Crystallographic Study on the Dioxygen Complex of Wild-type and Mutant Cytochrome P450cam

IMPLICATIONS FOR THE DIOXYGEN ACTIVATION MECHANISM*

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Two key amino acids, Thr252 and Asp251, are known to be important for dioxygen activation by cytochrome P450cam. We have solved crystal structures of a critical intermediate, the ferrous dioxygen complex (Fe(II)-O2), of the wild-type P450cam and its mutants, D251N and T252A. The wild-type dioxygen complex structure is very much the same as reported previously (Schlichting, L., Berendzen, J., Chu, K., Stock, A. M., Maves, S. A., Benson, D. E., Sweet, R. M., Ringe, D., Petsko, G. A., and Sligar, S. G. (2000) Science 287, 1615–1622) with the exception of higher occupancy and a more ordered structure of the iron-linked dioxygen and two “catalytic” water molecules that form part of a proton relay system to the iron-linked dioxygen. Due to the altered conformation of the I helix groove these two waters are missing in the D251N dioxygen complex which explains its lower catalytic activity and slower proton transfer to the dioxygen ligand. Similarly, the T252A mutation was expected to disrupt the active site solvent structure leading to hydrogen peroxide formation rather than substrate hydroxylation. Unexpectedly, however, the two “catalytic” waters are retained in the T252A mutant. Based on these findings, we propose that the Thr252 accepts a hydrogen bond from the hydroperoxy (Fe(III)-OOF) intermediate that promotes the second protonation on the distal oxygen atom, leading to O–O bond cleavage and compound I formation.

Cytochromes P450 (P450)† (1), ubiquitous heme-containing monooxygenases, utilize dioxygen to insert an oxygen atom into inert hydrocarbon substrates and play an important role in steroid biosynthesis, drug metabolism, and detoxification of xenobiotics (2) in the following reaction.

R-H + O2 + 2e– + 2H+ → R-OH + H2O

REACTION 1

Much of our understanding on P450 structure-function relationships derives from studies on P450cam, the camphor monooxygenase cytochrome P450 from Pseudomonas putida (3). This was the first P450 to be prepared in sufficient quantities for detailed studies (4), the first to be sequenced (5, 6), and the first to have its three-dimensional structure solved (7). It therefore is not surprising that much of what we now understand about the P450 catalytic cycle is based on studies with P450cam. Of particular interest is the dioxygen activation process wherein the ferrous dioxygen complex (Fe(II)-O2) (8) accepts an electron from the iron-sulfur protein, putidaredoxin, plus two protons, which results in heterolytic cleavage of the oxygen O–O bond leaving behind the Fe(IV)=O ferryl intermediate generally considered to be the active hydroxylating species (Fig. 1). Recent studies have identified the hydroperoxy intermediate in WT P450cam (9, 10), but as yet, the Fe(IV)=O has not been directly observed in any P450.

The P450cam crystal structure suggested that the highly conserved Thr252 in the I helix (Fig. 1) plays an important part of the dioxygen activation machinery. Not surprisingly, Thr252 was an early target for mutagenesis (11, 12). Replacement of Thr252 with an aliphatic residue leads to a loss in activity although the rate of NADH utilization does not decrease very much. In the Thr252 mutants reducing equivalents and protons are funneled to the production of peroxide rather than substrate hydroxylation, a process often called uncoupling (Fig. 1). A second conserved residue in the I helix is Asp251. In sharp contrast to the Thr252 mutants, replacement of Asp251 with Asn leads to a large drop in NADH consumption rate (13–15).

The crystal structures of the Thr252 → Ala (T252A) (17) and Asp251 → Asn (D251N) (16) mutants of P450cam in the ferric (Fe(III)) state provided the first hints as to how these mutations alter activity. The next major step in providing a structural underpinning to the dioxygen activation process was the structure of the ferrous dioxygen complex (18). An unexpectedly large change occurs in the I helix going from ferric to the ferrous dioxygen-bound state. There also is a significant rearrangement of water structure that provides a continuous hydrogen-bonded link between dioxygen, water molecules, and
Cytochrome P450cam Wild-type and Mutant Oxy Complexes

Fig. 1. A, the structure of P450cam highlighting the I helix and the catalytically important residues, Thr\textsuperscript{252} and Asp\textsuperscript{251}. B, the P450 oxygen activation mechanism. Electron and proton transfer to the ferrous dioxygen complex gives the Fe(III)-OOH hydroperoxy intermediate. A second protonation of the distal oxygen atom leads to heterolysis of the dioxygen O–O bond and formation of Fe(IV) = O, the active hydroxylation species.

Table I

| Data set          | O\textsubscript{2}-WT | O\textsubscript{2}-D251N | O\textsubscript{2}-T252A |
|-------------------|-----------------------|--------------------------|--------------------------|
| PDB ID            | 2A1M                  | 2A1N                     | 2A1O                     |
| Unit cell (Å, degree) | a = 67.06   | a = 67.01                | a = 67.26                |
|                   | b = 62.29             | b = 62.13                | b = 62.18                |
|                   | c = 95.64             | c = 95.06                | c = 95.22                |
|                   | β = 90.43             | β = 90.40                | β = 90.53                |
| Space group       | P\textsubscript{2}\textsuperscript{1} | P\textsubscript{2}\textsuperscript{1} | P\textsubscript{2}\textsuperscript{1} |
| Resolution range (Å) | 47.82–2.10 | 33.51–1.90               | 41.07–1.55               |
| Reflections (observed/unique) | 118,823/43,802 | 182,505/60,680 | 329,397/111,583 |
| R<sub>merge</sub><sup>a</sup> (%) | 10.7 (51.5) | 7.5 (44.7) | 3.9 (30.0) |
| R<sub>merge</sub><sup>b</sup> (%) | 10.5 (1.9) | 15.2 (2.1) | 27.8 (2.3) |
| Completeness<sup>a</sup> (%) | 94.0 (94.2) | 97.9 (94.4) | 98.4 (89.2) |
| Root mean square deviation bond length (Å) | 0.006 | 0.006 | 0.005 |
| Root mean square deviation bond angle (degree) | 1.3 | 1.3 | 1.3 |
| Number of water molecules | 449 | 507 | 509 |

<sup>a</sup> R<sub>merge</sub> = \[\frac{\sum[I_f - \langle I \rangle]}{\sum[I_f]}\] , where \(I_f\) is the intensity of an observation, and \(\langle I \rangle\) is the mean value for that reflection and the summations are overall reflections.

<sup>b</sup> Values for the highest resolution shell are in parentheses.

<sup>c</sup> R factor = \[\sum[F_o(h)] - [F_c(h)]/\sum[F_o(h)],\] where \(F_o\) and \(F_c\) are the observed and calculated structure factor amplitudes, respectively. R<sub>merge</sub> factor was calculated with 5% of the data.

Thr\textsuperscript{252}. Based on these findings, it was proposed that Asp\textsuperscript{251} and Thr\textsuperscript{252} form part of proton shuttle pathway that connects the surface to the dioxygen ligand. Given the functional consequences of the Thr\textsuperscript{252} and Asp\textsuperscript{251} mutants, it is very likely that the hydrogen bond network observed in the ferrous dioxygen complex is significantly changed or disrupted. Here we report the crystal structures of the ferrous dioxygen complexes of T252A and D251N mutants. Both structures reveal quite different and unexpected changes in active water site structure, which provides important new insights on the precise roles played by Thr\textsuperscript{252}, Asp\textsuperscript{251}, and the water molecule in the dioxygen activation process.

Materials and Methods

Protein Purification and Crystallization—WT and mutant P450cam were overexpressed in Escherichia coli and purified as described by Gunsalus et al. (4). Diffraction quality crystals of WT and the mutants were obtained as described previously (19). A somewhat different procedure was employed in forming the dioxygen complex from that described by Schlichting et al. (18) who used a high pressure oxygen cell. A single crystal first was transferred into a cryo buffer consisting of 50 mM Tris-Cl, pH 7.4, 0.4–0.6 M KCl, 1 mM L-camphor, 30% polyethylene glycol 4000, and 20% glycerol followed by reduction with 10 mM sodium dithionite for 10 min under anaerobic condition in a glove box. After reduction, the crystal was washed in the dithionite-free cryo buffer for 10 min under anaerobic condition and then soaked in the oxygen-saturated cryo buffer at −5 °C for 5 min. The crystal was flash-frozen and stored in liquid nitrogen until data collection.

Data Collection and Processing—X-ray data were collected in-house using an R-axis IV imaging plate system and rotating anode equipped with confocal optics (Osmic, Auburn Hills, MI) for WT and D251N or at the Stanford Synchrotron Radiation Laboratory on beamline 9-2 using a Quantum-315 CCD detector (Area Detectors Systems Corp., Poway, CA) for T252A and D251N. At the Stanford Synchrotron Radiation Laboratory 0.92 Å x-rays were used, and the beam was attenuated by about 50% to decrease the level of x-ray-induced reduction. Structures of the D251N derived from both in-house and Stanford Synchrotron Radiation Laboratory data were the same except for resolution and dioxygen ligand occupancy. The in-house data for the D251N was used, since the structure from the in-house data had better dioxygen occupancy/lower temperature factors, although synchrotron data extended about 50% to decrease the level of x-ray-induced reduction. Structures of the D251N and T252A were fitted to the electron density using the graphic program O, version 8.0 (22), and several rounds of electron minimization and temperature factor refinement were performed. The iron-ligand distances and geometry were not refined.

For 10 min under anaerobic condition and then soaked in the oxygen-saturated cryo buffer at −5 °C for 5 min. The crystal was flash-frozen and stored in liquid nitrogen until data collection.
RESULTS

Ferrous Dioxygen Complex of WT P450cam—The crystals used in this study belong to space group P2_1, with two molecules in the asymmetric unit. The previously reported structure of the WT dioxygen complex using the same crystal form shows that only one of these molecules has dioxygen bound (18). The method we employed for forming the dioxygen complex, a longer soaking period in dioxygen-saturated buffers rather than a brief exposure to dioxygen under high pressure, leads to both molecules having dioxygen bound as shown in Fig. 2. The unrestrained Fe–O bond length is 1.85 and 1.79 Å, while the Fe–O–O angle is 129° and 133° for molecules A and B, respectively. Temperature factors of the distal oxygen atom of the iron-bound dioxygen ligand, 21.0 and 22.7 Å² for molecules A and B, respectively, are significantly lower than that reported previously (18), 46.5 Å². Since temperature factors and occupancy are highly correlated, it appears that the method we used for preparing the oxy complex leads to higher occupancy and/or a more ordered structure. While both molecules in the asymmetric unit have oxygen bound with essentially no differences, we will focus on molecule B, since in the mutants the temperature factors for the dioxygen ligand are lower in molecule B leading to shaper electron density for both the ligand and surrounding residues and water molecules.

In the previous structure, one of the new water molecules introduced into the active site upon dioxygen binding (WAT902) is only 1.78 Å from an adjacent water molecule (WAT687) suggesting that each molecule is only partially occupied. Our simulated annealing omit maps again clearly show the presence of two water molecules, WAT234 and WAT453 (Fig. 2) equivalent to WAT902 and WAT687, respectively, in the previous structure. This network is considered critical for the proper delivery of protons to dioxygen for oxygen activation. Our present WT dioxygen complex structure also has structural changes in the I helix first elucidated by Schlichting et al. (18). In the ferric state the Asp251 peptide carbonyl is oriented perpendicular to the I helix axis, but in the ferrous dioxygen complex, the peptide reorients along the helical axis thus giving a more normal helical geometry. The peptide flip is accompanied by movement of Thr252. In the ferric state the Thr252 side chain OH group donates a hydrogen bond to the peptide carbonyl group of Gly248. In the dioxygen complex this hydrogen bond is weakened and Thr252 moves into position to interact with dioxygen at a distance of 3.1 Å. This movement also provides the additional room required for the two new water molecules, WAT453 and WAT234 (Fig. 2), that provide part of the hydrogen bonded network to dioxygen.

Ferrous Dioxygen Complex of the T252A Mutant—Electron density maps for the T252A mutant are shown in Fig. 2. The ligand is clearly visible and well ordered as is the surrounding solvent structure in the T252A mutant. Fig. 3A shows a superimposition of the WT (CPK coloring with light green carbons) and T252A (CPK coloring with yellow carbons) dioxygen complex structures. The new water molecules unique to the dioxygen complex structures are shown as small spheres. B, superimposition of the WT (CPK coloring with green carbons) and D251N dioxygen complex (CPK coloring with yellow carbons) structures.
Although the Asn251 peptide flips as in WT, in the mutant the change in water structure is more difficult to explain. Since the two catalytic waters are missing in D251N because the I helix does not open up to the extent found in the WT dioxygen complex, there is insufficient room for the two new waters found in the WT dioxygen complex.

**FIG. 4.** Possible hydrogen bond network for the P450cam hydroperoxy intermediate. The distances between heteroatoms are taken from molecule B of our WT dioxygen complex structure.

The two complexes, 160°. Considering that Thr252 is part of the dioxygen-water hydrogen bond network generally considered to be critical for dioxygen activation, it was very surprising to find such minor changes in water structure. These results show that Thr252 is not required to hold the active site water molecules in place and that uncoupling is not the result of a disordering of the active site water molecules.

**Ferrous Dioxygen Complex of the D251N Mutant—**The most interesting difference between the D251N and WT dioxygen complex structures is that the mutant is missing the two additional water molecules forming the hydrogen bonded network with dioxygen found in the WT dioxygen complex (Figs. 2C and 3B). The reason that these waters are missing is that the I helix does not undergo the same degree of movement found in WT. Although the Asp251 peptide flips as in WT, in the mutant the hydrogen bond between the Thr252 OH group and Gly248 peptide carbonyl oxygen atom remains intact at a distance of 2.8 Å but increases to 3.4 Å in WT. As a result, Thr252 in the mutant is further from dioxygen, 3.4 Å compared with 3.1 Å in WT. Since the I helix does not open to the extent found in the WT dioxygen complex, there is insufficient room for the two new waters found in the WT dioxygen complex.

**DISCUSSION**

Both the D251N and T252A mutants exhibit greatly diminished camphor hydroxylation activity, ~4% (11, 12) and 0.1% (13–15), respectively. However, T252A consumes NADH at about 85% WT rates and is highly uncoupled, since reducing equivalents and protons are funneled into the formation of hydrogen peroxide rather than camphor hydroxylation. In sharp contrast, the D251N mutant exhibits a greatly diminished rate of NADH consumption, which has been interpreted as a slower proton transfer to the iron-linked dioxygen (16).

Since these two mutants have quite different effects on enzyme activity, it is not surprising that the structural consequences of the two mutations also are different. The major differences are in the active site water structure. In the T252A dioxygen complex, the two “catalytic” waters found in the WT dioxygen complex are present but are missing in the D251N dioxygen complex. These results provide important insights on the role of water and Thr252 in catalysis. As already reported by Shlichting et al. (18), Thr252 makes a hydrogen bond with both catalytic waters and dioxygen ligand, which is required for proton transfer. The cause of uncoupling in the T252A mutant has been viewed as a disruption of the dioxygen-water hydrogen bond network resulting in release of peroxide rather than the timely addition of the second proton leading to O–O bond cleavage (Fig. 1). This scenario must now be reconsidered, since the T252A dioxygen structure shows that the two catalytic waters remain in place and are well ordered. Thus, Thr252 is not required for properly positioning the active site waters nor is a free side chain OH required, since Kimita et al. (23) showed that replacing the Thr252 side chain OH with a methoxy group, OCH3, does not lead to a significant change in activity. Taken together these results show that Thr252 does not serve as a hydrogen bond or proton donor in dioxygen activation. The one remaining role for Thr252 is to serve as a hydrogen bond acceptor from the hydroperoxy intermediate (Fig. 4). The iron-linked dioxygen accepts one proton and two electrons to form the hydroperoxy species. Two electrons enter into anti-bonding orbitals, πx and πy, which reduces bond order and elongates O–O distance from 1.21 to 1.49 Å (24). The hydroperoxy species with a longer O–O distance forms a new hydrogen bond with the Oγ of Thr252. The new hydrogen bond also increases proton affinity of the dioxygen distal oxygen atom. Therefore, the main role of Thr252 is to promote the addition of the second proton to the distal oxygen by accepting a hydrogen bond from the hydroperoxy intermediate.

For the D251N mutant, it is clear from kinetic isotope and proton inventory experiments (16) that water-mediated delivery of protons is important in P450cam and that the D251N mutation impaired the delivery of protons. The lack of ordered solvent near the oxygen ligand in the D251N dioxygen complex thus explains why the delivery of protons is so slow. Precisely why replacing Asp251 with Asn leads to such changes in solvent structure is more difficult to explain. Since the two catalytic waters are missing in D251N because the I helix does not open up to the extent found in the WT dioxygen complex, it is logical to conclude that Asp251 is somehow linked to the I helix conformational change required to position Thr252 and catalytic waters properly for dioxygen activation. We hypothesize that one reason for the lack of motion in the I helix in the mutant is due to the strong ion pairing between Asp251 and Lys178 and Arg186 (Fig. 3A). When the peptide of Asp251 flips upon oxygen binding, the strain imposed on the helix is relaxed by movement of the I helix that enables Thr252 to be positioned for interactions with dioxygen. This movement also provides room for the two new water molecules. How the strain energy is dissipated in the I helix is highly restricted, since Asp251 is strongly tied to Lys178 and Arg186. In the D251N mutant, however, these ion pairing interactions are lost and, hence, relaxation of the I helix strain resulting from the oxygen binding induced peptide flip has fewer restrictions. In other words, because the I helix has a larger range of freedom in the mutant, the energetic incentive to open up the helix and disrupt the Thr252 H-bond with Gly248 is lost and the I helix remains closed. Given this scenario, one main role of Asp251 is to provide conformational restrictions that ensures dioxygen binding leads to a repositioning of Thr252 and the entry of the two key water molecules required for dioxygen activation. It is also possible that Asp251 provides some long range electrostatic effects. For example, theoretical work has shown that the nature of the ligand coordinated to the P450 heme ferric iron is significantly affected by the protein electrostatic environment (25).

Finally, we mention some related work recently published on the P450oryF dioxygen complex (26). Unlike P450cam, there is no change in the I helix nor is there any change in solvent structure in going from the ferric to ferrous dioxygen-bound form with the exception of the displacement of one water by dioxygen. Most surprising was the lack of any solvent directly hydrogen-bonded to dioxygen, which lead to a proposed proton delivery network involving a peptide carbonyl oxygen atom.
rather direct delivery of the proton by water. The corresponding peptide carbonyl oxygen atom in P450cam belongs to Gly^{248}, which is 3.1 Å from the distal oxygen in our WT dioxygen structure (Fig. 4). While the ultimate source of protons for dioxygen activation is solvent, the direct proton donor to dioxygen appears not to be water in P450eryF.

Whether or not P450cam uses a similar carbonyl-mediated proton delivery system remains to be seen. However, the present work takes us a step closer to understanding how the highly conserved residues, Thr^{252} and Asp^{251}, work to promote oxygen activation. First, we propose that role of Thr^{252} is to stabilize the hydroperoxy intermediate, which favors addition of the second proton over peroxide release. Since the catalytic waters remain in place in the T252A mutant, electron and proton transfer to dioxygen remain fast as evidenced from the WT levels of NADH consumption (11, 12). Without stabilization of the hydroperoxy intermediate by Thr^{252}, however, peroxide release is favored over O–O bond cleavage. Second, the ion pairing between Asp^{251} and Lys^{176}/Arg^{186} ensures that relaxation of the I helix when the Asp^{251} peptide flips upon dioxygen binding results in the proper positioning of Thr^{252} and the two catalytic waters for dioxygen activation. Because the active site waters are absent proton delivery to dioxygen is slowed, which accounts for the slower proton and NADH consumption by the D251N mutant.

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