Modeling Cytochrome Oxidase: A Quantum Chemical Study of the O−O Bond Cleavage Mechanism

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Abstract: The mechanism of O−O bond cleavage at the binuclear center in cytochrome oxidase has been investigated by using hybrid density functional theory (B3LYP). A potential energy surface for the reaction step from compound A, with the O_2 molecule coordinated to heme a_3, to the bond-cleaved compound P is constructed. The features of the calculated potential surface agree well with experimental information on this reaction step. First, a free energy of activation of 15 kcal/mol is obtained, reasonably close to the value of 12 kcal/mol corresponding to the observed lifetime of compound A. Second, the calculations give a large entropy effect on the reaction rate, which explains the weak temperature dependence observed for the P formation reaction. Third, the calculated potential surface has no stable intermediate between A and P, in agreement with the experimental observation that compound A decays with the same rate as compound P forms. Fourth, the calculations show that the oxo−ferryl compound P together with a tyrosyl radical and a cupric−hydroxyl can be formed in a close to thermoneutral reaction, which gives support to the suggestion that the source of the fourth electron in the O−O bond cleavage reaction is the cross-linked tyrosine residue near the binuclear center. The calculations indicate, however, that a direct hydrogen-atom transfer from the tyrosine to the iron-coordinated O_2 molecule produces too high an energy barrier. We attribute this to weak electronic coupling between the tyrosine and the iron center. Instead, our work suggests that the rate-limiting step involves a hydrogen-atom transfer from a water molecule in the vicinity of the copper center, and that the actual O−O bond cleavage step requires an extra proton to be available in the vicinity of the tyrosine residue, possibly on the hydroxyl group of the heme a_3 farnesyl side chain. The reason the additional proton is needed for the O−O bond cleavage is discussed.

I. Introduction

About 2 billion years ago, photosynthetic organisms developed the ability to use water in the unlikely role of electron donor. The byproduct of this chemistry was molecular oxygen, which these organisms released as a waste product. With the appearance of significant amounts of O_2 in the atmosphere, a potent oxidant became available to drive biological processes, and conversion to a water/oxygen biochemistry took place. Today, oxygen is used as the oxidizing substrate in a multitude of enzymatic processes, including the synthesis of peptides, nucleic acids and fatty acids, signal transduction, drug detoxification, and cellular protection. The vast majority of biological oxygen consumption, however, is used in respiration. High-energy electrons, derived ultimately from food consumption, enter the respiratory chain at the level of NADH (with a reduction potential, $E_{m'}$, $= -0.33$ V) and are ushered through a series of energetically downhill steps that are coupled to ATP production. Oxygen ($E_{m'} = +0.82$ V) is used as the final electron acceptor to clear the respiratory chain for sustained electron transport and ATP production. Thus, a total of 1.15 V per electron is available to perform cellular processes, and current estimates indicate that the cell is able to harvest about 80% of this energy as ATP.

The enzyme that catalyzes respiratory oxygen reduction, cytochrome oxidase, or quinol oxidase in some primitive organisms, is a remarkable machine (for reviews, see refs 1−4). At the input level, electrons are transferred from cytochrome *c* ($E_{m'}$ +0.27 V) to a dimeric copper site (Cu_A) and then on to a low-spin heme (heme a). Intramolecular transfer from heme a to the binuclear center, comprising a heme (heme a_3) and a copper complex (Cu_B), see Figure 1, poises the enzyme for O_2 reduction. There is now an emerging consensus that the initial reduction of O_2 in the cytochrome and quinol oxidases is analogous to the one that occurs in the peroxidase class of enzymes (see refs 5 and 6). With two electrons in the binuclear center, one to convert heme a_3 from the ferric to the ferrous state and one to convert Cu_B from cupric to cuprous, O_2 can

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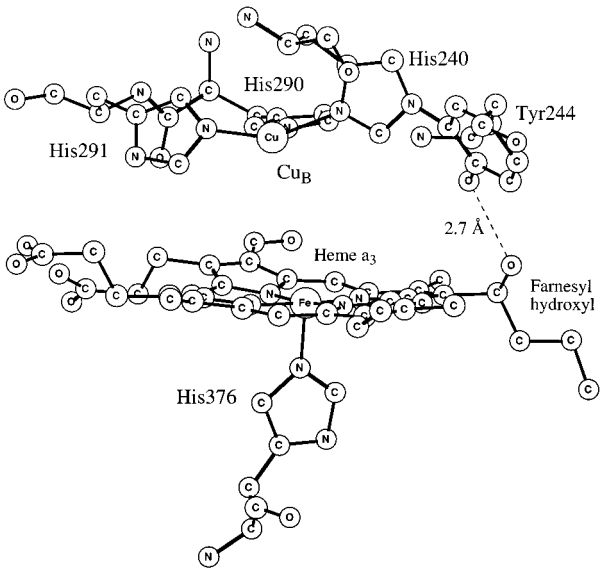

Figure 1. X-ray structure of the binuclear center.

bind to form an oxy complex (A). This oxy complex is activated to produce an O−O bond-cleaved form (P) of the enzyme.[7−13] In this species, the heme a_3 iron is oxidized to the Fe(IV)═O level, Cu_B is oxidized to the cupric state with OH^- bound, and the fourth electron is produced by chemistry in the protein that results in the formation of an amino acid radical.[9,14−18] Thus, the initial product of the interaction of O_2 with cytochrome oxidase produces a species that is formally equivalent to compounds I in the peroxidases.

The realization[19,20] that a tyrosine (Tyr244, beef heart numbering) in subunit I is cross-linked to one of the Cu_B histidine ligands, His240, has led to speculation that the tyrosine phenol side chain is the site of this radical.[13,21−24] Recent iodination work strongly supports this conjecture.[25] Establishing the structure of the P intermediate is crucial to understanding oxidase, as it has been shown that the enzyme plays a second key role in converting the electron currents in respiration to ATP, namely that it functions as a proton pump.[3,26,27] Although controversy swirls as to the precise nature of the coupling of electron motion to proton translocation (see, e.g., refs 28−31), it is clear that the reduction of the P intermediate drives much, if not all, of the proton translocation in the enzyme.

In recent work (briefly summarized in a meeting report,[32] we began detailed density functional calculations on the reduction of O_2 by the two-electron-reduced enzyme. In that report, we showed that two protons are needed for the reduction process, that the reaction of the reduced binuclear center and O_2 to produce oxo−ferryl, cupric−OH, and a tyrosyl radical is close to thermoneutral, and consistent with experimental observations,[13,33−35] that the reaction of the ferrous heme a_3−O_2 species is involved in the rate-limiting step. In this paper, we continue our study of the initial, O_2 bond-cleaving process. We present detailed calculations on the initial water-activating step and show that hydrogen atom abstraction from this bound water is required to protonate and reduce ferrous heme a_3−O_2 in the rate-determining step. We show that a true transition state can be found for this process and that the activation barrier that must be surmounted in its formation is consistent with experimental work. Entropy effects are shown to be important in this O_2-activating reaction, and we discuss the consequences of this in detail. Taken together, the results of our calculations provide a basis from which to consider the subsequent reduction/proton translocation reactions of the enzyme.

II. Computational Details

Calculations have been performed on models of the binuclear center in cytochrome oxidase, which is the site of O_2 reduction. As mentioned in the Introduction, preliminary results have been discussed in a conference report.[32] The X-ray structure of the binuclear center is shown in Figure 1.[19,36] The modeling aspects are discussed in the first subsection below, and the methods used are described in the second subsection.

a. Models. The binuclear center is composed of two parts, a copper (Cu_B) complex and an iron−heme (heme a_3) complex. Most of the calculations have to be performed on models including both metal centers. To elucidate reaction mechanisms implies a large number of calculations, which means that for practical reasons the models at present cannot have more than around 50 atoms.

In the largest model of the Cu_B complex, the three histidine ligands (see Figure 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 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 some cases, when the cross-linked tyrosine does not have to be included in the binuclear model, all three

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histidines can be modeled by imidazoles. Another aspect of the modeling is the overall charge of the complex. In the initial state for the O_2 activation, that is, in 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, yielding a neutral copper complex. The crystal structure shows that one of the histidine ligands (His290) is hydrogen-bonded to a chain of amino acid residues in the following way. His290 is hydrogen-bonded to the hydroxyl group of a threonine (Thr309), which in turn is hydrogen-bonded to the peptide carbonyl of a phenylalanine (Phe305). This means that there is a proton available that can make this imidazole a neutral ligand. It also means that this proton can be shifted toward the other end of the hydrogen-bonded chain, such that the His290 switches between being a neutral imidazole and a negatively charged imidazolate. The second histidine ligand (His291) is not hydrogen-bonded, and it is therefore most likely a neutral imidazole, in the same way as the axial ligand on heme a_3, His376, is considered as a neutral imidazole. The third copper ligated histidine (His240) is cross-linked to tyrosine at the second nitrogen position, and therefore it has to be a neutral imidazole. These structural features indicate that the Cu(I) oxidation state most likely is achieved through a plus-charge on the complex, although with a flexibility to switch toward a neutral complex with an imidazolate ligand. In the calculations described in this paper plus-charged model complexes are used. In the beginning of this study neutral models were also used, that is, with one imidazolate ligand on copper. Most energetic results were found to be rather insensitive to the protonation state of the copper ligands. Also for the heme a_3 complex, two models are used, one full but unsubstituted porphyrin and the other much smaller model consisting of an iron center with two chelating diformamidate ($NHCHNH^-$) ligands. In the large model a neutral imidazole replaces the axial histidine, and in the smaller model ammonia is used in the axial position. Only the smaller model is used in the binuclear calculations.

To evaluate the smaller models used for the binuclear center, calculations were performed on the individual metal complexes. In this way certain bond properties that are expected to be relevant for the reactions studied can be compared between the smaller and the larger models. One such property is the O−H bond strength of a water molecule loosely coordinated to Cu_B, yielding a Cu(II)OH complex when one of the O−H bonds is broken. The largest model, the positively charged $(Imidazole)_2$ (Imidazole-Phenol)Cu(I)$(H_2O)^+$ complex has a calculated water O−H bond strength of 83.4 kcal/mol. The binuclear model used in preliminary calculations on the particular reaction step where this O−H bond splitting occurs, $(NH_3)_3$ Cu(I)$(H_2O)^+$ yields a corresponding O−H bond strength of 87.7 kcal/mol. This difference of 4.3 kcal/mol in the water O−H bond strength is so large that it is used as a correction in the water splitting step when the small model is used. Another relevant property is the phenolic O−H bond strength of the cross-linked tyrosine, yielding a tyrosyl radical. In this case, the smaller model of the copper complex, $(NH_3)_2$ (Imidazole−Phenol)Cu(II)$(OH)^+$, yields an O−H bond strength that differ by only 0.6 kcal/mol from that of the largest model, $(Imidazole)_2$ (Imidazole−Phenol)-Cu(II)$(OH)^+$. Furthermore, the O−H bond in the (porf)Fe(III)OOH compound is found to be 76.9 kcal/mol using the large heme model, while the small model gives only 71.2 kcal/mol. This fairly large difference of 5.7 kcal/mol is mainly due to a too strong Fe−O bond in the parent (porf)Fe(II)$-O_2$ 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 heme compounds are quite similar for the two models. The Fe−O bond strength in (porf)Fe−OOH is found to be 30.9 kcal/mol in the large heme model and 32.1 kcal/mol in the small model. Similarly, the calculated Fe−O bond strength in (porf)Fe═O is 57.2 kcal/mol for the large model and 59.4 kcal/mol for the small model. Since these latter differences fall within the normal uncertainty of the B3LYP method, no corrections are used for these bond strengths. Thus, these model comparisons show that, if appropriate corrections are introduced, the smaller model complexes used in most of the binuclear calculations should be able to reproduce the results from the larger and more realistic models reasonably well. Furthermore, in one case both the large and the small model of the copper complex could be used to determine an activation energy, and as is described in section IV the results differ by only 2 kcal/mol. Similarly, small differences between ammonia and imidazole as histidine models were obtained in ref 37 where the mechanism of manganese catalase was studied.

One 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, while in other cases true changes in hydrogen bonding actually occur during a reaction step. The changes in hydrogen bonding are usually much smaller when going from a reactant to the immediately following transition state, which makes activation energies, in certain respects, simpler to determine than reaction energies. This means that the activation energies calculated should be considered as more reliable than the relative energies of the different intermediates. In one single case it was judged that the relative energy between two intermediates is best estimated using the large models of the separate metal complexes, and that is for the overall reaction energy of the A-to-P step. For another step, the relative energy of two intermediates could actually be calculated using both the binuclear model and taking the sum of the separate complexes. For that particular case the difference in relative energies thus obtained was less than 1 kcal/mol, indicating that it should be appropriate to mix results from the separate complexes with binuclear results. Finally, it should be noted that a model study like this one, investigating complicated biochemical reaction mechanisms, will unavoidedly have larger uncertainties in the calculated relative energies than if a simple gas-phase reaction is studied. However, the results from several similar theoretical studies of enzymes show that the accuracy is still enough for making important contributions to the elucidation of the reaction mechanisms, see for example ref 38

b. Methods. The calculations are performed in two steps. For each structure considered, a full geometry optimization is performed using the hybrid density functional B3LYP method.[39,40] In this first step, standard double-ζ basis sets are used for all light elements. For the metals (iron and copper) a nonrelativistic effective core potential (ECP) according to Hay and Wadt[41] is used. The valence basis set used in connection with this ECP is essentially of double-ζ quality. This basis set, which is labeled *lanl2dz* in the GAUSSIAN program,[44] is used also for the Hessian calculations, that is, second derivatives of the energy with respect to the nuclear coordinates. In this context it should be noted that although no restrictions are superimposed on the geometry optimizations, the optimized model structures agree reasonably well with the binuclear center of the enzyme. For example, in the two fully optimized transition states, the water cleavage and the final O−O bond cleavage transition states, which are the two most important structures determined in this study, the Cu−Fe distance is 5.6 and 5.3 Å, respectively. These values can be compared to crystal structure values

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which vary between 4.6 and 5.2 Å, depending on the state of the enzyme. 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. The inherent accuracy of the B3LYP method can be estimated from benchmark tests, in which the average error in the atomization energies for 55 small first- and second-row molecules is found to be 2.2 kcal/mol.[42] For transition metals there are no benchmarks due to the lack of accurate experimental numbers, but indications from normal metal−ligand bond strengths are that the errors are slightly larger, 3−5 kcal/mol.[43] All of the calculations are carried out using the GAUSSIAN program.[44,45]

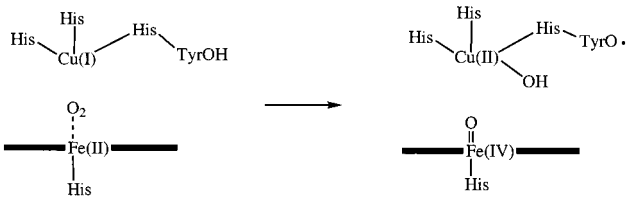
The activation energies are calculated as the energy difference between the reactant and a transition state structure. A full transition-state optimization requires the calculation of the Hessian. However, the different models used for the binuclear center contain between 35 and 55 atoms. This means that explicit Hessian calculations are very time-consuming, and therefore the Hessian has only been calculated for the most important models. In all cases approximate transition states are determined by freezing one or two coordinates at different values, optimizing all the other degrees of freedom, and determining the maximum (or saddle point) on the potential energy surface thus obtained. The main frozen parameter is the O−O bond distance of the O_2 molecule to be split. For some models an O−H bond distance also had to be frozen at different values for each O−O bond distance chosen. For the transition states of the most interesting models a full transition-state optimization, using an explicitly calculated Hessian, is performed using the approximate transition state as a starting structure. It turns out that the activation energies change by less than 2 kcal/mol going from the approximate transition states to the fully optimized ones. The calculated Hessians are also used to estimate zero-point, thermal, and entropy effects on the relative energies, applying the harmonic approximation. For this matter Hessians are calculated also for the corresponding reactant structures.

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.[46] 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. 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. The dielectric effects on the relative energies due to the surrounding protein are found to be rather small, which is usually the case for reactions where the charge state of the cluster is constant.

III. The O−O Bond Cleavage Reaction

The most crucial step in the full cytochrome oxidase cycle is the one that actually cleaves the O−O bond. This is probably also the step where quantum chemical calculations can contribute most toward a full understanding of the mechanism. For a reaction mechanism to be likely, there are certain energetic criteria that have to be fulfilled. First, since cytochrome oxidase is a very efficient enzyme, turning almost all of the O_2 reduction energy into ATP, each reaction step should be reasonably close to thermoneutral, otherwise energy would be wasted as heat.

Scheme 1



Second, there should be no high barriers involved in any step, otherwise the reaction would become too slow. The lifetime of the ferrous oxy compound A is 200 μs in the mixed valence form of the enzyme and about 30 μs in the fully reduced form of the enzyme,[47] with a surprisingly low-temperature dependence to form the next observed intermediate, compound P. From the slope of ln(k) versus 1/T (Arrhenius plot), the activation energy for the O_2 cleavage has been determined to be 6.4 kcal/mol.[47] To evaluate different suggestions for O−O bond cleavage mechanisms at the binuclear center quantum chemical methods can be used to determine the reaction and activation energies, and in this way decide which mechanisms are energetically feasible.

To cleave the O−O bond four electrons and four protons are needed, forming two water molecules. In the initial bond-cleaved product three of the four electrons are provided by the binuclear center itself, forming Fe(IV) and Cu(II). Concerning the source of the fourth electron there has been an extensive debate. On the basis of time-resolved resonance Raman spectroscopy on the mixed valence form of the enzyme an O−O bond cleavage mechanism resulting in the formation of a tyrosyl radical has been suggested.[13] More recent experiments indicate that a tyrosyl radical actually is formed in this step.[16,22,25] Thus, the initial products are suggested to be Fe(IV)═O, a tyrosyl radical, and a Cu(II) center, see Scheme 1.

The ferrous oxy compound A is formed by a reversible coordination of the O_2 molecule to the iron center of heme a_3. The ground state of compound A is an open shell singlet state, with a triplet state only about 1 kcal/mol higher in energy (as calculated using the small porphyrin model), indicating a very weak coupling between the two unpaired electrons (one on iron and one on the O_2 molecule). Using the large porphyrin model the coordination energy of the O_2 molecule at heme a_3 is calculated to be about 2 kcal/mol. In fact, separate calculations, using even larger basis sets than for the rest of the systems treated here indicate that the calculated O_2 binding energy should be even smaller, in good agreement with experimental estimates of this as a thermoneutral process.[48,49]

The O−O bond cleavage mechanism suggested in ref 13 starts with a bridging of the O_2 molecule between the iron and copper centers, forming an Fe(III)−O−O−Cu(II) peroxide type of structure. A concerted hydrogen-atom transfer from the tyrosine to the oxygen coordinated to copper is postulated to occur between the O_2 molecule and the cross-linked tyrosine residue, yielding the Fe(IV)═O oxo−ferryl, Cu(II)−OH hydroxyl, and neutral tyrosyl radical product of Scheme 1. To evaluate this proposed mechanism, the energy of the reaction described in Scheme 1 was calculated. Using the large model complexes the reaction in Scheme 1 was found to be exothermic by 4.4 kcal/mol, treating the iron and copper complexes separately. Thus,

(45) Frisch, M. J.; Trucks, G. W.; Schlegel, H. B.; Scuseria, G. E.; Robb, M. A.; Cheeseman, J. R.; Zakrzewski, V. G.; Montgomery, J. A., Jr.; Stratmann, R. E.; Burant, J. C.; Dapprich, S.; Millan, J. M.; Daniels, A. D.; Kudin, K. N.; Strain, M. C.; Farkas, O.; Tomasi, J.; Barone, V.; Cossi, M.; Cammi, R.; Mennucci, B.; Pomelli, C.; Adamo, C.; Clifford, S.; Ochterski, J.; Petersson, G. A.; Ayala, P. Y.; Cui, Q.; Morokuma, K.; Malick, D. K.; Rabuck, A. D.; Raghavachari, K.; Foresman, J. B.; Cioslowski, J.; Ortiz, J. V.; Stefanov, B. B.; Liu, G.; Liashenko, A.; Piskorz, P.; Komaromi, I.; Gomperts, R.; Martin, R. L.; Fox, D. J.; Keith, T.; Al-Laham, M. A.; Peng, C. Y.; Nanayakkara, A.; Gonzalez, C.; Challacombe, M.; Gill, P. M. W.; Johnson, B.; Chen, W.; Wong, M. W.; Andres, J. L.; Head-Gordon, M.; Replogle, E. S.; Pople, J. A.; *Gaussian 98*; Gaussian Inc.: Pittsburgh, PA, 1998.

(46) Wiberg, K. B.; Keith, T.A.; Frisch, M. J.; Murcko M. *J. Phys. Chem.* **1995**, *99*, 9072.

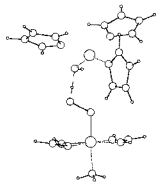
(47) Karpefors, M.; Ädelroth, P.; Namslaur, A.; Zhen, Y.; Brzezinski, P. *J. Am. Chem. Soc.* **2000**, in press.

(48) Chance, B.; Saronio, C.; Leigh, J. S. *J. Biol. Chem.* **1975**, *250*, 9226−923.

(49) Verkhovsky, M. I.; Morgan, J. E.; Wikström, M. *Biochemistry* **1994**, *33*, 3079−3086.

Calculated activation enthalpies

Water splitting step, yielding peroxide type intermediate



9.3 kcal/mol

O-O bond cleavage step, relative to peroxide type intermediate

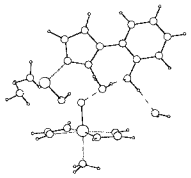
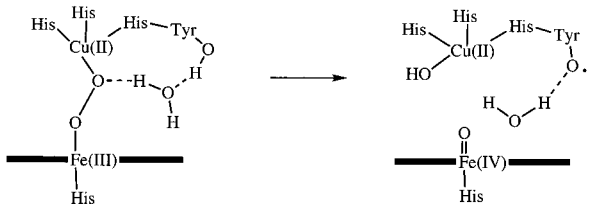
Type of model	Mechanism	Metal bridging	Non-bridging
	Water assisted	*>25* kcal/mol	*19.8* kcal/mol
	Water and proton assisted	not done	2.7 kcal/mol

Figure 2. Calculated activation enthalpies for different models and mechanisms. The values given as italics correspond to approximate transition states, and these do not include zero-point energies.

Scheme 2



the first criterion above, concerning the approximate thermoneutrality of the reactions is fulfilled for the overall reaction in Scheme 1.

A large number of calculations have been performed on models of the binuclear center in search for an O_2 bond cleavage mechanism at the cytochrome oxidase binuclear center that fulfills also the second energy criterion formulated above, that is, that there has to be a low enough activation energy. The most important results obtained in this process are summarized in Figure 2 and are discussed below. The main result of this search is a set of necessary prerequisites for making the O−O bond cleavage energetically feasible. It should be noted that there are possibly several slightly different versions of the detailed reaction scheme suggested here that fulfill the same criteria, and therefore further investigations are in progress. Apart from the energetic criteria it is necessary that the suggested mechanism agrees with other available experimental information, and therefore comparisons are made below to different kinds of experimental results for the O_2 bond cleavage reaction.

a. Water-Assisted Mechanisms. To evaluate the mechanism suggested in ref 13 a slight modification has to be introduced, since the O_2 molecule cannot simultaneously bridge between the two metals and form a hydrogen bond to the tyrosine for simple geometric reasons. However, an inserted water molecule can serve as a hydrogen-bonded link between O_2 and tyrosine, see Scheme 2. On the basis of spectroscopic data a water molecule has actually been proposed to be located in the vicinity of the copper center.[50] For such a system the same type of

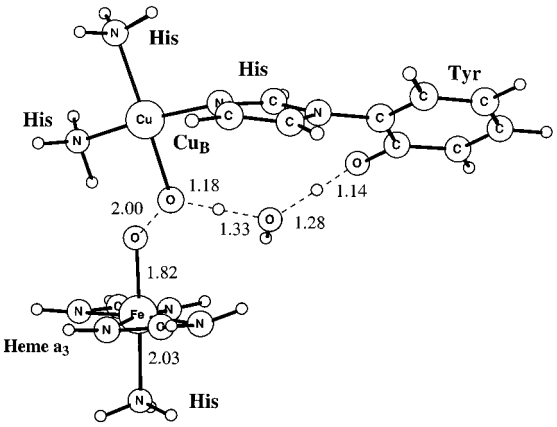
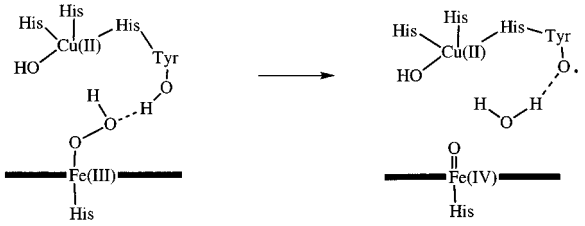


Figure 3. Water-assisted bridge-type splitting of the O−O bond.

Scheme 3



reaction as suggested in ref 13 can occur, with the only change that two coupled hydrogen atom transfers are needed to give the product of Scheme 1, one transferring a hydrogen atom from water to the bridging peroxide, and the other transferring a hydrogen atom from tyrosine to maintain the water molecule, see Scheme 2. In evaluating the activation energy for this type of reaction it was found that the energies for structures of the type shown in Figure 3, where the O−O bond is stretched and a hydrogen is approaching the copper coordinated oxygen, have very high energies, at least 25 kcal/mol above the bridging equilibrium. Thus, the energy in this region of the potential surface is much too high compared to the experimentally determined activation energy of 6.4 kcal/mol,[47] and therefore this type of mechanism was abandoned at an early stage of the investigation.

Another way to use the water molecule located in the vicinity of the binuclear center is to let it provide a proton to the O_2 molecule coordinated to heme a_3 in compound A. A water molecule as proton donor in the cytochrome oxidase O−O cleavage reaction has previously been suggested in ref 51 The calculations show that coupled to such a proton transfer, an electron transfer occurs from copper to the Fe−O_2 complex. This gives a nonbridging Fe(III)−O−OH peroxide starting structure for the O−O splitting step together with a Cu(II)−OH center (see further section IV below for this first reaction step). Very similar to the bridge mechanism discussed above, this Fe(III)−O−OH peroxide can hydrogen bond to the cross-linked tyrosine, and the O−O bond cleavage can be coupled to a hydrogen-atom transfer from the tyrosine, yielding the same product as in Scheme 2, that is, a water molecule, a tyrosyl radical and an oxo−ferryl heme as shown in Scheme 3.

The ground state of the (porf)Fe(III)−O−OH peroxide complex is a doublet and the Cu(II)−OH complex is also a doublet. For the combined binuclear center a ferromagnetic (triplet) coupling of these two spin centers is used in the calculations on the reactant and in the search for a transition

(50) Ralle, M.; Verkhovskaya, M. L.; Morgan, J. E.; Verkhovsky, M. I.; Wikström, M.; Blackburn, N. J. *Biochemistry* **1999**, *38*, 7185−7194.

(51) Konstantinov, A. A. *J. of Bioenerg. Biomembr.* **1998**, *30*, 121−130.

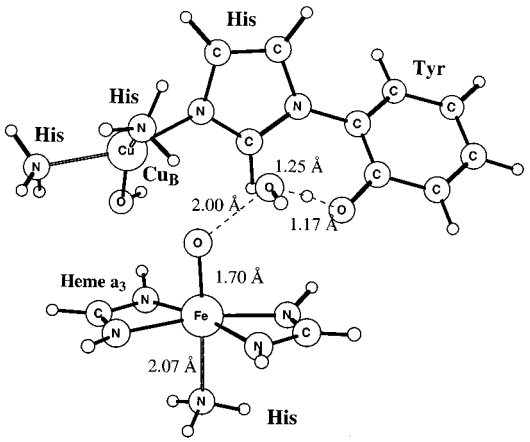
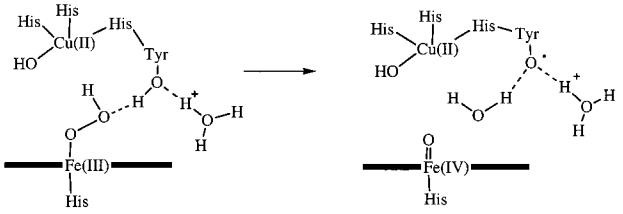


Figure 4. Structure of the approximate transition state for the nonbridging water-assisted mechanism of O−O splitting.

Scheme 4



state. In a few test calculations on similar models of the binuclear center an antiferromagnetic coupling of the spins located on the different metal centers are shown to give very similar results as the ferromagnetic coupling (see below).

Using such a nonbridging peroxide model of the reactant, a thorough search for the transition state for the O−O bond cleavage was performed. A two-dimensional search was made, and the two parameters used were the peroxide O−O distance and the O−H distance of the water molecule to be formed, that is, the distance between the distal oxygen in Fe−O−OH and the tyrosine hydroxyl proton. The potential surface in the region of an elongated O−O bond is very flat, and only an approximate transition state could be determined, having an O−O distance of about 2 Å, see Figure 4, and an energy about 20 kcal/mol above the peroxide type starting structure. Thus, for this mechanism also the calculated barrier was found to be much higher than the experimental activation energy of 6.4 kcal/mol.

The conclusion, thus far, is that neither the bridging nor the nonbridging form of the water-assisted mechanism gives barriers that are sufficiently low for cleaving the O−O bond at the experimentally observed rate. The reverse of the reaction in Scheme 3 can be compared to the similar water-splitting reaction in photosystem II (PSII), where a tyrosyl radical abstracts a hydrogen atom from a manganese-coordinated water molecule. Calculations on models of the PSII reaction, which is close to thermoneutral, show that this reaction has a much lower activation energy than the one obtained for the reaction in Scheme 3, the difference being on the order of 15 kcal/mol.[52] The higher barrier for the reaction in Scheme 3 is caused by the much weaker coupling between the water molecule and the metal center (iron), as compared to the water molecule in PSII, which is directly coordinated to the metal (manganese). The weak coupling, evident from the product side of Scheme 3, forces the creation of a radical on the hydroxyl group formed from the water molecule when a hydrogen atom is being transferred to the tyrosyl radical. Such a hydroxyl radical is

(52) Siegbahn, P. E. M., Blomberg, M. R. A. Unpublished data.

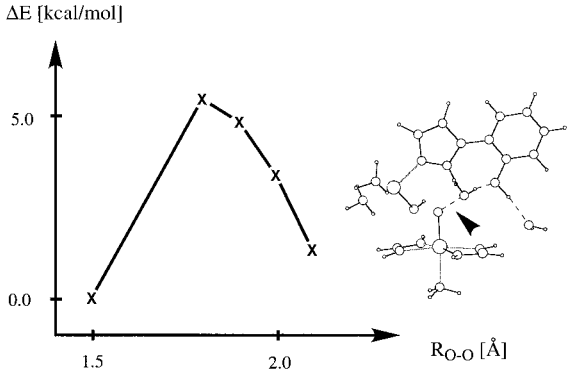


Figure 5. Pointwize determined potential surface around the O−O cleavage transition state for the water- and proton-assisted mechanism.

high in energy, which explains the large activation energy of the reaction in Scheme 3. In the PSII reaction there is no hydroxyl radical involved, but instead the radical character goes directly to the metal (manganese), which coordinates the water molecule.

b. Water- and Proton-Assisted Mechanisms. An alternative to the tyrosine as a source of the electron needed to cleave the O−O bond is to initially let the iron−porphyrin complex provide the electron. It is clear that the electronic coupling between the iron−porphyrin complex and the coordinated peroxide must be very strong. However, if the electron is taken from this source there is instead a problem of where to get the proton needed to form the product water. The calculations indicate that it is not possible to take the proton from tyrosine forming a tyrosinate, since the tyrosinate is too unstable in its position in the binuclear center. The solution to this problem suggested here is to simply add an additional proton to the binuclear site in the form of a protonation of the heme farnesyl hydroxyl group, which is located at the end of one of the proton channels (the K-channel) and which is hydrogen-bonded to the tyrosine according to the crystal structure. A protonation site of this type was modeled in the calculations by a protonated water molecule (H_3O^+), see Scheme 4. Protonation of the binuclear center at this stage is also in accord with experimental findings, indicating that a protonation occurs via the K-channel during the reduction phase.[53]

Initially an approximate transition state for the O−O splitting was determined starting from the Fe−O_2 H reactant in Scheme 4 and increasing the O−O distance successively in steps, optimizing all other degrees of freedom. The model of the binuclear center used for this investigation has a total charge of plus two, using two NH_3 ligands on copper. Only one parameter is found to be needed in this transition-state search, since the protons are automatically moved in the desired directions when the O−O bond is stretched. The resulting one-dimensional potential surface is shown in Figure 5. At an O−O distance of 1.8 Å a maximum on the is obtained, yielding a barrier of only 5.6 kcal/mol in the small basis set used in all geometry optimizations. In a geometry close to this point, a Hessian was calculated, and a full geometry optimization was performed. The fully optimized transition state gives the same activation energy as the approximate one, 5.6 kcal/mol in the small basis set. A large basis set calculation in this structure gives an energy that is 0.1 kcal/mol below that of the Fe−O_2 H reactant. The large energetic effect of the larger basis set indicates that the transition state might be shifted if an optimization is performed using the larger basis set. Therefore

(53) Mitchell, R.; Rich, P. R. *Biochim. Biophys. Acta* **1994**, *1186*, 19−26.

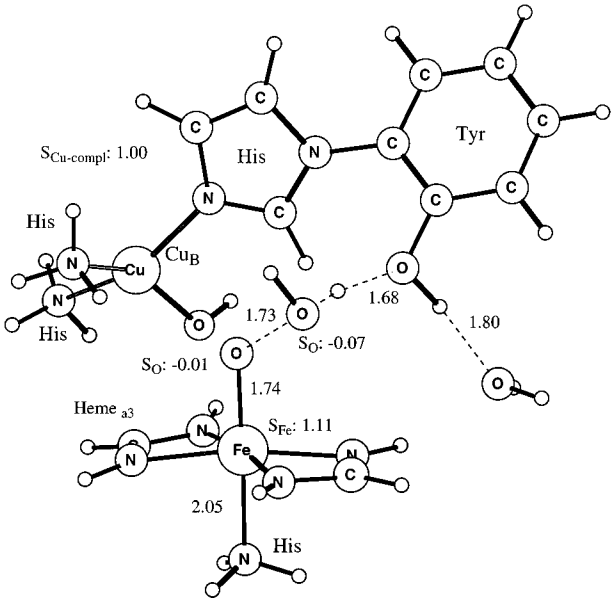
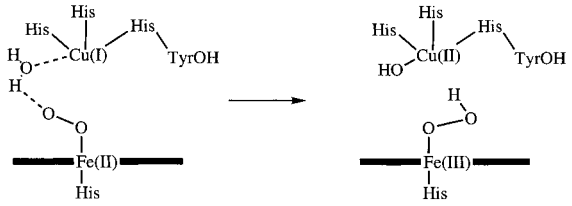


Figure 6. Optimized transition-state structure for the water- and proton-assisted mechanism.

Scheme 5



a new transition-state optimization was made using the large basis set on the oxygen atoms (including diffuse and polarization functions). This led to the optimized structure shown in Figure 6, and in the large basis set the activation energy is now found to be 2.4 kcal/mol. Including zero-point vibrational and temperature effects gives a free energy of activation of 3.4 kcal/mol. The entropy effects on this reaction step are found to be very small (only 0.6 kcal/mol). The $Fe-O_2$ H reactant has one spin on iron and another spin rather delocalized over the copper complex. The spin population at the copper complex is not changed during the O−O splitting reaction. In the transition-state region there is spin building up on the two peroxide oxygens. This spin is antiferromagnetically coupled to an increased spin on iron. The most important spin populations in the transition-state structure are shown in Figure 6. The transition state occurs very early in the reaction path, with an O−O distance that has increased only to 1.73 Å, and there is therefore only a small change of the spin compared to that of the $Fe-O_2$ H reactant. After the barrier is passed, for example, at an O−O distance of 1.90 Å, the spin on iron has increased to 1.52, the spin on the proximal and distal oxygens are −0.26 and −0.23, respectively, and there is still no spin on the tyrosine or the porphyrin. The calculations thus show that the product radical is not formed in the transition-state region but rather at a final stage of the reaction in Scheme 4, and the exact location of this radical should therefore not affect the activation energy.

Thus, the present calculations show that the O−O bond can be cleaved with a very low barrier relative to a peroxide starting point, if and only if both a water molecule and an extra proton are available at the binuclear center. In the following sections some further details of such a mechanism will be discussed. In the present section a water- and proton-assisted mechanism

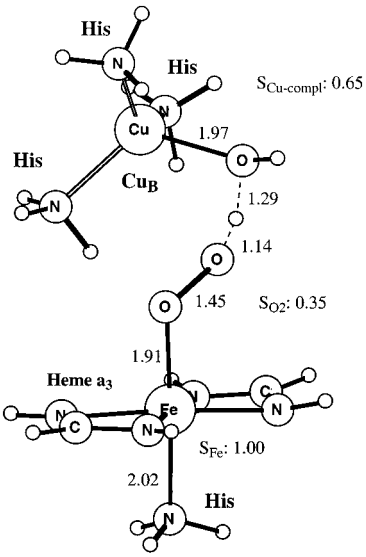


Figure 7. Optimized transition-state structure for the water-splitting step using ammonia ligands on Cu_B.

involving a nonbridging peroxide structure was investigated. It should be noted that a similar type of water and proton-assisted mechanism could be formulated also for the case when the reaction is initiated by the formation of a peroxide that is bridging between the two metal centers. Such a bridging mechanism is presently under investigation.

IV. Splitting the Water Molecule

The water- and proton-assisted mechanism proposed above is assumed to be initiated by the splitting of a water molecule, which has been proposed to be located in the vicinity of the Cu_B center.[50] The water molecule provides a proton to compound A and at the same time copper provides an electron. The product of this process would be Fe(III)$-O_2$ H + Cu(II)−OH, as described in Scheme 5. The reaction in Scheme 5 is investigated using a slightly different model of the binuclear center. Since the cross-linked tyrosine is not involved in this reaction step, it can be excluded from the model, and all three copper-coordinated histidines can be modeled by imidazoles. By using such a binuclear model the reaction in Scheme 5 is calculated to be 3.1 kcal/mol endothermic. In this value a correction of 5.7 kcal/mol for the too strong $Fe-O_2$ bonding as obtained using the small porphyrin model is included (see section IIa). As will be further discussed below, the calculations show that there is a large entropy effect on the water-splitting reaction, and therefore, if the free energy is considered, the reaction of Scheme 5 is found to be endergonic by 10.6 kcal/mol.

To determine the activation energy of the reaction in Scheme 5, a smaller model of the binulear center was first used, having three ammonia ligands on copper, see Figure 7. For this model an initial transition-state optimization was performed by distorting the structure step-by-step, starting with the reactant and approaching the product. Going from the reactant, the most natural parameter to be varied is the peroxide O−H bond to be formed. Freezing this distance at shorter and shorter values, thus moving the proton from the water molecule to the O_2 molecule, the energy goes up steadily. Going from the other side, starting with the Fe(III)$-O_2$ H peroxide and the Cu(II)−OH product and decreasing the O−H distance that leads to water formation and $Fe-O_2$, the energy also goes up steadily. The crossing point between these two potential surfaces gives an approximate

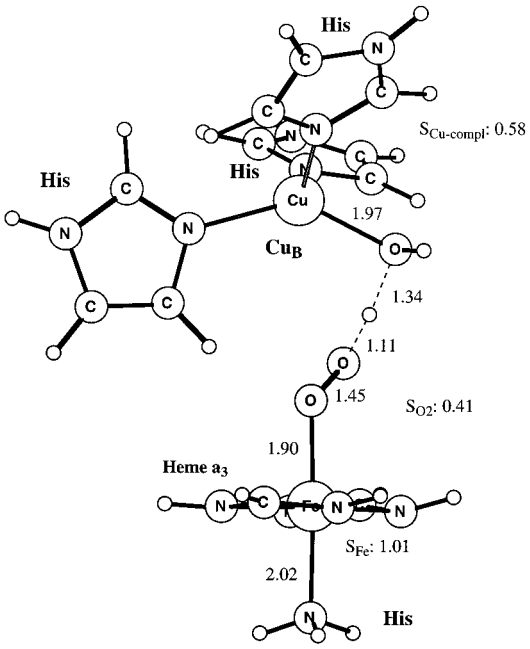


Figure 8. Optimized transition-state structure for the water-splitting step using imidazole ligands on Cu_B.

transition-state structure. The localization of the crossing point was further improved by freezing also the O−O distance at different values. In the approximate transition-state structure obtained in this way a Hessian was calculated and a true transition-state optimization was started. Somewhat surprisingly a fully optimized transition state could be obtained, despite the crossing of two different electronic states, having one imaginary frequency of 560 cm^{-1}, and with the struture shown in Figure 7. The energy in the fully optimized transition state is less than 2 (1.7) kcal/mol higher than in the approximate transition state using the geometry optimization basis set. The fully optimized transition state corresponds to an activation energy of 7.2 kcal/mol for this reaction step. In this type of situation with two crossing potential surfaces, there might be several states relatively close in energy. A single configuration method (like the B3LYP method) could then lead to a somewhat poorer description of the transition-state region than the equilibrium region, yielding a slightly too high calculated barrier for this reaction step. As mentioned above the reaction of Scheme 5 has to be corrected for errors in bond energies using the small models. Therefore, also the value for the activation energy has been corrected for this error, using half of the correction for the reaction energy. As was also mentioned above, the calculations give a large entropy effect for the water-splitting reaction, yielding a free energy of activation of 13.3 kcal/mol. Such a large entropy effect explains the surprisingly weak temperature dependence of this reaction step as obtained from kinetic measurements,[47] see further below.

In a second step, the fully optimized transition state in Figure 7 is used as a starting structure for a transition-state search using the larger model having three imidazole ligands on copper. The resulting fully optimized transition state is shown in Figure 8, and comparing Figures 7 and 8 it can be seen that the two models give very similar transition-state structures. The calculated activation energy is also very similar for the two models, differing only by 2.0 kcal/mol. The large model gives an activation enthalpy of 9.2 kcal/mol, and taking the entropy effect from the smaller model, the free energy of activation is calculated to be 15.3 kcal/mol.

Figure 9. Resonance structures in a protonated 1-propenol.

In Figures 7 and 8 the electronic structure in the transition state is given in the form of unpaired spin populations. The reactant of Scheme 5 has a Cu(I) electronic structure, with no spin population on copper, and the product has a Cu(II) electronic structure with one unpaired spin on the copper complex. As can be seen in Figures 7 and 8, at the transition state about half an electron is transferred from the copper complex to the iron complex. Furthermore, the proton is halfway between the copper-coordinated water molecule and the Fe−O_2 complex. This transition state therefore very clearly indicates that a hydrogen atom transfer is taking place.

The reactant of Scheme 5 has an open shell singlet ground state, with one spin on iron and one spin delocalized over the oxygen atoms. The state with a ferromagnetic (triplet) coupling of these two spins is about 1 kcal/mol higher in energy. As discussed above, the product of Scheme 5 has one spin localized on the iron center and one spin delocalized over the copper complex. An antiferromagnetic (singlet) coupling of the two metal-based spins turns out to be lowest in energy, again with the ferromagnetic (triplet) coupling only 1 kcal/mol higher in energy. The calculations on the reaction in Scheme 5 were performed for the antiferromagnetic coupling. Test calculations in a few points indicate that the ferromagnetic coupling would have given essentially the same result. This result is important since the rest of the calculations reported in this paper are actually performed for the corresponding ferromagnetically coupled states.

V. Source of the Proton: Possible Role of the Farnesyl Hydroxyl Group

It was shown above in section IIIb that an excess proton in the vicinity of the hydroxyl group of the cross-linked tyrosine gives rise to a substantial decrease of the O−O bond splitting barrier. Such a proton was modeled by H_3O^+ in the calculations. In the enzyme, the tyrosine residue is directly connected to the K-channel, which is supposed to transport protons from the inside of the membrane to the binuclear center during the reduction phase. In fact, the K-channel ends at the hydroxyl group of the hydroxyethylfarnesyl side chain on the heme a_3 porphyrin ring, and as indicated in the X-ray structure shown in Figure 1, this hydroxyl group is hydrogen-bonded to the tyrosine hydroxyl group. Due to resonances with the π-system of the heme-ring, the proton affinity of the farnesyl hydroxyl is expected to be large. The resonance structures can be illustrated using 1-propenol that is protonated on the hydroxyl group, see Figure 9. This type of resonance, reflected in the structure as an increased C−O bond length, leads to a stabilization of the positive charge, which results in an increased proton affinity as compared to the corresponding saturated compound. In the heme farnesyl case the double bond in propenol is replaced by the total π-system, and a much larger stabilization of the positive charge is obtained. In fact, in a calculation on a full iron−porphyrin model with a protonated hydroxyl group in the same position as the farnesyl hydroxyl, the water molecule leaves the heme. In more realistic calculations having a phenol group (modeling the tyrosine) hydrogen-bonded to the protonated hydroxyl group, the proton is located between the farnesyl hydroxyl and the tyrosine hydroxyl, and the farnesyl C−O bond is moderately stretched relative to the unprotonated species. These calculations support the hypothesis of a reasonably large

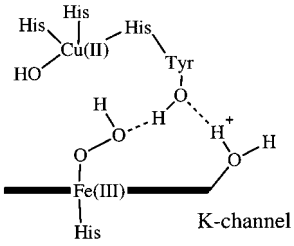


Figure 10. Model of the binuclear center including the farnesyl hydroxyl group.

pK_a value at this site (even if absolute pK_a values cannot be accurately calculated). It is therefore likely that this group is protonated at certain stages of the O_2 reduction process, for example in the reduced state (R), which is the state that binds the oxygen molecule. Through the hydrogen bonding between the farnesyl hydroxyl and the tyrosine hydroxyl, this proton is thus made available to the reaction center during the O_2-splitting step as shown in Figure 10. Interestingly, the farnesyl hydroxyl group is conserved in virtually all heme−copper oxidases. It might be argued that the binuclear center would become too highly charged (+2) by such a protonation of the farnesyl hydroxyl group. However, as mentioned in section II, one of the histidine ligands (His290) on copper is connected to a hydrogen-bonding chain, which might serve as a proton relay keeping the total charge on the binuclear center more or less constant. In this context it is particularly interesting to note that the threonine residue in this hydrogen-bonding chain has been shown in mutation experiments to be essential for the functioning of the enzyme.[54]

The hydrogen bonding between the cross-linked tyrosine and the farnesyl hydroxyl group is thus suggested to be important for lowering the O−O bond cleavage barrier. In fact, it could also be important for the localization of the radical in the final product. The calculations on Scheme 1 show that the formation of a neutral tyrosyl radical at the end of the O−O bond cleavage reaction gives a reaction that is close to thermoneutral. At this point of the reaction, therefore, it is most likely that the proton has left tyrosine, giving rise to a neutral tyrosyl radical, which is also in accord with the previously observed tyrosyl radicals in PSII and RNR.[55,56] One possibility here is that the high proton affinity of the farnesyl hydroxyl group makes the proton return there after the reaction.

The water molecule used to model the farnesyl hydroxyl group in the study of the water- and proton-assisted mechanism described above provides a site for an easily available proton in the vicinity of the tyrosine residue, but it is not basic enough to lower the ionization potential of tyrosine to become competitive with that of the heme−porphyrin ring. It was shown in a previous study[57] that the formation of a tyrosyl radical from tyrosine is energetically very unfavorable unless the tyrosine has a direct hydrogen-bonding contact with a reasonably strong base. Therefore, in the calculations described above on the reaction in Scheme 4, the radical in the final product is found on the porphyrin ring rather than on the tyrosine in the binuclear model. The farnesyl hydroxyl group is expected to be much more basic than the water molecule, and it could therefore decrease the ionization potential of the tyrosine so that it becomes lower than that of the porphyrin ring. In a separate calculation on an improved model of the ionized oxo−ferryl product, using a full porphyrin including the farnesyl hydroxyl group, hydrogen-bonded to a phenol hydroxyl group, the radical is actually found to be delocalized with 32% of the spin population on the phenol and the rest on the porphyrin, see Figure 11. This result shows that the exact location of the final product radical is very sensitive to the model used. Another possibility is that the extra proton at the end of the O−O bond cleavage reaction goes to the hydroxyl group at copper, which definitely would increase the probability for a tyrosyl radical product. In fact, the calculations indicate that the cupric hydroxyl group is a stronger base than the farnesyl hydroxyl group, and therefore it is more likely that the extra proton goes to the copper hydroxyl group at the end of the O−O bond cleavage. Combining the results of the present calculations with recent experimental observations,[16,22,25] the conclusion is that in the actual enzyme the product radical created in the O−O cleavage process most likely is localized to the cross-linked tyrosine residue, although, the calculations indicate that the location of the radical on tyrosine is not energetically significant. An alternative source of the fourth electron would be copper, as suggested by some authors.[6,13,22] However, the calculations on the present models indicate that a Cu(III) product of the O−O cleavage reaction is less favorable than the tyrosyl radical product by about 7 kcal/mol.

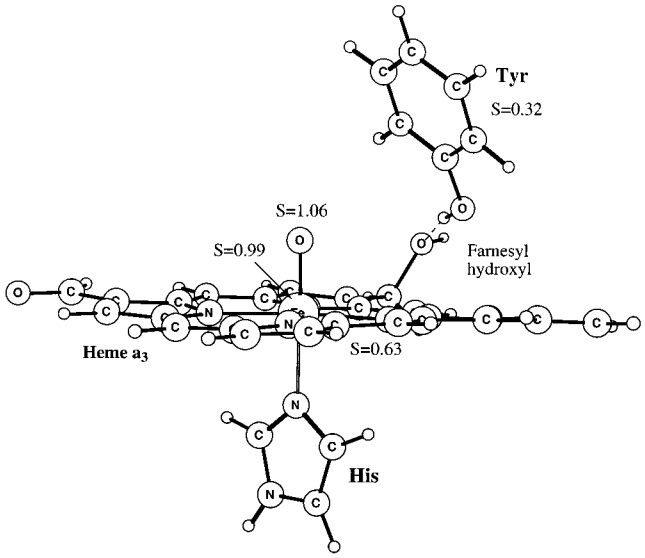


Figure 11. Spin populations for an improved model of the P_M state of the mixed valence enzyme, including the heme−tyrosine interaction.

VI. Mechanism for the A-to-P Transformation

At this point, the results obtained from the calculations, as described in the previous sections, are collected and a mechanism for the entire A-to-P transition is suggested. The energetic results of the calculations are summarized in Figure 12 in the form of a potential energy surface for the reaction, starting from the Fe(II)−O_2 compound A, itself formed through a reversible (thermoneutral) coordination of the O_2 molecule to the reduced enzyme (R).[48,49] The potential surface then goes all the way to P (also referred to as P_M) with an oxo−ferryl complex and a tyrosyl radical. As mentioned in section III above, the coordination of the O_2 molecule to the reduced enzyme (R) is calculated to be close to thermoneutral, in good agreement with the observed reversibility of this reaction step. The R-to-A step is not shown in Figure 12. As mentioned in section IV above, the calculations give an unusually large entropy effect on the water-

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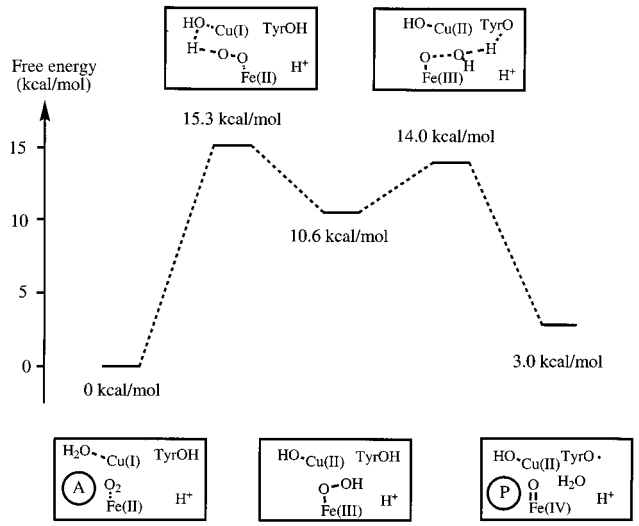

Figure 12. Calculated potential energy surface for the A-to-P transition.

splitting reaction step. Therefore, the free energy is used in the construction of the potential energy surface in Figure 12.

According to the mechanism suggested here, the rate-limiting step in the A-to-P transition is the initial cleavage of the water molecule, as can be seen from the potential surface in Figure 12. This step can be described as a hydrogen atom transfer from the $Cu-OH_2$ complex to the $Fe-O_2$ complex. The free energy of activation for this process is found to be 15.3 kcal/mol from the calculations. The experimental rate constant for this reaction corresponds to a free energy of activation of 12.4 kcal/mol, in reasonably good agreement with the calculated value. As mentioned in section III, the temperature dependence of the O_2 activation reaction is unexpectedly weak, resulting in an estimated activation energy of only 6.4 kcal/mol from an Arrhenius plot (the slope of $\ln(k)$ versus $1/T$).[47] The low-temperature dependence for the O_2 activation reaction can now be explained from the results of the calculations, as being due to an unusually large entropy effect. The calculated entropy effect is 6.0 kcal/mol, corresponding exactly to the difference between the experimental free energy of activation as obtained from the rate constant and the experimental activation energy as obtained from the temperature dependence of the reaction rate. The origin of the large entropy effect is the weak bonding of both the water molecule and the O_2 molecule in the reactant compound A. The calculations give a long Cu−O distance of 2.3 Å in compound A, and EXAFS data suggest a Cu−O distance of 2.5 Å,[50] indicating a very weak coordination of the water molecule to the copper center. This weak coordination in the reactant results in a large loss of entropy during the reaction where the firmly bound Fe−O−OH and Cu−OH species are formed. Such a loss of entropy should be typical for hydrogen atom transfer reactions from metal-coordinated groups, where the product is normally much more well-ordered due to the formation of new covalent bonds between the metal and the ligand. Furthermore, a kinetic isotope effect of 1.9 in D_2O is obtained experimentally for this process,[47] indicating that an internal hydrogen atom or proton transfer is involved in the rate-limiting step, also in agreement with the presently suggested mechanism, see Figure 8. The calculations give a somewhat too large kinetic isotope effect for the water-splitting step, 4.0 as compared to the experimental value of 1.9, which indicates a too strong coupling between the proton and electron transfer in the theoretical model. Very similar results for the kinetic isotope effect was recently obtained in model calculations for the water-splitting reaction in PSII.[52]

At this point one might ask if the binuclear center can accommodate simultaneously an O_2 and a water molecule in the manner required for the suggested mechanism. To investigate this question an O_2 and a water molecule were inserted into the crystal structure using the local $Fe-O_2$ and $Cu-OH_2$ structures obtained from the calculations. It turns out that this can be done in such a way that there is a perfect hydrogen-bonding distance between the two molecules. Furthermore, the crystal structure shows that the region of the binuclear center where the water molecule is supposed to be located to initiate the O_2 activation process is quite empty, except for a valine residue (Val243) at a distance of about 4.8 Å from the copper complex. Recent mutation experiments show that the replacement of this valine by isoleucine increases the lifetime of compound A,[58] which nicely supports the mechanism suggested by the present calculations. The bulky isoleucine would make it more costly for the water molecule to reach its position in the vicinity of the copper center, which is needed to initiate the O_2 activation, and thereby increase the barrier of the step that is rate-limiting according to the presently suggested mechanism.

The product of the rate-limiting water-splitting reaction step is an $Fe(III)-O_2$ H peroxide complex and a Cu(II)−OH complex. The best estimate of the relative free energy of this point is obtained by using the binuclear model complex having three imidazole ligands on copper (see Figure 8), which suggests that the energy is 10.6 kcal/mol above compound A. This value includes a large entropy effect of 7.4 kcal/mol. The calculations thus indicate that this $Fe(III)-O_2$ H peroxide complex is not a stable intermediate. From this point the O−O bond is cleaved with a low barrier. The only observable product of the O_2 activation, according to the presently suggested mechanism, is the bond-cleaved P state (also referred to as P_M), having an oxo−ferryl Fe(IV)═O state and a tyrosyl radical. This result is in accordance with time-resolved resonance Raman observations on the mixed valence form of the enzyme, showing that compound A disappears at the same rate as P_M appears.[13] 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 a species that can be labeled P_R. 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 almost the same structure as P_M, the main difference being that the tyrosyl radical is replaced by a tyrosine in P_R. This is consistent with the fact that P_M and P_R have very similar optical spectra.[59] It should here be noted that the formation of the observed P_R species when the fully reduced enzyme reacts with O_2 might very well occur by a somewhat different mechanism, not involving the tyrosyl radical, since in this case the electron can be taken directly from heme a during the O−O bond cleavage process. However, the final structure of P_R is likely to be the same one as described above. To start the cytochrome oxidase cycle 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 come in via the K-channel and protonate the farnesyl hydroxyl group. Protonation of the binuclear center via the K-channel during the reduction phase is also suggested by mutation experiments.[60−63]

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The reaction energy for the proposed mechanism for the A-to P-(P_M) transformation is obtained from the initial calculations on the reaction in Scheme 1, and assuming that the introduced water molecule and proton are bound by about the same amount before and after the O−O bond cleavage reaction, yielding a slightly exothermic reaction by 4.4 kcal/mol. To obtain the free energy of the reaction, the entropy effect has to be estimated. The entropy effect for the first water-splitting step was found to be 7.4 kcal/mol, and the entropy effect on the second, O−O splitting step was found to be small (as calculated for the barrier). The A-to-P step is therefore estimated to be endergonic by about 3.0 kcal/mol using the present models. This result is thus in reasonably good agreement with the thermoneutrality criterion. From experiments it is known that the reaction must be slightly exergonic, since at the end of the reaction there is no observable amount of compound A left. Rearrangements in the binuclear center, occurring at the end of the O−O bond cleavage reaction, and not included in the present model calculations, could give the needed driving force for the suggested mechanism of the A-to-P transformation. One such rearrangement could be the transfer of the extra proton to a stronger base, for example to the hydroxyl group on copper as mentioned above.

VII. Conclusions

In this paper the conditions for O−O bond cleavage at the binuclear center of cytochrome oxidase are investigated. One result of the calculations is that a water molecule needs to be present at the binuclear center when the O−O bond cleavage process is initiated. There is experimental support for such a water molecule.[50] There are two slightly different possible mechanisms, in which the water molecule plays somewhat different roles. In one case a bridging Fe−O−O−Cu peroxide intermediate is involved, and in that case the water molecule forms a hydrogen-bonding link between one of the oxygens and the cross-linked tyrosine. In the other case, the water molecule is split to form a nonbridging Fe−O−O−H peroxide intermediate, leaving a hydroxyl ligand on copper. The calculations indicate that both these types of peroxide structures are unstable and endothermic by several kcal/mol relative to compound A. The model calculations give the bridging peroxide structure slightly lower than the nonbridging one, but this difference is within the uncertainty of the calculated relative energies. The presence of the water molecule is, however, not enough to give an O−O bond cleavage barrier in accord with experimental rate measurements for the A-to-P transition. It was found that a protonation of the binuclear center, for example at the farnesyl hydroxyl group on the heme a_3 side chain, leads to a significant lowering of the activation energy. This water- and proton-assisted mechanism was studied for the nonbridging type of process. Variants of this mechanism, having the same general characteristics are presently under investigation. The protonation of the binuclear center is in accord with experiment, and in fact, the farnesyl hydroxyl group, which also is hydrogen-bonded to the cross-linked tyrosine, is located at the end of the K-channel, which is assumed to transport protons from the inside of the membrane to the binuclear center during the reduction phase. It is also interesting to note that this farnesyl hydroxyl group is conserved in virtually all heme−copper oxidases. With these two conditions fulfilled, a water molecule and a protonated farnesyl, a mechanism for the A-to-P transition could be formulated, which both is close to thermoneutral and has an activation energy in agreement with experiment. The final product is suggested to have a tyrosyl radical, and the calculations show that the O−O bond actually can be cleaved when the so-called mixed-valence form of the enzyme reacts with O_2, having no other electrons available than those of the Fe(II)−Cu(I) state of the binuclear center. The calculations further suggest that the A-to-P transition occurs in one step, without intermediates of sufficient lifetime to be detected. Finally, the calculations give a large entropy effect on the rate-limiting step, which rationalizes the low-temperature dependence of the reaction rate for the O_2 activation.

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