Uncoupling Oxygen Transfer and Electron Transfer in the Oxygenation of Camphor Analogues by Cytochrome P450-CAM

DIRECT OBSERVATION OF AN INTERMOLECULAR ISOTOPIC EFFECT FOR SUBSTRATE C-H ACTIVATION*

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The cytochromes P450 (P450) are a family of b-type heme monoxygenases that catalyze the activation of dioxygen for incorporation into a variety of organic substrates (1–7). Much of our present knowledge of the structure and mechanism of P450 enzymes comes from investigations of the bacterial P450-CAM isolated from Pseudomonas putida. P450-CAM catalyzes the highly stereoselective hydroxylation of its physiological substrate, (1R)-camphor, to form (1R)-5-exo-hydroxycamphor as the sole product (Reaction 1). In addition, the enzyme will hydroxylate related bi- and tricyclic molecules such as norcamphor (8–10), thiacamphor (11), adamantane (12), adamantanone (12), camphane (11), and 5,5-difluorocamphor (Reaction 2) (13) as well as epoxidize 5,6-dehydrocamphor (14) and 5-methylcamphor (Reaction 3) (15). Both enantiomers of camphor and 5-methylcamphor are oxygenated by the enzyme (15). Recently, it has been found to be capable of hydroxylating and epoxidizing a variety of molecules that bear no structural resemblance to camphor such as ethylbenzene (16), nicotine (17), 4-methyl-1-tetralone (18), styrene (19), 1,1,1-trichloroethane (20), thioanisole (21), and several others (22).

Four well characterized intermediates (1–4, Fig. 1) have been observed in the reaction cycle of P450 (1–7). The low-spin ferric resting state (1) becomes high-spin (2) upon substrate binding. Electron transfer from NADH via two-electron transfer proteins, putidaredoxin reductase (PdR) and putidaredoxin (Pd), yields the high-spin deoxyferrous state (3) that is capable of binding O2 to generate the oxyferrous state (4) or CO to produce the carbonmonoxy ferrrous inhibitor adduct (5). Addition of the second electron, the rate-limiting step in the cycle (23), is proposed to yield a ferric peroxide adduct (6a) which can be protonated on oxygen to give a hydroperoxide complex (6b). Protonation of the oxygen that already bears a proton and heterolytic cleavage of the peroxide O-O bond releases water and generates the proposed oxo-ferryl porphyrin radical intermediate (7). Intermediate 7 then transfers an oxygen atom to the hydrocarbon substrate, presumably by an oxygen rebound mechanism (24), to give the alcohol product and the P450 state 1. With (1R)-camphor, the cycle just described proceeds with nearly complete coupling (9, 15) of electron transfer (2 → 3 and 4 → 6) to oxygen transfer (7 → 1). Two modes of uncoupling electron transfer from oxygen transfer after addition of the second electron have been described (8, 16, 25). Protonation of 6b on the unprotonated oxygen would lead to release of hydrogen peroxide generating 2 and no oxygenated product (Path A, 6b → 2, Fig. 1, two-electron uncoupling). Alternatively, reduction of 7 by two electrons and concurrent protonation would give a second molecule of water (the “oxidase” reaction), regenerate 2 and produce no oxygenated product (Path B, 7 → 2, Fig. 1, four-electron oxidase uncoupling).

In an effort to probe the mechanism of dioxygen activation by P450-CAM through blockade of the normal site of reaction, we have synthesized camphor analogues with two fluorine atoms or an exocyclic olefin functionality at C-5. We have previously reported that 5,5-difluorocamphor is hydroxylated at C-9 to give the hydroxymethyl product (13) and that 5-methylcamphor is epoxidized to give the exo-epoxide product (15). Herein we describe in detail the substrate binding constants along with the electron, dioxygen, and substrate stoichiometries for the reactions of both (1R)- and (1S)-enantiomers of the difluoro and methylenyl substrates as well as for (1R)-9,9,9-d3-
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MATERIALS AND METHODS

Protein Purification—P450-CAM, Pd, and PdR were isolated from P. putida grown on (1R)-camphor. P450-CAM was purified by the method of Peterson and co-workers (26) with minor modifications (27). Pd and PdR were purified by published procedures (28).

Synthesis of Camphor Analogues—(1R)- and (1S)-camphor were purchased from Aldrich and used without further purification. The synthesis of (1S)-5,5-difluorocamphor (13), (1S)-5,5-difluorocamphor (13), (1S)-5,5-difluorocamphor (13), (1S)-5,5-difluorocamphor (13), (1S)-5,5-difluorocamphor (13), and (1R)-9,9-d13,5,5-difluorocamphor (29) have been described elsewhere.

Dissociation Constant Determination—Dissociation constants \( K_d \) were determined at 4°C as described previously (13) by titration of substrate-free P450-CAM (1.5 \( \mu \)M) with an aqueous 3–5 mM solution of substrate in 20 mM phosphate buffer containing KCl (100 mM). Binding was followed by monitoring the decrease in absorbance at 417 nm.

Enzyme Activity—Catalytic activities were determined from the rate of NADH oxidation. This was monitored as a loss of absorbance at 340 nm (\( e = 6.22 \text{mM}^{-1} \text{cm}^{-1} \)) as a function of time. Typical assay mixtures contained P450-CAM (0.05 \( \mu \)M), PdR (4.0 \( \mu \)M), Pd (10.0 \( \mu \)M), substrate (1.0 mM), NADH (0.5 mM), and KCl (100 mM) in 20 mM phosphate buffer at 25°C. Protein concentrations were chosen to optimize turnover rate.

Product Formation and Uncoupling—Reaction mixtures contained P450-CAM (0.5 \( \mu \)M), PdR (4.0 \( \mu \)M), Pd (10 \( \mu \)M), substrate (1.0 mM), and NADH (1.0 mM) with oxygen as the limiting reagent. NADH consumption was monitored as described above. Reactions were allowed to proceed for 15 min at which time a known amount of internal standard, 3-endo-bromocamphor, was added and the products extracted twice with \( CH_2Cl_2 \). The organic extract was concentrated under a slow stream of \( N_2 \) and analyzed by gas chromatography and gas chromatography/mass spectrometry. Uncoupling \( (H_2O_2 \text{ and/or } H_2O \text{ formed}) \) was calculated based on the difference between NADH consumed and product formed.

Oxygen Consumption—Oxygen consumption was continuously measured using a Clark-type electrode in conjunction with a Gilson 5/6 oxygraph. Concentrations for P450-CAM, Pd, PdR, NADH, and substrate are as specified under product formation and uncoupling. A full-scale deflection of approximately 280 \( \mu \text{mV} \text{ml}^{-1} \) was determined by the method of Robinson and Cooper (30). NADH oxidation was simultaneously monitored as above.

Determination of Hydrogen Peroxide Formation—Reaction concentrations were identical for all components to those described under the product formation and uncoupling section in a total volume of 3.0 ml. \( H_2O_2 \) production was monitored at time intervals by removing portions of the reaction mixture, quenching with 3% aqueous trichloroacetic acid, and performing a colorimetric determination at 480 nm as described by Atkin and Sligar (10). Values of \( H_2O_2 \) produced were derived from comparison to a standard curve prepared under identical conditions and are for 15-min reaction periods.

Instrumentation—Varian-Cary 210 or 219 spectrophotometers were used for UV-visible absorption spectral analysis. A Hewlett-Packard 5890A gas chromatograph with a stabilwax column (30 meter, 0.25-mm inner diameter) programmed at 70°C followed by a temperature increase to 225°C at 10°C/min and equipped with a Hewlett-Packard 3390A reporting integrator was used for product analysis. Electron impact mass spectra and gas chromatography/mass spectral data were obtained on a Finnegan Model 4521 spectrometer. High resolution mass spectrometry was performed on a VG Instruments 70SQ spectrometer.

RESULTS AND DISCUSSION

Binding Characteristics of Camphor Analogues—The major structural feature which distinguishes camphor-free cytochrome P450-CAM from the camphor-bound enzyme is the presence of six water molecules in the substrate binding pocket in the absence of camphor (31). Substrate binding expels these water molecules (31, 32) and converts the ferric heme iron from low-spin six-coordinate water-ligated (1, Fig. 1) to high-spin...
five-coordinate \( (2) \) (31, 32). The low- to high-spin conversion results in a shift of the Soret peak in the UV-visible absorption spectrum from 417 to 391 nm and the appearance of a peak at 646 nm. The absorption spectra of ferric P450-CAM with \( (1 \)S\)\)-substrate and the dimethin intensity at 646 nm. Incomplete conversion of the \( (1 \)S\)-substrate indicates for the \( (1 \)S\)-substrate and the dimethin intensity at 646 nm. Incomplete conversion of the substrate-bound enzyme by the shoulder seen near 417 nm and the diminished intensity at 646 nm. Incomplete conversion of the heme iron from low- to high-spin upon substrate binding, as seen with norcamphor and 5-bromocamphor, can be attributed to the inability of these substrates to completely displace the distal water ligand (33, 34). Identical spectra to those displayed in Fig. 2 for the \( (1 \)R\)- and \( (1 \)S\)-substrate-bound enzyme were observed with all the other \( (1 \)R\)- and \( (1 \)S\)-substrates, respectively (data not shown). These results indicate that the heme remains partially hexa-coordinate in the presence of the \( (1 \)S\)-substrates, but becomes penta-coordinate upon addition of the \( (1 \)R\)-substrates. Differences are also seen in the binding constants for the \( (1 \)R\)- and \( (1 \)S\)-substrates (Table I). The \( K_d \) values for the \( (1 \)R\)-substrates including \( (1 \)R\)-camphor, \( (1 \)S\)-5,5-difluorocamphor, and \( (1 \)R\)-5-methyl-5,5-difluorocamphor by the fully reconstituted NADH/PdR/Pd/P450-CAM system has been analyzed by gas chromatography, mass spectrometry, and NMR spectroscopy. \( (1 \)R\)-Camphor forms exclusively the 5-epoxy-alcohol (Reaction 1), \( (1 \)R\)-5,5-difluorocamphor yields primarily the 9-alcohol (Reaction 2), and \( (1 \)R\)-5-methyl-5,5-difluorocamphor produces the epo-exepoxide (Reaction 3). Table II contains a summary of the oxygenated products formed and their percent distribution. The absolute amount of hydroxylated camphor product formed with each substrate is dependent on the degree of coupling (Table I) and will be discussed below; with some substrates, the uncoupling products (hydrogen peroxide and, especially, water) are the major reaction products. \( (1 \)S\)-Camphor and \( (1 \)S\)-5-methyl-5,5-difluorocamphor produce predominantly the same organic product as their \( (1 \)R\)-enantiomers. With \( (1 \)S\)-5,5-difluorocamphor, however, the primary hydroxylated product is not the same as is formed with the \( (1 \)R\)-enantiomer. The new product is assigned to be 3-hydroxy-5,5-difluorocamphor based on the prominent \( M^+ \) at 28 ion in the mass spectrum that is typical of 3-hydroxyborborenanones (11). However, because of the small amount of this.

**Fig. 2.** Electronic absorption spectrum of \( (1 \)R\)-camphor bound (solid) and \( (1 \)S\)-camphor bound (dashed) cytochrome P450-CAM. Protein concentrations were 70 and 40 \( \mu \)M, respectively. Spectra were recorded at 4°C in 20 mM phosphate buffer, pH 7.4, containing 100 mM KCl and under conditions of saturating substrate concentration (1 mw).

**TABLE I**

| Substrate | Camphor | 5-Methylenycamphor |
|-----------|---------|---------------------|
|           | (1R)    | (1S)                |
| **Dissociation constant** | \( 1.6 \pm 0.3 \) | \( 2.6 \pm 0.3 \) |
| **NADH oxidation rate** | \( 2411 \pm 73 \) | \( 1400 \pm 69 \) |
| **Reaction stoichiometries** |                         |                     |
| NADH oxidized | \( 281 \pm 7 \) | \( 261 \pm 7 \) |
| \( \frac{O_2}{O_2} \) consumed | \( 275 \pm 3 \) | \( ND^a \) |
| \( \frac{NADH}{O_2} \) ratio | \( 1.02 \) | \( 1.07 \) |
| Products formed | \( 271 \pm 4 \) | \( 223 \pm 8 \) |
| \( \frac{H_2O_2}{O_2} \) formed | \( 8 \pm 2 \) | \( ND^a \) |
| Uncoupling, % | \( 4 \pm 3 \) | \( 11 \pm 4 \) |
| \( O_2 \) turnover number | \( 2364 \pm 72 \) | \( ND^a \) |

**TABLE II**

Regiospecificity for the oxygenation products of camphor and camphor analogues

| Substrate | Product(s) | Distribution % |
|-----------|------------|----------------|
| Camphor   | 5-epoxy-hydroxy | 100 |
| \( (1R) \) | 5-epoxy-hydroxy | 97.8 \( \pm 0.4 \) |
| \( (1S) \) | 2,5-diketone | 2.2 \( \pm 0.4 \) |
| 5-Methylenecamphor | 5-epoxy-epoxide | \( \sim 90 \) |
| \( (1R) \) | unidentified products | \( \sim 10 \) |
| \( (1S) \) | 5-epoxy-epoxide | \( \sim 96 \) |
| | unidentified products | \( \sim 4 \) |
| 5,5-Difluorocamphor | 9-hydroxy | 92.3 \( \pm 0.5 \) |
| \( (1R) \) | 6-hydroxy | 7.7 \( \pm 0.5 \) |
| \( (1S) \) | 9-hydroxy | 31.6 \( \pm 4.8 \) |
| | 3-hydroxy | 54.0 \( \pm 4.9 \) |
| | 6-hydroxy | 14.4 \( \pm 0.3 \) |
| 5,5-Difluoro-9,9-d(1)-camphor | 9-hydroxy | 50.5 \( \pm 3.6 \) |
| \( (1R) \) | 6-hydroxy | 49.5 \( \pm 3.6 \) |

\( a \) See text for discussion of product analysis.
product obtained, its proposed structure has not been confirmed by NMR analysis. All three fluorinated substrates produce an additional product that we propose to be 6-hydroxy-5,5-difluorocamphor based largely on the lack of (M⁺ - 28) and (M⁺ - 31) peaks characteristic of 3-hydroxy substituted camphors (11) and hydroxymethyl-substituted camphors (13), respectively. Once again, insufficient quantities were obtained for NMR analysis. The proposed 6-alcohol product is only a minor component with both enantiomers of 5,5-difluorocamphor, but it is formed in an equal amount to the 9-alcohol in the case of (1R)-9,9,9-d₃-5,5-difluorocamphor. This shift in the ratio of oxygenation products from 92:8 (9-alcohol:6-alcohol) in the d₀ case to 50:50 (as well as increased water production) in the d₃ case presumably results from metabolic switching due to the deuterium isotope effect. Similar observations have been made by Atkins and Sligar (8) with deuterated norcamphor.

In analyzing the crystal structures of both camphor-bound and camphor-free P450-CAM, Poulos and co-workers (31–33) have suggested that substrate specificity is achieved by specific interactions between the substrate, (1R)-camphor, and appropriately positioned amino acids in the substrate binding pocket. The most important interactions are a hydrogen bond between Tyr96 and the carbonyl oxygen on C-2 of camphor and van der Waals contacts between Val29₀ and the 8,9-geminal dimethyl groups of camphor and between Leu24₄ and the 10-methyl group of camphor. Like (1R)-camphor, (1S)-camphor reacts exclusively at the 5-exo-position. This suggests that the binding position of (1S)-camphor may still be oriented by the hydrogen bond between its carbonyl group and Tyr96. To maintain this hydrogen bond and also position C-5 in the same relative position as in (1R)-camphor requires that the protons on C-3 in (1S)-camphor occupy the position where the 10-methyl group was located in (1R)-camphor (Scheme 1). This modifies the protein-substrate van der Waals interactions; the incomplete spin shift upon binding (1S)-camphor indicates that the binding interaction is not optimal. Nonetheless, hydroxylation of (1S)-camphor still occurs at the exo position of C-5. The same arguments regarding regio- and stereoselectivity would seem to apply to the epoxidation of (1R)- and (1S)-5-methyl-6-enylcamphor. Interestingly, a recent study of the hydroxylation of (1R)- and (1S)-norcamphor showed that while C-5 is the primary site of hydroxylation for the (1R)-enantiomer, C-6 was preferred for (1S)-norcamphor (9).

For 5,5-difluorocamphor, blocking C-5 with fluorines shifts reaction off at carbon completely. The major product in the 1R case is the 9-alcohol. This is not surprising since C-9 is the second closest carbon to the heme iron after C-5 (32, 36). For the (1S)-enantiomer, the non-optimal binding and slow rate of oxygen transfer (Table III) lead to multiple reaction sites including the only example of C-3 hydroxylation observed herein (Table II).

NADH Oxidation Rates—As both electrons in the P450 cycle (Fig. 1) are ultimately derived from NADH, the rate of NADH oxidation reflects the rate of enzyme turnover when electron transfer is tightly coupled to oxygen transfer as with (1R)-camphor. However, as discussed above, uncoupling can occur at internal branch points in the reaction cycle; two-electron un-/coupling (Path A, Fig. 1) involves release of hydrogen peroxide while four-electron uncoupling (Path B, Fig. 1) leads to production of a second molecule of water (8, 16, 25). Therefore, the rate of NADH oxidation seen with such substrates not only reflects the diminished rate of oxygenated product formation but also the amount of water and hydrogen peroxide generated through uncoupling. The kinetic and stoichiometric data for the reaction of (1R)- and (1S)-camphor and for the five camphor analogues with the fully reconstituted NADH/PdR/Pd/P450-CAM system are summarized in Table I. The NADH oxidation rate for (1S)-camphor is approximately half the rate of the (1R)-enantiomer. However, in the cases of the methylenyl and difluoro substrates where the five position has either been modified or blocked, the rate of (1S)-enantiomers are about twice those of the (1R)-enantiomers. Looked at in a different way, fluorination of the substrate causes the NADH oxidation rate to decrease much more significantly within the 1R series (camphor, 5,5-difluorocamphor, and 5-methylcamphor) than within the 1S series.

With (1R)-9,9,9-d₃-5,5-difluorocamphor, the rate of NADH oxidation (557 min⁻¹) is actually higher than the rate for the non-deuterated substrate (420 min⁻¹) (Table I). Similar apparent inverse isotope effects have been reported for norcamphor by Sligar and co-workers (8). Once again, however, it is important to remember that with uncoupled substrates, the NADH oxidation rate reflects not only diminished oxygenated product formation but also two- and four-electron uncoupling. From the uncoupling and stoichiometry data in Table I, it is possible to calculate the rates of formation of oxygenated products (Table III). This analysis shows that the 9,9,9-d₃-substrate produces oxygenated products at a much slower rate than undeuterated 5,5-difluorocamphor. The apparent inverse isotope effect for NADH oxidation rates for the 9,9,9-d₃-substrate clearly results from the significantly increased amount of four-electron uncoupling observed with that substrate. The data in Table III also reveal that the rate of oxygenated product formation can be decreased by as much as 210-fold upon modification of the substrate.

To factor out the effects of four-electron uncoupling, it is convenient to define an O₂ turnover number by dividing the NADH oxidation rate by the NADH/O₂ consumption ratio (Table I). This provides a turnover number based on O₂ consumption. With one O₂ consumed per cycle of the enzyme, this value represents the number of cycles the enzyme carries out per minute. From these data, it is seen that d₀- and d₃-(1R)-difluorocamphor each turn over about 10-fold more slowly than (1R)-camphor.

Intermolecular Deuterium Isotope Effects—The rates in Table III are for the sum of the various products produced from a given substrate. These rates are determined under optimized turnover conditions which require a very large excess of Pd and PdR. Comparison of the rates of oxygenated product formation for 5,5-difluorocamphor versus the 9,9,9-d₃ molecule reveals an isotope effect of 6.4 (Table IV, part A). However, as mentioned above, the presence of the deuteriums on C-9 leads to a higher percentage of oxygenation on C-6. Recalculation of the isotope effect for d₀- and d₃-5,5-difluorocamphor based solely on hydroxylation at C-9 (Table IV, part A) leads to an isotope effect of 11.8. The isotope effect can be determined in a second way by using the quantities of products formed (Table I) upon oxygenation of d₀- and d₃-5,5-difluorocamphor under conditions where oxygen is the limiting reagent (and without as large an excess of Pd and PdR). As above, the amounts of products formed specifically at C-6 and C-9 can be calculated using the product distribution data in Table II. After that adjustment, the ratio of the amounts of oxygenated products formed at C-9 for the d₀
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TABLE III
Rates of formation of oxygenated products for substrates with P450-CAM

| Substrate                        | Oxygenated product(s) formed nmol/min/nmol P450 | Fold decrease relative to (1R)-camphor |
|---------------------------------|-----------------------------------------------|---------------------------------------|
| (1R)-Camphor                    | 2315                                          | 1.9                                   |
| (1S)-Camphor                    | 1246                                          | 15                                    |
| (1R)-5-Methylidencycamphor      | 156                                           | 5.7                                   |
| (1S)-5-Methylidencycamphor      | 405                                           |                                       |
| (1R)-5,5-Difluorocamphor        | 71.4                                          | 32                                    |
| (1S)-5,5-Difluorocamphor        | 48.9                                          | 47                                    |
| (1R)-9,9,9-d3,5,5-Difluorocamphor| 11.1                                          | 210                                   |

aOptimized rate of NADH oxidation (Table I) multiplied by the fractional coupling [percent coupling (100% – percent uncoupling; Table I) expressed as a fraction].

TABLE IV
Intermolecular isotope effect for reaction at C-9 in (1R)-5,5-difluorocamphor

| d3 substrate | 9,9,9-d3-substrate | Isotope effect kH/kD |
|--------------|--------------------|---------------------|
| A. Calculated from rate data (Table III) | | |
| Overall product formation rate (nmol/min/nmol P450) | 71.4a | 11.1a | 6.4 |
| Rate at C-9 (nmol/min/nmol P450) | 65.9b | 5.6b | 11.8 |
| Rate at C-6 (nmol/min/nmol P450) | 5.5d | 5.5d | |
| B. Calculated from stoichiometry data (Table I) | | |
| Overall amount formed (nmol) | 71c | 11c | 6.5 |
| Amount at C-9 (nmol) | 65.5d | 5.6d | 11.7 |
| Amount at C-6 (nmol) | 5.5d | 5.4d | |

aRates of oxygenated product formation, taken from Table III and determined using optimized turnover conditions.
bDerived by multiplying the overall product formation rate by the proportion of product observed at C-6 or C-9 (Table II).
cAmounts of oxygenated products formed, taken from Table I and determined under oxygen limiting conditions.
dDerived by multiplying the overall amount of product formed by the proportion of product observed at C-6 or C-9 (Table II).

and d3 substrate is 11.7 (Table IV, part B). In both experiments, the reactivity at C-6 is unaffected by the presence or absence of deuterium at C-9 (Table IV, parts A and B).

With 5,5-difluorocamphor, the significant slowdown in the rate of formation of oxygenated products (Table III) suggests that the rate-limiting step in the P450 cycle may have changed from electron transfer to oxygen transfer (which in turn is initiated by hydrogen atom abstraction). This conclusion is strengthened by the intermolecular isotope effect of 11.7 for hydroxylation of C-9 in 5,5-difluorocamphor (Table IV). With 5-exo- and 5-endodeuterated camphors, Gelb et al. (37) reported an intermolecular isotope effect of only 1.1–1.2 and concluded that oxygen transfer is sufficiently fast that the intrinsic isotope effect, kH/kD, for hydrogen atom removal is masked. The intermolecular isotope effect of 11.7 for oxygenation of C-9 in 5,5-difluorocamphor matches the intramolecular values of 11 or greater reported by Hjelmeland et al. (38) and Groves et al. (39) for aliphatic hydroxylation by liver microsomal P450. The fact that the intermolecular isotope effect is no longer masked suggests that the rate of hydrogen atom abstraction, which initiates oxygen atom transfer, has been slowed down sufficiently at C-9 in 5,5-difluorocamphor to replace the second electron transfer as the rate-limiting step for oxygenation at that site.

Reaction Stoichiometries and Uncoupling Reactions—Important additional information about the nature of the uncoupling of electron transfer and oxygen transfer is revealed by examination of the reactions between the substrates and the NADH/PdPd/P450-CAM system under conditions where the limiting reagent is dioxygen. (1R)-Camphor clearly exhibits a 1:1:1 ratio of NADH oxidized to O2 consumed to product formed (Table I). This is no longer true for (1R)-5-methylidencycamphor. Although the ratio of NADH oxidized to O2 consumed essentially remains 1:1 (1.07, Table I), the relative amount of oxygenated product formed is now less than 1. The system is uncoupled with increased levels of hydrogen peroxide making up for the decrease in oxygenated product formed. Interestingly, (1S)-5-methylidencycamphor has a rate of NADH consumption that is over 5-fold slower than (1R)-camphor, but it is only 9% uncoupled.

With the three difluoro substrates, the average amount of NADH oxidized is about 440 nmol/ml while the consumption of dioxygen remains about 260 nmol/ml. The amount of oxygenated product formed was also considerably lower, the system now being 83–98% uncoupled. The NADH/O2 consumption ratio (Table I) approaches 2.0 with increasing uncoupling. Relatively little hydrogen peroxide is formed. These observations combined with the apparent inverse deuterium isotope effect discussed earlier are consistent with four-electron oxidase uncoupling for the difluorocamphor substrates.

The exact causes for the uncoupling of the P450 reacation cycle are not known. Extensive studies by Raag and Poulos (33, 40) have failed to attribute it to any one factor. Uncoupling may result from less complementary enzyme-substrate fit or from the lack of the hydrogen bond between the substrate carbonyl and Tyr(96) since the hydroxylation of norcamphor and camphane, respectively, by P450-CAM are substantially uncoupled (11, 41). Uncoupling is not related to the fraction of low-spin ferric heme, since thiocamphor-bound P450-CAM and the camphor-bound Tyr(96)Phe P450-CAM mutant are only 65 and 59% high-spin, respectively, yet have a very high hydroxylation efficiency (100 and 98%, respectively) (11). An important factor that promotes uncoupling seems to be the presence of extra solvent water near the O-O bond undergoing cleavage. To avoid the two modes of uncoupling requires the precise delivery of two protons exclusively to the outer oxygen of the iron-bound peroxide to generate the oxo-ferryl state (6a → 6b → 7) and the prevention of electron/proton delivery to the oxo-ferryl state (to avoid 7 → 2). Gerber and Slijer (42) have proposed a distal charge relay system to accomplish the proper delivery of protons to the iron-bound peroxide oxygen. The “extra” water in
the active site during catalysis apparently provides an alternate source of protons to iron-bound peroxide, 6a and 6b, and the oxo-ferryl intermediate, 7, promoting water and/or hydrogen peroxide production. Removal of methyl groups from the substrate (as in norcamphor) or in mutant P450-CAM enzymes where active site valines are replaced with alanines (41) leads to uncoupling. Structures like ethylbenzene and 1,1,1-trichloroethane (16, 20), which certainly do not tightly fill the substrate binding pocket, also promote uncoupling. The results reported herein with 5-methylene camphor suggest that addition of a carbon atom to the camphor (the exocyclic olefin substituent at C-5) also leads to uncoupling.

Uncoupling also occurs when the potential substrate hydroxylation sites are blocked as has been observed in the present study with 5,5-difluorocamphor (Table I). Ullrich and coworkers (43) examined the reaction of perfluoro-ethane (16, 20), which certainly do not tightly fill the substrate binding pocket, also promote uncoupling. The results herein indicate that the mode of uncoupling can be varied using different 5-substituted camphor analogues. They presumably still go through the oxo-ferryl intermediate, 7 in Fig. 1, which is reduced to give a second molecule of water. The slowdown in the rate of oxygenated product formation (Table III) and the intermolecular isotope effect of greater than 11 for reaction at C-9 of 5,5-difluorocamphor (Table IV) suggests that the rate-limiting step in the P450 cycle may have changed from electron transfer to oxygen transfer with 5,5-difluorocamphor.

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