the increase in temperature.

Figure 5.7: UV-Visible spectra of D111A (A) L155R (B) at different temperature (20°C -70°C) in 50mM Tris-HCl buffer at pH 5.0
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 of the protein at different temperature. The far UV-CD spectrum of the protein showed characteristic β-sheet content in the secondary structure of the protein while the visible region CD spectra 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 has been depicted in Figure 5.8. The thermal unfolding data for the mutants are analysed considering it to be a two state unfolding process.

Figure 5.8: (A) Plot of ellipticity against temperature at 222 nm, 478 nm, and 530 nm for D111A mutant. (B) Plot of ellipticity against temperature at 222 nm, 478 nm, and 530 nm for L155R mutant.
This indicated that the secondary structure of the loop mutants of CuA unfolds later than the tertiary structure of the proteins. From temperature dependent CD study at the Far UV region, it is found that the mid point unfolding temperature are $76.3 \pm 0.2^\circ C$ for D111A and $79.1 \pm 0.2^\circ C$ for L155R mutant whereas the midpoint potential for the secondary structure of the wild type CuA from *Thermus thermophilus* is more than $100^\circ C$ (32).

Figure 5.9 shows the typical temperature dependent comparison of the mutants as well as CuA protein with the fraction unfolding data evaluated from the tertiary CD at neutral pH. The results indicate that the thermal stability of the mutants D111A and L155R decreases drastically as compared to the wild type CuA.

**Figure 5.9:** Plot of fraction unfolding against temperature at tertiary structure for wild type CuA (oxidized), D111A and L155R.
Thus, the replacement of the loop residues seems to affect the thermal stability of the protein largely. The midpoint unfolding temperatures are 67.1 ± 0.2⁰C for D111A and 64.3 ± 0.2⁰C for L155R, while earlier studies (32) showed that the midpoint unfolding temperature (Tm) for the wild type Cu₄ was more than 100⁰C at ambient pH for the reduced state, suggesting that the thermal stability decreases drastically with the mutation in the loop region of the Cu₄ protein.

5.2.7 Stability curve

The plot of the conformational stability (ΔG₄) of the protein versus temperature is very well known as the “protein stability curve” defined by the modified Gibbs–Helmholtz equation (34, 35). The nature of the stability curve depends on how the different thermodynamic parameters such as ΔHₘ, Tₘ, and ΔCₚ contribute to the conformational stability (ΔG₄) of the protein (36). As these thermodynamic parameters are related to the structure of the protein in folded and unfolded states, these values are often used to extract the structural information for the folded and unfolded states of the protein. The temperature dependence of the free energy of unfolding, ΔGₜ, data for the tertiary structure around the copper center in wild type Cu₄ and the loop mutants were fitted to the modified Gibbs Helmholtz equation (Eqn 2.5) (Figure 5.10).
Figures 5.10: Protein stability curves plotted for tertiary structure of the wild type CuA (■), D111A (○) and L155R (△) mutant. Each point in the stability curves represents the free energy of unfolding ($\Delta G_y$) at corresponding temperature. The solid line represents non-linear curve fits to Gibbs-Helmholtz equation (Eqn 2.5).

The stability curves for the tertiary structure of the loop mutants were found to be lower and narrower at all the temperatures as compared to the wild type CuA protein. The $\Delta C_p$ values for the D111A mutant (6.6 kJ mol$^{-1}$K$^{-1}$) and L155R mutant (6.3 kJ mol$^{-1}$K$^{-1}$) were found to be greater as compared to both the wild type CuA (2.45 kJ mol$^{-1}$K$^{-1}$). The midpoint entropy changes ($\Delta S_m = \Delta H_m / T_m$) for the D111A mutant (0.58 kJ mol$^{-1}$) and L155R mutant protein (0.59 kJ mol$^{-1}$) were also higher than that of the wild type CuA protein (0.234 kJ mol$^{-1}$) (28). The reduced $\Delta C_p$ and low $\Delta S_m$ values are known to broaden the stability curve (36). Hence the low thermal ($T_m$) and conformational stability ($\Delta G_c$) of the loop mutants were attributed to their greater $\Delta C_p$ and higher $\Delta S_m$ values.
5.2.8 pH induced conformational change

The loop mutants are purple colored protein having characteristic absorption bands in the visible region at 365, 477, 530, 790 nm like the wild type CuA protein from *Thermus thermophilus* (6). Increase or decrease in pH also did not immediately show any change in the spectra of the protein solution unlike in case of the *Paracoccus* CuA reported earlier (3). However, increase in pH of the wild type CuA solution was found to cause a slow but distinct change in the spectra indicating changes in the environment around the metal ion at high pH. Figure 5.11 (A) & (B) show the spectral changes in the absorption spectra for the loop mutants on increase or decrease in pH, which was markedly slow and the intensities of all the visible absorption bands were decreased with consecutive increase in intensity at ~ 320 nm with increase in pH. The protein solution was incubated for 48 hours under nitrogen atmosphere at each pH ranging from 2 to 12 to
ensure equilibrium at each pH. The nature of the buffer did not have any effect on the spectrum of the protein other than changing pH of the medium.

Figure 5.12A and 5.12B show the plot of maximum absorption for D111A and L155R mutants at 530 nm and 790 nm against the pH of the protein solution. This clearly shows the acid and alkaline transition of the loop variants as function of pH, which is reversible in nature indicating no depletion of Cu during the transitions.

Figure 5.12: The variation of absorbance of D111A mutant (A) with pH: ▼ at 794 nm; ■ at 530 nm; • at 478 nm; A358 nm and of L155R mutant (B) with pH 6.5 ▼ at 791 nm; ■ at 531 nm; • at 477 nm; A361 nm. Protein concentration: 35pM.

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The variation of CD of D111A mutant (C) with pH: A 329 nm; • at 468 nm; ■ at 521 nm and of L155R mutant (D) with pH: A 329 nm; • at 468 nm; ■ at 521 nm. Protein concentration: 50 μM.
Analogous observations of pH dependence in the visible CD spectra of the loop mutant proteins, where the intensities of CD bands at 521 nm and 468 nm were decreased with increase or decrease in pH (Figure 5.12C and 5.12D), support the possibility of conformational change at the dinuclear Cu site in both the mutants with the variation of pH (2-12).

The variation in the absorbance and CD at different wavelength as a function of pH could be analyzed by a one-site protonation/deprotonation equilibrium model (Eqn 4.1). The apparent pKₐ values were determined at 3.5 & 9.5 and 3.2 & 8.6 for D111A L155R mutant of Cuₐ protein respectively at 25°C.

5.3 Discussion

The modification in the β turns anchoring the active site of Cuₐ protein from Thermus thermophilus has limited effect on the active site structure of Cuₐ protein. In both the mutants D111A and L155R, a stable copper center with β barrel scaffold retained, which are redox active and are able to undergo intermolecular electron transfer. The UV/visible and CD spectra of the loop mutants D111A and L155R are remarkably similar to those of the wild type Cuₐ from Thermus thermophilus. Hence the optical and CD spectroscopic properties of the mutants indicate that the presence of binuclear copper active site with the dithiolato bridges, which seems basically to be anchored by the two β strands. Only slight difference in the Cys(S) → Cu interaction can be identified from spectroscopic data as the ratio between the A₄78nm and A₃60nm is different from that of the wild type Cuₐ protein. However EPR spectra showed significant changes for the mutants compared to the wild type. In case of the mutant D111A and L155R, four lines in the
EPR spectra are found instead of seven line split for the native CuA, which indicate that
the delocalization of the electron around the two copper atoms has vanished and the
unpaired electron has localized to one of the copper atom in the mutant proteins. That
means although the active site with two copper and thiolato bridge is constraint in case of
the mutants, yet the geometry of the active has been changed dramatically.

Both the mutants have the reduction potential value well within the range of the
copper containing proteins. The reduction potentials of the mutants have been increased
as compared to the wild type CuA protein at neutral pH. Thus the replacements of Asp111
in β turn 14 by Ala and L155 in β turn 17 by Arg results in increase in reduction
potential. Basically the reduction potential of the protein is tuned by the environment of
the active site of that protein (32, 37, 38). The site directed mutation on the β turns which
controlled the β strands to provide the active site in CuA protein from Thermus
thermophilus has effect on the active site geometry. Whether these variations are due to
subtle geometrical modifications, altered solvent exposure, or may be combination of
these two. The mutation of negatively charged amino acid residue Asp111 by a
hydrophobic residue Ala and a hydrophobic residue Leu155 by a positively charged
residue Arg in the C terminal β turns, the hydrophilicity/hydrophobicity possibly
involved in modulating the microenvironment of the active site as well as the reduction
potential of the metal center. However, replacing Asp111 by Ala, the hydrogen bond to
His114 disrupted (28, 39), as a result of which reduction potential should have largely
decreased (30). But it is surprising that reduction potential of D111A mutant was found
to increase ~70 mV from the wild type. Thus there are probably a number of contributing
factors to the observed influence of the mutation at the $\beta$ turns on the reduction potential of Cu$_A$ protein.

The thermal and conformational stability of the loop mutants have been decreased drastically as compared to the wild type Cu$_A$ protein from *Thermus thermophilus*. There is a decrease of $\sim$25-30$^\circ$C in the unfolding temperature for the oxidized mutant proteins from the native Cu$_A$ protein. There is a possibility that the mutation of Asp11 by Ala has disrupted the hydrogen bond network in the Cu$_A$ protein scaffold while the mutation of Leu155 by Arg has disrupted the salt bridge network of the protein, which is required for the stability of the protein (28). The difference in the pKa values for the mutants from the wild type Cu$_A$ protein can also be attributed to the subtle changes in the microenvironment of the dinuclear active site as a result of the mutation in the $\beta$ turns anchoring the active core.

An increase or decrease in pH also did not immediately show any change in the spectra of the protein solution. However, increase in pH of the protein solution was found to cause distinct change in the spectra indicating changes in the environment around the metal ion with the variation of pH. The spectral change on increase or decrease in pH was very slow and the intensities of all the visible absorption bands were decreased gradually. pH dependence conformational change are observed in the case of both the mutants, which are similar to the wild type (Chapter 4) but with different pKa values. The pH induced conformational transition was reversible in nature as observed in the restoration of 90% of the original spectrum of the protein on incubation for $\sim$48h. This supported that there was no release of free copper ion from the protein on increasing pH, which agreed with earlier reports on the mesophilic Cu$_A$ protein (3). Lappalainen et. al
showed that at pH 7.0, the CuA from *Paracoccus denitrificans* (Paracoccus CuA) shows absorption bands at 360, 480, 535 and 810 nm, which are similar to those of the wild type CuA from *Thermus thermophilus* (3). We have earlier shown (40) that increase in pH from 6.5 to 10 causes a pH induced conformational change in the Paracoccus CuA analogous to that observed in the present case for the wild type CuA from *Thermus thermophilus*. The \( pK_a \) for this transition in the Paracoccus CuA protein was 8.2 at room temperature, which is much lower than that observed in the wild type CuA from *Thermus thermophilus* (\( pK_a = 9.7 \)) (Chapter 4). Comparison of the spectral features of the loop mutants with those of wild type *Thermus* CuA and *Paracoccus* CuA shows different \( pK_a \) values for the loop mutants than the native CuA protein, which may be due to the subtle changes occurred in the loop region and induce the protonationation/deprotonation equilibrium.

**5.4 Conclusion**

Site directed mutation in the \( \beta \) turns which anchor the main \( \beta \) strands containing the active site of the electron transfer protein CuA protein from *Thermus thermophilus* results a subtle change in geometry of the active site. The thermal and conformational stability of the mutants drastically decreases as compared to wild type CuA protein due to the change in the loop nearby to the active site. These two mutations lead to increase in the reduction potential by \(-30\text{-}70\text{ mV}\). As a whole, this study shows the importance of the amino acid residues in the \( \beta \) turns nearby the active site of CuA protein, which is involved in tuning the stability and redox potential of the CuA protein.
5.5 References


