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MULTIPLE MECHANISMS OF CYTOCHROME P450-CATALYZED SUBSTRATE HYDROXYLATIONS

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SUMMARY - We have examined the 5-exo-hydroxylation of camphor by cytochrome P450 in [^{18}O] water/buffer solution. In the NADH/O_2-dependent reaction of the reconstituted multienzyme system, no ^{18}O-label is observed in the product alcohol. Similarly, in the m-chloroperbenzoic acid or cumene hydroperoxide supported reactions with ferric P450, solvent oxygen is not incorporated into hydroxycamphor. When the analagous reaction is carried out using iodosobenzene as the exogenous oxidant, however, the alcoholic oxygen of the product is derived entirely from the solvent. These results cannot be explained by equilibration of the iodosobenzene oxygen with solvent water before reacting with P450, and suggest a unique mechanism for iodosobenzene-supported P450 oxygenations. We propose two distinct mechanistic activities for cytochrome P450: a hydroxylase, and an oxene transferase, with the former encompassing the classic oxygenase as well as "peroxygenase" reactions.

Cytochrome P450 has been shown to catalyze the hydroxylation of unactivated alkanes according to the scheme: NADH + $[^{18}O]O_2$ + RH_2 → NAD^+ + $[^{18}O]OH^-$ + $[^{18}O]$RHOH. In the 11β-hydroxylase system, using $[^{18}O]O_2$, the source of the oxygen in the product alcohol was shown to arise primarily from atmospheric dioxygen, with little incorporation from labeled water (1). A significant advance in our understanding of the chemistry of P450 alkane hydroxylations occurred when it was demonstrated that exogenous peroxides or peracids can replace atmospheric dioxygen and the two reducing equivalents provided by pyridine nucleotide in their reaction with ferric (Fe^{3+}) P450 to generate hydroxylated substrate. When the peroxide reaction was conducted with hepatic $P450_{LM2}$ in the presence of oxygen-18 labeled water, a small but significant fraction of the label was incorporated into the product alcohol (2), but was

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Abbreviations: PhIO = iodosobenzene
PhI = iodobenzene
m-CPBA = m-chloroperbenzoic acid
FT-IR = Fourier transform infrared spectroscopy
m_i = mass ion

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attributed to an artifact or side radical oxidation process. More recently, iodosobenzene (PhIO) was shown to serve as an efficient oxygen donor in P450 catalyzed hydroxylations. Groves et al. (3) have documented the generation of a stable metal-oxo species in the reaction of chlorotetraphenylporphinatochromium (III) with iodosobenzene. The use of oxygen-18 water in these reactions led to virtually complete incorporation of label into the product epoxide. Since, under the organic solvent conditions employed, PhIO was reported not to undergo oxygen exchange, the facile incorporation of water label into the product was used to infer the generation of an exchanging oxychromium species. By analogy one would formally write the corresponding iron reaction as: $Fe^{3+} + PhIO \leftrightarrow [Fe^{V}= O]^{3+} + PhI \xleftrightarrow{H_2O*} [Fe^{V}= O*]^{3+}$, and, in fact, iron porphyrin catalysts will utilize PhIO to hydroxylate various substrates in model systems (4,5). We have used detailed $[^{18}O]$ H_2O exchange studies to probe the intermediate iron-oxygen states in P450 catalysis, using both the reconstituted NADH/O_2 dependent camphor hydroxylase system from Pseudomonas putida, as well as the reaction of ferric $P450_{cam}$ with the exogenous oxidants iodosobenzene, cumene hydroperoxide, and m-chloroperbenzoic acid. Our data demonstrate that the PhIO-dependent reaction is unique among the systems tested in exhibiting virtually complete incorporation of solvent water oxygen into the product alcohol. These results suggest the existence of two distinct mechanistic activities of P450: the classic hydroxylase, encompassing the peracid, peroxide and NADH/O_2 dependent reactions and characterized by a non-exchanging iron-oxo species, and a PhIO-dependent oxene transferase (6), which proceeds via an intermediate capable of facile oxygen exchange with solvent.

MATERIALS AND METHODS

Cytochrome P450, putidaredoxin reductase, and putidaredoxin were prepared from Pseudomonas putida (ATCC 29607) as described (7). Iodosobenzene was synthesized from iodobenzene, via the dichloride, as described, and the active oxygen content periodically determined by iodometry (8). Cumene hydroperoxide and m-chloroperbenzoic acid were obtained from commercial sources. Oxygen-18 water was obtained in 99% enrichment from Prochem, and diluted to obtain buffer of known isotope content. Camphor hydroxylation was assayed by gas chromatography on a six-foot 3% OV-17 column (Supelco) fitted to a Hewlett-Packard 5000 series G.C. Mass spectra were obtained on an HP 5985 GC/MS, with selective ion monitoring. Fourier transform infrared spectra were obtained as nujol mulls on a Nicolet 7199 FT-IR, using 100 transients. All enzyme turnover reactions were carried out in 80 mM potassium phosphate buffer, pH 7.0, 700 μM camphor, and 1 mM exogenous oxidant (when present). Reconstituted multienzyme turnover experiments were performed using saturating amounts of putidaredoxin reductase and putidaredoxin.

TABLE 1

Oxidant	Percent incorporation
NADH/O_2	0
m-CPBA	0
CHP	0
PhIO	99

For each reaction indicated, the numerical values correspond to the percent of product 5-exo-hydroxycamphor exhibiting incorporation of solvent oxygen into the alcohol moiety. Values are ±5%, and were determined from the m_i/m_i+2 ratio in the mass spectrum.

RESULTS

Iodosobenzene, m-CPBA, and CHP are all capable of reacting with ferric $P450_{cam}$ to yield 5-exo-hydroxycamphor and iodobenzene, m-chlorobenzoic acid, and cumenol, respectively. In all cases, protein destruction by the exogenous oxidant eventually terminates the reaction, usually yielding 3-7 complete turnovers at an oxidant concentration of 1 mM. Turnover reactions using these exogenous oxidants, or analogous reactions of the reconstituted multienzyme system, were conducted in $[^{18}O]$ H_2O, and the resulting hydroxycamphor analyzed for ^{18}O incorporation in the hydroxyl moiety. The data, shown in Table 1, demonstrate that iodosobenzene is unique in exhibiting virtually complete incorporation of solvent oxygen into the product. The extent of solvent oxygen incorporation into hydroxycamphor in the PhIO-driven reaction is independent of reaction time, with complete incorporation into the isolated product within 2.5 minutes, the earliest time tested. In contrast, the peracid, peroxide, and NADH/O_2 dependent reactions show virtually no incorporation of solvent oxygen into hydroxycamphor.

The significance of these results clearly depend on the assumption that PhIO does not undergo oxygen exchange with water before reacting with cytochrome P450. The limited literature references on this subject provide qualitative support for this assumption (3,9). To ensure the validity of our results, we have examined the oxygen exchange properties of iodosobenzene under physiological conditions using Fourier-transform infrared spectroscopy.

On overnight incubation of PhIO in $[^{18}O]$ H_2O, reproducible shifts in 3 bands, located at 1066 cm^{-1}, 735 cm^{-1}, and 690 cm^{-1} in $[^{16}O]$ PhIO, are observed (Fig. 1). These shifts provide a sensitive indication of the $^{16}O/^{18}O$ isotope ratio in PhIO. Thus, a sample of PhIO was incubated in a medium containing 20% $[^{18}O]$ H_2O, at the same pH and ionic strength as the enzyme reaction medium, for 2.5 minutes, immediately frozen and lyophilized,

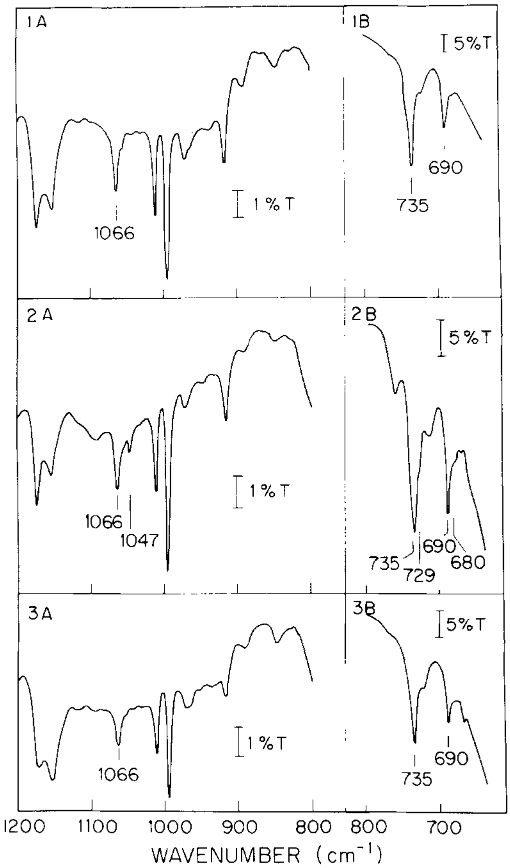

Figure 1: Infrared spectra of iodosobenzene samples. 1A,B: $[^{16}O]$ PhIO, showing oxygen isotope-sensitive absorptions at 1066, 735 and 690 cm^{-1}. 2A,B: PhIO isolated from 70% MeOH/30% H_2O solution, containing 20 atom percent $[^{18}O]$ H_2O. Note appearance of new bands at 1047, 729 and 680 cm^{-1}, demonstrative of an approximately 20/80 $[^{18}O]/[^{16}O]$ isotope ratio in PhIO. 3A,B: PhIO isolated from a 2.5 minute incubation in 5% MeOH/95% H_2O enriched 20 atom percent in $[^{18}O]$ H_2O, pH = 7.0, μ = 0.22. Absence of 1047, 729 and 680 cm^{-1} bands of comparable intensity to 2 indicate PhIO does not undergo rapid oxygen exchange with H_2O.

and an FT-IR of the dried sample obtained. Under these conditions PhIO shows no incorporation of ^{18}O from the solvent (Fig. 1), thus proving that the observed isotope incorporation into hydroxycamphor by the PhIO-dependent cytochrome P450 reaction must have an enzymatic basis.

DISCUSSION

It is widely accepted that the cytochrome P450 mixed-function oxidase system incorporates an oxygen atom from atmospheric dioxygen into the carbon skeleton of an organic substrate (1). Similarly, in the peroxide-dependent

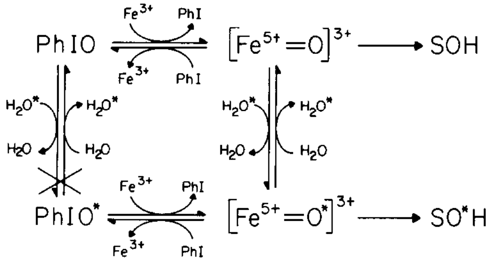

Figure 2: Postulated mechanism for incorporation of solvent oxygen into product (SOH) in the iodosobenzene-dependent hydroxylation of camphor by cytochrome P450.

reaction of ferric P450 the oxygen incorporated into the product is derived predominantly from the peroxide (2). The similarity of these two results might be expected, in view of the evidence of an alkyl-peroxide iron intermediate in the NADH/O_2 dependent reaction of P450 (10). Our data confirm these results for the camphor hydroxylase system, and demonstrate that the peracid-dependent hydroxylation of camphor by P450 also derives the hydroxyl oxygen from the oxidant, rather than the solvent. Drastically different results are obtained when iodosobenzene serves as the exogenous oxidant, however. In this case the hydroxyl oxygen in the product 5-exo-hydroxycamphor is derived from the solvent. Since we have unambiguously demonstrated that PhIO does not exchange oxygen with solvent water over the time-course of the reaction, the interaction of PhIO with the ferric heme of P450 either produces an intermediate that can readily exchange oxygen with solvent, in analogy with model studies using chromium tetraphenylporphyrin (3,11), or a substrate intermediate capable of capturing solvent must be generated. In this latter case, for example, production of a carbonium ion intermediate could capture OH^- to specifically produce 5-exo-alcohol, since carbonium ions of the bicyclic [2.2.1.] systems are not captured in the endo-position (12). A carbonium ion at the 5-position of camphor, however, might be expected to undergo facile rearrangement, which is not observed. In a more general sense, the fact that iron-porphyrin derivatives can utilize PhIO to hydroxylate and epoxidize substrate in the complete absence of H_2O argues against any mechanism involving the direct abstraction of a water oxygen (4,5). Therefore, these data strongly suggest that the iodosobenzene-dependent hydroxylation of camphor by cytochrome P450 proceeds through a unique intermediate capable of facile exchange with solvent water (Fig. 2), in analogy to the $[Cr^{V}=O]^{3+}$ structure suggested by model system investigations (3) and the recent observation that P450 catalyzes reversible oxygen transfer from iodosobenzene to iodobenzene (13). The lack of solvent oxygen incorporation into product in the peracid, peroxide, and NADH/O_2 dependent reactions suggest that they proceed through a different, non-exchanging iron-oxo intermediate, perhaps derived from homolytic O-O bond scission in the dioxygen intermediate (14). Thus, these results permit the delineation of two distinct mechanistic activities of cytochrome P450: first, the classic hydroxylase, encompassing the peracid, peroxide and native NADH/O_2 dependent reactions, and, second, a formal oxene transferase, which proceeds through an intermediate capable of facile oxygen exchange with solvent water. Additional experiments to better define these activities are currently in progress.

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