The site of the redox-linked proton pump in eukaryotic cytochrome *c* oxidases

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Abstract The electronic spectra of fully oxidized derivatives of *some* cytochrome *c* oxidase preparations are distinctly pH dependent. In general, the observed spectral shifts are greater in the case of pulsed derivatives compared to resting preparations and also, greater for preparations of the enzyme from shark skeletal muscle compared to beef heart. The low temperature near-infrared magnetic circular dichroism spectrum of the fully oxidized shark enzyme is *not* pH dependent in the experimental range, indicating the sensitivity of the visible region electronic spectrum to variation in pH to be due principally to changes at the heme a_3-Cu_B chromophore. The results are discussed in relation to proposed mechanisms of proton translocation in cytochrome *c* oxidase.

Key words: Cytochrome oxidase; EPR; MCD; Proton pump; Shark

1. Introduction

It has been inferred by Wikström [1] that the maximally observed H^+/e^- (i.e. proton pumped to electron transferred) ratio of 2 observed for cytochrome *c* oxidase is more suggestive of an intimately coupled mechanism involving the oxidation-reduction centers and reactants than mechanisms requiring essential protein conformational dynamics. Put another way, the mechanism by which electron transfer is coupled to proton translocation appears likely to involve principally changes in the first coordination sphere of one or more of the metal centers, rather than second or higher coordination sphere processes. Accordingly, hypotheses concerning this mechanism have emerged in which proton translocation is achieved by cycles of oxidation-reduction driven metal ion-ligand exchange processes. The original suggestion of Gelles et al. [2] that Cu_A is the site in question is no longer popular and more recently, heme a_3 has been nominated [3]. A feature that these and similar detailed hypotheses have in common is the postulated presence of at least one ligand species which may either be protonated, or alternately, bound to the key metal center in only one of its oxidation states. If such ideas have any basis in reality, then the effective pK_a of the ligand in question must be poised at around physiological pH. Consequently, it follows that if the oxidation level of the enzyme is held constant, then moderate shifts in pH in the vicinity of 7.4 can reasonably be expected to also effect the ligand exchange process.

In general, if a metal center undergoes a ligand exchange reaction, the spectral properties of the system will change in some way. Therefore, searching for pH-dependent behaviour in the spectra of derivatives of cytochrome *c* oxidase represents a viable method of both confirming the plausibility of certain hypothetical mechanisms of proton translocation and moreover, identifying the metal center(s) involved.

While the unidirectional nature of the proton pump is lost when cytochrome *c* oxidase is removed from the mitochondrial membrane, the same ligand exchange processes should occur during turnover of the enzyme in solution, provided the fully active structure has not been too severely compromised. In this paper we report the pH-dependent properties of the electronic spectra of some highly active preparations of cytochrome *c* oxidase obtained from beef heart and shark skeletal muscle. The observation of pH-dependent spectra is a function of the method of purification and age of the preparation, in addition to the particular derivative employed. The results are discussed in terms of the likely site of the proton pump in cytochrome *c* oxidase.

2. Experimental

Cytochrome *c* oxidase was prepared from the skeletal muscle of the Atlantic sharpnose shark (*Rhizoprionodon terraenovae*), scalloped hammerhead shark (*Sphyrna lewini*) and beef hearts by two methods. The first was the procedure of Wilson et al. at pH 7.4–7.5 [4] which is a variation on the Yonetani method [5] and also, the modifications developed by Baker et al. at pH 7.8–8.0 [6] to the Hartzell–Beinert preparation [7]. The standard spectrophotometric assays of oxidation–reduction activity [5] and cyanide binding kinetics [6] were used to characterize preparations. The pulsing protocol consisted of reducing the oxidase with sodium ascorbate at pH 7.4 and 20°C in the presence of a catalytic amount (2% relative to heme *a*) of bovine cytochrome *c* (Sigma) and then reoxidizing the enzyme with air in the presence of a catalytic amount (0.1% protoheme relative to heme *a*) of bovine catalase (Sigma) to prevent the formation of peroxide adducts.

Electronic absorption spectra were recorded on Perkin-Elmer λ5 and Varian DMS 100 spectrophotometers. Concentrations of cytochrome *c* oxidase were determined as total heme *a*, using $\varepsilon_{587} = 24\ mM^{-1} \cdot cm^{-1}$ for the pyridine hemochromagen [8].

EPR spectra were obtained using a hybrid instrument consisting of a Varian E109E console, used to provide the field modulation to a Bruker B-E 25 magnet, with an ER 082 power supply and B-H 15 field controller, plus a Varian E102 microwave bridge and V-453.3 cylindrical cavity. The spectrometer was fitted with an Oxford Instruments ESR 900 liquid helium flow cryostat.

MCD spectra were recorded using an Aviv Associates 41DS circular dichroism spectrometer in conjunction with a Cryomagnetics Incorporated cryomagnet. A '*single* spectrum' consists of data recorded in with the applied field in the forward direction minus the reverse field data, the difference being divided by two. In this manner, contributions

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Abbreviations: CHES, 3-(cyclohexylamino)-1-propanesulfonic acid; EDTA, ethylenediaminetetraacetic acid; EPR, electron paramagnetic resonance; HEPES, *N*-(2-hydroxyethyl)piperazine-*N'*-(2-ethanesulfonic acid); MES, 2-(*N*-morpholino)ethanesulfonic acid; MCD, magnetic circular dichroism.

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arising from natural circular dichroism are subtracted from the spectrum.

3. Results

In contrast to beef heart cytochrome *c* oxidase, which may be isolated in both resting and pulsed forms [9,10], the shark enzyme purified by either the Yonetani or Hartzell–Beinert method is always isolated in a resting form, exhibiting slow cyanide-binding kinetics. Following turnover, the shark oxidase exhibits rapid, monophasic cyanide-binding kinetics, indicating formation of a pulsed derivative [10]. The electronic absorption spectra of pulsed shark cytochrome *c* oxidase at pH 6.0 and 9.0 are shown in Fig. 1. The samples were prepared in the pulsed form as described in section 2 and then diluted to the required pH in additional buffer. These spectra clearly exhibit pH-dependent behaviour, which is reversible. When the samples used to obtain the data of Fig. 1 were returned to pH 7.4, the Soret maximum returned to 424–425 nm in both cases. These data, together with those obtained from various other oxidase preparations and/or derivatives, are collected in Table 1. Note that all fully oxidized derivatives of Hartzell–Beinert type preparations of both the beef and shark enzyme yielded pH-dependent spectra. On the other hand, the spectra of oxidized Yonetani type preparations exhibited much less significant sensitivity to pH, if any. Similar rather subtle effects on the spectra of the oxidized resting beef heart enzyme have been previously reported [11]. The spectra of fully reduced derivatives were clearly not pH dependent within experimental uncertainty.

The near-infrared MCD spectra of pD 6.4 and pD 9.4 samples of Hartzell–Beinert preparations of the resting shark oxidase at 4.2 K and 5.0 T are presented in Fig. 2. For several practical reasons, it proved impossible to obtain the analogous data for pulsed derivatives; that is, it could not be unequivocally established that, under the particular conditions required to record the MCD spectra, samples remained pulsed. The negative feature at 810 nm and the positive feature at 1580 nm are slightly red shifted, but otherwise strikingly similar to the same signatures observed in the spectra of the bovine enzyme and assigned to Cu_A and heme *a*, respectively [12]. The horizontal bar marked on Fig. 2 close to the 1580 nm feature represents the shift in this peak expected if it were to display the equivalent pH-dependent shift in energy exhibited by the Soret band of this derivative (Table 1). If there is any pH dependence to these spectra, it is insignificant in comparison with that displayed by the visible region spectra of oxidized Hartzell–Beinert derivatives listed in Table 1. It should also be mentioned that, within experimental uncertainty, the low temperature near-infrared MCD spectra of Yonetani type shark oxidase preparations (not shown) are the same as those of Fig. 2.

Table 1
Spectral properties of beef and shark cytochrome *c* oxidase derivatives

	Soret, α-band (nm)			
	pH 6.0	pH 6.5	pH 7.4	pH 9.0
Beef enzyme				
Yonetani[1]	417, 600	–	418, 600	420, 600
Hartzell–Beinert[2]	–	*423*, 600	*424*, 599	*427*, 600
Reduced[3]	443, 604	–	443, 603	444, 603
Shark enzyme				
Yonetani	422, 600	–	422, 600	423, 600
Resting H.-B.	*417*, 600	–	*422*, 600	*423*, 600
Pulsed H.-B.[4]	*415*, 600	–	*425*, 600	*427*, 600
Reduced	443, 604	–	443, 603	443, 604
Peroxide adduct[5]	428, 599	–	428, 600	428, 599

[1]Resting derivative as prepared. Stable from pH 5.5–10.0.
[2]Pulsed derivative as prepared [10]. Solutions develop turbidity at pH 6.0.
[3]Prepared by addition of excess solid sodium dithionite, or sodium ascorbate in the presence of a catalytic amount of cytochrome *c* to either type of enzyme preparation.
[4]Prepared from the Hartzell–Beinert type preparation as described in section 2.
[5]Prepared by addition of excess hydrogen peroxide to the fully reduced enzyme.

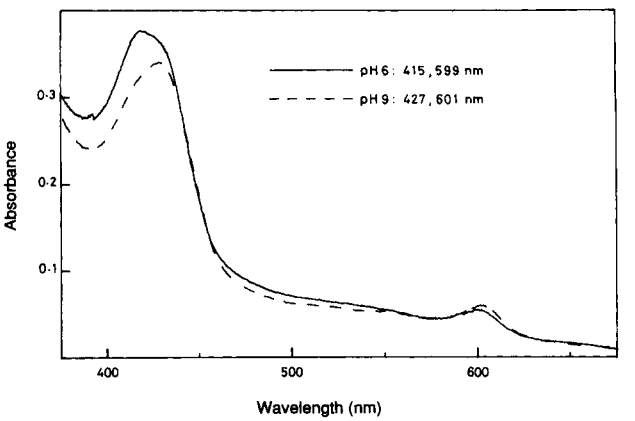

Fig. 1. Electronic absorption spectra of pulsed Atlantic sharpnose shark cytochrome *c* oxidase derivatives at 20°C, 3 μM enzyme concentration, 1 cm pathlength. Buffers were Na-salts, 1 mM in EDTA 0.5% (w/w) in Tween 80; 10 mM HEPES + 50 mM CHES, pH 9.0; 10 mM HEPES + 50 mM MES, pH 6.0.

The EPR spectra of cytochrome *c* oxidases exhibit a well-resolved feature attributable to the g_z component of the ferric heme *a* spectrum [7,12]. The small change observed in this signal upon taking the shark enzyme through a pulsing protocol is shown in Fig. 3. There is negligible pH dependence associated with this signal in either resting or pulsed forms of the enzyme as documented in Table 2.

The pH dependence of the activity of Hartzell–Beinert type shark cytochrome *c* oxidase is summarized in Table 3. At pH 6.0–7.5 (25°C) we find the turnover number of these preparations to be 460–470 s^{-1} (mol ferrocytochrome *c* oxidized/mol enzyme). This is significantly higher than the maximum value previously determined for Yonetani type preparations of shark oxidases [4,13]. At pH 9.0 there is no measurable activity under otherwise standard conditions. If, after being maintained at pH 9.0 for ca. 10 min, samples are returned to pH 6.0–7.5, then 80%–90% activity is typically recovered. With reference to the well documented pH dependence in the activity of the beef enzyme [14,15], the lack of activity at pH 9.0 observed for the shark oxidase is not surprising. However, the absence of any significantly increased activity in preparations of shark cytochrome *c* oxidase upon decreasing the pH from 7.5 to 6.0 was an unexpected result that will be the subject of future studies.

4. Discussion

To facilitate discussion of the results, a schematic representa-

tion of how a redox-linked proton pump might function is shown in Fig. 4. This is based on many of the considerations originally proposed by Gelles et al. [2]. For simplicity, the scheme translocates one proton for every electron transferred; rather than the observed two protons per electron transferred, which are associated with only two out of the four electrons required per reaction cycle [1]. The modifications to such a scheme necessary in order to achieve the experimentally established stoichiometry have been discussed by Rousseau et al. [3], but are irrelevant to the present arguments.

The relevant metal center (M), two substitution labile ligands (B and L), plus an array of negatively charged groups representing a 'proton conduit' to the metal center are envisaged to lie in a region of the protein defining a 'channel' which spans the mitochondrial membrane. The metal center may exist in one of two oxidation states, either oxidized (M^+), or one elctron reduced (M^0). Compared to L, the deprotonated ligand B^- has a preference for the metal center in its oxidized state. Compared to B^-, the ligand L has a preference for the metal center in its reduced state. It follows directly from these stipulations that one electron reduction of M^+ can lead to the translocation of a proton across the mitochondrial membrane, from matrix to cytosol, by the mechanism indicated in Fig. 4. In the absence of any protein conformational changes, other than the first coordination sphere processes depicted, the unidirectional nature of the proton pump seems to be guaranteed by two factors, which are not mutually exclusive. First, the protonated ligand LH^+ in structure IV is closer to the cytosol than to the matrix side of the membrane and therefore, the released proton is more likely to appear in the cytosol for steric reasons. Second, while the proton conduit to the coordination site consists of only two component bases as drawn, it could be much longer. Consequently, provided redistribution of protons amongst the conduit bases and matrix is faster than the ligand exchange reactions, arguments based on mass action additionally suggest that net movement of protons from the coordination site towards the matrix is unlikely while the difference in proton concentration either side of the membrane remains small.

Consider now the enzyme isolated from the mitochondrial membrane in micellar solution. For purposes of illustration, let us suppose that structure II in Fig. 4 is representative of the proton pumping site in the air-stable derivative obtained at physiological pH. If the pH is now raised, at some point the ligand L will become deprotonated and is then able to bind to the metal center. The ligand B^- may, or may not, be displaced; this does not matter, since in either case there will be a change in the coordination environment of M^+. Similarly, if structure

Table 2
EPR properties of shark cytochrome *c* oxidases

	g-value (g_z component of heme *a*)	
pH	Hammerhead	Sharpnose
Resting enzyme		
6.0	3.00	3.00
7.4	3.01	3.01
9.0	3.00	3.01
*Pulsed enzyme**		
6.0	2.98	2.98
7.6	2.98	2.98
9.0	2.99	2.98

*Prepared as described in section 2.

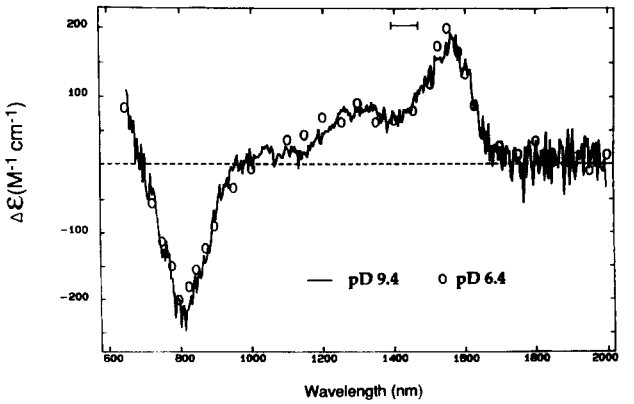

Fig. 2. Magnetic circular dichroism spectra of Atlantic sharpnose shark resting cytochrome *c* oxidase at 4.2 K and 5.0 T, 40 μM enzyme concentration, 0.8 mm pathlength. Deuterated buffers (Na-salts) were 0.5 mM in EDTA, 0.25% (w/w) Tween 80, 50% (v/v) d_3 glycerol; 5 mM HEPES + 25 mM CHES, pD 9.4; 5 mM HEPES + 25 mM MES, pD 6.4.

I were to better represent the air-stable derivative obtained, then lowering the pH would be expected to lead to the displacement of L by protonation of this ligand. Analogous arguments to these can be put forward for many component structures of schemes in which proton translocation is explained on the basis of oxidation–reduction driven ligand exchange reactions. That is, if some mechanism closely related to the hypothesis of Fig. 4 is correct, then it is unthinkable that at least some derivatives of isolated cytochrome *c* oxidase will not exhibit readily detectable pH-dependent spectral characteristics.

The data shown in Fig. 1 and some of that listed in Table 1 clearly exhibits pH-dependent behaviour. It should be stressed that all these data were collected on samples which were less than two hours old and maintained on ice prior to measurements. Many samples exhibited quite large spectral shifts when aged much longer than this, but significantly reduced pH-dependent behaviour. Fully oxidized derivatives of Hartzell-Beinert type preparations of both beef and shark cytochrome *c* oxidase show a marked sensitivity in the position of the Soret band to pH. Unlike the beef enzyme, shark oxidase does not undergo pH-dependent changes in aggregation state [13] and so, the observed spectral shifts cannot be attributed to factors linked to association-dissociation processes. Somewhat surprisingly, the Yonetani type enzyme preparations showed very little evidence of pH-dependent spectral behaviour.

The reproducibly observed sensitivity of the Soret band in some derivatives to pH firmly supports the plausibility of the type of mechanism given in Fig. 4 and furthermore, indicates heme *a* and/or the heme a_3-Cu_B pair to be candidates for the site of the proton pump. Moreover, as the enzyme seems to pump two protons during the transition from the ferryl intermediate to the oxidized form [1], the observation of pH-dependent spectra associated with the fully oxidized preparations reported here is to be expected. It is entirely reasonable to suppose that both intermediates involved in a proton translocation step will have pK_as close to physiological pH, but that the pK_a of the intermediate where the protons have been released will be below pH 7.4, whereas the other will be above pH 7.4.

The data of Fig. 2 is clearly not pH dependent within experimental uncertainty. Since the positive feature at 1580 nm is due

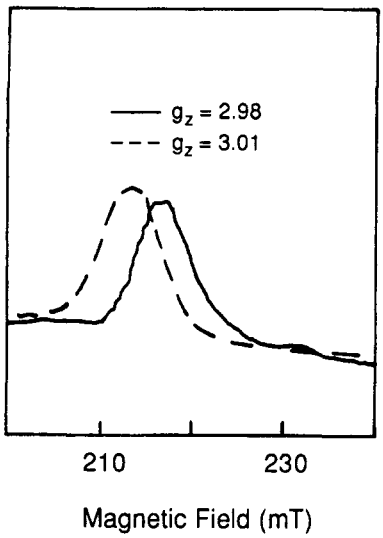

Fig. 3. X-Band EPR spectra of resting (– – –, pH 7.4, 65 μM enzyme) and pulsed (——, pH 7.6, 60 μM enzyme) forms of Atlantic sharpnose shark cytochrome *c* oxidase showing the g_z components of the heme *a* spectra. Buffers (Na-salts) were 50 mM HEPES, 1.0 mM in EDTA, 0.5% (w/w) Tween 80. Recording conditions were 12 K, 1 mW microwave power, 10 gauss modulation amplitude, 8×10^4 amplifier gain.

exclusively to heme *a* [12], this strongly suggests that the pH sensitive metal center must be the heme a_3-Cu_B pair. If one of the histidine ligands to heme *a* were to become deprotonated to form histidinate, then based on well-documented cases in which this occurs [16,17], a shift in the 1580 nm band to around 1350 nm is expected [18,19].

The absence of any significant pH dependence in the EPR signals associated with heme *a* in oxidized derivatives (Table 2) further supports this position. Simple rotation of the plane of one imidazole ring with respect to the other in a bis-histidine complex leads to very dramatic changes in the g_z signal, from around the observed position in Fig. 3 to off the scale on the low field side, with an accompanying reduction of intensity [20,21,22,23]. The absence of any pH dependence of this signal documented in Table 2 is very strong evidence that no such reorientation of the ligands to heme *a* occurs with change in pH.

The low energy near-infrared maxima like those at 1580 nm in Fig. 2 are usually assigned as porphyrin $a_{1u}(\pi)$ to iron (d_{yz}) charge-transfers and are known to be correlated with the g-value anisotropy in the EPR spectra of low-spin ferric hemes [18,19]. Consequently, the spectra of Figs. 2 and 3 reflect ground state properties of heme *a*. On the other hand, the Soret bands of most hemes correspond to an accidentally degenerate pair of transitions: $a_{1u}(\pi) \rightarrow e_g(\pi^*)$ and $a_{2u}(\pi) \rightarrow e_g(\pi^*)$ [24,25]. So, in principle, it could be argued that the pH-dependent spectra of Fig. 1 may be due to changes at heme *a* affecting only the energy of the e_g antibonding orbital of the porphyrin ring, which would not be expected to have any influence on the EPR and near-infrared MCD spectra. The problem with this point of view is that heme *a* is a low-spin center; that is, the bonding between the iron and its ligands has considerable covalent character with much mixing of nominally pure porphyrin and pure metal orbitals. Therefore, in reality, it is very difficult to envisage any interaction which could have a significant effect on the energy of the porphyrin orbitals of heme *a* without there being a measurable consequence to the energy of the iron orbitals. The observation that neither the near-infrared MCD nor the EPR spectra of the oxidized enzyme are sensitive to pH is, thus, extremely strong evidence that heme *a* is not the pH-dependent center.

The electronic spectra of the reduced cytochrome *c* oxidases from beef and shark are essentially the same and moreover, clearly not pH dependent (Table 1). The fully reduced enzyme contains heme a_3, uncoupled from Cu_B, quantitatively in a high spin ($S = 2$) state [26]; consistent with the five-coordinate consensus heme a_3 structure of this derivative, in which the proximal histidine is bound [27]. If the proposed proximal ligand to heme a_3 switching mechanism of Rousseau et al. [3] were correct, then by the current argument, it is reasonable to expect the spectra of fully reduced derivatives to be pH dependent, contrary to our observations (Table 1).

The distal side of heme a_3 is where oxygen and its reduced intermediates are bound during turnover [28]. While this site is undoubtedly involved in consumption of the 'scalar' protons required for generation of the product water, it is extremely difficult to envisage it simultaneously involved in 'vectorial' proton translocation by a ligand exchange mechanism. However, in the case of derivatives like the fully oxidized resting and pulsed enzyme where heme a_3 is magnetically coupled to Cu_B [29], presumably through bridging ligands, it is conceivable that ligand exchange processes at Cu_B might manifest themselves as perturbations in the spectral characteristics of heme a_3. Thus, by a process of elimination, the present data suggest Cu_B to be the site of the proton pump. This position is supported by the previous findings of Fabian and Malmström [11] that the electronic difference spectra of the cyanide and azide adducts of the enzyme are, respectively, pH dependent and pH independent; heme a_3 and Cu_B being, respectively, magnetically coupled and uncoupled in these two derivatives [12].

The possible reasons for the observed pH dependence in the activity of cytochrome *c* oxidase are complicated enough [14,15] that the lack of activity at pH 9.0 (Table 3) cannot be used to unambiguously support the plausibility of schemes like that shown in Fig. 4. However, given that electron transfer and proton translocation are coupled activities, it is entirely reasonable that the former is sensitive to pH. Furthermore, if the pH dependence of the electronic spectrum is indicative of the site of ligand exchange mediated proton pumping, as we have argued, then it would be of serious concern if the enzyme did not exhibit some significant change in activity within our working pH range.

The conclusion that Cu_B is the primary metal center involved

Table 3
Sharpnose shark cytochrome *c* oxidase pH-dependent steady-state kinetic parameters at 25°C

	pH 6.0[a]	pH 7.5[a]	pH 9.0[a]
k_{cat} (s^{-1})[b]	470 (±20)[c]	460 (±40)[c]	–[d]
K_m (μM)[b]	60 (±3)	58 (±4)	–[d]
k_{cat}/K_m ($M^{-1} \cdot s^{-1}$)[b]	7.8×10^6	8.0×10^6	–[d]

[a]100 mM sodium phosphate, 1.0 mM in sodium EDTA, 0.5% (w/w) in Tween 80 – see section 4.
[b]Beef heart cytochrome *c* employed in assays - see section 4.
[c]Numbers in parentheses represent standard deviations on the measurement.
[d]Samples maintained at pH 9.0 for ca. 10 min regained 80–90% activity when returned to pH 6.0–7.5.

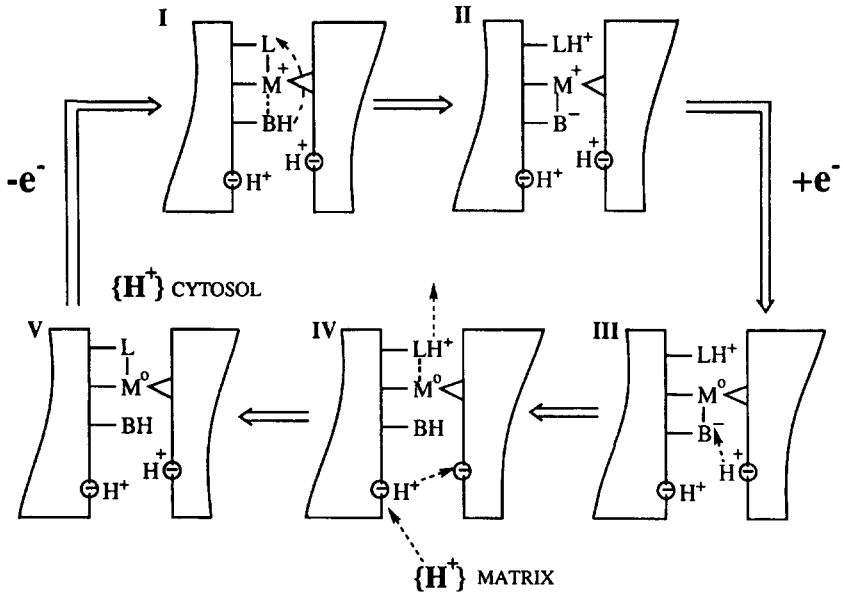

Fig. 4. Schematic representation of hypothetical mechanism coupling proton translocation to electron transfer processes. In each of the structures I through V, the matrix side of the membrane is below the metal center and ligands while the cytosol is above.

in proton translocation is appealing for some circumstantial reasons which should be mentioned. Recently, progress toward understanding the three dimensional structures of prokaryotic terminal oxidases, which are similar to the mitochondrially encoded subunits of eukaryotic cytochrome *c* oxidases, has been made through the mutant studies of Gennis and others [30,31,32]. In the resulting preliminary model structures, heme *a* and its axial ligands, together with heme a_3 and its proximal ligand seem to be associated with a comparatively rigid part of the structure. On the other hand, the ligand environment of Cu_B is less well defined and there appear to be several amino acid side-chains in the vicinity which might be able to undergo ligand exchange reactions. Furthermore, the stipulation that Cu_B is the center undergoing ligand exchange reactions of relevance to proton pumping activity does not conflict with the recent suggestion of Brunori et al. [27] that the pulsed to resting conversion process is mediated by detachment of the proximal ligand to heme a_3. Indeed, the fact that pH-dependent behaviour is observed in both pulsed and resting derivatives of the shark enzyme (Table 1) meshes rather well with this idea, since the same ligand exchange reactions at Cu_B can reasonably be expected to occur irrespective of whether the proximal heme a_3 ligand is attached or not.

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