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Coordination Dynamics of Heme-Copper Oxidases. The Ligand Shuttle and the Control and Coupling of Electron Transfer and Proton Translocation

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Results are presented which, taken with evidence developed by others, suggest a general mechanism for the entry and binding of exogenous ligands (including O_2) at the "binuclear site" (Cu_BFe_{a3}) of the heme-copper oxidases. The mechanism includes a "ligand shuttle" wherein the obligatory waystation for incoming ligands is Cu_B and the binding of exogenous ligands at this site triggers the exchange and displacement of endogenous ligands at Fe_{a3}. It is suggested that these ligand shuttle reactions might be functionally important in providing a coupling mechanism for electron transfer and proton translocation. Scenarios as to how this might happen are delineated.

INTRODUCTION

Heme-copper oxidases are the terminal oxidases of cellular respiration in all animals and plants as well as many simpler organisms. This class of enzyme has been estimated to be responsible for some 90% of the biological O_2 reduction on earth. Cytochrome oxidase (cytochrome aa_3 or CcO) is the most prevalent member of this class, all members of which appear to be structurally and functionally similar with respect to their O_2-activating apparatus. Cytochrome oxidase transfixes the mitochondrial membrane and catalyzes the four-electron reduction of O_2 by cytochrome *c* at turnover rates approaching $1000\,s^{-1}$. It also functions to conserve the energy of the redox reaction (ca. 0.5 V) by creating a transmembrane proton gradient. This is done through scalar uptake of protons from one side of the membrane in the course of the reaction $O_2 + 4H^+ + 4e^- \rightarrow 2H_2O$ and active vector translocation of an additional four protons across the membrane via redox-linked proton pumping.

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Understanding in molecular detail the mechanism of redox-linked proton pumping is one of the major issues in bioenergetics.[1-5] General issues of interest include how the oxidases effect facile electron transfer and O_2 activation, how corollary phenomena such as molecular switching and thermodynamic tuning of electron transfer occur, and how the energy of electron transfer is coupled to proton pumping to effect ion translocation against a concentration gradient. Our results,[6-25] together with advances by others in structural and functional understanding of heme-copper oxidases[5,26,27] and biological electron transfer,[28,29] have led us to suggest that labile coordination chemistry ("ligand shuttling") at the metal centers may play key roles in energy transduction in these enzymes.

Eukaryotic CcO contains two hemes and two copper centers. The two hemes A constitute cytochromes *a* and a_3. The copper centers are designated Cu_A and Cu_B. It appears that heme-copper oxidases have in common three analogous polypeptide subunits, designated I–III. In all known heme-copper oxidases, both of the hemes and Cu_B are located in Subunit I. Cu_A is located in Subunit II of the eukaryotic enzyme.

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Cytochrome *a* is 6-coordinate with axial endogenous protein ligands from histidine imidazole. Cytochrome a_3 has one endogenous imidazole ligand from a proximal histidine and has a labile distal coordination site available to bind exogenous or endogenous ligands. It is generally believed based on a large body of evidence[1,2,8,30,32] that cytochrome a_3 is the obligatory site of O_2 binding prior to reduction. Cu_B is very close to cytochrome a_3 on the distal side, forming the "binuclear site"; both Fe_{a3} and Cu_B are accessible to exogenous ligation.[9,33–35] Cu_A is not accessible to exogenous ligands and is magnetically remote from other metal centers. Certain prokaryotic oxidases, whose reducing substrate is other than cytochrome *c*, lack Cu_A entirely. In Cbo, for example, the reducing equivalents are provided by ubiquinol and Cu_A is not present in the enzyme. Inasmuch as Cbo pumps protons,[36] this calls into question the assertion[3] that Cu_A has a role in proton pumping. Present evidence suggests that the critical, conserved apparatus for energy transduction and storage by the enzyme is to be sought in Subunit I.

Not all heme-copper oxidases use heme A as their prosthetic groups. Cases in point include cytochrome ba_3 from *Thermus thermophilus* and cytochrome *bo* (Cbo) from *E. coli*. In ba_3 the 6-coordinate heme is protoheme. In Cbo the heme at the binuclear site is heme O, the same structure as heme A (including the farnesyl tail containing three unsaturated isoprenoid groups) except that the formyl has been replaced by a methyl group. The 6-coordinate heme may be either heme O or protoheme. Inasmuch as Subunit I or Cbo is fully functional and pumps protons, it seems clear that the formyl group has no essential role in proton pumping nor does the farnesyl chain have an indispensable function at the 6-coordinate site. However, no heme-copper oxidase characterized to date lacks a farnesyl-containing heme at the binuclear site, suggesting an essential functional role (unknown, at present) for the farnesyl at that site.

GENERAL CONSIDERATIONS

Key issues associated with the function of the heme-copper oxidases include the following. How does the enzyme recognize, admit, and control the binding of substrate? How is substrate activation accomplished? How do the proteins carry out facile electron transfer, and how do they modify or switch

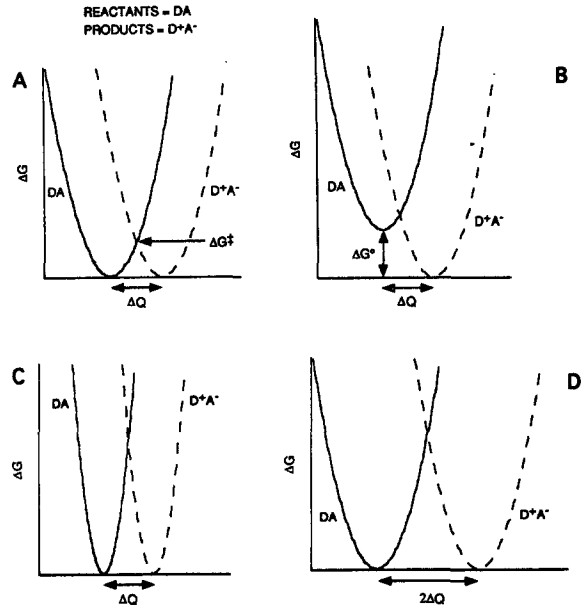


Fig. 1. Simplified (two-dimensional) schematic representation of the reactants' and products' harmonic ($\Delta G = kQ^2 + c$; force constant = k) potential surfaces and the reaction coordinate for electron transfer in different situations. (A) Zero ΔG^0; (B) nonzero ΔG^0, reorganization of nuclear coordinates (ΔQ) the same as in A; (C) zero ΔG^0, ΔQ same as in A, force constant twice that in A; (D) zero ΔG^0, ΔQ twice that in A, force constant same as in A.

electron transfer dynamics and thermodynamics in response to functional requirements and ambient conditions? How do they perform coupled functions such as energy storage by proton pumping?

Most of the aforementioned issues are directly tied to the factors that influence electron transfer dynamics. A protein may be able to control electron transfer and couple it to other events by controlling these factors. In the decades since the inception of the electron transfer theory of Marcus and Hush (Ref. 37 and references therein), these factors have become relatively well defined. They may be qualitatively understood by considering intersecting parabolas which represent the vibrational potential surfaces of the electron transfer reactants (DA, where D is the electron donor and A is the acceptor) and products (D^+A^-), shown in Fig. 1. The horizontal coordinate Q represents the coordinates of the nuclei of the D and A molecules and their surroundings, and the vertical coordinate represents free energy (ΔG) associated with the coordinates. The parabolas in Q, ΔG represent harmonic potential surfaces. Because of Franck–Condon restrictions on the electron transfer event, the reaction $DA \leftrightarrow D^+A^-$ can only occur (in the absence

of nuclear tunneling or the absorption of a photon) at the point where the parabolas intersect. Therefore, the lower locus of the potential surfaces represents the reaction coordinate for thermal D, A electron transfer, the intersection point of the reactants' and products' potential surfaces represents the activated complex, and the vertical distance between the minimum of the reactants' surface and the intersection point is the activation energy $\Delta G^{\ddagger}$ for electron transfer (Fig. 1). It is evident that the relative placement and the shapes of the reactants' and products' surfaces influence $\Delta G^{\ddagger}$ and thereby the electron transfer rate constant, k_{et}. This rate constant may be approximated from absolute rate theory as

$$k_{et} = \kappa kT/h[\exp(-\Delta G^{\ddagger}/RT)]$$

where κ represents the transmission coefficient (the probability of electron transfer one the activated complex is formed) and the other symbols have their usual meanings. The value of k_{et} is affected in the following ways. (A) The free energy of reaction ΔG^0 is the vertical displacement between the minima of the reactants' and products' surfaces; the size of this displacement clearly affects $\Delta G^{\ddagger}$ (Fig. 1B). The more favorable ΔG^0, the faster the reaction, up to the point $\Delta G^{\ddagger} = 0$. (B) The magnitude of the displacement ΔQ along the reaction coordinate (that is, the magnitude of the molecular reorganization) necessary to achieve the activated complex affects the height of the potential surface intersection point and thus $\Delta G^{\ddagger}$; the greater ΔQ, the slower the reaction (Fig. 1D). (C) For any fixed ΔQ, the greater the force constants of the molecular coordinates that have to be reorganized to reach the activated complex (the steeper the parabolas; Fig. 1C), the greater will be $\Delta G^{\ddagger}$ and the slower the reaction. (D) The electron transfer probability κ is directly related to k_{et}, and is determined by overlap (H_{rp}) between reactants' and products' wave functions which in turn is influenced *inter alia* by the effective D-A distance in relationships that have received much theoretical discussion but have only recently begun to be established experimentally.[28,29] At short electron transfer distances, κ may be effectively unity, but at longer distances it falls off exponentially with distance, such that the average distance dependence for nonadiabatic electron transfer in proteins is 1.7 Å per factor of 10 in k_{et}.[28] The D-A distance at issue, however, may not be the most direct distance,[29] but instead the route through covalent bonds, hydrogen bonds, and through space that gives the largest H_{rp} and therefore the greatest value of κ. Thus, the making or breaking (e.g., under protein conformational control) of a hydrogen bond or, especially, a covalent bond along the *electron transfer* path between D and A may make a very large difference in k_{et} without changing any of the parameters that affect $\Delta G^{\ddagger}$ as described above and also without changing the *direct* D-A distance. (E) Finally, the foregoing discussion assumes that H_{rp} is always much less than $\Delta G^{\ddagger}$, such that (even if $\kappa \approx 1$) $\Delta G^{\ddagger}$ is always determined by the energy difference between the reactants' potential minimum and the surfaces' crossing point. This need not be the case for "strongly adiabatic" reactions such as those in which bond making or breaking accompanies the electron transfer event. Examples of this might be the formation of HO_2^- from O_2 or the formation of peroxide bringing two metal centers from dioxygen bound to one. In cases like these the splitting of the potential surfaces at the nominal intersection point may be large and, in addition to $\kappa = 1$, $\Delta G^{\ddagger}$ is actually diminished and k_{et} accelerated because H_{rp} is of the same magnitude as $\Delta G^{\ddagger}$ and subtracts from it.

The point to the foregoing for our purposes is that the coordination chemistry of redox-active transition metals in proteins offers a direct elegant way of affecting k_{et} by purposeful changes in any or all of the factors mentioned above. It is clear that the ligands bound to a metal ion will generally affect the free energy of redox reactions, the force constants of the inner coordination sphere, and the magnitude of the ΔQ reorganizations necessary to reach the activated complex. The making or breaking of a metal–ligand bond can form a through-bond path to another redox center or cause such a path to be discontinuous. Phenomena such as protonation or coordination to metals can engender "strong-overlap" electron transfer reactivity and thus cause nominally slow redox reactions to become fast. In addition to electron transfer, the chemistry of the ligands themselves is often profoundly affected by coordination to metals and by the metals' redox chemistry. An obvious case in point is the acid–base reactivity of a ligand, which is enormously sensitive to whether the ligand is coordinated to a metal, the identity of the metal and, for a given metal, the oxidation state. There are numerous examples of this, a dramatic one being the behavior of the peptide bond. The R–(C=O)–(N–H)—R′ structure does not deprotonate significantly in concentrated NaOH in the absence of a transition metal, but loses

a proton with a pK of 7 or less when given the opportunity to coordinate to a metal as deprotonated peptide, a change in acid dissociation constant approaching 10 orders of magnitude. It is clear that the control of electron transfer reactivity by coordination of ligands, and the converse control of ligand reactivity by coordination and electron transfer, offer rich options for coupling the dynamics and energetics of these types of reactions.

Our results indicate that binding of an exogenous π-acid ligand (e.g., CO, O_2) to Cu_B triggers changes in the coordination sphere of Fe_{a3}. Specifically, an endogenous ligand L binds to the distal (Cu_B) side of the heme when the exogenous ligand binds to Cu_B. In response to this, the bond between Fe_{a3} and the proximal histidine is broken. Wse infer that L was formerly bound to Cu_B and was displaced by the incoming exogenous ligand. The importance of this finding is that the coordination spheres of both of the metals at the binuclear center are more labile than previously suspected. Once this is recognized, it seems likely that this "ligand shuttling" activity may be important in elements of the enzyme's reactivity other than the binding of exogenous ligands, namely in the electron and proton transfer reactions and in the coupling of two. Firstly, the number, identity, and spatial disposition of ligands influences every aspect of the electron transfer reactivity of a metal: the reaction free energy, the reorganizational free energy, the displacement along the reaction coordinate, and (by influencing the effective distance between electron donor and acceptor) the electron transfer probability. Secondly, the acid–base properties of a ligand are profoundly affected by binding to a metal and by the identity and oxidation state of the metal to which the ligand is bound. The potential is clear that the enzyme uses these elegant options based on the coordination chemistry of the metal centers to effect functional control and redox-linked proton translocation.

COORDINATION DYNAMICS OF THE HEME-COPPER BINUCLEAR SITE

We have made an extensive study in our laboratories of the coordination dynamics of the binuclear site of the heme-copper oxidases. Primarily we have studied the photodissociation and recombination reactions of the CO complexes and the response of the protein structure to these events. Photodissociation of CO forms the basis of the "flow-flash" techniques which were developed by Gibson and co-workers[38,39] and the low-temperature "trapping" techniques derived therefrom.[40] It is essential to understand the consequences of CO photodissociation in order to understand whether the O_2 reactions observed by flow-flash adequately represent the physiological O_2 reactions which occur in the absence of CO. Secondly, O_2 as a substrate of CcO is first a ligand and only later an electron transfer entity, and the early events in the physiological function are ligation reactions. Inasmuch as CO and O_2 have extensive similarities in their coordination chemistry, the reactivity of CO as a ligand toward CcO is likely to model that of O_2. To the extent that this is the case, the coordination dynamics of CO can be studied and functionally relevant conclusions drawn without the complications superimposed by the electron transfer reactions of O_2.

Control of Ligand Binding to the Metal Centers

We have substantial species-comparative kinetics data on CO photodissociation and rebinding of various heme-copper oxidases.[7,13,23] Table I presents these data. All of the oxidases examined conform to the same general mechanism (Scheme 1) wherein CO in solution first binds in a pre-equilibrium with Cu_B, and is then transferred to the heme in a subsequent step. At 1 atm CO (1 mM [CO] in solution) the observed pseudo-first-order rate constants (k_{obs}) for the formation of Fe_{a3}-CO span only a factor of 10. However, the range spanned by the stability constants of Cu_B-CO (K_1) and the rate constants for transfer of CO from Cu_B to Fe_{a3} (k_2) is much larger. The k_{obs} values ($k_{obs} \approx K_1 k_2[CO]$) are similar because the variations in K_1 and k_2 tend to compensate one another. Why this is the case and what functional significance this may have are matters for speculation at present. The activation parameters are for k_2 and show that, with the exception of cytochrome ba_3, which is unique in several respects,[25] the rate of transfer from Cu_B to heme a_3 tends to be controlled by the activation entropy. The rate constant k_{-1} is for the loss of CO from CuB-CO into solution, and is related by microscopic reversibility to k_1, the CO on rate from solution, by $k_1 = K_1 k_{-1}$. From this is can be seen that for cytochrome *o* alone k_1 is much slower than diffusion controlled (*vide infra*).

The Nature of Conformers: Cytochrome ba_3

FTIR studies[7] of the CO complexes of cytochrome ba_3 show that there are at least two major

Table I. Kinetics Data

Oxidase[a]	$k_{obs}(s^{-1})^b$	$K_1\ (M^{-1})^c$	$k_2\ (s^{-1})^c$	$\Delta H^{\ddagger d}$	$\Delta S^{\ddagger}$ (eu)	$k_{-1}\ (s^{-1})^c$
Bovine aa_3	90	87	1030	10	−12	7×10^5
P. denit. aa_3	42	53	780	11	−9	1×10^6
E. coli bo	60	400	190	11	−13	500
T. t. caa_3	40	3900	50	11	−16	2×10^4
T. t. ba_3	8	> 10,000	8	15	−5	?

[a] *P. denit.* = *Paracoccus denitrificans*; T. t. = *Thermus thermophilus*;
[b] At 1 atm (= 1 mM) Co;
[c] For definition, see Scheme 1;
[d] kcal/mol.

conformers (C—O stretching frequencies 1983 and 1974 cm^{-1}) present in almost equal proportion. The relative populations of the conformers are temperature dependent. The 1974 cm^{-1} conformer is favored at higher temperature, and the thermodynamic parameters for the interconversion are $\Delta H = +0.3$ kcal/mol and $\Delta S = +1.3$ cal/mol K. Clearly the conformational change cannot be a large one, yet the C—O frequencies are significantly affected (even more are the Fe—N(Im) frequencies, *vide infra*).

Our RR studies of ba_3 show that the axial Fe—N(his-Im) stretching mode in the reduced unliganded enzyme is also split into two peaks, indicating (as in the CO complex) that two major conformers are present. The relative intensities are temperature dependent with the same thermodynamic parameters as those of the C—O stretch noted above. Thus the splitting of the C—O and Fe—N peaks seems to be due to the same conformational effects. Inasmuch as the frequency splitting in the Fe—N stretches (193 and 213 cm^{-1}) is 10% of the frequency itself compared to 0.5% in the C—O stretch, we may infer that the conformational change has its greatest effect on the proximal side of cytochrome a_3. Despite the large effect on the Fe—N frequency, the tiny thermodynamic changes mean that the conformational change cannot be large in the global-structural sense. It is possible that simple rotation of the imidazole plane about the Fe—N bond axis might alter both the Fe—N and C—O frequencies as observed, and also act as a control mechanism for the coordination or redox chemistry of the heme. The mechanism by which the protein controls the chemistry of the binuclear heme-copper site is an important issue and, as we shall see, the proximal coordination chemistry of cytochrome a_3 may be central.

Control of Ligand Entry: Cytochrome bo

Following the fast events in the photodissociation of Cbo, the equilibration of Cu_B–CO with CO in solution occurs with a rate constant of ca. 500 s^{-1}. This is very slow compared to the aa_3 oxidases (Table I), which are near $10^6\ s^{-1}$. From this, the bimolecular rate constant for the formation of Cu_B–CO calculated from K_1 is ca. $2 \times 10^5\ M^{-1}s^{-1}$, compared to (3–5) $\times 10^7\ M^{-1}s^{-1}$ for aa_3. The latter rate constants approach the diffusion controlled limit. The calculated rate constant for Cbo, however, is at least a factor of 100 slower than diffusion controlled; we would believe this number to be in error were it not for the following results.

From the Gennis group we have two mutants of Cbo which lack ligands for Cu_B and thus fail to incorporate this metal center. If it is actually the case, as we propose, that Cu_B acts as the "gateway" to active site, or the obligatory waystation for the binding of exogenous ligands to the heme, we should not observe saturation in the rebinding of CO by these mutants. Indeed, we do not; the k_{obs} for CO rebinding is linear in [CO] up to at least 20 mM (20 atm). The second-order rate constants for binding of CO to the heme in these mutants are in the range (2–4) $\times 10^5\ M^{-1}s^{-1}$. These rate constants are quite similar to those calculated above for the binding of CO to Cu_B in wild-type Cbo. Also they are similar to the CO binding rate constants of myoglobins, which require substantial protein conformational change to allow exogenous ligands to approach the heme. The implication is that $2 \times 10^5\ M^{-1}s^{-1}$ may be the real rate constant for the formation of Cu_B–CO in Cbo, and that Cbo (and by implication other heme-copper oxidases) can exert protein conformational control over the entry of exogenous ligands, in addition to the control implicit in the ligand shuttle.

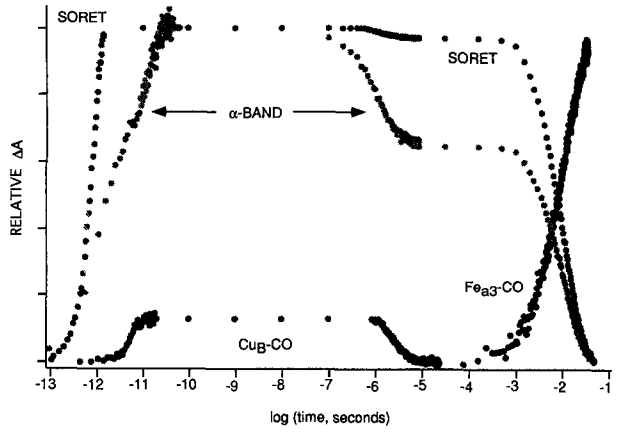
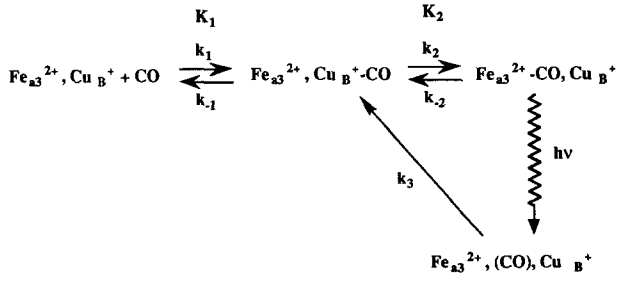


Fig. 2. Plot showing the electronic absorption and TRIR dynamics of CcO–CO from 100 fs to 100 ms, twelve orders of magnitude in time. See text.

General Mechanism and Functional Significance

We have shown[9,13,23] that the more obvious aspects of the CO dynamics are summarized in Scheme 1:



We have suggested that Scheme 1 also applies to the binding of O_2 by the enezyme, and subsequent results by others support this suggestion.[41–43] This begs the question, however, why the enzyme should interpose such mechanistic complexities in the way of binding the physiological substrate O_2 at the site at which electron transfer occurs. If Scheme 1 is relevant to the O_2 mechanism, the question of its functional purpose must be addressed.

In fact, we have found that Scheme 1 is not detailed enough to account for some important aspects of the CO dynamics.[13,23] Figure 2 is a log(time) representation of the CO photodissociation/recombination dynamics observed by transient UV-Vis and infrared probes over 13 orders of magnitude of time. While Scheme 1 and the data in Table I account for the recombination kinetics on the millisecond time scale and the ultrafast dynamics of the Soret absorbance, as well as the appearance and disappearance of the infrared absorbance due to Cu_B–CO, they account neither for the behavior of the α-band between 1 ps and 10 μs nor for other details observed in the time-resolved spectroscopies.[13,14,25] We have argued[13,23] that Fig. 3 is a minimal mechanism necessary to accommodate all of the facts.

The importance of Fig. 3 is that is suggests previously unsuspected lability in the coordination spheres of Cu_B and Fe_{a3} as a consequence of entry of the exogenous ligand into the protein and its binding at the binuclear site. Binding of CO to Cu_B causes the release of a ligand (L) previously bound to Cu_B; L then binds to Fe_{a3} on the distal side, displacing the proximal histidine. The rate-determining step for transfer of CO from Cu_B to Fe_{a3} is the rate of Fe–L bond scission. We do not know the identity of L, but spectroscopic evidence and chemical arguments suggest that it is not imidazole. When L departs from Fe_{a3} and CO binds to the heme, the proximal histidine rebinds as well. We have designated this behavior of the binuclear site the "ligand shuttle." We have demonstrated that this occurs in the fully reduced and mixed-valence forms of CcO. We have some evidence that other exogenous ligands such as cyanide and NO engender the ligand shuttle, and others[41–43] have recently published evidence that O_2 behaves similarly. There are ample if largely indirect indications that ligand shuttling may occur in other oxidation states of CcO, for example in the cyanide-binding reactions of the resting enzyme[44] and in the structural changes responsible for the "fast-slow" transition.[6,45]

We suggest that the ligand shuttle and related labile coordination chemistry may be a common feature of the reactivity of heme-copper oxidases, and we further suggest that these reactions may be important contributors to the function of the enzymes. Some ways that the ligand shuttle might be functionally significant are the following, as we discuss in more detail elsewhere.[25]

(A) Regulation. The ligand shuttle might prevent the enzyme from binding an incoming O_2 until the binuclear site has been "told," e.g., by the conformational effect, that the enzyme is ready for another redox turnover cycle.

(B) Redox Thermodynamics. It is well known[5] that the metal centers of CcO exhibit anticooperativity in their redox potentials. That is, for example, the reduction of one heme makes the other more difficult to reduce, notwithstanding evidence that the two hemes are ca. 20 Å apart. A change in ligation at one metal center induced by a redox reaction at the other could be the mechanism of anticooperativity.

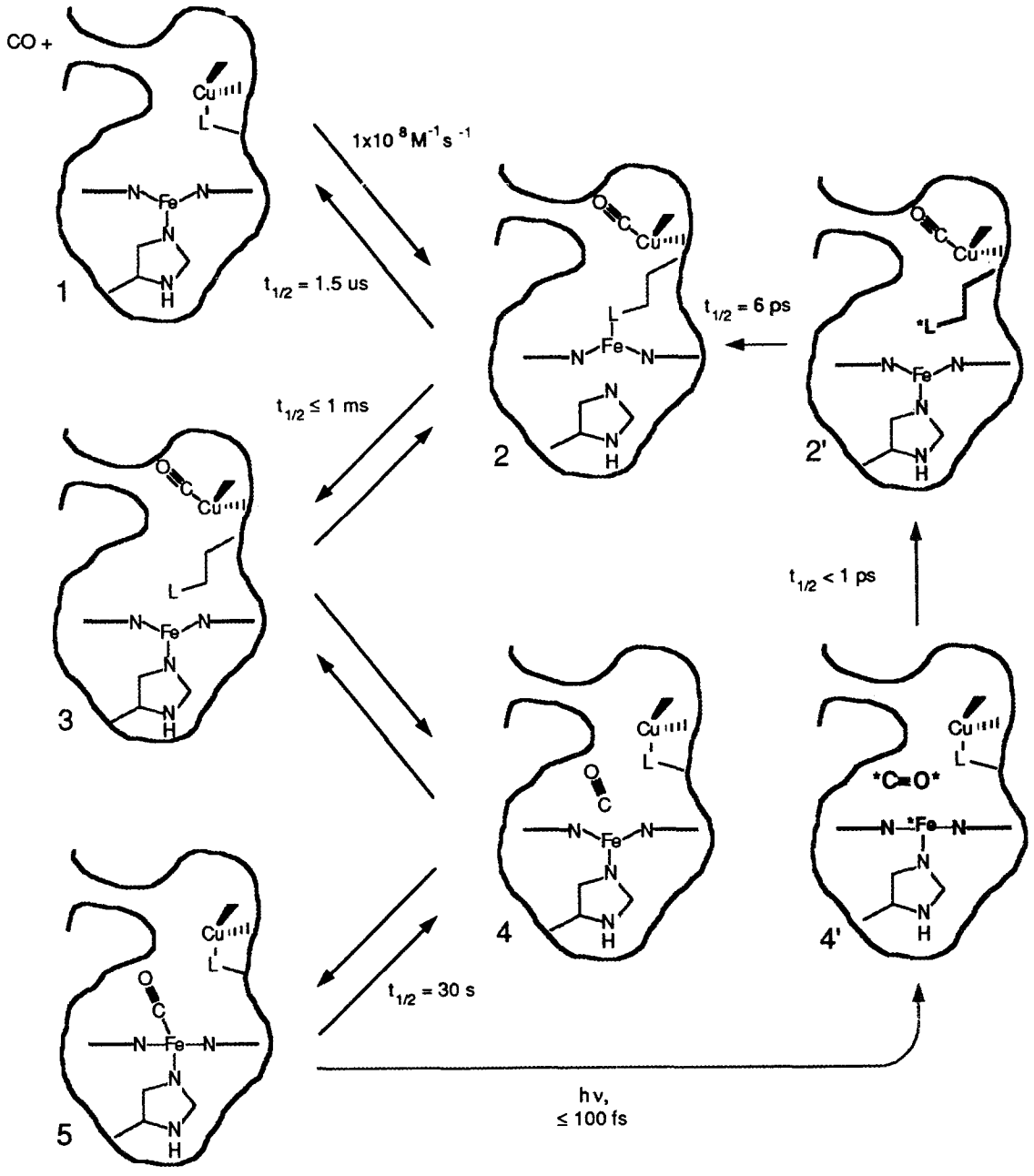


Fig. 3. The mechanism of photodissociation, thermal recombination, and thermal dissociation of CcO–CO. The right-hand path is the photochemical one, which differs from the thermal in that the photodissociated CO has excess kinetic energy and the heme is vibrationarily "hot" in species 4′ and 2′. This nonthermal energy is dissipated by the time species 2 forms, and the photochemical and thermal plates are the same thereafter. Adapted from Ref. 13.

(C) Redox Dynamics. Through all of the factors enumerated in the "General Considerations" section, changes in the coordination spheres of the metal centers *via* ligand shuttle reactions may modify and purposefully control rates of electron transfer.

(D) Electron Transfer Switching. The ligand shuttle combined with structural inferences from mutant work on Cbo[26,27] provides a clear scenario in molecular detail as to how electron transfer between cytochrome *a* and a_3 might be turned on and off.

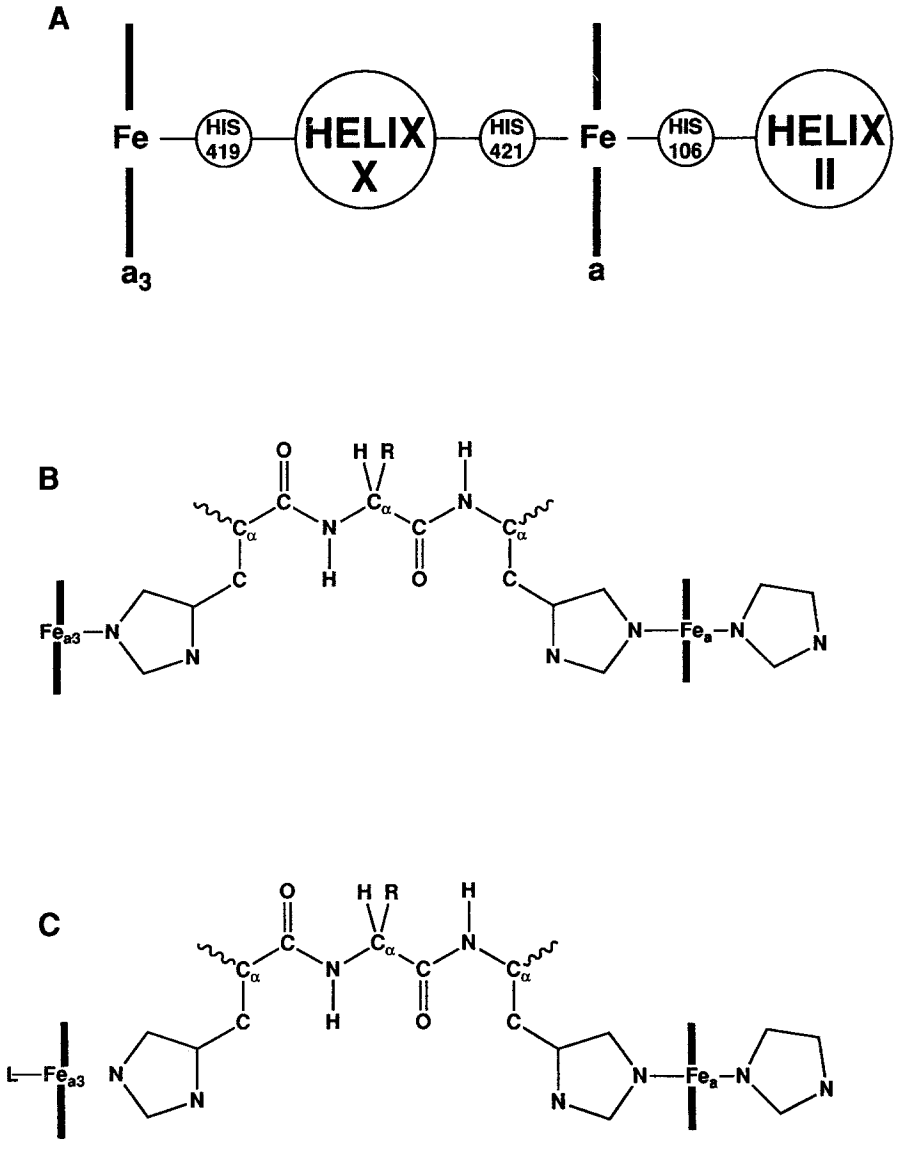


Fig. 4. (A) The arrangement of the helices that contain the heme ligands, and the hemes themselves, of heme-copper oxidases (according to the model given in Ref. 98). The histidine residue numbers are from Cbo. (B) The microscopic structure connecting cytochromes *a* and a_3, given the model shown in A. (C) The "ligand shuttle" intermediate that we propose (Species 2 in Fig. 12; see text and Refs. 13 and 19). The cleavage of the Fe_{a3} to the proximal histidine bond and the binding of distal L are shown.

Evidence suggests that the *a* and a_3 hemes are on opposite sides of the same helix (helix X) bound to histidines only two residues apart.[27] This is shown in Fig. 4A. This gives a direct through-bond path for $a \leftrightarrow a_3$ electron transfer that is 16 covalent bonds long from one ion atom to the other, as shown in Fig. 4B. Using the equations of Dutton *et al.*[28] and the through-bond distance shown in Fig. 4B, we calculate a rate constant for electron transfer between *a* and a_3 of $1.9 \times 10^5 s^{-1}$. The measured value is $2.5 \times 10^5 s^{-1}$.[42] We consider this to be remarkably good agreement that lends significant credibility to this model. Furthermore, as discussed above, our evidence suggests that in certain ligand shuttle intermediates the Fe_{a3}–(proximal histidine) bond is broken. Thus, in this intermediate the electron transfer pathway com-

prises 16 covalent bonds and one through-space jump as shown in Fig. 4C. Assuming that the cleavage of this bond results in a through-space jump for electron transfer equal to the Fe–N(Im) bond distance (ca. 2 Å). The predicted rate constant for electron transfer between a and a_3 is $3000\,s^{-1}$. In Fig. 4B, Fe_{a3} is probably displaced ca. 0.5 Å toward the proximal histidine; if, in Fig. 4C, Fe_{a3} is displaced a similar distance toward distal L and the nonbonded proximal histidine does not move, the through-space jump is ca. 3 Å and the predicted rate constant for $a \leftrightarrow a_3$ electron transfer becomes $200\,s^{-1}$. This is the electron transfer switching mechanism that we suggest. This is clearly an intriguing idea as to how metalloproteins in general might switch intraprotein electron transfer dynamics without large protein conformational changes; indeed, the direct distance between the electron transfer partners need not change.

(E) Proton Translocation. In the "general considerations" section we pointed out the connection between coordination and Brønsted acid–base reactivity of ligands. Figure 3 shows the binding and dissociation of ligands remote from one another (e.g., across a heme) in reponse to the ligand shuttle. If these ligands have labile protons the ligand shuttle might result, for example, in proton release on one side of a heme and uptake on the other. Such coordination-linked protonation/deprotonation reactions might, if coupled appropriately to protein conformational changes, effect net proton translocation.[19] A schematic representation of how this might occur is shown in Fig. 5. The scheme assumes *a priori* that L, the ligand that is transferred between Cu_B and Fe_{a3} in the ligand shuttle, has a labile proton. In response to binding of a ligand X (either an exogenous ligand such as CO or O_2 or an endogenous group), L is transferred from Cu to Fe. Because of the charges and the acid–base properties of the metals, L is protonated when bound to Cu but deprotonated when bound to Fe. In (3) this proton is lost to the "surroundings," which may be intra- or extra-protein. In (4) a protein conformational change has occurred that prevents proton exchange with the surroundings. At the same time, a H-bonded pathway forms which does not exist in the other species and which allows the proximal histidine (now more acidic because it is bound to Fe) to protonate L (now more basic because it has dissociated from Fe). In (5) the protein has returned to the conformation wherein the proton communication with the surroundings is restored and the H-bonded pathway around the heme is broken. The proximal histidine then reprotonates from the surroundings using a different proton channel than that used in (3). Note that, while electron transfer reactions may be directly involved in these processes, they need not be. The effects on electron transfer dynamics in the various ligand shuttle species postulated in (D) above are sufficient to couple electron transfer and proton translocation in this scenario. However, as noted in (F) below, direct coupling could be a factor as well.

(F) Coupling of Protonation/Deprotonation and Electron Transfer. The energy of charge separation is fundamentally related to the magnitudes and signs of the charges to be separated. Thus the acid dissociation constant of a coordinated ligand with a labile proton is strongly related to the charge (oxidation state) of the metal to which it is bound. This relationship affords an attractive mechanism for directly coupling the energy of an electron transfer reaction to proton transfer in order to displace protons against a concentration gradient.

Mechanisms for regulating ligand binding to heme-copper oxidases are at play that have nothing directly to do with the ligand shuttle; these were noted in an earlier section. One example is protein conformational control. While the rate constant for binding CO to Cu_B of CcO is approximately diffusion controlled, the analogous rate constant for Cbo is similar to the rate constant for binding CO to myoglobin, where it is known that significant rearrangement of the protein structure is necessary to admit CO to the active site. This shows that control of ligand entry into the protein by structures remote from the metal centers is potentially important. While this may be an intrinsic difference between CcO and Cbo, it may also be that this mode of control is accessible to all of the heme-copper oxidases depending upon conditions. Another way of regulating ligand binding is via the magitude of the pre-equilibrium binding constant K_1 for the binding of the exogenous ligand to Cu_B. For CO, this stability constant varies by more than a factor of 200 among the oxidases we have studied. It may be that the oxidases modify the ligand affinity of Cu_B depending on the ambient conditions (e.g., oxygen tension) under which the various oxidase must operate, and that Cu_B is used for this purpose because its coordination options are richer than those of the heme. Alteration of the ligand affinity of Cu_B may be ephemeral as well as permanent. We have clear evidence that the CO binding constant of Cu_B is much smaller in the heme-CO form of the oxidases than in

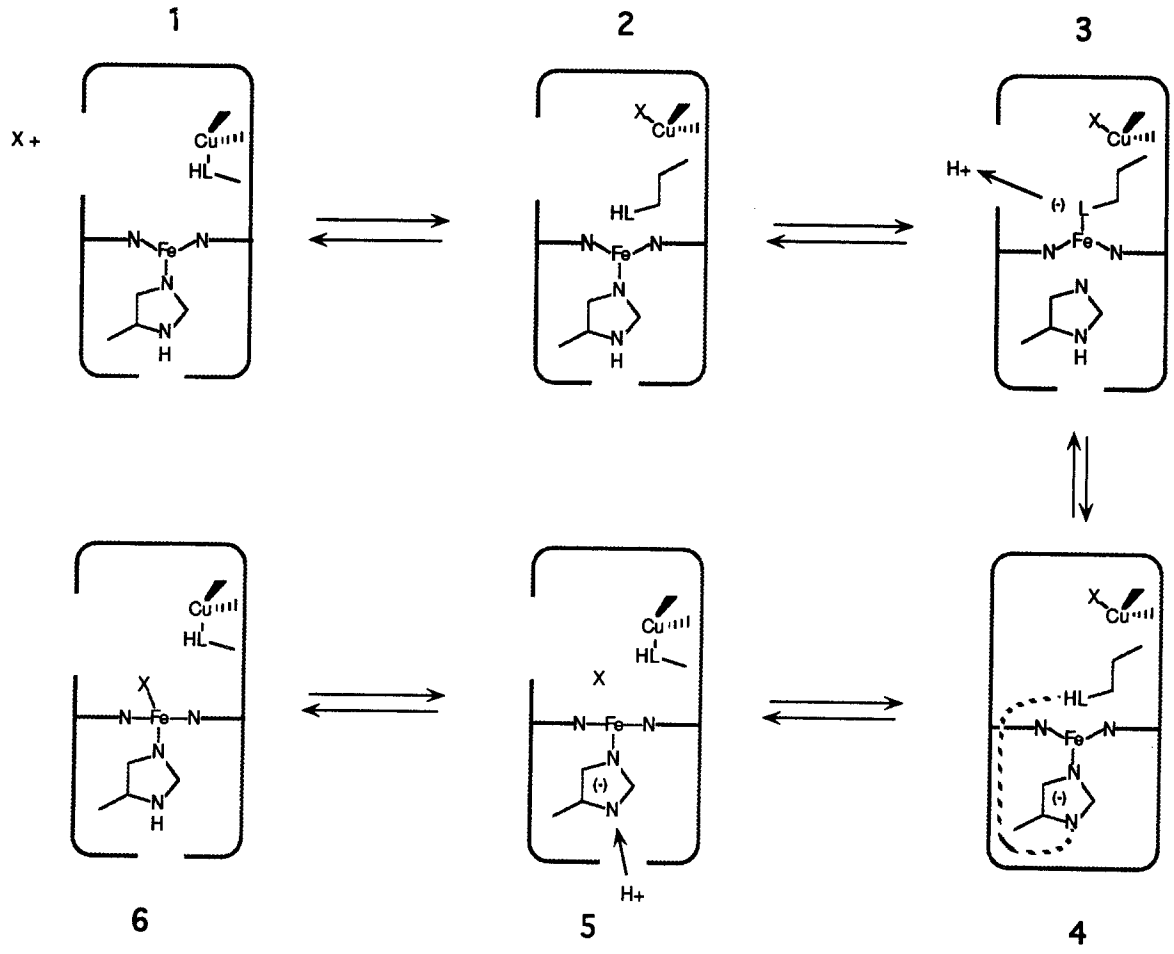


Fig. 5. Schematic representation of a microscopic proton translocation scheme based on the ligand shuttle reactions that we infer. Adapted from Ref. 25.

the unliganded form. It is also clear that comparisons among different oxidases from different organisms are necessary to assess the range of behavior of these systems.

The mechanism of the observed (5) redox anticooperativity between the hemes is not clear. It is beginning to be clear for the first time, however, that action (e.g., ligation) at one heme affects the spectroscopy of the other.[46] Thus once more the coordination chemistry offers a plausible control mechanism.

CONCLUDING REMARKS

We have attempted here to put forward hypotheses from which predictions can be made that are experimentally testable. It is, however, important to distinguish the models presented here that are backed by significant experimental evidence from those that are admittedly speculative at present. The mechanistic scheme shown in Fig. 3 derives from extensive spectroscopic and dynamics studies (reviewed in Ref. 25) that could only be alluded to here. Likewise the structural models shown in Fig. 4 have solid experimental support developed by others.[27] We feel confident that the conclusions concerning the electron transfer rates of the various species in Fig. 4 are valid, at least as relative numbers, and the potential for the enzymes to switch electron transfer on and off using this mechanism is real. Whether the oxidases actually use this mechanism functionally is a question that must be (and can be) settled experimentally. The proton translocation scheme in Fig. 5 is speculative but experimentally testable.

For over a century, the heme-copper oxidases have presented fascinating puzzles to scientists concerned with cellular respiration. Recent developments

have dramatically advanced our molecular understanding of these systems. Nevertheless, these developments only serve as a context within which to frame the truly penetrating questions that address the central issues concerned with mechanisms in bioenergetics. Finding the answers to these questions will continue to challenge us for the foreseeable future.

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