FEBS Letters 588 (2014) 545–548

FEBS Letters

journal homepage: www.FEBSLetters.org

Mutations in the D-channel of cytochrome *c* oxidase causes leakage of the proton pump

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ARTICLE INFO

Article history:
Received 31 October 2013
Revised 6 December 2013
Accepted 9 December 2013
Available online 31 December 2013

Edited by Peter Brzezinski

Keywords:
Cytochrome oxidase
Proton pumping
Computational modeling

ABSTRACT

It has experimentally been found that certain mutations close to the entry point of the proton transfer channel in cytochrome *c* oxidase stop proton translocation but not the oxygen reduction chemistry. This effect is termed uncoupling. Since the mutations are 20 Å away from the catalytic center, this is very surprising. A new explanation for this phenomenon is suggested here, involving a local effect at the entry point of the proton channel, rather than the long range effects suggested earlier.

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 for the A-family of these enzymes is

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

where H_i^+ are protons taken from the N-side of the membrane and H_o^+ are protons translocated, or pumped, across the membrane from the N-side to the P-side [1a]. The membrane gradient created drives the synthesis of ATP, where the energy of food consumption and respiration is stored. The X-ray structure for the A-family of CcO has been solved for two types of bacteria [2,3] and also for a mammalian species [4]. 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. 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 two of the eight protons follow the K-channel, and all protons being pumped follow the D-channel [1b]. There is still no consensus on the proton pumping mechanism. In particular, there are many quite different suggestions for how gating, required to direct the protons either to the BNC or for pumping, is achieved [1c,5–7]. In common for these mechanisms is that the pump loading site (PLS), where the protons are stored before being pumped to the P-side, should be somewhere in the region of the heme-a_3 propionates, and also that the gating point, at which the protons are directed to the BNC or to the PLS, is situated in the region of Glu286 (*Rhodobacter sphaeroides* numbering). This residue is about 20 Å from the entry point of the D-channel. Still, and extremely surprisingly, it has been found that certain mutations close to this entry point can specifically affect the proton gating [8,9]. For example, mutating Asn139 (see Fig. 1) to an Asp stops pumping, but allows the chemistry at the BNC to occur at an almost normal rate. This type of mutant is referred to as uncoupled. The uncoupling of proton pumping can obviously not be explained by assuming that proton transfer through the D-channel is stopped by the mutation. Instead, there have been two major types of attempted explanations for how the rate of proton transfer through the D-channel could be affected by the mutations. Even though the distance between the mutation and Glu286 is very long (about 20 Å), either a direct electrostatic effect from the mutation could modulate the pK_a value of Glu286, or the mutation could cause a long range structural change in the Glu286 region [10,11]. More recently, a third explanation has been given, suggesting that a continous hydrogen-bonded chain through the D-channel should be essential for proton pumping, and that this should be disturbed by the mutations [8b].

In the present communication, an entirely new and different explanation for the uncoupling of proton pumping caused by the mutations in the D-channel is given. The starting point is to try to find a local origin of the uncoupling, caused by effects in the region close to the mutations. The reason is that we consider it unlikely that the mutations could lead to sufficiently large electrostatic or structural effects on the distant Glu286 to cause uncoupling at that

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0014-5793/$36.00
http://dx.doi.org/10.1016/j.febslet.2013.12.020

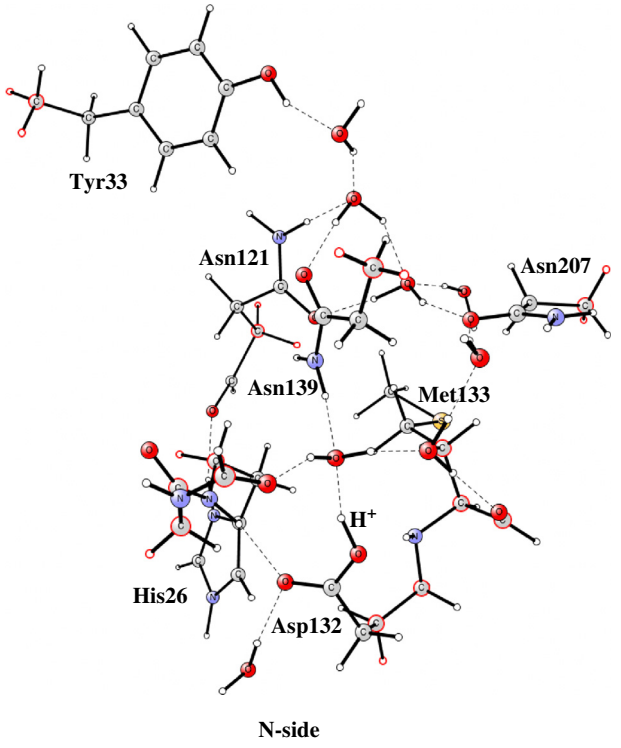

Fig. 1. Model for the lower part of the D-channel used for the present calculations. The proton being transferred is marked H^+. Atoms kept fixed are circled in red. The entry point for the D-channel is Asp132. The structure models the proton located at the minimum **M**, see text.

point. The absence of a structural effect is supported by the X-ray structure for the Asn139Asp mutant in *R. sphaeroides* [8b], even if it is noted that the corresponding mutation in *Paracoccus denitrificans* shows some minor changes of hydrogen bonding in the Glu286 region [12]. It is actually not difficult to imagine that a local effect close to the mutation could affect the pumping, since the rate of proton transfer through the D-channel is not only given by the energy at the transition state, supposedly located close to Glu286, but also by the energy of the resting state for the proton. The rate-limiting barrier for the proton transfer is the energy difference between these two points. This means that if the resting state for the proton before it moves through the D-channel is close to the position of the mutations, a local effect of the mutations can certainly have consequences for the pumping. Previously, the resting state for the proton has mainly been considered as being in the water medium on the N-side.

Another point where the present approach differs from the earlier ones, concerns the direction of the change in proton transfer rate caused by the mutation. Previously, mostly explanations involving a slower rate of proton transfer through the D-channel have been considered. The argument has been that the proton must be prevented from reaching the PLS to stop the pumping. The detailed pumping mechanism presented by us earlier [9], shows that it is also possible to prevent pumping by *increasing the back-leakage* from the PLS. In fact, preventing leakage is one of the most demanding aspects for a functioning proton pump. A major result of the previous studies was that a too fast proton transfer to the PLS is very likely to lead to leakage. Indeed, a surprisingly slow rate for the proton to reach the PLS, corresponding to a barrier of 10.8 kcal/mol, has been found experimentally [1d]. It can be added that the present suggestion of leakage in the mutants is consistent with a recent, experimentally based, suggestion [13].

The general idea to explain the uncoupling effect of the mutations is thus to keep the energy of the transition state for proton transfer the same as for the wildtype enzyme, but to raise the energy for the resting state. Since the energy of the proton on the N-side is the same for the wildtype and the mutants, the only reasonable way found to achieve a higher rate for the mutants is to assume that there should be an *energy-well* (**M**) for the proton in the wildtype, which becomes less deep or absent in the mutants. A place inside the D-channel where the proton is more bound than on the N-side is thus necessary.

To test the above idea, calculations were performed for both the wildtype and for two mutants, both involving mutations of Asn139. In the first case, this residue is replaced by a threonine, and in the second case by an aspartic acid. From the absence of significant distortions at the place of the mutation [8b], the aspartate is assumed to be protonated to keep the charge the same as in the wildtype enzyme. A model of the region around the mutation containing 140 atoms was used, see Fig. 1. The methods used are described in the supporting information. The purpose of the calculations is not to prove the suggested mechanism but instead to show that it is a valid possibility.

Since absolute pK_a values are not reliably evaluated using the present rather limited models, the conclusions have to rely on calculated relative energies. From results with various charges of the model, a neutral model was the only one that gave reasonable energies. This means that Asp132, at the entry point of the D-channel, is protonated with the proton pointing towards the N-side and with His26 neutral. It should be noted that this is not the resting state, see further below. The reverse protonation, with His26 protonated instead, was found to be higher in energy. The actually calculated values can be summarized in the following way (the interpretations are given below). To initiate proton transfer through the D-channel, a proton is added to His26. For the wildtype enzyme, the repulsion from the added proton makes Asp132 rotate 180°, with its proton now close to Asn139, see Fig. 1. The rotation leads to a lowering of the energy by 8.0 kcal/mol. For the Thr mutant, the corresponding value is 7.6 kcal/mol and for the Asp mutant it is 5.3 kcal/mol. From the calculations, relative pK_a values of His26 for the starting structure (with the proton pointing towards the N-side) compared to the wildtype enzyme, could also be evaluated for the mutants. For the Thr mutant, the pK_a value of His26 is 1.5 lower (2.0 kcal/mol) than for the wildtype, while for the Asp mutant it is 0.8 (1.1 kcal/mol) lower than for the wildtype.

The above results are best interpreted in the following way. First, one absolute pK_a value has to be chosen for the wildtype enzyme, as a reference point for the calculated relative values. This reference value was chosen for His26 in the situation when the proton is added, and when Asp132 is protonated with the proton pointing towards the N-side. A low pK_a value of His26 of 3.3 was chosen since there is a close lying proton on Asp132. Any value between 3.0 and 5.0 could have been chosen, and the effects discussed below would still be qualitatively the same. This means that with a proton on His26 the energy is higher by 5.0 kcal/mol compared to the situation with a proton on the N-side at pH = 7. As Asp132 is rotated (with its proton), the energy goes over a barrier ($\mathbf{TS}_N$) and then to a minimum **M**, see Fig. 1, with an energy of −3.0 kcal/mol at point $\mathbf{M_1}$. This is obtained from the computed energy difference of −8.0 kcal/mol between $\mathbf{M_1}$ and the point with the proton on Asp132 pointing towards the N-side at +5.0 kcal/mol. The point (**M**) is the *energy-well* described above. It should be noted that this minimum is not located at the same position as the one suggested previously by model calculations [14]. From the corresponding calculated values for the Thr mutant, this indeed means a less deep well of only −0.6 kcal/mol. For the Asp mutant, the energy well disappears. The *barrier* for proton transfer to the PLS via the transition state labeled $\mathbf{TS}_G$ (supposedly situated close to Glu286), should according to a previous analysis of experiments

for the wildtype enzyme be 10.8 kcal/mol [1,7] when there is an electron on heme a. This means that the *energy* at $\mathbf{TS}_G$ should be (10.8–3.0) = 7.8 kcal/mol. The assumption made here is that the mutants are too far away from $\mathbf{TS}_G$ to affect its energy and should therefore have the same energy of 7.8 kcal/mol at this point. The *barriers* should then be 8.4 (7.8 + 0.6) kcal/mol for Thr and 7.8 (7.8 + 0) kcal/mol for Asp. This means that the transfer of the proton to the PLS should be 60 times faster for the Thr and 160 times faster for the Asp mutant. After the proton has passed $\mathbf{TS}_G$ the energy goes down to −5.0 kcal/mol, followed by a reprotonation of **M** from the N-side (going down −3.0 kcal/mol), leading to an energy for a state with a proton at the PLS ($\mathbf{M_2}$) of −8.0 kcal/mol taken from the previous study. The suggested energy profile for proton transfer from the N-side to the PLS is shown in Fig. 2. The reference point in the figure, set to zero, is with a proton on the N-side. The corresponding profile for the Thr mutant is shown in red in the figure.

One of the most important conclusions from the previous analysis of the kinetic experiments [1,7], is that the energy of $\mathbf{TS}_G$, as well as the energy of the PLS, is affected by the presence of an electron on heme a. The diagram without an electron on heme a is drawn in Fig. S1 in the supporting information. It was concluded in one of the studies that the structure for $\mathbf{TS}_G$ should be *positively* charged [7]. A similar modulation of the proton transfer barrier by the redox state of heme a was predicted in a mathematical/kinetic modeling study [15]. The energy of 7.8 kcal/mol in Fig. 2 is taken from the situation when there is an electron on heme a. In a very critical leakage situation, the electron on heme a is transferred to the BNC and neutralized by a proton, leading to a danger for leakage back to the N-side, since the energy for a state with a proton at the PLS is raised to a higher level, from −8.0 in Fig. 2 to −0.9 kcal/mol in Fig. S1 (point $\mathbf{M}_2$ in the diagrams). Since $\mathbf{TS}_G$ is positive, its energy is also raised, by a similar amount from +7.8 to +15.2 kcal/mol, which will prevent leakage. For the Thr mutant the corresponding energies, without an electron on heme a, are +1.5 for the PLS and +15.2 kcal/mol for $\mathbf{TS}_G$. While the back-leakage barrier for the wildtype of (15.2 + 0.9) = 16.1 kcal/mol will be enough to prevent back-leakage from the PLS to the N-side, this will not be the case for the mutant with a barrier of only (15.2–1.5) = 13.7 kcal/mol. This means that leakage should increase for the Thr mutant by a factor of 60 and for the Asp mutant by a factor of 160, leading to the result that there should be essentially no pumping for the mutants, as found experimentally. At the same time, there is no reason why the proton transfer to the BNC should be stopped. On the contrary, the faster transfer through the D-channel could even lead to a faster chemistry.

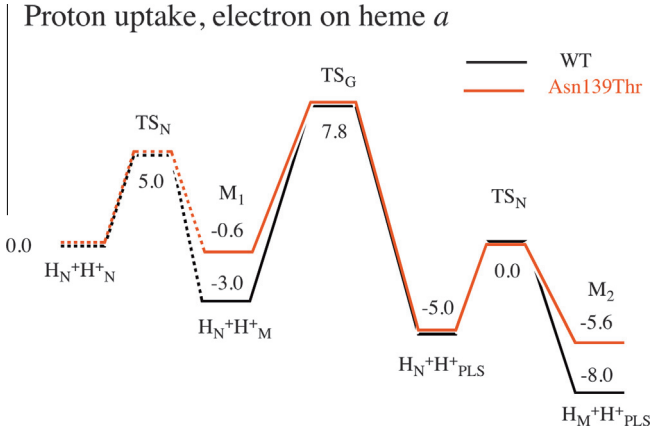

Fig. 2. Energy profiles for the wildtype enzyme in black and for the Asn139Thr mutant in red. The diagrams are drawn for the case with an electron on heme a. Under each energy level there is a description of the protonations at that point. N is the N-side, **M** the energy-well and PLS the pump-loading site.

To fully explain the experiments, there is one additional energetic requirement for the proton transfer through the D-channel: the minimum **M** should not be reprotonated before the proton has reached $\mathbf{TS}_G$, because this would lower the barrier also for the back-leakage. This is fulfilled if two conditions are met. First, there has to be a barrier for the reprotonation of **M** (at $\mathbf{TS}_N$) and second, the energy must increase rather quickly after the proton has left the **M** region to move towards the Glu286 region. The sum of these energies (the barrier and the energy increase) should be higher than the proton transfer barrier at $\mathbf{TS}_G$. These two conditions should be easily met by the enzyme. The calculated barrier for the rotation of Asp132 for the wild-type enzyme is 5.0 kcal/mol, actually quite close to the energy when His26 becomes protonated. This barrier could be slightly increased if the protonated His26 swings in and forms a salt-bridge to the deprotonated Asp132 as the proton leaves the **M** region. Such a salt bridge has been implicated in earlier modeling studies [16].

The question of how mutations close to the entry point of the D-channel, can stop proton pumping, while maintaining the rate of the chemistry at the BNC, has been addressed using a simple idea. The idea is that the uncoupling of proton pumping could be explained if there is a minimum **M** for the proton transfer close to the place of the mutation, which is deeper for the wildtype enzyme than for the mutants. The relative pK_a values obtained from model calculations support this idea. This makes the assumption of a long range effect of the mutations unnecessary. To optimize the capture of protons from the N-side, it has probably been found advantageous to have a minimum close to the entry point of the D-channel.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.febslet.2013.12.020.

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