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On the Mechanism of Proton Translocation by Respiratory Enzyme[1]

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The protonmotive function of the respiratory heme-copper oxidases is often described as the sum of two separate mechanisms: a proton pump *plus* an incomplete Mitchellian redox loop. However, these two functions may be mechanistically intertwined so that the *uptake of protons to form water* during the reduction of O_2 *is a crucial part of the proton pump mechanism itself.* This principle can be deduced from thermodynamic, kinetic, mechanistic, as well as from structural considerations, and was first proposed in conjunction with a histidine cycle model of proton translocation [Morgan, J. E., Verkhovsky, M. I., and Wikström, M. (1994). *J. Bioenerg. Biomembr.* **26,** 599–608]. However, histidine cycle models go much further to suggest chemical details of how this principle might be applied.

1. COUPLING BETWEEN PROTON TRANSLOCATION AND DIOXYGEN REDUCTION

Coupling between the driving and the driven reaction is crucial in a redox-linked proton pump. It is achieved if the two reactions have a common intermediate in much the same way as chemical, conformational, and electrochemical intermediates were envisioned to couple respiration to oxidative phosphorylation (Boyer *et al.*, 1977). The heme-copper oxidases catalyze a set of reactions that involve electron transfers from the donor (cytochrome *c* or a hydroquinol) to the low-spin heme, and from the latter to the binuclear heme iron-copper site (see Regan *et al.*, this issue p. xxx), *plus* O_2 reduction at the latter site. Research in the past two decades has led to the view of the catalytic cycle of these enzymes shown in Fig. 1 (cf. Babcock and Wikström, 1992; Ferguson-Miller and Babcock, 1996). Note that the P_R state in this scheme, formed primarily after acceptance of the third electron, is spectroscopically identical to a P state (P_M, not shown) that is formed more slowly on spontaneous decay of the oxy species (A) when no third electron is available (Morgan *et al.*, 1996).

Although many details and important structures remain to be elucidated, the major endergonic reactions of proton translocation in the cycle can been associated to the P→F and F→O transitions based on both equilibrium (Wikström, 1989) and kinetic (Verkhovsky *et al.*, 1997) data. These transitions, which may include a number of substeps, are each coupled to the translocation of *two protons* across the dielectric. Although this imposes important restrictions on the mechanism, it does not imply that protons are necessarily released into the aqueous medium in synchrony with these steps. The major electrochemical *work* of H^+ pumping is done in these steps, but H^+ release (or uptake) into the aqueous media surrounding the membrane may be significantly delayed (cf. Heberle and Dencher, 1991). Therefore, the report that protons are not released into the medium in the P→F step, but only at ca. 1 ms after addition of O_2 to the reduced enzyme (Nilsson *et al.*, 1990; Oliveberg *et al.*, 1991), does not necessarily contradict our view.

[1] The amino acid numbering in this paper refers to subunit I of the cytochrome *c* oxidase from bovine heart mitochondria.

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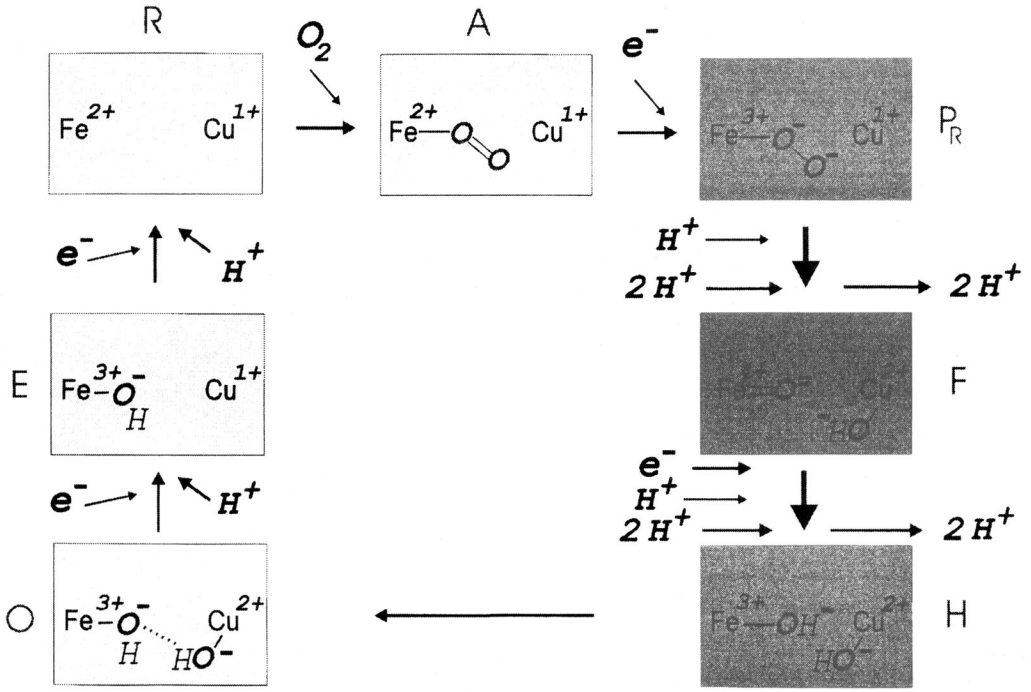


Fig. 1. Catalytic cycle of the heme-copper oxidases. The scheme emphasises division into a reductive (left) and an oxidative (right) half-cycle, of which only the latter is associated with proton translocation.

2. PRINCIPLE OF COUPLING

If protons were allowed to flow freely into the binuclear site to form water, a large amount of available free energy would be dissipated. Protonation of the oxygen reduction site is the consequence of the oxygen reduction chemistry, which in turn is initiated by electron transfer into the site. The protonation is a highly exergonic event as seen by the following considerations.

$$\text{Fe (IV)} = O^{2-} + e^- \rightarrow \text{Fe (III)–}O^{2-} \qquad (1)$$

In this reaction, electron transfer to an oxyferryl species is expected to lead to a ≈9 unit increase of pK_a of the oxygen ligand (Williams, 1985).

Similarly, in

$$\text{Fe(II)–O=O} + e^- \rightarrow \text{Fe(III)–}O^-\text{–}O^- \qquad (2)$$

(see Fig. 1) there is again accumulation of negative charge that requires protonation for the chemistry to proceed (in this case scission of the O–O bond). Note that this principle is independent of the precise structure of the P itermediate (see e.g., Kitagawa and Ogura, 1997 this issue, p. xxx, and below). In both examples, *electron transfer into the binuclear site converts a strong acid function into a weak one, providing the driving force for proton uptake.* Proton consumption must be gated to capture that energy, which brought us to the concept that *it may be the uptake of protons that constitutes the "common intermediate" between O_2 reduction chemistry and proton translocation* (Wikström *et al.*, 1994; Morgan *et al.*, 1994). Independently, Rich (1995) made a very similar conclusion, which he based on the principle of electroneutrality. Although the chemistry of the P→F and F→O steps must be different, it seems reasonable to invoke a proton-translocating mechanism common to both. A unifying feature is the uptake of one consumed proton in each step (Fig. 1; Mitchell *et al.*, 1992; Mitchell and Rich, 1994), although this stoichiometry may vary with $\Delta\mu_H{}^+$ (Wikström *et al.*, 1997). Proton requirement from the *i*-side of the membrane is hence shared by both the chemical and the proton translocation mechanisms, which may provide the key principle of coupling. Simplifying, the coupling may be achieved as follows:

a. H^+ uptake from the *i*-side is driven by the accumulation of electronic charge at the binuclear center.

b. The H^+ cannot reach the oxygen chemistry (gating), but is instead bound to a pump element, which is not protonically connected to the binuclear site.

c. The pump element has alternative access to protons on the *i*- and *o*-side of the membrane.

d. Protonation of the pump element from the *i*-side is stabilized electrostatically (high pK_a) by the charge in the binuclear site.

e. The proton gate opens, resulting in H^+ flux from the *i*-side of the membrane into the binuclear site, where the charge is annihilated and the chemistry completed.

f. As a consequence, the protonated pump element is destabilized (pK_a decreases), releasing H^+ into the *o*-phase.

3. THE PUMP ELEMENT—A POSSIBLE DUAL ROLE

The pump element must have access to two discrete proton connectivities relative to the protonic sidedness, i.e., with the aqueous *i*- and *o*-sides of the membrane, respectively. The switch between these orientations may either involve protein structures around it, or in the simplest case, a conformational change of the pump element itself (Wikström and Krab, 1979; Wikström, 1982).

After protonic loading of the pump element in response to electron transfer into the binuclear site, the protonated element switches to an output position. Now, the proton gate to the binuclear site should open. The simplest way to achieve this is that *in its input position the pump element itself contributes to the barrier against H^+ transfer to the binuclear site.* The switch to the output position required for the directionality of pumping then simultaneously opens the gate for H^+ transfer into the binuclear site. Thus, the orientation of the pump element may determine not only its own protonic sidedness, but it could also provide the gate for protons to the binuclear site.

H^+ uptake completes the chemistry at the binuclear center and the pump element is deprotonated in its output position. It would then have to switch to the input position, both to be reprotonated and to close the H^+ conduction path into the binuclear site. Reorientation in the protonated state must be effectively prevented, which may be achieved in several ways. The pK_a may be sufficiently lowered by the annihilation of the negative charge in the binuclear site, causing essentially complete deprotonation (thermodynamic control). It may have lower energy in the input state only when deprotonated, so that a back-transition in the protonated state would be unlikely (thermodynamic control). Finally, the transition barrier may be high when the element is protonated but low when deprotonated, preventing backflux kinetically.

4. PATHWAYS OF PROTON CONDUCTION

In earlier views of two possible proton-conducting pathways in the heme-copper oxidases, the division of labor was proposed to be between "pumped" and "consumed" protons, respectively (Iwata *et al.*, 1995). However, in the mechanism described above, protons enter the pump element and the binuclear site from the *i*-phase *via the same gated pathway.* Recent experimental findings indeed suggest that all protons taken up during the oxygen reduction phase of the catalytic cycle (see Fig. 1) are transferred via the so-called D-channel. On the other hand, mutation of residues in the so-called K-channel prevents reduction of heme a_3, but has little or no effect on oxygen reduction (Konstantinov *et al.*, 1997 and this issue p. xxx; Brzezinski and Ädelroth, this issue, p. xxx). Reduction of the binuclear site metals is coupled to the uptake of two protons from the *i*-phase. Hence, it is plausible that at least one is taken up via the K-channel, possibly to protonate metal-bound OH^- ligand(s) (Fann *et al.*, 1995; Fig. 1). However, reduction of Cu_B is much faster than reduction of heme a_3, in a K-channel mutant (Jünemann *et al.*, 1997; Rich *et al.*, this issue, p. xxx), so that in this case accompanying proton uptake could also occur via the D-channel.

From this would follow that perhaps six or even seven of the eight protons taken up during a complete turnover may enter through the D-channel, while only two (or one) may enter via the K-channel. However, caution must be exerted because a block in a proton-conducting pathway might not reveal inhibition in single-turnover experiments. In multi-turnover experiments, on the other hand, which utilize H_2O_2 as substrate ("peroxidase cycle," cf. Konstantinov, this issue), two protons will be delivered to the binuclear center with the H_2O_2, which may artificially bypass the function of a proton channel.

Proton uptake through the K-channel would have to be controlled, or much slower than through the D-channel, to prevent premature protonation of the binuclear site. Enzyme turnover velocity is largely determined by the rate of reduction of the binuclear center, and this is determined by the rate of proton uptake (Verkhovsky *et al.*, 1995; but see Brunori *et al.*, this issue, p. xxx). In contrast, proton uptake via the D-channel must be able to occur much faster than turnover to be compatible with the fast partial reactions

of the oxygen reduction chemistry. This kinetic difference between the channels might suffice to prevent short-circuiting. Alternatively, the K-channel may not ordinarily be a proton conductor at all, but may instead transfer OH^- ions out of the binuclear site, as recently suggested by Rottenberg (1996).

5. STRUCTURE OF THE D-CHANNEL AND FUNCTION OF GLU-242

The D-channel has been defined by site-directed mutagenesis experiments combined with the crystal structures (Iwata *et al.*, 1995; Tsukihara *et al.*, 1996). Key components are at least asp-91 (Thomas *et al.*, 1993; Fetter *et al.*, 1995; Verkhovskaya *et al.*, 1997), asn-80, asn-98 (Garcia-Horsman *et al.*, 1995), ser-157, and several bound water molecules (Riistama *et al.*, 1997; Hofacker and Schulten, 1998). Recent theoretical work has shown that making and breaking of H-bonds between bound water molecules and polar amino acid side chains limit H^+ transfer through such a channel (Pomès and Roux, 1996), which is consistent with the mutagenesis data. Three additional water molecules appear to link the channel to the carboxylic group of glu-242 by hydrogen-bonding (Riistama *et al.*, 1997; Hofacker and Schulten, 1998; Fig. 2). Replacement of glu-242 by a cysteine abolishes proton translocation in the bo_3 enzyme, while replacement with an aspartic acid does not, demonstrating the importance of this site in proton translocation (Verkhovskaya *et al.*, 1997). Tethering the side chain of glu-242 by H-bonding or an ionic interaction to a nearby arg or lys by mutagenesis also abolishes proton translocation. This suggests that *the side chain of glu-242 may have to move between input and output states during proton translocation,* and that it is in protonic contact with the D-channel only in the former state (Riistama *et al.*, 1997). Glu-242 might therefore function not only as a proton conductor, but also as a control valve.

6. IDENTITY OF THE PUMP ELEMENT

We can thus account for the movement of the pumped protons to the residue glu-242, some properties of which (apparent mobility; short distance from the binuclear site) fulfill the requirements for a pump element. Iwata *et al.* (1995) suggested that protons may be carried from glu-242 to the *o*-phase via one of the propionates of heme a_3 (cf. Hofacker and Schulten,

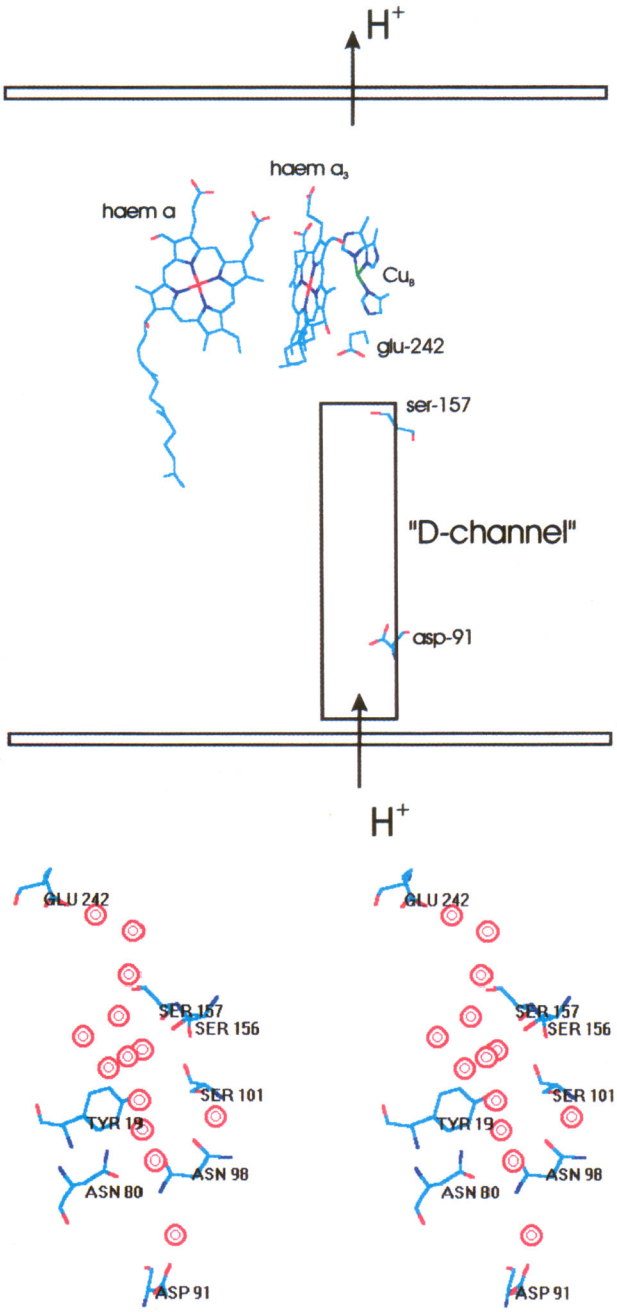
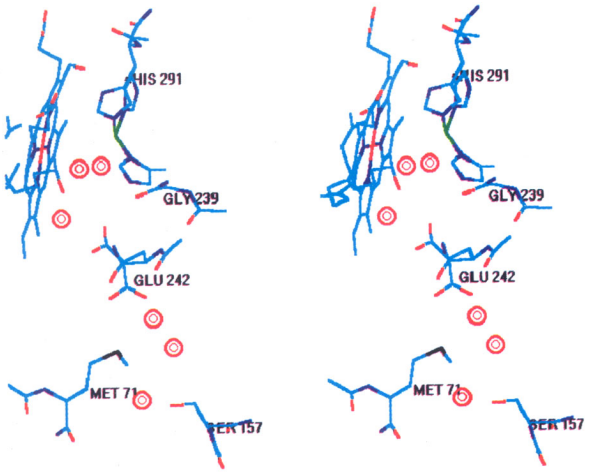


Fig. 2A, B. The proton-conducting D-pathway (from Riistama *et al.*, 1997). A, overall view; B, stereo view of key residues in the D-channel in a projection similar to A; C, stereo view of proposed connectivity between the upper part of the D-channel (at ser-157), glu-242, and his-291. Putative input and output conformers of glu-242 are indicated simultaneously. Heme a_3 and Cu_B (green) are also shown. Projection similar to A. Red circles in B and C indicate oxygens of predicted water molecules.



1998; Kannt *et al.*, this volume). Saari *et al.* (1980) had already suggested that the heme *a* propionates are directed toward the *o*- phase of the membrane, as verified by the X-ray structures (Iwata *et al.*, 1995; Tsukihara *et al.*, 1995). A heme *a* propionate (or a residue in its vicinity) becomes protonated at high protonmotive force and could thus be close to the output H^+ channel, or be involved more directly in the pump mechanism (Saari *et al.*, 1980; Wikström, 1982). However, it is not easy to envisage how the glu-242 side chain could make *two* shuttle rounds to a propionate group to conform to the $2H^+/e^-$ stoichiometry. Neither is it clear how such a mechanism would be coupled to the oxygen reduction chemistry. Hence, while a heme propionate may well be involved in proton pumping, this is not in itself sufficient to explain the mechanism.

FTIR data have suggested connectivity between the strongly H-bonded carboxylic acid group of glu-242 and a histidine ligand of Cu_B, possibly his-291 (Puustinen *et al.*, 1997). Calculations based on the X-ray structure suggest that 3–4 bound water molecules may connect the vicinity of the Nε of his-291 to the putative output position of the glu-242 side chain (Riistama *et al.*, 1997; Fig. 2). This water array could therefore be an essential part of the pump mechanism, and could conduct protons from glu-242 to his-291. His-291 is therefore a candidate for the pump element, in which case glu-242 transfers protons to it as well as to the binuclear site, from the D-channel. The movement of glu-242 could help to prevent proton leakage back from the histidine.

The same water array could also connect the glu-242 to the Δ propionate of heme a_3, although this would require some movement of the propionate carboxylic group from its position in the crystal structure.

7. THE HISTIDINE CYCLE

The fundamental postulate proposed as a basis for this model was the *direct linkage between uptake of water protons and proton translocation* (Wikström *et al.*, 1994; Morgan *et al.*, 1994; Rich, 1995). This led us to propose, in addition, a detailed mechanistic scheme of how the protons might be carried across the dielectric by dissociation and movement of a Cu_B histidine ligand. The basic idea is thus displacement of pumped protons by proton uptake by the oxygen reduction chemistry, while involvement of a histidine ligand is a far more speculative concept, the details of which can vary significantly. For example, Iwata *et al.* (1995) proposed a version where the histidine ligand dissociates on reduction of the binuclear site. This disagrees with recent electrostatic calculations, and with the X-ray structure of the reduced enzyme (Kannt *et al.*, this issue, p. xxx). In the original model (cf. Fig. 3) histidine dissociates only when the third electron is

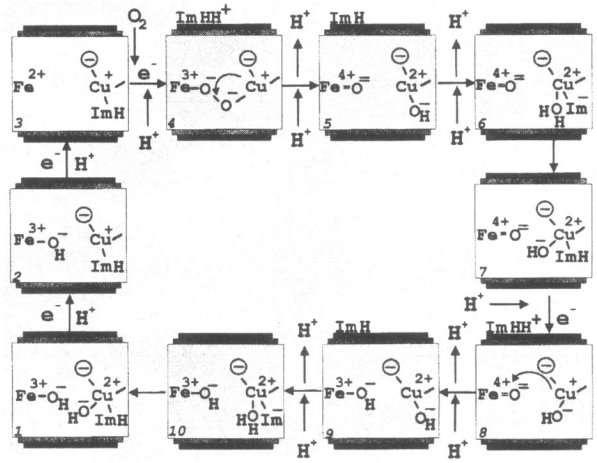


Fig. 3. Histidine cycle. Cu_B is depicted with two permanent ligands, a neutral imidazole (his−240; only the bond to copper is shown), and an imidazolate (his−290; circled minus sign). The third imidazole ligand (his−291) is labile, and indicated as ImH, Im^-, or $ImHH^+$ depending on its state of protonation. It is depicted above the box when in a protonic output position; then, proton transfer is unhindered to the binuclear site chemistry. Inside the box the Im is bound to Cu_B and proton uptake into the binuclear site is prevented. For an alternative mechanism to steps 3→5, see the text.

transferred to the binuclear site, which is consistent with these recent findings.

Figure 3 summarizes a recent version of this model. In this scheme the "moving histidine" is neutral when bound to a stable tetragonal Cu_B conformation (we now consider the moving histidine to be his-291; cf. above). The oxidized binuclear site (state 1) would usually be electroneutral, possibly due to two metal-bound OH^- ligands and an imidazolate ligand (his-290) of Cu_B, which could be stabilized by donation of a H-bond from thr-309, as proposed by Iwata *et al.* (1995). Reduction by two electrons leads to proton uptake (or OH^- extrusion) to form two water molecules. Transfer of the third electron to the binuclear site is accompanied, and electrically stabilized, by H^+ uptake from the water array (cf. above) to the Nε of his-291, which dissociates from Cu_B. The imidazolium cation ($ImHH^+$) formed could be stabilized by a π-cation interaction with the nearby trp-236, and swings to a protonic output position in a domain near the heme a_3 propionates, acidic residues, and several bound water molecules (Tsukihara *et al.*, 1996; Hofacker and Schulten, 1998; Wikström *et al.*, 1998). The water array should now rearrange to provide a proton-conducting path into the binuclear site, promoted by the movement away of the his-291 side chain.

In step 4→5 (Fig. 3) fast electron transfer from the trigonal Cu_B^{1+} forms an unstable trigonal Cu_B^{2+} state with minimal reorganization energy (Marcus and Sutin, 1985). Scission of the O–O bond is coupled to uptake of H^+, and the leaving OH^- group binds to Cu_B^{2+}, which must reorganize toward the stable tetragonal configuration. Simultaneously, the pK_a of $ImHH^+$ is lowered for electrostatic reasons with proton release into the output channel.

Alternatively, if the O–O bond is already broken in P, as suggested by Raman data (Kitagawa and Ogura, 1997 and this issue, p. xxx), this could occur by H atom transfer from tyr-244 forming initially a high-potential tyrosine radical (Ostermeier *et al.*, 1997). This idea has evolved from the finding that tyr-244 may be covalently bonded to the Cu_B ligand his-240 (S. Yoshikawa, personal communication; Ostermeier *et al.*, 1997; Kannt *et al.*, this issue, p. xxx). In this case reduction of the radical by the third electron, rather than O–O bond scission, is the driving force for proton translocation. The 4→5 step of Fig. 3 would then be replaced by protonation from the *i*-side of the formed tyrosinate (with proton release to the *o*-side from $ImHH^+$), by which one arrives at state 5 also by this alternative mechanism.

Reorganization of the trigonal Cu_B geometry in state 5 could move the OH^- ligand close to the position occupied originally by the Nε of his-291. Here, proximity of the OH^- ligand to the array of water molecules is expected to favor its protonation due to the increase in polarity (cf. Canary *et al.*, 1995). Deal and Burstyn (1996) report a pK_a of about 7 for water ligands of aqueous cupric complexes with two or three nitrogenous ligands, in which protonation of OH^- is associated with an increase in overall charge from +1 to +2. State 5 (Fig. 3) has zero charge, which changes to +1 on protonation of the OH^-, which would be expected to be associated with a much higher pK_a value (Tommos and Babcock, 1997). This protonation of OH^- (step 5→6), in turn, induces charge imbalance, with expulsion of the proton from ImH, and return of the his-291 ligand as Im^- to resume electroneutrality. In this process the Im^- could be protonated by the water ligand to finally form the stable tetragonal Cu_B state (state 7). The next pumping cycle (F→O) is analogous, and triggered by input of the 4th electron.

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