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Energy diagrams and mechanism for proton pumping in cytochrome *c* oxidase

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Abstract

The powerful technique of energy diagrams has been used to analyze the mechanism for proton pumping in cytochrome *c* oxidase. Energy levels and barriers are derived starting out from recent kinetic experiments for the O to E transition, and are then refined using general criteria and a few additional experimental facts. Both allowed and non-allowed pathways are obtained in this way. A useful requirement is that the forward and backward rate should approach each other for the full membrane gradient. A key finding is that an electron on heme a (or the binuclear center) must have a significant lowering effect on the barrier for proton uptake, in order to prevent backflow from the pump-site to the N-side. While there is no structural gating in the present mechanism, there is thus an electronic gating provided by the electron on heme a. A quantitative analysis of the energy levels in the diagrams, leads to Prop-A of heme a_3 as the most likely position for the pump-site, and the Glu278 region as the place for the transition state for proton uptake. Variations of key redox potentials and pK_a values during the pumping process are derived for comparison to experiments.

Keywords: Cytochrome c oxidase; Theory; Proton pumping; Diagram; Mechanism

1. Introduction

Cytochrome *c* oxidase (CcO) is the terminal enzyme in the respiratory chain. It catalyzes the reduction of dioxygen to water and couples this reaction to the translocation of protons across the mitochondrial (or bacterial) membrane. The reaction catalyzed is

$$O_2 + 8H_i^+ + 4e^- \rightarrow 2H_2O + 4H_o^+$$

where H_i^+ are protons taken from the inside (N-side) of the membrane and H_o^+ are protons translocated, or pumped, across the membrane from the N-side to the outside (P-side) [1]. The translocated protons drive the synthesis of ATP where the energy of food consumption and respiration is stored. The general present view is that all four transitions, one for each electron, proceed in basically the same way, with one proton consumed in the dioxygen chemistry and one proton translocated. The present study specifically concerns one of these transitions, the transition from O to E, but should be reasonably valid also for the other transitions, see below, however.

The X-ray structure of CcO has been solved for two types of bacteria [2,3] and also for a mammalian species [4]. A schematic picture of the enzyme, showing the most important pumping elements, is shown in Fig. 1. There are four redox centers in the enzyme, Cu_A and heme a which function as electron transport cofactors, and Cu_B and heme a_3 which form the binuclear center (BNC), the active site for dioxygen reduction. The first redox center in the electron transfer chain is Cu_A, which obtains the electron from a cytochrome *c* on the outside of the membrane. From Cu_A the electron is transferred to heme a and then further to the binuclear center. Protons are taken up from the N-side to the binuclear center and for pumping along two different pathways, the D and the K channels. It is believed that at most one of the eight protons follows the K channel [5]. All four protons being pumped and at least three of the protons consumed in the dioxygen reduction should thus follow the D channel. Since there are indications that the K-channel proton is taken up in the O to E transition [6], this transition could be somewhat different from the other ones. However, this could be due to a possible difference between an inactive O state and the active O state,

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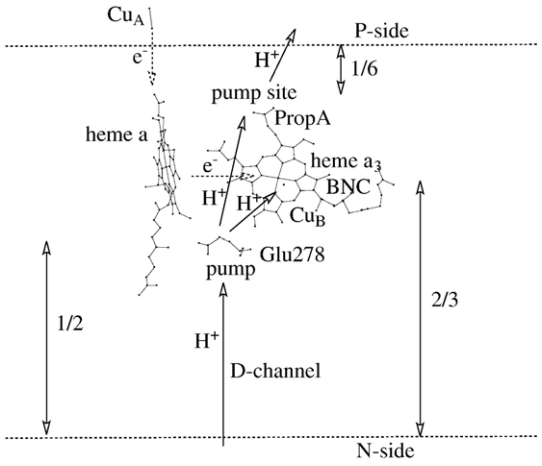

Fig. 1. Schematic picture of the most important pumping elements in CcO.

which was used in the kinetic experiments discussed below [7]. In Fig. 1 a few other groups are also shown. One of them is the heme a_3 propionate Prop-A, which has been suggested to play the role of the "pump-site", used as a temporary storage of protons on the translocation path. Another one is Glu278, which has been suggested to be in a critical region defining the transition state for proton transfer, and will therefore be termed "the pump" in the discussion below.

The mechanism for proton pumping in CcO is one of the most important mechanisms in biochemistry that still remains to be understood. Several suggestions have been made [8–17], each one probably containing some part of the true mechanism. For example, the role of the propionates of the hemes as pump-sites, and of the region around Glu278 as the pump, have been suggested, but there are also many other suggestions. The most difficult part to understand is how the protons can be allowed to move across the membrane from the N-side to the P-side against the potential gradient, but must not be allowed to move the other way, which is thermodynamically more favorable.

A major step towards a deeper understanding of the proton pumping mechanism was taken recently when detailed kinetic measurements for the O to E transition were made [7]. The present theoretical study is built upon the findings and interpretations in that paper. Most importantly, the sequence of events leading to pumping is taken directly from the interpretations in that work. The sequence suggested is initiated by an electron transfer to heme a from the outside via Cu_A. The next event was suggested to be a proton uptake from the N-side to a protonatable group, which leads to an equilibration of the electron between heme a and heme a_3 of the BNC. Another proton from the N-side was then interpreted to be transferred to the BNC completing the chemistry of that transition. Finally, in the rate-limiting step it was suggested that a proton is being pumped to the P-side. There has long been strong evidence for proton uptake upon reduction of heme a [10–15]. A general picture for pumping which starts by proton uptake to a protonatable group upon heme a reduction, followed by expelling the proton to the P-side as the next proton goes to the binuclear center, has therefore become the most common model for proton pumping. However, the recent kinetic experiments are probably the best evidence to date, that pumping actually proceeds in this way.

Many proton pumping mechanisms with minor modifications of the above scenario have been suggested, but there is one rather recent mechanism which is quite different from the others [16,17]. In that scheme, suggested on the basis of structural changes observed in a mutant enzyme and measurements for the P to F transition, the initial event is an electron transfer to the binuclear center, creating a high affinity for a proton. In the next step a proton goes from Glu278 to the binuclear center. The deprotonation of Glu278 leads to a conformational change, which results in an increase of the pK_a value of a protonatable group near prop-D on heme a_3, and therefore a fast uptake of a proton from the N-side. When Glu278 is reprotonated from the N-side, the structural change relaxes, and the proton near prop-D is expelled to the P-side.

The kinetic experiments also give a good guide-line to some of the intermediate energy levels in the process. For example, by the disappearance of one state and the appearance of another one, it can be concluded that the latter state is more than −3 kcal/mol lower in energy, but a more precise value cannot be obtained. This information is therefore not enough to fully describe a pumping mechanism. To reach the final mechanism, an iterative fine-tuning is needed, as described below, where the requirements for pumping and consumption are fulfilled both with and without a membrane gradient. The energy levels in the diagrams do therefore not critically depend on the absolute accuracy of the kinetic measurements. It should be clarified here that it is not strictly required that pumping occurs with the full gradient. What is required is that the chemistry at the binuclear center is equally fast or faster than the backflow of protons from the outside with the maximum gradient, otherwise the gradient would never reach this value.

To fully describe a functional pumping mechanism is an extremely difficult and complicated task. In this paper the powerful technique of energy diagrams is used to grasp the complexity of the process with all its necessary details. Energy diagrams have not been used much in the context of proton pumping, but a few attempts have been made. In some of the first cases, hypothetical diagrams were constructed to illustrate possible pumping mechanisms [18]. Some progress was reached in this way, the major one being the realization that a large barrier is needed for the final step where a proton is pumped from the pump-site to the P-side. This barrier needs to be on the limit of what is possible for a turnaround in milliseconds, in order to prevent protons from going the wrong way from the P-side to the N-side. However, many problems could not be overcome in these early approaches, due to too many possibilities to be investigated, and a diagram that would actually lead to pumping was not found.

Another type of approach is one where all relevant pK_a-values and redox potentials are computed. This was first attempted based on quantum chemical DFT calculations [19,20]. Even though this led to useful insight into the pumping mechanism, the absolute accuracy of the calculations was not sufficient for convincingly demonstrating an energy scheme that

leads to pumping. One problem is that quantum chemical models have to be limited in size. Molecular mechanical models do not have this problem since these calculations are much faster. Several studies have been performed using such an approach, and one of the main conclusions from those studies is that the His291 ligand of Cu_B plays the role of the pump-site [21,22,23]. The general mechanism for pumping was in those studies found to be similar to the main mechanism mentioned above, with the first proton taken up from the N-side going to the pump-site, followed by the ejection of this proton to the P-side as the second proton is taken up to the BNC. In one of those studies [22] a detailed kinetic model was built for generation of the membrane gradient, and in another one [23] a partial energy diagram was constructed for the proton exit step. In another theoretical approach, a Monte Carlo simulation method was developed which allowed simulations over milliseconds [24,25]. It was concluded that the critical proton transfer across Glu278 has to be concerted and not stepwise, if there should be a reasonably small barrier. Recently, calculations of a large number of possible pathways with barriers and thermodynamics, were made using this technique [26]. The results are still being analyzed for a possible pumping mechanism. Furthermore, electrostatic methods have been used to determine pK_a values and proton uptake for all residues during the individual stages of reduction of CcO [27], but the proton pumping mechanism was not addressed. A full functional energy diagram for proton pumping has not been found in any of these theoretical studies.

2. Energy diagrams

In this section the construction of the energy diagrams for proton pumping in CcO will be described. In the first subsection the experimental background is given. The analysis of the pumping mechanism starts out from the recent kinetic measurements for the O to E transition [7], and the sequence of events leading to pumping is directly taken from that study. The discussion is furthermore based on the X-ray structure of the enzyme [2,4]. Another experimental fact used is that the pumping cycle turns around in a millisecond. It is here assumed that the non-allowed pathways therefore should have rates on the order of 100 ms or higher. This means that the barriers for the non-allowed pathways should be $\geq$16 kcal/mol while the allowed pathways without membrane gradient should be $\leq$14 kcal/mol. The choice of 16 kcal/mol (100 ms) is a reasonable but somewhat arbitrary choice. However, it turns out that any value chosen in the allowed 15–18 kcal/mol range, gives a very similar picture of pumping with nearly identical critical barriers, see below. Another important experimental fact used is that the maximum membrane potential measured is 0.20 V [28]. This means that the chemistry occurring in the BNC must function up to this potential and be equally fast or faster than the backflow of protons from the P-side. Since the backflow of protons is limited by a barrier set to be 16 kcal/mol, as stated above, and this barrier is essentially independent of the membrane gradient, the allowed pathways should have barriers approaching 16 kcal/mol for the full membrane gradient. In fact, the reason the membrane gradient cannot exceed 0.20 V should be that backflow is equally fast as the forward reaction at this value. It turns out that this puts such severe restrictions on the energy levels and barriers that most of them will be very accurately specified. It is even possible, as shown in Section 3, to construct a reasonable energy diagram based almost solely on this requirement. Obviously, the diagrams will also depend on general assumptions of how pumping should be achieved. At the present stage, the simplest possible solutions will be preferred. When there are other, more complicated, solutions possible, these will just be mentioned in the text. The energy diagrams without and with a membrane gradient are discussed in two subsections below.

The notation of the states in the energy diagrams contains three protons and one electron. The inclusion of three protons is the minimum requirement, since one proton should be consumed in the chemistry at the binuclear center (BNC), one should be pumped to the outside (P-side), and the third proton is needed to describe the forbidden pathways for a proton initially on the outside. A proton on the inside (N-side) is labeled H_i^+, one on the outside H_o^+, one on the pump-site H_p^+, and one in the binuclear center H_B^+. The electron is similarly labeled with e_c^- if it is on cytochrome *c*, e_a^- if it is on heme a, and e_B^- if it is in the BNC. The initial state with two protons on the inside, one on the outside and an electron on cytochrome *c* is then $H_i^+H_i^+e_c^-H_o^+$. The desired end-point is $H_B^+H_o^+e_B^-H_o^+$. Each energy level is given a roman number, which is the same one as used in the description of the experiments.

The first useful energy that can be derived from known data is the driving force for proton pumping. Without any gradient across the membrane, the overall driving force for the catalytic cycle is obtained from the difference in redox potentials of the (donor) cytochrome *c*, which is taken to be the same as the one for heme a (0.27 V) [7], and the (acceptor) O_2/H_2O (0.80 V), and is therefore 4*(0.80–0.27)=2.12 eV (48.9 kcal/mol). This includes also the binding and cleavage of dioxygen which is here estimated to be −5 kcal/mol based on calculated values [19]. With four cycles of proton pumping, including the step O to E discussed in more detail here, the average driving force for each cycle will then be (48.9–5)/4=11.0 kcal/mol. With a maximum gradient of 0.2 V (4.6 kcal/mol), and with two charges moved across the membrane for every electron, the driving force with full gradient is 11.0–2×4.6=1.8 kcal/mol.

2.1. Kinetic experiments

As already mentioned above, the recent detailed kinetic experiments of the O to E transition [7], gives a deeper understanding of the pumping process and provides an excellent starting point for the present investigation. The results and interpretations of these experiments are shown schematically in Fig. 2. The experiments were initiated by an electron injection to Cu_A. After 10 μs, an equilibrium was observed between the electron on Cu_A (state I′) and on heme a (state II), with a 30 to 70% distribution of the electron. After 150 μs, a redistribution of the electron was detected with 40% remaining on heme a (state III), and with 60% on heme a_3 in the BNC (state IV). This

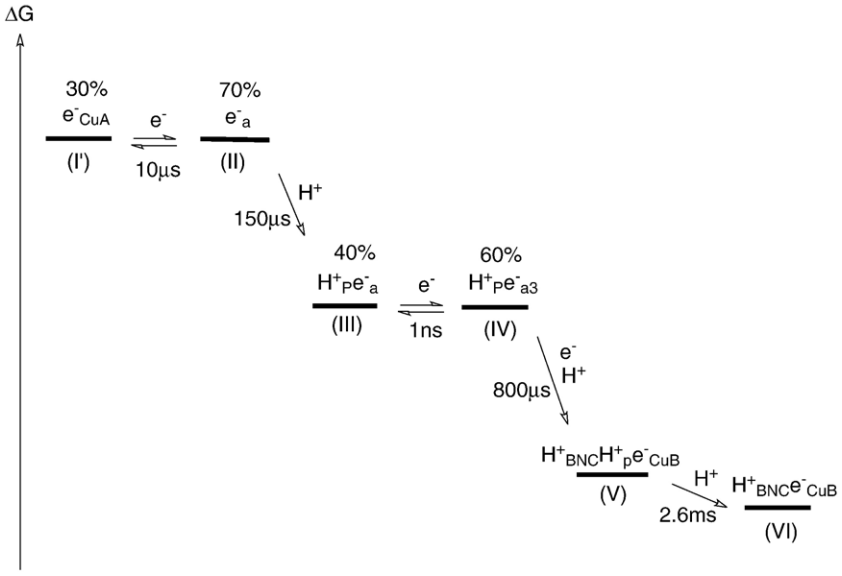

Fig. 2. Schematic picture of the results and interpretations of the kinetic measurements for the O to E transition [7].

transition was found to be accompanied by a proton motion perpendicular to the membrane, interpreted to be a proton uptake from the N-side to a protonatable group in the membrane. After an additional 800 μs, the electron disappears from both heme a and heme a_3, and is now on Cu_B (state V). Also this transition is accompanied by a proton motion perpendicular to the membrane, interpreted to be an uptake from the N-side to the BNC. Finally, in the rate-limiting step requiring 2.6 ms, a proton was interpreted to be pumped to the P-side (state VI). These results will be used as a starting point for the diagrammatic analysis described below.

2.2. Energy diagrams without a membrane gradient

In this subsection, the energy levels and barriers for the energy diagrams used in Figs. 3 and 4 are described. For the barriers between the different levels, the experimental rates are transformed to free energy barriers using transition state theory,

$$k = \kappa k_B T/h^* \exp(-\Delta G^{\ddagger}/RT)$$

where $\Delta G^{\ddagger}$ is the free energy barrier height and the transmission coefficient κ is set to 1.0. It should in this context be noted that the barrier heights are very accurately determined by the rates due to the exponential dependence, and the analysis does therefore not critically depend on the accuracy of the rate measurements. For example, the rate limiting step has a measured rate of 2.6 ms. If it would be 1 ms, this would only affect the barrier height by 0.6 kcal/mol, which is such a small amount that it has almost no influence on the mechanistic discussion. In a later section, relevant redox potentials and pK_a values will also be derived. These values are not used in the construction of the diagrams, but are given here mainly as a background for discussing the pumping mechanism and assess the credibility of the energies in the diagrams. The values derived could also be useful in future experimental tests of the mechanism.

2.2.1. Energy levels

As a starting point, approximate energy levels can be taken from the kinetic experiments. They can only be more precisely specified once a full energy diagram with membrane gradient has been constructed. The energy levels and barriers will then be adjusted to fulfill the requirement that the maximum membrane potential is 0.20 V. For the first state I, $H_i^+H_i^+e_c^-H_o^+$, the energy is set to zero. Experimentally, the redox potentials of cytochrome *c* and heme a are very similar and the electron transfer between them therefore puts the resulting level II also at zero energy. In state II, the electron is now in equilibrium between Cu_A and heme a.

After the next transition, which requires 150 μs, two states were found to be in equilibrium, III and IV. This transition was interpreted as a proton uptake from the N-side to a protonatable site in the membrane. The electron is now observed on heme a_3, and since it has completely left Cu_A, states III and IV must be at least −2.7 kcal/mol down in energy from II. Since the measurements were made at pH 8, the energy levels of III and IV will be lowered further by one pK_a unit at pH 7, down to or below −3.4 kcal/mol. In order to avoid a too high barrier (higher than 16.0 kcal/mol) from IV to V with membrane gradient (see discussion below) these levels will be placed at −5.0 kcal/mol, see Figs. 3 and 4.

In the transition from IV to V a second proton is taken up from the N-side in an 800 μs event. It was interpreted to go to the binuclear site and probably protonates an OH ligand of Cu_B while the electron at heme a_3 moves to reduce the copper. No significant electron occupancy is now observed in either heme, which means that state V is at least −2.7 kcal/mol below state IV under the experimental conditions (pH 8). At pH 7 the energy of state V will be −3.4 kcal/mol or more lower than state IV. This puts the energy level of state V at −8.4 kcal/mol or lower. To make the barrier for the back reaction from VI to V 16.0 kcal/mol, as was chosen for the non-allowed paths (see above), the value of −8.9 kcal/mol will be used for V. Another limiting value for the

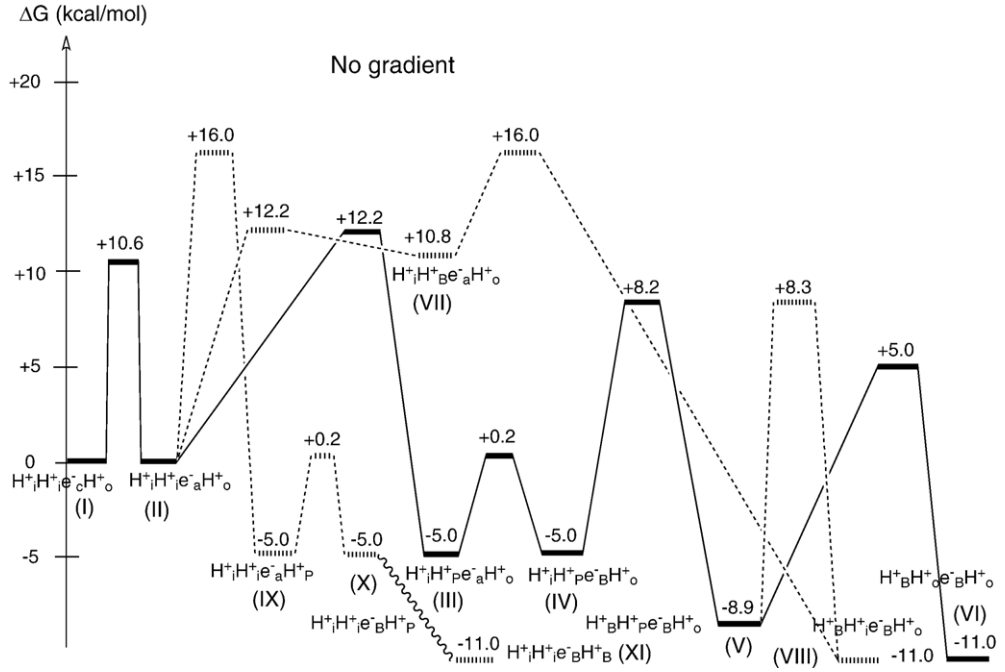

Fig. 3. Energy diagram for proton pumping without any membrane potential. Three protons and one electron is included. Full lines show the suggested pathway. Dashed lines show non-allowed pathways.

energy of state V can also be obtained from the fact that state V has to be higher than state VI also with membrane gradient, which means that state V must be higher than −10.2 kcal/mol.

2.2.2. Barriers

To fully define the preferred path requires assignment of energy barriers as well. Nearly all of the ones along this path can be obtained from the experiment described above. Using transition state theory, the first transition from I to II will have a barrier of 10.6 kcal/mol, which corresponds to the observed time constant of 10 μs. For the next transition between II and III (time constant 150 μs) the corresponding barrier is 12.2 kcal/mol. Reaction II to III is a pure heme–heme electron transfer which due to the short distance should occur in about one nanosecond

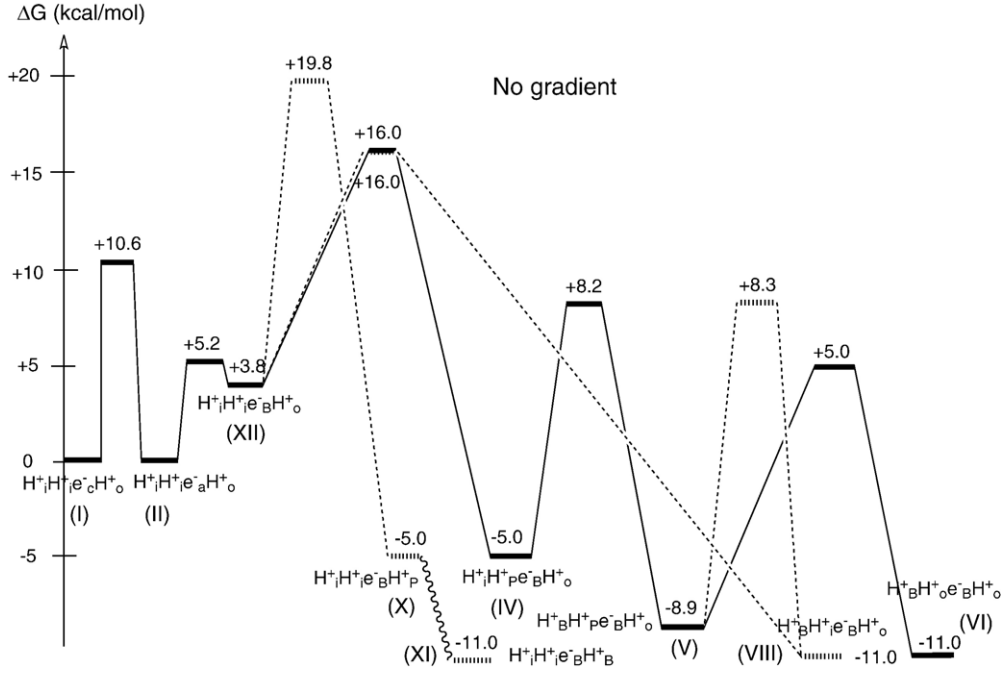

Fig. 4. Energy diagram for proton pumping without any membrane potential. Three protons and one electron is included. Full lines show the suggested pathway. Dashed lines show non-allowed pathways.

[29–31], which corresponds to a barrier of 5.2 kcal/mol. IV to V (τ=0.8 ms) has a barrier of 13.2 kcal/mol, and the final rate-limiting step V to VI (τ=2.6 ms) a barrier of 13.9 kcal/mol.

If an energy diagram should explain the pumping mechanism, it is not enough to describe a pathway that gives the desired result. It is at least as important to show that the pathways which do not lead to pumping have prohibitively high barriers. As mentioned above, the rate-limiting barrier for the allowed pathway is 13.9 kcal/mol, from V to VI, giving a turnaround on the order of milliseconds. A reasonable assumption is then that the non-allowed pathways should all have barriers of 16 kcal/mol or higher (corresponding to a time constant on the order of 100 ms from transition state theory). In principle it is not strictly required that all non-allowed pathways must have barriers higher than 16 kcal/mol. A slightly lower barrier can sometimes be sufficient, as exemplified below, if the competing allowed pathway at that point has a barrier lower than 14 kcal/mol. However, for simplicity the same requirement is here used for all of the non-allowed pathways.

In the context of defining the non-allowed pathways, a general assumption has been made. This is that only certain pathways are considered possible for proton transfer, namely the ones that are used for consumption and pumping. All other pathways are assumed to have prohibitively high barriers in both directions. Furthermore, it is assumed that proton uptake to both the pump-site and the BNC have to pass the same transition state structure. This means, for example, that if a proton should move from the pump-site to the BNC it has to go over the same TS structure as the one going from the N-side to the pump-site. In other words, there should be no low-barrier shortcut for the protons to go between the pump-site and the BNC. These requirements should be rather easy to fulfill in the evolution of the enzyme. Instead, the difficulty lies in allowing proton transfer along a path with low barriers in the forward direction only under certain well-defined circumstances, while disallowing it under other circumstances, and to prevent leakage in the reverse direction. These non-allowed pathways are the only ones discussed below. It is, of course, possible that the enzyme has found a more complicated solution to the pumping problem, with several different paths and different TS structures, but at the present stage only the simplest possibilities are considered.

The first non-allowed pathway discussed here is the one where a proton from the P-side at level II goes back to the pump-site leading to level IX. The barrier for this step should be identical to the one going back from VI to V, which is 16.0 kcal/mol. This barrier therefore fulfills the criterion that the barrier for a non-allowed pathway should be 16 kcal/mol or higher. From level IX, levels X and XI can be reached by an initial heme–heme electron transfer followed by a proton transfer from P to the BNC. The energies of IX, X and XI are given from the discussion above to −5.0, −5.0 and −11.0 kcal/mol, respectively. The barrier between IX and X is 5.2 kcal/mol, since it should be the same as the one between III and IV, while the barrier between X and XI should be the same as the one between V and VIII, 17.2 kcal/mol. This latter barrier is in practice not required, since the initial barrier along this pathway is prohibitively high, and it is therefore not shown in the diagram in Fig. 3. The details of this path are shown in a diagram in the supporting material.

Two more non-allowed pathways are shown in Fig. 3. One of them is the pathway from V to VIII, in which a proton is going back from the pump-site to the N-side rather than going to the P-side at the stage when both an electron and a proton have reached the BNC. This back-leakage reaction is quite critical and turns out to put strong restrictions on the character of the transition state for proton transfer. The back-reaction from VIII to V is similar to the forward reaction from II to III, which has a barrier of 12.2 kcal/mol, the difference being that for II to III there is an electron on heme a. If the barrier was unaffected by the presence of the electron on heme a, the back-reaction from VIII to V would therefore also have a barrier of 12.2 kcal/mol. In turn, this means that the barrier in the forward reaction from V to VIII would be only 10.1 kcal/mol, and pumping would not work. A large effect on the barrier from an electron on heme a is thus a major requirement for pumping. Since the electron on heme a stabilizes a proton on the pump-site in III by 7.1 kcal/mol, see below in Section 2.4, it needs to stabilize the transition state structure by a similar amount.

From the above it is clear that an electron on heme a must have a dramatic lowering effect on the barrier for proton transfer if pumping should be achieved. From this, two conclusions can be drawn. First, the transition state for proton transfer must be positively charged. Second, the local transition state for proton transfer must lie relatively close to heme a. This is most easily achieved if the barrier for proton transfer is determined by moving a proton to the transition state area, which should be close to heme a, everything else remaining the same. When the gradient is discussed below, it is furthermore found to be advantageous if the TS for proton transfer is located closer to the N-side than the pump-site is. Glu278 is a site that is located at a proper position to define such a transition state area. Since the pK_a of Glu278 is quite high, it is already protonated and cannot accept another proton. The proton should therefore go to a nearby molecule, like a water molecule, at the TS, and the barrier height for proton transfer should thus be determined by the pK_a value of this molecule. It is also important that the character of the TS remains the same even with a full membrane gradient, and this is satisfied with a sufficiently high pK_a for Glu278. A lower limit will be given below. It should furthermore be noted that with a high pK_a value for Glu278, the barrier height will not depend on this value, but only on the pK_a value of the water molecule close to it, where the proton resides in the TS. For these reasons, Glu278 is assigned as the area for the transition state for proton pumping, sometimes referred to as the “pump”. It is interesting to note that a transition state of the type described above has recently been suggested based on model calculations [24]. A minor problem in the context of the character of the pump, as described above, is to explain how the measured rate for the IV to V transition could be so similar to the one for the II to III transition. With a positively charged TS, a larger difference than 1 kcal/mol for the barrier heights is expected due to the repulsion from the proton on the pump-site in the IV to V transition. A possibility

could be that the proton leaves the pump-site during the IV to V transition, temporarily or permanently, further away from the pump as the proton from the N-side approaches the Glu278 region. Only a detailed optimization of the transition state for this process could resolve this problem.

A transition state in the area of Glu278 fits the present energetics very well. Still, another site for the TS can obviously not be ruled out. For example, one possibility could be that the TS for proton uptake from the N-side to the pump-site is defined by the passage of a proton over Prop-D of heme a_3. This could be a natural place for a TS. There are two complications with the use of a TS at Prop-D. First, as will be discussed below, it is much further to the P-side than Glu278 is, which means that the barriers for pumping will be more strongly affected by the membrane gradient. Second, a TS in the Prop-D area for proton transfer to the pump-site requires another TS for proton transfer to the BNC. Both of these problems may be possible to overcome, but at the present stage only the simplest possibility will be considered.

Another assumption about the transition state, which was extensively tried here, is one where the barrier is determined by a local deprotonation of Glu278 into GluO- and H^+, close to each other, i.e. without any change of charge of the TS. However, this would lead to back-leakage from V to VIII, since the local barrier would be essentially unaffected by an electron on heme a.

With the above definition of the pump as the region of Glu278, the barrier between V and VIII is set to be the same as the one for the back reaction from III to II. This can be done since the pump is at about the same distance from heme a as the pump-site is. The reactant (III) for this back-transfer with a proton on the pump-site, and the TS where the proton has moved to this position, should therefore be about equally strongly affected by the electron on heme a. The barrier between these positions for the proton should therefore be unaffected, and therefore similar to the barrier between V and VIII where there is no electron on heme a. This means that this back-reaction, in sharp contrast to the forward reaction, is unaffected by the electron on heme a. The barrier should therefore be about 17.2 kcal/mol and is set to this value. It should be noted that the electron at BNC does not have any effect on the V to VIII step, since its charge is compensated by a proton.

In the third non-allowed pathway from II via VII to VIII, the proton first moves to the BNC followed by the electron from heme a. This pathway is not observed in the kinetic experiment and, following the general analysis made here, the barrier should therefore be at least 16 kcal/mol (see below, however). There are a few possibilities to satisfy this requirement. The choice adopted here is to set the endergonicity for the proton transfer to BNC, without an electron there, to 10.8 kcal/mol (level VII). With the additional barrier of 5.2 kcal/mol for the heme to heme electron transfer (see above), this leads to a total barrier of 16.0 kcal/mol, see Fig. 3. Quantum chemical calculations actually do give an endergonicity of 10 kcal/mol for this type of proton transfer [20], but this could be an overestimate since a rather small model was used. A slightly lower total barrier of 15 kcal/mol between VII and VIII could also work, since the competing allowed pathway from II to III only has a barrier of 12.2 kcal/mol and is essentially irreversible when III is reached. This choice would reduce the endergonicity requirement to 9.8 kcal/mol for the II to VII step. It could be reduced further by making the barrier higher for the heme to heme electron transfer in VII to VIII. If this electron transfer barrier instead was set to 8 kcal/mol, the required endergonicity for II to VII would be down to 5 kcal/mol, which may be a more realistic value than 10.8 kcal/mol.

Another possibility to satisfy the requirement of a high barrier between II and VIII is to put the proton transfer barrier between II and VII (from the inside to the BNC) to 16 kcal/mol. A problem in this context is that the proton transfer barrier from IV to V (also from the N-side to the BNC) was experimentally determined to be only 13.2 kcal/mol. There are two differences between these situations. The first one is that in IV to V a proton on the pump-site increases the barrier by 1.0 kcal/mol, indicating that the barrier between II and VIII on this ground should be only 12.2 kcal/mol. The second difference is that in II to VII the electron is on heme a, while it is on the BNC in IV to V. This difference has been suggested to cause an increase of the barrier through an unfavorable water alignment towards the pump-site [32]. If this increase would be 4 kcal/mol, it would lead to a barrier between II and VII higher than 16 kcal/mol.

Another diagram with possible paths is shown in Fig. 4. When level II has been reached, these paths start out with an electron transfer from heme a to the BNC, in contrast to the previous diagram which at level II starts out with a proton uptake from the N-side to the pump-site. The electron transfer to the BNC defines level XII, which should be endergonic by ≥ 3 kcal/mol, since the electron is experimentally observed not to be on heme a_3 in state II. There is one pathway, in particular, which has to be prevented once state XII is reached, and this is the subsequent proton transfer to the BNC to reach state VIII, which would shortcut pumping. The local barrier from XII to VIII for this proton transfer should be the same as the one observed between II and III of 12.2 kcal/mol. To reach altogether ≥ 16.0 kcal/mol for the barrier from II to VIII, state XII therefore has to be placed at $\geq +3.8$ kcal/mol. For this reason level XII is put at 3.8 kcal/mol. The barrier between II and XII should be 5.2 kcal/mol as in the corresponding cases above.

From XII there are three options. In the first case, a proton will go from the N-side to the pump-site to reach level IV, which in principle could be an allowed pathway, therefore drawn with a full line in Fig. 4. However, the overall barrier for this transfer should be $(3.8+12.2)=16.0$ kcal/mol, which should make this pathway too slow in practice. A smaller value than +3.8 kcal/mol is not possible. A higher value is possible, but is here considered to give a too large effect of the electron on heme a_3 on the pK_a value of the protonatable site in the BNC [19]. Continuing along the fully drawn pathway from IV leads over to exactly the same paths as discussed for the diagram in Fig. 3.

In the second option from level XII, a proton should be taken up from the N-side and go to the binuclear center, to level VIII at −11.0 kcal/mol, with an overall barrier again of $(3.8+12.2)=16.0$ kcal/mol, making this pathway non-allowed as already

discussed above. In the third case a proton from the P-side goes to the pump-site, from level XII to X, and then on to level XI by a proton transfer from the pump-site to the BNC. The barrier from XII to X should be 16.0 kcal/mol since it should be the same as the one between VI and V. Adding the energy of level XII at +3.8 kcal/mol makes the overall barrier prohibitively high at +19.8 kcal/mol. Levels X and XI were already discussed in the previous diagram and were placed at −5.0 and −11.0 kcal/mol, respectively. The barrier from X to XI is not needed since this pathway is already non-allowed.

A diagram showing a more complete picture of the non-allowed paths without a membrane gradient is available in the supplementary material.

2.3. Energy diagram with a full membrane gradient

The main principle used here in translating the energy levels for the intermediates from the case without to the one with a full membrane gradient is quite simple. The full membrane gradient potential is 0.20 V, equivalent to 4.6 kcal/mol of energy. This means that for a proton on the P-side the energy is raised by this amount. The gradient is furthermore assumed to be linear from the P-side to the N-side, which means that for a proton position located 2/3 of the way from the N-side, an energy of $2/3 \times 4.6 = +3.1$ kcal/mol is added. For an electron on a corresponding distance from the N-side, the energy is instead lowered by the same amount. Heme a and BNC are located at this distance from the N-side. The best possibility for the assumed pump-site is the prop-A group on heme a_3 (see further below), and therefore the pump-site is assumed to be located 5/6 of the way from the N-side, giving a gradient effect of +3.8 kcal/mol. This leads to the energy levels as shown in Fig. 5. The energy levels shown in Fig. 4 are not drawn in the diagram with full membrane gradient, since the pathways shown there were already forbidden without the gradient, and the barriers will just increase with the gradient.

The way the barriers should be translated to the case with a full membrane gradient is less obvious. In order to do this, the positions and characters of the transition states for proton transfer are needed. As mentioned above, only one TS structure for proton transfer to or from the N-side is considered in the present analysis. This means that when a proton is taken up from the N-side to go either to the pump-site or to the BNC, it has to pass the same TS structure. There should furthermore not be any shortcut for a proton going from the pump-site to the BNC. In a previous section, this transition state was defined as one where a proton has been moved to the pump, everything else staying essentially the same. As already mentioned, the pump is suggested to be located close to Glu278, which is at about half the distance from the N-side to the P-side. This means that for a proton at this position, like in the TS, an energy of +2.3 kcal/mol should be added.

With the definition of the pump as the region around Glu278, which defines the TS for proton uptake from the inside, there is only one more proton transfer TS that needs to be defined. This is the TS for the final rate-limiting step, where a proton is pumped to the P-side. For simplicity it is here assumed that the character of this TS is the same as the one for the pump, which means that the TS is charged by +1, from a proton motion to the TS region, everything else remaining the same. Since it is important that the reverse barrier for proton leakage from the P-side remains high at all instances of the pumping, this barrier should not be affected by an electron on heme a or in the BNC. A large distance between this TS to both heme a and BNC is therefore required. To disallow leakage, the barrier from the P-side was set to

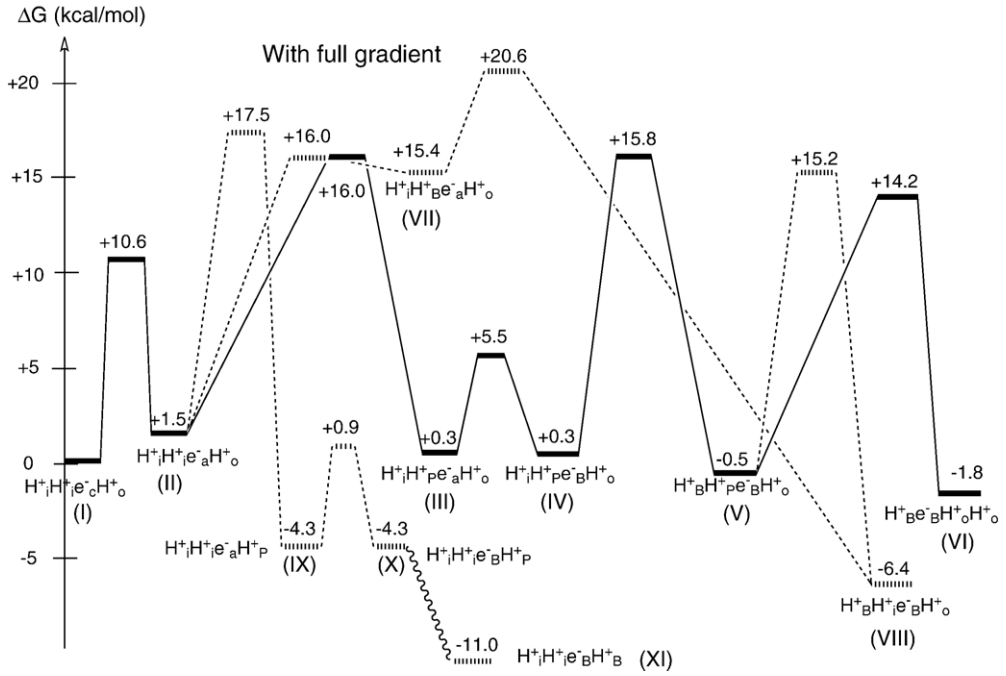

Fig. 5. Energy diagram for proton pumping with full membrane potential (0.20 V). Three protons and one electron is included. Full lines show the suggested pathway. Dashed lines show non-allowed pathways.

16.0 kcal/mol without gradient. Ideally, this barrier should not be lowered by the gradient. This is fulfilled by positioning the TS at the boundary to the P-side. Some flexibility is obviously allowed. For example, if the TS is positioned further down in the membrane the barrier would be somewhat lowered by the membrane gradient. This would require that the barrier from the P-side would be set to a larger value than 16.0 kcal/mol without gradient, etc. However, the flexibility is not high. Changes of significantly more than 1.0 kcal/mol will have chain effects on the rest of the energy diagram, which would be difficult to repair.

As discussed above, a particularly critical barrier is the one on the non-allowed pathway between V and VIII. This pathway describes a proton transfer back from the pump-site, when a proton and electron has already reached the BNC. With the pump-site at 5/6 the distance and the pump at 1/2 the distance from the N-side, the barrier for V to VIII should decrease by 1.5 kcal/mol. Since the barrier without gradient is 17.2 kcal/mol, the barrier with gradient will be 15.7 kcal/mol. Even though this is slightly below 16 kcal/mol, it is slower than the alternative pathway with pumping towards the P-side. It is clear that the positioning of the pump and the pump-site are very critical for preventing this back-reaction. Within the present framework, there are not many options for choosing the pump at a different site than the region around Glu278 or to choose the pump-site far away from Prop-A of heme a_3.

It is critical for the mechanism described in the diagrams, that the character of the TS at the pump will remain the same even when the membrane gradient is applied. To make sure that this is the case for the full gradient, the pK_a of Glu278 should be sufficiently high so that it never decreases below the pH of the N-side of 7.0. Since Glu278 is positioned 1/2 of the way from the N-side to the P-side of the membrane, this means that its pK_a without any gradient should be $\geq 7+1/2\times 4.6/1.4=8.7$. For this limiting value the effective pK_a should approach 7.0 for the full gradient. The measured pK_a for Glu278 of ≥ 9.5 [33] fulfills this criterion.

A diagram showing a more complete picture of the non-allowed paths with a membrane gradient is available in the supplementary material.

2.4. Redox potentials and pK_a values

This discussion of the pK_a values and redox potentials will only concern the case without a membrane gradient. The analysis starts out from the redox potential of heme a, measured to be 0.27 V before the pumping process has started [7]. After proton uptake to the pump-site, the energy decreases by −5.0 kcal/mol, see level III in Fig. 3, indicating an increase of the redox potential of heme a by 0.22 V to 0.49 V. The pK_a value of the pump-site thus becomes $7+5.0/1.36=10.7$ with the electron on heme a. The pK_a value before the pumping process has started can be obtained from the energy difference of 2.1 kcal/mol between levels V and VI in Fig. 3 as, $7-2.1/1.4=5.5$. An electron on heme a thus raises the pK_a of the pump-site by $(10.7-5.5)=5.2$ units, i.e. a stabilization of a proton on the pump-site by 7.1 kcal/mol.

From the energy levels in Fig. 4 it is also possible to obtain the redox potential for heme a_3, which is the electron acceptor in the binuclear center in the 150 μs phase. The energy difference between levels II and XII of +3.8 kcal/mol, leads to the redox potential of heme a_3 before the proton has reached the pump-site of $0.27-3.8/23.1=0.11$ V. Since states III and IV are approximately isoergonic, the redox potential of heme a_3 is the same as that of heme a after the proton has reached the pump-site, i.e. 0.49 V, an increase by 0.38 V. With an electron at heme a_3 the energy decreases by −8.8 kcal/mol upon uptake of a proton from the N-side to the pump-site (level XII to IV), which means that the pK_a value of P is raised to $7+8.8/1.36=13.5$. This corresponds to an increase of $(13.5-5.5)=8.0$ units, and a stabilization of a proton on the pump-site by 10.9 kcal/mol.

From the effects on the pK_a value of the pump-site by an electron on heme a (+5.2 units) and by one on heme a_3 (+8.0 units), it is possible to suggest a likely position for the pump-site. If it is assumed that there is only one pump-site, the ratio of the effects of 1.5 indicates that the pump-site should be 1.5 times closer to heme a_3 than to heme a. Measuring the distances from one of the oxygens of Prop-A of heme a_3 to the iron-centers of the two hemes, leads to a distance ratio of 1.7 making it an excellent candidate for being the pump-site, particularly considering the somewhat arbitrary choice of distances. By the same procedure, Prop-D has a distance ratio of only 1.1, and the δ-nitrogen of the Cu_B ligand His291 a ratio of 2.3, which makes both of them unlikely candidates for being the single pump-site at the present stage. In summary, the positioning of the pump-site at ProP-A of heme a_3 relies on three different energy differences taken from the diagrams in Figs. 3 and 4. The first one is the energy difference between levels V and VI of −2.1 kcal/mol, giving the pK_a value of the pump-site at the start, before the pumping process is initiated. The second one is the energy difference between levels II and XII of +3.8, giving the difference in redox-potentials between heme a and heme a_3 at the start. The third one finally, is the energy difference between levels II and III of −5.0 kcal/mol, giving the pK_a value of the pump-site after the electron has reached heme a. Another positioning of the pump-site would require that one or more of these values are changed quite substantially. For example, the most uncertain energy level involved here, level XII, would have to be raised by more than 5 kcal/mol to give a ratio corresponding to the position of His291. Such a large change, resulting in a pK_a value of about 17 for the pump site when the electron is in heme a_3, is quite unlikely.

3. Alternative derivation

The derivation of the energy diagrams discussed above, started out from the kinetic experiments, and then made adjustments as the consequences became clear for the barriers for the allowed and non-allowed pathways, and also for the effects of the membrane gradient. In retrospect there is an alternative way to derive approximate energy diagrams, which is useful to consider for judging the final accuracy of the diagrams. As above, the lower limit for the barriers for the non-allowed pathways are again set to 16.0 kcal/mol, including the

one for leakage of protons from the P-side. Furthermore, it is assumed that at full gradient (0.20 V) the forward and backward flow of protons occurs at the same rate. The argument is that it is the equality of the rates in the forward and backward directions that defines the maximum gradient which is achievable. The simplest assumption is then that all of the proton transfer barriers in the forward direction are also 16.0 kcal/mol with full membrane gradient. Concerning the energy levels of the intermediates with full membrane gradient, it is important to note that the total driving force is very small, only −1.8 kcal/mol. This leads to rather strict requirements on the energy levels. For example, all the energy levels must be higher than level VI at −1.8 kcal/mol to prevent back-flow of protons from the outside. The energy levels of the intermediates cannot be much higher than a couple of kcal/mol above zero, either. For example, from the character of the local barrier between IV and V it follows that it has to be higher than the one between II and III, requiring that level IV is lower than level II. This means that levels II, IV and V all have to be close to zero in energy. The positioning of level III has little or no impact on the mechanism. To start with, level II is set as above to +1.5 kcal/mol since I and II are experimentally degenerate without gradient. From these requirements it is here assumed that the levels from II to VI go down linearly in energy. This puts level III at +0.7 kcal/mol, level IV at −0.2 kcal/mol, and level V at −1.0 kcal/mol. Another assumption, like setting all intermediates at the same energy level, would not make a significant difference for the energy levels or the mechanism. The electron transfer barriers are not critical for the mechanism and are set to about +10 and +5 kcal/mol for I to II and III to IV, respectively. This fully defines an energy diagram for the preferred pathway with full membrane gradient, and it is shown in Fig. 6. With the same conclusions about the pumping elements as used in the construction of the original diagrams, a new diagram can then be derived without membrane gradient. These conclusions were, that the pump-site is at Prop-A of heme a_3 and that the pump is in the Glu278 area, defining the proton transfer TS to or from the N-side. The pump-site is thus at 5/6 the distance from the inside, BNC and heme a at 2/3 the distance, and the pump at 1/2 the distance. With this, the corrections for the membrane gradient are identical to the ones used before. The full energy diagram without membrane gradient is also shown in Fig. 6.

The energy differences between the new diagram without gradient in Fig. 6 and the old one in Fig. 3, derived on the basis of the kinetic experiments, are very small. The energies of I, II and VI were set as before and are therefore identical. For the other energy levels the differences are +0.4 kcal/mol for III, −0.5 kcal/mol for IV, and −0.5 kcal/mol for V. For the barriers the differences are 0.0 kcal/mol for II to III, +0.5 kcal/mol for IV to V, and +0.5 kcal/mol for V to VI. Since the new diagrams were set up based on rather general principles and assumptions, it is argued here that the large similarities between these barriers and those measured, give very strong support for the latter.

A general energy diagram for the case of a maximum gradient of 0.24 V instead of 0.20 V can also easily be constructed. In that case the total driving force will be 0.0 kcal/mol instead of −1.8 kcal/mol, and level II will be at +1.8 kcal/mol instead of +1.5 kcal/mol. Again assuming a linear dependence on the energy levels will put III at +1.4 kcal/mol, IV at +0.9 kcal/mol, and V at +0.5 kcal/mol. The barrier point between V and VI will now be at +16.0 kcal/mol. The other proton transfer barriers are set to +16.0 kcal/mol as above. The same procedure as for the gradient of 0.20 V, will for the gradient of 0.24 V then lead to new barriers for the case without membrane gradient. These will differ from the measured ones by −0.8 kcal/mol for II to III, by −1.2 kcal/mol for IV to V, and

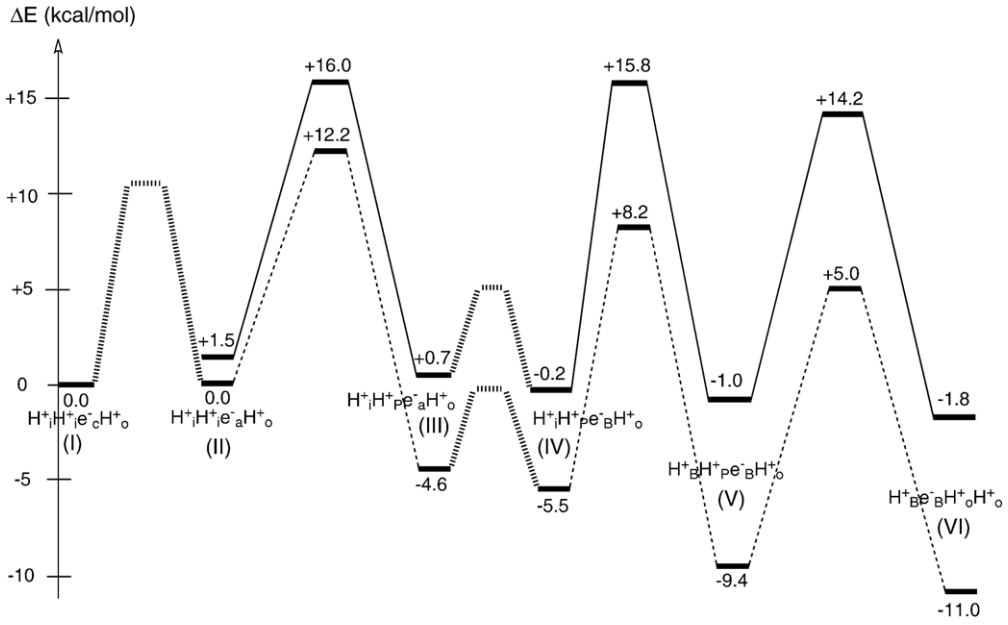

Fig. 6. Energy diagrams derived in an alternative way (see text). Full line shows the diagram with full gradient (0.20 V), the dashed one without gradient. The hashed barriers for the electron transfers are taken directly from experiments.

by +0.7 kcal/mol for V to VI. Again, the differences are rather small, although slightly larger than the ones given above for a maximum gradient of 0.20 V.

4. Discussion

In the previous sections diagrams have been described, which quantitatively determine a pumping mechanism in CcO, with allowed and non-allowed pathways. The sequence of steps in the pumping process has been taken from the suggestions convincingly made in recent kinetic experiments on the O to E transition [7]. Rather similar suggestions had been made before [10–15], and the general features of the process could therefore be termed the standard picture of pumping in CcO. The process is initiated by an electron transfer from cytochrome *c* to heme a via Cu_A. The electron on heme a attracts a proton from the N-side to the pump-site. After electron transfer from heme a to the BNC, another proton is taken up from the N-side, this time to the BNC. In the final step, the proton on the pump-site is expelled to the P-side and the process is completed.

An analysis of allowed and non-allowed pathways using the energy diagrams leads to a detailed understanding of most parts of the pumping process. The picture that emerges is the following. Assuming a single pump-site, it is identified as being at a place where the distance ratio to heme a and to the BNC is around 1.5, which fits very well for prop-A of heme a_3. The propionates of heme a are very unlikely as the single pump-site, as is prop-D of heme a3. The His291 ligand of CuB has a distance ratio of 2.3 which also makes it unlikely as the pump-site. The pump-site should have a pK_a of 5.5 before the electron has reached heme a, derived from the energy difference between levels V and VI in Fig. 3, and this fits general assumptions concerning prop-A of heme a_3. When the electron is transferred from cytochrome *c* to heme a, the pK_a of the pump-site increases to 10.7, which is derived from the exergonicity of −5.0 kcal/mol for this process (II to III), which consists of an uptake of a proton from the N-side to the pump-site. When the proton has reached the pump-site, the experimental observation was made that the electron equilibrates between being on heme a and the BNC. This places the redox-potential after the proton uptake at 0.49 V for both heme a and the BNC. Before the proton transfer the redox-potentials were 0.27 V and 0.11 V, respectively, the first one from experiments, the second one derived from the energy levels II and XII. With the electron at the BNC, the pK_a of the pump-site increases to 13.4, derived from the energy difference between levels XII and IV, which corresponds to a proton uptake to the pump-site when the electron is at BNC.

The uptake of a proton from the N-side to the pump-site passes a relatively high barrier of 12.2 kcal/mol. Several observations and requirements contribute to a suggestion for the character and positioning of the transition state for this proton uptake. The most important observation is that an electron on heme a must lower the barrier for the proton uptake significantly. This follows from considerations of the non-allowed pathway from V to VIII, which describes a proton transfer back to the N-side from the pump-site, at a stage when both a proton and an electron have already reached the BNC. It is concluded that if this reaction should be prevented, the stabilizing effect of an electron on heme a on the pump-site (7.1 kcal/mol) and on the TS should be approximately the same. This would give a large lowering effect on the barrier for the forward, allowed, reaction from II to III, since there is no corresponding stabilizing effect on the reactant II from an electron on heme a. A consequence of this is that the TS for proton transfer should be positively charged and be rather close to heme a. The most likely position for the transition state is the region around Glu278, which is the position in the D-channel closest to heme a. This position is furthermore closer to the N-side than alternative choices like Prop-D on heme a_3, which means that the barrier for the allowed pathway should not be raised too much by the membrane gradient. The picture of the TS is thus that a proton, coming from the N-side or from the pump-site, should at the TS have reached a position close to Glu278, with no other changes occurring.

An interesting consequence of the character of the TS for proton transfer, described above, is that the barrier for proton uptake to the pump-site or to the BNC will be as high as (12.2+7.1)=19.3 kcal/mol without an electron on heme a or in the BNC. This barrier is so high that it would prevent proton uptake at the time-scale of the pumping process. This conclusion by itself is not of direct use for the pumping mechanism described here, but is a consequence of it.

In the next step, after a proton has been taken up from the N-side to the pump-site, a proton from the N-side should go to the BNC, completing the chemistry that drives the pumping process. The uptake of this proton should go over a similar transition state, in the area of Glu278, as the one when the previous proton went to the pump-site. The barrier is higher due to the repulsion from the proton at the pump-site. When the proton has reached the BNC, the pK_a of the pump-site should go back to its original value of 5.5, since the electron at the BNC is now fully screened. The chemistry at the BNC has thus created a situation where there is a driving force for pumping the proton at the pump-site to the P-side, but also one for sending this proton back to the N-side. This is a critical situation with strict requirements for the ability of pumping, as discussed above. The mechanism for directing the proton to the P-side is to have an intrinsically very high barrier for moving the proton between the pump-site and the N-side. The only way to allow a proton to pass in the forward direction over this TS is to have an electron (unshielded) either on heme a or on the BNC. In the backward direction, the barrier will always stay high. After the chemistry is completed at the BNC, and the proton resides on the pump-site, the barrier will thus be too high to go back to the N-side, and the proton would have to go to the P-side.

The final step, where the proton is pumped from the pump-site to the P-side, was experimentally found to have a large barrier with a rate of 2.6 ms without membrane gradient. A large barrier follows from the fact that the transfer in the reverse direction, from the P-side to the pump-site, must be prevented at all times. The barrier for the forward and backward direction will differ by at most the size of the forward driving force. Since the driving force is extremely small at the limit of the full

gradient, the barrier is therefore required to be quite high also for the forward direction. It should be emphasized, that in general it is not required that all intermediates in a catalytic cycle must be higher in energy than the product. In most cases, the presence of a low-energy intermediate will only slow down the rate of the cycle. However, in the present case of proton pumping, where the competing process with protons going in the opposite direction is energetically more favorable, the only solution found here for directing the process in the right direction is to use the small driving force for this purpose, and this leads to the requirement that no intermediate has an energy lower than the final point.

A few factors contribute to the quite accurate positioning of the energy levels in the diagram. The first one is the very small total driving force for the full cycle of only −1.8 kcal/mol with membrane gradient, which requires that all intermediates have higher energies than this value. The second fact is the almost full disappearance of state II in the II to III transition, and the third one is the similar disappearance of state IV in the IV to V transition in the kinetic measurements [7]. These facts require that levels III and IV without the gradient have to be between −3.4 and −6.8 kcal/mol, and level V between −6.8 and −10.2 kcal/mol. The next constraint on the energy levels is introduced by the choice of 16 kcal/mol for the back-flow of protons from the P-side to the pump-site. This constraint was imposed to limit the rate for non-allowed pathways to 100 ms, compared to the rate of 1 ms for the allowed pathway. This fixes energy level V to −8.9 kcal/mol, and levels III and IV to be between −3.4 and −5.5 kcal/mol. The remaining small flexibility has been used to optimize the function of the enzyme.

For the case with full membrane gradient the choice of 16 kcal/mol as the lower limit for the non-allowed pathways has the following consequences. It limits uptake of protons from the P-side to the pump-site, which would destroy pumping. Chemistry with full pumping becomes achieved with a barrier of 16.0 kcal/mol. The barrier at level V for avoiding pumping becomes 15.7 kcal/mol. The barrier for letting protons through from the P-side to the N-side, which would destroy the gradient, becomes 17.0 kcal/mol. The barrier for taking the BNC proton from the P-side and perform the chemistry, becomes 16.0 kcal/mol. The latter pathway would not build up a gradient, but would not destroy it either. It is interesting to note that another choice than 16 kcal/mol for the lower limit of the barrier for uptake of protons from the P-side to the pump-site would not change any of these four barriers significantly. In principle any lower limit value in the range 15–18 kcal/mol is allowed by the constraints given by the experimental observations in the transitions II to III and IV to V. The only choice which would change the rate of pumping is 18 kcal/mol, which would increase the rate-limiting barrier by 0.9 kcal/mol to 16.9 kcal/mol. Again, the choice of 18 kcal/mol is the only one that would change the barrier for avoiding pumping at V with a decrease from 15.7 to 14.6 kcal/mol. Overall, the critical barriers are very stable to the choice of barrier from the P-side to the pump-site.

As described above, the energetics derived from general principles, as shown in Fig. 6, is remarkably consistent with the overall mechanism and the kinetic measurements. It appears that the only alternative would be if there could be a completely different mechanism, which was also consistent with the kinetic measurements. Considerable thought was therefore devoted to investigate if this is possible. As already mentioned, most mechanisms suggested follow essentially the same sequence of events as used in the mechanism adopted here. However, there is one pumping mechanism suggested which is basically different [16,17]. It should be pointed out that this alternative mechanism was suggested based on measurements for the P to F transition, and there is a possibility that the mechanism for this transition could be different than the one discussed here for the O to E transition. In the alternative mechanism, which is initiated by an electron transfer to the BNC, the first proton goes to the BNC. This proton transfer leads to a structural reorganisation, which is then used for pumping. It is rather difficult to match the experimental kinetic observations for the O to E transition with this alternative mechanism. In the experiments an initial proton transfer should occur before the electron transfer. With the alternative mechanism, this transfer would then have to be one which is neither to the pump-site nor to the BNC, which still leads to the observed equilibrium between III and IV. One such possibility could be that a proton goes up along the K-channel and moves closer to the BNC, thereby increasing the redox potential for the BNC to the same value as for heme a, leading to an equilibrium between two states. The effect on the redox potential of heme a should be smaller, and after this proton transfer the two redox potentials should thus be similar. In the next step in the alternative mechanism, a proton should first move to the BNC. This should cause a structural change which increases the pK_a of the pump-site, such that a rapid transfer of a proton from the N-side to the pump-site occurs. In the experiments, this rapid transfer might be hidden behind the much slower proton transfer to the BNC, which requires the reorganisation. It is therefore possible that only one state is observed, which should then correspond to state V. To be consistent with the experimental observations, it is thus required that this transfer to the pump-site is extremely rapid, which should require a well defined pathway such as the one in the lower part of the D-channel. In the final rate-limiting step, the proton is pumped to the P-side, just as in the mechanism described in the diagrams. A problem with this interpretation of the experimental results is that it does not agree with the relative magnitude of the charge translocations observed in the two first steps, with the initial charge transfer being 40% larger than the second one [7].

Another possibility to interpret the kinetic experiments, that might possibly be in line with the alternative mechanism (with some modifications), could be that the transition from II to III is a proton transfer from the N-side to the BNC, again leading to a structural reorganisation as described above. State III would then have an electron on heme a and a proton on the BNC. In state IV, the electron should move from heme a to the BNC, followed by a rapid transfer of a proton from the N-side to the pump-site. States III and IV should have the same energy, as required from the experimental observations, but should then not be interconverted by a simple electron transfer but with a combined electron transfer and a proton transfer to the

pump-site. All of this is in principle possible, but the next step from IV to V would then be difficult to match with the experimental observations. It could in principle be just the reverse of the structural reorganisation occurring in the II to III transition, but there appears to be no reason why this should destroy the original equilibrium between two states, as observed in the experiments. Instead, two states, V and V′, should be in equilibrium, where V has an electron on heme a and V′ an electron on the BNC, but this is not in line with what is observed. This type of mechanism would also require that the proton transfer to the BNC leading to III should be so exergonic that it could simultaneously afford a costly structural reorganisation, which appears quite unlikely. In most models for pumping, like the one adopted here, it is instead assumed that the combined proton and electron transfer to the BNC is what supplies the energy for proton pumping, but this would not be possible with this version of the alternative mechanism. Furthermore, it should be noted in this context that the alternative mechanism will also have to stand the tests involved in making a consistent energy diagram, as has been done here for the standard mechanism. This type of test is very critical, and even small deviations may be enough to disregard a mechanism. The present very accurate agreement between the standard mechanism, as displayed in the diagrams, and experimental observations, is therefore an extremely strong support for that mechanism. This type of test has not yet been done for the alternative mechanism but will be a future project. In summary, the alternative mechanism may possibly be made to be consistent with the experimental observations, but it does at the moment appear as much less likely than the standard mechanism followed here.

The diagrammatic analysis of the standard model for proton pumping in CcO has been shown to be remarkably consistent with recent kinetic measurements. Since the general features of the diagram can be obtained from general principles, see Fig. 6, the present analysis is a very strong support for the measurements. The general analysis started out from the requirement that the chemistry at the BNC should occur faster than or equally fast as the backflow of protons from the P-side when the full membrane gradient of 0.20 V has been reached. It should be noted that it was not required that pumping to the P-side should occur at this limit. Still, the diagram actually suggests that pumping will occur all the way to the full gradient. At the point in the diagram (level V in Fig. 6), where the proton could choose to go back to the N-side rather than to the P-side, the barrier is lower towards the P-side. One event that has not been treated in the present analysis is when the next electron reaches heme a. There is nothing in the present mechanism that would prevent this from happening too early. If this happens when a proton still resides on the pump-site, there will be no pumping. However, the chemistry at the BNC would occur as usual and would continue to build up the gradient. It is possible that this is actually what happens in this case. Another possibility is that the rate of the flow of electrons is regulated outside CcO, so that the electrons on average only arrive at a time when pumping is already completed. There are obviously also other questions remaining in the mechanism but it is hoped that the present diagrams should represent the major features of a plausible pumping mechanism with quantitative accuracy.

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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.2007.06.009.

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