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Free energy profiles for H^+ conduction in the D-pathway of Cytochrome c Oxidase: A study of the wild type and N98D mutant enzymes

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Abstract

The molecular mechanism for proton conduction in the D-pathway of Cytochrome c Oxidase (CcO) is investigated through the free energy profile, i.e., potential of mean force (PMF) calculations of both the native enzyme and the N98D mutant. The multistate empirical valence bond (MS-EVB) model was applied to simulate the interaction of an excess proton with the channel environment. In the study of the wild type enzyme, the PMF reveals the previously proposed proton trap inside the channel; it also shows a high free energy barrier against the passage of proton at the entry of the channel, where two conserved asparagines (ASN80/98) may be essential for the gating of proton uptake. We also present data from an investigation of the N98D mutant, which has been previously shown to completely eliminate proton pumping but significantly enhance the oxidase activity in *Rhodobacter sphaeroides*. These results suggest that mutating Asn98 to negatively charged aspartate will create an unfavorable energy barrier sufficiently high to prevent the overall proton uptake through the D-pathway, whereas with a protonated aspartic acid the proton conduction was found to be accelerated. Plausible explanations for the origin of the uncoupling of proton pumping from the oxidase activity will be discussed.

Keywords: Cytochrome c Oxidase; Multistate empirical valence bond (MS-EVB); Free energy calculation; Proton pumping

1. Introduction

Cytochrome c Oxidase (CcO), the membrane-bound terminal enzyme of the respiratory chain, catalyzes the reduction of di-oxygen to water in a process coupled to the translocation of four protons across the cell membrane ($O_2 + 8\,H^+ + 4e^- \rightarrow 2\,H_2O + 4\,H^+$) (for recent reviews, see refs [1–3]). Up to seven out of the eight protons taken up from the cytoplasmic side of the membrane are transferred into the enzyme by the so-called D-pathway that leads approximately half way into the membrane and ends at the well conserved Glu242 residue (bovine enzyme numbering is used throughout). However, it is not clear how the protons are distributed either toward the exit pathway as pumped protons or toward the catalytic site as chemical protons. A number of hydrophilic residues from several transmembrane helices are thought to contribute to this pathway and play essential roles for the proton translocation. The route for protons is lined by Asp91, Asn80, Asn98, and Tyr19 before a large cavity is reached in the protein interior situated with Ser156 and Ser157 as the possible hydrogen-bonding partners [4,5] for the water chain that eventually directs the proton to Glu242 (Fig. 1).

Due to its high capacity for proton transport (PT), the D-pathway has been a target of intensive analysis. A range of computational approaches have been used to explore the detailed mechanism governing the PT, including conventional molecular dynamics (MD) simulations [6–8], multistate empirical valence bond model (MS-EVB2) MD simulation [5] and continuum electrostatics calculations [9]. Notable successes of these methods include defining the water-filled, polar residues aiding the proton conducting D-pathway and estimating the free energy landscape for proton permeation through this pathway when the Glu242 residue is deprotonated.

The present study is a continuation of our earlier work [5] where the MS-EVB2 model was employed to investigate the effect of the protonation state of Glu242 on the distance of proton

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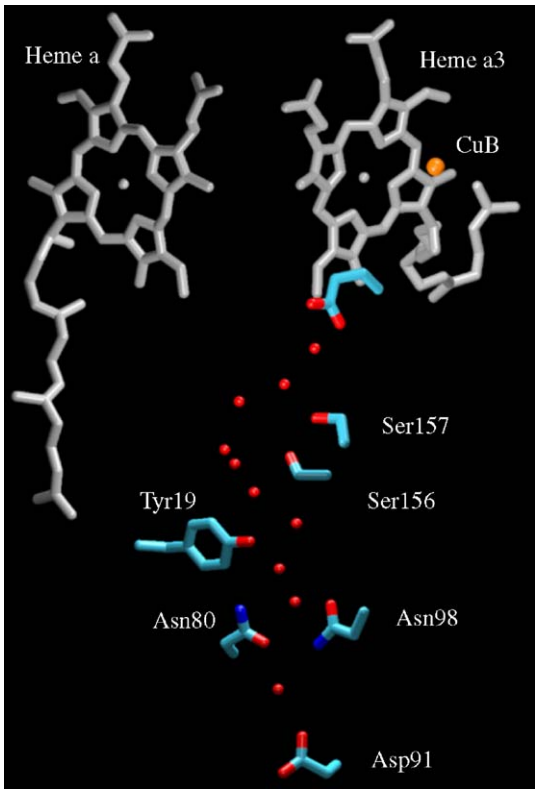

Fig. 1. Heme a and a3, CuB, and the residues of the D-pathway. Water molecules are shown in red. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

propagation in the D-pathway. On the basis of the MS-EVB2 simulations and free energy calculations, a possible two-step PT mechanism was proposed, which involves a wide channel region at a distance of 6–7 Å away from residue Glu242 as a proton trapping site. The essential theme of this mechanism is that the "proton trap", primarily ascribed to the wide pore, may divide the PT along the D-pathway into a biphasic process; whether or not the second step PT takes place depends on the protonation state of Glu242. In the current work, the MS-EVB2 model is further applied to calculate the potential of mean force (PMF), i.e., free energy profile, associated with the PT through the D-pathway between the deprotonated Asp91 and the protonated Glu242. The results strongly support our proposal that with a protonated glutamatic acid situated at locus 242, the second PT step becomes impeded due to the presence of a free energy well located at the proton trap region. The PMF also reveals an unfavorable energy barrier against the passage of proton at locus 80/98 near the entry of the channel, where two conserved asparagines may be essential for the gating of proton uptake.

Mutations of some residues within the D-pathway result in severe inhibition of proton pumping and enzyme turnover rate [10–13]. In order to determine the nature of the structural changes induced by mutations and to understand how these changes may lead to an alteration of PT in the D-pathway, specific amino acid residues lining the D-pathway have been chosen for computational mutations in this study, with the rationale of introducing mutations that will change the electrostatic properties of the pathway interior, for example, from hydrophilic groups to hydrophobic groups or from neutral groups to charged groups. The influence of site-directed mutations on the channel conductance was further exemplified by a specific case study: the N98D mutant (N139 in *Rhodobacter sphaeroides*; N131 in *Paracoccus denitrificans*), in which Asn98 is replaced by an aspartic acid. The site-directed mutagenesis experiments have shown that this mutation yields an enzyme that completely abolishes the proton pumping but significantly enhances the oxygen reduction activity [14]. The molecular basis underlying the cause of the uncoupling of proton pumping from the oxidase activity is not well understood and merits further examination. For this purpose, the PMF experienced by an excess proton as it is transported along the N98D mutated D-pathway was calculated, and on the basis of the results structural and functional features, plausible explanations for the influence of the mutation will be offered.

The paper is organized as follows: first, the MS-EVB2 model and the related concepts will be briefly introduced, followed by the description of the simulation protocol. In the subsequent section, the results of the PMF calculations in the wild type enzyme and N98D mutant will be presented and discussed respectively. Conclusions from this work will be given in the final section.

2. Methods

2.1. MS-EVB2 simulations of PT in wild type and mutant CcOs

The primary goal of this work is to elucidate the molecular nature of PT in the D-pathway of bovine heart CcO. One well-established and computationally practical approach for describing the PT in biological systems is the multistate empirical valence bond (MS-EVB) model [15–18] and its second generation model MS-EVB2 [18]. Its reliability has been demonstrated in various studies involving protein channels [5,19–23] and solution phases [15–18]. Presented here is only a brief description of the essential features of the MS-EVB methodology. In an EVB model, the state of a chemical reaction can be described as a linear combination of a number of independent localized valence bond states $|i>$:

$$|\psi>=\Sigma c_i|i> \tag{1}$$

The MS-EVB potential energy surface is defined as the electronic ground-state wave function that is obtained by solving the eigenvalue problem:

$$c^T Hc=E_0 \tag{2}$$

Here, c is the ground state eigenvector with elements $c_i[i=1,N]$; N is the number of EVB states. In bulk aqueous systems, when the population of the highest occupied state, c^2_{max}, is ~0.5, the excess proton assumes a Zundel cation structure. An Eigen cation, having an amplitude $c^2_{max}\approx 0.65$, is more hydronium-like and slightly more stable than Zundel cation in bulk water [16,17]. In Eq. (2), E_0 represents the lowest eigenvalue; H is the EVB Hamiltonian with matrix elements $h_{ij}=<i|\hat{H}|j>$. The diagonal matrix elements depend on the nuclear degrees of freedom of the system and can be described by molecular mechanics force fields. The off-diagonal elements are then empirically chosen to reproduce as best possible the actual potential energy surface, typically as determined from *ab initio* calculations or experimental results. After one determines the MS-EVB potential surface, the forces on the system nuclei can then be generated from the ground state in Eq. (2) using the Hellmann–Feynman theorem. For a complete description of this model, see the available literature [15–18]. The interactions between hydronium and the surrounding water molecules were modeled here using the MS-EVB2 parameter set [18].

The coordinates of CcO were retrieved from Protein Data Bank (PDB) entry 1V54. The same simulation system as described in ref. [5] was used in this study. Briefly, a reduced model was adopted, represented by subunit I with all the pore water molecules in the X-ray structure, 5 Å water shells on either sides, an excess

proton and a counter ion Cl^- for total charge neutrality. The α carbon atoms whose distances to all of the water molecules in the D-pathway are greater than 5 Å were harmonically tethered during the simulation to keep the structural integrity. The absence of constraints along the proton conduction pathway allows for minor structural rearrangement of protein side chains. MS-EVB2 simulations were carried out in the same way as previously described in ref. [5]. Glutamic acid 242 was protonated if not specially stated otherwise, and the rest of the residues remained in their default protonation state. Water molecules were modeled using the flexible TIP3P potential, and the equation of motion was integrated using a time step of 1 fs, which is consistent with the water hydrogen vibrational modes. The system was simulated at a constant temperature of 300 K in the constant NVT ensemble maintained by a Nose–Hoover thermostat with a relaxation constant of 0.2 ps. Long range electrostatic interactions were calculated using the Ewald summation method, and the cutoff radii for both Lennard–Jones interactions and the real-space Coulomb interactions were 10 Å. For a detailed description of the simulation model and protocol, see ref. [5].

The mutants were prepared from the wild type CcO system (equilibrated for 1 ns) by mutating appropriate residues. The systems were then subjected to at least 500 ps equilibration to adapt the modified side chains positions to the mutant environment using the same protocol as for the wild type system.

2.2. Potential of mean force (PMF) calculations

The one-dimensional potential of mean force, or free energy profile, of an ion along the channel axis is a fundamental concept in modeling ion permeation. To construct the PMF of proton conduction through the D-pathway in both wild type and mutant CcO, umbrella sampling was employed. In this study, the PMF describes the overall free energy profile along the z axis of the PT pathway. The protonic center of excess charge [24] of the system was tethered by means of virtual one-dimensional spring acting along the z axis with spring constant varying from 1 to 20 kcal/mol/Å^2. The tethering points for the umbrella potentials were equally distributed 0.25 Å apart, each requiring about 50 windows of 800 ps MD simulation time to adequately sample the range of motion of the proton during its conduction through the channel. The spatial distributions of the center of excess charge along the z axis for each window were then combined using the one dimension WHAM algorithm [25] to form the free energy profile. The self-consistent set of equations was iterated until the convergence tolerance was $<10^{-4}$ kcal/mol. Detailed protocol and information regarding the application of umbrella sampling in free energy calculations can be found in refs [5,25,26].

The MD simulation program software DLPOLY [27] was employed with the Amber force field parameter set [28]. The simulations were performed in parallel on eight 1.5-GHz Intel Itanium 2 processors, and the required time for a 1 ns simulation was about 12 days. The simulation trajectories were then analyzed with tools either from the GROMACS package [29,30] or local code. Computer aided structure analysis were performed using the VMD software [31].

3. Results and discussion

3.1. Proton conduction in the D-pathway of CcO

The PMF for proton permeation along the D-pathway of CcO was constructed from the umbrella sampling trajectories, as shown in Fig. 2. The error in the free energy is typically less than 1 kcal/mol, and in most cases of the calculations performed in this work, structures in the free energy profile can be reproduced in smaller sampling data sets, and only those features are commented upon below. The center of excess charge (CEC) was initially at z of approximately −18 Å, corresponding to the location of Asp91 that is fixed as deprotonated because of its close proximity to the bulk region. As the proton propagates along the D-pathway, a 4 kcal/mol free energy barrier against the passage of proton is observed at Asn80 and Asn98, which lie close to the entry of the channel, and are of prime importance for the maintenance of the proton translocation capacity. Their essential role has been affirmed by the fact that nonconservative mutations of Asn80 and Asn98 have resulted in significant alteration of the proton pumping behavior [11,14,32,33]. It also appears that some of these mutants exhibited an ideal decoupled phenotype.

Asparagine has high propensity to hydrogen bond since the amide group can accept two and donate two hydrogen bonds. In CcO, the pore of the D-pathway can be interrupted by side chain-side chain interactions of these two bulky residues so as to form a gate, controlling further proton permeation. Structural studies, confirming this speculation, suggest the channel may indeed be blocked by them. The amide group of Asn98, orientated almost parallel to the membrane plane, is hydrogen-bonded to the O_δ group from Asn80. This configuration also ensures that in each of the two Asn residues at least one of the two H_δs is facing the channel and, hence, is available as a hydrogen bond donor to water (see Fig. 3). As seen from the MS-EVB2 simulations, the passage of a proton across the channel was not feasible without displacing the Asn side chains regardless of the protonation state of Asp91. Evidence from the visual inspection of trajectories indicates that the energy barrier is perhaps caused by the proton attempting to break through the asparagine gate. Further data analysis confirms that along the umbrella sampling coordinate the location where the hydrogen bonds dissociate exactly corresponds to the peak on the PMF. These results suggest that the conformational change of these two asparagine side chains is necessary for unblocking the proton. Although one can be reasonably certain that the opening of the gate in the D-pathway involves a conformational change of the Asn80 and Asn98 side chains, the possibility of other conformational changes cannot be excluded, such as an increase of pore radius induced by movement of the neighboring helices. However, this kind of long time-scale motion may not be directly accessible with conventional MD simulation which is limited to the multi-nanosecond time scale at the present time.

The role of Asn80 and Asn98 was further assessed by replacing them with various hydrophobic groups or nonconservative hydrophilic groups, such as valine, alanine, serine or aspartic acid

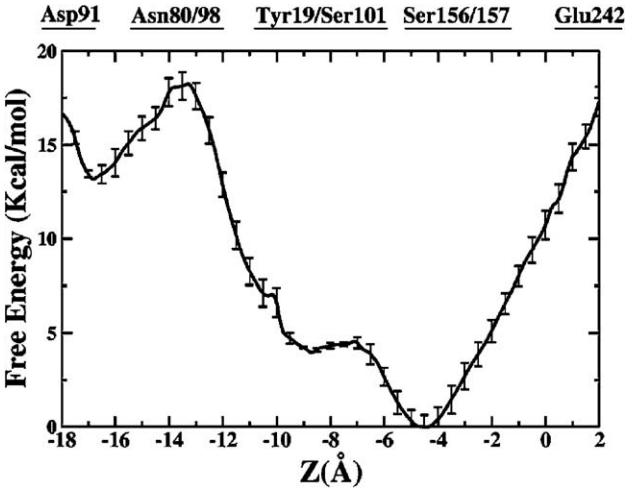

Fig. 2. Free energy of the excess proton in the D-pathway of wild type CcO as a function of z axial position for the simulation with the Glu242 residue in its protonated state.

(Fig. 3). Conventional MD simulations showed that in the wild type enzyme a crystallographic water molecule bridges the gap between the amide group from Asn98 and the carboxylate oxygen from Asp91, the residue that has been shown to be a key residue for proton pumping in the D-pathway [11,12]. Substantial structural differences were noted between the wild type CcO and mutants shortly after the MD simulations were carried out (Fig. 3). Upon replacing Asn80 and Asn98 with either hydrophobic groups (Fig. 3a and b) or negatively charged aspartic acid (Fig. 3d), Asp91, the initial proton acceptor for the D-pathway, completely re-orientates its carboxylate group by pointing directly towards the bulk water. The water molecule travels ~3 Å towards residue Asp91 to preserve their hydrogen bonding, which leads to a situation where the hydrogen bonding connection between Asp91 and the residue at 98 can no longer be established. Such conformational rearrangements in these mutants are likely to be relevant to their loss of proton translocation [11,33]. In contrast, such rearrangements are not observed in the other mutant structure in which Asn98 was changed to a serine, as depicted in Fig. 3c, indicating the necessity of a neutral polar residue at this site for effective proton pumping. Furthermore, the reorientation in the mutants increases the exposure of Asp91 to the bulk water,

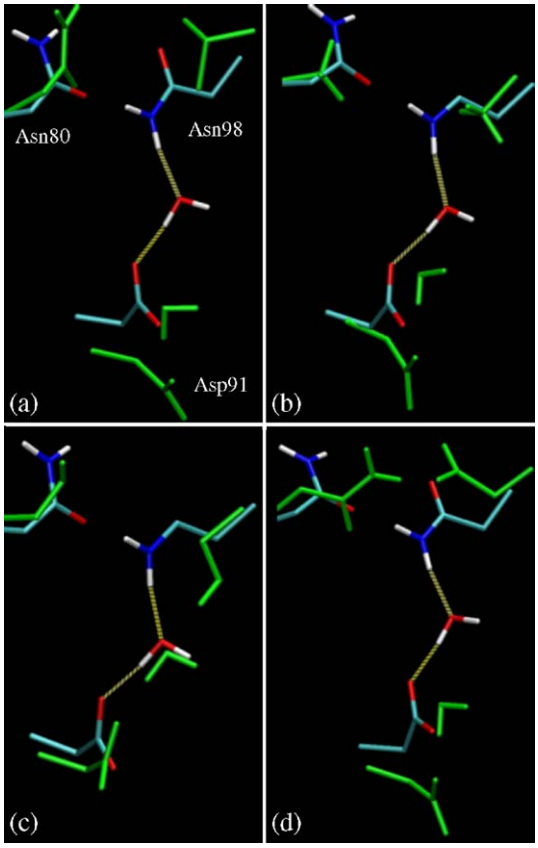

Fig. 3. The structure near Asp91 in the wild type CcO and mutants. The residues in the native CcO are represented in licorice mode, and colored by residue ID number. The residues in mutants are colored in green. (a) N98V; (b) N80A/N98A; (c) N80S/N98S; (d) N98D. The crystal water molecule bridges Asn98 to Asp91 via hydrogen bonding as shown by dotted lines and constitutes part of the proton path. The image was generated with VMD software. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

thus possibly decreasing its pK_a value and eventually slowing the rate of proton uptake into the D-pathway. Although recent continuum electrostatic calculations of the N98V variant showed a slight upward shift of the pK_a values of residue Asp91 (from 3.15 to 3.85) [9], those calculations are based on the static, not dynamic, conformational structure and certain small changes in peptide conformation in response to the presence of mutated groups cannot be taken into account. The effects of Asn98 mutation on the pK_a of Asp91 will need to be determined by site-directed mutagenesis and require further experimental investigation. Our results suggest that Asn80 and Asn98 may play a role in controlling the access of solvent protons farther into the D-pathway via gating as well as maintaining the tight connection to Asp91.

Beyond Asn80/98, the free energy profile generally exhibits a downward trend, indicating that the PT occurs spontaneously in the D-pathway (Fig. 2). A shoulder, spanning from z of −10 to −6 Å, is related to the formation of hydrogen bonds between the excess proton and the OH groups of Tyr19 and Ser101, which are well conserved and noted to be within the pathway [34]. One important feature of the free energy curve of the proton propagating through the channel is the free energy well centered at $z \approx -4$. Consistent with our previous publication [5], the free energy well is observed at the proton trap region, which is the 2.5-Å-radius wide channel region and is proposed to reserve an excess proton when Glu242 is protonated. This proton trap region has been previously shown to be a potential important feature for the continuous and efficient proton conduction in the D-pathway. As shown in Fig. 2, the well is deep enough to trap a proton tightly, and therefore is in accord with the reduced mobility of the proton in this region observed in the MS-EVB2 simulations [5].

The crucial role of Glu242 in the proton translocation through CcO is experimentally well established [6,13,33,35,36]. In the present work, an examination of the computational mutant E242A is additionally provided. Results from the MS-EVB2 simulation of the E242A mutant are quite similar to that of the WT enzyme with the protonated Glu242: the proton transport still can take place, but the excess proton only diffuses halfway along the channel direction and became relatively immobilized at the proton trap region (see supplementary data.). The PMF and the mutant study complements our previously published results on simulations of proton translocation in CcO with Glu242 in the different protonation state as well as the presence of a proton trap inside the D-pathway [5]. We therefore conclude that the deprotonation/protonation reaction at Glu242 is the primary driving force for the translocation of the channel proton from the trap region to Glu 242. The role of Glu242 was also recently discussed by Olsson et al. [37], who proposed a concerted PT mechanism that does not require Glu242 to be protonated/deprotonated, i.e., pushing a proton from the glutamic acid group to a water molecule occurs concurrently with a PT from its adjacent water molecule to the same acid. As found in the present work, it is not easy for the proton to move closer to Glu242 (at $z=2$ Å) when it is protonated because of the significant increase of free energy as seen from the PMF. In order for protons to approach Glu242, it would seem that the residue must firstly release its proton and become transiently deprotonated. But with a high pK_a value of 9.4 [38], there is an apparent need for an increased negative charge of the binuclear

center or another protonatable residue in the exit region with an even higher pK_a value, presumably the PRDa3 group from Heme a3 [32]. An extension of our MS-EVB model to include these possibilities is the focus of ongoing research.

Analysis of the excess proton and channel interactions in the MS-EVB2 simulations complements the information obtained from the PMF. One representative MS-EVB trajectory is shown in Fig. 4. This figure depicts the protonic CEC along the z axis as a function of time. Hydrogen bonding interaction between the proton and the residues along the channel wall is a major factor in stabilizing proton solvation structures. In Fig. 4, three semi-stationary regions are seen. The positions of two semi-stable intermediate states coincide with the location of the hydrophilic groups and are distributed along the pore: one at the Asn80 and Asn98, another at Tyr19 and Ser101. The proton–protein interactions are highly favorable at the 2.5-Å-radius wide channel region, where two OH groups from Ser156 and Ser157 exhibit strong hydrogen bonding interaction with the proton donor hydronium and act like a clamp holding the excess proton in position and eventually impeding its diffusion further along the pathway. The favorable interaction between the proton and the exposed sidechain's electric dipole moment has the effect of retarding the proton transport. Indeed, on the basis of a triple mutant study (Y19F/S156A/S157A), it was found that the proton transfer across the D-pathway occurred at a considerately faster rate when the proton was not able to interact electrostatically with the channel wall (see supplementary material). In that sense, the D-pathway may not necessarily rely on polar residues to create a favorable basin of attraction for protons. Instead, they might just serve a structural purpose to stabilize a single long water chain [39], which, when formed, facilitates fast transfer of protons by the Grotthuss mechanism.

3.2. A special case study on the decoupled N98D mutant

It has been reported previously that specific-site mutations in the D-pathway lead to a completely inactive proton pump and in most cases severely diminish the oxygen reduction activity due to inhibition of proton uptake through the D-pathway (for examples, see refs. [10,13,33]). However, the situation is very different for the N98D mutant. It has been shown that when Asn98 was mutated to an aspartate, the mutant CcO exhibited either 100% oxidase activity (in P. denitrificans) or two times higher activity than the wild type enzyme (in R. sphaeroides) but was not able to pump protons [14,33]. Therefore, the N98D mutant can be considered as being truly decoupled. Earlier attempts have been made to understand the basis for the uncoupling of proton pumping from the oxygen reduction activity. Namslauer et al. [32] reported that the N98D mutation results in raising the effective pK_a of Glu242 by 1.6 pH units with respect to a pK_a of 9.4 in the wild type CcO, which may contribute to the lack of proton pumping. Very recently Brändén et al. [40] found that replacement of Asp91 (Asp132 in R. sphaeroides) by Asn in the N98D mutant can restore the Glu242 pK_a to the original value and give recoupling of the proton pumping, indicating the effects of mutations are of an electrostatic nature. Given the fact that the double mutation (D91N/N98D) moves the carboxylic group a short distance (~5 Å) from the entrance of the pathway up toward Glu242 this observation is perhaps not surprising. Similar findings were also made in Cytochrome bo3 of *Escherichia coli* and suggested that the D-pathway requires an acidic group and potentially hydrogen-bonding amide residues in the vicinity of the entrance in order for proton translocation and pumping to occur normally [11]. It thereby remains uncertain to what extent the recovery of proton pumping activity in the double mutant presented by Brändén et al. is directly linked to the restoration of Glu242 pK_a. Furthermore, the previous experimental investigations have been concentrated on the branching point Glu242 and beyond; the alteration of proton conduction rate in the course of the D-pathway between Asp91 and Glu242 has not been taken into account. A Monte Carlo simulation study recently completed by Olsson and Warshel [41] has provided a framework of the overall pumping process and suggested that the stopping of proton flow to the exit path may be due to the large stabilization (an energy shift of ≈1 kcal/mol) of protons in the D-pathway that increases the effective barrier to the PRDa3 site. However, the difference is insignificant, and the hypothesis needs to be validated by studies that incorporate an explicit atomistic model. In this section, an effort is described to gain further insight into the effects of the N98D mutation on the uncoupling of proton pumping; in particular, to investigate the energetic consequences of the N98D mutation on the proton permeating explicitly through the D-pathway. Toward this goal, a PMF calculation of the proton translocation in the D-pathway of the N98D mutant with the key residues of Glu242 and Asp91 both in the deprotonated state has been performed.

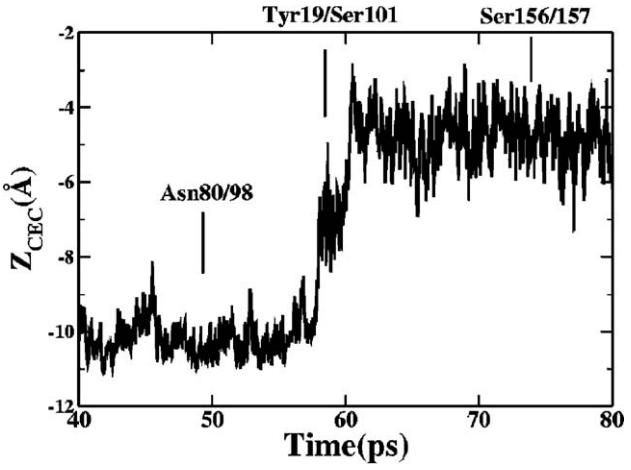

Fig. 4. Time evolution of the protonic center of excess charge (CEC) along the z axis in the simulation with protonated Glu242. Residues forming hydrogen bonds with the proton are listed for each semi-stationary region of the trajectory.

It should be noted that in the wild type CcO the asparagine side chain has a very high pK_a (>17), and cannot become protonated or deprotonated under any biologically relevant pH conditions. This property speaks against its explicit participation in the proton transfer. Whereas in the case of N98D mutant, with an acidic residue at position 98 it has the potential to be directly involved in PT. Nevertheless, the present work considered a proton pathway that only included water molecules as proton acceptor due to the present parameterization of the MS-EVB2 model. In order to give

plausible explanations for the influence of the N98D mutation, two possibilities have been addressed herein: (1) In view of its close proximity to the cytoplasmic surface of the D-pathway it is likely that the introduced aspartic acid (Asp98) is negatively charged, i.e., in the deprotonated state. This view is also in accordance with the interpretations of experimental results by Pfitzner et al. [33], who attributed the lack of proton pumping in the N98D mutant to the introduction of a negatively charged carboxylate moiety and the consequent alteration of the hydrogen bonding pattern; (2) recent electrostatic calculations of N98D mutants show the pK_a values of Asp98 varying from 13.4 to 5.7, depending on the effective dielectric constants [9], thus indicating Asp98 may be protonated. This being the case, it is possible that the introduced aspartic acid may be transiently involved in the PT pathway, and play a role of proton donor/acceptor.

As a preliminary, the structural changes induced by the mutations were reviewed. Comparison of native and mutant configurations shows no significant changes in the protein structure. The backbone conformations and, for the most part, the side chain conformations of the mutants are essentially the same as in the native enzyme. The most pronounced change, as described previously in this section (Fig. 3), occurs in the vicinity of Asp91 when another negatively charged aspartic acid is introduced. The displacement in the peptide side chains and the reorganization of the H-bonds is expected to affect the structure and dynamics of hydrogen-bonded water chain within the path. It appears from a visual examination of trajectory that the D-pathway water chain is interrupted and becomes discontinuous between the deprotonated Asp98 and Ser156/157 shortly after the simulation was carried out, and adequate proton transfer connectivity along the path cannot be achieved.

The PMFs for the translocation of an excess proton along the D-pathway of N98D mutants, as calculated with umbrella sampling, are shown in Fig. 5. At $z=-10$ Å, the protonic center of excess charge is located on average above Asp98 near the bottom of the D-pathway, whereas a numerical value of $z=2$ Å corresponds to configurations in which the excess proton is located at a position within a hydrogen-bonding distance of the carboxylic acid group of Glu242. For comparison, the PMF profile obtained from earlier simulations [5] of the wild type channel with Glu242 deprotonated is also presented in Fig. 5.

With Asp98 in the deprotonated state, unlike in the wild type D-pathway where the proton experiences no barrier traveling to Glu242, the passage of proton in the mutant is accompanied by a steady increase of free energy resulting in a barrier of 8 kcal/mol peaking at $z=-1.5$ Å. This barrier reflects the effect of modifications of electrostatic interactions between the excess proton and the charged residues along the channel. More specifically, the excess proton in the center of the channel is not "solvated" significantly while the solvation of a proton at both the bottom and top of the pathway has a significant contribution from the cation-attractive environment created by the ionized residues Asp98 and Glu242. Thus, it is perhaps not surprising that this barrier, correlated to the electrostatic effects originating remotely from position 98, is located approximately half way into the pathway at a wide channel region previously defined as a proton trap. The main function of this region in the N98D mutant may now lie in the exclusion of protons rather than being a "proton reservoir" as in the native enzyme, but the change in proton conductivity is not caused by direct electrostatic interactions of the excess proton and polar residues located adjacently to the proton trap region.

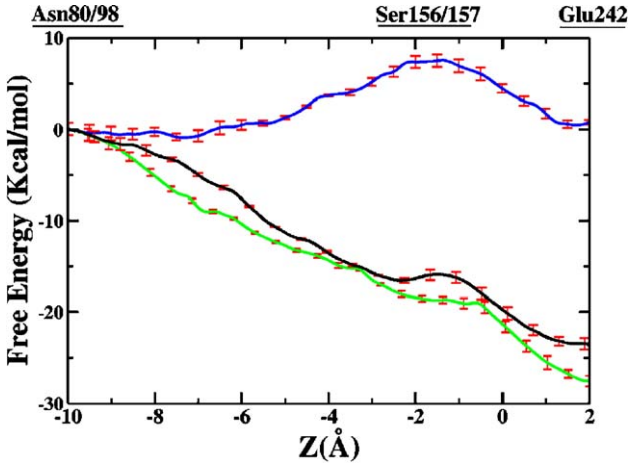

Fig. 5. Potential of mean force (PMF) for proton translocation along the z axis of the D-pathway in the native enzyme and N98D mutants. Blue and green lines correspond to the profiles with deprotonated Asp98 and protonated Asp98, respectively. Black line shows the PMF profile of the wild type channel. The error bars come from calculating the free energy separately from the first and second halves of each MD trajectory. In all simulations here, the Glu242 residue is deprotonated. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

It is known that the presence of negatively charged residues "decorating" the intracellular entryway of K^+ channels can be related to unusually larger conductance rates [42]. This may not be the case in the D-pathway. It is suggested by our results that upon introduction of an additional negative charge at position 98 the free energy of transfer of a proton inside the D-pathway of CcO can be significantly increased to a barrier sufficiently high to block proton transport. Since the D-pathway is used for uptake of both the substrate protons, which are transferred to the catalytic side, and pumped protons, which are transferred to the exit path, both proton pumping and oxygen reduction activity will be sufficiently reduced in such a case. This effect may also apply to the recently examined G204D mutant (R. sphaeroides CcO numbering) [43], in which the substitution of glycine 204 by aspartate leads to a 98% reduction in enzyme turnover rate and complete elimination of proton pumping, though mutating with phenylalanine or tryptophan has been found to have normal proton pumping and only moderately reduced enzyme turnover rate [33]. Inspection of crystal structures indicates that residue G204D lies 4 Å above Asn98 in a polar region containing a number of hydrophilic groups and crystal water molecules and hence likely to be charged.

Experimental studies have determined that the transition rates between the intermediate states, which are associated with proton uptake from the bulk solution, were either the same or two times faster in the N98D mutant [32]. This suggests that the cause of the uncoupling of proton pumping from the oxidase activity in this mutant was not due to inhibition of proton uptake through the D-pathway but from a different origin. On the other hand, results from the present computational studies demonstrate that replacing

Asn98 by a negatively charged Asp will impair the proton uptake through the D-pathway by creating a considerable energy barrier. Taken together, these observations suggest that the inserted aspartic acid residue at locus 98 may have a high pK_a and would appear to be protonated in the oxidized enzyme. It may be likely though that a permeating proton could approach Asp98 so closely via the bridging water molecule as to cause an alteration in the side-chain pK_a to trigger a transient deprotonation event.

In the following, the same initial configurations were used as a starting point in the second PMF calculation except that the Asp98 was assumed to be protonated. The result of the calculations is depicted in Fig. 5, colored in green. As one can see, neutralizing Asp98 leads to significant change in the free energy profile so the conduction of proton is essentially barrierless. The overall shape of the profile is highly similar to the one in the native channel but with a less pronounced shoulder around $z=-1$ Å. The shoulder in the native channel, corresponding to the proton trap region, was suggested to slow down the proton propagation along the path. The fact that it appears less pronounced in the mutant channel can certainly give rise to a rapid conduction. In fact, as seen from the MS-EVB simulations, the proton initially placed above Asp98 diffuses somewhat faster in the mutant channel than in the native channel. Simulations with different initial starting configurations have also been performed to ensure that the observed behavior is not an artifact. Moreover, the total free energy of transfer of a proton to Glu242 changes from −24 kcal/mol in the native enzyme to −28 kcal/mol in the N98D mutant, indicating an increased proton affinity through the D-pathway to Glu242 upon addition of another acidic amino acid. Similar findings were also made in ref. [41] suggesting protons are more stabilized in the N98D mutated D-pathway. Although one should bear in mind that the absolute energy values represented here are subject to error and must be treated with caution, these results support the notion that replacing Asn98 with a neutrally charged aspartic acid will have an impact on the proton potential energy profile, and may consequently raise the proton conduction rate along the D-pathway.

The introduced aspartate may have a high pK_a and can potentially be a proton-donating group explicitly involved in the proton transfer events by releasing a proton on one side and rapidly accepting another proton from Asp91 via the bridging water molecule. It is therefore conceivable that its direct participation in the PT pathway may facilitate the proton conduction by providing another carboxylic side chain in addition to Asp91 that can rapidly shuttle protons. Since the explicit participation of ionizable residues such as Asp and Glu is not taken into account in this study, the present work considered the course of the D-pathway that only requires the water molecules as the proton acceptors. Further investigations with the direct participation of Asp98 should be undertaken to consider an increased proton conduction rate in the N98D mutated channel.

The D-pathway is required to deliver two different types of protons during different steps of the reaction cycle. Its dual role suggests the highly conserved Glu242 acts as the switch which directs the substrate and pumped protons along different trajectories via side chain conformational isomerization. An earlier suggestion of a mechanical model [14] postulates that this switch may be held in a certain position in the N98D mutant so that all protons coming from the D-pathway are directed to the binuclear center. Supporting this consideration, it is plausible that a faster proton replenishment by the mutant via the D-pathway as suggested here may short circuit the proton pumping by not allowing time for some critical geometrical rearrangement to occur at the pump elements in close association with the pumping pathway, presumably the ion pair of the conserved arginine (R438 in bovine CcO; R482 in R. sphaeroides CcO) and the propionate group of Heme a3 given the fact that their dissociation can be triggered by electron transfer [44]. Therefore, the pump element might not be able to deliver its protons to the output side, but rather the "pumped protons" would be delivered to the binuclear center for oxygen reduction; the oxidase activity is enhanced while the proton pumping is eliminated. An alternative explanation for the uncoupling of proton pumping from the oxygen reduction chemistry in the N98D mutant is equally possible: Perhaps there is no such mechanistic separation to distinguish these two types of protons, rather, their fate is decided on momentary needs. Since the proton pumping mechanism competes kinetically with the highly exergonic proton consumption of the oxygen reduction, the normal function of proton pumping may require a proton to be transferred to Glu242 at a certain rate. It is very likely that the efficient proton pumping requires a finely tuned dynamics of the proton transfer through the D-pathway to allow an accurate timing of the events associated with the chemical reaction of oxygen reduction to water and the proton pumping. Either delayed or enhanced proton replenishment via the D-pathway might eventually lead to the decoupling. Future research concerned with the aspects of explicit protonation/deprotonation of Glu242 and PT beyond Glu-242 will be needed to distinguish these possibilities and refine the mechanistic picture of the proton pumping.

4. Conclusions

The present work has explored the energetic behavior of an excess proton propagating along the D-pathway of both the native and the N98D mutant forms of CcO. In the study of wild type channel, the results reveal some elements similar to our original assertion [5]: the PMF shows a deep free energy well at Ser156/157 that significantly favors the presence of the excess proton and traps the proton tightly when Glu242 is protonated. The PMF also reveals a free energy barrier opposing the passage of proton at Asn80/98, which is postulated to possibly be involved in gating of proton uptake into the D-pathway.

Through a comparative study of a proton diffusing along the N98D mutated pathway, it was demonstrated that the presence of negatively charged group inside the vestibular cavity of the D-pathway will significantly change the energy landscape and contribute to opposing the passage of protons. The analysis from an energetic perspective provides the valuable insight into the mechanism of PT in the N98D mutated D-pathway that the introduced aspartate residue may provide another carboxylic acid side chain in addition to Asp91 and Glu242 that can directly participate in the PT process and aid in the shuttling of protons, thus leading to an increased conductivity of the D-pathway. Since

the internal electron transfer rates are tightly coupled to the proton transfer, the electron transfer may be able to compete kinetically with the enhanced proton transfer. In turn, this could possibly short circuit the proton pumping and cause its uncoupling from the oxygen reduction chemistry. The present calculations were restricted to inside of the channel and did not evaluate the energy for moving the proton from the bulk region to the channel mouth. The effect of the N98D mutant on the apparent pK_a of Asp91 and the rate of proton uptake into the D-pathway is a topic that merits further investigation.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.bbabio.2006.05.028.

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