Reduced Oxy Intermediate Observed in D251N Cytochrome P450cam[†]

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ABSTRACT: Cytochrome P450s are ubiquitous heme proteins responsible for various oxidative metabolic processes. The overall rate-determining step in the catalytic cycle of native cytochrome P450cam is the reduction of the dioxygen complex, which has made detection of catalytic intermediates after this reduction impossible. However, for the site-specific mutant D251N cytochrome P450cam (which affects proton transfer near the catalytic center), the overall rate-determining step occurs *after* the reduction of oxy-P450. As a consequence, we have observed in the UV−visible spectrum during catalytic turnover a new intermediate that is one electron reduced from oxy-P450 with an intact dioxygen bond.

The family of heme protein monooxygenases known as cytochrome P450 plays a critical role in the synthesis and degradation of many xenobiotics and physiologically important compounds (Ortiz de Montellano, 1995a; Sono et al., 1996). Some of the products formed by these enzymes have also been implicated as causative agents in a number of cancers (Gonzales & Gelboin, 1994; Shimada et al., 1996). Intermediates involved in dioxygen activation have been proposed (Fruetel et al., 1992, Gruenke et al., 1995, Loida & Sligar, 1993) from the production of superoxide or peroxide upon loss of catalytic efficiency. The mechanism of dioxygen activation is the least understood step in P450 catalysis (Figure 1), which begins with the reduction of the dioxygen complex (oxy-P450, **4** in Figure 1). The stabilization and observation of the intermediates formed during dioxygen activation are of tremendous interest from both the chemical and biological perspectives.

Gerber and Sligar (1992), along with Shimada et al. (1990), showed that the turnover rate for camphor hydroxylation by D251N P450cam was 1−2 orders of magnitude slower than native P450cam.[1] They noted that stabilization of one of the intermediates involved in dioxygen activation during D251N P450cam catalysis was one possibility after kinetic analysis of the observable individual steps showed that the overall rate-determining event occurred after formation of oxy-P450, **4**, but before formation of the substrate-bound ferric state, **2**. During enzymatic turnover, D251N P450cam shows a significantly red-shifted UV−visible spectrum (Figure 2) compared to other catalytic intermediates of P450, like **1**−**4** and **7** in Figure 1 (Gerber & Sligar, 1994). The oxidation state of P450cam responsible for this spectral intermediate, however, was never determined. Gerber and Sligar (1994) concluded that the spectral intermediate was
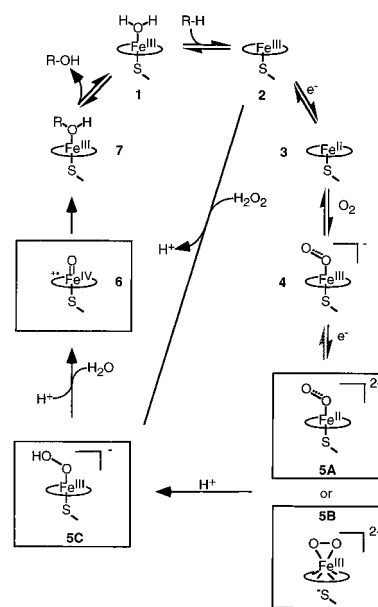

FIGURE 1: Catalytic cycle of cytochrome P450. Numbers correspond to intermediates cited in text. Boxed intermediates are proposed, but no catalytic form has been observed.
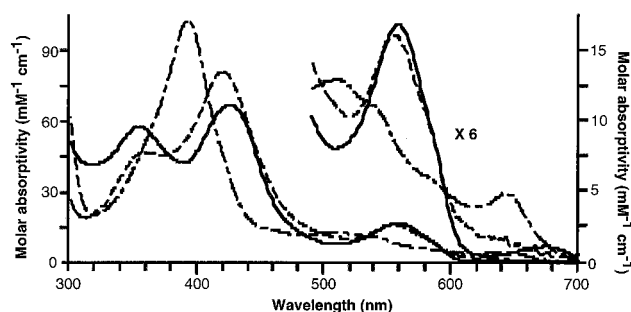

FIGURE 2: Comparison of intermediate optical spectra to other known intermediates that could possible be observed during turnover. (− − −) Oxy-P450 D251N, which is the same as wild type (**4**); (−) D251N spectral intermediate; (− - −) ferric substrate bound (**2**). Product bound complex (**7**) or ferric substrate free (**1**) are not observed under saturated substrate conditions.

either a reduced oxy-P450 intermediate or a complex of putidaredoxin and oxy-P450cam. We have now established the oxidation and oxygenation state of the cytochrome D251N P450cam spectral intermediate.

[1] Abbreviations: P450cam, cytochrome P450cam (CYP 101) isolated from *Pseudomonas putida* and recombinantly expressed in *Escherichia coli*; Pd, putidaredoxin from *P. putida* expressed in *E. coli*; Pdr, putidaredoxin reductase from *P. putida* expressed in *E. coli*; EPR, electron paramagnetic resonance; DPPH, α,α′-diphenyl-β-picrylhydrazyl; GC, gas chromatography; CO, carbon monoxide; Oxy-P450, the ferrous-dioxygen intermediate (**4** in Figure 1) of cytochrome P450.

EXPERIMENTAL PROCEDURES

Materials and Sample Preparation. Wild-type and D251N cytochrome P450$_{cam}$, putidaredoxin, and putidaredoxin reductase were separately cloned, expressed, and purified from *Escherichia coli* as previously reported (Gerber & Sligar, 1992). Purity of all proteins were within 95% of reported R_z ratios (Gunsalus & Wagner, 1978). Samples that monitored the D251N P450$_{cam}$ spectral intermediate by the reconstituted enzyme system (referred to also as the multiple-turnover method) were prepared as previously reported (Gerber & Sligar, 1994). All measurements were done at pH 8.0, 50 mM potassium phosphate, and 500 μM camphor.

For single-turnover reactions, an anaerobic atmosphere was maintained within a Coy Products environmental chamber under nitrogen with a 5% hydrogen atmosphere and palladium catalyst. Putidaredoxin and P450$_{cam}$ each were reduced with a 10-fold excess of dithionite and anaerobically purified. A mixture of 2.1 equiv putidaredoxin and 1 equiv of P450$_{cam}$ was used at a P450$_{cam}$ concentration of 100 μM. The ratios of putidaredoxin to P450$_{cam}$ were the smallest they could be to produce 1 equivalent of 5-hydroxycamphor relative to P450$_{cam}$. Kinetic reactions were initiated at 22 °C by the addition of oxygen and terminated appropriately for each analysis technique. Control samples were prepared anaerobically.

Methods of Kinetic Analysis. UV−visible spectra were recorded with 0.01 cm path-length cuvette. The cuvette was loaded under anaerobic conditions, initial spectrum recorded, oxygenated, resealed, and spectra were recorded at 30 s intervals. Equivalents of the spectral intermediate were quantitated by observing the change in absorbance and calculated from the ferric high spin complex (**2**) minus the spectral intermediate difference spectrum.

Samples for EPR analysis were flash frozen in liquid nitrogen at appropriate times. Measurement of reduced Pd-based EPR resonances used an X-band Bruker ESP 300 EPR spectrometer with the temperature held at 100 ± 0.5 K with a liquid nitrogen cryostat. Accurate and simultaneous measurement of frequency was obtained by monitoring a crystalline DPPH sample in a double resonator cavity (TE$_{104}$). A small signal, accounting for less than 0.1% of the double-integrated area, of reversed phase was shown to be from the DPPH sample in the second resonator cavity. Relative concentration of reduced Pd was obtained by double integration of derivative spectra. The amount of oxidized Pd was assumed to be inversely proportional to the concentration of reduced Pd.

Reactions for GC analysis were quenched with an equal volume of 2 M potassium hydroxide, extracted with chloroform, and dried with MgSO$_4$. GC was performed on a 30 m DB-5 capillary column with a temperature program run initially from 70 °C for 2 min, ramp rate at 10 °C/min, to 230 °C for 5 min. Retention times for these reactions were 4.9 min, camphor; 12.8 min, 5-hydroxycamphor; 13.2 min, 3-bromocamphor. Signals were integrated and compared to an internal standard of 3-bromocamphor.

UV−Visible Spectral Methods. Samples for observation of the spectral intermediate were generated either by a putidaredoxin−dithionite reduction as above, by multiple-turnover method, or by the initial formation of oxy-P450$_{cam}$ from the reduced deoxy P450$_{cam}$ solution at 4 °C and the addition of reduced putidaredoxin. UV−visible spectra were recorded before more than 5% product formation had occurred.
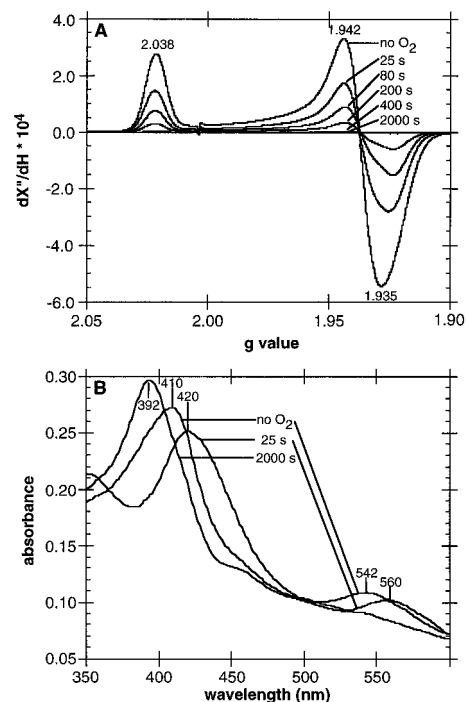

FIGURE 3: Reaction of reduced D251N P450$_{cam}$ and reduced Pd with dioxygen at 22 °C, 50 mM potassium phosphate, pH 8.0, 500 μM camphor. (A) 100 K X-band EPR and (B) UV−visible with 0.01 cm path length cuvette.

Photolysis experiments were initiated at 4 °C with a 500 mJ laser pulse at 532 nm. Observation optics and experimental setup are as previously reported (McLean et al., 1996). Geminate yields were recorded at 435 nm for the spectral intermediate ($\Delta\epsilon = 18.2$ mM^{-1} cm^{-1}) and at 430 nm for oxy-P450$_{cam}$ ($\Delta\epsilon = 8.95$ mM^{-1} cm^{-1}). Oxy-D251N P450$_{cam}$ was formed as above at 4 °C, while the spectral intermediate was formed by the multiple-turnover method.

CO addition to samples prepared by the multiple-turnover method was stabilized as above. CO was bubbled through each stoppered cuvette prior to recording spectra. Percent ferrous carbon monoxide intermediate formed was then determined from the calculated difference spectrum of ferrous carbon monoxide P450 minus the spectral intermediate.

RESULTS AND DISCUSSION

In order to determine the nature of this spectral intermediate, we coupled kinetic measurements with three techniques: UV−visible spectrophotometry to observe the spectral intermediate, EPR to observe reduced putidaredoxin, and GC to observe the hydroxylated product, 5-hydroxycamphor. In this way, we monitored the relative concentrations of these species simultaneously during the course of a single catalytic cycle (Figure 3). Reactions were initiated by the rapid addition of oxygen to a mixture of reduced putidaredoxin and reduced deoxy P450$_{cam}$, **3**. Oxygenation of this sample resulted in the formation of oxy-P450$_{cam}$, **4**, in the first 5 ms (Brewer & Peterson, 1988; Pederson et al., 1976). The reaction in question (clockwise in Figure 1 from **4** to **2**) was initiated by the interaction of reduced Pd with this rapidly formed oxy-P450$_{cam}$, **4**.

Formation of the new spectral intermediate is required for catalysis. As shown in Figure 4, at 25 s, we observed the formation of 0.95 equiv of the spectral intermediate, but only 0.08 equiv of 5-hydroxycamphor. The sum of the amount of the spectral intermediate and 5-hydroxycamphor at 25 s
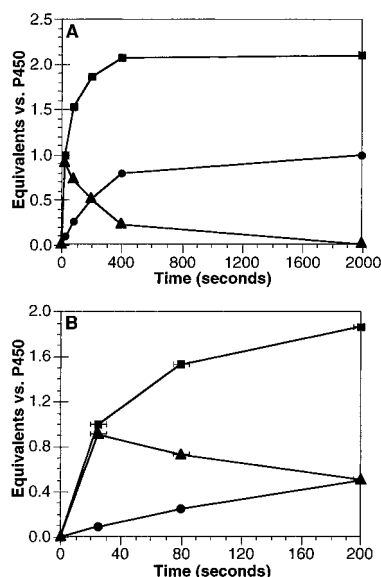
FIGURE 5: Kinetic models for oxidation of Pd overlaid on experimental data (■); (− - −) kinetic rate for 5-hydroxycamphor formation, $k = 0.0035$ s^{-1}; (− − −) kinetic rate for autooxidation of putidaredoxin, $k = 0.007$ s^{-1} (Sligar et al., 1974); (—) kinetic rate for 1.0 equiv of oxidized putidaredoxin formed in 1 s and 1.1 equiv autooxidize at the same rate above, $k = 0.007$ s^{-1}.

FIGURE 4: Kinetics of single-turnover reaction with a (A) full time course and (B) 0−200 s expansion of time course. Circles are 5-hydroxycamphor determined by GC, triangles are equivalents of intermediate determined by UV−visible, and squares are oxidized Pd determined by the intensity of reduced Pd EPR signal. Errors on measurements are within the area of symbol unless denoted by an error bar.

was essentially the same as the amount of 5-hydroxycamphor formed after 30 min. After 30 min, 99% of 5-hydroxycamphor was formed relative to the initially reduced P450$_{cam}$, which had returned quantitatively to ferric substrate bound (**2**), thus confirming mass and reductant balance in the single catalytic cycle. The direct correlation between the spectral intermediate decay and 5-hydroxycamphor formation, combined with the mass balance, shows that the spectral intermediate is on the path of the catalytic cycle.

Reduction of oxy-P450 (**4** to **5A** and **B** in Figure 1) is not the rate-determining step in D251N P450$_{cam}$ catalysis. The rate of formation of oxidized putidaredoxin is rapid: at 25 s, 1.0 equiv of oxidized putidaredoxin was formed, which is consistent with the final amount of product and initial amount of the spectral intermediate observed. The best model (Figure 5) of these kinetics[2] is the rapid initial oxidation (within the first second) of 1.0 equiv of reduced putidaredoxin and followed by a slow decay ($k = 0.007$ s^{-1}) of the remaining reduced putidaredoxin at the same rate as normal Pd autooxidation (Sligar et al., 1974). The small discrepancy at 25 s between the calculated and observed amounts of oxidized Pd in Figure 5 probably arises from less than expected Pd autooxidation since the dioxygen concentration was subsaturating in this preparation.[3]

We are able to establish that the added reducing equivalent in our spectral intermediate is present in the oxy-iron heme

moiety by two different techniques. First, the UV−visible spectrum of the spectral intermediate (Figure 2) shows significantly larger differences than those previously noted from oxy-P450. The Soret band (350−450 nm, which arises from a $\pi - \pi^*$ transition of the heme) of the spectral intermediate of D251N P450$_{cam}$ is considerably red-shifted compared to oxy-P450, and the spectrum of D251N oxy-P450 is the same as native oxy-P450. The Soret of the spectral intermediate is more like that of a p-type hyper spectrum than oxy-P450 and is similar to that of the ferrous carbon monoxide P450$_{cam}$ complex (Gouterman, 1978; Hanson et al., 1976). Correlations among a wide range of metalloporphyrin spectra (Momenteau & Reed, 1994; Wang & Brinigar, 1979) suggest that red-shifting of both the Soret and visible bands is consistent with an increased electron density on the ligands of the spectral intermediate, compared to oxy-P450.

The change in the electronic structure of the spectral intermediate is confirmed by its photochemistry, which is distinct from that of oxy-P450$_{cam}$. Irradiation of D251N oxy-P450$_{cam}$ with a laser pulse results in relatively rapid loss of the bound O_2 with a geminate yield of ~70%; in contrast, irradiation of the spectral intermediate shows no photolytic activity (or geminate recombination >99.6%). The spectral intermediate's lack of observed photolytic activity shows that there is a major change in the electronic structure from oxy-P450. At the simplest level, these results suggest that the spectral intermediate is not a result of a Pd-based perturbation of D251N oxy-P450.

Proton transfer could be the rate-determining step in D251N P450$_{cam}$ catalysis. The pH profile of enzymatic turnover (Gerber & Sligar, 1994) and kinetic solvent isotope studies with native P450$_{cam}$ (Aikens & Sligar, 1994) both suggest that proton flow becomes significantly slower upon neutralization of aspartate 251. Gerber and Sligar (Gerber, 1993) also reported that there is no significant difference in peroxide-supported turnover (i.e., the "peroxide shunt") of D251N from native P450$_{cam}$. This argues that the new intermediate precedes O−O bond heterolysis (**5C** to **6**) since O−O bond heterolysis must also occur in peroxide-supported catalysis (Tajima et al., 1993; Egawa et al., 1994; Ortiz de Montellano, 1995b). In the hydrogen peroxide and alkyl-hydroperoxide supported catalysis, a ferric hydroperoxo (**5C**) or similar ferric alkylperoxo intermediates are proposed to be formed under nonphysiological conditions (Balch, 1992; Blake & Coon, 1981; Tajima et al., 1993, 1995; Yamaguchi et al., 1993). EPR and UV−visible signals for ferric hydroperoxo intermediates are well established from a variety of nonphysiological protein-based intermediates (Blake &

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[2] If the rate-determining step was the second electron transfer (**4** to **5A**−**B**), the kinetics of oxidized Pd formation would be expected to be a mixture of the rate of 5-hydroxycamphor formation (− - − in Figure 5) and autooxidation of excess reduced Pd (− − − in Figure 5). However, if the rate-determining step is *beyond* the second electron transfer (for example, rate-determining step is **5A**−**B** to **5C** or **5C** to **6**), there should be a formation of 1.0 equiv (vs P450) of oxidized Pd in the first second with a slow continued formation of oxidized Pd derived from autooxidation of excess (1.1 equiv vs P450) reduced Pd (— in Figure 5).

[3] The concentration of dioxygen in air-saturated buffer is only 250 mM, while the Pd concentration is 210 mM. The rate constant for Pd autooxidation with air saturated buffer at this concentration is 0.007 s^{-1}. Subsaturated dioxygen concentration would significantly slow this process since it is a second-order reaction.
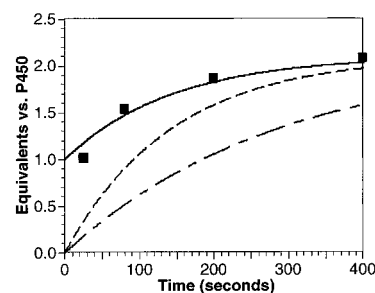
Coon, 1981; Davydov et al., 1991; Kappl et al., 1985; Nyman & Debrunner, 1991; Tajima et al., 1993) and observed model compounds (Balch, 1992; Tajima et al., 1995; Yamaguchi et al., 1993). The spectral intermediate observed with D251N is distinct from these species in both its UV−visible spectra and EPR spectra (Balch, 1992; Blake & Coon, 1981; Davydov et al., 1991; Kappl et al., 1985; Nyman & Debrunner, 1991; Tajima et al., 1993, 1995; Yamaguchi et al., 1993), suggesting the rate-determining step is the first proton transfer (**5A** and **B** to **5C**).

Addition of CO to the spectral intermediate gives a 95% yield of the Fe(II) CO complex, which is normally formed by the ligand exchange with oxy-P450, **4**, or by addition of CO to deoxy ferrous P450, **3**. The reaction of CO with the spectral intermediate takes place before 5% of product formation occurs. Since we have shown that the spectral intermediate is one electron reduced from oxy-P450, we suggest that in D251N P450$_{cam}$ catalysis, the second electron transfer (**4** to **5A** and **B**) is reversible (with the electron acceptor in this case being oxidized Pd). These observations confirm that the spectral intermediate is somewhere before the second proton transfer (**5C** to **6**).

At this time, we cannot yet make further suggestions as to the electronic nature of the spectral intermediate. We note that the only two model compounds isoelectronic and isoprotic with this intermediate occur before the first proton transfer (**5A−B** to **5C**). The side-bound ferric peroxide porphyrin complexes of Valentine (Burstyn et al., 1988) would be similar to the possible intermediate **5B,** while end-bound cobalt(III) superoxide complexes (Smith & Pilbrow, 1981; Tovrog et al., 1976) would be isoelectronic to (but of different charge than) the proposed intermediate **5A**.

In conclusion, we have shown that a newly observed catalytic intermediate is formed from the D251N mutant of P450$_{cam}$. The intermediate is a reduced oxy-P450 species, **5**. Kinetic and spectroscopic evidence shows that the second electron transfer (**4** to **5A** and **B** in Figure 1) is not the rate-determining step in D251N P450$_{cam}$ catalysis. Differences in the electronic properties of this reduced oxy-P450$_{cam}$ intermediate suggest that reduction of the oxy-iron heme has occurred. Carbon monoxide reactivity and the lack of similarity to a previously characterized ferric hydroperoxide intermediate suggests that the rate-determining step in D251N cytochrome P450$_{cam}$ catalysis is the first proton transfer (**5A** and **B** to **5C**) and the reduced oxy intermediate is the first example of a completely deprotonated hemeprotein intermediate at this oxidation state.

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