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Structure and dynamics of a proton shuttle in cytochrome *c* oxidase

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Abstract

Protein-assisted transport of protons across the bioenergetic membrane is mediated by hydrogen-bonded networks. These networks involve titratable amino acid residues of membrane-spanning protein assemblies as well as internal water molecules. In cytochrome *c* oxidase, the so-called D-channel defines such a network for the uptake of protons from the cytoplasmic side of the membrane. It has been proposed that conformational changes of a Glu residue are required for the establishment of a proton linkage from the channel into the active site. The thermodynamic basis for the conformational isomerization of this residue is investigated using simulated annealing and free energy molecular dynamics simulations. The results support the existence of metastable conformations of the side chain, and their interchange through local structural fluctuations of neighboring residues and nearby internal chains of water molecules. The conformational isomerization of both protonated and unprotonated states of Glu, coupled with the reorganization of hydrogen bonds, suggests a kinetically competent mechanism for proton shuttling. © 1998 Elsevier Science B.V.

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Proton translocation across biological membranes is required for the establishment of pH gradients. The resulting chemiosmotic force constitutes the central principle of bioenergetics. Unlike that of other ions, the transport of protons does not require the net diffusion of atomic or molecular species but can instead take place according to a 'Grotthus mechanism' involving chemical exchange of hydrogen nuclei along hydrogen bonds forming extensive networks [1]. Because of the extended and complex nature of such networks, the molecular properties and detailed mechanisms governing H^+ translocation in proteins have remained elusive. A high level of detail is required to understand how proton pumping arises, and how it is coupled to redox reactions. In recent years, the elucidation of a number of three-dimensional structures of transmembrane protein assemblies involved in proton translocation has opened the way to detailed mechanistic studies through the analysis of such hydrogen-bonded networks.

Conceptually, the molecular theory underlying proton transfer along hydrogen-bonded chains (HBC), or 'proton wires,' involves two complemen-

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tary and distinct steps: proton transfer along pre-existing hydrogen bond(s), and conformational changes involved in the reorganization of the hydrogen-bonded network. This is because the passage of a proton along an oriented HBC leaves the chain in the opposite orientation. Before the H^+ translocation cycle is completed, the reorientation of the chain must take place [2].

Three-dimensional structures of proton-translocating proteins provide invaluable but sometimes incomplete information. Wherever hydrogen bonds are missing in the connectivity of a putative proton wire, two questions may be asked: could intervening water molecules left undetected at the resolution of the structure constitute bridging links in an otherwise fragmented HBC? Could thermal fluctuations and conformational changes of the protein account for the formation of continuous or alternative HBCs implicated in the translocation?

Understanding the basis for the possible role of water in proton wires is an active field of research. Water chains constitute tractable models of proton wires, and the best-characterized proton wire to date is the single-file chain of water molecules filling the gramicidin pore. Experimental measurements have shown that H^+ conduction in gramicidin is much faster than even the diffusion of water molecules through the pore [3], and theoretical studies have begun to provide useful insight on the balance of forces governing the fast (and passive) conduction of protons by gramicidin [4,5]. Furthermore, relay models of protons by buried water molecules have been proposed for several systems of bioenergetic relevance. For example, there is evidence for the involvement of buried water molecules in the bacteriorhodopsin proton pump [6]. Proposals have also been made for a functional role of hydrogen-bonded water chains detected in high-resolution crystals of the photosynthetic reaction center [7] and of the lumen-side domain of cytochrome *f* [8].

Cytochrome *c* oxidase is an example where both the presence of functional internal water molecules and the conformational isomerization of titratable amino acid side chains appear to be required for the net movement of protons. Spectroscopic and mutagenesis studies [9,10] indicate that a Glu side chain in subunit I (residue 242 in the numbering scheme of bovine heart cytochrome *c* oxidase) is implicated in the relay of protons from the cytoplasmic (matrix) side to the binuclear center (via the so-called 'D-channel'). On the basis of the two structures published to date [11,12], however, this residue is surrounded by largely nonpolar cavities and does not appear to be part of a protein HBC. Theoretical approaches have shown that water molecules may occupy these cavities and largely complete the HBC [9,13]. However, the crystallographic conformation of Glu^{242} of bovine heart cytochrome *c* oxidase suggests that in the fully oxidized form of the enzyme (X-ray state), the carboxylic acid group of this Glu residue forms a hydrogen bond only with water in the proposed input side for protons, as shown in Fig. 1. After protonation from water molecules in the input channel, the delivery (output) of protons to the side of the binuclear center, therefore, would necessitate the conformational isomerization of Glu^{242} to form hydrogen bond(s) with water molecules in the output channel, as proposed earlier [9,13]. Once deprotonated, and after reverting to its input conformation, the residue would be ready to pick up a new H^+.

We have investigated the thermodynamic basis for proton shuttling by Glu^{242} in bovine heart cytochrome *c* oxidase, using molecular dynamics and free energy simulations of subunit I. In addition to the crystallographic structure [12], the positions of 16 bound water molecules were determined in a previous study [9] using a statistical mechanical method [14]. The potential energy parameters of the protein and heme groups were provided by the CHARMM force field [15], version 22 [16], while the TIP3P model was used for water [17]. In the binuclear center, Fe_{a_3} was in its ferrous state, and the propionates of heme a_3 were deprotonated. Asp^{364}, which is in contact with propionate A of heme a_3, was protonated. Cu_B was modeled in the cupric state with a hydroxide ion as its oxygenic ligand. The calculations were repeated with and without proton on the carboxylic acid group of Glu^{242}. The local dynamics of all amino acid residues with at least one atom within 5 Å, and all four water molecules within 7 Å from C_γ of Glu^{242} was investigated, with all other atoms of subunit I fixed in their crystallographic conformation. The dielectric constant was taken as 1, and the cutoff radius for nonbonded interactions was 20 Å.

In order to identify stable conformations of Glu^{242}, simulated annealing was used to generate uncorre-

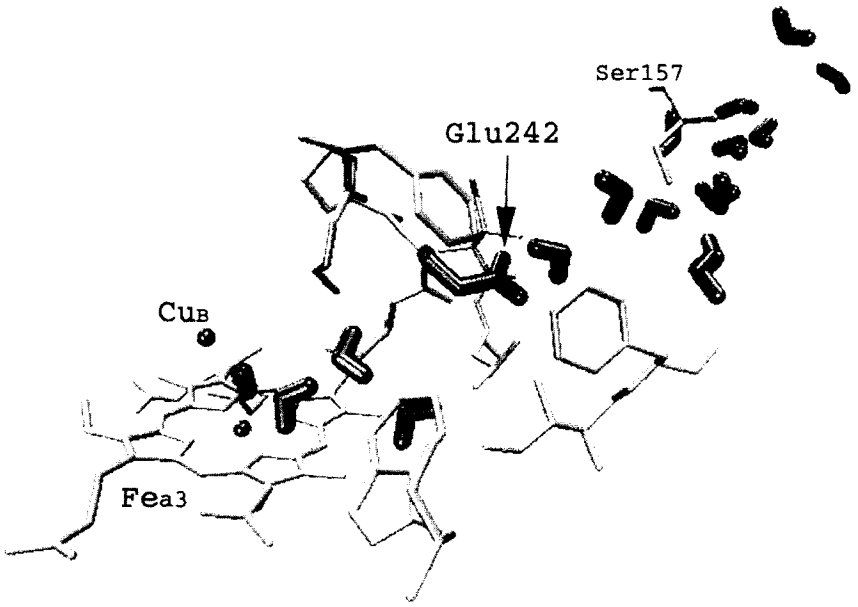

Fig. 1. Structure of the proposed proton wire. Glu^{242} sits at a bottleneck between two hydrogen-bonded water chains. These bound waters are highlighted, as well as the side chain of Glu^{242}. The D-channel is at the top right hand corner and includes Ser^{157}. The binuclear center, which comprises heme a_3 and Cu_B, is at the bottom left hand corner. The residues near Glu^{242} are also shown. By swinging from right to left, the carboxylic group of Glu could relay protons from the D-channel (input) to the binuclear center (output).

lated conformations. Twenty structures were recorded at 0.5-ps intervals from a 10-ps simulation at a temperature of 5000 K, and the conformations were then subjected to gradual cooling over 7 ps, and to subsequent energy minimization. During simulated annealing dynamics, harmonic potential energy functions were imposed to restrain the position of heavy atoms other than those of Glu^{242} and water molecules to the vicinity of their crystallographic position. This procedure led to the characterization of four and three distinct conformers, respectively, for the unprotonated (Glu^-) and protonated (GluH) states of Glu^{242}. In either case, the conformers are distinguished by a combination of different torsional angles as well as by different hydrogen-bonding properties (see Fig. 2).

With small deviations from the crystallographic conformation, both protonated and unprotonated forms of Glu can exist in conformations where the carboxylic acid group is hydrogen bonded, respectively, with input and output water molecules, consistent with earlier proposals of its possible proton shuttling role [9,13]. Interestingly, the unprotonated Glu^- possesses two 'bridging' conformations, B_{IN} and B_{OUT}, where it makes H bonds with both input and output water molecules in addition to the IN and OUT conformations where it is hydrogen bonded only to input and output water, respectively. In the torsional space, these bridging conformations are intermediate between the IN and OUT conformations. In contrast, in the absence of the carboxylate charge, the protonated form of Glu can only form a hydrogen bond either with the input channel (IN and IN′ conformers) or with the output channel (OUT conformer).

To evaluate the relative stability of the various conformations, and the activation energies that need to be overcome in the isomerizations, free energy simulations were performed. The isomerizations were characterized by the three torsional angles of the Glu side chain. Of these, it is mostly χ_2 and χ_3 which define the distinct conformations (see Fig. 2). Thus, for Glu^-, isomerization from IN to B_{IN} to B_{OUT} to OUT followed $(\chi_2,\chi_3)\simeq(50,166)$ to $(30,255)$ to $(-65,325)$ to $(-70,430)$ in the two-dimensional space defined by χ_2 and χ_3 dihedral angles (in degrees). Similarly, for GluH the isomerizations from IN to IN′ to OUT followed $(\chi_2,\chi_3)\simeq(63,164)$ to $(31,252)$ to $(-84,332)$. The free energy contours, or their projection along χ_3 shown in Fig. 2, were each obtained from 34 equilibrium simulations at 300 K, from which the equilibrium distributions of χ_2 and χ_3 were computed. Different starting configurations

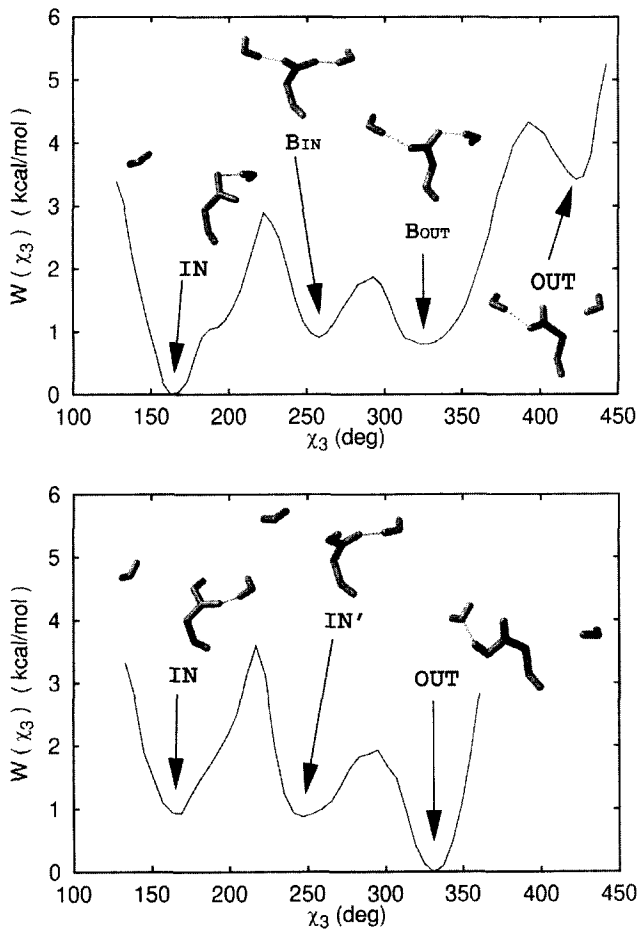

Fig. 2. Free energy profiles for the conformational isomerization of (top) unprotonated and (bottom) protonated Glu^{242}. In the figures depicting each conformer, the input and output water molecules are shown, respectively, at the right and left of Glu^{242}, with hydrogen bonding indicated by dashed lines. In a proton shuttle, Glu^- could become protonated from the input side either in its IN, B_{IN}, or B_{OUT} conformation. GluH could lose its proton to output waters in its OUT conformation.

were used. The total simulation time used to generate these data was 530 and 236 ps, respectively, for Glu^- and GluH. During some of these simulations, harmonic constraints were imposed on these torsions to improve the sampling across free energy barriers, and the unbiasing of these so-called 'umbrella simulations' was performed with the WHAM algorithm [18,19]. The resulting curves (Fig. 2) constitute the potential of mean-force or reversible work for the isomerizations.

It must be stressed that the isomerizations involve only small displacements of the nearby amino acid residues, as well as of the neighboring water molecules. They do not require large scale motions of the protein, but instead can occur with local fluctuations and within small deviations from the X-ray structure, except for the carboxylic group of Glu^{242}, which swings by 3 Å over the IN–OUT flip.

The relative thermodynamic stability of the various conformers is given by the relative free energies of the well bottoms (Fig. 2). Apart from the OUT conformation of Glu^-, all the conformations of Glu^- (and all those of GluH) are within 1–2 kcal/mol from each other. Although the error bars of such calculations are large and accumulate along the reaction coordinate (of the order of 0.5 kcal/mol between adjacent wells), these results indicate that because free energy differences are small, all conformations are likely to be significantly populated—with the exception of the unprotonated OUT conformer, which appears to be much less likely than the other conformers of Glu^-. The analysis of the hydrogen bond lengths with input and output waters indicates that, on average, Glu^- forms stronger H bonds with the input water (2.8 and 3.1 Å, respectively, for input and output waters in the B_{OUT} conformation). This is important because it contributes to the relative destabilization of the OUT conformation of Glu^-.

Perhaps the most significant feature of the free energy profiles is that the activation barriers separating consecutive conformers, which rise to between 1 and 3 or 4 kcal/mol in height, are small to moderate. This suggests that conformational isomerizations exchanging hydrogen bonds between Glu^{242} and the input and output water molecules may occur sufficiently rapidly to be consistent with the kinetics of proton transport in cytochrome *c* oxidase.

Based on these results, one can propose a detailed isomerization mechanism for the shuttling of protons from the input to the output side by Glu^{242}. Glu^- could in principle receive an input H^+ in either of its IN, B_{IN}, or B_{OUT} conformations, all three of which engage in strong hydrogen bonding with the nearest input water molecule. In the first two cases, the protonation would leave GluH in its IN or IN′ conformation, respectively. In both cases the carboxylic OH would be facing the input side. Isomerization to OUT would first be required before H^+ can be released to the output water channel. In contrast, if Glu^- was instead protonated in its B_{OUT} conformation near $(\chi_2,\chi_3)\simeq(-65,325)$, the resulting GluH

would spontaneously lose its hydrogen bond with the input water molecule and adopt the OUT conformation at $(\chi_2,\chi_3)\simeq(-84,332)$. While moderate activation energies are required in the first two processes, the last process involves no conformational isomerization of Glu^{242} and could in principle constitute a fast mechanism for the relay of protons from the D-channel to the binuclear region. Once deprotonated, the conformations of Glu^- could again rapidly equilibrate between IN, B_{IN}, and B_{OUT}, before picking up a new proton.

It should be noted that the calculations reported above focus on the conformational isomerization of Glu^{242} and do not address the entire cycle sketched above. In particular, they do not provide information on the respective proton affinities of the various conformers. To determine the relative proton affinity of groups involved in the pathway(s) of H^+ translocation, alternative protonation and charge or redox states of many other groups of the enzyme, particularly in the binuclear center, must be characterized in various conformational states and at various stages of the enzyme redox cycle. We estimate the electrostatic contribution of a unit-charge change in the redox state of the binuclear center to the relative energies of the IN and OUT conformers of unprotonated Glu^{242} to be within 1 kcal/mol[1]. Because this value lies within the error bars for the complete IN–OUT isomerization obtained in the present study, our conclusions on the nature and the feasibility of the conformational isomerization of Glu^{242} should hold for different charge distributions in the enzyme catalytic site.

While the driving forces for H^+ translocation include electrostatic coupling, the existence of thermodynamically and kinetically competent pathways involving successive hydrogen bonds is also essential. Furthermore, as noted in a recent theoretical study of pK_a shifts in bacterial cytochrome *c* oxidase [20], the protonation and deprotonation of intervening residues such as the conserved Glu may constitute transient events in the shuttle cycle. Thus, the interplay of equilibrium and dynamic properties in the mechanism of proton wires deserves further characterization. For example, it is possible that the relative rates of specific proton transfer and conformational exchange steps involved in the Grotthus mechanism help control the overall directionality of proton translocation against a transmembrane proton-motive force in proton pumps.

The present study suggests that the conformational isomerization of the Glu^{242} side chain is consistent with a proton shuttling role in bovine heart cytochrome *c* oxidase as was suggested in recent experimental [9,10] and theoretical studies [9,13]. More generally, this study also illustrates how local structural fluctuations of a protein and of bound waters may contribute to a detailed molecular mechanism for proton translocation.

[1]Calculated as the upper bound of the difference in pairwise Coulombic interaction potentials obtained from representative conformations of the IN and OUT states of Glu^-, upon varying by one unit charge the point charge at Fe_{a_3}, Cu_B, and the oxygenic ligand of Cu_B, assuming a dielectric constant of 4 in the intervening medium.

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