Structure of a Model Transient Peroxide Intermediate of Peroxidases by ab Initio Methods

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Received May 21, 1996. Revised Manuscript Received August 30, 1996[⊗]

Abstract: Peroxidases are oxidative metabolizing heme proteins that require hydrogen peroxide to be transformed to the catalytically active, compound **I**, species from the ferric resting state. Although a peroxide complex has been proposed as a key intermediate in this reaction, this intermediate is too transient to have thus far been definitively characterized. Results of previous molecular dynamics (MD) simulations of a peroxide complex with cytochrome C peroxidase (CCP) indicated that peroxide forms a stable complex and binds in a nonsymmetric, end-on mode in which the oxygen atoms systematically exchange places as ligands for the iron. These results provided support for a plausible pathway from the peroxide complex to compound **I**, involving the participation of nearby histidine and arginine residues. To further explore the reliability of this bonding description, we report here, for the first time, the use of ab initio methods to determine the optimized geometry and stability of a peroxide complex with a model heme peroxidase. Two stable minima were identified, with binding energies of 9.7 kcal/mol. In both of these complexes, the peroxide binds in an end-on mode but with alternative oxygen atoms as the Fe ligand. No minimum corresponding to a bridged structure could be found. The two end-on minima are connected by a low-energy ridge. These results provide confirmation of three mechanistically important characteristics found in the previous 300 K MD simulations of a peroxidase−HOOH complex: (i) formation of a stable peroxide complex, (ii) binding of peroxide in an asymmetric end-on mode, and (iii) dynamic interchange between the oxygen atoms that bind to the Fe, implying a small energy barrier between them.

Introduction

Peroxidases are ubiquitous oxidative metabolizing heme proteins widely distributed in plants, fungal, and bacteria but also found in mammalian species. As shown schematically in Figure 1, the common characteristic of peroxidase oxidations[1] is the requirement of stochiometric amounts of peroxide that transforms the ferric-heme resting state, via a putative peroxide intermediate, to the catalytically active, compound **I**, Fe═O, ferryl species, two oxidation states above the ferric resting state. While both the resting state and compound **I** species are stable enough to have been extensively characterized, there is only indirect kinetic and spectroscopic evidence[2,3] for the formation of the transient peroxide intermediate. The nature of this intermediate, the mechanism of formation of compound **I** from it, and the role of the nearby residues in its formation are currently among the least understood aspects of peroxidase oxidations.

Based on the first known crystal structure of a peroxidase, cytochrome C peroxidase (CCP), a mechanism of compound **I** formation from the putative peroxide intermediate and the postulated role of the nearby His and Arg residues in it has been proposed by the crystallographers who solved the structure.[5] As shown schematically in Figure 1, in this mechanism, the proximal histidine is proposed to function as an acid−base catalyst by abstracting a proton from the proximal oxygen and transferring it to the distal peroxide oxygen, resulting in a neutral precursor to product formation (the first two protein assisted steps in Figure 1). In the last protein assisted step of this proposed mechanism, facile O−O bond cleavage, with water as the leaving group, assisted by electrostatic stabilization of this neutral leaving group by nearby Arg has been suggested. The observed structural features of the protein formed the main basis for this hypothesis, particularly the proximity of the distal imidazole and arginine to a putative peroxide ligand of the heme iron in CCP.

To further explore the plausibility of this pathway, using the X-ray structure of CCP, we have previously reported molecular dynamics simulations of this putative CCP−peroxide intermediate.[4] The results indicated the formation of a stable CCP−HOOH complex, providing additional support for this intermediate, previously inferred from kinetic and spectroscopic data.[2,3] In this species, the peroxide was found to bind to the Fe as a ligand in a bent end-on geometry, rather than a bridged structure. In addition, during the MD simulations at 300 K, a rapid exchange of oxygen atoms as ligands of the heme iron was observed, with equal probability of each oxygen serving as a ligand, indicative of a low-energy barrier between them.

The dynamic behavior found also provided direct support for a proposed mechanism of compound **I** formation shown in Figure 1 and the postulated role of the nearby His and Arg residues in it.[5] Specifically, in the simulations, the peroxide hydrogens were found to form H-bonds with the N_ϵ of the histidine. As the O atoms of the peroxide alternate as ligands to the iron, the histidine alternate as a partner in H-bonds with

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⊗ Abstract published in *Advance ACS Abstracts,* October 15, 1996.
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S0002-7863(96)01723-4 CCC: $12.00

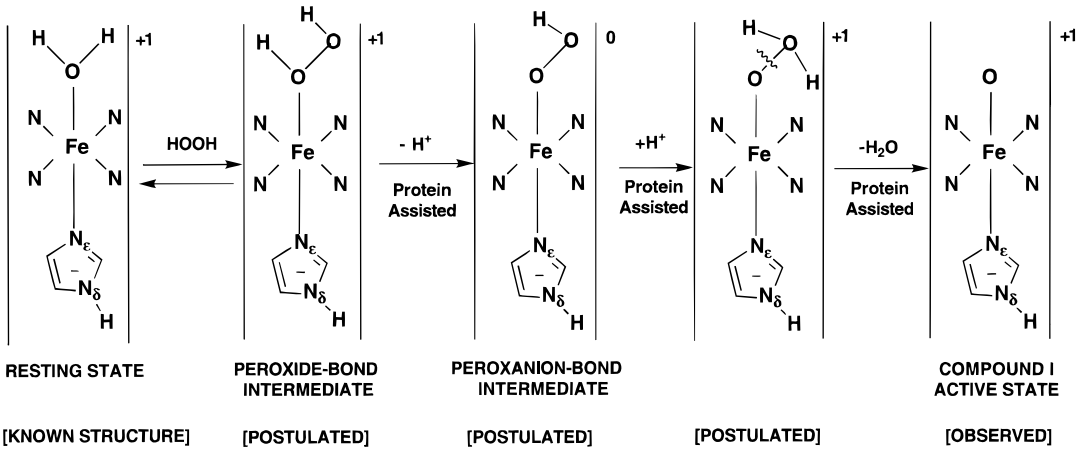

Figure 1. Proposed pathway for transformation of peroxidases from the resting state to the catalytically active oxidation species.

the distal and proximal peroxide hydrogen atoms. This dynamic H-bonding interaction between the imidazole of the His and the two peroxide hydrogen atoms supports the proposed function of the His as an acid−base catalysis by abstracting a proton from proximal oxygen and transferring it to the distal peroxide oxygen, leading to the formation of a product precursor complex. The proposed electrostatic stabilization of the neutral leaving group by the Arg is supported by the finding that the distal peroxide oxygen remains near the cationic Arg HNe during the simulation.

These previous studies were performed using the capabilities and force field in the Amber suite of programs.[6] The validity of the mechanistic inferences obtained from it depends to a large extent on whether the peroxide does indeed form a stable complex with the heme iron and whether the bonding mode obtained for the peroxide during the MD simulations is reliable. Neither of these key properties can be verified by experimental methods at the present time. However, it is now possible to use the techniques of ab initio quantum chemistry in order to further characterize these transient peroxidase−peroxide complexes. To this end, we report here for the first time ab initio quantum chemical studies of a model ferric heme peroxidase−peroxide complex. Specifically, we have calculated the optimized structures and interaction energy of the peroxide with the ferric heme unit and compared these results with those found in the MD simulations. These calculations include quantum effects that cannot easily be incorporated in the empirical force field, particularly for transient species for which there is no independent structural information. The results obtained substantiate the finding of a stable peroxide complex with two equally preferred end-on binding modes with either oxygen atom as the ligand for the iron and a small energy barrier separating them. In addition, no evidence could be found for a stable bridged structure.

Model, Method, and Procedures

In this study an iron−porphyrin complex with an imidazole ligand was used as the model 5-coordinated peroxidase. This imidazole is conserved as the distal axial ligand of the iron in all known peroxidase structures with the exception of chloroperoxidase and is considered the prototypical axial ligand of peroxidases.[7] The geometry of the five-coordinated species used was that of the heme unit in the resting state of CCP[5] with the substituents of the protoporphyrin IX ring replaced by H atoms. Neutral forms of the imidazole and peroxide ligand were used, resulting in a net charge on the complex of +1. The calculations were done for a sextet state of the peroxide complex. There is

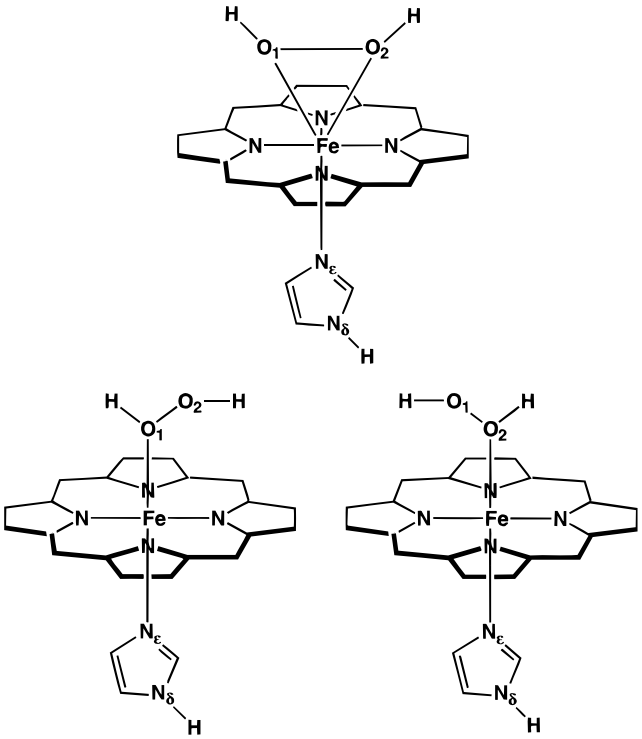

Figure 2. Three possible binding modes of peroxide to the model heme ferric peroxidase.

experimental evidence from electron spin resonance measurements for a sextet ground state in the resting state of peroxidases.[8] Furthermore, companion calculations of the water−porphyrin−imidazole complex (Loew and Dupuis, to be published) of the same type as the one described here yielded a sextet lowest energy state.

The calculations were performed using a spin-restricted open-shell Hartree−Fock (ROHF) wave function, with a 6-31G basis set[9] for the porphyrin, a 6-31G** basis set for the imidazole and the peroxide ligands, and a compact effective core potential basis set for Fe.[10] The net atomic charges reported here are from a Mulliken population analysis. All the calculations reported here were carried out with the HONDO 95 computer program.[11]

Three modes of binding of the peroxide to the iron were investigated. As shown in Figure 2, they are two end-on structures with different oxygen atoms as ligands for the iron and a bridged structure with the

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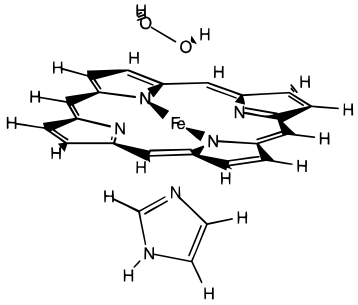
Table 1. Optimized Geometry and Electronic Structure of Model Ferric Peroxidase−Peroxide Complex



geometry	electronic structure
$r(\mathrm{Fe-O_1}) = 2.46$ Å	$q_{\mathrm{Fe}} = +2.55$
$r(\mathrm{Fe-N_\epsilon}) = 2.18$ Å	$q_{\mathrm{O1}} = -0.41$
$r(\mathrm{Fe-N_{pyr}}) = 2.05$ Å	$q_{\mathrm{O2}} = -0.29$
$r(\mathrm{O_1-O_2}) = 1.40$ Å	$q_{\mathrm{N\epsilon}} = -0.81$
$r(\mathrm{H-O_1}) = 0.95$ Å	$q_{\mathrm{Npyr}} = -1.23$
$r(\mathrm{H-O_2}) = 0.95$ Å	
$\angle(\mathrm{Fe-O_1-O_2}) = 118°$	$q_{(\mathrm{ImH})} = -0.07$
$\angle(\mathrm{N_\epsilon-Fe-O_1}) = 175°$	$q_{(\mathrm{HOOH})} = +0.06$
$\angle(\mathrm{N_{pyr}-Fe-N_\epsilon}) = 95°$	$q_{(\mathrm{porph})} = -1.5$
$\angle(\mathrm{N_{pyr}-Fe-O_1}) = 88°$ (82°)[a]	
$\tau(\mathrm{O_2-O_1-N_\epsilon-C}) = 15°$	$\sum_i d_i = 5.17$
$\tau(\mathrm{C-N_\epsilon-Fe-N_{pyr}}) = 44°$	
$\tau(\mathrm{O_2-O_1-Fe-N_{pyr}}) = 120°$	$\Delta E_{\mathrm{binding}} = -9.7$ kcal/mol
$\tau(\mathrm{H-O_1-O_2-H}) = 120°$	
$\tau(\mathrm{Fe-O_1-O_2-H}) = 92°$	

[a] There are two pairs of values of this angle because of the slight tilt of the $\mathrm{H-O_1-O_2}$.

two oxygen atoms equidistant from the iron. The starting geometry of the end-on peroxide complex was constructed from the water complex geometry. Full geometry optimization of the complex resulted in the end-on structure which is a local minimum on the potential energy surface.

The calculated structural parameters of the peroxide fragment (O−O = 1.40 Å; H−O−O bond angle = 102°; H−O−O−H torsion angle = 120°) are in good agreement with the experimental molecular parameters of an isolated peroxide molecule (O−O = 1.45 Å; H−O−O bond angle = 100°; H−O−O−H torsion angle = 119°). Thus, for the purpose of the present study that focuses on the description of the interaction of the intact peroxide ligand with the rest of the porphyrin molecule, rather than on peroxide chemistry, the level of theory used should provide reliable answers to the questions addressed.

The binding energy was obtained from a single-point supermolecule calculation in which the $\mathrm{Fe-O_1}$ separation was increased tenfold from its optimized value of 2.25 Å in the end-on structure to 22.5 Å.

The search for a bridged structure started from a half-way structure defined in a Linear-Synchrotron-Transit pathway connecting the two mirrored end−on structures. The distance between the iron and the center point of the $\mathrm{O_1-O_2}$ bond was frozen to specific values and all other coordinates were allowed to relax. The energies of six bridge structures with Fe−bridge distances ranging from 2.44 to 2.81 Å were then calculated from optimized geometries with this constraint.

Results and Discussion

Two stable optimized end-on binding modes of peroxide to the model ferric heme peroxidase were obtained with equal energies. The geometry of the optimized end-on peroxide complex is given in Table 1. The $\mathrm{Fe-O_1}$ bond length is 2.46 Å and the $\mathrm{Fe-O_1-O_2}$ bond angle is 118°. The axial ligands are nearly cis with an $\mathrm{O_2-O_1-N_\epsilon-C}$ torsion angle of 15°. The HOOH torsion angle is essentially the same as in the gas-phase geometry of peroxide itself. The torsion angle $\mathrm{Fe-O_1-O_2-H}$ is close to 90° such that the $\mathrm{O_2-H}$ bond is almost parallel to the plane of the porphyrin. Thus the H atom bound to $\mathrm{O_2}$ is leaning over the porphyrin ring. This geometrical arrangement reflects the electrostatic interaction between the H atom of the peroxide and the N atoms of the porphyrin which are found to have a substantial negative charge of −1.23, as indicated by the population analysis given in this table. This peroxide conformation is to be contrasted with the one found in molecular dynamic simulations of the full protein CCP−peroxide complex. In these simulations, the neighboring distal histidine residue provided an alternative binding site for the H atom in the end-on geometry. Our "gas-phase" calculations did not include the histidine residue. It would be interesting to do a more complete ab initio calculation with a model histidine residue to determine if the same H-bonding interaction is obtained as in the full protein simulation.

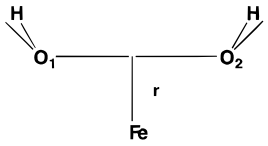
The net charges on the key atoms and major fragments of the peroxide−peroxidase heme complex are also given in Table 1. We see from this table that, although the oxygen ligand of the peroxide and the nitrogen ligand of the imidazole have a large negative charge, both axial ligands are essentially neutral. There is, however, considerable charge transfer from the iron to the porphyrin ring, resulting in a porphyrin ring that is very nearly a dianion and an iron with a charge close to the formal ferric ion value of +3. The charge transfer from the iron to the porphyrin ring is mainly from the Fe 4s orbitals. The d orbital occupancy of 5.17 found is very close to the ideal value of 5 for a sextet state of the ferric ion.

Comparison of the optimized Fe−O distance from the ab initio and Amber calculations reveals the Fe−O distance found in the optimized geometries of the model compound by the ab initio methods is longer (2.46 Å) than that found for the full protein complex or model compound by the empirical energy based methods (2.12 Å). There is unfortunately no independent structural data for the peroxide complex with which to compare these results. Both methods however consistently predict the same increase in the Fe−O bond length of 0.15 Å in going from the resting state water complex to the peroxide complex.

The ab initio binding energy of the peroxide to the five-coordinated ferric imidazole peroxidase model was calculated in the supermolecule approximation as the single point energy difference between the optimized peroxide complex and the fragments (peroxide and 5-coordinated heme) at large distances and was found to be 9.7 kcal/mol. The supermolecule was not allowed to relax to account for the absence of the peroxide. Thus, the binding energy calculated is somewhat larger than the energy that would have resulted from re-optimization of the supermolecule.

The finding of a stable peroxide complex by ab initio methods is an important validation of the results of the previous calculation for the full CCP−peroxide complex using the Amber potential. In that study, the energy of interaction of the peroxide with the entire protein was found to be 20 kcal/mol. Although these values were obtained by very different methods, the difference of 10 kcal/mol between the ab initio binding energy of the peroxide to the model heme compound (9.7 kcal/mol) and the binding energy of the peroxide to the entire protein (20 kcal/mol) is consistent with additional interactions found between the peroxide and the protein in the MD simulations. Specifically, in the protein complex the peroxide H atoms form H-bonds with the imidazole of the histidine and there are also electrostatic interactions between the peroxide and the cationic arginine residue. These local interactions of the peroxide with the nearby polar residues in the distal binding pocket can account for the 10 kcal/mol additional binding energy. The results of the two studies taken together form a consistent description of three essential features of the peroxide−peroxidase complex.

Table 2. Calculated Energies[a] of Bridged Structures of Model Peroxide−Peroxidase Complexes at Different Distances, *r*, of the Iron from the Center of the O_1-O_2 Bond



r (Å)	*E* (kcal/mol)	*r* (Å)	*E* (kcal/mol)
2.44	+9.8	2.60	+6.8
2.49	+8.7	2.65	+6.1
2.55	+7.6	2.81	+4.7

[a] Energies relative to unconstrained optimized end-on complex. Energies calculated from constrained optimization of bridged complexes with fixed values of *r*.

They (i) reinforce the finding that the peroxide forms a stable complex as a ligand for the heme iron, (ii) demonstrate that peroxide interaction with the heme unit itself is enough to allow stable complex formation, and (iii) indicate that peroxide interaction with the protein enhances this stability.

The energies of the six bridged structures relative to the optimized end-on structure obtained by constrained energy optimization are shown in Table 2. As shown in this table, these energies decrease with increasing distance between the iron and the center point of the O_1-O_2 bond. The bridged structures thus belong to a ridge between the two mirrored end-on structures, with a possibility of a minimum energy structure on the ridge, corresponding to a transition state, with an Fe-bridge distance greater than 2.8 Å and a relative energy <4.7 kcal/mol. However, a fully relaxed geometry optimization initiated from the constrained bridge structure at a distance of 2.81 Å progressively lost its bridge character with a corresponding increase in end-on character and continuous energy decrease. The existence of a low-energy ridge between the two end-on minima is consistent with the dynamic exchange between these two minima found in the 300 K MD simulations of the full protein complex.

The calculations reported here were performed at the Hartree−Fock level of theory that does not take into account electron correlation effects. Thus it is important to have some criterion for assessing the quality of the results presented. In this regard, the finding of primarily electrostatic interactions between the iron−heme unit and the peroxide is the most reassuring, since for strong electrostatic interactions, the Hartree−Fock model is known to provide an essentially correct description of the electronic structure of molecules and complexes. The addition of electron correlation is expected to yield a somewhat shorter $Fe-O_1$ bond distance and alter the calculated interaction energies by a few kcal/mol but not to alter the major findings of this study and the comparisons made with the previous results.

Conclusions

In this study, ab initio SCF-ROHF quantum chemical methods have been used for the first time to characterize a putative transient peroxide−peroxidase intermediate thought to play a key role in the oxidative metabolism of substrates by peroxidases. It has long been known that peroxide is required to transform the resting state of these oxidative heme proteins, peroxidases, to the catalytically active compound **I**, ferryl Fe═O species and that at least one intermediate is formed prior to compound **I** formation. There is both kinetic and spectroscopic evidence for such an intermediate. However, it has proven extremely difficult to characterize the nature of this intermediate and the pathway to formation of compound **I** from it, using either experimental or theoretical methods alone. In a previous study, profiting from the first known crystal structure of cytochrome C peroxidase, we have performed MD simulations that provided support for a stable CCP−peroxide complex with dynamic properties that indicate a pathway to compound **I** formation and implicate the distal histidine and nearby arginine residue in its formation. The validity of these results depends crucially on the reliability of the finding of a stable complex with an end-on mode of peroxide binding to the heme iron and of facile dynamic interchange between the two oxygen atoms of the peroxide as a ligand for the iron. The ab initio quantum chemical calculations performed here provide further support for these two crucial findings. Although differences were obtained in the detailed geometry of the end-on complexes, two stable peroxide complexes with end-on geometries with each oxygen atom as a ligand for the iron were obtained. No minimum corresponding to the bridged structure was found, which was also the case for the previous calculation. The binding energy for the end-on peroxide complex obtained in the ab initio calculations of the model complex and in the previous empirical based Amber calculations of the peroxide−CCP complex both clearly indicate that a stable peroxide intermediate is formed. It also appears that interactions of the peroxide with the heme unit itself are enough to form the stable complex, but that interactions of the peroxide with nearby distal residues of the protein considerably enhance this stability. In addition, the two end-on minima were found to be separated by a small energy ridge consistent with the facile interchange of these two minima found in the 300 K MD simulations. These results then provide further evidence for the plausibility of a peroxide intermediate and for the postulated pathway to formation of compound **I** from it.

Acknowledgment. Support for this work from NSF grant MCB 9305619 (G.H.L.) is gratefully acknowledged.

JA961723E