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MECHANISMS OF HYDROXYLATION BY CYTOCHROME P-450: EXCHANGE OF IRON-OXYGEN INTERMEDIATES WITH WATER[1]

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SUMMARY - Highly-purified rat liver microsomal cytochrome P-450 converted cyclohexane to cyclohexanol in the presence of iodosobenzene. Oxygen from ^{18}O-iodosobenzene was not incorporated into cyclohexanol but oxygen from $H_2{}^{18}O$ was readily incorporated. Cytochrome P-450 catalyzed the facile exchange of oxygen between iodosobenzene and water but neither cytochrome P-420 nor the apoenzyme did. Under these conditions cytochrome P-450 readily incorporated oxygen from $^{18}O_2$ into cyclohexanol in the presence of NADPH-cytochrome P-450 reductase and NADPH. The results are interpreted in a mechanism in which cytochrome P-450 forms a common hydroxylating species in the presence of iodosobenzene or O_2 plus NADPH.

A microsomal mixed-function oxidase system containing cytochrome P-450[2] catalyzes the oxidation of a wide variety of organic compounds to polar metabolites. The mechanism by which enzymes of this class accomplish hydroxylations and other oxidative reactions has been a subject of great interest (1). The suggestion that the hydroxylating species involved in such reactions is a perferryl complex has been advanced by studies with monooxygenated oxidants such as ϕIO, which can replace O_2 and physiological electron donors (2-5). These studies support the hypothesis that the activated iron is bound to a single atom of oxygen. Studies with model non-iron metalloporphyrins and ϕIO have also been carried out (6-8).

Recently Heimbrook and Sligar (9) have shown that oxygen from $H_2{}^{18}O$ is incorporated into the hydroxylated product during the reaction of camphor with a

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[2] Abbreviations used include: P-450, cytochrome P-450; P-420, cytochrome P-420; ϕIO, iodosobenzene; and ϕI, iodobenzene.

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bacterial P-450 in the presence of ϕIO but not with O_2 and physiological electron donors. We have carried out investigations using purified liver microsomal P-450s in the ϕIO- and NADPH-supported systems in order to reconcile the differences found in oxygen exchange for these systems with a perferryl hydroxylating species postulated to be common to both pathways. These experiments are reported here and a mechanism is presented which rationalizes these observations.

MATERIALS AND METHODS

ϕIO was purchased from ICN-K and K Laboratories, Plainview, New York. ϕI^{18}O was prepared by treatment of iodobenzene dichloride with $H_2{}^{18}O$. $^{18}O_2$ and $H_2{}^{18}O$ were puchased from Merck Isotopes, St. Louis, Missouri. d_{12}-Cyclohexane (99.5% atomic excess) was purchased from Sigma Chemical Co., St. Louis, Missouri. Cumene hydroperoxide was obtained from Matheson, Coleman, and Bell, Norwood, Ohio. Liver microsomes were prepared from phenobarbital-treated rats, and P-450 (fraction PB-B_2) and NADPH-P-450 reductase were purified to electrophoretic homogeneity as described elsewhere (10). P-450 was converted to P-420 by heating for 5 min at 100°C in the presence of 1% (w/v) sodium dodecyl sulfate. P-450 was converted to apo-P-420 by dialysis versus 100 volumes of 50 mM Tris-HCl buffer (pH 7.7) containing 1% (w/v) sodium dodecyl sulfate and 5% (v/v) 2-mercaptoethanol (two changes of buffer, 8 h per dialysis). Excess sodium dodecyl sulfate was removed from P-420 and apo-P-420 by extensive dialysis.

Typical incubations were carried out at 23°C in 20 mM potassium phosphate buffer (pH 7.7) using 10 mg microsomal protein ml^{-1}, 10 mg bovine serum albumin ml^{-1}, 8 μM P-450, P-420, or apo-P-420, 8 μM NADPH-P-450 reductase, 5 mM ϕIO, 1 mM cumene hydroperoxide, 10 mM cyclohexane, and an NADPH generating system consisting of 0.5 mM NADPH, 10 mM glucose-6-phosphate, and 1.0 I.U. glucose-6-phosphate dehydrogenase ml^{-1} as indicated. When P-450 and NADPH-P-450 reductase were utilized together, L-α-dilauroylglyceryl-3-phosphorylcholine was added at a concentration of 30 μM. Reactions involving NADPH-P-450 reductase were carried out for 20 min and reactions involving ϕIO were carried out for 2 min. $^{18}O_2$ was introduced into evacuated samples from a manifold and O_2 was analyzed for excess ^{18}O in each sample (prior to incubation) by gas chromatography/mass spectrometry using a molecular seive column at 30°C. Cyclohexanol was extracted into an equal volume of ether and analyzed for excess ^{18}O by gas chromatography/mass spectrometry using a 3% OV-17 column at 80°C. The peaks at m/e 101 and 103 (M + 1) were used in calculations. Levels of cyclohexanol produced were determined by gas chromatography (flame ionization detector) with the same column, using n-octanol as an internal standard. ϕIO was extracted into three volumes of CH_2Cl_2. CH_2Cl_2 was removed in vacuo and samples were analyzed for excess ^{18}O by direct probe desorption mass spectrometry. The peaks at m/e 221 and 223 (M + 1) were used in calculations. All mass spectrometry utilized a Ribermag R-10-10 instrument in the chemical ionization mode with isobutane as the ionization gas.

RESULTS

P-450 catalyzed the conversion of cyclohexane to cyclohexanol in the presence of ϕIO as well as with NADPH, O_2, and an optimal concentration of NADPH-P-450 reductase (Table 1). No significant kinetic deuterium isotope effect was observed in either case.

TABLE 1

Conversion of cyclohexane to cyclohexanol

System	Turnover number, min^{-1}
P-450, NADPH-P-450 reductase, NADPH, O_2, cyclohexane	30
P-450, NADPH-P-450 reductase, NADPH, O_2, d_{12}-cyclohexane	34
P-450, φIO, cyclohexane	19
P-450, φIO, d_{12}-cyclohexane	15

Turnover numbers are expressed as nmol cyclohexanol produced per min per nmol P-450. Results are presented as means of duplicate experiments and are not corrected for P-450 destruction.

When cyclohexane was hydroxylated in the presence of P-450, NADPH-P-450 reductase, NADPH, O_2, and $H_2{}^{18}O$, none (< 3%) of the oxygen in cyclohexanol was derived from water. Similar results were obtained when the reaction was supported with cumene hydroperoxide. However, when the reaction was carried out in the presence of φIO and NADPH was absent, 86% (± 16%) of the oxygen in cyclohexanol was derived from water.

Cyclohexane was incubated with either microsomes or purified P-450 in the presence of $\phi I^{18}O$ (67% atomic excess). The isolated cyclohexanol did not contain any detectable ^{18}O (< 3%). Similar results were obtained when P-450s purified from β-naphthoflavone-treated rats (10) or phenobarbital-treated rabbits (11) were used to hydroxylate cyclohexane. Other experiments showed that the cyclohexene oxide and cyclohexen-3-ol derived from cyclohexene were devoid of ^{18}O under similar conditions.

P-450 was incubated with cyclohexane in the presence of NADPH-P-450 reductase and NADPH under an $^{18}O_2$ atmosphere (atomic excess ranged from 18 to 57% in individual experiments). The data indicated that 114% (mean of several experiments) of the oxygen in cyclohexanol was derived from $^{18}O_2$. A similar experiment with microsomes indicated that 87% of the oxygen in cyclohexanol was derived from $^{18}O_2$. When cyclohexane was hydroxylated by either purified P-450 or microsomes in the presence of φIO or cumene hydroperoxide, none of the oxygen in cyclohexanol was derived from $^{18}O_2$.

TABLE 2

Exchange of oxygen of $\phi I^{18}O$ with H_2O

Conditions	Atomic excess ^{18}O in isolated $\phi IO \pm SD$
$\phi I^{18}O$	67 ± 14
$\phi I^{18}O$ plus buffer	66 ± 17
$\phi I^{18}O$ plus P-450	34 ± 7
$\phi I^{18}O$ plus P-450 plus cyclohexane	30 ± 1
$\phi I^{18}O$ plus albumin	74 ± 13
$\phi I^{18}O$ plus P-420	67 ± 10
$\phi I^{18}O$ plus apo-P-420	65 ± 7

The above observations suggested that exchange of the oxygen of ϕIO with water occurred under the incubation conditions. The exchange was studied by isolating ϕIO after treatment under several conditions and the results are shown in Table 2. About one-half of the oxygen of ϕIO was exchanged within 2 min in the presence of P-450 regardless of whether cyclohexane was present or not. The exchange did not occur rapidly in buffer or in the presence of albumin, P-420, or apo-P-420.

DISCUSSION

The results are in accord with those of Heimbrook and Sligar (9), who showed that oxygen from $H_2{}^{18}O$ was quantitatively incorporated into 5-exo-hydroxycamphor upon incubation of camphor with ϕIO and a bacterial P-450. We have also demonstrated that oxygen from $^{18}O_2$ is not incorporated into the hydroxylated product (cyclohexanol) and that P-450 readily catalyzes the exchange of oxygen between ϕIO and water. These results are discussed in relationship to the scheme shown in Figure 1.

Steps a-i are drawn on the basis of the work of others (1) for the hydroxylation in the presence of O_2 and physiological electron donors, with substrate (RH) binding, one electron reduction, O_2 binding, a second one electron reduction, rearrangement, and release of water to give the perferryl intermediate (Fe^{V}=O) in the presence of substrate. Hydrogen atom abstraction (g) precedes product formation (h) and release (i). In the ϕIO mechanism, ferric P-450 binds ϕIO and forms the perferryl intermediate associated with

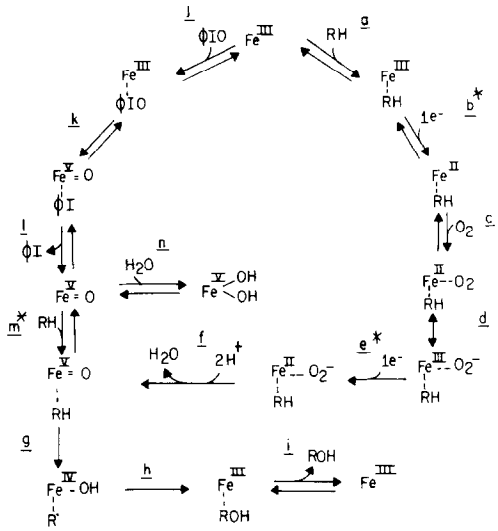

Figure 1. Proposed mechanisms for P-450 catalyzed hydroxylations involving NADPH, NADPH-P-450 reductase, and O_2 (reactions a-f) or ϕIO (reactions j-n). Reaction steps g-i are viewed as common to both systems. RH indicates cyclohexane in these experiments. Steps denoted with asterisks are postulated to be rate-limiting. See text for discussion.

ϕI (steps j and k). ϕI dissociates in step l to give free Fe^{V}=O, which is postulated to equilibrate rapidly with water (step n). The free perferryl intermediate can bind the substrate RH (step m) to form the hydroxylating species common to the pathway shown in steps a-i. If ϕI serves as RH, steps m, g, h, and i are identical to the reverse reactions shown in steps j-l. We have previously demonstrated that ϕ^{125}I is converted to ϕ^{125}IO under these conditions (5).

The extensive incorporation of oxygen from water into cyclohexanol suggests that step n is rapid. Step g appears to be relatively rapid, as judged by the lack of intermolecular deuterium isotope effects observed in both the ϕIO and NADPH-O_2 incubation systems (Table 1); thus, the perferryl intermediate is committed to catalysis when formed in the presence of RH. Thus, step m must be slow. Under the conditions used here, P-450 catalyzed the ϕIO-dependent hydroxylation of cyclohexane at a rate of 19 min^{-1} (Table 1) and the exchange of oxygen between ϕIO and water at a rate of 170 min^{-1} (Table 2). Oxygen transfer from the perferryl intermediate to ϕI appears to be more rapid than methylene hydroxylation. Steps such as b and e are postulated to be limiting in the NADPH-supported reaction.

Thus, we feel that the exchange of oxygen in the φIO-supported pathway is the result of the formation of the free perferryl intermediate. These results are in accord with the results obtained by Groves and his asociates with Mn^{V}=O and Cr^{V}=O porphyrin intermediates (6,8). These complexes exchange oxygen freely with water but are committed to catalysis in the presence of substrates which can be hydroxylated. Although the suggestion of Heimbrook and Sligar (9) that a distinct hydroxylating species is formed in the presence of φIO cannot be dismissed, the scheme presented in Figure 1 and the postulates about the relative kinetics of the steps involved can explain all of the data obtained to date using a single perferryl intermediate.

Although oxygen from water is not incorporated into monohydroxylated products produced by P-450 in the presence of hydroperoxides (vide supra, ref. 9), discrepancies have been noted between the products of P-450-supported reactions in the presence of hydroperoxides and NADPH and O_2 (12,13). We have not focused upon the relationship of these systems, although we postulate that rate-limiting steps occur prior to the formation of the substrate-bound perferryl intermediate, if this intermediate is common to the hydroperoxide pathway. Data have been presented elsewhere to support the similarity between the φIO pathway and the pathway involving NADPH and O_2 (4). We favor the view that these two latter pathways proceed through a common hydroxylating species and that differences in exchange with water are the result of differences in steps leading to this species and the kinetics of these steps.

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