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Why is the reduction of NO in cytochrome c dependent nitric oxide reductase (cNOR) not electrogenic?

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ABSTRACT

The membrane-bound enzyme cNOR (cytochrome c dependent nitric oxide reductase) catalyzes the reduction of NO in a non-electrogenic process. This is in contrast to the reduction of O_2 in cytochrome c oxidase (CcO), the other member of the heme-copper oxidase family, which stores energy by the generation of a membrane gradient. This difference between the two enzymes has not been understood, but it has been speculated to be of kinetic origin, since per electron the NO reduction is more exergonic than the O_2 reduction, and the energy should thus be enough for an electrogenic process. However, it has not been clear how and why electrogenicity, which mainly affects the thermodynamics, would slow down the very exergonic NO reduction. Quantum chemical calculations are used to construct a free energy profile for the catalytic reduction of NO in the active site of cNOR. The energy profile shows that the reduction of the NO molecules by the enzyme and the formation of N_2O are very exergonic steps, making the rereduction of the enzyme *endergonic* and rate-limiting for the entire catalytic cycle. Therefore the NO reduction cannot be electrogenic, i.e. cannot take electrons and protons from the opposite sides of the membrane, since it would increase the endergonicity of the rereduction when the gradient is present, thereby increasing the rate-limiting barrier, and the reaction would become too slow. It also means that proton pumping coupled to electron transfer is not possible in cNOR. In CcO the corresponding rereduction of the enzyme is very exergonic.

1. Introduction

Nitric oxide reductase (NOR) is a membrane-bound enzyme catalyzing the two-electron reduction of nitric oxide (NO) to nitrous oxide (N_2O), according to Eq. (1):

$$2NO + 2H^+ + 2e^- \rightarrow N_2O + H_2O. \quad (1)$$

This reaction is one of the steps in the denitrification pathway, in which nitrite (NO_3^-) is transformed to dinitrogen (N_2). The NOR enzyme is a member of the heme-copper oxidase superfamily, which also contains cytochrome c oxidase (CcO), the terminal enzyme in the aerobic respiratory chain. CcO catalyzes the four-electron reduction of molecular oxygen, according to Eq. (2):

$$O_2 + 4H^+ + 4e^- \rightarrow 2H_2O. \quad (2)$$

In both NOR and CcO the catalytically active site is a binuclear center (BNC) with a heme group and a non-heme metal ion, which is iron (Fe_B) in NOR and copper (Cu_B) in CcO.

Both these reduction processes have high reduction potentials, 1.18 V (NO reduction) and 0.80 V (O_2 reduction), leading to exergonic reduction reactions using, for example, cytochrome c with a reduction potential of 0.25 eV as electron donor. In aerobic respiration, the reduction of molecular oxygen occurs in such a way that a substantial part of the energy released in the exergonic reaction is stored as an electrochemical gradient over the mitochondrial or bacterial membrane. The membrane gradient is used by ATP synthase to transform ADP to the energy rich compound ATP. The build up of the electrochemical gradient in CcO occurs in two different ways. First the chemical reaction of water formation is electrogenic, i.e. the electrons and the protons in Eq. (2) come from opposite sides of the membrane. The electron donor, cytochrome c is located on the P-side (intermembrane space or periplasm), and the protons are taken from the other side of the membrane, the N-side (matrix or cytoplasm). Secondly, coupled to the exergonic water formation, protons are pumped all the way from the N-side to the P-side, which increases the efficiency of the energy conservation. In contrast, for another member of the heme-copper oxidase superfamily, the cytochrome c dependent nitric oxide reductase (cNOR), it is established that the electrons and the protons for the reduction of nitric oxide described in Eq. (1) are taken from the same side of the membrane (the P-side (periplasm)), i.e. this reaction is not electrogenic, and this enzyme does not pump protons either [1–3]. The reason for this difference between cNOR and CcO has not been understood, and it is quite surprising considering the fact that, counted per electron Reaction (1) is more exergonic than Reaction (2), suggesting that the free energy released should be enough for both electrogenic chemistry of water

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formation and proton pumping [3]. It has been speculated that due to the toxicity of the NO molecule, gradient generation is avoided, since it would slow down the transformation of NO into the less toxic N_2O molecule [2,3]. However, since the overall reaction is very exergonic, the energy is more than enough for both electrogenic water formation and proton pumping without decreasing the reaction rate. Before the character of the rate limiting step is known, it is not possible to judge how the generation of a membrane gradient would affect the rate of the reaction.

Apart from the cytochrome c dependent cNOR, discussed above and the main target of the present study, the family of nitric oxide reductases has another subfamily, qNOR, which has quinol as electron donor. The cNOR family is experimentally most well characterized, and the results mentioned above about electrogenicity concern this type of NOR. In 2010 the crystal structure was solved for the cNOR of the *Pseudomonas aeruginosa* bacterium at 2.7 Å resolution [5]. In the crystal structure there seems to be no proton paths present between the N-side (cytoplasm) of the membrane and the BNC, corresponding to those present in the different CcO families [4], while possible proton paths connecting the BNC with the P-side (periplasm) have been localized [3,5,6]. These observations support the interpretation that the NO reduction in cNOR is not electrogenic. In 2011 also the crystal structure of a member of the qNOR family, from *Geobacillus stearothermophilus*, was solved at 2.5 Å resolution [7]. One important difference between the two structures solved so far, is that the qNOR enzyme has a distinct water filled channel connecting the BNC with the N-side. This finding has lead to speculations that in the case of qNOR the reduction of nitric oxide actually might be electrogenic, since the protons could be taken up from the N-side via this water channel [7].

In a recent computational study of the cNOR reaction a free energy profile was produced, showing that the part of the reaction leading to the formation of the nitrous oxide from the two-electron reduced enzyme (**4** to **1**, see the scheme in Fig. 1) is very exergonic. This implies that the reduction steps (**1** to **4**), leading to water formation, actually are endergonic even without any gradient present, and directly involved in the rate-limiting steps of the entire catalytic cycle [8]. This is very different from the reduction of molecular oxygen, where the O–O bond cleavage (**R** to $\mathbf{P}_M$, see the scheme in Fig. 2) is only slightly exergonic and all reduction steps ($\mathbf{P}_M$ to **R**) are more or less exergonic. Therefore it is here suggested that, although the NO reduction in cNOR is exergonic enough, the reason for this reaction being non-electrogenic is kinetic, and it is caused by the large exergonicity of the N_2O formation. The fact that the cNOR enzyme is located in a membrane where other enzymes build up a gradient, further stresses the importance of a non-electrogenic nitric oxide reduction, which otherwise would become too slow when the gradient is present.

It should be stressed that it is the energetic requirement for electrogenicity and proton pumping that can be investigated here. Electrogenic chemistry of water formation, i.e. taking electrons and protons from the opposite sides of the membrane, and proton pumping, i.e. transport of protons from one side of the membrane to the other, are two different processes that both contribute to the generation of an electrochemical gradient. As mentioned above both of these processes are present in CcO, where it is also generally agreed that the proton pumping is coupled to the electron transfer steps. The idea here is to construct the free energy profiles for the reduction processes in cNOR and CcO for the situation without electrochemical gradient present. It should be noted that there is no energy cost connected with proton pumping across the entire membrane when there is no gradient. The effects of the gradient on the energetics can then be estimated, realizing that only reaction steps involving the motion of charge perpendicular to the membrane are affected. Both electrogenic water formation and proton pumping occur in such a way that charges are moving against the gradient, i.e. those reaction steps correspond to an extra energy cost when the gradient is present, and they will be less exergonic (or more endergonic). If the gradient in this way affects the energetics of the rate limiting step of the catalytic cycle it would affect the overall rate of the reaction. The rate limiting step is defined as the step that has the highest barrier (including endothermicity) relative to the lowest previous intermediate, and it can be located anywhere in the catalytic cycle. The main point here is to explain why the water formation chemistry in cNOR is not electrogenic. Conclusions can also be drawn about proton pumping occurring with the same type of mechanism as in CcO, i.e. coupled to the electron transfer steps. The possibility of other types of proton pumping mechanisms, not coupled to electron transfer, is not discussed here. With the type of results presented here, it can be concluded that the absence of proton pathways in the enzyme is an effect of the energetically prohibitive electrogenicity, not the other way around. In other words, proton paths from the N-side to the BNC are not needed in cNOR.

In the present study, new conclusions are drawn from the free energy profiles for nitric oxide reduction in cNOR and O_2 reduction in CcO obtained from new and previous quantum chemical calculations [8,9]. The comparison between the two reactions is made to clarify and strengthen the conclusions drawn for the NO reduction process. Additional calculations had to be performed on CcO to make the comparison feasible. Even if cNOR is the main target of the present study, potential differences between cNOR and qNOR suggested on the basis of experimental observations are also discussed with reference to the calculated energy profile for NO reduction.

2. Models and methods

Quantum chemical calculations are performed on models of the BNC in both cNOR and CcO (aa_3) to describe the main steps in each catalytic cycle, compare the reaction schemes shown in Fig. 1 and in Fig. 2. The models and methods are the same as used in previous similar studies [8,9], and they are only shortly summarized here.

The binuclear active sites (BNC) in nitric oxide reductase and cytochrome c oxidase are quite similar, with a heme group in close vicinity of a histidine ligated metal complex, containing a copper ion (Cu_B) in CcO and a non-heme iron (Fe_B) in NOR. The models used in the calculations were chosen in reasonably equivalent ways for the two systems, and they are shown in Fig. 3. The model of cNOR, which contains a heme b_3 group is based on the *P. aeruginosa* [5] structure, and the model of CcO (aa_3), which contains a heme a_3 is taken from the *Rhodobacter sphaeroides* [10] structure. The models are mainly made up of the metal ions and their first shell ligands, but a few second shell ligands are also included, see Fig. 3. A few atoms are fixed to the X-ray coordinates during geometry optimizations to maintain some constraints from the surrounding protein, and those

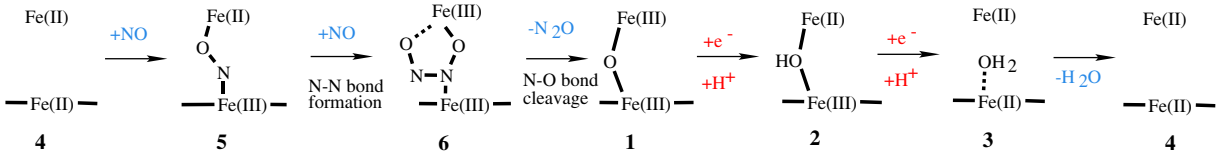

Fig. 1. Catalytic cycle of NOR starting from the two-electron reduced state labeled **4** according to the mechanism suggested in Ref. [8]. The labeling of the intermediates follows the previous study [8].

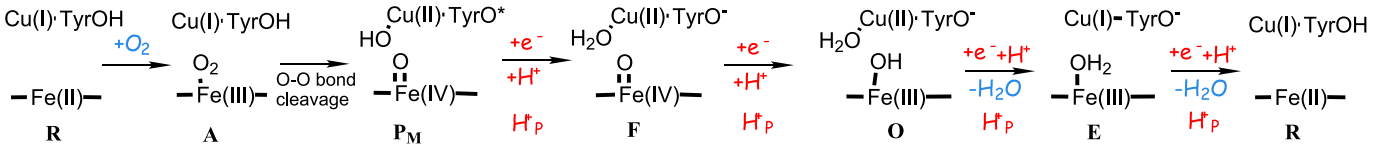

Fig. 2. Catalytic cycle of CcO starting from the two-electron reduced state **R**. The notation H^+_P corresponds to pumped protons.

are marked with red circles in Fig. 3. The cNOR model has about 130 atoms (depending on the state) [8], and the CcO model about 144 atoms [9]. The total charge is +1 for all states considered here for both models.

Relative enthalpy values are obtained from density functional calculations using the B3LYP* functional [11], which is a modification of the original B3LYP functional [12] and a large basis set (cc-pvtz(-f) for main group elements plus lacv3p+ for the metal ions [13]). Zero point effects from Hessian calculations, empirical dispersion effects according to a formula by Grimme [14], and solvent effects using the self consistent reaction field (SCRF) approach as implemented in Jaguar with a dielectric constant of four are included. To obtain free energies the entropy effects are estimated to be mainly due to the loss or gain of translational entropy when binding or releasing small gaseous molecules (10.8 kcal/mol for O_2 and NO, and 11.1 kcal/mol for N_2O) [8]. The calculated relative free energies reported here for the cNOR reaction agree with those reported in Ref. [8], while the values for the CcO reaction differ from those previously reported for the same model in Ref. [9] by the inclusion of zero point and dispersion corrections in the present values. Furthermore, a correction of 4.6 kcal/mol is introduced here, raising the energy of the heme a_3-Fe(III) states. This correction is introduced to improve the agreement with experiment for the O_2 binding step in CcO. The general DFT accuracy in this type of calculations has been estimated to 3–5 kcal/mol [15,16].

To construct energy diagrams for the full catalytic cycles of cNOR and CcO the energetics of the reduction steps has to be calculated. Since it is not possible to calculate accurate absolute reduction potentials and pK_a values using the present methods, a procedure has been developed where the calculated relative energies for the entire cycle are adjusted to fit the total reaction energy as obtained from experimental reduction potentials for the electron donor and acceptor [17–21]. The immediate electron donor to the BNC in cNOR is heme b, with a midpoint reduction potential of 0.345 V [22], and the potential for the reduction of two molecules of nitric oxide to nitrous oxide and water is 1.177 V. Taking into account that two electrons are involved this gives an experimental exergonicity of 38.4 kcal/mol for one catalytic cycle in cNOR. Similarly, a total exergonicity of 51.0 kcal/mol is obtained for CcO from the values 0.25 V for cytochrome c and 0.8 V for the reduction of O_2 to water taking into account that four electrons are involved in this case [23]. It is noted that the immediate electron donor to the BNC in CcO, heme a, has a reduction potential (0.21 V) very close to cytochrome c, used here to be consistent with what is generally done for CcO [23], and therefore the energetic picture for CcO would be the same if the reduction potential of heme a was used.

Apart from the calculated relative energies of the intermediate states in the reduction processes, important barriers are also included in the energy diagrams. For the cNOR reaction the barriers are obtained either from calculations (bond formation and bond cleavage) or from experiment (proton transfer), as described in Ref. [8]. For the CcO reaction all barriers are taken from experiment. The O–O bond cleavage barrier of 12.4 kcal/mol is deduced from the life-time of compound **A** using transition state theory [24]. The proton and electron transfer of each reduction step is described in a simplified and qualitative way with one rate limiting barrier for each reduction step, taken to be approximately 13 kcal/mol in correspondence with the experimentally known rates for the different reduction steps being in the 100 to 1000 μs range.

3. Results and discussion

As a background to the discussion of nitric oxide reductase, the energetics of O_2 reduction in cytochrome c oxidase will be presented in the first subsection below. In the second subsection the main results of the present study will be presented, i.e. implications from the computed free energy profile for NO reduction in cNOR will be discussed. To simplify the comparison between the two reactions, the two-electron reduced state is taken as reference state in both cases. In the final subsection recent experimental findings for qNOR will be commented on.

3.1. O_2 reduction in cytochrome c oxidase (CcO)

The main steps in the catalytic cycle of O_2 reduction in CcO are summarized in the scheme in Fig. 2. The calculated relative free energies of the intermediates generate the energy profile shown in Fig. 4, having the two-electron reduced state, labeled **R**, as reference point. It should be noted that this energy profile corresponds to the situation when there is no electrochemical gradient present. To make the profile more complete, an estimated proton transfer barrier is added in each reduction step, see the Models and methods section. The calculated relative

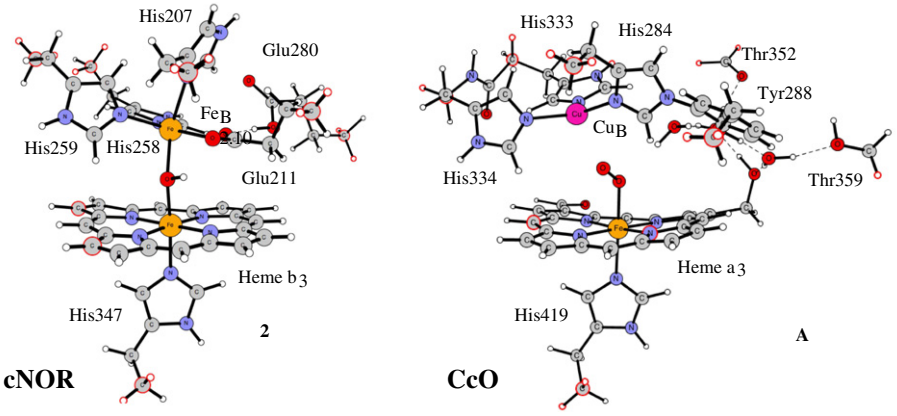

Fig. 3. BNC models used in the calculations for cNOR (left) and CcO (right) [8,9]. The atoms with red circles are fixed in geometry optimizations.

energies agree with the general picture obtained from experimental results for the reduction potentials involved [25], and with previously published computational results [17,18,21]. The initial steps when starting from the two-electron reduced state **R**, the binding (**A**) and cleavage ($\mathbf{P}_M$) of molecular oxygen are weakly exergonic, with a free energy of −5.3 kcal/mol. In these steps the four electron reduction of oxygen occurs, with the electrons taken from the active site in the enzyme, compare the scheme in Fig. 2. The rereduction of the enzyme (from $\mathbf{P}_M$ to **R**) occurs in four reduction steps, in which an electron and a proton (for the water formation) are delivered from cytochrome c (via heme a) and the bulk, respectively. In each reduction step one proton is pumped across the membrane, with no energy cost when there is no gradient present. The first two reduction steps after the O–O bond cleavage, the reduction of the tyrosyl radical to tyrosinate ($\mathbf{P}_M$ to **F**) and the reduction of Fe(IV) to Fe(III) (**F** to **O**) are both quite exergonic, while the following two reduction steps, yielding Cu(I) (**O** to **E**) and Fe(II) (**E** to **R**), respectively, are much less exergonic, see Fig. 4. Most important in the present context is that the four reduction steps together are exergonic by as much as 45.7 kcal/mol (42.1 kcal/mol if the reduction potential of heme a would have been used instead of cytochrome c).

As mentioned above the energy profile in Fig. 4 corresponds to the situation without any gradient across the membrane. When there is an electrochemical gradient present, all charge transfer processes occurring against the gradient correspond to an energy cost, and those steps will become less exergonic and contribute to maintain the gradient. This is how the energy storing is achieved. In CcO both the electrogenic chemistry of water formation, moving electrons and protons against the gradient to the BNC, and proton pumping against the gradient from one side of the membrane to the other, correspond to moving charge against the gradient. Therefore, when there is a gradient present, the exergonicity of these steps will decrease. The maximum gradient in CcO is known to be 200 mV [23], which means that moving one charge against the full gradient costs 4.6 kcal/mol. One reduction step occurring in an electrogenic way, corresponds to moving one charge against the gradient, and therefore costs 4.6 kcal/mol extra compared to the relative energies without gradient presented in Fig. 4. Furthermore, if one proton is pumped in each reduction step, there is another cost of 4.6 kcal/mol in each reduction step. This means that all the reduction steps together become 18.4 (4 × 4.6) kcal/mol less exergonic due to the electrogenic water formation. Taking also the cost of proton pumping into account, another 18.4 kcal/mol has to be subtracted from the exergonicity, assuming one proton pumped per electron. With a total exergonicity in all four reduction steps of 45.7 kcal/mol without gradient, there is still an exergonicity of 8.9 (45.7 − 18.4 − 18.4) kcal/mol in the rereduction process at full gradient, assuming that one proton per electron is pumped. It can be mentioned that it is generally agreed that less than four protons per reduced oxygen molecule are pumped at full gradient [26], leaving a somewhat larger exergonicity. Furthermore, there might be other difficulties connected with the shape of the energy profile in Fig. 4 and the proton pumping mechanism, but the main point made here is that the four reaction steps where the enzyme is rereduced together lead to a quite exergonic process in CcO, also with the gradient present.

3.2. Cytochrome c oxidizing nitric oxide reductase (cNOR)

In cNOR the electrons used to reduce nitric oxide are delivered by cytochrome c on the P-side of the membrane, and the protons are taken up from bulk water. As mentioned above, it has been found that, in contrast to CcO, there is no electrochemical gradient built up over the membrane in cNOR, which has been interpreted to show that the electrons and the protons are taken from the same side of the membrane (the P-side), and also that there is no proton pumping [1–3,6]. The crystal structure obtained recently seems to confirm this conclusion, since there are no proton channels found between the BNC and the N-side [5]. In both cNOR and CcO there are four redox active metal centers, which in cNOR consist of three heme groups and one non-heme iron, Fe_B. One of the heme groups, a heme b_3, and Fe_B constitute the catalytically active binuclear center (BNC). The two other heme groups, heme c and heme b, are used to transport

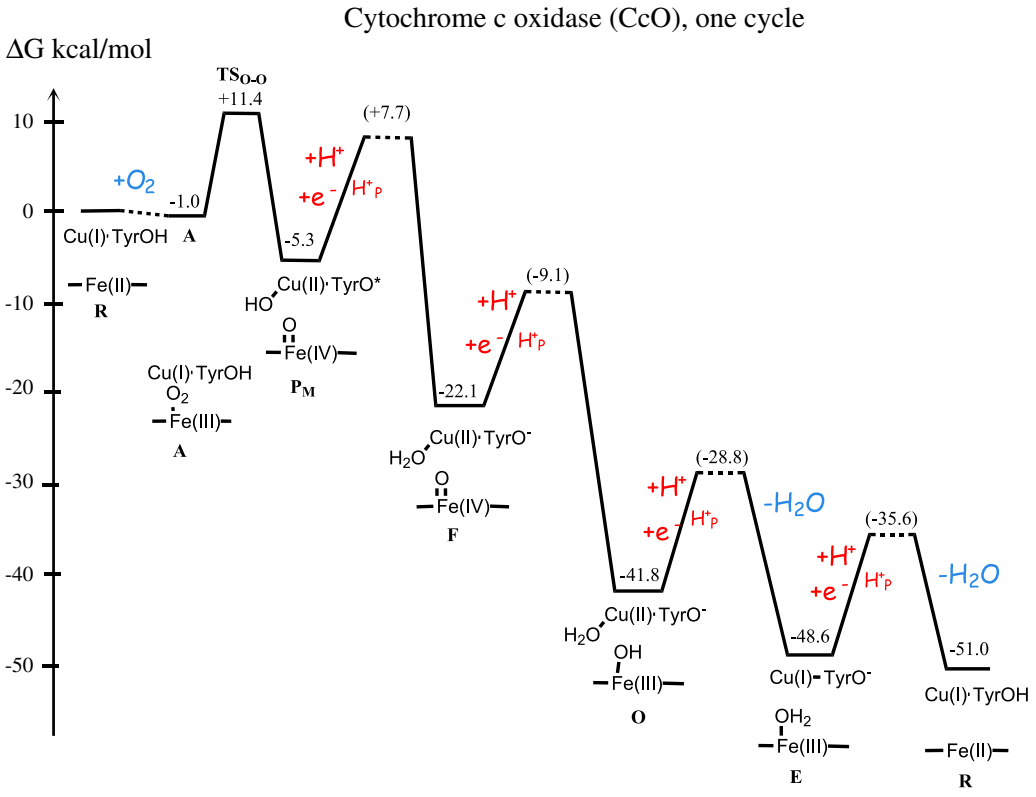

Fig. 4. Calculated free energy profile for the catalytic cycle of CcO starting from the two-electron reduced state **R**. The notation H^+_P corresponds to pumped protons.

the electrons from cytochrome c to the BNC where the chemistry occurs. The immediate electron donor to the BNC is heme b, and therefore the energetics of the catalytic cycle is calculated with respect to the reduction potential of heme b.

The catalytic reaction, forming N_2O and water from two NO molecules, occurs in several steps, involving the formation of an N–N bond, cleavage of one of the N–O bonds and two steps each of electron and proton uptake. This is a more complicated chemical reaction than the O_2 reduction in CcO, and it is not obvious in which order the different steps occur, and what the structures are of different intermediates. Several suggestions have been put forward, and they have often been classified according to their type of coordination of the NO molecules to the Fe ions in the BNC, and are therefore labeled cis:b_3-, cis:Fe_B- and trans-mechanisms, respectively [5,8]. In a recent computational study a mechanism of the cis:b_3-type was suggested on the basis of results from quantum chemical calculations on a model built on the cNOR structure [8]. The mechanism suggested is the one presented in the scheme in Fig. 1 and the calculated free energy profile for this reaction is given in Fig. 5. This mechanism was shown to agree with a substantial part of available experimental data, and the results from the calculations strongly dismiss other mechanistic suggestions [8].

The free energy profile for NO reduction in cNOR shown in Fig. 5 differs substantially from the corresponding profile for O_2 reduction in CcO shown in Fig. 4. In the initial steps of the catalytic cycle, starting from the two-electron reduced state **4**, the two NO molecules bind to the BNC and the N–N bond is formed in a cis-hyponitrite intermediate **6**. Already this part of the cycle, which also involves the two electron reduction of the NO molecules by the enzyme, is found to be exergonic by 24.4 kcal/mol (from **4** to **6**). From the cis-hyponitrite intermediate the N_2O molecule is formed and released in a very exergonic (by 28.1 kcal/mol) non-redox process, ending with **1**, yielding a total exergonicity for this first part of the reaction (from **4** to **1**) of as much as 52.5 kcal/mol. This means that the rereduction of the BNC and the water formation is *endergonic* by 14.1 kcal/mol, which is very different from CcO, where the total rereduction process is *exergonic* by 45.7 kcal/mol, as discussed above. It can be noted here that the rereduction of the enzyme occurs very similarly in cNOR and CcO, with each electron transfer coupled to a proton transfer, ensuring electroneutrality, and in both cases leading to water formation. The endergonic reduction process in cNOR refers to the energy difference between the oxidized intermediate **1** and the two electron reduced state **4**, see Fig. 5. The calculated relative energies furthermore indicate that the first reduction step (**1** to **2**) is exergonic, which makes the slowest reaction step even more endergonic, by 19.8 kcal/mol (from **2** to **4**). The fact that the rate-limiting step of the rereduction process in cNOR is as high as 19.8 kcal/mol already without the gradient present, makes it impossible for the enzyme to take up the protons from the N-side of the membrane, i.e. for the reaction to be electrogenic. Taking the protons from the N-side implies that, with the gradient present from other reactions in the membrane, the protons have to move against the gradient, which would make the reduction steps even more endergonic. Since the endergonicity of the rereduction is part of the rate limitation, this would increase the barrier and the reaction would become significantly slower, i.e. too slow. Obviously, proton pumping similar to that in CcO, which is coupled to the electron transfer, could also not be afforded in this situation, since it would also add to the rate limiting barrier. Still, the electrons have to move against the gradient, from heme c located on the P-side to heme b inside the membrane. This process is slightly exergonic (by 0.8 kcal/mol), an energy not included in the energy profile in Fig. 5, and which is barely enough to make the electron transfer to heme b close to thermoneutral when the gradient is present. Thus, the calculated free energy profile indicates that the NO reduction in cNOR has to occur in a non-electrogenic way, otherwise the reaction would become too slow as soon as the gradient starts to build up (from other processes in the membrane). This result is partly related to a previous suggestion that the reason that cNOR does not pump protons is connected to low proton affinities in the BNC during the NO reduction. That might indicate that the driving force for

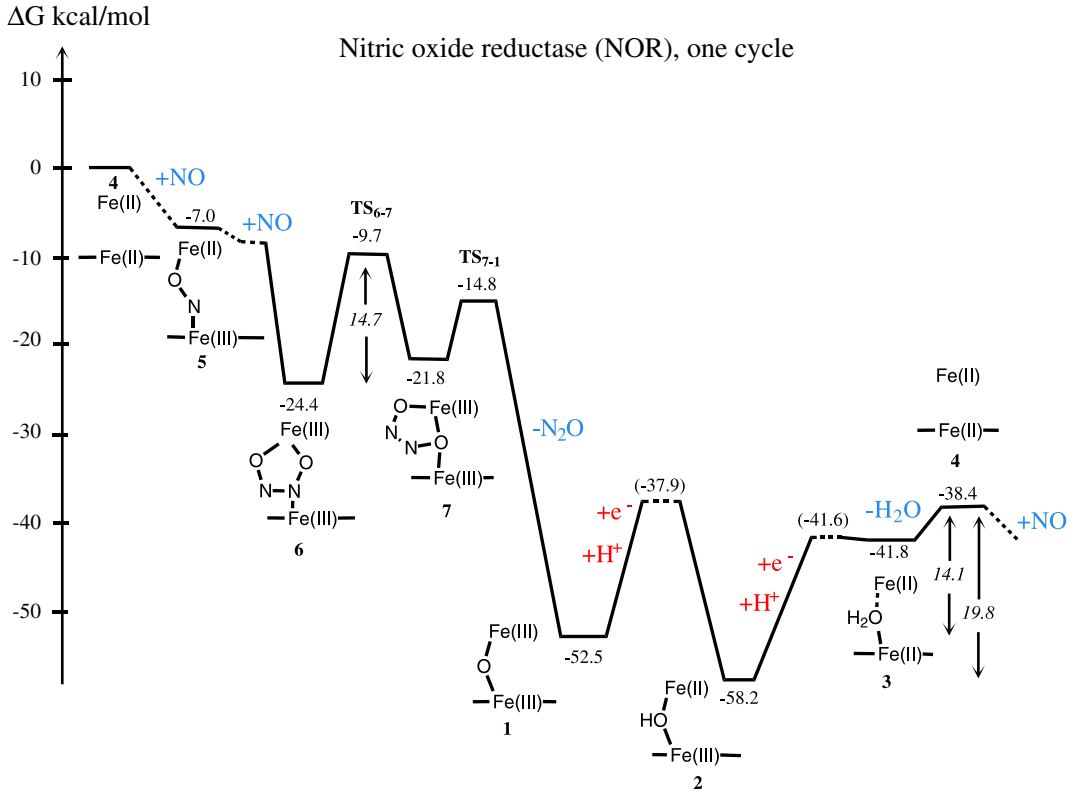

Fig. 5. Calculated free energy profile for the catalytic cycle of cNOR starting from the two-electron reduced state labeled **4**, according to the mechanism suggested in Ref. [8]. The labeling of the intermediates follows the previous study [8].

proton transfer into the BNC is low, which in turn would make proton transfer against the gradient difficult [3]. This suggestion was based on the fact that low proton affinities were obtained in a previous computational study on the NO reduction process [27]. The present study shows that not only the proton transfer, but the entire rereduction process is endergonic, and most importantly, directly responsible for the rate-limitation of the entire catalytic cycle. Furthermore, it shows that the reason for the endergonicity and rate-limitation is that the N_2O formation is very exergonic.

The calculated free energy profiles thus seem to give a convincing explanation to the non-electrogenicity of the NO reduction in cNOR. To further strengthen the conclusions the reliability of the calculated relative free energies should be evaluated. The overall energies of the entire reduction processes are adjusted to fit to experimental reduction potentials, as mentioned in the Models and methods section. However, the shapes and the details of the energy profiles are obtained entirely from the calculations. The relative accuracy in the calculated energetics for the NO and O_2 reduction processes can be estimated by comparisons to experimental reduction potentials. The difference in the experimentally determined reduction potentials for Reactions (1) and (2) is 8.7 kcal/mol (0.377 V), and the computed difference using the present approach is very similar, 7.9 kcal/mol, showing that the accuracy in the computed energetics for the two chemical reactions should be quite similar. Obviously, the shapes of the free energy profiles depend also on the redox properties of the metal ions in the BNC. Again, a comparison can be made between the two systems, cNOR and CcO. The experimental difference in reduction potentials between heme b_3 in cNOR and heme a_3 in CcO is about 7.5 kcal/mol, with the lower value for heme b_3. The calculated difference in electron affinity for five-coordinated Fe(III) between the two heme models used in the calculations (see Models and methods section), is 4.0 kcal/mol. Furthermore, to obtain the CcO energy profile in Fig. 4, a correction of 4.6 kcal/mol was introduced for the heme a_3 reduction potential, as mentioned in the Models and methods section. This leads to an effective difference in reduction potential between the two heme models used to obtain the energy profiles (Figs. 5 and 4) of 8.6 kcal/mol, quite close to the experimental value of 7.5 kcal/mol. Since the shape of the calculated free energy profile for the O_2 reduction in CcO agrees quite well with experimental information [25], it can be concluded that the shape of the calculated energy profile for the NO reduction should also be quite reliable, although in the NO case there is less experimental knowledge regarding the details of the energetics of the enzymatic reaction to compare with.

There remains some uncertainty in the calculations for the redox properties of the non-heme iron (Fe_B). The calculated difference in electron affinities between Fe_B and heme b_3, as obtained from the reaction steps in the catalytic reaction, can be compared to the experimentally obtained difference in reduction potential between the same metal ions in the BNC of cNOR, indicating that the calculated value for Fe_B might be about 4 kcal/mol too large. If a correction to decrease the reduction potential for Fe_B is introduced in the calculated energy profile in Fig. 5, intermediate **2** will be raised relative to intermediate **1**. However, such a decrease in reduction potential should also lower intermediate **6** (and all following points) by approximately the same value, and therefore would essentially not affect the calculated endergonicity. It can be noted that the calculated endergonicity of 19.8 kcal/mol is somewhat higher than the rate limiting barrier of about 16 kcal/mol as obtained from the observed turnover rate for cNOR in *Paracoccus denitrificans* [28]. A possible explanation for this slight overestimation of the rate limiting barrier is that the release of the water molecule (step **3** to **4**) actually might occur concerted with the following NO binding, which could lower the rate limiting barrier by a few kcal/mol, a process which is very difficult to describe computationally.

It is interesting to note that a first indication of an endergonic reduction process in cNOR can be obtained from the experimental reduction potentials [29]. As mentioned above, the immediate electron donor to the BNC is heme b with a reduction potential of 0.345 V, while the BNC metal ions have been found to have lower reduction potentials, 0.06–0.08 V for heme b_3 and 0.32–0.08 V for Fe_B [22,30]. For a pure two electron reduction process the experimental potentials indicate an *endergonicity* of 9–12 kcal/mol. A corresponding value for CcO, considering two electrons, and based on the experimental reduction potentials for heme a, heme a_3 and Cu_B would be an *exergonic* reaction by about 7 kcal/mol.

Finally, it is also interesting to note that experiments on cbb_3 cytochrome c oxidase indicate that with NO as the substrate the protons seem to be taken from the P-side instead of from the N-side as when O_2 is reduced in the same enzyme [31]. A reason for this reversal of the proton uptake could be that with the gradient present, the proton uptake from the N-side during NO reduction becomes too slow according to the results presented above. However, it should be noted that in order to draw any definite conclusions about those experimental observations, calculations should be performed on NO reduction in a model of the CcO–BNC, since the cofactors are different and the resulting mechanism and energy profile might differ from the present ones.

3.3. Quinol oxidizing nitric oxide reductase (qNOR)

As mentioned in the Introduction, the structure of qNOR is quite different from that of cNOR in one aspect, namely that in qNOR there is a channel filled with water, connecting the BNC with the N-side of the membrane, which can be interpreted to indicate that the qNOR enzyme works electrogenically [7]. Assuming that heme b in qNOR has the same reduction potential as heme b in cNOR, the same energy diagram as in Fig. 5 should be valid also for qNOR. This might indicate that the conclusions above for cNOR should hold also for qNOR. However, there is another difference between the two enzymes which could be important, namely that the ultimate electron donor is quite different. In cNOR the electron donor is cytochrome c, with a reduction potential of 0.26 V. The electrons are transferred to heme b via another heme group, heme c, with a reduction potential quite close to that of heme b, 0.31 V and 0.345 V, respectively. In qNOR, which lacks the heme c cofactor, the electron donor to heme b is a quinol, with a binding site located close to heme b and at approximately the same level in the membrane [7], and the quinol has a very low reduction potential, e.g. −0.08 V if a menaquinone is used. The location of the quinol binding site means that the electron transfer occurs more or less perpendicular to the gradient, i.e. with no extra cost. At the same time, the protons on quinol have to leave when the electrons leave. Since the quinol is located rather close to the P-side it is natural to expect that the protons leave to the P-side, and with a gradient present this represents the same cost as moving electrons from the P-side to the BNC level. Thus if the protons needed for the water formation are taken up via the water channel from the N-side and the quinol protons leave to the P-side, this is equivalent to an electrogenic reaction taking the electrons and the protons from opposite sides of the membrane and moving the charges against the gradient, present from other reactions in the membrane. The low reduction potential of the quinol means that the electron transfer from the quinol to heme b is exergonic by about 10 kcal/mol. If nothing else happens during this electron transfer the energy is lost and an electrogenic process at the BNC in qNOR is still not possible. On the other hand, if the electron transfer from the quinol to heme b is directly coupled to substrate proton uptake from the N-side to the level of the BNC, and also to the expulsion of the quinol protons to the P-side, the electron transfer energy can be used. The cost for moving the protons against the gradient can be taken from this energy, i.e. the electron transfer will be less exergonic with a gradient present. With a gradient of 200 mV, the cost of moving one charge across the membrane against the gradient is 4.6 kcal/mol, which is easily afforded by the 10 kcal/mol exergonic electron transfer. In this case there would be no change in the energy profile in Fig. 5 due to the gradient. Evidently, it is also

possible that the NO reduction in qNOR occurs in a non electrogenic manner, either by leaving the quinol protons to the same side as the substrate protons are taken from (P- or N-side), or by actually using the quinol protons to make water, which would mean that both electrons and protons move perpendicular to the gradient.

4. Conclusions

The heme-copper oxidase superfamily contains two main types of enzymes, cytochrome c oxidase (CcO) and nitric oxide reductase (NOR). CcO catalyzes the four electron reduction of molecular oxygen as part of the aerobic respiratory chain, in an electrogenic reaction coupled to proton pumping, which leads to an efficient storage of the excess energy into an electrochemical gradient across the mitochondrial or bacterial membrane. NOR catalyzes the two electron reduction of nitric oxide as part of the denitrification chain. Experiments on the cytochrome c dependent subfamily of nitric oxide reductase, cNOR, have shown that, although the NO reduction per electron is more exergonic than the O_2 reduction, it is non-electrogenic. The mechanism for NO reduction has not been known, and the reason for the non-electrogenicity in this reaction has not been understood. Recently, based on density functional theory calculations, a mechanism for the NO reduction was suggested, shown to be in agreement with a large part of the experimental observations [8]. By comparing the calculated free energy profile for the NO reduction according to this new mechanism with the corresponding profile for O_2 reduction in CcO, the difference between the two systems can rather easily be understood as a kinetic effect.

The calculations show that the binding of the NO molecules together with formation and release of N_2O (from **4** to **1**) is a very exergonic process (by 52.5 kcal/mol), which leads to an endergonic rereduction of the enzyme active site to complete the catalytic cycle (from **1** to **4**). Furthermore, the rereduction process is found to be rate-limiting for the entire catalytic cycle. The rereduction steps, which include all electron and proton transfers to the BNC, are the only ones affected by the gradient. Thus *if* the reaction were electrogenic, the rereduction steps would become even more endergonic with a gradient present, raising the rate limiting barrier and making the reaction too slow. Therefore the NO reduction process has to be non-electrogenic and the electron transfer steps can not be coupled to proton pumping. The O_2 reduction process is found to be very different, with a strongly exergonic rereduction process, which still is exergonic with the gradient present in spite of both electrogenic water formation and proton pumping. The large exergonicity of the initial steps of the NO reduction process in cNOR can be divided into two parts. The first part is the formation of the cis-hyponitrite intermediate (from **4** to **6**), which is exergonic by 24.4 kcal/mol. Since this part of the reaction involves the reduction of the two NO molecules by the enzyme active site, the energetics depends on the reduction potentials in the BNC. The unusually low reduction potentials of the cNOR–BNC, 0.06–0.32 V, as compared to 0.34–0.39 V for the CcO–BNC, ensure that the binding of the toxic NO molecules is fast and irreversible. With higher reduction potentials in the cNOR–BNC the cis-hyponitrite intermediate would be less stable. This, in turn, would decrease the endergonicity of the rereduction part. However, most likely it would also introduce a barrier for the NO-binding process, which would increase the rate-limiting barrier, which can be seen if two cycles are considered. It would therefore still not be possible to have an electrogenic process. The other part of the exergonicity in the initial steps is the exergonic formation and release of N_2O from the cis-hyponitrite intermediate (from **6** to **1**), exergonic by 28.1 kcal/mol, which is pure chemical in nature without change in any oxidation states, and should not be particularly affected by the reduction potentials in the BNC. Considering the other electron transfer cofactors, it can be noted that the ultimate electron donor is the same (cytochrome c) for cNOR and CcO. The difference in the accessory cofactors transporting the electrons from cytochrome c to the BNC, heme c and heme b in cNOR, and Cu_A and heme a in CcO, is rather small, 0.31–0.345 and 0.245–0.21 V, respectively. The slightly higher reduction potentials of the cNOR cofactors, which leave less energy to the catalytic cycle in the BNC, might be chosen to ensure fast electron transfer from cytochrome c to the accessory cofactors in the enzyme.

The conclusions drawn above about NOR concern the subclass cNOR with cytochrome c as electron donor. The other subclass qNOR might be different, since it has a very low-potential electron donor, a quinol, which is located close to heme b. For this type of enzyme an electrogenic process might be possible, but only if the electron transfer between the quinol and the accessory heme b cofactor is coupled to all proton transfer steps perpendicular to the membrane. In that case there will be no extra endergonicity in the BNC reaction due to the gradient.

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