A REDUCED OXY INTERMEDIATE OF CYTOCHROME $P450_{cam}$ INVOLVED IN DIOXYGEN ACTIVATION

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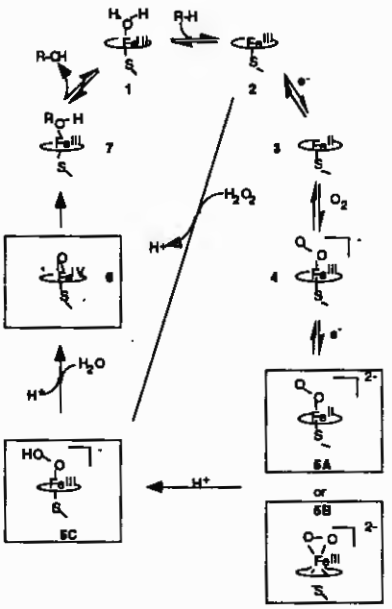
Cytochrome P450s are implicated in a wide variety of biochemical oxidations[1] and have been keen interest in the chemical community for 25 years.[2] Direct observation of intermediates involved in dioxygen activation and hydrocarbon oxidation have been the focus of many investigations, but no catalytically competent intermediates have been identified.[3] The catalytic rate prohibiting the observation of intermediates past oxy P450 (**4** in Figure 1) was thought to be the second electron transfer (**4** to **5AB**).[4,5]

Figure 1



Numbers refer to intermediates cited in text. Boxed intermediates are proposed and have never been directly observed.

A number of methods have been examined for the detection of previously unobserved catalytic intermediates of cytochrome P450 that are involved in dioxygen activation or substrate activation. Peroxide diffusion into polymer entrapped films of cytochrome $P450_{cam}$ at -10 °C showed no changes other than peroxide derived heme bleaching. Electron transfer from a pendantly attached $Ru(bpy)_3^{2-}$ to cytochrome $P450_{cam}$ was hoped to be used to speed up the reduction of oxy P450, however wild type and A113C/C136S/C334S $P450_{cam}$ showed no photoinduced electron transfer. The wild type case was thought to be due to the 28 Å distance between the donor and acceptor, while the mutant case was due to local unfolding of the protein and energy transfer to the heme. A mutation (D251N $P450_{cam}$) that slowed the overall catalytic rate, while maintaining full catalytic coupling, was examined. In addition to slower catalytic rates, that were 1 to 2 orders of magnitude slower than wild type,[6] the P450 exhibited a new UV-visible spectrum under catalytic turnover that was red shifted from all other catalytic intermediates.[7] The D251N $P450_{cam}$ mutation provided a method for detection of a previously unobserved catalytic intermediate.

The new UV-visible signature was shown to arise from a reduced oxy intermediate, which was one electron reduced from oxy P450. Kinetics of intermediate formation, by UV-visible, camphor hydroxylation, by GC, and oxidation of reduced putidaredoxin, by EPR, were measured in parallel for the reaction of ferrous deoxy P450 and reduced putidaredoxin with oxygen. This reaction initially formed oxy P450, which subsequently reacted with the reduced putidaredoxin. Within the first 25 seconds the intermediate had been fully formed, less than 10 % 5-hydroxycamphor had been formed, while 1.0 equivalent of reduced putidaredoxin was oxidized. The kinetics of reduced putidaredoxin oxidation fit to a model where 1.0 equivalent of reduced putidaredoxin was oxidized within the first 25 seconds ($k > 0.2$ sec^{-1}), while the remaining equivalents of reduced putidaredoxin are autooxidized at the same rate as reported ($k = 0.007$ sec^{-1}).[8] These results clearly demonstrate that the reduction of oxy P450 (which was the rate determining step for the wild type enzyme)[4,5] was not the rate determining step in D251N $P450_{cam}$ catalysis. CO reactivity and observation of an O-O vibration in the resonance Raman spectrum clearly demonstrated that the intermediate was before O-O bond heterolysis but after the reduction of oxy P450. This then showed that this new intermediate was a reduced oxy intermediate.

Various spectroscopic and reactivity studies were examined to further characterize this reduced oxy intermediate. Resonance Raman spectroscopy demonstrated that the additional electron density was not on the O-O bond, which conferred with the lack of observed EPR signal, due to the characteristic spectra of ferric hydroperoxo[9-11] and η^2-ferric peroxo[12-14] porphyrin complexes. These observations clearly ruled out the possibility of the reduced oxy intermediate being a ferric hydroperoxo intermediate. This then defined the reduced oxy intermediate as occurring before the first proton transfer. The additional electron was not observed on the heme iron by resonance Raman, by the lack of change in the heme marker bands relative to oxy P450,[15-16] but is an effect similar to the iron nitrosyl intermediates of P450[17,18] and other hemeproteins.[19] Occupation of the additional electron on a sulfur based molecular orbital was consistent with various theoretical models,[20,21] marked increases in the geminate recombination rate, and a new band observed at 394 cm^{-1} in transient resonance Raman experiments. The lack of nucleophilic reactivity, along with solely peroxide branchpoint uncoupling with various substrates, concurs with the additional electron residing on a sulfur based molecular orbital.

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