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Review

The mechanism for proton pumping in cytochrome c oxidase from an electrostatic and quantum chemical perspective ☆

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ABSTRACT

The mechanism for proton pumping in cytochrome c oxidase in the respiratory chain, has for decades been one of the main unsolved problems in biochemistry. However, even though several different suggested mechanisms exist, many of the steps in these mechanisms are quite similar and constitute a general consensus framework for discussing proton pumping. When these steps are analyzed, at least three critical gating situations are found, and these points are where the suggested mechanisms in general differ. The requirements for gating are reviewed and analyzed in detail, and a mechanism is suggested, where solutions for all the gating situations are formulated. This mechanism is based on an electrostatic analysis of a kinetic experiment fior the O to E transition. The key component of the mechanism is a positively charged transition state. An electron on heme *a* opens the gate for proton transfer from the N-side to a pump loading site (PLS). When the negative charge of the electron is compensated by a chemical proton, the positive transition state prevents backflow from the PLS to the N-side at the most critical stage of the pumping process. The mechanism has now been tested by large model DFT calculations, and these calculations give strong support for the suggested mechanism. This article is part of a Special Issue entitled: Respiratory Oxidases.

1. Introduction

Cytochrome c oxidase (CcO) is the terminal enzyme in the respiratory chain, located in the inner mitochondrial or bacterial membrane. In this enzyme molecular oxygen is reduced to water by electrons delivered from cytochrome c in the outer cytosol, the P-side of the membrane, and protons delivered from the inside, the N-side of the membrane. The fact that the electrons and protons are taken from different sides results in the generation of an electrochemical gradient across the membrane, storing some of the energy from the exergonic oxygen reduction. This energy, in the form of the gradient, is used by ATP-synthase to make ATP, the energy currency of the cells. The cofactors responsible for electron transfer and the oxygen chemistry are shown in Fig. 1 for cytochrome c oxidase belonging to class A. The electrons are transferred from cytochrome c via a dinuclear copper complex, Cu_A, and a heme group, heme *a*, to the binuclear center (BNC) consisting of another heme group, heme a_3 and a mononuclear copper complex, Cu_B, where the redox chemistry occurs, forming two water molecules for each O_2 molecule consumed.

Already in 1977, Wikström discovered that the redox chemistry in CcO is coupled to a proton pump. For each O_2 molecule consumed, an additional four protons are translocated across the entire membrane against the electrochemical gradient [1]. This proton translocation further contributes to the gradient buildup and thus to the efficiency of the energy storage. The molecular mechanism for the proton pumping against the gradient is far from obvious, and still remains controversial [2]. Many different mechanisms have been suggested, but the nature of the gates that separate the protons consumed in water formation from the protons being pumped has not been understood [3]. Progress in elucidating the pumping mechanism was for several years partly hampered by the belief that only two of the four reduction steps were coupled to proton pumping [4], implying that for those steps, two protons should be pumped per electron (apart from the proton taken up for the chemistry). A mechanism of that type is clearly extremely difficult to realize, and it was never successful. Still, it was rather early possible to formulate some general elements of a pumping mechanism, which are included in most of the mechanisms that have been suggested. One such element is the assumption that the electron transfer into CcO is coupled to a proton transfer into a pump loading site (PLS), where the protons to be pumped are temporarily stored, and which is separated from the site where the oxygen chemistry occurs [5,6]. The driving force for the proton transfer is assumed to be an electrostatic interaction with the incoming electron. After the transfer of a proton to the PLS, another proton is taken up for the chemistry in the BNC. The second general element of the pump mechanism is the assumption that the electrostatic repulsion from this second proton will expel the proton at the PLS out of the enzyme to the P-side [5,6]. These two assumptions,

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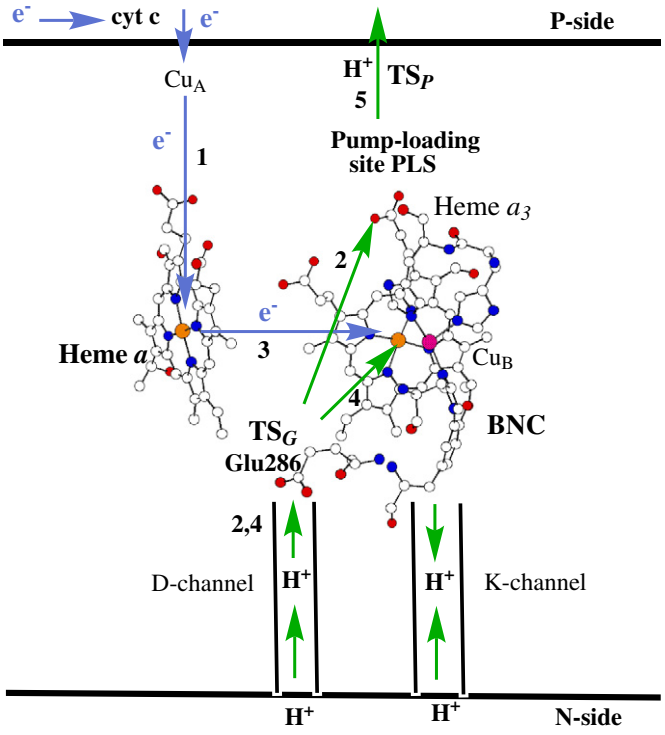

Fig. 1. Overview of electron and proton transfer in cytochrome c oxidase (class A).

the initial uptake of the pump-proton into a pump loading site, and the removal of this proton by electrostatic repulsion from the chemical proton (i.e. the proton that takes part in the chemistry), can be considered as necessary components of a pump mechanism, but they give no information of the nature of the gates, that govern the protons in different situations to go in the right direction, in particular, to move against the electrochemical gradient. However, it can be noted that already these rather general elements of a pumping mechanism point out the three most important gating situations [5,6]. The first gate is needed to guide the first proton to the PLS and not to the water production site at the BNC. Since proton pumping corresponds to a translocation of a proton from the N-side to the P-side, there is also a gate needed that prevents the PLS proton to be taken up from the P-side of the membrane. Furthermore, a gate is needed that prevents the PLS proton to move back to the N-side of the membrane when it is destabilized by the chemical proton.

An important step forward was taken in 1998 when Michel challenged the idea of only two pumping steps [7], which initiated further investigations and finally led to a new interpretation of experimental data suggesting that one proton is pumped in each of the four reduction steps [8]. With one proton being pumped for each electron transferred, it is natural to assume that the general mechanisms for proton consumption and proton pumping are similar for all four reduction steps. This should make it possible to construct a general pumping mechanism in more detail. Several mechanisms have been suggested, and most of them contain the two main electrostatic elements mentioned above. However, the mechanisms differ in several details concerning both the actual order of elementary proton and electron transfer steps, and in the nature of the different gates suggested.

Essentially all different pumping mechanisms suggested start with the transfer of an electron from cytochrome c into the CcO enzyme. In the earliest suggested mechanisms, e.g. the histidine cycle by Wikström and coworkers in 1994 [5], and the mechanism described by Rich et al. in 1996 [6], it was assumed that the electron was transferred all the way to the BNC before the pump-proton was transferred to the PLS. However, in 1998 Michel presented an explicit mechanism where the pump-proton was taken up already when the electron is at heme *a*, i.e. before it arrives at the BNC [7]. Coupling between reduction of heme *a* and proton pumping had in more rudimentary forms been suggested before, e.g. by Babcock et al. in 1983 [9]. The idea that it is the electron transfer to heme *a* that triggers the next step, the uptake of the pump-proton, has been adopted as an important ingredient in several more recently suggested mechanisms [8,10–13], and the analysis of the recent kinetic experiments for one reduction step (O to E) by Wikström and coworkers supports this mechanism [14]. That idea actually makes it much easier to construct a gating mechanism that prevents this first proton to go to the BNC, which would waste the energy to be conserved by the proton pumping. One suggestion is the water-gated mechanism, where it is assumed that the position of the electron at heme *a* governs the hydrogen bonding between a chain of water molecules so that the first proton is prevented to go to the BNC [8]. Another suggestion is that the endergonicity of proton transfer to the oxidized BNC results in a too high barrier for completing the chemistry at the BNC. Instead, there first has to be an uptake of a proton to the PLS [11,12], to allow the electron to go to the BNC. Only at that stage can the chemistry be completed. Surprisingly, some quite recently suggested pumping mechanisms still pursue the idea that the first step is electron transfer to the BNC [2,15]. In fact, to our knowledge, no specific gating mechanism has in that case been suggested that prevents the first proton to perform the very exergonic transfer to the reduced BNC, but it has still been assumed that there exists such a kinetic gate [15]. In a fundamentally different mechanism, suggested by Brzezinski and coworkers, the chemical proton is actually assumed to go to the reduced BNC before the pump-proton is taken up [16,17].

The second step, the protonation of the PLS can occur either via the transfer of an "extra" proton [11,12,18,19], or by a charge separation process where a conserved glutamic acid (Glu286 in *Rhodobacter sphaeroides* notation), situated in the proton transfer D-channel (see Fig. 1), delivers its acidic proton to the PLS as suggested in several mechanisms [2,13,15,20,21]. The extra proton might be available in the D-channel [22] or it can be taken up all the way from the N-side of the membrane. In the charge separation mechanism, the Glu286 is suggested to be reprotonated in a following step. Clearly the two mechanisms can be considered as two extremes of a similar process, where the Glu286 is first deprotonated and then becomes reprotonated either more or less concertedly, or more slowly, by another proton from the N-side [12]. The assumption of the character of this proton transfer step will have implications for the possibility to prevent back leakage of the pump-proton at a later stage, which will be discussed below [11,12]. An important observation from the kinetic experiment [14] mentioned above, is that the barrier for this proton transfer step is quite high, about 11 kcal/mol using transition state theory [12]. This rather high barrier actually turns out to be necessary for making a gating mechanism possible. The specific location of the PLS is not known, but there are different suggestions, most of them locate the PLS in the vicinity of the propionate groups of the two hemes. A general requirement is that this position has to be close enough to electrostatically interact with the electron in heme *a* and/or the BNC. To make the interaction large enough, the dielectric constant must be rather small ($\epsilon = 3$–4) [11,12].

If the electron is at heme *a* when the PLS becomes loaded with a proton, the next step should be transfer of the electron to the BNC. A commonly adopted requirement for the PLS is that it is located closer to the BNC than to heme *a*, and therefore it is the PLS proton that triggers this electron transfer. It is also possible that the pump-proton moves from a location close to heme *a* to one close to the BNC, in concert with the electron transfer. When the BNC is reduced, it is favorable for a second proton to be taken up from the N-side of the membrane and to move into the BNC to perform the chemistry. This exergonic step is made even more exergonic when it is followed by the removal of the pump-proton from the PLS. However, from a thermodynamic point of view there is no advantage for the pump-

proton to go to the P-side of the membrane. On the contrary, when there is a gradient present it would be thermodynamically more favorable if the proton would move back to the N-side. Therefore, there has to be a kinetic barrier preventing the proton from going back to the N-side. Here it is important to note that the fact that the chemical proton and the pump-proton are taken up via the same pathway, the D-channel, is not enough to prevent the pump-proton to leak back to the N-side. As illustrated in Fig. 2, when the chemical proton is in the common part of the D-channel, it is quite far from the PLS-proton, and the repulsion between the two protons is therefore only about 5 kcal/mol (using $\epsilon = 4$), which is not enough to counterbalance the attractive force on the pump-proton from the electron in the BNC, which is about 10 kcal/mol (using $\epsilon = 4$) [11]. The repulsion is large enough only when the chemical proton is more or less at the BNC-site, but in that position it will not hinder the pump-proton to leak back. This argument is essentially independent of the value of ϵ. It should also be noted that, due to microreversibility, the fact that the Glu286 is protonated cannot prevent the back leakage as has been suggested [8,13,15], see further below. Two explicit suggestions for back leakage barriers are the positively charged transition state [11,12] and the Glu286 rotational transition state [21], which will be further discussed below. In this context, it should also be noted that the transfer of the pump-proton from the PLS to the P-side of the membrane has to be rather slow, since there has to be a barrier in this region that prevents the pump-proton to go in the opposite direction from the P-side to the PLS when heme *a* becomes reduced in the first step. This slow rate is also in agreement with the experimental observations in the kinetic experiments mentioned above [14].

There are also other suggestions for proton pumping mechanisms that differ more substantially from the ones briefly mentioned above [10,16,23,24]. A common theme for those mechanisms is that, apart from the electrostatic effects, long range conformational changes also play important roles.

In summary, what should be clear from the description above is that based only on experimental results, it has not been possible to reach consensus on the mechanisms for proton pumping in cytochrome c oxidase. In fact, not even the detailed order of the elementary steps of electron and proton transfer is agreed upon. Concerning the nature of the gates forcing the protons to move against the electrochemical gradient it should be noted that most of the suggested pumping mechanisms actually do not even have suggestions for these gates. The main purpose of the present paper is to show that there is in fact one rather complete pumping mechanism suggested, which also has computational support for the nature of the gating.

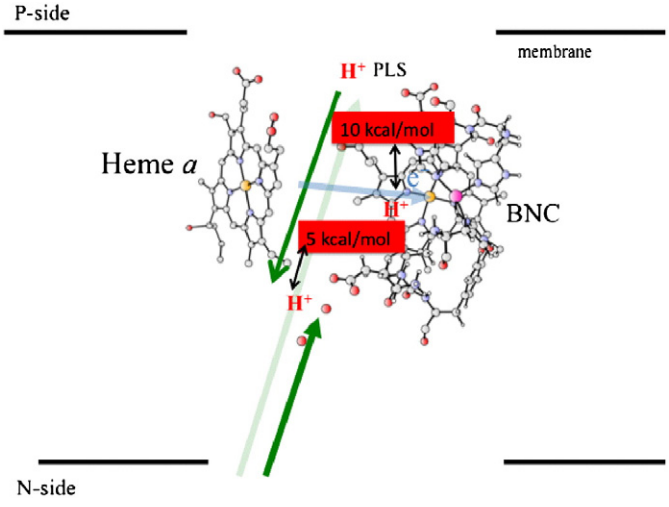

Fig. 2. The electrostatic repulsion on the pump-proton is large enough only when the chemical electron is close to its final site in the BNC, where it cannot prevent back leakage of the pump-proton to the N-side.

2. Results and discussion

Below, some of the suggested mechanisms for proton pumping in CcO will be described in detail, and, in particular, different types of computational support for the suggestions will be presented. One of the suggested mechanisms is more complete than the other ones, and it will be presented first in more detail [11,12,25,26]. This mechanism has several components in common with the other mechanisms discussed afterwards.

2.1. Main pumping mechanism

The essential properties of the suggested mechanism were presented in 2007 [11] in connection with an analysis of the results from the kinetic experiment for one reduction step [14]. Based on the time-resolved experimental results, an energy diagram for the elementary steps of electron and proton transfer could be constructed [11,27], the black curve in Fig. 3, where transition state theory was used to translate the measured life-times to barrier heights. The barriers obtained for the individual steps are high enough to satisfy transition state theory, and they are low enough to make the elementary steps microscopically reversible, which is a prerequisite for this type of mechanistic investigation [28,29]. The energy diagram in Fig. 3 only gives a starting point for a pumping mechanism, since it only describes the energetics of the actually occurring pathways. To explain the different gating situations, energy barriers for the non-allowed reaction pathways, not leading to pumping, also have to be included. As will be discussed below, it is possible to construct such barriers with certain assumptions of the nature of the gates. A barrier height of about 16 kcal/mol is taken as high enough to prevent a reaction step to occur. The details of the pumping mechanism are illustrated in Fig. 1 with arrows and numbers on the different steps, and in the more detailed picture of the active site in Fig. 4. The step numbering is also shown in the energy profile in Fig. 3.

In step 1 (I to II, referring to the states in Fig. 3) an electron is transferred from cytochrome c, via Cu_A to heme *a*. This is a fairly slow electron transfer step of 10 μs corresponding to an experimental barrier of 10.6 kcal/mol. The electron on heme *a* raises the pK_a-value of a pump-loading site (PLS) in the vicinity of the BNC. The increased pK_a leads to a proton uptake from the N-side via the D-channel to the PLS in step 2 (II to III), with an experimental barrier of 10.8 kcal/mol. As mentioned above it is not known where the PLS is, but it is here suggested to be at propA of heme a_3, or in the water cluster close to the propionates of heme a_3, see Fig. 4. In this step the first gating situations appear. First, the proton must be taken from the N-side and not from the P-side, which is labeled gate A in Fig. 3. This requirement can be fulfilled by a permanent barrier between the PLS and the P-side, as suggested already by Rich et al. in 1996 [6] and in the early attempts to construct energy diagrams for the pumping mechanism [30]. Actually, the slow release of the pump-proton in the final step (5) (V to VI), observed in the kinetic experiments [14], demonstrates the presence of such a barrier, see Fig. 3. The reverse of this experimental barrier is 16 kcal/mol and it corresponds to the uptake of protons from the P-side, i.e. the forbidden path labeled gate A in Fig. 3. The exact location of the transition state corresponding to this barrier, labeled TS_{P-A}, is not known, except that it has to be somewhere between the heme a_3 propionates and the outside of the membrane, see Figs. 1 and 4. This is the simplest gate, since it does not have to change height during the entire reaction. The second gate needed at the same stage of the reaction, labeled gate B in Fig. 3, is one that forces the proton coming through the D-channel to avoid going to the BNC, but instead to continue to the propionate region and the PLS. If the electron was already in the BNC, as assumed in some

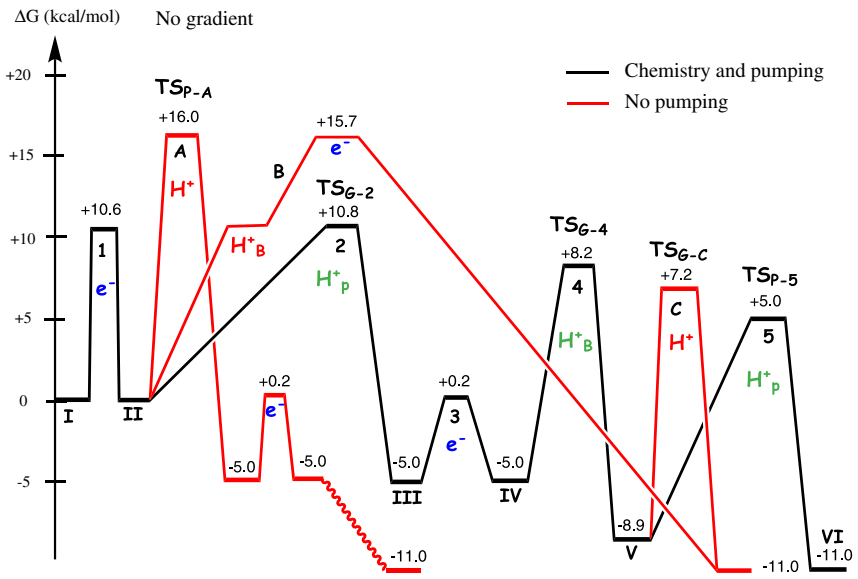

Fig. 3. Energy diagram showing both allowed (black) and forbidden (red) reaction paths. Steps 1 to 5 are defined in Fig. 1.

pumping mechanisms [2,15], it would be very exergonic for the proton to go to the BNC, and it is difficult to imagine the nature of a gate that could prevent this to occur. However, with the electron at heme *a*, as in the present mechanism, it is actually endergonic for the proton to go to the BNC, about 11 kcal/mol as estimated from quantum chemical calculations [11,31]. Adding on top of this endergonicity, the barrier for electron transfer between heme *a* and heme a_3 of 5 kcal/mol (see step 3 (III to IV) in Fig. 3) gives a total barrier of about 16 kcal/mol, which is enough to prevent the first proton to go to the BNC [11,25]. In addition, the redox potentials of the electron acceptors in the BNC are too low for the electron to move from heme *a* before the proton is in the PLS. Thus, with barriers of about 16 kcal/mol for both gate A and gate B, the proton prefers to go

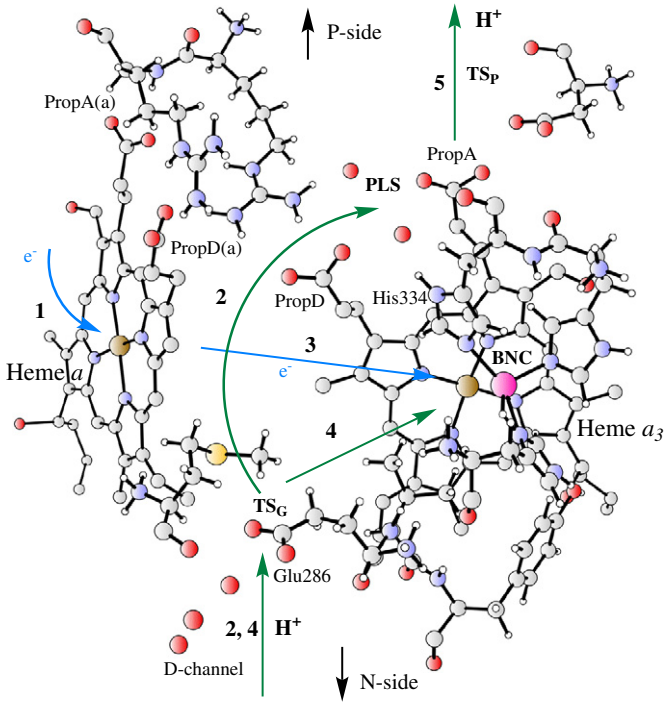

Fig. 4. Closer look at the pathways for protons and electrons. Note that some residues are omitted for clarity.

from the N-side to the PLS with a barrier of 10.8 kcal/mol as measured experimentally, and with an exergonicity of 5.0 kcal/mol, also derived from the experiment [11,14].

As mentioned in the Introduction, there are different suggestions for the character of the proton transfer step 2 (II to III). In the present mechanism it is assumed that this transfer occurs mainly in the form of an extra proton in the D-channel passing the Glu286 and several water molecules to the heme a_3 propionates. Whether Glu286 during this process becomes deprotonated and quickly reprotonated, or if the proton just moves between a chain of water molecules is a minor issue. The main point is that there is an extra positive charge traveling along the reaction path. The importance of this assumption will be discussed below in connection with step 5 (V to VI) and gate C. It is further assumed that the transition state for this proton transfer is in the vicinity of Glu286, and it is labeled $TS_{G\text{-}2}$. In the quantum chemical calculations described below, it is found that the energy difference between having the proton in $TS_{G\text{-}2}$, i.e. in the water cluster near Glu286 on the one hand, and having the proton in the PLS, i.e. near propA of heme a_3 on the other hand, is 15.4 kcal/mol, very close to the corresponding energy difference of 15.8 kcal/mol between the $TS_{G\text{-}2}$ and the point labeled III in the energy diagram derived from experiment, and which corresponds to the protonated PLS, see Fig. 3. These computational results strongly support the possibility of this type of proton transfer with an extra proton, and thereby the nature of the gate C to be discussed below. The alternative mechanism suggested for this proton transfer step, where the Glu286 proton is first transferred all the way to the PLS and where the glutamate is reprotonated only in a subsequent step, was also investigated using the same quantum chemical model system. Preliminary results indicate that such a charge separation step would be quite endergonic, and can probably only occur with a very high barrier for moving the glutamic proton along the chain of water molecules toward the heme a_3 propionates, see further below.

In step 3 (III to IV), the electron is transferred from heme *a* to the BNC, since the proton in the PLS increases the redox potential of the electron acceptors in the BNC. The electron in the BNC in turn increases the pK_a of the proton acceptors in the BNC (O^{2-} or OH^-, depending on the reduction step), and in step 4 (IV to V) the chemistry for this reduction step is completed by the uptake of a proton from the N-side to the BNC, with an experimental barrier of 13.2 kcal/mol and an exergonicity of 3.9 kcal/mol [11,14]. The path from the N-side to Glu286 for the chemical proton is assumed to be identical to

the one for the pump-proton. At, or near, Glu286 there should be a branching point, at which the pump-proton takes one way and the chemical proton another one. In contrast to the situation for the proton transfer to the PLS in step 2 (II to III), in step 4 (IV to V) with the electron in the BNC, the chemical proton should go directly toward the negative charge, which increases the driving force for the glutamic acid proton to start moving before the new proton has arrived [12]. In fact, with the proton present in the PLS a purely positively charged transition state for the transfer of the chemical proton would be too high. Therefore this proton transfer is most likely somewhat different than the one of the pump-proton, and involves a larger degree of charge separation, see further the discussion in Ref. [12]. This also gives an important role for the conserved Glu286, and can explain why mutations of the Glu286 essentially eliminates enzyme turnover [32]. In this step (4 (IV to V)) another gating situation appears (not shown in Fig. 3), since the proton going to the BNC must not be the one at the PLS, but instead one coming from the N-side via the D-channel. This is rather easily achieved if it is assumed that both protons (the one in the PLS and the one from the N-side) would have to pass the same transition state point in the region of the Glu286. With the electron in the BNC the pK_a of the PLS is clearly above the pH of the N-side (point IV in Fig. 3 is at −5 kcal/mol), and therefore the barrier will be lower for the proton coming from the N-side than for the one coming from the PLS.

At this point, labeled V in Fig. 3, the negative charge in the active site is quenched, and the pK_a of the PLS is back to its lower value. Therefore, the pump-proton should be expelled to the P-side of the membrane in step 5 (V to VI) with an experimental barrier of 13.9 kcal/mol and an exergonicity of 2.1 kcal/mol, completing the pumping of one proton. This step (5) involves one of the most demanding gating situations, labeled gate C in Fig. 3, since the pump-proton must not move back to the N-side via the D-channel, which is thermodynamically more favorable when the electrochemical gradient is present. This means that, in contrast to the gating situation A when the uptake of the pump-proton in step 2 (II to III) should occur via the transition state $TS_{G\text{-}2}$ and not via $TS_{P\text{–}A}$, in the gating situation C the proton must be expelled via $TS_{P\text{-}5}$ and not via $TS_{G\text{–}C}$, implying that the relation between the barrier heights of TS_G and TS_P must have changed. This is where the assumption about the positive character of $TS_{G\text{-}2}$ for the uptake of the pump-proton enters. In gating situation A, when step 2 is about to occur, there is a negative charge at heme *a* which is close enough to stabilize the positive charge in $TS_{G\text{-}2}$, making the barrier low enough for the proton to pass. However, when the chemistry is completed there is no negative charge present, and the positively charged $TS_{G\text{–}C}$ will be high enough to prevent the back leakage of the pump-proton [11,12]. This explains the fairly high forward barrier in step 2, since the stabilizing effect from the electron can only be of the order of a few kcal/mol, and cannot by itself lead to gating. The height of the barrier in gate C given in Fig. 3, 16.1 kcal/mol, is obtained by using the simple assumption of a positive TS, together with Coulomb's law, the crystal structure and the experimental value for the barrier of step 2 [11,14]. Since the distance between heme *a* and the suggested locations of either TS_G or PLS are rather similar, the two positions will be stabilized by a similar amount by the electron in heme *a*, and the back leakage can be roughly estimated by the reverse barrier of step 2. On the other hand, for a neutral transition state, as occurring in a charge separation type of process, the back leakage will be much lower, only 8.7 kcal/mol (10.8–2.1), as illustrated in Fig. 5 [33]. The barrier for a positively charged transition state has been investigated by quantum chemical model calculations, and below, it will be described how they are used to further establish the results from the simple electrostatic considerations [26]. It should be noted that a similar idea with a positively charged transition state for the pump-protons that would prevent back leakage to the N-side was earlier suggested by Warshel and coworkers [18], who later abandoned this idea [20].

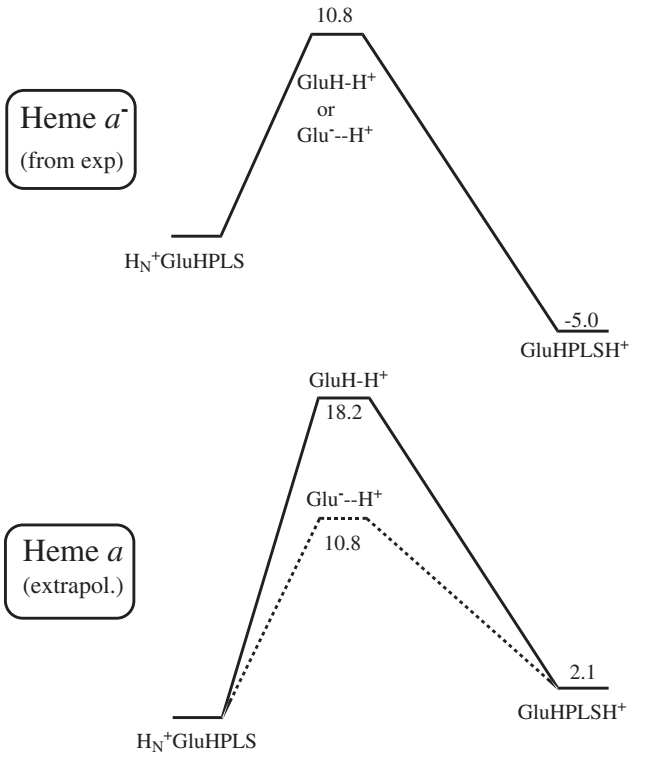

Fig. 5. Energy profiles for proton transfer between the N-side of the membrane (H_N^+) and the PLS. The lower profiles illustrate two different extremes for the character of the TS: the full curve corresponds to a positively charged TS, and the dotted curve corresponds to a neutral charge separation type of TS, which is essentially not affected by the electron in heme *a* [33].

Essentially the same procedure occurs four times, one for each electron, with the main variation that in one or two steps, the K-channel is used instead of the D-channel for the proton involved in the chemistry.

2.2. Other pumping mechanisms

In 2003 Wikström and coworkers formulated the so called water-gated mechanism for proton translocation in CcO [8]. Some aspects of this mechanism are incorporated into the mechanisms described above, first that the pump-proton is transferred to the heme a_3 propionate region when the electron is at heme *a*, and second, that the pump-proton in this position triggers the electron transfer from heme *a* to the BNC. The following steps, proton transfer to the BNC and ejection of the pump-proton to the P-side, are also the same in the two mechanisms, as in most of the suggested mechanisms. One of the differences between the mechanism in the previous subsection and the water-gated mechanism is the character of the proton transfer processes, where Wikström and coworkers emphasize full charge separation for both the pump-proton and the chemical proton, yielding intermediates with a negatively charged Glu286 and with the proton either in the PLS or in the BNC. This is in contrast to the mechanism in the previous subsection, where particularly the pump-proton is mainly transferred as an extra proton and where there is no significant amount of charge separation. However, the main differences between the mechanisms are in the nature of the gates. In the water-gated mechanism it is suggested that the electric field from the electron in heme *a* orients the water molecules for proton transfer from Glu286 to the PLS. After the electron transfer to the BNC, the water molecules would reorient to provide a proton path to the BNC. The idea is that the hydrogen bonding pattern of the water molecules should prevent the first proton to go to the BNC, i.e. taking

care of the gating situation A in Fig. 3. Molecular dynamics simulations on the picosecond time scale actually indicated such a redox controlled shift of hydrogen bonding pattern [8]. However, the energetic importance of the shift in hydrogen bonding is unclear. Taking into account the typical strength of this type of hydrogen bonds, on the order of 5 kcal/mol, and estimates of charge-dipole interactions at these distances, it appears quite unlikely that this shift in hydrogen bonding would be the only source of gating. Furthermore, recent simulations on more elaborate computational models result in other hydrogen bonding patterns of the water molecules, not leading to gating [34].

In 2008 Wikström and coworkers presented another gating mechanism with the glutamic acid (Glu286) as a valve in the proton pump in CcO [35]. This mechanism is mainly concerned with a gating situation which was only briefly discussed above (not indicated in Fig. 3), where the pump-proton in the PLS has to be prevented from leaking back when the reduced BNC is being protonated. As mentioned above, in the mechanism suggested by Wikström and coworkers, the proton transfers to both the PLS and the BNC are assumed to occur via a charge separation process where the Glu286 is deprotonated in the first step. Thus, when the reduced BNC is to be protonated, the Glu286 is negatively charged and the reprotonation must not occur from the protonated PLS, but rather from the N-side of the membrane. It is suggested that both kinetic and thermodynamic asymmetries in the position of the Glu286 side chain play important roles in preventing such a leak, making the Glu286 work as a valve in the proton pump [21,35]. The results from molecular dynamics simulations are the basis for this suggestion, showing that the Glu286 side chain flips between two positions, one “down” position where it is in contact with the N-side of the membrane via the D-channel, and one “up” position, where it is in contact with a cluster of water molecules leading either to the PLS or to the BNC [35]. The energetics of the two conformations was found to depend on the redox states of the cofactors, as well as the protonation state of the Glu286. The flip between the conformations was found to be very fast. The results from the simulations together with a set of constraints and assumptions, were used to incorporate the dynamics of the Glu286 side chain into the free energy diagram for one reduction step, published earlier in connection with the analysis of the kinetic experiments [21,27]. It should here be noted that the results from a thorough computational analysis by Cui and coworkers do not support the role of the Glu286 as a robust gating valve [34,36]. Furthermore, it is quite clear from the energy diagram presented in Ref. [21] that the flip of the Glu286 side chain, corresponding to an energy change of at most 4 pK units, would only describe a fraction of the energetics of the proton transfer and would not by itself prevent any back leakage of the protons from the PLS. In fact, an extra barrier of at least 10.6 kcal/mol and “which must find a different explanation” had to be introduced in the diagram [21] to prevent the pump-proton in the PLS to leak back to the unprotonated Glu286. In a later study, combined molecular dynamic simulations and continuum electrostatic calculations showed that the hydrogen bonding of the heme a_3 A-propionate to Asp407 dissociates via a rotation when heme a is reduced, yielding an increased pK_a value of propA and making it a likely position for the PLS [37]. The rotation of the propionate side chain was found to be connected with a barrier, and the reverse of this barrier was estimated to be at least 8 kcal/mol with heme a oxidized. It was suggested that this was close enough to the missing 10.6 kcal/mol to be sufficient to prevent the back leakage of the PLS proton. However, the simulations of the forward reaction were performed for the unprotonated propA, while the reverse rotation would occur for a protonated propA, and it is therefore not clear that this would give a reasonable estimate of the barrier for the reverse reaction. Furthermore, quantum chemical calculations on the models described below, indicate that protonation of the heme a_3 propA does not seem to be connected with such a side chain rotation, making the suggested gating mechanism rather unlikely. In fact, preliminary calculations on the largest models described below, indicate that it is a disadvantage for the protonated heme a_3 A-propionate to dissociate the hydrogen bond to Asp407, and that rather than lowering the energy, such a structure is connected to an energy cost of at least 10 kcal/mol.

Finally, the gating situation C in Fig. 3, i.e. when the pump-proton in the PLS is expelled from the enzyme, was not discussed in connection with the free energy diagram presented in Ref. [21]. However, from the energy diagram presented in Fig. 4 in that paper, the barrier for back leakage of the pump-proton to the N-side can be estimated to be only 7.3 pK units, to be compared to the barrier for pumping to the P-side of 10.2 pK units in the same diagram. This estimate is obtained by noting that the state labeled Vc (this is when the chemistry has occurred, but before the pump-proton is expelled) corresponds to the state labeled IIIc, but without the electron in heme a. Since there is no stabilizing negative charge in Vc the energy is raised to 1.7 pK units above the zero level. The value of 1.7 pK units is obtained by comparing state Vc and VI in the diagram. Then, going backwards from $+1.7$ pK units at position IIIc in the diagram to the N-side, the barrier is $9.0-1.7=7.3$ pK units, assuming that the transition state point itself is not affected by the redox state of the cofactors, which should be the case for a charge separation type of transition state [33]. In summary, this type of gate, with Glu286 as a valve, does not appear to prevent back-leakage.

In 2004 Propovic and Stuchebrukhov suggested a pumping mechanism for CcO based on continuum electrostatic calculations and stressing the importance of kinetic gating [38,39], and basically the same mechanism was still pursued in 2010 [15]. In contrast to the two mechanisms discussed above where the transfer of the pump-proton is coupled to reduction of heme a, in this mechanism a proton is loaded into the PLS when the electron moves from heme a to heme a_3. The proton is taken from the Glu286 which afterwards is reprotonated. In another step, a second proton transfer occurs, this time from Glu286 to the BNC for the chemistry. It is argued that proton transfer from Glu286 to the PLS is faster than proton transfer from Glu286 to the BNC, and therefore the first proton should go to the PLS and the second one to the BNC. There are arguments presented, referring to the structure, for this type of kinetic gating, but there are no calculations or estimates of any barrier heights to support the suggestion. As in most suggested mechanisms, in the next step the pump-proton is assumed to be expelled by repulsion from the chemical proton, and again it is argued that due to kinetics this will occur to the P-side and not to the N-side. A qualitative diagram was drawn showing a high barrier for back leakage to the N-side [40] but without explanation for the source of this kinetic gating. It is, in fact, even suggested that the pump-proton leaves the PLS before the Glu286 is reprotonated, and still moves against the gradient toward the P-side rather than toward the negatively charged Glu286. It is also argued that the second, chemical proton blocks the back transfer of the pump-proton to the Glu286 [15,39], which is not possible due to microscopic reversibility, as discussed above. The main specific suggestion in this pumping mechanism concerns the location of the PLS, which is different from all other suggestions (apart from the early and very different histidine cycle mechanism by Wikström and coworkers [5]). Based on pK_a values from the electrostatic calculations, it is concluded that the most likely pump loading site is His334 (*R. sphaeroides* notation), which is coordinated to Cu_B and hydrogen bonding via a water molecule to both propionates of heme a_3, see Fig. 4. However, the accuracy of this type of pK_a calculation is uncertain, and significantly different results for the same site at the same stage of reduction have been obtained by different researchers [38,39,41–43]. A detailed analysis of the kinetic experiment for one reduction step [14] was interpreted to give further support for His334 as a possible pump loading site [44]. In another study, possible proton exit channels in CcO were investigated, i.e. the path from the PLS to the P-side of the membrane, using similar electrostatic calculations and free energy evaluations [40]. It was estimated that the forward

barrier for the pump-proton from the PLS toward the P-side should be 140 meV (3.2 kcal/mol) and the backward barrier 400 meV (9.2 kcal/mol). It was concluded that this barrier should hinder proton transfer from the P-side to the PLS. However, with such a low barrier as 9.2 kcal/mol, the proton transfer from the P-side would be quite fast, on the order of μs. It should be noted that the study was published in 2005, before the experimental results showing that the forward barrier for the pump-proton is much higher than the estimated 3.2 kcal/mol, see Fig. 3 (step 5).

In 2005 Warshel and coworkers made a first attempt to calculate free energy profiles for proton and electron transfer in CcO [18]. This led to a sketch of a gating mechanism where the electron in the BNC stabilizes the positive charge during the uptake of the pump-proton, while the lack of negative charge in the BNC when the pump-proton is expelled leads to an increase of the same barrier, preventing back leakage to the N-side, somewhat similar to the first mechanism discussed above. The methodology has been further developed using Monte Carlo simulations on a millisecond time scale to calculate more accurate energy profiles for proton translocation pathways [20,45]. The methodology has been applied to investigate the energetic effects of mutations on the proton transfer processes, and to explain important experimental observations in mutational studies of the pumping mechanisms in CcO [28,45].

Already in 2006 Cui constructed an energy diagram for one reduction step in CcO including both the allowed reaction path with chemistry and proton pumping and two important forbidden pathways not leading to pumping [46]. The diagram is purely hypothetical without values or specific suggestions for the nature of the gates, but it illustrates the most important gating situations in an elegant way. In 2009 extensive computational investigations were performed by Cui and coworkers to determine different factors influencing the pK_a-value of the important Glu286 [36]. The elaborate computational model for CcO thus developed was recently used to examine some of the suggested mechanism for proton pumping, and it was concluded that neither Glu286 rotation nor water reorientation is likely to constitute key gating elements [34].

In 2008 Pomes and coworkers summarized a scheme for the elementary steps of proton pumping in CcO [13], which coincide very closely with the one discussed above as advocated by the Wikström group. Continuum electrostatic calculations were used to show that the pK_a-value of the Glu286 residue varies with the redox states of the cofactors in a pattern supporting the suggested scheme. The questions about the gates required to guarantee the directionality of the proton transfer were not addressed. The only gating mechanism mentioned is the proposal that the protonation of the Glu286 eliminates the possibility of proton back leakage through the D-channel [13], which is not possible, as discussed above. In a later study [47] free energy simulations were used to study conformational gating in the D-channel. However, this study was only concerned with the entrance of the D-channel and the effects on the proton uptake by mutations at the very entrance.

In 2008 Fee and coworkers performed quantum chemical calculations on models of a B-type cytochrome c oxidase aiming at a chemical mechanism for proton pumping [48]. A detailed study of the entire catalytic cycle partitioned into 14 different steps was presented, and it was suggested that the histidine directly hydrogen binding to the heme a_3 propA (His411 in *R. sphaeroides* notation) plays the role of the PLS. However, there is no gating included in the quantum chemical models used.

In 2008 Xu and Voth used the multi-state empirical valence bond molecular dynamics method to simulate the transfer of an excess proton in a chain of water molecules between Glu286 and heme a_3 propD [19]. The energy variation along the path was found to be rather small, 2–4 kcal/mol, and can therefore not describe any gating mechanism.

A recent (2011) QM/MM study by Varotsis and coworkers of the redox controlled proton transfer in cytochrome c oxidase will not be discussed here since the energetics obtained appears to be unrealistic with very large energy differences of about 70 kcal/mol for different structures, and artificial spin populations on the heme a_3 propA in some states [49].

2.3. Quantum chemical calculations

Quantum chemical methods have been used to investigate the chemistry and the catalytic cycle in cytochrome c oxidase, see e.g. Ref. [33] and references therein. However, until very recently it was considered impossible to construct quantum chemical models that could give more direct information on the energetics of the proton pumping in terms of reaction barriers involved in the gating mechanisms. Therefore, the recently published [26] DFT (B3LYP) calculations on large models consisting of about 250 atoms were the first ones trying to shed light on the gating mechanisms using a quantum chemical approach. The most important results from that study will be summarized below, together with some new results using even larger models of 390 atoms. The computational methods and models are described in the Appendix below. It should be noted here that the quantum chemical calculations are performed for the situation without electrochemical gradient. But as was shown in the previous study based on electrostatic considerations [11], the effects of the gradient can easily be added to the energy profile, leading to an equilibrium situation with no net proton pumping with full gradient.

The main purpose of the quantum chemical study in Ref. [26] was to try to calculate relative energies for the forbidden reaction paths, those not leading to proton pumping, which are more accurate than those shown in Fig. 3, and which are obtained from simple electrostatic considerations [11,12]. Since it is difficult to calculate accurate absolute pK_a-values, the only realistic goal is to obtain relative energies for limited parts of the proton pathways within the protein. Thus, a model was built containing Glu286 with its closest surrounding, considered to describe the TS_G-region, and the heme a_3 propA with its closest surrounding, considered to describe the PLS region. To study the effects on the energetics of the electron, heme *a* was also included in the model, yielding a total of 250 atoms [26].

The reliability of the 250-atom quantum chemical model could be tested by comparisons to some of the experimentally observed results. First, as described above, in several suggested mechanisms, the electron on heme *a* is assumed to increase the pK_a-value of the PLS, triggering uptake of the pump-proton. From the analysis of the kinetic experiment on one reduction step, it was estimated that this effect should be 5–7 kcal/mol [11,27]. This estimate is also obtained by simply concluding that the PLS, wherever it is, should not be protonated at the onset of the cycle, indicating a pK_a of 4–5 at most, and that after the electron uptake the PLS should be protonated, with a pK_a of at least 9. The calculated effect of heme *a* reduction on the pK_a-value of the assumed PLS (heme a_3 propA) using the 250-atom quantum chemical model was 6.5 kcal/mol, in very good agreement with the estimates based on experiment [26]. A second experimental quantity that could be calculated is part of step 2 on the observed reaction path, namely the energy difference for moving the proton from $TS_{G\text{-}2}$ to the PLS (state III), with heme *a* reduced, i.e. the reverse barrier for uptake of the pump-proton. With the assumption that there is an extra proton in the $TS_{G\text{-}2}$, a value of 16.4 kcal/mol was obtained for this energy difference, very close to the value derived from experiment, 15.8 kcal/mol (see Fig. 3). It could be concluded that the model should give reasonably reliable results for relative proton affinities, and also that the mechanism with an extra proton should be realistic. Therefore the model was used to calculate the barrier for the forbidden, not observed, reaction step, gate C in Fig. 3, corresponding to the energy difference between state V, with the proton in PLS, and $TS_{G\text{–}C}$ with heme *a* oxidized. The calculated value, still assuming an extra proton, was 15.3 kcal/mol, in very good agreement with the value shown in Fig. 3, 16.1 kcal/mol as estimated from simple

electrostatic considerations. Thus, the calculated value of 15.3 kcal/mol for the gate C barrier gives strong support for the suggested mechanism with a positively charged transition state. The same model was also used to calculate the relative energy of moving the Glu286 acidic proton itself to the PLS, an energy which would be involved as part of a barrier in several suggested mechanisms. The calculated value with heme *a* reduced was 20.3 kcal/mol, which strongly disfavors such a mechanism. However, see further below.

The model calculations in Ref. [26] basically support the suggested mechanism with a positive transition state for preventing back leakage of the pump-protons. However, there are a few aspects of the 250-atom model which make the conclusions somewhat uncertain. First, the calculated energy difference for moving the Glu286 proton to the propA of heme a_3, 20.3 kcal/mol, corresponds to a pK_a difference of almost 15 units, which is certainly not realistic. Furthermore, the calculated value depends strongly on the dielectric constant used in the calculations, decreasing to 13.6 kcal/mol if ϵ is increased from 4 to 10. This shows that the calculated value is uncertain and indicates that the model might be too small or in some sense unbalanced for obtaining this charge separation energy. It should be noted, though, that the results obtained for the mechanism with an extra proton, do not have the same problem. The values for this mechanism are quite stable with respect to the choice of ϵ decreasing by less than 2 kcal/mol if ϵ is increased from 4 to 10. Still, the model used is rather limited in the description of the surroundings of both Glu286 and the heme a_3 propionates. For example, the entire Mg-complex located close to the heme a_3 PropA is missing. Finally, the model cannot describe the detailed pathway of a proton moving between Glu286 and the PLS. To include the possibility to follow the proton pathway, not only a few water molecules have to be added, but also those amino acid residues that might interact with these water molecules.

Due to the uncertainties in the 250-atom model described above, it was decided to perform new calculations with a larger more realistic model. The basic idea in the mechanism with a positively charged transition state for proton transfer to the PLS, is that a positive charge in any of the two regions, around Glu286 (taken to be the location for the positively charged TS_G) and around the PLS, should be stabilized or destabilized by a similar amount from the presence or absence of an electron in heme *a*, since the distance to the heme *a* iron ion is about the same for the two regions. This means that the reverse barrier of step 2 should be approximately the same as the forward barrier of gate C, since the points III and V are identical apart from the electron in heme *a*, and as long as the (charge compensated) chemistry in the BNC is not considered. This is in agreement with the calculations using the 250-atom model, 16.4 kcal/mol was obtained for the reverse barrier of step 2, with the electron in heme *a*, and 15.3 kcal/mol for the gate C, without electron. Thus, the effect of the electron was established with the 250-atom model, and the new model therefore does not have to include heme *a*. Instead the description of the Glu286 and the heme a_3 propionate regions can be significantly improved, and also the connecting region. The amino acids included in the new model are listed in the Computational details and models section, and they are also indicated in Fig. 6.

Crystal structures of CcO show a large number of water molecules inside the protein, particularly in the D-channel for proton transfer, and in the region of the heme a_3 propionates. However, there is no clear hydrogen bonding connection between Glu286 at the end of the D-channel and the heme a_3 propionates (or the BNC) where the protons are supposed to move. Still, it is generally assumed that in the working enzyme there are water molecules available, which are not seen in the X-ray analysis. Therefore a number of water molecules were added to the quantum chemical model in this region. The results reported here are with six water molecules added, apart from the water molecules from the crystal structure, leading to a model with 390 atoms. This is the largest model investigated so far, and it can be noted that the main results are not very sensitive to the exact

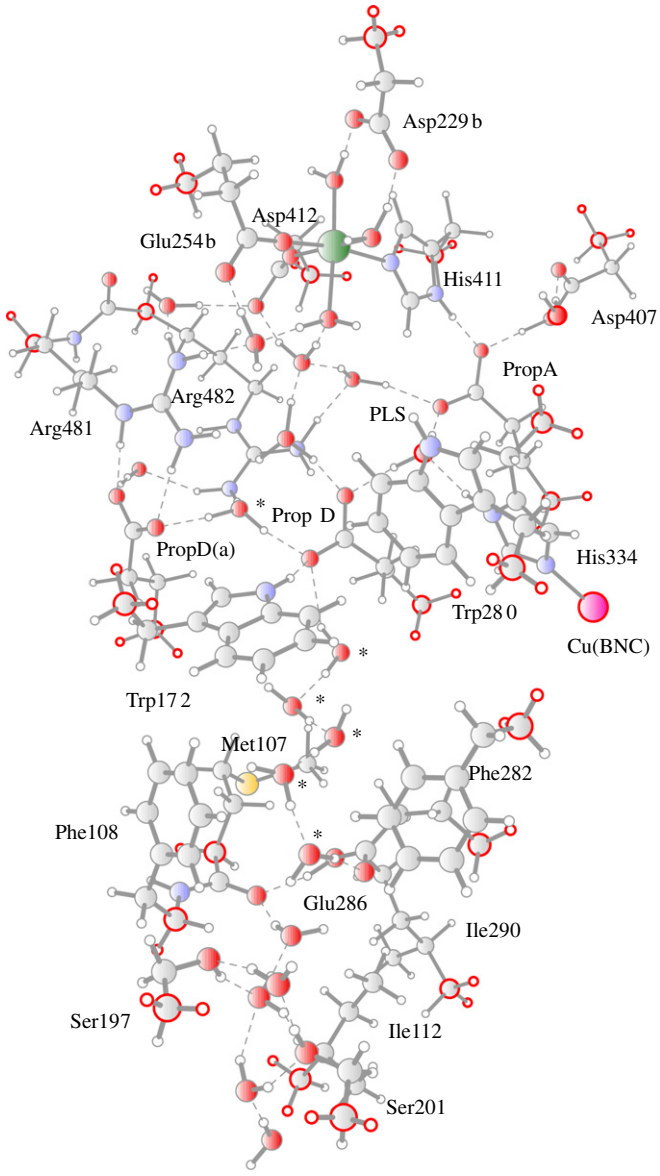

Fig. 6. The large model with 390 atoms. The asterisks mark the added water molecules. The atoms with a red circle are fixed from the crystal structure during geometry optimization.

number of water molecules included. A model with only five water molecules added gives quite similar results. The binding energies of the added water molecules were calculated, and they were found to be in the range between 14 and about 20 kcal/mol, which means that they are all bound relative to bulk water (14 kcal/mol is used as a reference for bulk water). Therefore, there should be no extra energy cost associated with inserting the water molecules into the models. Dispersion effects have been found to be important for the binding of small molecules in proteins, and they contribute on the average about 4 kcal/mol to the binding energies of the added water molecules [50,51]. The extra water molecules are marked with asterisks in Fig. 6. A difficulty with these added water molecules, not appearing in the crystal structures and therefore supposedly quite labile, is to find the configuration with the lowest energy for each state investigated. The search for global minima is still in progress, and therefore the results presented here are somewhat preliminary.

An unrealistic result obtained with the 250-atom model was the high energy cost of about 20 kcal/mol for transferring the acidic proton of Glu286 to the heme a_3 propA. With the new 390-atom model,

this value decreases to the much more reasonable value of 9.9 kcal/mol. Furthermore, the large dielectric effect has almost disappeared, the value is changed to 9.2 kcal/mol when ϵ is increased from 4 to 10. The large change of this relative energy, from 20 to 10 kcal/mol is easy to understand, since the extra water molecules stabilize the negatively charged glutamate, when the proton is in the PLS, more than they do for the neutral protonated glutamic acid. The starting structure for the unprotonated glutamate is taken from the alternative orientation of the Glu286 side chain observed in a mutant structure [52], which means that the oxygens point more "upwards" toward the propionates than in the wild-type structure. The calculated energy cost of 9.9 kcal/mol would be only part of the proton transfer barrier for the pumping mechanisms where deprotonation of the Glu286 is the first step. Preliminary calculations indicate that in the process of moving the proton from Glu286 to heme a_3 propA an even higher barrier occurs, of the order of 20 kcal/mol, which would indicate that this type of mechanism might not be energetically feasible. However, the calculated energy cost of moving the proton from Glu286 to heme a_3 propA of 9.9 kcal/mol corresponding to a pK_a difference of 7 units might still be somewhat too large, indicating that these calculated relative energies could be overestimated. Calculations are in progress to further establish the energetics of this type of process.

The results for the charge separation process are clearly improved for the larger model, in particular the fact that the ϵ dependence of the results has more or less disappeared. Therefore, also the mechanism with an extra proton was reinvestigated using this large model. As it turns out the calculated barrier for gate C with an extra proton becomes 15.4 kcal/mol using the 390-atom model, essentially identical to the result for the 250-atom model. Also this value is quite stable to the choice of dielectric constant, decreasing to 14.2 kcal/mol when ϵ is increased from 4 to 10. The structure for the approximate transition state is shown in Fig. 7. Thus, the mechanism with an extra proton, giving a positively charged transition state for transfer of the pump-proton, is supported also by the new, larger model. However, the very high similarity of the calculated values for the two models should be considered as fortuitous.

As described above, one objective with the design of the present large model was to try to calculate the energetics of a reaction path for moving the proton all the way from the PLS to the Glu286 (TS_{G-C}), corresponding to the gate C reaction path. As it turns out, with this model, the energy is quite insensitive to the exact position of the proton in the vicinity of the heme a_3 propA. The energy for putting the proton on propA or on one of the water molecules just above only varies within about 1 kcal/mol. Moving the proton to the heme a_3 propD oxygen hydrogen bonding to Trp172, increases the energy by about 5 kcal/mol with respect to the lowest point close to PropA. The energy increases by another 5 kcal/mol, to about 10 kcal/mol, when the proton is moved to one of the water molecules in the chain just "below" propD, and then increases further to 15.4 kcal/mol when the proton is located on the water molecule directly hydrogen bonding to Glu286, see Fig. 7. This is assumed to be the highest point on the proton path, since the model used does not allow the calculation of reasonably accurate energies for moving the proton further down the D-channel. In this investigation of the proton path, Glu286 is never deprotonated, the proton is just moving between the water molecules and the glutamate side chain is pointing in the same direction ("downwards") in all structures. In calculations on a slightly smaller model, with one less water molecule, other structures of the glutamate side chain were also tried, but they did not change the energetic picture. It can also be noted, that when the proton passes the heme a_3 PropD the structure of the propionate chain is only slightly changed to make it possible to insert a water molecule between the propionate oxygens and the Arg482, which seems to be much less of a structural change than the ones suggested in earlier computational investigations [19,20]. Using a model similar to the 250-atom model, but without heme *a* (just keeping its negatively charged D-propionate) the explicit energetics of insertion of a water molecule between the D-propionate of heme a_3 and Arg482 was investigated. It was found that such a water insertion was very close to thermoneutral, irrespective of the protonation state of Glu286, arguing against the suggestion that such a water insertion would be connected with the storage of some of the chemical energy [16]. The flexibility of the heme a_3 PropD side chain indicated by the present study is also in line with computational results obtained by Cui and coworkers [36].

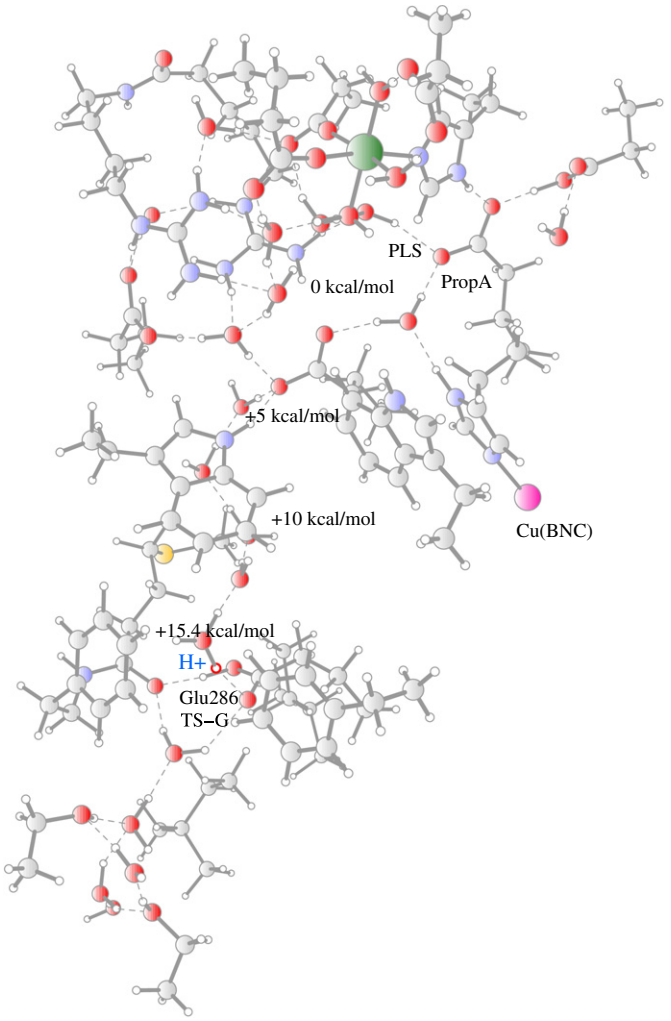

Fig. 7. Approximate transition state for proton transfer between the N-side and the PLS using the large model with 390 atoms. The extra proton is marked in red. Approximate relative energies for proton motion from the PLS to the TS_{G-C} are also given.

There is one significant change in the results as compared to the smaller 250-atom model. For the smaller model a rather high barrier was found for moving the proton between propA and propD on heme a_3 [26], while the larger 390-atom model gives no signs of such a barrier. The barrier obtained seems to be an artifact of the missing Mg-complex with all its ligands in the smaller model, and it is therefore interpreted as a problem with the smaller model rather than with the new one. There is, however, a potential problem with the present larger 390-atom model, which is connected to the fact that charged groups are located rather close to the boundary of the model (the carboxylate Mg-ligands), which introduces certain boundary problems. In the presently discussed results such problems are avoided by not allowing the proton to move close to the outer boundaries.

3. Summary

An analysis of the generally accepted steps of proton pumping in cytochrome c oxidase identifies three main critical gating situations.

The first one is trivial and concerns the problem that the proton must not be taken from the P-side to the PLS. This gating is simply handled by a transition state with a barrier, sufficiently high to block protons from the P-side, and sufficiently low to allow protons to go to the P-side from the PLS region at a reasonable rate (ms). The difference in height of these barriers is given by the driving force in the forward direction. When the driving force becomes zero for the full gradient, equilibrium has been reached.

The second gating situation is the most difficult one to account for. It concerns the step when the PLS proton should be expelled to the P-side by electrostatic repulsion from the second, chemical proton going to the BNC. The problem is that a sufficient force to push the PLS proton is not reached until the chemical proton has essentially reached the BNC. This means that without a gate, the D-channel is open for the PLS proton to go back to the N-side. The solution to this gating situation suggested here, is that there is a transition state for proton transfer between the N-side and the PLS which is positively charged. This means that when there is an electron on heme *a*, the gate is open due to the electrostatic stabilization of the transition state from the electron. On the other hand, when the negative charge of the electron is compensated by the chemical proton at the BNC, the gate is locked, since there is no longer any stabilization of the transition state, and the PLS proton is therefore forced to go to the P-side rather than to the N-side.

The third gating situation concerns the problem that the first proton taken up should go to the PLS and not to the BNC. This is solved first by having a very low pK_a at the oxidized BNC, i.e. before the electron has reached that point, and second by having low redox potentials for the electron acceptors in the BNC before the proton has reached the PLS. Furthermore, when the BNC is reduced, the chemical proton is taken from the N-side to the BNC rather than from the PLS, since in this situation the pK_a is lower on the N-side.

A quantitative diagram for proton pumping in CcO was first reached by an electrostatic analysis of experimental kinetic experiments for the O to E reduction step in the catalytic cycle. In order to obtain a mechanism with atomistic details, quantum chemical calculations on large cluster models have been performed. So far, these calculations support the electrostatic analysis.

Acknowledgement

We are grateful to Peter Brzezinzki for valuable discussions.

Appendix A. Computational details and models

Hybrid density functional, B3LYP [53], calculations have been performed on large models of parts of the proton pathway in cytochrome c oxidase, constructed from one of the crystal structures [54]. The structures were fully optimized, except for some atoms fixed from the crystal structure, using the double zeta basis set labeled lacvp in the Jaguar program [55]. The energies reported were obtained using the triple zeta plus polarization basis set, lacv3p**, in single point calculations and include an empirical dispersion correction to the energy [50]. Solvent effects from the surrounding protein were also included, using the self consistent reaction field approach with a dielectric constant of 4.0, in accordance with previous experience [56]. All energies reported are pure electronic energies with solvent effects included but without zero point or entropy effects. The calculations were performed using the Jaguar program [55].

An approximate proton path was obtained in such a way that the moving proton was fixed at different points along the path between heme a_3 PropA and the water cluster hydrogen bonding to the Glu286. In some cases a local minimum was obtained and in other cases an O – H bond length had to be fixed.

The large model was constructed by extracting the following amino acids from the crystal structure: Met107, Phe108, Ile112, Trp172, Ser197, Ser201, Trp280, Phe282, Glu286, Ile290, His334, Asp407, His411, Asp412, Arg481 and Arg482, all from subunit a. From subunit b Asp229 and Glu254 are included. The arginines are positively charged and the carboxylate amino acids are negatively charged, except for Glu286 and Asp407 which are protonated and therefore neutral. From heme a_3 both propionate chains were included in the model and from heme *a* only the D-propionate. In the earlier study it was shown that the A-propionate of heme *a* most likely is protonated [26], and since it then has no charge effect it was not included in the present model. The doubly charged Mg ion is included as well as a singly charged Cu ion. The latter represents the basic charge of the BNC. Several water molecules from the crystal structure are included in the model, together with six extra water molecules as discussed in the text. This gives a model of 390 atoms in a closed shell singlet state. The total charge is 0 for the case with an extra proton and −1 without it.

Cartesian coordinates for the most important structures are given in the Supplementary data.

Appendix B. Supplementary data

Supplementary data to this article can be found online at doi:10.1016/j.bbabio.2011.09.014.

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