A Role for Thr 252 in Cytochrome P450*cam* Oxygen Activation

Danni L. Harris* and Gilda H. Loew

Contribution from the Molecular Research Institute, 845 Page Mill Road, Palo Alto, California 94304

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Abstract: Molecular dynamics simulations of the ferrous dioxygen bound form of wild type cytochrome P450*cam* were performed and the results analyzed to reveal the time-dependent interactions of T252 with surrounding residues as well as with the bound oxygen. The results indicate a time-dependent bimodal interaction of T252 with both G248 and the terminal oxygen of the bound dioxygen. The hydrogen bonding interaction of T252 with these two moieties is "anticorrelated" in the sense that the breaking of the T252–G248 hydrogen bond is concurrent with formation of the T252–dioxygen interaction. These simulations support the probability of a role of T252 in stabilization of the initial dioxygen bound complex and promotion of subsequent formation of compound I previously indicated by several experimental studies.

Introduction

Cytochrome P450*cam* is a monooxygenase which catalyzes the hydroxylation of camphor in the bacterium *Pseudomonas putida*. It was the first member of a ubiquitous family of cytochrome P450 isozymes for which a crystal structure was reported.[1] Extensive experimental investigation of this and other isozymes during the past 30 years has helped to elucidate the steps in the cytochrome P450 enzymatic cycle thought to be common to all isozymes. Figure 1 indicates these steps schematically and shows cytochrome P450's hydroxylate substrates via an enzymatic cycle which involves (i) entry of the substrate, (ii) displacement of most or all of the substrate cavity water, (iii) a change of ferric heme spin state upon displacement of the sixth ferric heme water, which facilitates (iv) a change in the redox potential toward one-electron reduction, via a complex electron transport system, (v) a one-electron reduction to a ferrous heme followed by (vi) entry and binding of molecular oxygen to the ferrous heme, (vii) a second one-electron reduction, (viii) formation of the putative reactive intermediate, compound I, a radical cation, and (ix) hydrogen radical abstraction by the ferryl oxygen from the substrate followed by radical recombination to produce the hydroxylated products and regeneration of the resting state of the enzyme.

The ferrous dioxygen cytochrome P450 is the last stable state in this enzymatic cycle. The next step, vii, appears to be coupled to a proton transfer event that is partially rate limiting to the overall reaction, since it is the only step with a significant solvent kinetic isotope effect.[2] Unlike the peroxidases, another family of metabolizing heme proteins for which there is direct evidence for a compound I radical-cation perferryl oxygen species as the reactive intermediate, the corresponding species has not yet been directly identified or characterized for the cytochrome P450's. A major difficulty is that the steps subsequent to the second electron reduction leading to product formation are very rapid, making trapping of the reactive intermediate a challenging task. Consequently, while steps i–vii are well established, the precise nature of the ultimate oxidative form of the enzyme has not been firmly established. However, current evidence, including

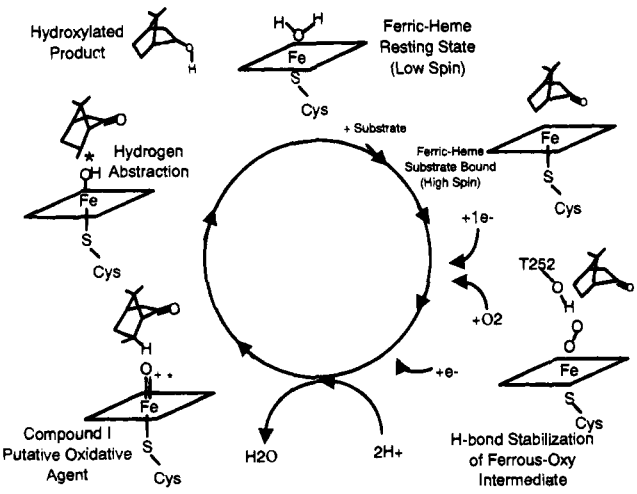

Figure 1. Schematic representation of the major steps in the P450*cam* enzymatic cycle (decoupling steps not shown).

isotope substitution kinetic data,[3] is consistent with this species being similar to the compound I/radical-cation ferryl oxygen species found in peroxidases.

In addition to the nature of the reactive intermediate itself, its mechanism of formation from the ferrous dioxygen species is also still under active investigation. Among the major unresolved questions are (i) the role and identity of the binding site residues, if any, involved in the formation of the reactive intermediate and (ii) the concertedness of the second electronic reduction and O–O bond cleavage to form the ferryl oxygen species. One common hypothesis is that hydrogen bonding and/or proton donation stabilizes the initial ferrous dioxygen complex and facilitates the O–O bond cleavage in the twice reduced state. Sequence alignments of P450's indicate that the threonine residue is a highly conserved residue at sequence location 252.[4] In P450*cam*, this threonine forms part of the dioxygen binding groove in the substrate binding site. Consequently, speculation about the identity of the putative proton donor stabilizing the

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bound dioxygen has focused on Thr 252. To test this idea, a series of site specific mutations of Thr 252 have been generated and the effect on the relative percentage of hydroxylated camphor and hydrogen peroxide produced has been monitored.[5,6] Production of hydrogen peroxide is a competitive pathway with formation of compound I and product formation. The greater the percentage of peroxide production the greater the inhibition of compound I formation. For mutations which replace the Thr at position 252 with a non-hydrogen bonding side chain, e.g. T252A and T252V, the enzymatic reaction shifts to primarily production of hydrogen peroxide (e.g., T252A: 6% ROH/83% H_2O_2[6,7]). P450*cam*'s with Thr or Ser at position 252 primarily promote formation of hydroxylated product (Wild Type (T252): 97% ROH/3% H_2O_2; T252S (S252): 81% ROH/15% H_2O_2). These studies definitively indicate the importance of a hydrogen bonding amino acid at this position in determining the enzymatic reaction.

Recently, the postulated role of the protein in compound I formation has been extended to not only include a hydrogen bond between the reduced dioxygen complex and the T252 hydroxyl group but extend to the Asp 251 as a proton donor to the T252 either directly or through a bridging water molecule.[8,9] While this model has been invoked as one possible explanation of the magnitude of the kinetic solvent isotope effect and the shape of the proton inventory curves in this system, there is no direct evidence for it.

The goal of the study described here was to further probe the postulated role of Thr 252, in compound I formation, by performing molecular dynamic simulations of the P450*cam* system in three states in its enzymatic cycle: (i) the substrate bound ferric state, (ii) the ferrous dioxygen state, and (iii) the hypothetical compound I state. In each of these states, we investigated the nature and time dependence of nonbonded H bonding networks involving T252 with the specific purpose of elucidating the manner in which T252 and other residues and waters in the binding sight cavity might play a role in stabilizing the bound dioxygen state of the enzyme and facilitating the formation of the hypothetical compound I form of cytochrome P450's.

Methods

The geometries used for the molecular dynamic simulations of P450*cam* in the substrate bound ferric, ferrous dioxygen, and compound I states were based on the ferric substrate bound cytochrome P450*cam* crystal structure[10] and the CO bound complex crystal structure.[11] The initial orientation of the dioxygen was taken to be similar to that of the CO in the CO bound structure in which the oxygen atom is in a groove between G248 and T252. The force constants for the oxygen bound to iron were estimated from the Weiss structures of a model dioxygen complex[12] and resonance Raman data from Bangcharoenpaurpong et al.[13]

Charges for the dioxygen bound heme were taken from Mulliken charges derived from an INDO/S/CI calculation of the $S = 0$ state for a model P450 system with a methyl mercaptate replacing the cysteinate. These calculations, that included 391 doubly excited configurations interacting with the SCF closed shell configuration, led to a ^1A ground state, stabilized relative to the lowest triplet state by 14 700 cm^{-1}. These results are very similar to those obtained from a previous INDO/S/CI calculation for a model ferrous dioxygen hemoglobin active site with imidazole as the sixth iron ligand. In that system, inclusion of configuration interaction depresses the diamagnetic ^1A state by 10 626 cm^{-1} below the lowest lying ^3A state.[14] The charge on the terminal oxygen in the model P450 ferrous dioxygen state was found to be −0.45 e while that on the oxygen atom directly interacting with the iron was −0.26 e. The parameterization for the ferryl state is as described elsewhere,[15] and the charge parameterization and AMBER atom types for it as well as the once reduced dioxygen bound form of the cysteinate heme are given as supplementary materials submitted with this paper.

The atomic charges for camphor were obtained from electrostatic potential derived charges from AM1 calculations, employing the method of Singh and Kollman with three shells and a point density of 5 points/Å on each surface.[16] All other charges and force field parameters were taken from AMBER 4.0.[17]

Beginning with the P450*cam* crystal structure, including all crystallographic waters, and using a heme model appropriate to the enzymatic state considered (dioxygen or ferryl oxygen added to the heme center as required), each of the substrate bound P450 structures in this study was energy minimized using AMBER 4.0 using 100 steps of steepest descents followed by sufficient steps of conjugate gradients to reduce the gradient to ≈0.8 kcal/Å. All glutamic, aspartic, lysine, and arginine residues were charged residues. The protonation state of histidine was assigned on the basis of the proximity of acidic/basic residues within a 4 Å radius of the histidine nitrogens. The positions of the hydrogen atoms attached to oxygen and nitrogen were then modified by short dynamics runs of 5−10 ps at 350 K. The structure was equilibrated for 15 ps at 300 K by loosely coupling the system to a heat bath and employing a series of gradually decreasing harmonic coordinate constraints. The system was then further equilibrated for about 25 ps before accumulation of dynamics data for analysis. Following the equilibration procedure, molecular dynamics runs were continued for 125 ps.

Nonbonded interactions were truncated at a relatively long distance of 15.0 Å in order to minimize the large forces associated with truncations in the nonbonded interactions. In order to compensate for the absence of water solvating the protein, we employed a radially dependent dielectric constant of $D = r$, where r is the interatomic distance for the atom pair electrostatic contribution being computed. The use of this relatively long nonbonded cutoff and a radially screened dielectric constant resulted in retention of all crystallographic waters in the simulation and minimal contraction of the protein. The radius of gyration of the protein was found to be ca. 21.1 Å following minimization and equilibration and did not change significantly during the full protein simulations. This radius of gyration was similar to that found in short simulations of the protein with a 5 Å solvent shell (21.4 Å), employing a dielectric constant of unity. The use of a radial dielectric is essential for conservation of energy in conjunction with the Berendsen coupling algorithm[18] in simulations not including water of solvation.[19]

Results and Discussion

Figure 2 shows the dynamic behavior of the T252 hydroxyl proton−G248 carbonyl oxygen distance for camphor bound to

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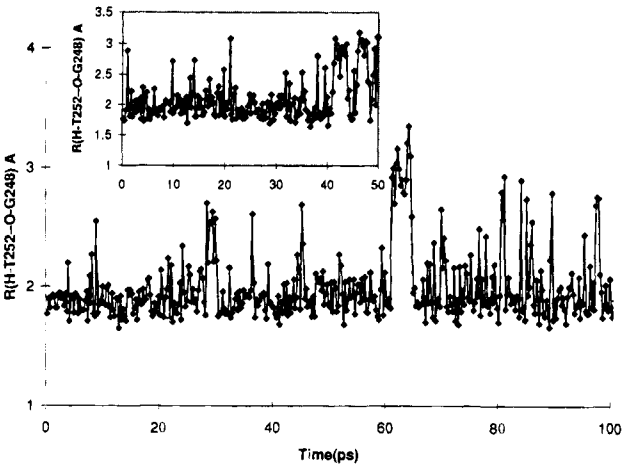

Figure 2. T252 (hydroxyl hydrogen)−G248 (carbonyl oxygen) distance time series for P450*cam* with heme in compound I form (main figure) and ferric heme form (inset), both with camphor bound in the substrate binding site.

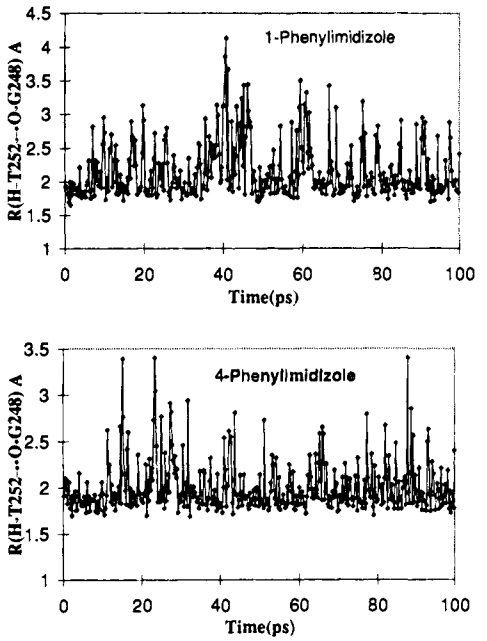

Figure 3. T252 (hydroxyl hydrogen)−G248 (carbonyl oxygen) distance time series for P450*cam* (ferric heme form) with 1-phenylimidazole (top) and 4-phenylimidazole bound in the substrate binding cavity.

the P450*cam* enzyme in the ferric heme (inset) and compound I enzymatic states. Note that, despite short-lived large amplitude fluctuations in the hydrogen bond length, this hydrogen bond between the hydroxyl hydrogen of T252 and the carbonyl oxygen of G248 is dynamically stable in the two states that occur before and after the ferrous heme dioxygen bound state in the enzymatic cycle (cf. Figure 1). The simulations of Paulsen and Ornstein on P450*cam* also illustrate the retention of this hydrogen bond.[20] The formation of this T252 hydroxyl−G248 hydrogen bond, rather than a T252 amide−G248 bond, is a contributing but not sole factor of the distortion of the I helix which seems to be ubiquitous in the P450*cam* structures.[1,21] In addition, as shown in Figure 3, this hydrogen bond seems to be a feature typical not only of the natural substrate bound state but also of inhibitors that have potential hydrogen bond donating

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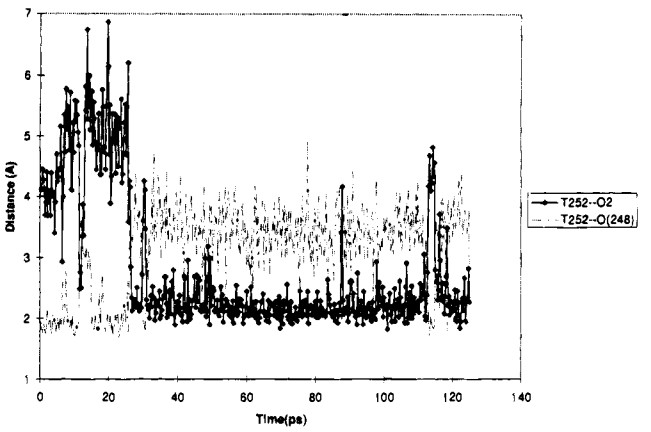

Figure 4. T252 (hydroxyl hydrogen)−G248 (carbonyl oxygen) and T252 (hydroxyl hydrogen)−terminal oxygen (O2) distance time series for P450*cam* for the ferrous heme dioxygen bound form of the enzyme.

and accepting groups bound as ligands to the ferric form of the enzyme. Thus, even when heme iron bound ligands are present, which are potential H-bond donors or acceptors, such as the 1- and 4-phenylimidazole inhibitors, their interactions are insufficient to perturb the T252−G248 hydrogen bond.

In contrast to that in the substrate or ligand bound ferric and compound I species, the dynamic behavior of the Thr 252 is qualitatively different in the substrate bound ferrous dioxygen state. As shown in Figure 4, initially in the energy-minimized and thermalized system, the T252 hydroxyl−G248 carbonyl hydrogen bond is intact. However, as shown in this figure, after about 30 ps, the terminal oxygen atom interacts instead with the T252 hydrogen of the hydroxyl group. The figure also clearly shows the anticorrelation in the hydrogen bonding between T252 and the bound dioxygen and between T252 and the G248 carbonyl oxygen. The G248−T252 hydrogen bond distance increases as the T252 hydroxyl hydrogen−terminal oxygen hydrogen bond distance decreases. Thus, these simulations indicate that the charge density and the proximity of the terminal oxygen atom of the bound dioxygen to the T252 hydroxyl group are sufficient to induce a hydrogen bond switch of the T252 from the G248 to the terminal oxygen atom. This hydrogen bond persists throughout the remainder of the 125 ps simulation.

Shown in Figure 5 are the initial energy-optimized positions (left panel) of the residues on the distal side of the heme near the bound dioxygen in the ferrous dioxygen form of the enzyme. This figure illustrates that the T252−G248 hydrogen bond is initially present in the simulation. The interaction between the terminal oxygen of the dioxygen bound to the heme iron and the OH group of T252 is clearly shown in the snapshot taken at the end of the 125 ps simulation in Figure 5 (right panel). These results provide additional compelling evidence for the inferences made from experimental studies of the possible role of Thr 252 both in preventing autoxidation of the dioxygen complex and in aiding O−O bond cleavage to form compound I. However, as seen in Figure 5, the carboxyl of Asp 251 maintains its stable salt bridge with Lys 178 and Arg 186 throughout these simulations and there is no evidence from these simulations that it interacts with the T252 hydroxyl group.

Conclusions

Simulations of ligand bound cytochrome P450*cam* systems in the ferric and compound I forms of the enzyme indicate that the hydroxyl hydrogen of T252 forms a stable hydrogen bond with the carbonyl oxygen of G248 in both of these species. In marked contrast, in the ferrous dioxygen form of the enzyme,

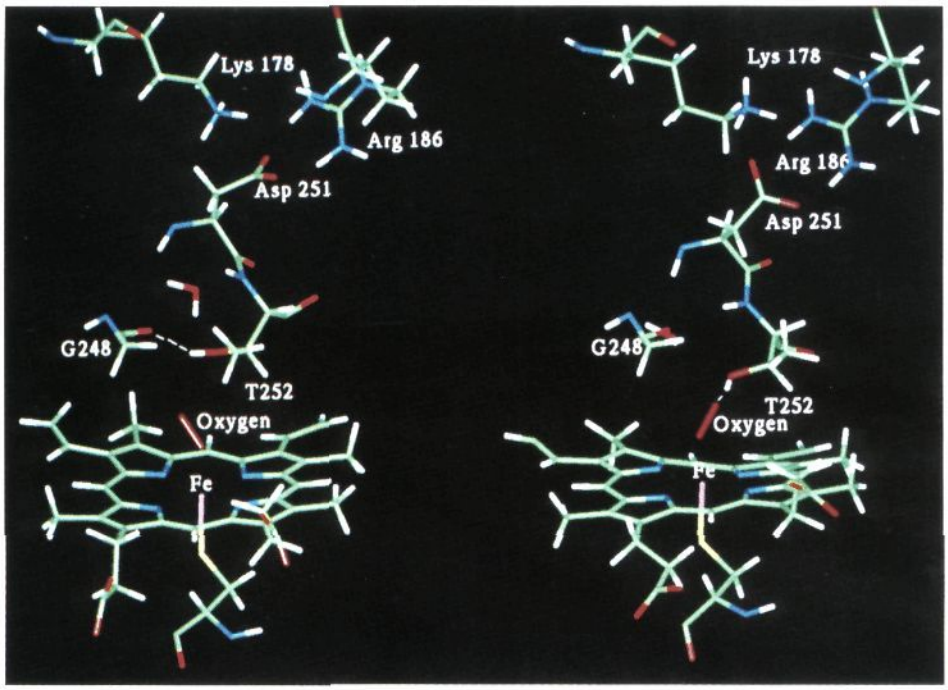

Figure 5. (left) T252−G248 hydrogen bond (1.79 Å) in the energy-minimized starting structure of ferrous heme with dioxygen bound (camphor not shown). (right) Rotated dioxygen position at 125 ps showing the T252−terminal oxygen hydrogen bond (2.13 Å at this particular point in the trajectory) that forms initially at 30 ps in the trajectory.

the hydrogen bond between T252 and G248 diminishes during the simulation, a result that is linked to simultaneous formation of T252 interactions with the terminal oxygen atom of the dioxygen. This preference of T252 for the terminal oxygen over the Gly 248 is manifest after 30 ps of MD simulations and persists during the remainder of the 125 ps simulation. The formation of this stable interaction can be understood as a direct result of the response of the hydroxyl side chain of T252 to the negative charge distribution and proximity of the terminal O-atom. Comparison of this dynamic behavior of the highly conserved T252 in the ferrous dioxygen state with three other forms of the cytochrome P-450*cam* enzyme, prior and subsequent to the binding of molecular oxygen, thus provides unbiased evidence for the postulated role of the highly conserved T252 residue in the P450 family in the stabilization of the ferrous dioxygen form of the enzyme. While such an interaction has been postulated for the twice reduced bound oxygen species,[3,9] it appears to be already present in the ferrous dioxygen form and would be strengthened by the addition of the second electron, particularly if the charge density on the terminal oxygen is increased by this further reduction.

Although these results lend direct support to the role of Thr 252 in compound I formation, as deduced from the mutant studies, they do not support a role for Asp 251 in a charge relay system as a proton donor to Thr 252. In these simulations, the conformation of the side chain of Asp 251 remains very similar to that of the energy-optimized one, corresponding to a stable salt bridge between it and the charged side chains of Lys 178 and Arg 186. The deprotonated form of Asp 251, assumed to be the dominant form at physiological pH, cannot, in any case, act as a direct proton donor to Thr 252. In its deprotonated form, it would be a proton acceptor directly competing with the terminal oxygen atom of the bound dioxygen and hence would impede rather than enhance the role of Thr 252 in stabilizing this form and contributing to O−O bond cleavage of the twice reduced form. However, there is no evidence from the simulations reported here that the side chain of Asp 251 does, in fact, undergo the conformational change necessary to interact with Thr 252, in preference to its very favorable electrostatic interactions with the neighboring cationic residues (cf. Figure 5). The only evidence for significant conformational change in the Asp 251 side chain we have observed thus far is in an MD simulation we recently performed for a 4-phenylimidazole inhibitor complex with the ferric form of the cytochrome P450*cam*.[22] This result was obtained, however, only after a water, not identified in the crystal structure, was added to the binding pocket. Thus it is still possible that, if there are bound waters strategically located in the once or twice reduced dioxygen bound state, they could be part of the postulated charge relay system that involves both Thr 252 and Asp 251 in compound I formation. However, there are no such bound waters in the most relevant X-ray structure known thus far, that of the CO bound state of the wild type enzyme. Since our simulations without the addition of water find no role for Asp 251, the results thus far confirm only the role of Thr 252 in the formation of the putative compound I reactive species of cytochrome P450.

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Supplementary Material Available: Two listings of AMBER Prep files (6 pages). This material is contained in many libraries on microfiche, immediately follows this article in the microfilm version of the journal, and can be ordered from the ACS; see any current masthead page for ordering information.

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