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Regioselectivity in the Cytochromes *P*-450: Control by Protein Constraints and by Chemical Reactivities[1]

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Three alicyclic compounds (D-camphor, adamantanone, adamantane) were found to be hydroxylated by the cytochrome *P*-450 isoenzymes *P*-450$_{cam}$ and *P*-450$_{LM2}$. With *P*-450$_{cam}$ as the catalyst only one product was formed from each of the substrates: 5-*exo*-hydroxycamphor, 5-hydroxyadamantanone, and 1-adamantanol. With *P*-450$_{LM2}$ as the catalyst, two or more isomeric products were formed from each substrate: 3-*endo*-, 5-*exo*-, and 5-*endo*-hydroxycamphor; 4-*anti*- and 5-hydroxyadamantanone; and 1- and 2-adamantanol. The products from *P*-450$_{cam}$ hydroxylations were found to be isosteric with one another, suggesting that each of them was attacked at a topologically congruent position within a rigid enzyme–substrate complex. The distribution of products from *P*-450$_{LM2}$ hydroxylations, on the other hand, were similar to the distributions expected during solution-phase hydroxylations. Thus, it would appear that the complex which *P*-450$_{LM2}$ forms with its substrate allows considerable movement of the substrate molecule, such that most of the hydrogens in the substrate are exposed to the enzymatic hydrogen abstractor. Under these conditions, the distribution of products more nearly reflects the rank order of chemical reactivities of the various hydroxylatable positions, with only a moderate protein-based steric constraint being expressed. These suggestions were also evident in the tightness of binding of the substrates to the two enzymes and in the magnitude of coupling between the substrate binding and the spin-state equilibria. Thus, the product from *P*-450$_{cam}$-catalyzed hydroxylation may be predicted by a consideration of the relation of the topology of the prospective substrate to that of D-camphor. The products from *P*-450$_{LM2}$-catalyzed hydroxylations, on the other hand, may be approximately predicted from the chemical reactivities of the various abstractable hydrogens in the prospective substrate.

The cytochromes *P*-450[2] comprise a family of ubiquitous enzymes whose function is to introduce a hydroxyl group into an organic compound. The reasons for such functionalization of the organic molecule are as diverse as the biological locations of the enzymes themselves. For instance, *P*-450$_{cam}$ hydroxylates D-camphor as the first step in the catabolism of this terpene when *Pseudomonas putida* is grown on camphor as the sole carbon source (9). On the other hand, the mammalian adrenal cytochromes *P*-450 are involved in several hydroxylations of the steroid nucleus during steroidogenesis (9). Still another example is the hydroxylation of various lipophilic xenobiotics by mammalian liver

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[2] Abbreviations used: *P*-450$_{cam}$, soluble, camphor-hydroxylating cytochrome *P*-450 isolated from *Pseudomonas putida*; *P*-450$_{LM2}$, rabbit liver microsomal cytochrome *P*-450 inducible by *in vivo* pretreatment with phenobarbital; *P*-450$_{LM4}$, rabbit liver microsomal cytochrome *P*-450 inducible by *in vivo* pretreatment with β-naphthoflavone; *P*-450, generic term encompassing all forms of cytochrome *P*-450; GC–MS, gas chromatography–mass spectrometry.

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microsomal cytochromes for the purpose of providing a suitable nucleophilic functional group for subsequent hydrophilic derivatization by conjugating enzymes such as UDP-glucuronyl transferase.

As we would expect, the substrate specificities of the various *P*-450 enzymes vary widely. However, since the basic chemical transformation is the same among them all, we can inquire as to the relative roles of the substrate-binding site on the protein and the intrinsic chemistries of the substrate carbon skeleton and of the *P*-450 reactive oxygen intermediate in determining which molecules and, just as importantly, which positions on those molecules are hydroxylated. In this paper, we have examined the hydroxylation of three substrates by two *P*-450 isozymes. The substrates (camphor, adamantanone, and adamantane) were chosen such that both enzymes exhibited hydroxylase activity toward them. We report two near extremes of substrate specificity and regioselectivity. The selection of substrates and hydroxylation sites by *P*-450$_{cam}$ is strongly controlled by the substrate-binding site, while *P*-450$_{LM2}$ shows little selection among substrates and a regioselectivity suggestive of chemical rather than protein control.

MATERIALS AND METHODS

Chemicals. Three of the hydroxycamphors, the 5-*exo*- and 5-*endo* epimers, and 6-*endo* were a gift from Dr. I. C. Gunsalus. 3-*exo*-Hydroxycamphor was prepared by zinc dust reduction of 3-ketocamphor (camphoroquinone) as described by Kreiser (11). 5-Ketocamphor and adamantane-2,4-dione were prepared by Jones oxidation of 5-*exo*-hydroxycamphor and of 4-hydroxyadamantanone. Authentic samples of 4-*syn*-, 4-*anti*-, and 5-hydroxyadamantanones were the generous gift of Dr. James Henkel, Department of Medicinal Chemistry, University of Connecticut. Dilauroyl glyceryl-3-phosphorylcholine, isocitric acid, and $NADP^+$ were purchased from Sigma Chemical Company. All other chemicals were purchased from Aldrich Chemical Company.

Enzymes. Previously described procedures were followed for the preparation of electrophoretically homogeneous *P*-450$_{cam}$, putidaredoxin, and putidaredoxin reductase (10); *P*-450$_{LM2}$ and *P*-450$_{LM4}$ (1); and NADPH-cytochrome *P*-450 oxidoreductase (3). Catalase and isocitric dehydrogenase were purchased from Sigma.

Enzyme-substrate titrations. With *P*-450$_{LM2}$, a solution of the protein (1 nmol) and dilauroyl glyceryl-3-phosphorylcholine (50 μg) in 1.0 ml of potassium phosphate buffer (0.1 M, pH 7.4) at 25°C was titrated with small aliquots of a methanolic solution of the substrate. Optical spectra in the region 350 to 500 nm were collected with a Varian–Cary 219 spectrophotometer interfaced to an Apple II Plus microcomputer. Difference spectra were calculated by subtracting the original spectrum from the subsequent substrate-perturbed spectra, using 405 nm as an isosbestic point. Plots of the reciprocal of the magnitude of the difference spectrum versus the reciprocal of the substrate concentration were linear, with correlation coefficients from 0.991 to 0.998. From such plots apparent dissociation constants for the enzyme–substrate complexes and maximal mole fractions of high-spin heme were calculated. In the case of tight binding substrates, corrections for substrate depletion effects were applied by computer iteration. Substrate titrations of *P*-450$_{cam}$ were performed as previously described (6).

Enzymatic hydroxylation reactions. Hydroxylation reactions involving *P*-450$_{LM2}$ or *P*-450$_{LM4}$ utilized an NADPH-generating system consisting of isocitric dehydrogenase (0.4 units), $NADP^+$ (100 μM), isocitric acid (5 mM), and magnesium chloride (10 mM). Catalase (6000 units) was included to prevent the accumulation of hydrogen peroxide. Reaction mixtures contained *P*-450$_{LM2}$ or *P*-450$_{LM4}$ (1.0 nmol), NADPH-cytochrome *P*-450 oxidoreductase (3.0 nmol), dilauroyl glyceryl-3-phosphorylcholine (50 μg, added as a sonicated suspension in water), potassium phosphate (100 μmol, pH 7.4), one of the substrates (added as a concentrated solution in methanol), catalase, and the NADPH-generating system in a total volume of 1.0 ml. Reactions were initiated by the addition of isocitric dehydrogenase and were allowed to proceed for 3 to 6 h at 25°C. Accumulation of product in such reactions was linear with time for at least 3 h. The generating system prevents the accumulation of $NADP^+$, which inhibits reductase, and the catalase removes H_2O_2, which can destroy *P*-450. In these circumstances, rates will be constant until one of the substrates becomes depleted or until product inhibition becomes important. These effects do not occur for hours with the rates observed here. The observed regioselectivity was invariant with the time period of reaction. Thus, product yields were maximized by allowing the reaction to run to completion. Rate measurements were, of course, made within linear reaction times. Control reactions were also run in which the cytochrome *P*-450 reductase was omitted. No background levels of the reported products were present in these controls. Substrate concentrations were: D-camphor, 5 mM; adamantanone, 8 mM; and adamantane, 0.3 mM.

Hydroxylation reaction mixtures involving *P*-450$_{cam}$ contained *P*-450$_{cam}$ (0.5 nmol), putidaredoxin (2.2

nmol), putidaredoxin reductase (0.5 nmol), and potassium phosphate (100 μmol, pH 7.0) in a total volume of 1.0 ml. Reactions were initiated by the addition of NADH (200 nmol) and were conducted at 20°C. Substrate concentrations were all 1 mM with $P\text{-}450_{cam}$.

At the end of the reaction periods an appropriate amount of a suitable internal standard was added and the products were extracted with 1 ml of chloroform. The standards used were *p*-chlorobenzyl alcohol with camphor and adamantanone, and 1-phenylethanol with adamantane. The chloroform solutions were concentrated by evaporation under nitrogen and subjected to gas chromatographic analysis on both a polar and a nonpolar column, using flame ionization detection. Column A (polar) was 10% Carbowax 20 M on Supelcoport (80/100). Column B (nonpolar) was 3% OV-17 on Supelcoport (80/100). Both were 1/8 in. by 6 ft stainless-steel columns. Gas chromatographic peaks were quantitated with a Hewlett-Packard 3390A Integrator. Gas chromatography–mass spectrometry was performed on a Hewlett-Packard 5992 instrument, using 3-ft versions of Columns A or B.

Oxidation of secondary alcohols to ketones for structural identification purposes was accomplished by use of the Jones reagent (0.1 M sodium dichromate in 2.5 M sulfuric acid). The alcohol sample in chloroform (0.1 ml) was shaken with Jones reagent (0.2 ml) for a few minutes at room temperature until the orange color had changed to green. The sample was centrifuged and the aqueous layer was removed. The chloroform layer was washed once with water and then subjected to gas chromatography.

RESULTS

Hydroxylation of D*-camphor.* Ten isomeric hydroxycamphors could in principle result from enzymatic hydroxylation of D-camphor. These are 3-*exo*-, 3-*endo*-, 4-, 5-*exo*-, 5-*endo*, 6-*exo*, 6-*endo*-, 8-, 9-, and 10-hydroxycamphor. Exposure of D-camphor to the reconstituted $P\text{-}450_{cam}$ enzyme system leads to the accumulation of a single product which has been rigorously shown to be 5-*exo*-hydroxycamphor (5). Under the conditions described here, this product is produced as a catalytic rate of 60 mol/mol $P\text{-}450_{cam}$/min. Greater rates may be obtained by manipulation of putidaredoxin concentrations and extrapolation of measured values to infinite redoxin concentration. However, moderate concentrations of the redoxin were used here in the interests of conservation of enzyme preparations. When a reconstituted $P\text{-}450_{LM2}$ enzyme system is substituted for the $P\text{-}450_{cam}$ system, three products accumulate which GC–MS demonstrates to be hydroxycamphors (designated A, B, and C; see Fig. 1). Gas chromatography on both polar and nonpolar columns (Table I) as well as their mass spectra (Table II) demonstrated hydroxycamphors B and C to be 5-*exo*- and 5-*endo*-hydroxycamphor, respectively. The evidence strongly indicates hydroxycamphor A to be 3-*endo*-hydroxycamphor. The mass spectrum of A showed a substantial $M^+ - 73$ peak (m/z 95) due to loss of hydroxyketene followed by methyl, indicating the hydroxyl group to be in the 3 position, adjacent to the carbonyl. An appreciable $M^+ - 18$ peak (m/z 150) due to loss of water would indicate a 3-*endo*-hydroxyl stereochemistry since a 3-*exo*-hydroxyl has no readily abstractable hydrogen atoms nearby. Furthermore, oxidation of the mixture of A, B, and C by the Jones reagent gave two ketocamphors D and E, which were shown by gas chromatography (Table I) and GC–MS (Table II) to be 3-keto- and 5-ketocamphor, respectively, with the aid of the authentic compounds. Also, the mass spectrum of authentic 3-*exo*-hydroxycamphor was distinct from that of C. However, in the absence of an authentic sample of 3-*endo*-hydroxycamphor one cannot be absolutely certain of our assignment. As seen in Table

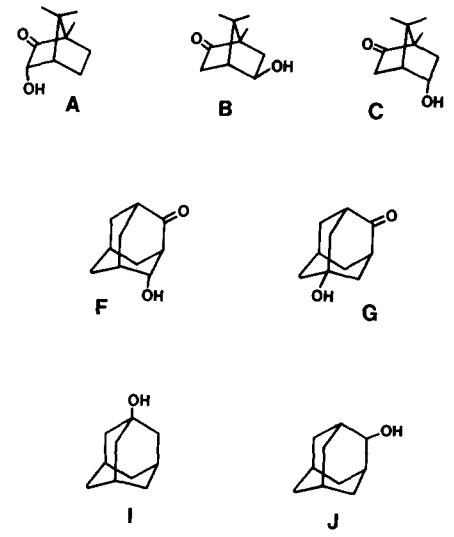

FIG. 1. Hydroxylation products with $P\text{-}450_{LM2}$.

TABLE I

GAS CHROMATOGRAPHIC DATA

Compound	Retention times, min (at °C) Carbowax 20 M	OV-17
3-*exo*-Hydroxycamphor	2.90 (180)	1.70 (150)
5-*exo*-Hydroxycamphor	5.64 (180)	2.77 (150)
5-*endo*-Hydroxycamphor	7.28 (180)	3.18 (150)
6-*endo*-Hydroxycamphor	—	3.5 (150)
Hydroxycamphor A	2.90 (180)	1.79 (150)
Hydroxycamphor B	5.68 (180)	2.75 (150)
Hydroxycamphor C	7.28 (180)	3.17 (150)
3-Ketocamphor	2.15 (180)	2.18 (150)
5-Ketocamphor	1.96 (180)	1.90 (150)
Ketocamphor D	2.15 (180)	2.18 (150)
Ketocamphor E	1.93 (180)	1.82 (150)
4-*syn*-Hydroxyadamantanone	10.30 (200)	7.84 (150)
4-*anti*-Hydroxyadamantanone	9.32 (200)	6.98 (150)
5-Hydroxyadamantanone	6.18 (200)	5.94 (150)
Hydroxyadamantanone F	9.24 (200)	8.09 (150)
Hydroxyadamantanone G	6.14 (200)	5.72 (150)
Adamantane-2,4-dione	4.99 (200)	7.39 (150)
Adamantanedione H	4.98 (200)	7.41 (150)
1-Adamantanol	6.44 (140)	2.12 (130)
2-Adamantanol	9.69 (140)	2.75 (130)
Adamantanol I	6.44 (140)	2.11 (130)
Adamantanol J	9.68 (140)	2.74 (130)

III, 5-*endo*-hydroxycamphor was by far the predominant product, with the 3-*endo*- and 5-*exo*-isomers being about equally formed. Small amounts of two or three compounds not present in no-enzyme controls were detected. Their total amount was only about 7% of the total, but because of their low yields they could not be identified. None of the product peaks had a gas chromatographic retention time similar to that of 6-*endo*-hydroxycamphor (Table I). The overall catalytic rate of camphor hydroxylation by $P\text{-}450_{LM2}$ was 1.3 mol/mol $P\text{-}450_{LM2}$/min.

A third *P*-450 isozyme, $P\text{-}450_{LM4}$, was allowed to oxidize D-camphor as well. In this case, the rate was exceedingly low, only 0.006 mol/mol $P\text{-}450_{LM4}$/min. The only product detected was 5-*exo*-hydroxycamphor, although, in view of the amount of product, small amounts (10–20%) of other isomeric hydroxycamphors could have escaped notice.

Hydroxylation of adamantanone. Because of the symmetry of the adamantanone skeleton (C_{2v} symmetry), only five isomeric hydroxyadamantanones are possible, neglecting enantiomers. These are 1-, 4-*syn*-, 4-*anti*-, 5-, and 6-hydroxyadamantanone. The 4-*syn*- and 4-*anti*-hydroxyadamantanones may exhibit enantiomerism, but the present analytical methods would not distinguish enantiomers. Adamantanone was hydroxylated by $P\text{-}450_{cam}$ to a single product, hydroxyadamantanone G, at a rate of 52 mol/mol $P\text{-}450_{cam}$/min. $P\text{-}450_{LM2}$, on the other hand, produced two hydroxyadamantanones F and G, in a 3:2 ratio (Table III). The rate was 1.9 mol/mol $P\text{-}450_{LM2}$/min. Fortunately, authentic samples were available to us for three of the five possible hydroxyadamantanones. Gas chromatographic retention times (Table I) and mass spectra (Table II) indicated that F and G were 4-*anti*- and 5-hydroxyadamantanone, respectively. Deconvolution of the mass spectra in order to rule out the possibility that F and G were other isomers such as the 1- or 6-isomers was not feasible due to the complexity of fragmentation pathways inherent in the symmetric tricyclic skeleton. However, Jones oxidation of the mixture of F and G produced an adamantanedione H and left G unchanged. H was identified as adamantane-2,4-dione by gas chromatography (Table I). This proved that G is a tertiary alcohol while F is a 4-hydroxyadamantanone. Since the gas chromatographic behavior and mass spectra of 4-*syn*- and 4-*anti*-hydroxyadamantanone were similar (cf. Tables I and II), we cannot be absolutely sure of our assignment that F is 4-*anti*-hydroxyadamantanone as opposed to the *syn* stereoisomer. However, the assignment that G is the 5-hydroxy isomer is certain.

Hydroxylation of adamantane. The adamantane skeleton has an even higher symmetry than does adamantanone (T_d symmetry). As a consequence, only two isomeric adamantanols (1- and 2-adamantanol) are possible, neither of which may exhibit enantiomerism. As before, $P\text{-}450_{cam}$ produced a single product, I, identified as 1-adamantanol. The gas chromatographic technique used would have detected the

TABLE II

MASS SPECTROMETRIC DATA

Compound	Prominent Ions, *m/z* (% Abundance)
3-*exo*-Hydroxycamphor	168 (26%), 125 (36%), 95 (20%), 84 (75%), 83 (91%), 70 (52%), 41 (100%)
5-*exo*-Hydroxycamphor	168 (32%), 153 (13%), 125 (41%), 124 (25%), 123 (23%), 111 (100%), 109 (25%), 107 (27%)
5-*endo*-Hydroxycamphor	168 (23%), 153 (69%), 125 (29%), 124 (42%), 123 (41%), 111 (88%), 109 (75%), 108 (100%), 107 (98%), 93 (67%)
Hydroxycamphor A	168 (29%), 150 (14%), 135 (58%), 125 (46%), 95 (31%), 84 (93%), 83 (100%)
Hydroxycamphor B	168 (35%), 153 (4%), 125 (50%), 124 (27%), 123 (21%), 111 (100%), 109 (15%), 107 (27%)
Hydroxycamphor C	168 (13%), 153 (36%), 125 (8%), 124 (10%), 123 (10%), 111 (31%), 109 (37%), 108 (100%), 107 (54%), 93 (59%)
3-Ketocamphor	166 (12%), 138 (19%), 123 (19%), 110 (11%), 95 (100%), 83 (51%)
5-Ketocamphor	166 (87%), 151 (11%), 138 (11%), 123 (71%), 109 (81%), 107 (25%), 95 (41%), 69 (100%)
Ketocamphor D	166 (50%), 138 (32%), 123 (49%), 110 (26%), 95 (100%), 83 (76%)
Ketocamphor E	166 (99%), 151 (21%), 138 (20%), 123 (80%), 109 (96%), 95 (27%), 69 (100%)
4-*syn*-Hydroxyadamantanone	166 (20%), 148 (18%), 138 (31%), 120 (12%), 109 (10%), 96 (40%), 79 (100%)
4-*anti*-Hydroxyadamantanone	166 (24%), 148 (14%), 138 (46%), 120 (13%), 109 (18%), 96 (73%), 79 (100%)
5-Hydroxyadamantanone	166 (26%), 148 (10%), 108 (22%), 95 (100%), 79 (14%)
Hydroxyadamantanone F	166 (21%), 148 (14%), 138 (47%), 120 (15%), 109 (19%), 96 (70%), 79 (100%)
Hydroxyadamantanone G	166 (20%), 148 (9%), 108 (19%), 95 (100%)

Note. Electron-impact mass spectra were recorded on various authentic standards and on cytochrome *P*-450 reaction products using a Hewlett-Packard 5992 gas chromatograph–mass spectrometer. The ionization energy was 14 eV.

presence of less than 1% of 2-adamantanol because of the good separation (see Table I). However, none was detectable. The catalytic rate was 43 mol/mol $P\text{-}450_{cam}$/min. Also, as before, $P\text{-}450_{LM2}$ gave more than one product, specifically I and J, which were identified by gas chromatography (Table I) as 1- and 2-adamantanol. Jones oxidation of the mixture of I and J gave 2-adamantanone and unchanged I. The catalytic rate was 1.6 mol/mol $P\text{-}450_{LM2}$/min.

Substrate-binding phenomena. Dissociation constants for the various enzyme–substrate complexes were measured by monitoring the substrate-induced spectral change as the enzyme was titrated with substrate (14). The spectral change arises from a perturbation of the ligand field experienced by the heme iron, resulting in a change in the equilibrium constant between the high-spin and low-spin states (15). The maximal spectral change (ΔA_{max}) at saturating substrate concentration was extrapolated from iterative double-reciprocal plots of substrate concentration versus absorbance change. The maximum change in the mole fraction of high-spin heme was calculated by dividing ΔA_{max} by the differential extinction coefficient ($\Delta\epsilon$) and the total heme concentration. The total mole fraction of high-spin heme (X^{HS}_{max}) is given by the sum of this change and the initial high-spin mole fraction (X^{HS}_0). Thus, the new, substrate-perturbed equilibrium constant between high- and low-spin heme can be calculated from X^{HS}_{max}.

$$X^{HS}_{max} = [\Delta A_{max}/(\Delta\epsilon/[\text{heme}])] + X^{HS}_0$$

$$K_{eq} = \frac{[\text{high-spin}]}{[\text{low-spin}]} = \frac{X^{HS}_{max}}{1 - X^{HS}_{max}}$$

$P\text{-}450_{cam}$ bound its ketonic substrates tightly with dissociation constants in the low micromolar region, while the hydrocarbon adamantane bound less tightly. Camphor, the natural substrate, is well known to cause nearly a complete shift of

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