0.2 Abstract

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Introduction:

Cytochrome c oxidase (CcO) is the ubiquitous terminal respiratory enzyme present in the inner mitochondrial membrane of eukaryotes and in the periplasmic membrane of prokaryotic systems (1). It mediates trans-membrane electron transfer from cytochrome c in outside the membrane to molecular oxygen bound at the membrane embedded catalytic site of the enzyme (1, 2). The overall enzymatic reaction is

\[ O_2 + 4e^- (\text{cytc}) + 8H^+ \text{in} \rightarrow 2H_2O + 4H^+ \text{out} \]

This electron transfer process is coupled to the active proton pumping activity of the enzyme from inside to outside of the membrane generating the proton motive force (2-4).

The mammalian CcO contains 13 subunit; first four of which are encoded by mitochondrial DNA. The bacterial enzyme, on the other hand consists of 4 subunits that have high sequence homology with the first four subunits of the eukaryotic mitochondrial counterparts (5, 6). Certain bacteria also contain ubiquinol oxidase as terminal oxidase, which bears close similarity to the cytochrome c oxidase. The first two subunits of CcO viz., subunit I and subunit II contains the main functional units pertaining to the electron transfer and proton pumping activity of the enzyme. Subunit I is a transmembrane protein containing a six coordinated low spin heme (heme \(a\), iron protoporphyrin a complex), and a binuclear site formed by a copper center (CuB) lying close to a five coordinated high spin heme (heme \(a_3\)). This heme \(a_3\)-CuB binuclear center forms the catalytic site of the enzyme and molecular oxygen binds at the axial position of the iron in heme \(a_3\). Subunit II of CcO contains a dinuclear copper center, CuA (Cu\(^{1+}\).....Cu\(^{15+}\)) at the solvent exposed domain of the enzyme (6-8). Reduced cytochrome c binds at the surface of the subunit II of CcO during the enzymatic function, and transfers an electron to the CuA center. This is
then is followed by intramolecular electron transfer from the CuA center of the subunit II to the heme \( a \), and finally to the binuclear heme \( a_2-CuB \) site of the subunit I of the enzyme.

The main functional features of cytochrome c oxidase can be classified as, (a) intermolecular electron transfer from cytochrome c to the CuA site in the subunit II of cytochrome c oxidase, (b) intramolecular electron transfer from CuA to heme \( a \), and heme \( a \) to the binuclear \( a_2-CuB \) site, (c) reduction of oxygen to water at the binuclear heme \( a_2-CuB \) site, (d) transmembrane active proton pumping driven by the electron transport, and (e) translocation of the water produced by reduction of oxygen. These processes are interrelated to each other and understanding of the biological function of this complex enzyme would require detail mechanistic understanding of all of these processes, which is a subject of immense interest.

The crystal structures of the enzyme from bacterial as well as mammalian sources have been reported which revealed several important structural aspects of the enzyme (6-8). However, the specific functional role of the dinuclear CuA center in the enzyme is still not unambiguously understood.

Cytochrome c552 is a highly thermostable c-type heme protein from a Gram-negative eubacterium *Thermus thermophilus* HB8 (9). This water soluble, 14.4 kDa periplasmic class I cytochrome c is known to be involved in the terminal step of the bacterial respiratory electron transfer process. The binding of heme cofactor through axial ligands in the heme protein not only helps in the folding but also known to play significant role towards the conformational stability of the protein (10). Emphases were given to modulate different functions in the thermophilic cytochrome c552 by
substitution of one of the axial ligand (methionine) by alanine (11) and one distal residue (valine) above the heme by aspartic acid. The high thermostability of cytochrome c552 has the advantage to engineer this protein to create an artificial thermostable peroxidase. Cytochrome c552 can be transformed into a thermally stable artificial peroxidase by rational modification based on the molecular mechanisms of natural peroxidases.

The goal of the present thesis is to obtain more detailed insight in interactions between redox proteins and electrodes and the mechanisms of electron transfer. In addition to this, the influences of the protein environment on the redox properties of the active site have been considered. The possible influence of the promoter in the electron transfer of the protein to the electrode was also considered, because redox enzymes do not often give an unambiguous and reversible electrochemical response.

The present thesis describes the studies of the structure function relationship of the soluble domain of the subunit II containing CuA site in the complex respiratory enzyme cytochrome c oxidase by various spectroscopic and electrochemical methods. This thesis also describes the enhanced peroxidase activity of cytochrome c552 double mutant V49D/M69A at high temperatures. This thesis is comprised of seven chapters. A chapter wise brief description has been given below.

A brief account of the work presented in this thesis

Chapter 1: Introduction

This chapter gives a brief introduction of the respiratory chain and electron transfer proteins. The basic electron transfer theory for long range electron transfer processes and the direct electrochemistry of the metalloproteins are reviewed in this chapter. It also
gives a description of peroxidases and its mechanism of function. This chapter represents the overview the importance of the whole thesis work.

Chapter 2: Materials and Methods

This chapter is about the Materials used for carrying out the experiments during PhD work. It also makes a portrait of some basic principles and methods in biochemistry and biophysics that were developed and applied during PhD work.

The results of the thesis are discussed in the following five chapters:

Chapter 3: Direct electrochemistry of CuA protein from cytochrome c oxidase of Thermus thermophilus in presence of surfactants

Cytochrome c oxidase is ubiquitous enzyme involved in the terminal step of respiratory electron transfer process. The unique binuclear copper center containing bis-dithiolato bridges form a valence delocalized [Cu\(^{1+5}\)-Cu\(^{1+5}\)] state of the metal center located at the subunit II of cytochrome c oxidase. This metal center acts as the electron entry site of the enzyme and accepts electrons from cytochrome c. Direct electrochemistry of this binuclear copper center containing the water soluble protein obtained by genetically truncating the membrane bound part of the subunit II from Thermus thermophilus was achieved by confining the protein on glassy carbon electrode surface in presence of various surfactants. Totally reproducible, Nernstian responses are obtained with CuA. The redox potential and the electrochemical response were enhanced prominently in case of cationic surfactant CTAB indicating that the nature of the surfactant has a significant effect on the microenvironment of the protein-electrode interface. The results have been used to understand the mechanism of electron transfer from cytochrome c to the copper center during the enzymatic reaction.
Chapter 4: pH-Induced transition in CuA domain from Thermus thermophilus: An electrochemical approach

Direct electrochemistry of this CuA domain obtained by genetically truncating membrane bound part of the subunit II of cytochrome c oxidase from Thermus thermophilus has been examined to understand the redox properties of the metal center in the protein. At room temperature, CuA exhibits a reversible reduction at a neomycin sulphide modified glassy carbon electrode (50mM Tris, pH 6.5) with $E^\circ = 0.26\text{V vs NHE}$. In the present study, we have also demonstrated the electrochemical behavior of CuA in terms of reduction potential ($E^\circ$), which is sensitive to the acid/base equilibrium dynamics. CuA protein was found to be its native state at pH 6.5, while at lower and higher pH region, the redox properties of CuA protein changed considerably. Three distinct redox forms were identified, which interconvert with change in pH. The low pH conformation probably involves disruption of the CuA active site and protein unfolding whereas the alkaline conformation probably corresponds to a structural change in the metal active site of the protein leading to formation of a high pH form of the protein. Two distinct pKa values were observed at 3.5 and 9.7. These results were used to understand the mechanism of electron transfer from cytochrome c to CuA domain and CuA domain to the low spin Heme $a_3$ in the mitochondrial chain.

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

Two loop-mutants of the purple copper protein CuA from Thermus thermophilus have been constructed and characterized. The copper centers of both the variants appear to be similar with the native CuA, although the variants exhibit diminished stability. The absorption spectra and the circular dichroism spectra of the loop variants display similar
features at room temperature. The reduction potentials of the two stable variants are all higher than that of wt Cu$_A$ protein. In contrast, the most significant effect in the variants is the four line EPR spectra with higher $g_z$ value accompanied by a sizable increase in $A_z$, to the contrary to the seven line spectra of wild type Cu$_A$ protein. The reduced forms of the loop-mutants D111A and L155R also showed pKa values 3.5 & 9.5 and 3.2 & 8.6 respectively which are distinctly different from that of the native Cu$_A$ protein. The results are interpreted by taking into consideration that the modification in the loop residues is intimately connected with the stable $\beta$-sandwich structure of the cupredoxin which gives better understanding to the conformational and structural stability of the protein linked to the remarkable changes in the cupredoxin fold. These results were also used to understand the Cu$_A$ active site for mediating biological electron transfer.

Chapter 6: Spectroscopic and electrochemical characterization of engineered mononuclear variants in binuclear Cu$_A$ from *Thermus thermophilus*

Spectroscopic and electrochemical characterization of a purple Cu$_A$ center engineered into the blue copper protein from *Thermus thermophilus* is presented. Bridging cysteine ligands of the Cu center in the Cu$_A$ protein were replaced with serine and histidine, and the variants (C149H and C153S Cu$_A$ mutant proteins) were characterized by electrochemistry, as well as electronic absorption (UV-vis), circular dichroism (CD), electron paramagnetic resonance (EPR) spectroscopic techniques. The replacements resulted in dramatically perturbed spectroscopic properties, indicating that the cysteines play a critical role in maintaining the structural integrity of the Cu center. The replacements at different cysteine residues resulted in different perturbations, because the two cysteines are geometrically symmetrical in the primary coordination sphere with respect to the two copper ions in the native Cu$_A$ protein. The ICPAES data for copper
analysis indicated that the protein binds one copper ion per protein. The UV-visible, CD, EPR and electrochemistry of the engineered blue CuA are strikingly similar to other native blue Cu centers, indicating that they all share similar geometric and electronic structures. Both the C149H and the C153S variants have one copper center with slight tetragonal distortion which suggests the rearrangement of the ligand around the copper active site. This difference in mutation effects at different cysteines may be due to different constraints exerted on the two cysteines by hydrogen-bonding patterns in the ligand loop of the protein scaffold.

Chapter 7: Engineering of Thermus thermophilus Cytochrome c552: Thermally Tolerant Artificial Peroxidase

The site specific mutants of c552 from Thermus thermophilus were designed to introduce residues that could act as acid-base catalysts near the active site to enhance the peroxidase activity. The Val49 in the distal heme pocket of c552 was found to be located at a position almost equivalent to the Glu183 that is involved in stabilization of the ferryl heme intermediate in chloroperoxidase (CPO). The Val49 residue of c552 was mutated with aspartic acid (V49D) and Met69 with alanine (M69A) simultaneously that could potentially form hydrogen bond with hydrogen peroxide and facilitate formation and stabilization of the putative redox intermediate of the peroxidase cycle. The double mutant V49D/M69A was shows better peroxidase activity than its wild type, single mutant M69A even at higher temperature than 50°C. The optimum temperature for the peroxidase activity of the c552 double mutant was found to be 70°C, making it a high-temperature peroxidase. The results have been discussed in the light of understanding structure function relationship of these heme proteins and the creation of a thermally stable artificial peroxidase. We demonstrate that cytochrome c552 (cyt c552) from Thermus
thermophilus HB8 can be transformed into a thermally tolerant peroxidase by a design process modeled on the catalytic mechanism of peroxidases. At temperatures above 50°C, the enzymatic activity of the engineered cyt c552 surpasses that of a myoglobin variant that is known to exhibit the highest activity among artificial peroxidases. In future our protein V49D/M69A may be a promising thermostable artificial peroxidase.

It can be concluded that the interaction of redox enzymes with the glassy carbon electrode is determined primarily in presence of surfactants with different polarity. The charge of the surfactant played role in acquiring reversible electrochemical responses for the protein on the electrode surface. A flexible promoter that can adjust its shape to fit both the protein surface and the electrode surface gives the best results. The irreversible denaturation of the protein on the electrode surface was overcome by using surfactant as promoter. The electrostatic and hydrophobic interactions probably play an important role in this process. The electrode thereby becomes gradually more accessible for electron transfer in presence of surfactants. A good promoter therefore not only forms a flexible bridge that compensates the electrostatic repulsion, but must also protect the protein from the hydrophobic patches on the electrode. The dependence of the redox potentials of CuA on pH, measured with electrochemistry showed the existence of different conformational change of CuA. Neomycin sulphate apparently has a function as promoter for CuA protein in this pH dependent electrochemical study. The redox potential as well as thermal stability of a protein is always influenced pH of the solution, the active site environment and also by the charges of the peptide. The mutagenic studies of CuA protein showed that the redox potential of CuA vary with the mutation in the loop region, which exerts subtle changes in the active site of the CuA. The thermal stability also decreases with the
mutation in the loop region of Cu₄ protein. Simultaneously spectroscopic and
electrochemical measurements of the mutants replacing the bridging cysteines in the
active core of the Cu₄ showed that a mononuclear blue copper protein can be engineered
inside the dinuclear Cu₄ protein scaffold. Moreover, a thermally tolerant artificial
peroxidase mimic was created successfully by rational protein engineering in cytochrome
c₅₅₂, which is an electron transfer protein in nature.

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