Protonmotive Mechanism of Heme-Copper Oxidases

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The mechanism of coupling of proton and electron transfer in oxidases is reviewed and related to the structural information that is now available. A "glutamate trap" mechanism for proton/electron coupling is described.

INTRODUCTION

There have been a wide range of ideas on possible coupling mechanisms in protonmotive oxidases, and most were formulated before specific structural information had become available. These have been well reviewed elsewhere and will not be covered again here. Instead, we will focus on a model that has originated (Rich, 1995) from considerations of charge balancing requirements and which has been extended to include proposals of specific atomic details in the light of the available crystallographic information.

INTERNAL CHARGE BALANCING AND PROTONMOTIVE MECHANISM

An important aspect of coupled proton and electron transfer in the oxidases concerns the observation that conversion between the stable catalytic intermediates of the binuclear center reaction cycle generally involves binding of protons equal to the number of negative charges added so that the net internal charge change is zero. A need for such charge counterbalance of stable intermediates within regions of proteins of low dielectric strength has relevance not only to cytochrome oxidase and cytochrome *bo* and other ionmotive systems, but also to soluble enzymes with occluded reaction cores (Rich, 1996).

The tendency for charge compensation is due to minimization of free energy and can occur by a variety of processes. The dominance of protonation changes in such minimization in the binuclear center has been established empirically for a range of conditions (Rich *et al.*, 1996) and is imposed by the details of the surrounding protein structure. The pH-dependences of midpoint potentials of the metals during classical redox potentiometry are also roughly consistent with proton-linked charge counterbalance, although these are complicated by an intricate electrostatic linkage between several of the metal centers and multiple protonation sites (reviewed in Rich and Moody, 1997). However, counterbalance by protonation changes cannot be assumed in all conditions. It is of course inevitable that pathways to stable intermediates must pass through unstable transition states which are not charge balanced. Furthermore, even for the most stable intermediates, conditions are to be expected where other factors outweigh protonation changes in minimizing energy. Conditions where strict charge counterbalance may not hold in stable states of the oxidases have been described recently (Capitanio *et al.*, 1997). If, as is suggested below, these protonations are central to the coupling mechanism, then such conditions might adversely affect coupling efficiency.

The kinetics of charge-associated protonations can be important factors that are additional to the

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[2] Abbreviations: P and N phases, the positive and negative aqueous phases; O, P, F, and R, the oxidized, peroxy, ferryl, and reduced forms of the binuclear center (regardless of redox states of Cu_A and heme *a*); CO, carbon monoxide.

microscopic electron transfer rate constants in controlling equilibrium constants and observed rates of reaction. For example, the cobinding proton is critical in controlling the binding constants and rate constants of anionic ligands in the distal heme pocket of horseradish peroxidase under some conditions (Meunier and Rich, 1997b). Control of reaction rates through limitations of proton movement rates might be particularly important if the proton has to move through the protein structure in order to arrive at its binding site, as is likely to be the case for the protonation sites associated with the deeply-buried binuclear center.

Charge-linked protonations are likely to be central to the coupling mechanism of several of the more complex ionmotive enzymes. In such a general mechanism (Fig. 1), electron transfer into a region of locally low dielectric strength is electrically counterbalanced by the uptake of a proton (or of a different cation in enzymes coupled to transfer of other ions). The electron then passes to an acceptor, A (in the case of oxidases, this acceptor is oxygen), at a position which is separated from the protons. The reduced form of the acceptor (an oxide in the case of oxidases) requires protonation and this provides a strong driving force. However, the charge-counterbalancing protons are spatially separated from the acceptor—the system may be said to be "electrostatically locked" in a charge-balanced state incapable of geminate production of protonated, reduced acceptor—so that further protons

must be taken up from solution. As they are taken up from the N phase,[2] these "substrate protons" (H_S^+) promote exit of the counterbalancing translocated protons (H_T^+) into the aqueous P phase. In Fig. 1, the entire pathway for H_T^+ is shown to be physically separated from (but electrostatically influenced by) H_S^+ and the acceptor chemistry. However, a common entry channel for H_S^+ and H_T^+ is also possible, provided that there is an adequate means of separation of H_T^+ from the oxide products.

GATING OF THE TRANSLOCATED PROTONS

Critical to the mechanism outlined in Fig. 1 is the nature of the "gating" process by which the charge-counterbalancing H_T^+ are taken up from the N phase in the initial reduction step, but are repulsed into the P phase during the final chemical transformation. Uptake from the N phase may be ensured by a large permanent activation barrier for passage of protons through the channel to the P phase, the energy required to overcome the barrier being supplied by chemical transformation and protonation of substrate. For complex enzymes whose electron transfer pathway involves multiple steps, sidedness of proton uptake may be governed by the spatial position of the electron entry step(s); in the cases of cytochrome oxidase, for example, the electron
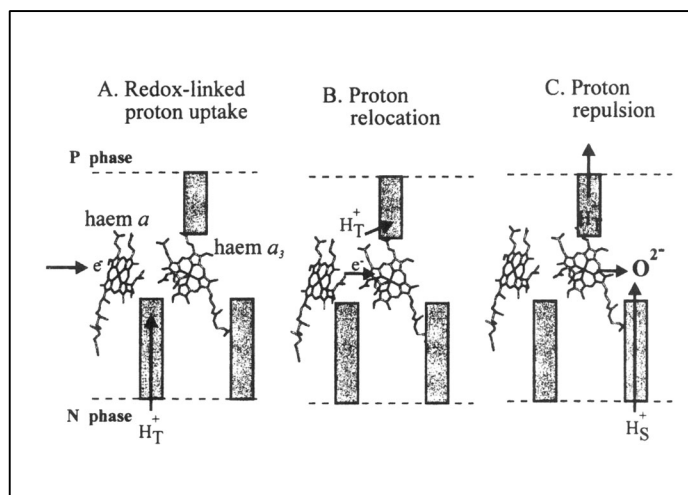

Fig. 1. Coupling of ion translocation to electron transfer in a protonmotive enzyme of low internal dielectric strength. The model is a general one for an enzyme with two sequential redox centers and separate channels for entry of translocated and substrate protons (H_T^+ and H_S^+). The oxidase redox centers are hemes a and a_3 and the protonatable product of reduction of the acceptor is an oxide, O^{2-}.

is transferred to the binuclear center via heme a, and this transient heme a reduction may ensure protonation only from the N phase, a feature incorporated into the "glutamate trap" model below.

A number of possible mechanisms may be envisaged to ensure repulsion of H_T^+ into the P phase. If a common entry route is shared by H_S^+ and H_T^+, electrostatic interaction with the incoming substrate protons may itself be enough, if the N phase pathway for H_T^+ is blocked by entry of H_S^+ although additional mechanisms are required to prevent access of the H_T^+ to the chemical transformation site. Electrostatic interaction with incoming H_S^+ and/or transformation of the oxygen intermediates could promote movement of H_T^+ away from the oxygen intermediates and towards the P phase at the same time.

If the route for H_T^+ is always physically separated from the oxygen chemistry and the channel for H_S^+, then only the problem of gating need be considered. This could be driven in response to internal changes in electrostatic potentials as the electrons and H_S^+ move within the structure and onto oxygen metabolites, and might involve relocation of H_T^+ and/or bond rearrangements within the channels which change their proton conductivities.

IDENTITY OF PROTONATION SITES AND TRANSFER ROUTES

Possible protonation sites and routes were identified both in the predicted (Fetter *et al.*, 1995; Hosler *et al.*, 1993) and determined structures of subunit I (Tsukihara *et al.*, 1996; Iwata *et al.*, 1995). The clearest candidates for stable protonation sites include the histidine ligands to the metal centers, the heme propionates and residues close to them (such as H368, D369, and ligands to the bound magnesium; see Fig. 2), and conserved protonatable residues K319, E242, and Y244 (for clarity, all numbering refers to the homologous position in the bovine sequence, even when an enzyme from another organism is discussed).

Two possible pores of relatively hydrophilic residues that may act as proton channels have also been noted in the bacterial structure (Iwata *et al.* 1995). One of these ("pore B" or the "K channel") appears to lead from the N phase via K319 to the binuclear center whereas a second ("pore A" or the "D channel") appears to lead from D91 close to the N phase to the conserved E242. A putative route
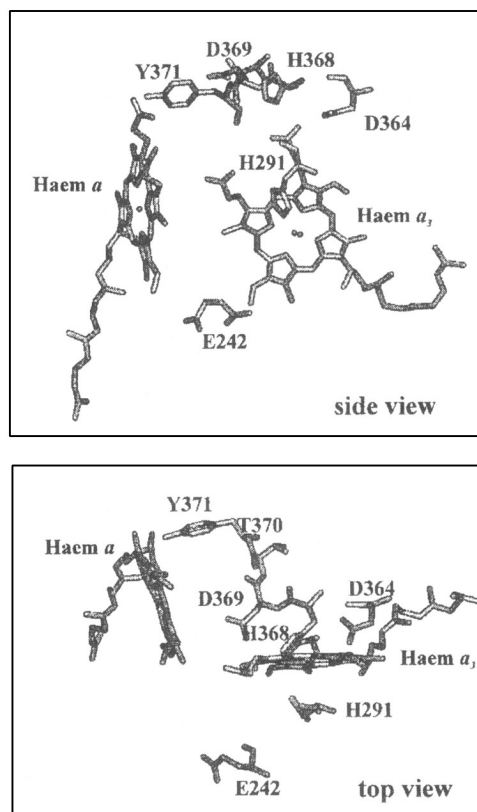

Fig. 2. Possible stable protonation sites in cytochrome c oxidase. The structures have been drawn from the coordinates of the bovine enzyme published in Tsukihara *et al.* (1996) and numbering of residues is also according to the bovine subunit I sequence. The top view is a perspective from the membrane and the bottom view from the cytochrome c side of the membrane.

was also suggested for proton exit into the P phase involving residues around the bound magnesium and the interface between subunits I and II (Iwata *et al.*, 1995), although its connectivity with the other pores was unclear. In contrast, this same structure to the P phase was proposed in (Tsukihara *et al.* 1996) to be a channel to allow water escape. Possible routes for proton transfer to the P phase were proposed through an extended hydrogen-bonded structure between helices III and IV, part of which overlaps with the postulated "pore B" in (Iwata *et al.*, 1995), or via a hydrogen-bonded network extending across the entire protein and located primarily between helices XI and XII and passing close to heme a (although this latter structure is absent in bacterial homologues). Several routes for entry of oxygen to the binuclear center from the hydrophobic membrane phase have also been described (Tsukihara *et al.*, 1996). It seems inevitable that some of these path-

ways and residues must play essential roles in the catalytic reaction cycle and the coupled proton transfer mechanism.

A MOLECULAR MECHANISM FOR PROTON TRANSLOCATION: THE GLUTAMATE TRAP

Two protons are bound when the stable peroxy state is formed from the oxidized enzyme (Mitchell and Rich, 1994) and we assume that these protons (and two others at later steps) are translocated, in response to uptake of H_S^+ for water generation, as outlined in Fig. 1.

The "glutamate trap" model addresses the questions of the specific sites involved, the basis for their separation from the oxygen intermediates, and the nature of the gating to the P phase, in the light of the crystal structure information, and has been developed (Rich, 1997) from the more abstract charge-balancing model in (Rich, 1995). Heme a has associated redox-linked protonation site(s) (Moody and Rich, 1990) and it is proposed that a key one of these is the conserved E242, which becomes protonated from the N phase through "pore A". Besides allowing heme a to become reduced more easily, this protonation may be necessary before electron transfer to the binuclear center can proceed. A shift of the electron density from the heme a to the binuclear center may well cause a relocation of this proton to a site more strongly redox-linked to the binuclear center. This electron transfer-linked sequential relocation of the proton could form a basis for the gating mechanism by which subsequent exit into the P phase is ensured. At one stage of the cycle (during conversion of P into F), maximally three protons must be accumulated transiently in the protein through the glutamate trap and this has a bearing on the atomic identity of the groups. The residues H368, D369, and possible ligands to the bound magnesium are close to the heme a_3 propionates and are reasonable candidates both for H_T^+ accumulation (particularly since there is a precedence for redox linkage of residues similarly placed with respect to the heme propionates in other heme systems (Smulevich *et al.*, 1991; Moore, 1996)) and for the start of the pathway to the P phase. Their physical separation from the oxygen intermediates may be ensured simply by their being separated by a proton-impermeable intervening structure, and the gating is provided by the dynamics of the glutamate residue which conducted them to this region and whose protonation capabilities and, perhaps, orientation are

governed by the heme a redox state. When an oxide product is formed [presumably during the P → F and the F → O steps (Babcock and Wikström, 1992)], uptake of H_S^+ is required for its conversion into water. The most clear candidate for H_S^+ uptake is "pore B," although recent data (see below) raise questions on this route.

A "histidine cycle" mechanism (Wikström *et al.*, 1994), originally suggested before the crystal structure information was available, also addresses the issues of gating and spatial separation. A histidine ligand to the Cu_B was proposed to be a critical binding site for H_T^+. A double protonation of this histidine from imidazolate to imidazolium was envisaged in response to changes in the oxygen intermediates, followed by a conformational change of the residue so that the protons were unavailable to the oxygen intermediates themselves and instead were ultimately ejected into the aqueous P phase at later stages in the catalytic cycle. Some support came from the observation, at least in the bacterial crystal structure (Iwata *et al.*, 1995), that one of the histidine ligands to Cu_B (equivalent to H290 in the beef heart structure) was disordered and, therefore, might be able to adopt several conformations. However, further scrutiny of the structure has highlighted a more likely possible route for protonation of H291 (also a ligand to Cu_B) via "pore A," E242, and some predicted water molecules (Wikström *et al.*, 1997; Wikström *et al.*, this volume).

EXPERIMENTAL PROGRESS

Even before the crystal structures appeared, it was already clear from studies of a range of site-directed mutants (Hosler *et al.*, 1993) that residues in pores A and B are essential for the catalytic function of the oxidases, and the first such studies were generally consistent with independent roles with "pore A" exclusively conducting H_T^+ and "pore B" conducting H_S^+. However, further analyses of effects of some of these mutant enzymes have complicated this simple view, and do not yet provide an unambiguous assignment of their roles (see also Brzezinski and Ädelroth, and Konstantinov, this volume).

DATA RELATING TO THE CONSERVED GLUTAMATE AND "PORE A"

The possible proton pathway in the bacterial structure leading from D91 at the N phase to E242

(Iwata et al., 1995) is not so clear in the bovine structure since E242 does not appear to be connected to the rest of the network of hydrogen bonding groups that may transfer the protons (Tsukihara et al., 1996). However, flexibility of the glutamate residue and the possible role of water molecules in this region might circumvent the apparent block in the bovine structure. Furthermore, it has been shown that D91N and related mutations not only decrease the catalytic activity but also lead to a loss of proton pumping associated with this remaining activity (Fetter et al., 1995; Thomas et al., 1993b), supporting a role in the transfer of H_T^+. Effects of other mutations in pore A have not given a consistent effect. For example, mutations equivalent to N163A and T167A (in R. sphaeroides) have no effect on the enzymatic activity (Mitchell et al., 1996) whereas mutation of residues equivalent to N80 and N98 in E. coli had effects similar to the D91N mutation (García-Horsman et al., 1995). In R. sphaeroides, a mutation equivalent to S157A decreases the enzymatic activity by half but does not prevent proton translocation (Mitchell et al., 1996).

Mutations of E242 itself can have a dramatic inhibitory effect on activity. For example, the equivalents of E242Q in E. coli and R. sphaeroides are completely inactive (Verkhovskaya et al., 1997; Mitchell et al., 1995) and the E242A equivalent in E. coli retains only 5–10% activity (Svensson-Ek et al., 1996; Thomas et al., 1994). Turnover in these mutants was so low that effects on associated pumping could not be determined. However, other mutations in cytochrome bo of E. coli retained sufficient activity so that proton translocation could be assessed (Verkhovskaya et al., 1997): conservative substitution to aspartate retained proton translocation whereas mutation to cysteine resulted in its loss.

A number of studies have probed more specifically the reasons for loss of activity. In the E242A mutant of E. coli, the reaction of the fully-reduced cytochrome oxidase with oxygen, using the flow-flash technique, has been studied (Svensson-Ek et al., 1996). The formation of the P intermediate seemed to be unaffected, whereas the subsequent phase of electron transfer to produce the F form was strongly impaired. In a separate study (Konstantinov et al., 1997), the time-resolved generation of membrane potential associated with the F → O step was monitored in the R. sphaeroides enzyme. In the wild type, kinetic phases and relative amplitudes could be equated with electron transfer to the binuclear center and with the linked

movements of H_S^+ and H_T^+. None of these could be detected in the E242Q and D91N mutant enzymes.

The flow-flash data point to a requirement for the glutamate residue in the P → F transition and the electrogenic measurement (provided that the F form had been generated, a factor which was not possible to establish) to a requirement in the F → O step. Since proton translocation is likely to be associated specifically with these steps (Wikström, 1989), these data are consistent with a role for E242 in the H_T^+ pathway.

In a study of the E242Q mutant in R. sphaeroides (Meunier and Rich, 1997a), we observed that a major fraction of the binuclear center was trapped in an unreactive state with spectral features which were not characteristic of the E, P, nor F reaction intermediates but were similar to compound A, a form which is produced transiently on binding oxygen to ferrous heme a_3 (Orii, 1988; Chance et al., 1975). However, normally this species is rapidly converted to other oxygen intermediates at room temperature and is photolabile (Babcock et al., 1984) whereas the trapped species was stable and non-photolabile. Hence, its nature remains to be established. This form would presumably not be observed in the kinetic experiments described above, but clearly requires scrutiny if the nature of the mutations is to be properly understood. Redox titration of the cyanide-ligated form of this enzyme showed that the redox-linked protonations associated with heme a were more weakly linked, a finding that was also observed in a mutant of the yeast enzyme with an altered residue (I66N equivalent) that is in close proximity to E242. These data support a role for E242 as one of the sites that is redox-linked to heme a, a feature incorporated into the "glutamate trap" model. However, this point remains equivocal, particularly as recent FTIR measurements suggest that this glutamate may always remain in a protonated state (Puustinen et al., 1997).

DATA RELATING TO THE CONSERVED LYSINE AND "PORE B"

"Pore B" is a good candidate for the transfer of H_S^+ into the binuclear center. Residues S255, K319, T316, the hydroxyl group of heme a_3, and Y244 can clearly form, together with water molecules, a hydrogen bonded structure connecting the N phase and the binuclear center. Although K319 does not appear to be hydrogen bonded in the Paracoccus enzyme, conclusions on such functions from structure alone are

inconclusive since flexibility of residues and roles for bound water could alleviate such apparent breaks in proton conducting ability.

Several mutants of conserved, protonatable residues in "pore B" have been obtained for cytochrome *bo* from *E. coli* and the aa_3 type oxidase from *R. sphaeroides* where the binuclear center appears to be spectrally intact but the catalytic activity is drastically decreased (Hosler *et al.*, 1996; Thomas *et al.*, 1993a; Fetter *et al.*, 1995). Yet, associated proton translocation in T359A (Fetter *et al.*, 1995) appears to be normal. Also unaffected in the K319M equivalent in *R. sphaeroides* (Jünemann *et al.*, 1997) are the redox-linked protonation properties of heme *a* and Cu_B, suggesting that this residue is not a stable charge-linked protonation site. Furthermore, reactions of the oxidized and reduced forms of the binuclear center with cyanide, formate, peroxide, and CO are normal, arguing against a function for "pore B" in ligand conduction.

Perhaps most surprising is the demonstration in flow-flash experiments that the reaction of oxygen with fully reduced forms of such mutants can proceed through to the F form in a wild-type manner (Svensson *et al.*, 1995). Furthermore, all electrogenic reactions involved in the F → O transition are also normal in mutants equivalent to K319M and T316A in *R. sphaeroides* (Konstantinov *et al.*, 1997). In both types of experiments, uptakes of both H_S^+ and H_T^+ are expected to be required, arguing against a critical role for "pore B" in proton transfers in the oxidative reaction steps.

The most striking effect of these K319 mutations is on the rate and extent of reduction of the fully oxidized state (Hosler *et al.*, 1996), and it is clearly this which impairs catalytic turnover. We have studied this block further (Jünemann *et al.*, 1997) and find that it is reduction primarily of heme a_3 that is inhibited. Up to one, but not two, electrons can enter the binuclear center (at least on a seconds time scale). Hence, there is a problem with formation of the doubly-reduced (and doubly protonated) binuclear center (the R state) which is the intermediate required for productive reaction with oxygen to produce the P intermediate (Babcock and Wikström, 1992). Interestingly, the P state could be formed at relatively normal rates in the mutant by direct reaction with hydrogen peroxide or with CO/ oxygen, showing that thermodynamic accessibility of the P state (or of the R state, which is part of the reaction pathway with CO/oxygen) is not in itself the problem.

Overall the above results, although emphasizing the importance of the "pore B" structure, cast doubts on a simple static role as a pathway for transfer of H_S^+ (or ligands generally) into the binuclear center throughout the reaction cycle. This led Konstantinov and co-workers to suggest that the proton channels in the oxidase structure may not conduct exclusively H_S^+ and H_T^+ but may change their roles at different stages of the catalytic cycle (Konstantinov *et al.*, 1997). We have instead proposed (Jünemann *et al.*, 1997) that "pore B" may act as a "dielectric well" rather than as a conduction pathway for proton uptake. Such a structure may be contrasted with a "proton well" (Mitchell, 1968) by being proton impermeable but with a high effective dielectric constant (e.g. by having an extended hydrogen bonding network between the binuclear center and the N phase), and would provide a means of dielectric relaxation that reduces the otherwise prohibitive energy cost of the transient charge-uncompensated states that must occur prior to formation of the stable, charge-compensated ones. Hence, disruption of "pore B" hinders formation of an unstable transient before the R state (for example, an uncompensated state in which both electrons have entered the binuclear center but full compensation by protonation has not occurred) and so blocks the route to R formation. If such intermediates can be circumvented, for example when the enzyme is reacted with hydrogen peroxide (which can donate electrons and protons at the same time), or if the enzyme is prereduced (and so is preprotonated), then relatively normal behavior can be observed.

DATA RELATING TO THE P PHASE CHANNEL FOR H_T^+

There has been little experimental progress on identification of the route for passage of H_T^+ from their binding sites into the P phase. If such protons do indeed pass through the E242 residue, then this does focus possibilities. Evidence for internal proton rearrangement associated with reversed electron transfer from the binuclear center to heme *a* has been reported (Ådelroth *et al.*, 1995; Hallén *et al.*, 1994) and we presently interpret this to be a manifestation of the H_T^+ movement between E242 and the stable sites more strongly linked to the binuclear center metals. We have favored stable protonation sites at the heme a_3 propionates, the bound magnesium, and nearby protonatable residues H368 and D369. In the histidine cycle model (Wikström *et al.*, 1997) these residues are proposed to provide the exit route for H_T^+ at stable

binding sites on H291, the ligand to Cu_B. Data on effects of mutations of these residues are presently sparse: in general mutations at positions D364, D369, and H368 (Fetter *et al.*, 1995; Hosler *et al.*, 1994; Verkhovskaya *et al.*, 1997; Qian *et al.*, 1997) have no dramatic effect on activity and do not result in a loss of proton pumping activity. Some of these residues were proposed in (Iwata *et al.*, 1995) to be candidates for the transfer channel to the P phase, although these same residues were considered for unspecified reasons to be a water channel in Tsukihara *et al.* (1996) and structures more distant from the binuclear center for proton transfer to the P phase were suggested.

CONCLUSIONS AND PROSPECTS

The interpretation of the data from mutant enzymes is still ambiguous on a number of key points and there are, anyway, several caveats to such interpretation. First of all, mutant enzymes are often heterogeneous, with the mixture of forms dependent on preparation methods. Such heterogeneity is often not recognized and yet the behavior of just one subfraction might give misleading information on the natural role of the modified amino acid. Furthermore, different mutations in structures such as extended proton channels might not be expected to give consistent effects: for example, lowering of proton conductivity may not always occur because of generation of alternative routes, even if a residue critical for conduction in the natural pathway is removed. These possibilities may well explain some inconsistencies in the available literature. Nevertheless, the present data are consistent overall with central roles for the conserved glutamate residue and its associated "pore A" and with an essential function of some type for "pore B" at least in the reduction of the oxidized enzyme. To some extent, ideas on coupling mechanism are generally converging around these features. Whether "pore B" operates as a proton channel only during the first part of the catalytic cycle or whether it acts, as we have suggested, as a "dielectric well" throughout the cycle cannot be resolved from currently available data. Likewise, other crucial differences between working models such as the identity of the stable protonation sites for H_T^+, the essence of the gating, and the pathways for entry of H_S^+ and exit of H_T^+ will also have to await further experimental input from a range of kinetic and spectroscopic methods.

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