

O–O bond splitting mechanism in cytochrome oxidase

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Abstract

Hybrid density functional theory (DFT) calculations have been used to investigate different mechanisms for O–O bond splitting in cytochrome oxidase. It is shown that the requirement for a low activation barrier for the O–O bond splitting is that two protons, apart from the tyrosine hydroxyl proton, are available at the binuclear center. A mechanism is suggested for the transformation from a species with a molecularly coordinated O₂, to an O–O cleaved species with an oxo-ferryl group. The mechanism has a calculated activation barrier in reasonable agreement with experimental estimates, and the overall reaction is close to thermoneutral, in line with the requirement that the energy wasted as heat should be minimized. The rate limiting step in the mechanism occurs at the initial Fe–O₂ intermediate, consistent with experimental observations that the decay of the oxy intermediate parallels the increase of the oxo product. The formation of a radical at the cross-linked tyrosine–histidine structure is a possible source for one of the electrons required in the bond cleavage process. Possible sources for the two protons are discussed, including a suggested key role for the hydroxyl group on the farnesyl side chain of heme a₃. © 2000 Elsevier Science S.A. All rights reserved.

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1. Introduction

Cytochrome oxidase catalyzes the four-electron reduction of O₂ to water in the terminal reaction in aerobic metabolism. This process clears the respiratory chain for the sustained redox chemistry that drives proton translocation and ultimately ATP formation. As befitting its crucial function in bioenergetics, cytochrome oxidase reduces dioxygen with low overpotential and with remarkably fast rates, transferring up to 1000 electrons per second to O₂. Moreover, as Wikström has shown, the free energy made available in O₂ reduction is used in the uphill translocation of protons across the membrane, thus contributing directly to the chemiosmotic gradient that is used for ATP synthesis [1].

The mechanism of O₂ reduction by oxidase has been actively pursued by a number of groups (for reviews, see Refs. [2–4]) and a variety of intermediates have been observed. Two of these, termed the P and F species, are of particular interest, as these have been implicated in the

pumping mechanism [5]. Recent work by Michel [6] and by Wikström and coworkers [7] has highlighted both the uncertainties and the progress in our understanding of the underlying mechanism by which exergonic electron transfers are coupled to endergonic proton motion. One of the key issues in advancing our insight into the molecular mechanism of proton pumping is to establish the molecular structures of the species that couple redox free energy into ion motion. This structural information is also essential in understanding how oxidase is able to cleave the oxygen–oxygen bond with low driving force and without the occurrence of potentially cytotoxic intermediates.

Accordingly, intense effort has been devoted to providing structural insight into the P and F intermediates. There is general agreement that F is a ferryl-oxo species that occurs at the three-electron reduced state of the oxygen substrate [2–4], but considerable controversy has swirled about the P species, which is formally at the two-electron reduction level [2]. The optical absorption properties of P and F in the visible region are clearly distinct, which suggests that the chemical structures at heme a₃ in the two intermediates are different. Thus, initial hypotheses for the structure of P centered around a bond-

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intact, heme a_3 -bound, peroxide species in either bridging or non-bridging interaction with Cu_B (e.g., Refs. [2,8,9]). Weng and Baker carried out optical work on the P and F species that are formed by peroxide reaction with the oxidized enzyme, however, and concluded that both P and F are ferryl-oxo species [10]. This conclusion was supported subsequently by spectroscopic data from Watmough et al. [11], Fabian and Palmer [12], and Wang et al. [13]. Raman spectroscopic work by Proshlyakov, Kitagawa and their coworkers gave clear vibrational evidence for a ferryl-oxo structure at heme a_3 in the hydrogen peroxide-generated P intermediate [14–16]. The link between the P species formed in the peroxide/oxidized enzyme reaction and that generated by oxygen reaction with reduced forms of cytochrome oxidase was made in time-resolved Raman studies of the reaction of O_2 with the two-electron reduced enzyme [17]. The results showed that P is formed on the 150 μs time scale, as expected from earlier optical studies [18–20], and that it has a ferryl-oxo structure with the same (Fe(IV)=O) vibrational frequency as that detected in the hydrogen peroxide process. Thus, there appears to be an emerging consensus that both P and F are bond-cleaved, ferryl-oxo intermediates.

If this is the case, then there is an immediate problem, as the cleavage of the O=O bond at the level of P requires an additional redox equivalent beyond those present in the binuclear center. Oxidation of the heme a_3 macrocycle to produce a porphyrin π -cation radical can be ruled out, as the Raman and optical properties of P are inconsistent with those that would be expected for a ring-oxidized species [3,17]. Fabian and Palmer suggested oxidation of Cu_B to the trivalent state [12]. Although this would be unprecedented in a biological system, it remains a possibility. More likely, however, is that the necessary reducing equivalent is provided by the unique, H240–Y244 cross-linked structure that has been observed recently in the bovine [21] and paracoccus [22] enzymes [17,23,24]. This species is ideally positioned relative to the O_2 binding site at heme a_3 to provide both an electron and a proton in the bond cleavage chemistry to produce the P species.

In the work described here, we have used computational methods to explore the feasibility of and plausible mechanisms for the formation of a bond-cleaved P structure. Our results highlight the role of protons in this process and provide insights into means by which the free energy available in the reduction of dioxygen is conserved in the bond cleavage process. The reduction of the protein-bound species that occur in P is used in driving proton pumping during subsequent steps in the reaction cycle.

2. Computational details

The O_2 activation in cytochrome oxidase occurs at the binuclear center shown in Fig. 1. The main components of the binuclear center are: the heme a_3 , the Cu_B , the

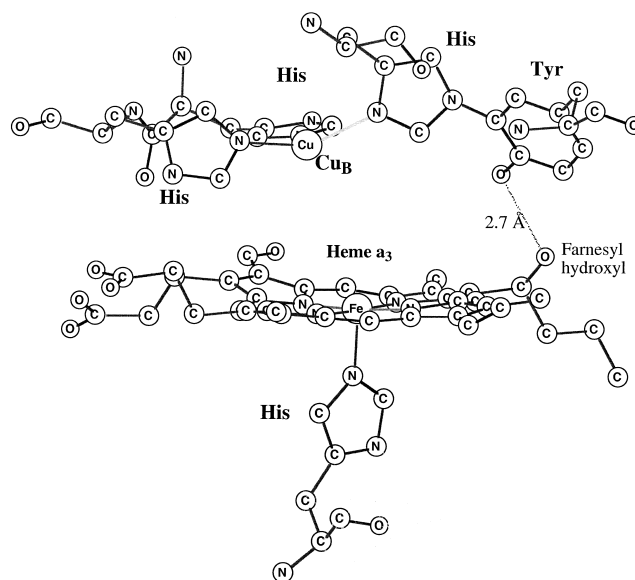


Fig. 1. X-ray structure of the binuclear center in cytochrome oxidase from the bovine enzyme (PDB structure 2OCC [21]).

histidine ligands of copper and iron, and the cross-linked tyrosine. In the basic model of the binuclear center, used in the quantum chemical calculations, the heme group is replaced by an iron with two chelating diformamidate (NHCHNH^-) ligands, while all histidines, except that covalently linked to the tyrosine, are replaced by ammonia. The cross-linked histidine–tyrosine is replaced by a cross-linked imidazole–phenol. Calculations were also performed on the separated iron or copper complexes, in which case somewhat larger models could be used, with imidazoles for all histidines and a full unsubstituted iron-porphyrin for the heme group. Those calculations are mainly used to verify that the smaller models reproduce most of the relevant properties of the more realistic models reasonably well. The details of those comparisons are given below, as are the details of the quantum chemical methodology.

2.1. Models

In the large model of the Cu_B complex the three histidine ligands (see Fig. 1) are represented by imidazoles and the cross-linked His–Tyr residue is represented by a cross-linked imidazole–phenol unit. In calculations on the combined binuclear center such a model of the copper complex would lead to a too large system. Instead, two of the histidines are modeled by ammonia ligands, and only the cross-linked histidine is modeled by an imidazole. In the initial state for the O_2 activation, the reduced state of the enzyme (R), the binuclear center has a Cu(I) center. This oxidation state of Cu_B can be achieved either by a plus charge on the copper complex, or by deprotonation of one of the histidine (imidazole or ammonia) ligands. Both types of models have been used, although with a

dominance for the plus-charged model. To evaluate the smaller models, certain bond properties that are expected to be relevant for the reactions studied are compared to the corresponding results for the larger more realistic models. One such property is the O–H bond strength of the cross-linked tyrosine, yielding a tyrosyl radical. The small model, $(\text{NH}_3)_2(\text{Imidazole-Phenol})\text{Cu}(\text{II})(\text{OH})^+$, yields a phenolic O–H bond strength that is 0.6 kcal/mol larger than the largest model, $(\text{Imidazole})_2(\text{Imidazole-Phenol})\text{Cu}(\text{II})(\text{OH})^+$. The small model, furthermore, turns out to be quite insensitive to the ligand protonation state. The neutral $(\text{NH}_3)(\text{NH}_2)(\text{Imidazole-Phenol})\text{Cu}(\text{II})(\text{OH})$ complex gives a phenolic O–H bond strength that is almost identical to the protonated model of the same size, only 0.5 kcal/mol smaller.

For the heme a_3 complex the large model has a full but unsubstituted porphyrin and the smaller model consists of an iron center with two chelating diformamidate (NHCHNH^-) ligands. This small porphyrin model has been used previously to model biological heme systems [25,26]. In the large model a neutral imidazole replaces the axial histidine, and in the smaller model ammonia is used in the axial position. The O–H bond in the $(\text{porf})\text{Fe}(\text{III})\text{OOH}$ compound is calculated to be 5.7 kcal/mol weaker when the small heme model is used as compared to when the large heme model is used. This fairly large difference is mainly due to a too strong Fe–O bond in the parent $(\text{porf})\text{Fe}(\text{II})\text{-O}_2$ compound as calculated for the small heme model. This value is considered so large that it is used as a correction in relevant cases when the small heme model is used. On the other hand, the Fe–O bond strength in other oxygen heme compounds is found to be quite similar for the two models. The Fe–O bond strength in both $(\text{porf})\text{Fe}\text{-OOH}$ and $(\text{porf})\text{Fe}\text{=O}$, as calculated using the small model, is found to be within 1–2 kcal/mol of the corresponding large model value. Since these latter differences fall within the normal uncertainty of the B3LYP method, no corrections are used for these bond strengths.

The conclusion from these model comparisons is that, for most purposes, the smaller model complexes, which are the only ones that can be used in models of the combined binuclear center, reproduce the results from the larger and more realistic models reasonably well.

A difficulty in studying the mechanisms for O_2 activation in cytochrome oxidase is to describe the hydrogen bonding situation for different structures in a balanced way. In the enzyme, the hydrogen bonding is expected to be saturated during the whole reaction while, in the model systems, only parts of the hydrogen bonding opportunities are present. In some cases the models used introduce new and artificial hydrogen bonding, which can be difficult to keep constant during the reaction. The changes in hydrogen bonding are usually larger when comparing different equilibrium structures than when going from a reactant to an immediately following transition state, which makes

reaction energies, in certain respects, more difficult to determine than activation energies. Therefore, special care has to be taken when relative energies of different equilibrium structures are calculated. Fortunately, the relative energy of different intermediates can be obtained in two ways, either by using the separate copper and iron complexes or by using the binuclear model. In most cases the results obtained in these two ways agree reasonably well, indicating that no large artificial hydrogen bonding effects have been introduced. In this context, one could also ask the question of how well the optimized model structures agree with the binuclear center of the enzyme, considering the fact that no restrictions are imposed on the geometry optimizations. In the crystal structure of Fig. 1, the Fe–Cu distance is 4.89 Å, and in the binuclear models used in the present investigation the optimized Fe–Cu distances are surprisingly constant, varying only between 4.4 and 4.6 Å. The distance between copper and the tyrosine oxygen is the same in all models, 5.3 Å, as compared to 5.7 Å in the crystal structure. Finally, the distance between iron and the tyrosine oxygen obviously varies much more, both longer (up to 7 Å) and shorter (down to 4.5 Å) distances than the 5.8 Å of the crystal structure being obtained. However, such variations in the structure are expected to have a very small impact on the relative energies. Considering the crude nature of the binuclear models, as compared with the full binuclear center with the protein surrounding, the structural resemblance between the models and the true enzyme has to be considered surprisingly large, and it should be sufficient for the present purposes.

2.2. Methods

The quantum chemical calculations are performed in two steps. For each structure considered a full geometry optimization is performed using the hybrid density functional B3LYP method [27–30]. In this first step, standard double zeta basis sets are used for all light elements. For the metals (iron and copper) a non-relativistic effective core potential (ECP) according to Hay and Wadt [31] is used. The valence basis set used in connection with this ECP is essentially of double zeta quality. In a second step, the energy is evaluated for the optimized geometries using larger basis sets including diffuse functions and a single set of polarization functions on each atom. This final energy evaluation is also performed at the B3LYP level. All the calculations are carried out using the GAUSSIAN program [32].

The procedure commonly used in our group to search for transition states in enzyme models containing transition metals is the following. A guessed reaction coordinate is frozen at different values, optimizing all other degrees of freedom at each value of the frozen parameter. If this procedure gives a maximum on the potential energy surface, the molecular structure at such a maximum can be

used as a very good starting structure for a transition state search using a calculated Hessian, i.e. the second derivatives of the energy with respect to the nuclear coordinates. In fact, it is our experience that such a final transition state optimization commonly leads to very small changes in the structure and particularly in the activation energy. In some cases it is not possible to use a single parameter as the reaction coordinate, since it does not lead to the desired product. In such a case a two-dimensional search for a transition state structure has to be used, freezing two parameters independently at different values and searching for a saddle point. In the present study the main frozen parameter is the O–O distance of the O₂ molecule to be split. For all mechanisms except the one labeled the two proton mechanism and containing a H₃O⁺ unit, a two-dimensional search had to be performed, using the tyrosine O–H bond distance as the second parameter. The models used for the binuclear center in all different mechanisms investigated contain more than 50 atoms. This means that it is not feasible to calculate the Hessian, which is needed for the final transition state optimization. Therefore, the activation energies given here are those obtained directly from the freezing procedure. Of course, this represents an uncertainty in the results, but it should be noted that the conclusions of this paper are drawn on the basis of comparisons between activation energies differing by as much as 25–30 kcal/mol, which is far more than the uncertainty introduced by the inaccuracy in the transition state search.

The surrounding protein is treated as a dielectric medium. The approach used for calculating the dielectric effects is the self-consistent isodensity polarized continuum model (SCI-PCM) as implemented in the GAUSSIAN-94 program [33]. The default isodensity value of $0.0004e/B^3$ was used, which has been found to yield volumes very close to the observed molar volumes [33,34]. The dielectric constant of the protein is the main empirical parameter of the model and it was chosen to be equal to 4 in line with previous suggestions for proteins [35]. The dielectric effects on the relative energies due to the surrounding protein are usually small for reactions where the charge state of the cluster is constant.

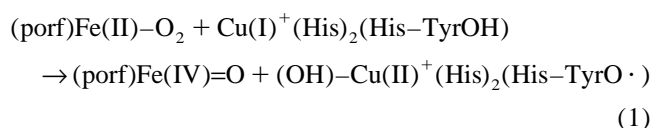
3. Mechanisms for O–O splitting

The O–O bond cleavage in cytochrome oxidase is coupled to a complicated flow of electrons and protons through the mitochondrial membrane. Although there is a large amount of experimental data available, the information is still insufficient to determine how many protons and electrons are involved in the O–O bond cleavage step, and this is one of the main questions when searching for a possible mechanism. Using quantum chemical methods the reaction energy and the activation energy can be determined for different possible reaction mechanisms for

O–O bond cleavage. For a mechanism to be likely, the following criteria should be fulfilled: (i) each reaction step should be reasonably close to thermoneutral not to waste energy as heat; and (ii) there should be no high barrier involved. A large number of calculations have been performed on different models of the binuclear center, and the results from these calculations are briefly summarized below, with emphasis on these two criteria.

3.1. Preliminary investigations

The starting point of this study is the mechanism for O–O splitting suggested in Ref. [17]. This mechanism is initiated by the O₂ molecule bridging between the iron and copper centers and simultaneous hydrogen bonding to the cross-linked tyrosine. When the O–O bond is broken, a concerted hydrogen atom transfer is considered to occur, moving the hydroxyl hydrogen of tyrosine to the bridging oxygen atom which binds to copper. The product of such a reaction is thus a Fe=O oxo-ferryl, a Cu(II)–OH hydroxyl and a neutral tyrosyl radical. The reaction energy for the reaction



is estimated, using the smaller models and separated iron and copper complexes, to be exothermic by 3 kcal/mol. Thus, the first criterion above, concerning the approximate thermoneutrality of the reaction, seems to be fulfilled for such a reaction mechanism. With regard to the activation energy, it turns out that the type of transition state suggested in Ref. [17] is not possible, since the O₂ molecule cannot simultaneously bridge between the two metals and be hydrogen bound to the tyrosine for simple geometric reasons, which means that the distance between the tyrosyl hydroxyl group and the bridging oxygens is too long. On the other hand, if a water molecule is inserted between O₂ and the tyrosine, the same type of reaction can occur, involving two coupled hydrogen atom transfers (Fig. 2), and gives the same product. However, the activation energy for the type of transition state sketched in

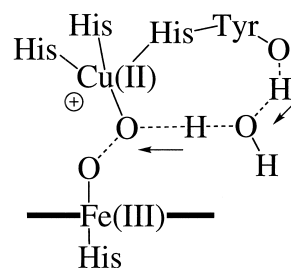
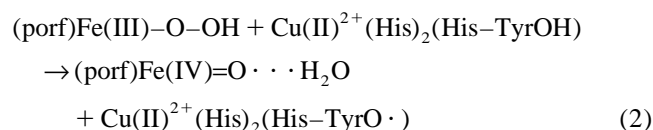


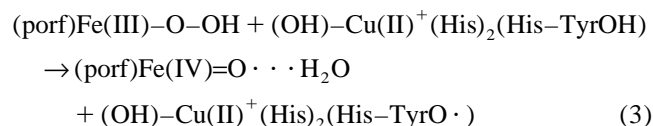
Fig. 2. Sketch of the transition state for the first mechanism investigated. The arrows indicate hydrogen atom transfer. The barrier is estimated to be more than 25 kcal/mol.

Fig. 2 is found to be at least 25 kcal/mol, which is much too high compared to experiments.

The next mechanism tried starts from compound A, with the molecular oxygen coordinated to heme a_3 . A proton is added to the binuclear center, at the O_2 molecule (see below), which leads to a Fe–O–O–H peroxide starting structure for the O–O splitting step. Similar to the mechanism described above, a hydrogen atom is considered to be transferred from tyrosine to the peroxide, concerted to the O–O bond splitting. This reaction yields a water molecule, besides the oxo-ferryl product and the tyrosyl radical:



The exothermicity for this reaction step is calculated to be 4 kcal/mol, using the combined binuclear center model. But again the barrier is found to be much too high, 31 kcal/mol. A possible source of the ‘extra’ proton in this reaction scheme is a water molecule, which, on the basis of spectroscopic data, has been proposed to be located in the vicinity of the copper center [36]. Taking a proton from such a water molecule leaves a hydroxyl group, which can become a ligand on copper. Including such a OH ligand on copper changes reaction (2) to



The energies for reaction (3) are almost identical to those of reaction (2), the exothermicity is changed to 6 kcal/mol and the barrier is found to be 26 kcal/mol. The type of transition state obtained for this reaction mechanism is sketched in Fig. 3. In conclusion, none of the mechanisms investigated so far gives a sufficiently low barrier for splitting the O–O bond.

At this point the question could be raised of whether it is at all possible for the binuclear center to split the O–O bond *before* the arrival of the third electron from heme a, a question that has been discussed extensively [17]. To shed

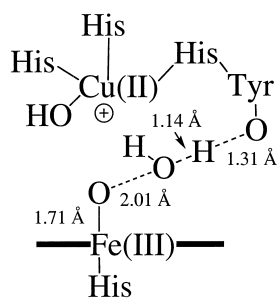
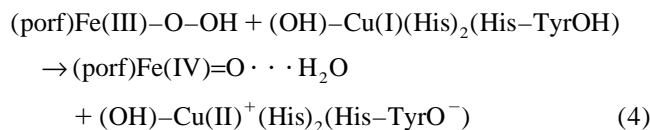


Fig. 3. Sketch of the transition state for the mechanism initiated by cleavage of a water molecule near copper. The barrier is estimated to be 26 kcal/mol.

some light on this issue, an electron was introduced into reaction (3), and calculations were performed for the reaction

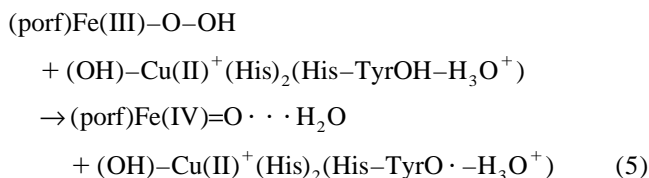


The only difference between the reactants in reactions (2) and (3) is that there is a Cu(I) center in reaction (3), while there is a Cu(II) center in reaction (2). This means that for the mechanism described in reaction (4) one of the electrons that are needed to break the O–O bond can be taken from copper and, therefore, this mechanism gives no tyrosyl radical. Instead, only a proton is taken from tyrosine, and tyrosinate is formed. For this mechanism there is no barrier for splitting the O–O bond. On the other hand, the reaction is found to be quite exothermic, 36 kcal/mol, indicating that the O–O splitting has raised the electron affinity by a large amount (about 30 kcal/mol when comparing reactions (3) and (4)). This means that the condition of thermoneutrality is not fulfilled if the third electron arrives before the O–O splitting. On the other hand, it should be possible to transfer the third electron *after* the O–O splitting in a thermoneutral process by properly tuning the redox potentials of the hemes. In this way the energy of the oxygen reduction can be saved and be used for proton translocation. It would have been useful to obtain theoretical estimates of these redox potentials but, unfortunately, they are difficult to compute accurately. No direct comparison between theory and experiment for these quantities is therefore possible at this stage. In conclusion, the calculations show how the high electron affinity of the dioxygen molecule is transformed to a high electron affinity of the binuclear center by essentially thermoneutral splitting the O–O bond. This reaction is thus a prerequisite for driving electron transfer in the respiratory chain.

3.2. The ‘two proton mechanism’

In a final attempt to find a low barrier mechanism for splitting the O–O bond, another proton was added to the binuclear center, starting from the mechanism of reaction (3). This proton is considered to be hydrogen bonding to the tyrosyl hydroxyl group and in the calculations is added in the form of a H_3O^+ molecule. Compared to the first mechanism investigated which has no other protons than the hydroxyl proton of the tyrosine residue involved, this mechanism has two ‘extra’ protons, and it will therefore be referred to as the ‘two proton mechanism’. The presence of such protons at the binuclear center in the reduced state (R) is well in line with experimental observations showing that protonation of the binuclear center occurs via the K-channel during the reduction phase [37]. In the next

section a possible site for the second ‘extra’ proton is discussed. The reaction to be studied can thus be written



Using a combined binuclear center model for the reactant in (5) and increasing the O–O distance successively the energy increases rather slowly. It turns out that already for an O–O internuclear distance of 1.9 Å the barrier is passed and the energy is decreasing. The optimized structure near the transition state (O–O 1.8 Å) is shown in Fig. 4. The energy and wave-function dependence on the O–O distance shows that, in this region, two different surfaces (with the same total spin but with different electronic structure) have avoided crossing with each other. The crossing occurs at about 1 kcal/mol above the (porf)Fe(III)–O–OH reactant, indicating that there is essentially no barrier for the O–O splitting step. The electronic structure of the binuclear center for the reactant in reaction (5) can be described in the following way. There are two unpaired spins, one residing on iron, with very little delocalization over the iron ligands, and one residing on the copper complex, delocalized over the copper center, the hydroxyl and imidazole ligands. During O–O splitting, very little happens at the copper complex. Instead, after the curve crossing is reached, spin builds up on the two oxygens of the O₂ molecule, which is caused by the splitting of the O–O bond. The spin on the oxygens due to

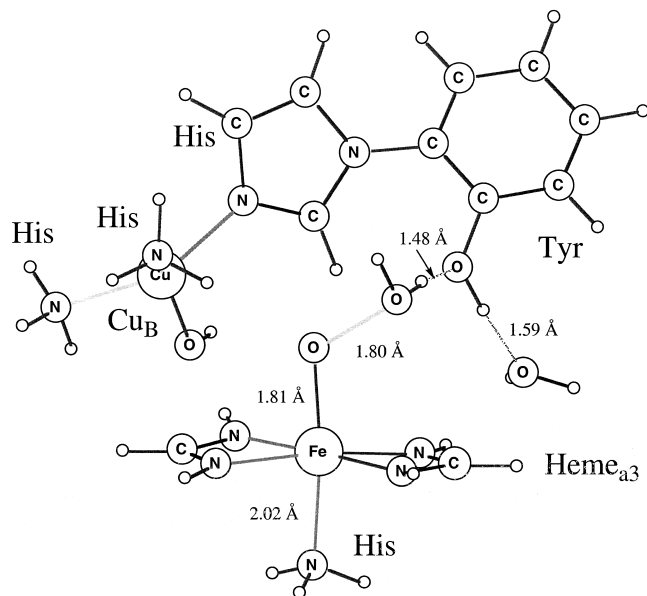


Fig. 4. Structure of the model system close to the transition state for O–O splitting according to the two proton mechanism. The barrier counted from the peroxy complex is estimated to be about 1 kcal/mol.

the breaking of the covalent bond is partly compensated by an electron transfer from the iron atom, forming Fe(IV). The spin that remains on the oxygens is antiferromagnetically coupled to an increased spin on the iron atom, corresponding to an involvement of the Fe(IV) oxidation state. The radical created in the product, in reaction (5) indicated as a tyrosyl radical, is thus not formed in the curve crossing region, but rather at a later stage of the reaction. The location of this radical, therefore, does not affect the activation energy. As it turns out, for the model used in the calculations the final product radical appears on the heme group rather than on tyrosine. It has previously been shown [35] that a tyrosyl radical cannot be created unless the tyrosine has a direct hydrogen bonding contact with a reasonably strong base (see below).

In conclusion, the calculations have shown that, with two protons, besides the tyrosine hydroxyl proton available at the binuclear center, the O–O bond can be split with no or a low barrier relative to a peroxy starting point. The calculations have also shown that all other mechanisms tried yield very high barriers for the O–O splitting, in the range of 25 to 30 kcal/mol.

3.3. Possible role of the farnesyl hydroxyl group

As discussed in the previous section, one of the protons needed to give a low barrier for O–O splitting could be taken from a water molecule proposed to be located near the copper center [36]. The second proton, which in the calculations was introduced as H₃O⁺, could in the real system arrive at the cross-linked tyrosine via the K-channel, which is assumed to transport protons from the inside of the membrane to the binuclear center. In fact, the K-channel ends at the hydroxyl group on the hydroxyethylfarnesyl side chain of the heme a₃ porphyrin ring. This hydroxyl group is furthermore close enough to be hydrogen bonded to the tyrosine hydroxyl group, which is indicated in the X-ray structure shown in Fig. 1. Thus one possibility would be that the farnesyl hydroxyl is protonated in the R state, which is the state that binds the oxygen molecule, and through hydrogen bonding between the farnesyl hydroxyl and the tyrosine hydroxyl, this proton is made available to the reaction center during the O₂ splitting step. Preliminary calculations indicate that the proton affinity of the farnesyl hydroxyl is substantial, which supports the hypothesis of this group being protonated at certain stages of the O₂ reduction process. The high proton affinity of the farnesyl hydroxyl group is due to resonances with the π-system of the heme-ring, which is shown in the optimized structure as a significantly increased C–O bond length in the protonated species. In fact, in the free heme, protonation of this hydroxyl group leads to the formation of a water molecule. It is furthermore interesting to note that the farnesyl hydroxyl group is conserved in virtually all heme–copper oxidases.

The calculations indicate that the role of the extra

proton, possibly located on the farnesyl hydroxyl group, would be to lower the energy in the O–O bond breaking region. This is done in the following way. As the O–O bond increases, the distal oxygen in the reactant of reaction (5) has to receive the second proton needed for water formation. This proton is taken from the tyrosine hydroxyl, which is hydrogen bonded to the distal oxygen of the Fe–O–OH reactant. But such a proton movement, from the tyrosine OH group to the Fe–O–OH hydroxyl group, is found to be too expensive *if there is not* an extra proton in the vicinity, which can go in and replace the tyrosine proton (see Fig. 4), as shown by the calculations described in the previous sections. This is where the postulated proton on the farnesyl hydroxyl group comes in, since it is perfectly located to replace the tyrosine proton in the transition state region. Thus, in this region the proton has left the farnesyl hydroxyl. In the product, on the other hand, where a radical is formed, supposedly on the cross-linked tyrosine, the proton is expected to return to the farnesyl hydroxyl, leaving a tyrosyl radical that is neutral in accordance with the previously observed tyrosyl radicals in PSII and RNR [38,39].

In the model used to study the two proton mechanism the farnesyl hydroxyl group is replaced by a water molecule, uncoupled to the small porphyrin model complex (see Section 2). Even though this model provides the necessary initial binding of a proton, it is clear that certain properties of the assumed product of the O–O splitting reaction (5) are not very well described by such a model. One property that is definitely not well described is the relative ionization potentials (IP) of the porphyrin ring and the tyrosyl side chain. The water molecule hydrogen bonded to the tyrosine is a much weaker base than the farnesyl hydroxyl group, and therefore the IP of the tyrosine will be too high in the model. On the other hand, the porphyrin model used is expected to have a too low IP, since increasing the delocalized π -system should increase the IP of the heme; compare Ref. [35]. One effect of such an imbalance in the calculated IPs is that, in the model calculations, the radical actually appears on the porphyrin, rather than on the tyrosine. However, in a separate model calculation for the ionized oxo-ferryl product using a full

porphyrin including the farnesyl hydroxyl group, hydrogen bonded to a phenol hydroxyl group, the radical is delocalized such that 0.3 of the spin population is found on the phenol and the rest on the porphyrin. This result shows that the exact location of the radical is very sensitive to the model used and cannot be definitely determined by the calculations. However, combining the energetic results from the calculations presented above with recent experimental observations [40], the conclusion is that the radical is *most likely* localized to the tyrosine in the O–O splitting product in the actual enzyme.

3.4. Proposed mechanism for the A to P transformation

The results obtained from the calculations, as described in the previous section, indicate that the actual O–O splitting process, going from an Fe–O–OH peroxy complex to the final product, has almost no barrier. This result may appear to be in disagreement with experiment, where an activation energy of 6–7 kcal/mol has been determined from the temperature dependence of the rate of P_M formation [41]. However, the experimental value concerns the entire process going from compound A, with a more or less unperturbed O_2 molecule coordinated to the reduced enzyme (R) with Fe(II) and Cu(I) centers, to the products. In contrast, the calculated barrier is counted from the Fe–O–OH peroxy complex. A simple way to rationalize these results is to conclude that the Fe(III)–O–OH peroxy complex is not a stable intermediate. Indeed, preliminary calculations show that the formation of the Fe(III)–O–OH + Cu(II)–OH intermediate from A, i.e. Fe(II)– O_2 + Cu(I)– H_2O , is endothermic by about 9 kcal/mol. In the present model, this energy would thus approximate the height of the activation barrier for the entire O–O splitting reaction as initiated from the dioxygen adduct compound A, in reasonable agreement with the experimental estimate [41]. These results taken together lead to the possible mechanism for O–O cleavage sketched in Fig. 5, starting from compound A, itself formed through a reversible (thermoneutral) coordination of the O_2 molecule to the reduced enzyme (R) [42,43], and going to P, or rather P_M , with an oxo-ferryl complex and a tyrosyl radical, via a

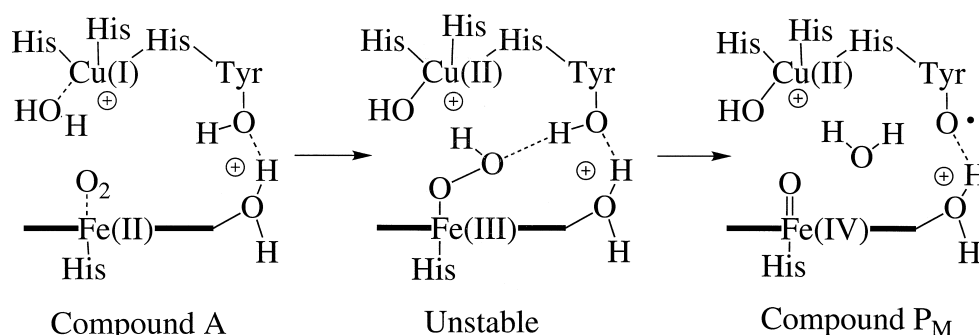


Fig. 5. Proposed O–O splitting mechanism.

transition state corresponding to a peroxy-type structure. An important aspect of the oxo-ferryl P_M species is that, for this structure, the electron affinity is large enough to induce the transfer of the third electron from heme a to the binuclear center, forming the P_R species. This electron eliminates the radical hole on the tyrosine, and will thereby make the extra proton leave the farnesyl hydroxyl group, forming a neutral tyrosine. Thus P_R will have the same structure as P_M , the only difference being that the tyrosyl radical is replaced by a tyrosine and the farnesyl hydroxyl group is unprotonated. This is consistent with the fact that P_M and P_R have very similar optical spectral properties [44]. To start all over with a new O_2 molecule, the binuclear center first needs to be reduced. In one of the reduction steps a new proton should enter via the K-channel and protonate the farnesyl hydroxyl group. To investigate at what stage such a protonation is likely to occur, the proton affinity of the farnesyl hydroxyl group is presently calculated for different oxidation states of iron. Protonation of the binuclear center via the K-channel during the reduction phase is also suggested by mutation experiments [45–47].

The reaction energy for the mechanism proposed in Fig. 5, i.e. going from A to P (P_M), is best estimated using the results for reaction (3). For reaction (3), which corresponds to going from the unstable peroxy intermediate in Fig. 5 to the oxo-ferryl P_M species with a tyrosyl radical, an exothermicity of 6 kcal/mol is calculated. Taken together with the estimate that the unstable peroxy state is about 9 kcal/mol above compound A as discussed above, this yields a 3 kcal/mol endothermic reaction in going from A to P_M , which is reasonably close to the expected thermoneutral reaction. It should be noted that the model system actually used to determine the O–O splitting barrier for the two proton mechanism in fact gives a somewhat too exothermic reaction, 9 kcal/mol going from A to P_M . One reason for this is the fact that the IP for the heme model is expected to be too small, as discussed above. Another problem is that it is rather difficult to correctly describe the changes in hydrogen bonding occurring between species A and P_M . The changes in hydrogen bonding are much smaller when going from the reactant to the transition state, which makes the barrier height, in certain respects, simpler to determine than the reaction energy. In conclusion, the A to P_M transition is suggested to be close to thermoneutral.

Regarding the structure and energy of the transition state for the A to P reaction, the results are much more preliminary, since the main objective of the calculations has been to find a mechanism for which the actual O–O splitting step is not prohibitively high. The peroxy-type structure suggested in Fig. 5 is one of several possibilities. In fact, it is more likely that a preceding transition state, corresponding to splitting the water molecule near the copper center, is the highest energy structure. In that case the barrier would be higher than the estimated 9 kcal/mol

for the endothermicity of peroxide formation. However, 9 kcal/mol is only a rather crude estimate and there are actually some indications that the energy of this structure should be somewhat lower. A preceding transition state could therefore be low enough to agree with the experimentally determined activation energy of 6–7 kcal/mol [41] for the A to P transition. Another possibility is a reaction where both protons are transferred to the O_2 molecule *in concert* with the O–O bond breaking. However, none of these possibilities, which are presently under investigation, are likely to change the present estimate of the barrier significantly.

4. Conclusions

To investigate the mechanisms for O–O bond splitting in cytochrome oxidase, possible intermediates and transition states for different models of the binuclear center were calculated. The calculations show that the O–O bond can be split at the two electron level in a close to thermoneutral reaction, yielding an oxo-ferryl product and a tyrosyl radical. The calculations also indicate that the location of the radical on tyrosine is only of minor energetic significance, since for certain types of models the radical appears on the porphyrin. This result can be compared to the situation in heme peroxidases, for which the location of the radical formed in the O–O bond splitting step varies among different enzymes. In certain enzymes (such as horseradish peroxidase, peanut peroxidase and pea cytosolic ascorbate peroxidase) the radical in compound I is found on the porphyrin, while in cytochrome *c* peroxidase it is found on a proximal tryptophan residue. Rather than a direct energetic role in the O–O bond splitting process, the tyrosyl radical in cytochrome oxidase is more likely to play a special role in proton pumping which is coupled to O_2 reduction. The calculations also show how the O–O splitting process increases the electron affinity of the binuclear center.

A large number of attempts using different models had to be made in order to find an O–O splitting mechanism with a low enough activation barrier. Two different models for the initial structure of the actual O–O splitting step were used, either an Fe–O–O–Cu bridging structure or an Fe–O–O–H structure. In the latter structure the proton is assumed to come from a water molecule in the vicinity of the copper atom. Taking a proton and an electron from the cross-linked tyrosine can, in both these cases, yield an oxo-ferryl group and a tyrosyl radical in a close to thermoneutral way. However, the activation barrier is in both cases found to be prohibitively high, on the order of 25 kcal/mol. On the other hand, if one more proton is made readily available to the binuclear center, the activation barrier for splitting the O–O bond in a preformed Fe–O–O–H compound more or less disappears. The activation energy for the entire O–O splitting process,

from the A to the P species, is instead suggested to be determined by the formation of the Fe–O–O–H structure. This is consistent with the experimental observation that Fe(II)–O₂ decay is paralleled by the appearance of Fe(IV)=O [17]. The second proton required for the O–O splitting is suggested to reside on the farnesyl hydroxyl group, which is hydrogen bonded to the cross-linked tyrosine. This hydroxyl group is at the end of the K-channel, along which protons can migrate from the inside of the mitochondrial membrane to the binuclear center.

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