Cytochrome c oxidase: The unique enzyme at the terminal step of electron transfer in the respiratory chain

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The terminal step of the respiratory chain involves a multiple subunit enzyme called cytochrome c oxidase. This enzyme spans the inner mitochondrial membrane with active sites on both sides of the membrane and mediates transmembrane electron transfer coupled with active proton pumping across the membrane. Cytochrome c oxidase catalyses the most crucial and only irreversible step in the respiratory process where electrons from cytochrome c are transported from outside the inner mitochondrial membrane to reduce molecular oxygen into water. The present article gives a brief description of the molecular structure of this complex membrane protein in the light of the functioning of the active sites of the enzyme.

THE cellular respiration process involves a series of electron transfer enzymes which transport the reducing equivalents obtained from metabolism of food stuffs, to reduce molecular oxygen into water. In the case of eukaryotic cells, these processes take place inside mitochondria. The electron transfer enzymes, commonly called complexes I, II, III and IV reside in the inner mitochondrial membrane. Electron transfer from complex I to complex IV in the outerside of the inner mitochondrial membrane takes place along the downhill of electron potential[1]. The terminal step of electron transfer involves reduction of molecular oxygen by the complex IV. The complex IV in respiratory chain is a multi-subunit membrane bound enzyme called cytochrome c oxidase (CcO). This enzyme spans the inner mitochondrial membrane with active sites at either side of the membrane. Molecular oxygen diffuses into the cell and binds at the active site of cytochrome c oxidase inside the inner mitochondrial membrane (matrix side). A schematic diagram of the electron transfer pathway from complex I to complex IV in the inner mitochondrial membrane is given in Figure 1. Electron from complex III is transported by the small electron carrier protein, cytochrome c, to cytochrome c oxidase and it enters the enzyme at the cytosolic or outer side of the inner mitochondrial membrane (Figure 1). This follows a transmembrane electron transfer across cytochrome c oxidase by which an electron from outside of the membrane reaches the inside of the membrane at the active site of cytochrome c oxidase where molecular oxygen is attached and causes reduction of dioxygen to water. The overall reaction involved is:

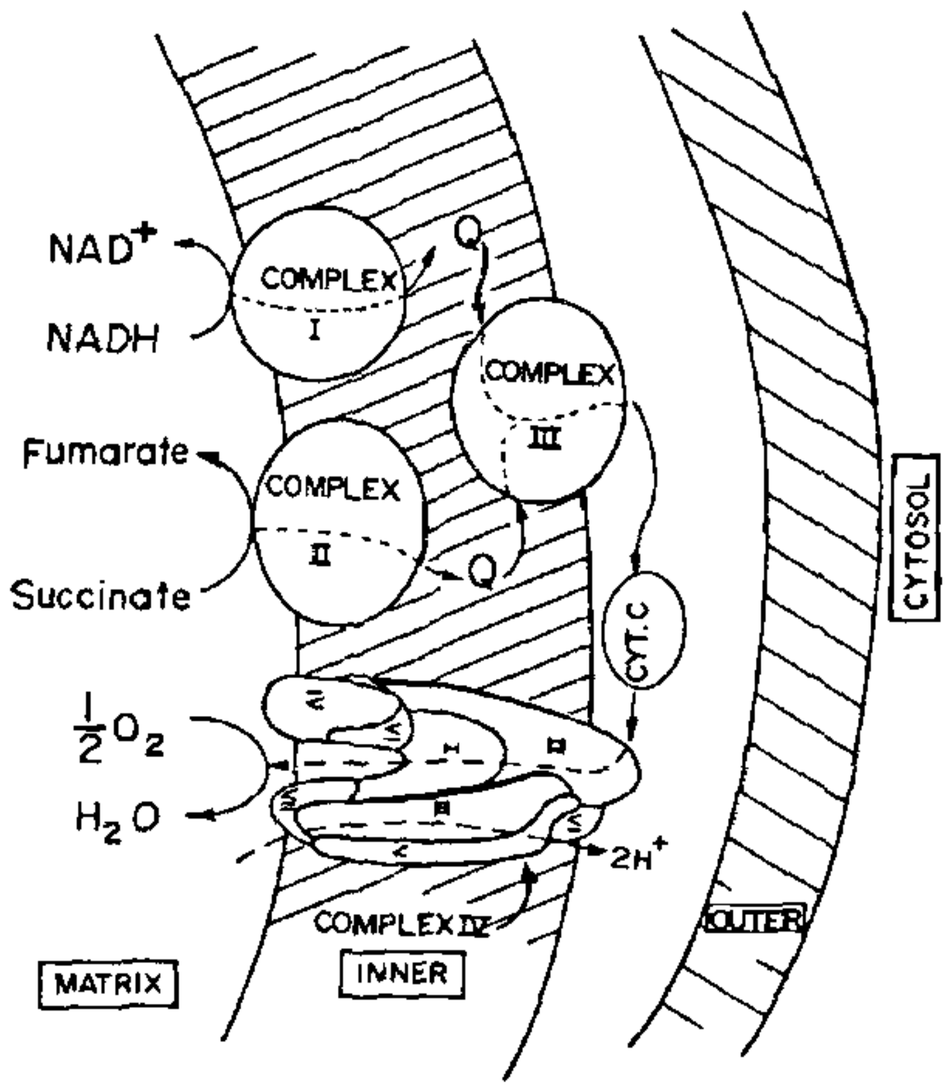

Figure 1. Schematic of the respiratory chain in the mitochondrial membrane. The subunits of the mammalian complex IV (CcO) embedded in the lipid bilayer are also shown.

$$4H^+ + 4e^- + O_2 \rightarrow 2H_2O.$$

The whole process of transmembrane electron transfer by complex IV has been shown to be associated with an active vectorial proton pumping from inside to outside of the mitochondrial membrane[2,3]. The resulting pH gradient and associated potential gradient have been shown to be the driving force for the synthesis of ATP from ADP by ATP synthase[1]. ATP is stored as the energy currency of the cell.

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The terminal step of electron transfer by cytochrome c oxidase has attracted extensive studies in recent years. Over the last few years several review articles[1-8], books[9-11] and even some journals have devoted entire volumes[12] to papers concerned with the structure and function of this enzyme. The interest in studies on cytochrome c oxidase is two-fold. Firstly, the overall process of transmembrane electron transfer across the 'non-conducting' peptide backbone from cytochrome c (1 electron donor) to dioxygen (4 electron acceptor) offers an interesting mechanistic problem. Further, this enzyme catalyses the most vital and only irreversible step in the respiratory chain. Secondly, this enzyme is crucial to energy conservation. Its role in energy conservation is the most important part of its function involving generation of redox linked proton gradient across the mitochondrial membrane which is also of great interest with reference to Mitchell's chemiosmotic theory[13]. Irregularities in the cytochrome c oxidase distribution in cells may give rise to several fatal diseases. Pathological studies show[14] that myopathies, myopathies with renal dysfunction, cardiomyopathies and encephalomyopathies can be observed due to defects in cytochrome c oxidase. Babies born with a severe cytochrome c oxidase deficiency in skeletal muscle at birth suffer from the so-called 'floppy baby' syndrome. In some cases it has been observed that a 'floppy baby', if survived, becomes capable of synthesizing cytochrome c oxidase to a normal level in the deficient skeletal muscles which indicates that synthesis and regulation of this enzyme is highly essential for healthy growth of the living beings.

The present article outlines some of the structural features of the enzyme in the light of the unique biological functioning of the system, especially on the mechanistic aspects of the transmembrane electron transfer by cytochrome c oxidase, coupled with vectorial proton pump across the membrane.

Subunit composition and structure of the enzyme

Electron microscopy and imaging studies on cytochrome c oxidase showed that the general shape of the monomeric cytochrome c oxidase looks like a 'Y', with the stalk of the 'Y' extending up to 50 Å to the cytoplasmic side of the inner mitochondrial membrane[1]. The arms of the 'Y' cross the lipid membrane and penetrate ~20–30 Å inside the mitochondrial matrix. A schematic diagram of cytochrome c oxidase embedded in mammalian mitochondrial membrane is shown in Figure 1. Although cytochrome c oxidase occurring in all aerobic organisms have the same basic structure and active centres, the number of polypeptide subunits in the enzyme varies with organisms. The enzyme from bacterial source contains at most three subunits while there are as many as thirteen different subunits in mammalian cytochrome c oxidase. Bovine heart cytochrome c oxidase is one of the most extensively studied cytochrome c oxidases from mammalian source. The first three subunits (called subunits I, II and III) of mammalian cytochrome c oxidase are coded by mitochondrial DNA and they play the most vital role in the enzyme while other subunits are coded by nuclear DNA[4]. The nuclear coded subunits possibly play some regulatory roles in the enzyme activity. The three subunits of bacterial cytochrome c oxidase are homologous to the mitochondrial coded subunits (I, II, III) of mammalian enzyme. Subunit I of cytochrome c oxidase has 12 membrane-spanning helixes, moreover, it has a large degree of sequence homology with myoglobin[5]. Subunits II traverses the membrane only twice, with the major part near the C-terminal remaining outside the membrane and it bears significant homology with 'blue' copper proteins, such as azurin and plastocyanin[5]. Figure 1 shows the relative positioning of different subunits in the membrane- bound cytochrome c oxidase (complex IV). Comparison between cytochrome c oxidase of several sources showed that there are six histidine residues in subunits I and two histidines and two cysteines in subunit II fully conserved in all the species. These amino acid residues serve as ligands to the metal centres in the enzyme. Subunits III of cytochrome c oxidase has five helixes spanning the membrane. It does not contain any redox active metal ion. It was previously believed that this subunit might have some role in the proton pumping activity of the enzyme. However, recent studies[15] using site-directed mutagenesis of this subunit indicate that this subunit has no role of proton pumping. However, this subunit is possibly important for folding of subunits I and II in the enzyme.

The molecular weight of monomeric bovine cytochrome c oxidase is 204,000. In mitochondria as well as in reconstituted vesicles this enzyme, however, exists as dimers[4]. Dimeric form of the enzyme is possibly necessary for its efficient proton-pumping activity *in vivo*. However, monomeric enzyme occurring in lauryl maltoside solution shows significant electron transfer activity. In the case of dimeric species the extended parts of the enzyme in the cytosolic side possibly makes a groove for binding of cytochrome c.

Prosthetic groups of the enzyme

The active centres of cytochrome c oxidase are characterized by specific redox behaviour linked with its function. There are two heme *a* moieties, called heme *a* and heme a_3 and two copper centres, called Cu_A and Cu_B constitute the redox centres of the enzyme. Figure 2 gives a schematic diagram of the active centres in the enzyme. Heme *a* is a substituted porphyrin complex (called porphyrin *a*) of iron where the position 8 is

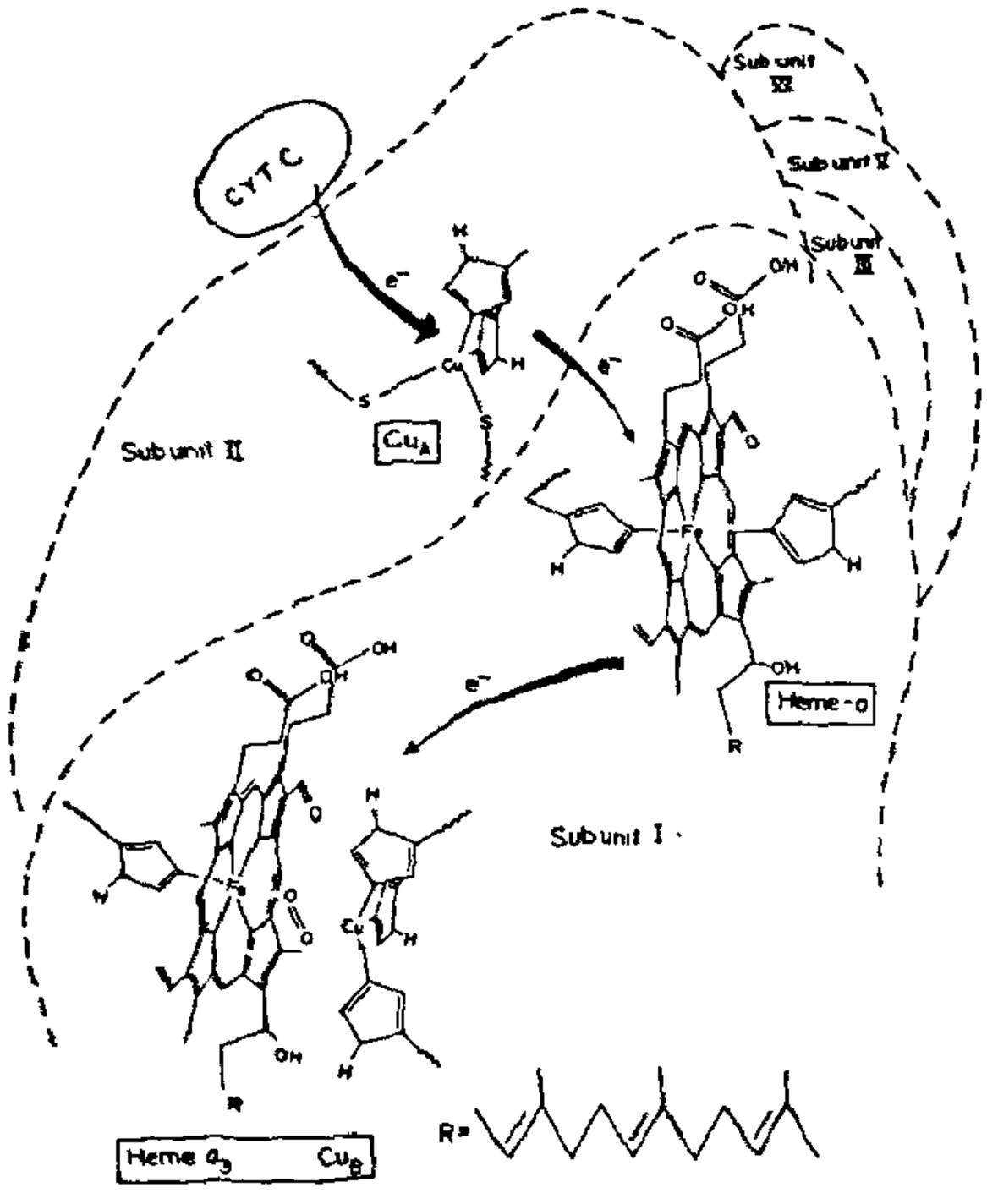

Figure 2. Structures and probable relative disposition of the active metal centres in CcO. Electron transfer path way in cytochrome c oxidase is also shown schematically.

occupied by a formyl group and a long isoprenoid chain is attached at position 2 of the porphyrin ring (Figure 2). Although heme *a* and heme a_3 contain the same porphyrin *a* ligand, the axial ligands to iron in these two centres are different. Both the heme *a* and heme a_3 centres reside on subunit I with the porphyrin *a* plane almost perpendicular to the membrane. Heme *a* is a low-spin ($S = 1/2$) iron (III) species at the native state with two histidines, H61 and H378 of subunit I axially coordinated to the metal ion. Heme a_3 has one histidine residue (H376 of subunit I) bound axially. Cu_B also resides on subunit I with three histidine residues bound to it (H240, H290, H291). Heme a_3 and Cu_B have been proposed to be bridged by μ-peroxy group (–Fe–O–O–Cu–) in the active enzyme forming the binuclear active centre for oxygen binding. Cu_B and heme a_3 are ~ 4 Å apart from each other which facilitated the formation of the bridged complex. Cu_A has been shown[8] to reside on the non-helical part of subunit II and it is ligated to two histidines (H161, G204) and two cysteine (C196, C200) residues of this subunit in the native state. Recently, some reports indicated that there may be two coppers present in the Cu_A site[3]. The distance between heme *a* and Cu_A in the enzyme is ~ 10 Å while heme *a* is ~ 16 Å away from heme a_3. Apart from these metal active centres, recent studies[5] indicate that there is another copper, called Cu_X present along with a zinc or magnesium ion in the enzyme. However, the specific roles of these metal centres in the function of cytochrome c oxidase have not yet been unambiguously established.

Electron transfer pathway

Cytochrome c is the physiological electron donor to cytochrome c oxidase. Cytochrome c oxidase has a high-affinity binding site for cytochrome c. The enzyme can also bind to a second cytochrome c at a low-affinity site. The interaction of cytochrome c with high-affinity site involves six or seven lysine residues near the heme edge of cytochrome c. Electrostatic interaction between the positively charged lysine residues of cytochrome c and the negatively charged surface residues of cytochrome c oxidase is important in binding of cytochrome c to cytochrome c oxidase. However, hydrophobic interaction between residues of the two proteins also seems to play a role in the tight binding of cytochrome c to the oxidase. Several studies on chemically modified cytochrome c oxidase suggest[16] that carboxyl groups in the subunit II of cytochrome c oxidase are involved in cytochrome c binding. These studies indicate that the cytochrome c binding site in cytochrome c oxidase is near the Cu_A centre of subunit II. Binding of cytochrome c causes some conformational change in cytochrome c oxidase which might be important for tight binding of the proteins for efficient electron transfer. The binding of cytochrome c is stronger with the dimeric enzyme compared to the monomeric one indicating that the high-affinity binding site in cytochrome c oxidase is a form of a groove formed by the extended parts of the two monomers in the assembly[14]. The cytochrome c binding site in the dimeric cytochrome c oxidase possibly involves the subunit II of one monomer and subunit III of the second one. Cytochrome c binding site in subunit II of cytochrome c oxidase is at a distance of ~ 5 Å from the Cu_A site. Fluorescence energy transfer studies suggested[17] that the distance between cytochrome c and heme *a* is 20–25 Å. Previous kinetic studies suggested[5] that the initial electron entry into the enzyme occurs through heme *a* which is then followed by a fast equilibration with Cu_A. The rate constant for electron transfer from cytochrome c to heme *a* was determined to be $\sim 5 \times 10^6/M/s$. The rate of electron equilibration between heme *a* and Cu_A was found to vary over a wide range from 100/s to 17000/s, possibly depending on the overall state of the enzyme. Recent transient absorption spectroscopic studies[18] of cytochrome c binding to bovine cytochrome c oxidase complex with O_2, however, showed that Cu_A is the initial electron acceptor

on the oxidase from cytochrome c. The effect of cytochrome c on redox state of heme *a* is explained by rapid equilibration of heme *a* with Cu_A. Figure 2 depicts the proposed pathway of electron flow between the redox active sites in cytochrome *c* oxidase.

Electron transfer to the heme *a* site is followed by a series of complex intramolecular electron transfer steps which finally carry the electron to the binuclear Cu_B-heme a_3 site. Reduction of oxygen takes place at the binuclear centre which involves several transient states such as μ-peroxy (–Fe–O–O–Cu–) and ferryl (–Fe=O) intermediates. The rate of electron transfer from the heme *a* to the binuclear site is very slow (~ 1 – 2/s) and it is the rate-determining step in the whole respiratory process. The intramolecular electron transfer between heme *a* and the binuclear site depends on the state of the enzyme. A number of different types of enzymatic states of cytochrome c oxidase have been detected spectroscopically, including an oxygenated form, resting enzyme and the pulsed form. The oxygenated form possibly consists of oxygen bound at the binuclear site in the absence of reducing equivalents. The resting form is obtained upon long standing in the absence of oxygen or electron. The pulsed form of the oxidase is generated by reaction of the reduced enzyme with oxygen. The intramolecular electron transfer rate for the pulsed form is very fast while that for the resting species is slow. However, only the pulsed form of the enzyme is possibly present in intact mitochondria *in vivo*.

It has been shown that modified bovine cytochrome c oxidase which has been specifically changed near the Cu_A site, still shows significant electron transfer activity[8, 19, 20]. However, such modifications at Cu_A site stop the proton pumping activity of the enzyme. There are several mechanisms proposed for the intramolecular electron transfer in cytochrome c oxidase. It was previously believed that the transfer of electron from the Cu_A and heme *a* site to the binuclear centre would require a large change in conformation involving movement of the redox sites sited close to each other at the intermediate steps. Recent studies[4, 21] however, support the view that the electron transfer in cytochrome c oxidase is highly coupled to a vectorial proton pump in the opposite direction. These studies[21] indicate that small fluctuations in the helical structures associated with the redox changes in the active centers would be sufficient for allowing electron flow across the protein mediated possibly by several aromatic residues. Modification of the Cu_A site or reduction of this centre also increases the rate of cyanide binding to the heme a_3–Cu_B site to several orders of magnitude. Reduction of the heme *a* and/or Cu_A site possibly causes a conformational change which converts the binuclear oxygen binding site from a 'closed' to an 'open' conformation has this facilitates internal electron transfer.

Proton pumping activity

It has now been unambiguously proved that cytochrome c oxidase acts as a redox-linked proton pump, with stoichiometry of four protons translocated for every O_2 consumed[22]. Several models have been proposed to understand the mechanism of coupling of the redox activity with the proton translocation in this enzyme. In these models a proton is taken up or rejected as the metal ions alternate between two valence states, oxidized and reduced. In order to have an efficient coupling between the electron transfer and proton translocation, there must exist some gating mechanism in the system. A conformational switching is associated with the gating of the proton channel. The conformational coupling mechanism suggests[23] that the enzyme may exist in two conformations E_1 and E_2 representing the input and output state for electron and protons. Rapid electron transfer from heme *a* and Cu_A to the binuclear centre causes change in conformation to an open state (E_2) and the conformational change to the initial state (E_1 E_2) is then coupled to proton pumping when both heme *a* and Cu_A sites are reduced and the enzyme is doubly protonated.

The rate-limiting step in cytochrome c oxidase activity is considered to be the conformational change from E_1 to E_2 rather than internal electron transfer itself. However, the question of whether heme *a* or Cu_Aor both redox centres together are involved in the gating mechanism remains undecided. Wikström *et al.*[9] proposed a general mechanism of redox-linked proton translocation in cytochrome c oxidase which involves a cubic scheme of eight states as shown in Figure 3. Any vectorial transition from the top face of the cube in Figure 3 to the bottom face must proceed via protonation of the linked acidic groups. The proton in this case may or may not be directly attached to the metal centres. Direct and indirect coupling mechanisms would be followed despending upon whether the acidic group and the metal centre are closed to each other or not in this enzyme. This scheme

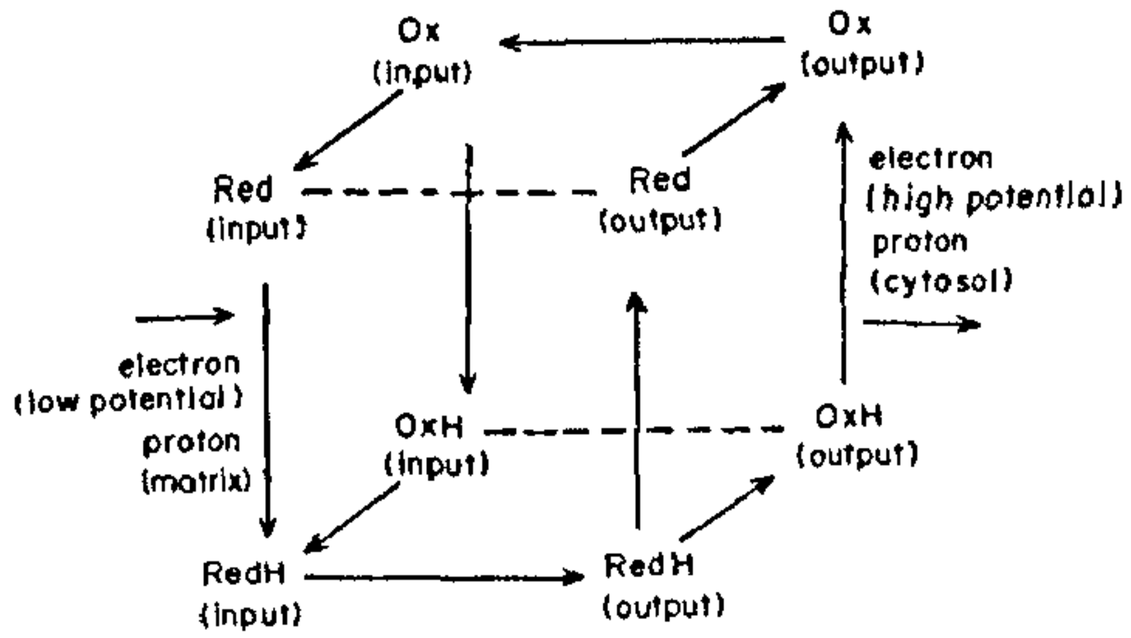

Figure 3. Eighth state model for redox-linked proton translocation by cytochrome c oxidase proposed by Wikstrom.

of redox-linked proton pump, although does not provide any direct mechanistic explanation, does provide a very useful thermodynamic and kinetic picture of the whole process.

One of the most popular mechanisms for gated proton translocation across biological membrane is based on the movement of proton along the hydrogen-bonded chain (HBC) network in the protein matrix[24]. The amino acids such as histidine, tyrosine, glutamic acid, lysine etc., provide the polar groups for the HBC formation. Figure 4 depicts a general scheme for such HBC mechanism for proton transfer coupled to conformational transition in the enzyme. According to this mechanism, the hydrogen bonds along the HBC are present in a particular configuration which minimizes their total potential energy in the resting state (E_1, Figure 4 *a*) of the enzyme. A conformational change occurs (Figure 4 *b*) due to redox change at the metal sites. This alternative conformation is such that it allows protons to move along the HBC to the other side of the bonds. The site for activation of conformational transition to the HBC can either be the heme *a* or the Cu_A redox centres. Controlled heat treatment of cytochrome c oxidase inactivates the proton pumping property of the enzyme, but this heat-treated species is capable of transferring electron across the membrane[8,21]. It has been shown that heat treatment modifies the Cu_A site which decouples the proton pumping activity from electron transport property of the enzyme[8,21]. Heat treatment or partial reduction of the Cu_A site induces the 'open' state of the heme a_3–Cu_B site. Based on these observations Chan and co-workers proposed[8] that reduction of the Cu_A in cytochrome c oxidase causes a ligand exchange by a tyrosine residue

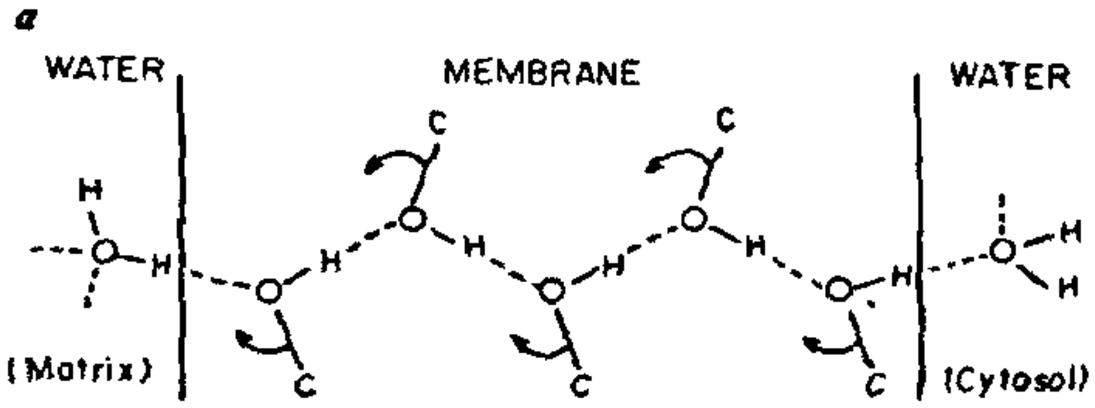

Figure 4. Mechanism of proton movement across membrane by HBC. *a*, Configuration before proton translocation; *b*, configuration after proton translocation across membrane.

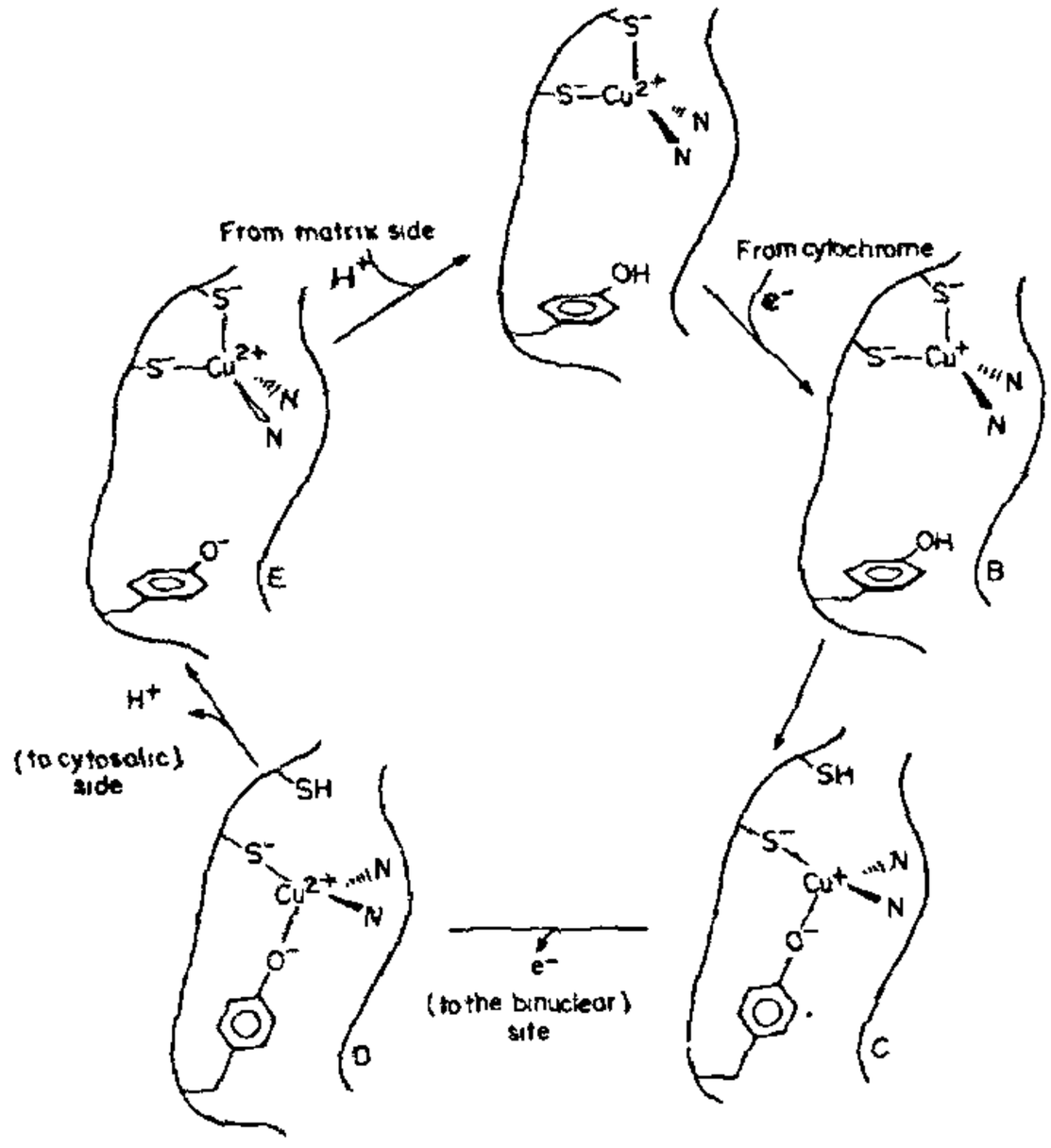

Figure 5. Schematic representation of Cu_A-based mechanism for redox linked proton translocation.

for a cysteine group. This change in ligation has been proposed to be linked with the HBC transitions associated with a proton translocation from one residue to the other which provide the mechanism for vectorial uptake of protons from the matrix side and subsequent release of proton to the cytosolic side during enzyme turnover. Figure 5 shows the Cu_A-based mechanism for redox-linked proton translocation proposed by Chan and co-workers[8]. Babcock and Callahan[25] however, suggested that the formyl group of heme *a* is hydrogen bonded to a tyrosine residue in subunit I which is linked with the proton translocation along HBC in cytochrome c oxidase. Electron transfer from heme a to the binuclear site is associated with changes in this hydrogen bonding which acts as a gate for the proton pump. The cubic scheme (Figure 3) proposed by Wikström has also recently been explained[3] in terms of redox linked proton pumping associated with the Cu_B site.

Conclusions

Although research on various aspects of cytochrome c oxidase has been going on since several decades, many of the fundamental problems of structure-function properties of this enzyme are still not resolved. Studies on cytochrome c oxidase have, in fact, opened up a new field of research which included almost all instrumental

and theoretical techniques available for chemical and biochemical studies. Several new findings have been added to the understanding of the mechanism of energy transduction by conformational coupling. We have[20,21] studied time-resolved tryptophan fluorescence of this enzyme using picosecond pulsed dye laser, to monitor any structural changes in the enzyme. Our studies indicate that this technique can be used to probe conformational fluctuations in the enzyme during the enzyme turnover. However, a critical evaluation of how the enzyme transfers electrons from cytochrome c to molecular oxygen and couples this redox reaction to transmembrane proton pumping have still to await a high-resolution crystal structure of cytochrome c oxidase. Several attempts are being made to determine high-resolution crystal structure of this complex membrane protein in different laboratories all over the world. The three-dimensional structural information will forward the field and provide necessary framework for interpretation of various kinetic and structural data obtained from solution studies. All these *in vitro* studies would finally aid to our understanding of the biological functioning of the enzyme in the respiratory chain in conjugation with other substrates and enzymes in the intact mitochondria *in vivo*.

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