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Could Cu_B be the site of redox linkage in cytochrome *c* oxidase?

(proton pump/electron transfer/mitochondria/respiration/oxygen reduction)

RANDY W. LARSEN, LIAN-PING PAN, SIEGFRIED M. MUSSER, ZHUYIN LI, AND SUNNEY I. CHAN*

Arthur Amos Noyes Laboratory of Chemical Physics, California Institute of Technology, Pasadena, CA 91125

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ABSTRACT **This paper explores the proton pumping function of cytochrome *c* oxidase [ferrocytochrome-*c*:oxygen oxidoreductase (EC 1.9.3.1)] based upon redox linkage at the "high-potential" Cu_B center. A model is proposed that is derived from a redox-linked ligand exchange mechanism previously described for the Cu_A site. Qualitative analysis of this mechanism indicates that such a mechanism is feasible. However, the relatively short distance between Cu_B and cytochrome a_3 implies that the uncoupling electron transfers are quite facile. In addition, the position of the Cu_B center with respect to the inner mitochondrial membrane argues against redox linkage at the Cu_B site.**

The respiratory systems of aerobic organisms consist of a series of membrane-associated protein complexes that utilize a redox potential gradient to drive the synthesis of ATP. One of these enzymes that directly participates in energy transduction during respiration is cytochrome *c* oxidase (1). One of the most intriguing aspects of the catalytic function of cytochrome *c* oxidase involves the ability of the enzyme to *actively* translocate protons across the inner mitochondrial membrane. In fact the enzyme represents a unique class of proton-transport proteins. Despite intensive research efforts spanning the past decade, neither the mechanism of proton pumping nor the metal center(s) associated with the redox linkage has been identified. It is widely believed, however, that one of the low-potential metal centers (either cytochrome *a* or Cu_A) is the site of the redox linkage in cytochrome *c* oxidase (2, 3). The suggested involvement of a low-potential metal center in the redox linkage is based, in part, upon the fact that the transfer of an electron from the low-potential metal centers to the dioxygen reduction site constitutes the largest free energy change of the catalytic cycle in the enzyme. Studies by Wikström and Casey (2) of whole mitochondria appear to support this hypothesis.

Room-temperature resonance Raman (4, 5) and optical absorption (6) studies of single turnovers of the enzyme in the fully reduced cytochrome *c* oxidase have, however, provided indirect evidence for an alternative site for the redox linkage. The implication is that Cu_B may function as a distinct one-electron transfer site similar to the low-potential metal sites. If this is the case, then electron transfer from Cu_B to the dioxygen intermediates formed at the Fe_{a3} may be linked to proton translocation in cytochrome *c* oxidase.

The idea of Cu_B participating in redox-linked proton translocation is not new and has been considered by Wikström (7). It is the purpose of this study to further explore the possibility of a Cu_B redox-linked proton pump based upon the rules previously developed for redox-linked proton translocation in cytochrome *c* oxidase (8).

Electron Transfer Pathways in Cytochrome *c* Oxidase

Room-temperature resonance Raman studies by Han *et al.* (4) and Varotsis and Babcock (5) of the early events in the oxidation of the fully reduced enzyme by O_2 indicate that the initial electron transfer involves the rapid (<30 μs) oxidation of cytochrome *a* producing a two-electron-reduced dioxygen intermediate while Cu_B and presumably Cu_A remain in their cuprous state. Varotsis and Babcock (5) further suggest that the oxidation of cytochrome *a* is biphasic with the slow phase of oxidation (>500 μs) producing an $Fe^{4+}_{a3}{=}O/Cu^{1+}_B$ species as the four-electron-reduced intermediate. These results are summarized in Fig. 1.

These observations are consistent, in part, with earlier low-temperature EPR studies by Blair *et al.* (11), which demonstrated that a branched electron transfer pathway exists between the low-potential metal centers and the dioxygen reduction site. These authors observed that the initial electron transfer to O_2 in the fully reduced enzyme involves the oxidation of ≈30% of the cytochrome *a* sites and ≈70% of the Cu_A centers. The latter electron transfer pathway produces a species at the dioxygen reduction site that is formally at the three-electron level of dioxygen reduction. This species also gives rise to an unusual EPR resonance that has been assigned as a hydroperoxide-bridged cupric/ferrous intermediate and indicates that electron transfer from Cu_B to the dioxygen reduction site and structural rearrangement at the binuclear center have preceded electron transfer from the low-potential sites (at least in this point of the branched pathway) at low temperatures. At this point, it is not clear whether the same O–O bond cleavage chemistry takes place with the other branch of the electron transfer pathway, as dioxygen reduction is significantly more facile in this branch and is essentially complete before the chemistry can be monitored even at low temperature.

A possible difference in the dioxygen chemistry between the two branches of the electron transfer pathway in the single turnover experiment is not unexpected and is certainly germane to the problem under discussion here. However, since the binding of O_2 to cytochrome a_3 occurs rapidly once both cytochrome a_3 and Cu_B are reduced, it is unlikely that any significant population of the fully reduced form of the enzyme is maintained during respiration. In addition, thermodynamic considerations (12) indicate that the only electron transfer steps associated with proton translocation involve the one-electron reduction of the two-electron-reduced dioxygen intermediate (compound C) and the subsequent one-electron reduction of the three-electron-reduced dioxygen intermediate (oxyferryl). Thus, the initial rapid oxidation of cytochrome *a* observed in the time-resolved resonance Raman data most likely represents an uncoupling electron transfer step since it is only the second electron transferred to dioxygen.

Cu_B as the Site of Redox Linkage

Fig. 2 depicts a mechanistic model for redox linkage based on the Cu_B center. The model is similar to the redox-linked ligand exchange proposal that has been put forth based on the

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*To whom reprint requests should be addressed.

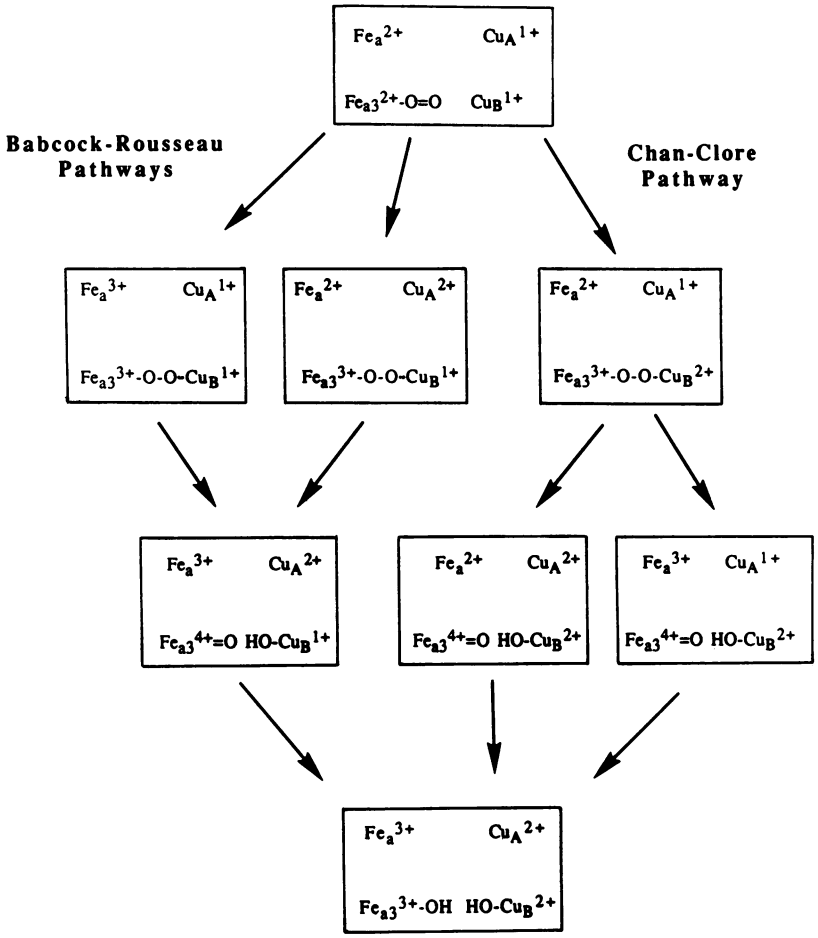

FIG. 1. Possible electron transfer pathways during the oxidation of fully reduced cytochrome *c* oxidase by dioxygen. The Chan–Clore pathway is based upon data presented in refs. 9 and 10. The Babcock–Rousseau pathways are derived from room-temperature resonance Raman data presented in refs. 4 and 5.

low-potential Cu_A as the site of redox linkage (13). In the initial state of the pump (Fig. 2*A*), Cu_B is coordinated to four ligands consisting of three histidines and an amino acid, R_3, in a square planar geometry. The square planar geometry is typical of four-coordinate Cu^{2+} complexes. (A six-coordinate distorted octahedral structure is also a viable alternative and would not alter the mechanism described here.) Upon electron reduction the square planar geometry becomes unstable and a ligand exchange reaction takes place, in which R_4 replaces R_3, to form a four-coordinate tetrahedral Cu^{1+} structure (Fig. 2*B*), the tetrahedral geometry being more stable for four-coordinate Cu^{1+} complexes. By coordinating to the Cu^{1+} ion, the protonated form of R_4 becomes more acidic and the deprotonated form of R_3 becomes more basic. A proton can then be transferred from R_4 to R_3 (Fig. 2*C*), a process that can be facilitated by the coordinated histidines. Subsequent oxidation of the Cu^{1+} ion allows the copper site to revert back to the square planar geometry with R_3 replacing R_4 (Fig. 2*D*). As with R_4, R_3 is more acidic when coordinated to Cu_B. As with R_3, R_4 is more basic when it is not coordinated. Clearly, for the redox-linked ligand exchange reaction to be coupled to vectorial movement of a proton, the electron transfer from Cu_B^{1+} to the high-potential dioxygen reduction site must be linked to some conformational event gating the proton flow. It also seems clear that the proton of the acidic R_3 must be transferred to a series of amino acids that form the proton "channel" linking the elements of redox linkage to the cytosol. In addition, it is essential that the amino acid(s) at the input side of the proton channel be more basic than the deprotonated, unligated form of R_4 to ensure that proton transfer occurs in the proper direction. Similarly, it is paramount that the deprotonated R_4 becomes protonated by a "matrix" proton to complete the cycle (step *D* to *E*). A proton channel from the matrix to the pump site is probably required here, since the dioxygen reduction site exists in a hydrophobic environment and is not directly exposed to the solvent (14). If so, it may take a number of turnover cycles before a matrix proton reaches the pump site. Presumably, protons in the proton channel migrate vectorially in sequence toward the pump site to replenish the proton "hole" that is formed at R_4 after each pumping cycle.

The mechanism described above fulfills all the requirements necessary for Cu_B to act as the site of redox linkage in cytochrome *c* oxidase. The ligand rearrangement of the pump site effectively participates in electron gating. It is assumed that the exchangeable ligand, R_3, is positioned between the Cu_B site and the low-potential electron donor(s) and that the exchangeable ligand, R_4, is positioned between Cu_B and the electron acceptor, cytochrome a_3. The electron transfer distance for reduction of Cu_B by the electron donor(s) will be shortened when R_3 is coordinated to Cu_B. In the square planar input state, Cu_B^{2+} can then be readily reduced by the donor, but the corresponding oxidation of the square planar Cu_B^{1+} would be less favored. Similarly, when R_4 is coordinated to Cu_B in the tetrahedral output state, oxidation by the acceptor is favored and reduction of the tetrahedral Cu_B^{2+} complex is less favored.

The average maximal H^+/e^- stoichiometry of this pump model is 0.75. This value is obtained by considering the

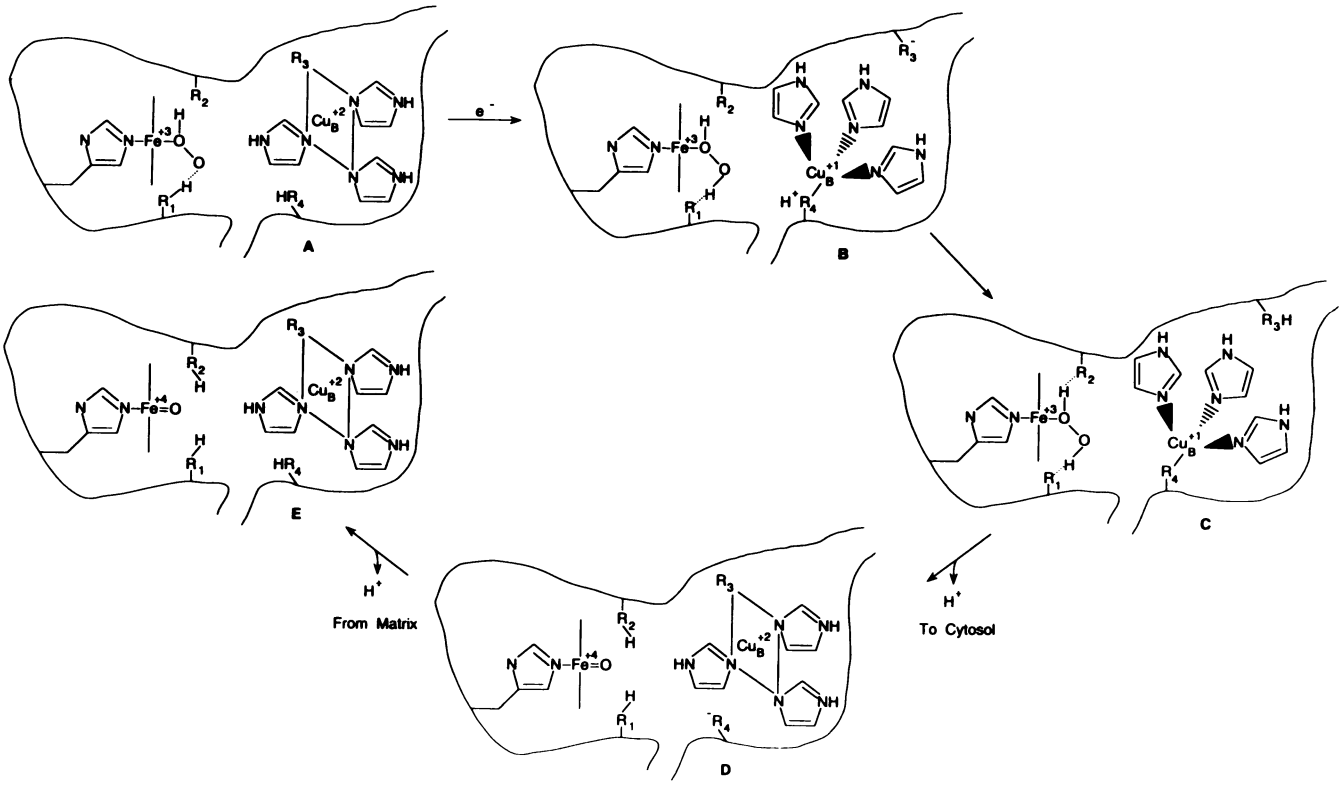

FIG. 2. Redox-linked ligand-exchange proton pump model localized at the high-potential Cu_B center. Openings at the top and bottom of each diagram represent the ends of proton-conducting channels leading to the cytosol and to the matrix, respectively. Diagram shows one complete cycle of the Cu_B pump.

potential electron transfer pathways from ferrocytochrome *c* to the dioxygen reduction site during turnover. The first electron transferred passes through one or both of the low-potential metal centers and reduces the high-potential Cu_B site. This electron is not transferred to cytochrome a_3 until a second electron is transferred from the low-potential sites to cytochrome a_3. At this point, the binding of dioxygen takes place. Accordingly, proton pumping does not occur during the first half of the turnover cycle. The second half of the cycle begins with the transfer of an electron from Cu_B to the Fe^{2+}_{a3}—O═O adduct. Here a single proton is translocated. The remaining two electrons are subsequently transferred in turn via the low-potential centers to the Cu_B site, and electron transfers to the peroxidic and oxyferryl intermediates at the cytochrome a_3 site lead to the pumping of two additional protons. The above model for redox linkage at the Cu_B center assumes a "local" stoichiometry of one proton translocated for every electron that passes through the Cu_B site and does not take into account any indirect proton translocation via a conformationally linked remote site. Thus, the possibility of indirect coupling between the Cu_B pump site and a second pump site is not discounted.

The proton input and output states are based upon the two exchangeable ligands R_3 and R_4. In the input state, R_4 is not ligated to Cu_B and has access to protons only from the matrix (via a proton channel). In the output state, R_3 becomes protonated and has access only to the cytosol (again via a proton channel). The increase in positive charge on the copper ion (from Cu^{1+} to Cu^{2+}) increases the kinetic barrier for passive back-diffusion of protons across the osmotic barrier formed between the proton channels following reoxidation of the Cu_B center to avert a futile cycle.

It has been pointed out that for active transport processes, it is a kinetic advantage for the affinity of the transported substrate to be lower on the output side than on the input side (15). Previous studies have demonstrated that a proton pump model based on ligand exchange or rearrangement achieves a kinetic effect similar to cooperative electron and proton binding through pK_a changes associated with the exchangeable ligands (in this case R_3 and R_4). In the input state, a high pK_a for R_4 (>9) would ensure rapid protonation from the matrix. Rapid deprotonation of R_3 in the output state is, however, directly linked to the strongly exergonic conversion of Cu_B from the tetrahedral to the square planar geometry.

This type of model, therefore, requires that the input and output conversions of Cu_B be strongly exergonic when the site is reduced and strongly endergonic when the site is oxidized. This implies that the redox potentials of the site will tend to be matched to the redox potential of the donor and acceptor. Previous studies of the Cu_A proton pump model, which assumes that electron transfers are rate-limiting steps in the catalytic cycle, indicate that pumping efficiencies of 0.3–0.6 can be obtained with electron fluxes of 10–100 s^{-1} (13).

The free energy available for proton translocation involving Cu_B may be realized in the electron transfer from Cu_B to cytochrome a_3. The redox potentials of the intermediates formed during the reduction of dioxygen to water have been found to be 801 mV for the two-electron-reduced dioxygen (compound C) and 939 mV for the three-electron-reduced dioxygen (oxyferryl) (16). The driving force associated with the electron transfer from Cu_B to cytochrome a_3 is, then, ≈460–560 mV, assuming a value of 340 mV for the redox potential of Cu_B in the square planar geometry (12). The coupling electron transfer, however, occurs from the tetrahedral Cu_B complex. The redox potential of Cu_B is presumed to be significantly higher than 340 mV since, in the tetrahedral geometry, the redox potential of this site must be more closely matched to that of cytochrome a_3 for the electron transfer. The redox potential of the tetrahedral form of Cu_B

could be as high as 500 mV, leaving 300–400 mV of redox energy to drive proton translocation. This redox energy would be sufficient to translocate a single proton across the 200-mV electrochemical potential spanning the inner mitochondrial membrane (17).

Coupling of Redox Linkage at Cu_B to the Dioxygen Reduction Reaction

If Cu_B is to act as the site of redox linkage in the proton pump of cytochrome *c* oxidase, certain constraints must also be placed on the dioxygen reduction cycle of the enzyme. Specifically, the intermediates formed during the reduction of dioxygen to water must be localized on the heme of cytochrome a_3 and cannot involve bridging interactions with Cu_B. It is widely believed, however, that at least one intermediate (compound C) consists of a bridging peroxide between the heme of cytochrome a_3 and Cu_B. This assignment is based, in part, on the premise that a bridged intermediate could circumvent the unfavorable thermodynamics of the formation of H_2O_2 from O_2 via two single-electron transfer steps. Both the resonance Raman and magnetic circular dichroism spectra of this intermediate are consistent with the assignment of this species as an intermediate, or low-spin, peroxide-bound cytochrome a_3 complex (9, 18). In addition, low-temperature EPR has provided evidence for both electrostatic and magnetic interactions between Cu_B and the Fe_{a3}—O—O intermediate formed at the three-electron level of dioxygen reduction. Blair *et al.* (13) have argued that this three-electron-reduced dioxygen is a cupric hydroperoxide with the peroxide bridged between a ferrous cytochrome a_3 and cupric Cu_B. On the other hand, room-temperature time-resolved resonance Raman studies by several laboratories (4, 5, 10) have failed to detect any vibrational bands associated with an Fe_{a3}—O—O—Cu_B complex in compound C in single-turnover experiments. This would suggest that a peroxide-bridged intermediate may be formed only when Cu_B is in a cupric state. Thus, if the second reducing equivalent (to dioxygen) arises from the oxidation of Cu_B, a bridged peroxide species would be formed at the binuclear center. Within the context of the redox linkage model outlined earlier, the sequence of electron transfers to the various dioxygen intermediates anchored at cytochrome a_3 is thus crucial for redox-linked proton translocation.

On the other hand, if the formation of a bridged peroxide is not involved in the turnover cycle of the enzyme, and specifically proton pumping, the following alternative mechanism for the four-electron reduction of O_2 by cytochrome *c* oxidase suggests itself (Fig. 3). The first step in this mechanism (step 1) involves the binding of O_2 to a ferrous cytochrome a_3. Upon formation of the dioxygen adduct, significant delocalization of electron density from the iron to the bound dioxygen takes place to give a superoxide-like species. Similar structures have been hypothesized for the dioxygen-bound forms of other heme proteins such as hemoglobin and myoglobin (19, 20). Transfer of a second electron to the Fe_{a3}–O_2 adduct then produces a peroxide-bound cytochrome a_3 (step 2). This reaction could be facilitated by the presence of acidic amino acids, such as R_1 and R_2, which can donate protons to stabilize the ferric hydroperoxide complex. The third electron transferred forms the ferryl iron porphyrin complex through heterolytic cleavage of the O—O bond (step 3). Heterolytic O—O cleavage of peroxide-bound porphyrins has been shown to be readily catalyzed by acidic solvents (21). Finally, the fourth electron transferred reduces the ferryl intermediate to the Fe_{a3}—OH^- complex (step 4), which is similar to that observed in "pulsed" cytochrome *c* oxidase (22). Other mechanisms for the catalytic cycle of cytochrome *c* oxidase that do not involve bridging intermediates have also been proposed (23, 24).

Potential Drawbacks to Cu_B as the Site of Redox Linkage

Although the above proton pump model incorporates all of the necessary processes required for a redox-linked proton pump, other factors—including uncoupling electron transfer reactions and the position of Cu_B with respect to the inner mitochondrial membrane—must also be considered. Uncoupling reactions are those processes in which redox linkage at the "pump" site is bypassed (i.e., occurs in the absence of the driven proton transfer reaction). It has been pointed out that uncoupling reactions are more exergonic than their coupled counterparts (12).

Recent studies have revealed certain potential uncoupling pathways in the oxidation of the fully reduced enzyme by O_2. The appearance of the oxidized form of cytochrome *a* prior to the oxidation of either Cu_A or Cu_B centers would indicate that electron transfer between cytochrome *a* and the dioxygen reduction site represents an uncoupling pathway, assum-

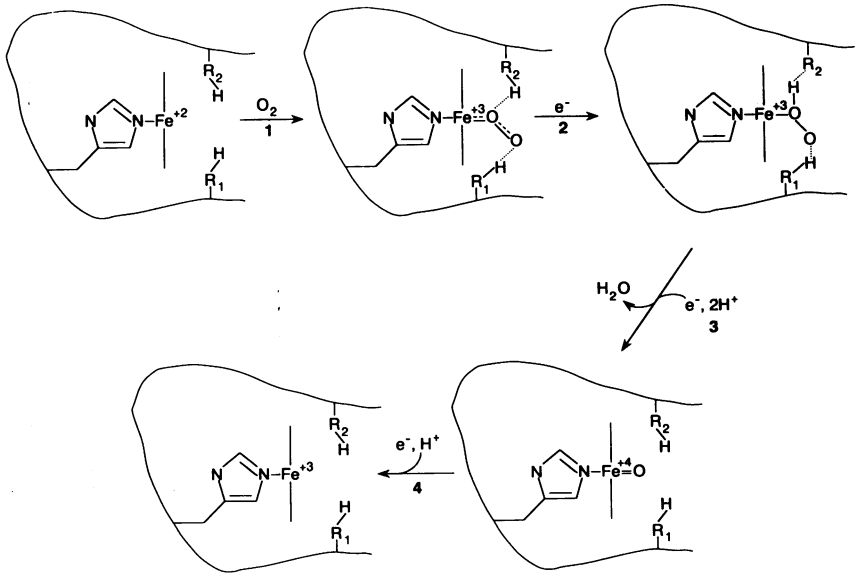

FIG. 3. Mechanism for the reduction of dioxygen to water localized on cytochrome a_3 and not involving bridging interactions with Cu_B.

ing that either Cu_A or Cu_B is the site of redox linkage. By reducing all four metal centers in the enzyme, electrons are placed in states not normally occupied in respiring mitochondria; that is, uncoupling pathways become populated.

As was discussed earlier, the gating of electron transfer to and from the pump site involves, as one component, a conformational state (input state) in which the distance between the pump site and the electron donor is minimized and a corresponding output state in which the distance between the pump site and the electron acceptor is minimized (output state). However, extended x-ray absorption fine structure (EXAFS) data indicate that the distance between Cu_B and cytochrome a_3 in the "resting" form of the enzyme (in which Cu_B is presumed to be in a square planar geometry characteristic of the input state) is only on the order of 3 Å (22). This small distance would not allow for any significant changes in the distance between the input and output states. Thus, the ratio of coupling to uncoupling electron transfer rates cannot be large and there is an intrinsic low pump efficiency inherent with this model of the proton pump (13).

Another drawback to redox linkage at the Cu_B site relates to the positioning of the metal center with respect to the inner mitochondrial membrane. Malatesta *et al.* (25) have shown that respiratory control imposed by the electrical component of the transmembrane gradient is experienced through the membrane and affects electron transfer between cytochrome *c* and the dioxygen reduction site. Because the electron transfer between Cu_B and the heme of cytochrome a_3 takes place in the same region of the membrane, little or no respiratory control of redox linkage could be obtained. It has also been suggested (26) that a protonic fault created on the matrix side of the membrane would not migrate efficiently, due to the facile fault recombination on the cytosolic side of the membrane. A model of redox linkage based on Cu_B, such as that outlined above, would predict H^+/e^- stoichiometries that are, at best, only weakly sensitive to various conditions of respiratory control over a broad range of electron transfer activities, contrary to experimental observation.

Conclusion

The focus of this discussion is to examine, qualitatively, the potential role of the "high-potential" Cu_B site in the proton pumping activity of cytochrome *c* oxidase. A model has been constructed based upon a redox-linked ligand exchange reaction similar to that previously proposed for the Cu_A site. A qualitative assessment of such a model indicates that such a mechanism is feasible and experimental evidence exists which is, at least indirectly, consistent with this type of model. However, since the distance between Cu_B and cytochrome a_3 is small, it is reasonable to assume that the uncoupling electron transfer pathways are quite facile. This difficulty, along with the location of the Cu_B in the membrane, argues against redox linkage at the Cu_B site. The drawbacks of the proposed model do not, however, exclude Cu_B as being one component of a potential two-center pump in which Cu_B acts as an input site for matrix protons and electron gating takes place at one of the low-potential metal centers—i.e., Cu_A or cytochrome *a*.

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