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Current Issues in the Chemistry of Cytochrome *c* Oxidase

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Some contemporary issues relevant to the chemistry of mammalian cytochrome *c* oxidase are discussed. These include the optical properties of heme A and the spectroscopic consequences of the differences in side-chain substitution compared to heme B; a common fallacy concerning the electrostatic exchange interaction between cytochrome a_3 and Cu_B; the question of the number and location of the copper components of the enzyme; and the mode of binding of ligands such as cyanide and azide.

KEY WORDS: Optical spectra; exchange coupling; binuclear center; ligand binding.

INTRODUCTION

In this article I intend to comment on a number of aspects of cytochrome oxidase folklore and chemistry. These were selected with two criteria. First are those topics which can be a source of confusion to those individuals not familiar with the properties of this enzyme and to those whose background in physical chemistry is limited. The second are some issues of contemporary interest.

It is useful to start with a glossary of terms that are used when referring to this enzyme in one or another of its redox states (Table I); Table I also gives some spectral characteristics of the various forms.

Oxidized enzyme, sometimes called fully oxidized, is enzyme in which the four redox active metal centers are in their common higher oxidation state; 3^+ for iron and 2^+ for copper. Likewise *reduced* enzyme, sometimes called fully reduced, has all four metal centers in their common lower oxidation state; 2^+ for iron and 1^+ for copper. *Partially reduced* enzyme, usually called *mixed-valence*, has some metal centers in their higher oxidation state and the remainder in their lower oxidation state; the most common examples are the mixed-valence carbon monoxide derivative, the mixed-valence cyanide derivative, and the mixed valence formate derivative (Table I).

There are a number of forms of oxidized enzyme. *Fast* enzyme is enzyme that reacts relatively rapidly with cyanide ($k \sim 2\,M^{-1}\,sec^{-1}$); *slow* enzyme reacts at about 1% of this rate. Fast enzyme is a stable form of the derivative called "pulsed" which is obtained when enzyme has been subjected to a cycle of reduction followed by reoxidation (usually with dioxygen) under conditions in which the production of hydrogen peroxide is avoided (dithionite is a common reductant for this enzyme; it reacts with dioxygen to produce O_2^- which dismutes to produce H_2O_2). *Oxygenated* enzyme is enzyme which has been subjected to a cycle of reduction and reoxidation under conditions in which hydrogen peroxide is produced. *Resting* enzyme was originally taken to mean the immediate product of the purification procedure. However, today it is used to refer to a form of the enzyme which is less reactive in a variety of chemical reactions, notably ligand addition and internal electron transfer; it can be equated with the slow form. Note that until recently most preparations were a mixture of the fast and slow species; however, procedures now exist for the preparation of the enzyme which behaves as pure fast form and for conversion of the fast enzyme to the slow form (Baker *et al.*, 1987).

Finally, we have the *open* and *closed* forms of the enzyme. The basis for this distinction is that the rate

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Table I. A Glossary of Terms Frequently Encountered in the Cytochrome Oxidase Literature

Oxidized or fully Oxidized:	$a^{+3}a_3^{+3}Cu_A^{+2}Cu_B^{+2}$
Fast:	A preparation of enzyme that reacts (relatively) rapidly with cyanide. Identical with "pulsed" (see below) (λ_{max} = 424 nm).
Slow:	A preparation of enzyme that reacts slowly with cyanide. Identical with "resting" (λ_{max} = 417 nm).
Resting:	Originally taken to be the enzyme as isolated but now used to describe a preparation which is less than fully active.
Pulsed:	Enzyme subjected to a cycle of reduction and reoxidation under conditions which minimize or avoid formation of hydrogen peroxide.
Oxygenated:	Enzyme subjected to a cycle of reduction and reoxidation under conditions of hydrogen peroxide formation. Probably an adduct with H_2O_2 in which the oxidation state of the binuclear center is not clearly defined (λ_{max} = 428 nm).
Reduced or fully reduced:	$a^{+2}a_3^{+2}Cu_A^{+1}Cu_B^{+1}$ (λ_{max} = 443 nm)
Mixed-valence CO:	$a^{+3}a_3^{+2}$-CO $Cu_A^{+2}Cu_B^{+1}$ (split Soret, no 655 nm band)
Mixed-valence formate:	$a^{+2}a_3^{+3}$-HCOOH $Cu_A^{+1}Cu_B^{+2}$ (split Soret, 655 nm band, no 830 nm band)
Mixed-valence cyanide:	$a^{+2}a_3^{+3}$-CN $Cu_A^{+1}Cu_B^{+1/+2}$ (split Soret, no 655 nm band, no 830 nm band)

The *closed* form refers to the fact that the *oxidized* enzyme reacts either slowly (*fast form*) or very slowly (*slow form*) with cyanide.
The *open* form refers to the fact that *partially reduced* enzyme reacts 10^3–10^6 times more rapidly with cyanide than the oxidized form.

of the reaction of both the fast and slow forms with cyanide is actually quite slow; enzyme which is partially reduced reacts at least three orders of magnitude faster than the fast form. Consequently, the oxidized enzyme is said to be in the closed conformation. Upon partial reduction a structural change, plausibly the jettison of the putative bridging ligand, leads to the large increase in rate (Nicholls *et al.*, 1972). The enzyme is then said to be in the open conformation. Fully reduced enzyme is also said to be in the closed form because the rate of reaction with cyanide is once more lowered, by about a factor of 10. However, this decrease is comparable to that expected in going from a reaction of CN^- with Fe^{+3} to CN^- with Fe^{+2}, and thus this kinetic basis for invoking the second structural change in going from partially reduced to fully reduced enzyme is not justified.

THE OPTICAL PROPERTIES OF HEME A

Depending upon whether it is oxidized or reduced, the color of cytochrome oxidase varies from brown to green. This color arises from the two heme centers present in the enzyme. Both of these heme centers contain heme A and have been the origin of considerable confusion as to the optical properties of the individual hemes.

Typical hemes such as B (as found in myoglobin, cytochrome *b*) and heme C (as found in cytochrome *c*) are said to exhibit spectroscopic four-fold symmetry; this implies that the peripheral substituents do not appear to affect in any important way the intrinsic four-fold symmetry of the porphyrin. Consequently, the optical properties of these hemes follow the classical optical pattern of an intense blue feature (the Soret band, or B band) and the less intense visible feature (the $\alpha\beta$ bands, the Q band). Thus the replacement of saturated substitutens (e.g., thioether) on pyrroles I and II by unsaturated substituents (e.g., vinyl)—as occurs in going from heme C to heme B—only leads to a small red shift in the optical spectrum. Hemes that follow this pattern are often denoted as D_{4h}, the group theoretical symbol for the effective four-fold symmetry of the porphyrin. By contrast, the effect of the peripheral substituents in heme A are quite dramatic, and have a major influence upon the way in which we must interpret these spectra.

In heme A the vinyl on pyrrole I is replaced with a saturated farnesyl-ethyl substituent, and the methyl on pyrrole IV is oxidized to the formyl group. These changes differ from those present in heme B where the two unsaturated substituents are located on adjacent pyrolles and thus do not introduce any distinction between the x- and y-directions on the heme; in heme A the two unsaturated substituents (vinyl on pyrrole II and formyl on pyrrole IV) are opposite from one another and, as a consequence, the conjugation is more extensive along the II–IV axis than it is along the I–III axis. These two changes lead to a marked lowering in the symmetry of the heme, from four-fold as in hemes B and C to two-fold in heme A.

The effects on the opical spectrum are profound; (i) instead of the simple α,β pattern typical of D_{4h} cytochromes, most of the visible intensity is transferred into the α-band which is also shifted substantially to lower energy (as expected from the four-orbital model of Gouterman, 1978). Hence the absorption maximum of ferrous cytochrome *a* is at 605 nm rather than 550–566 nm. Furthermore, the β-band is much less pronounced than it is in D_{4h} hemes, having lost

intensity to the α-band. (ii) The extremely intense mcd A term normally associated with the α-band of low-spin ferrous hemes such as cytochrome *a* is missing. This mcd term requires a symmetry which is three-fold or higher and this symmetry is lacking in heme A. (iii) The polarization ratios ($I_{\parallel}/I_{\perp}$) of the resonance Raman lines are typical of depolarized modes (0.75–1.0) rather than the "anomalous" polarization values 0.75 to infinity found with high-symmetry hemes (Woodruff *et al.*, 1982).

Good evidence that the optical properties of heme A are well behaved is the demonstration that the visible spectra of both oxidized and reduced CcO can be reproduced by 1 : 1 mixtures of the spectra of heme A model compounds (Carter and Palmer, 1982). Thus, an equimolar mixture of high-spin and low-spin ferric heme A model compounds resembles oxidized CcO while the equimolar mixture of high-spin and low-spin ferrous heme A models resembles reduced CcO. It should be noted that the individual contributions of cytochromes *a* and a_3 to the optical spectra of oxidized and reduced CcO was obtained more than 25 years ago by Vanneste (1966); while the conclusions of his analysis were viewed with suspicion for a number of years, there is now general agreement that his results are essentially correct.

Addition of weak-field ligands such as formate to CcO cause a blue shift in the Soret band while strong-field ligands such as cyanide cause a red shift. These shifts are conventionally interpreted as a result of a shift in the spin state of cytochrome a_3 to high-spin (the blue shift) or low-spin (the red shift). It is indeed true that changes in spin state of the indicated type lead to such spectral shifts. However, it must be noted that not all shifts in the Soret band can be interpreted as simply. In the case of cyanide, there is little ambiguity because the formation of the low-spin a_3 is supported by the loss of the 655 nm band and by the expected changes in mcd, Raman, and magnetic susceptibility. But red and blue shifts can be induced by other means, changes in pH for example, and in such cases the relevant physical methods do not indicate any changes in spin state. The best example is the conversion of fast to slow enzyme which results in a 6–7 nm blue shift in Soret band. Magnetic susceptibility measurements of these two forms of the enzyme give no evidence for a low-spin to high-spin conversion (Day *et al.*, 1993), nor is support for this idea to be found in the mcd and Raman measurements. The precise origin of such shifts is, however, not known. Among the plausible alternatives are (i) blue shifts due to the appearance-disappearance of a formal negative/positive charge at the end of the porphyrin ring; (ii) one of the unsaturated β substituents is moving into/out-of conjunction with the porphyrin ring π-system and lowering/raising the energy of the transition. Another possibility is raised by the finding that cytochrome a_3 appears to be a mixture of intermediate-spin and high-spin states in fast enzyme but exclusively high-spin in slow enzyme (Day *et al.*, 1993). The observed optical changes might well be related to this difference in magnetic composition.

Contrasted to the marked changes in the nature of the essentially porphyrin transitions is the normal behavior of those transitions which are ligand-to-metal charge-transfer bands; i.e., porphyrin → iron transitions. These are two of these. The first is found between 1000–2000 nm and is the porphyrin → d_{yz} transition of low-spin, ferric cytochrome *a*. The wavelength of this transition (Thomson *et al.*, 1985) is consistent with the belief that the iron in this cytochrome is coordinated to two histidine residues. The second optical band is found at about 650 nm. This is the porphyrin → d_{z2} transition of high-spin ferric cytochrome a_3. It is visible as an inflection on the low-energy side of the α-band; however, its mcd characteristics are much better resolved. There is some evidence that the properties of this transition are affected by the oxidation state of Cu_B (Mitchell *et al.*, 1991).

EXCHANGE INTERACTIONS

Another aspect of the physical properties which is often incorrectly understood is the consequences of an antiferromagnetic interaction, such as that which exists between Cu_B and cytochrome a_3 in the oxidized cyanide derivative. In this instance, we have the $S = \frac{1}{2}$ cytochrome a_3, coupled to the $S = \frac{1}{2}$ Cu. This coupling leads to two spin states with $S = 0$ and $S = 1$, respectively. Because the former is more stable, the coupling is said to be antiferromagnetic; the strength of this interaction is quantified by the parameter *J*. At low temperatures ($kT << J$) all of the molecules are in the $S = 0$ state and the observed magnetic moment of zero reflects this. At high temperatures ($KT >> J$) we have thermal distribution between the two coupled states, and this is where the confusion arises.

The degeneracy of the $S = 0$ state is 1 and that of the $S = 1$ state is 3. The total degeneracy is 4 and the observed magnetic moment will be $\frac{1}{4}$ of $S = 0$ plus

$\frac{3}{4}$ of $S = 1$; that is, $\frac{1}{4}(0) + \frac{3}{4}(8) = 6$ Bohr magnetons. This value is the same as that which would be exhibited by cytochrome a_3 and Cu_B in the absence of the exchange interaction and thus it is often assumed that the coupling has been broken. In reality it has not. The simplest demonstration of this is the fact that no EPR signal is observed. The reality is that the coupling is still present; this interaction is much stronger than that of the paramagnetic electrons within the two metal centers with the magnetic field, and hence the EPR measurement still perceives the system as having integer spin. Because the measured susceptibility is identical with that appropriate to an uncoupled system, it does not follow that the coupling is no longer present and, as a corollary, that cyanide is no longer functioning as a bridge between a_3 and Cu_B.

WHAT IS THE COPPER CONTENT?

The copper center denoted Cu_A has been of ongoing considerable interest initially because it was recognized as having unusual chemical properties, but more recently because of suggestions that it might be a center for proton translocation. The plot thickens with the recent discovery that the characteristic EPR of Cu_A is also found in a second copper protein, namely nitrous oxide reductase (Kroneck *et al.*, 1990), and in this later case there is eidence that the EPR is due to the mixed-valence Cu(II)–Cu(I) state. At first sight such a view might appear heretical in the context of CcO but recent developments suggest that this is plausible.

The copper content of CcO has been the subject of a number of studies in the past five years, first by Einarsdottir and Caughey (1985), then Bombelka *et al.* (1986), Steffens *et al.* (1987), and more recently by Oblad *et al.* (1989) and Pan *et al.* (1991). There is not good agreement between the results although it is agreed that preparations of the enzyme contain more than two equivalents of copper with some investigators tending toward a value of 3 while the remainder finding about 2.5 copper per enzyme. For example, Oblad *et al.* have analyzed a large number of preparations obtained over a number of years and obtain a mean ratio of Cu : Fe of 1.42 ± 0.30 while Zn : Fe ratios were 0.518 ± 0.094. EPR integrations of Cu_A yielded 0.88–1.22 spin per cytochrome *a* (deduced from the area under the $g = 3$ EPR feature of cytochrome *a*) with a mean value of 1.06 Cu_A/cytochrome *a*. This is significantly higher than the traditional value of ca. 0.8 which is usually reported. Similar chemical data has been reported on Buse and Steffens (1986).

On the other hand, other workers believe that there are only 2.5 Cu per enzyme, implying that the dimer is the functional form. More recently, Pan *et al.* (1991) found a stoichiometry of 5 Cu/4 Fe/2 Zn/2 Mg but that several treatments, incubation at mildly alkaline pH or depletion of subunit III, reduces these values to 4 Cu/4 Fe/2 Zn/2 Mg without any obvious effect on catalytic activity. They did claim to have seen the EPR spectrum of Cu_x at $g = 1.96$ in fully reduced enzyme, but this is almost certainly an iron-sulfur signal, probably from a contamination by a small amount of succinate dehydrogenase. This being the case, it is possible that their analysis for "oxidase-intrinsic" iron was too high, reflecting the contribution from succinate dehydrogenase (this enzyme contains nine iron atoms per molecule). The putative Cu_x signal represented about 0.1 Cu which would predict a contribution of about 0.4 Fe (only the 2Fe2S component would have been observed by EPR). If this is correct, then the Cu : Fe ratios obtained by Pan *et al.* must be revised up by about 1.25, yielding a value in the neighbourhood of 1.5, close to that found by Oblad *et al.* and others. However, the Zn : Fe and Mg : Fe ratios would also have to be increased, which would make these values out of line with ratios of 1 : 1 which everyone agrees upon.

There is no simple way to reconcile these results and prejudice inevitably takes over. I find myself prejudiced to the higher number for the following three reasons. First, there has never been a satisfactory accounting of the anomalously low EPR integral for Cu_A which is obtained when the chemically determined copper content is assumed to represent two equivalents of copper per mole of enzyme. The anomalous value disappears when the copper content is taken to represent three equivalents per mole enzyme. Second, the proposal that the characteristic EPR of Cu_A is due to a mixed-valence Cu(II)–Cu(I) system (Kroneck *et al.*, 1990) provides a nice explanation for the EPR lineshape observed at 9 GHz and the unusual pattern of hyperfine splittings observed at 3 GHz (Fronczis *et al.*, 1979). The original efforts to rationalize these splittings using metal–ligand interactions was deemed unsatisfactory by the original workers and it is impressive how easily one can reproduce the low-frequency spectra with a mixed-valence Cu(II)–Cu(I) system. The absence of these splitting at 9 GHz

can then be explained as a result of *g*-strain, a common phenomenon in the EPR of metal centers of proteins. Third, in developing a protocol for the reproducible removal of Cu_A from CcO, we consistently find that the intensity of the copper EPR increases significantly during all incubation conditions. This increase is due to the formation of a new copper (II) species with a characteristic Type II EPR spectrum. By resolving the total EPR signal at $g = 2$ into the separate contributions of the new Type II and residual Cu_A components, we consistently find that the quantity of Type II species found is about twice the amount of Cu_A lost (A. Jain and G. Palmer, unpublished data). This increase in copper signal occurs under conditions where the heme a_3-Cu_B center is intact. This observation provides strong support for the idea that the Cu_A site is actually a delocalized Cu(II)–Cu(I) pair and that the dislodging of Cu_A disrupts the binuclear copper center and renders Cu_x EPR-detectable.

WHERE IS CYANIDE BOUND?

Cyanide is a potent inhibitor of cytochrome oxidase activity. It was originally believed that cyanide bound to cytochrome a_3; indeed Keilin and Hartree's (1939) original discovery of cytochrome a_3 was due, in part, to the optical changes observed following addition of cyanide to reduced enzyme. More recently, the location of cyanide as a bridge connecting Cu_B and cytochrome a_3 has become the dogma, based in part on the observation that cyanide converts cytochrome a_3 to the low-spin state and substantially reduces but does not abolish the magnetic coupling to Cu_B. Parenthetically it might be noted that the nature of this coupling is ambiguous with both the ferromagnetic (Thomson *et al.*, 1981) and antiferromagnetic (Tweedle *et al.*, 1978; Barnes *et al.*, 1991) alternatives having their advocates.

The assignment that cyanide bridges the metals of the binuclear center was recently challenged by Yoshikawa and Caughey (1990) who instead have proposed that cyanide is bound exclusively to Cu_B. This suggestion was based in part on the finding that the stretching frequency of cyanide attached to CcO is at $2151\,cm^{-1}$, a frequency asserted to be inconsistent with a bridging location for cyanide. Additionally, it was noted that CcO reduced with 1 electron in the presence of cyanide exhibited two peaks, at 2151 and $2131\,cm^{-1}$, respectively; it was assumed that 1-electron enzyme is homogeneous and completely lacked the presumed bridge between iron and copper and thus the $2151\,cm^{-1}$ mode could not be due to a bridging cyanide. Finally, it was felt that the narrow linewidth of the observed IR mode was inconsistent with a bridging mode for cyanide.

We have confirmed Yoshikawa and Caughey's infrared data (Li and Palmer, 1993) but believe that the arguments used to eliminate the bridging mode of cyanide do not survive scrutiny. First, for metal-cyanide complexes with six or less *d*-electrons the bond to the metal ion is through carbon and tripositive metal ions which typically give rise to a C–N stretch around $2130\,cm^{-1}$. Metals with more than 6 *d*-electrons typically bind through nitrogen; in this case dipositive metal ions exhibit a C–N stretch around $2130\,cm^{-1}$. When cyanide is present as a bridge, these frequencies are expected to increase by approximately $30\,cm^{-1}$ (Shriver *et al.*, 1965) with one instance of a ferrous cupric cyanide bridge being observed at $2108\,cm^{-1}$; this frequency would be expected to be at ca. $2180\,cm^{-1}$ in the ferric analog. Thus, the infrared frequency of $2151\,cm^{-1}$ found with CcO is clearly larger than that anticipated for either mononuclear copper or iron and is reasonable for a cyanide coordinated as Fe–C=N–Cu species.

The presence of two cyanide stretches in 1-electron reduced CcO is to be expected. Cyanide-treated enzyme reduced with one electron has a composition which is approximately 40% cytochrome a^{+2}, 25% Cu_A^{+1}, with the balance of the electron presumably on Cu_B, thus accounting for the finite amount of low-spin a_3-CN signal which is detected by EPR (Garcia-Iniguez, 1980). The 65% of the enzyme with the electron present on either Cu_A or cytochrome *a* should still have cyanide occupying the bridging location, while in the remainder of the enzyme the electron is present on Cu_B, the bridge is presumably broken, and cyanide becomes the terminal ligand to cytochrome a_3. It thus appears that the mode at $2151\,cm^{-1}$ reflects bridging cyanide while the mode at $2131\,cm^{-1}$ is due to cyanide bound terminally to a_3.

Finally the possibilities for ligand disorder would appear to be much less in a bridging geometry, and I find it more plausible to expect a bridging rather than a terminal cyanide to exhibit a narrow linewidth.

It thus seems that the original basis for concluding that cyanide is not a bridging ligand is not well founded. Indeed this conclusion also seems to have been reached by Tsubaki and Yoshikawa (1991) though the basis for this change in position was not discussed (but see Caughey *et al.*, this volume).

WHERE IS AZIDE BOUND?

Sodium azide is another common inhibitor of cytochrome oxidase. Even though it can be classed as a moderately strong ligand for heme iron the spectral changes that follow addition of azide to CcO are remarkable insofar as they are very modest. Thus, the absorbance changes in the Soret region are small and somewhat variable (Li and Palmer, 1993), while there are essentially no changes in the mcd spectrum in the Soret, in striking contrast to the behavior of cyanide.

Thus, it may not have been surprising when Yoshikawa and Caughey (1992) proposed that azide was bound to a nonmetal site in the fully oxidized enzyme. This conclusion was based on their finding that the IR mode of the CcO-azide compound shifted in the correct direction but did not split when $^{15}N_3$ was used in place of $^{14}N_3$; they presumed that such a splitting must necessarily exist when azide is bound to a metal. We have confirmed their data with $^{14}N_3$ but unlike them find that the use of $^{15}N_3$ leads to a broadened IR mode consistent with a splitting of about $9\,cm^{-1}$ (Li and Palmer, 1993). In fact the original presumption of Yoshikawa and Caughey (1992) is invalid, for Pate *et al.* (1989) have examined the mixed isotope splitting in a series of model copper complexes and shown that the splitting obtained with $^{15}N_3$ ranges from $< 2\,cm^{-1}$ to $> 17\,cm^{-1}$ depending upon whether the Cu center is either a μ-1,3 or a μ-1,1 bridged derivative, with mononuclear terminal copper falling in between.

We find that the IR mode of azide is eliminated by cyanide, regardless of whether the enzyme is treated with cyanide before or after treatment with azide. On the other hand, neither formate nor thiocyanate eliminate the azide IR absorption. Indeed, when thiocyanate and azide are both added to CcO, the IR modes of both species can be seen simultaneously. It thus appears that azide, like cyanide, bridges cytochrome a_3 and Cu_B while thiocyanate and formate bind elsewhere on the binuclear center, most plausibly at Cu_B.

DO THE DIFFERENT FORMS OF CcO HAVE DIFFERENT CONFORMATIONS?

The evidence that there may be various conformations of CcO is rather circumstantial. Clearly the loss of the bridge on reduction of the binuclear center must lead to some kind of structural rearrangement, but this change might be very localized. On the other hand, the fast and slow forms of the enzyme clearly have different accessibilities to D_2O, for in the former there is a clear shift in the formyl mode of cytochrome *a* while in the latter it is absent (Schoonover *et al.*, 1988). At the time this result was obtained, the intimate relationship of cytochrome *a* to the binuclear center was not known and as the primary event in the fast-to-slow transition occurred at the binuclear center, the change in accessibility to D_2O was taken as evidence of some kind of global conformation change. However, with the newer knowledge of the connectivity between cytochrome *a* and the binuclear center reviewed elsewhere in this volume (Hosler *et al.*), the structural changes that accompany the fast-to-slow transition may still be quite localized.

Interestingly, from measurements of the effect of high pressures on the optical properties during catalysis, Kornblatt (1988) has deduced that there are very large changes in volume, probably associated with the reduction of cytochrome *a* or Cu_A. Such large changes have two obvious origins. The first is that there is a large conformational change associated with electron transfer, the second is that turnover is accompanied by the movement of 5–10 water molecules between ordered and disordered environments. In this context it is worth remembering that Gray and Malmstrom (1989) have suggested that electron transfer from cytochrome *a* to cytochrome a_3 is inherently rapid but requires an enabling conformational change.

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