Substitution of eq A-5 into eq A-6 yields the quadratic

$$[ESG] = \{(K_d^{app} + [E_0] + [S_0]) - \sqrt{(K_d^{app} + [E_0] + [S_0])^2 - 4[E_0][S_0]}\}/2 \quad (A-7)$$

Of course, if $[S_0] \gg [E_0]$, then the solution reduces to a hyperbola:

$$[ESG] = \frac{[E_0][S_0]}{K_d^{app} + [S_0]} \quad (A-8)$$

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Deuterium Isotope Effects in Norcamphor Metabolism by Cytochrome P-450$_{cam}$: Kinetic Evidence for the Two-Electron Reduction of a High-Valent Iron–Oxo Intermediate

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ABSTRACT: The kinetics of NADH consumption, oxygen uptake, and hydrogen peroxide production have been studied for norcamphor metabolism by cytochrome P-450$_{cam}$. The kinetic deuterium isotope effects on these processes, with specifically deuteriated norcamphor, are 0.77, 1.22, and 1.16, respectively. Steady-state UV–visible spectroscopy indicates that transfer of the second electron to the dioxy ferrous P-450 is the rate-limiting step, as it is when camphor is the substrate. The inverse deuterium isotope effect for NADH consumption is consistent with an isotope-dependent branching between monooxygenase and oxidase activity, where these reactivities differ in their NADH:oxygen stoichiometries. However, no isotope-dependent redistribution of steady-state intermediates was detected by isotopic difference UV–visible spectroscopy in the presence of norcamphor. The kinetic isotope effects and steady-state spectral results suggest that the high-valent iron–oxo hydroxylating intermediate $[FeO]^{3+}$ is reduced by NADH and the physiological electron-transfer proteins to afford water.

The four-electron reduction of molecular oxygen to form 2 equiv of water is traditionally ascribed to non-heme, copper-containing oxidases (Malmstrom et al., 1975), copper-containing monooxygenases (Hamilton et al., 1972), and cytochrome oxidase, a structurally complex two-heme and two-copper enzyme (Caughey et al., 1976; Poynton & Schatz, 1975). It also has been suggested that this type of oxidase activity occurs with various cytochrome P-450's (Staudt et al., 1974; Zhukovr & Archakov, 1982), and recently it was demonstrated that several purified hepatic P-450's catalyze this reaction without reduction of intermediate hydrogen peroxide, although peroxide formation was detected in all the isozymes studied (Gorsky et al., 1984). Recently, we reported that, in the presence of the substrate analogue norcamphor, the soluble cytochrome P-450$_{cam}$, isolated from *Pseudomonas putida*, exhibits an efficient four-electron oxidase activity in addition to the expected monooxygenation of substrates (Atkins & Sligar, 1987). Hydrogen peroxide formation was minimal compared to water production in this complete reconstituted system containing P-450$_{cam}$, putidaredoxin, putidaredoxin reductase, and NADH. Furthermore, a deuterium isotope effect on the ratio of monooxygenase/oxidase activity was observed, which led to the proposal of a common intermediate for both activities. Since chemical steps of the P-450 reaction cycle subsequent to formation of the dioxy ferrous intermediate ($P\text{-}450^{rs}_{O_2}$)[1] demonstrate a strong commitment to catalysis and are expected to be irreversible (Harada et al., 1984), and since the only isotopically sensitive step would be hydrogen (deu-

[1] Abbreviations: $P\text{-}450^{rs}_{O_2}$, dioxy ferrous form of P-450; Pd, putidaredoxin; Fp, putidaredoxin reductase; $(V_{max})_{O_2}$, steady-state velocity of oxygen consumption with saturating levels of the appropriate substrate; $(V_{max})_{H_2O_2}$, steady-state velocity of hydrogen peroxide production with saturating levels of these substrates; $(V_{max})_{NADH}$, corresponding steady-state velocity of NADH oxidation; I, *exo,exo*-norcamphor-*5,6-d*$_2$; II, *exo,exo*-norcamphor-*3,3,5,6-d*$_4$; Tris, tris(hydroxymethyl)aminomethane; GC, gas chromatography.

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terium) abstraction by the compound I type iron–oxo species, we proposed that the oxidase activity of cytochrome P-450$_{cam}$ resulted from the two-electron reduction of this intermediate by NADH and the associated electron-transfer proteins. Cytochrome P-450$_{cam}$ possesses no active site acid/base groups nor additional metal centers (Poulos et al., 1985, 1986) and therefore represents an interesting contrast to the more "traditional" multinuclear metal-centered oxidases with respect to the mechanism of four-electron reduction of dioxygen.

In this communication we have examined the stoichiometry of norcamphor and quantitatively determined the kinetics of oxygen and NADH comsumption and of hydrogen peroxide formation. While the kinetic deuterium isotope effects on atmospheric dioxygen consumption and hydrogen production are small and greater than one, the effect of substrate isotopic substitution on the rate of NADH consumption is significantly larger and inverse. This apparent paradox is consistent with a kinetic model in which the hydroxylating intermediate, presumably $[FeO]^{3+}$, undergoes two-electron reduction to form oxidase-derived water.

Materials and Methods

Enzymes and Enzyme Reactions. Cytochrome P-450, putidaredoxin (Pd), and putidaredoxin reductase (Fp) were purified by previously established procedures (Gunsalus & Wagner, 1978). Substrate-free P-450 was prepared immediately before use by passage over a Sephadex G-25 column equilabrated with 50 mM Tris, pH 7.4. The 2-mercaptoethanol was removed from the stock putadaredoxin solution by passage over a Bio-Gel P-4 column in the same buffer immediately prior to use and was essential for reliable peroxide assays. NADH consumption was determined by measuring the change in absorbance at 340 nm with an extinction coefficient of 6.22 cm^{-1} mM^{-1}. The reaction, in a total volume of 1 mL, contained 0.89 μM P-450$_{cam}$, 4.2 μM putidaredoxin, 1.81 μM putidaredoxin reductase, 50 mM KCl, 5 mM substrate, and 225 μM NADH in 50 mM Tris, pH 7.4. O_2 consumption was monitored by a Clark electrode using a full-scale deflection as a change in O_2 concentration of 223 μM, at 23 °C. These reactions, in a volume of 1.48 mL, were identical with the "NADH reactions " with respect to concentration of substrate and KCl, but the enzyme concentrations were halved and the NADH concentration was increased to 1.5 mM. These changes resulted in a longer period of "linear" kinetics and reduced the observed rate of O_2 consumption to allow for more easily measurable rates. A series of "fast" reactions were also performed, in which the enzyme concentrations were increased to those described for the NADH reactions, in order to observe the entire range of O_2 consumption. For determination of H_2O_2, a single reaction of 6 mL was performed with 3.5 μM P-450, 4.0 μM Pd, 3.0 μM Fp, 50 mM KCl, 5 mM substrate, and 0.9 mM NADH. At various times, 1-mL aliquots were removed and added to 2 mL of ice-cold 3% trichloroacetic acid. After 15 min the samples were centrifuged to remove precipitated protein, and 1 mL of the supernatant was added to a cuvette containing 1 mL of 10 mM ferrous ammonium sulfate and 0.2 mL of 0.5 M potassium thiocyanate. After 2 min, the A_{540} was measured and the concentration of H_2O_2 was estimated from a standard curve prepared in an identical manner with known amounts of H_2O_2 present. Stock H_2O_2 concentrations were determined as described elsewhere (Cotton & Dunford, 1973). Optical measurements were obtained on an HP 8450 UV–vis spectrophotometer.

Deuteriated Norcamphors. The *exo,exo*-norcamphor-*5,6-d_2* (I) was prepared by previously established methods (Korvola & Malkonen, 1974). Briefly, this was accomplished by catalytic deuteriation of 5-norbornen-2-ol (Aldrich) in 95% ethanol at 2 atm, with 5% Pd on charcoal as catalyst. The resulting norborneol-d_2 was subjected to modified Jones oxidation (Muller & Blans, 1979). Identity of the product was confirmed by GC and mass spectral comparison to commercially available norcamphor (Aldrich). Mass spectral analysis indicated 97.7% d_2, 1.5% d_1, and 0.8% d_0. GC indicated greater than 98% purity.

The *exo,exo*-norcamphor-*3,3,5,6-d_4* (II) was also prepared from I by established procedures (Tidwell, 1970). The *exo,exo*-norcamphor-*5,6-d_2* was heated to 45 °C in dioxane/D_2O 60:40, where the D_2O was 40% NaOD. After 12 h the mixture was extracted with ether and purified by silica gel column chromatography (ether/petroleum ether 1:2). Mass spectral analysis indicated 34.0% d_4, 51.1% d_3, and 14.8% d_2. Since exchange at the 3-exo position is at least 100-fold faster than that at the endo position (Tidwell, 1970), the d_3 component of this mixture is >95% 3-exo-d. This compound was greater than 97% pure, with respect to norcamphor, on the basis of GC.

Mass spectral data were obtained on a VG 7070E mass spectrometer in line with an HP 5700 gas chromatograph equipped with a 30-m DB-5 capillary column. The spectrometer was interfaced with a HP 11250 multispec data system. Deuterium content was determined in the ion-selective monitoring mode, where ions m/z 113, 112, 111, 110, and 109 and m/z 115, 114, 113, 112, and 111 were monitored for I and II, respectively. Optical measurements were performed on an HP 8450 UV–vis spectrophotometer. Steady-state difference spectra were obtained with 4.5 μM P-450, 4.0 μM Pd, 2.0 μM Fp, 100 mM KCl, and 1.0 mM substrate. A single reaction mixture was divided equally between two cuvettes, and an optical balance was obtained. Reaction was initiated by addition of NADH, final concentration 800 μM, to the sample cuvette, followed by repetitive scanning of the 390–600-nm range, at 5-s intervals with integration times of 3 s. Isotopic difference spectra were obtained in an identical manner, except the sample was divided between cuvettes before addition of substrate. Norcamphor or deuteriated norcamphor (II) was then added to reference and sample cuvettes, respectively, and NADH was added simultaneously to both vessels. The samples were rapidly mixed and repetitively scanned as before.

For experiments including exogeneously added H_2O_2 or superoxide, stock solutions of 0.1 mM H_2O_2 or KO_2 were prepared immediately prior to use in Tris buffer or methanol, respectively. The appropriate amount was then added immediately prior to initiation of the reaction with NADH. Due to the extremely short half-life of superoxide in aqueous environments (Ingraham & Meyer, 1985), the actual concentration is probably less than the initial concentration reported in Table II.

Results

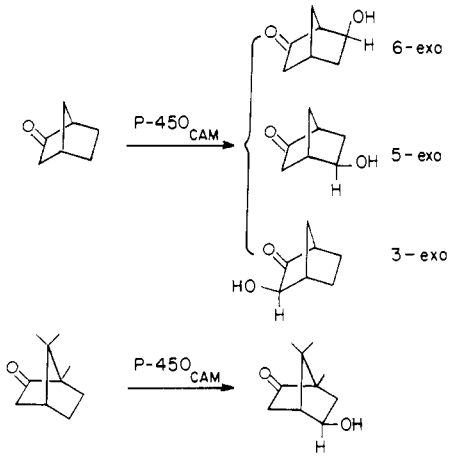
Background. We have previously demonstrated that norcamphor affords three hydroxylated products when metabolized by the reconstituted P-450$_{cam}$ system (Atkins & Sligar, 1987) and that the relative amount of each product is altered upon deuterium substitution at various positions on the norcamphor skeleton. The products are 3-*exo*-, 5-*exo*-, and 6-*exo*-hydroxynorcamphor (Scheme I). The 5- and 6-alcohols are the predominant metabolites and are formed in nearly equal amounts, with the 3-alcohol being a minor product. Deuteriation at the 5- and 6-positions of norcamphor results in the 3-alcohol being preferentially produced. It was also

Table I: Rates and Kinetic Deuterium Isotope Effects for Norcamphor Metabolism by P-450$_{cam}$[a]

substrate	nmol/(min·nmol of P-450)					
	$(V_{max})_{O_2}$	$(^HV/^DV)_{O_2}$	$(V_{max})_{H_2O_2}$	$(^HV/^DV)_{H_2O_2}$	$(V_{max})_{NADH}$	$(^HV/^DV)_{NADH}$
norcamphor	16.8 ± 0.5		5.9 ± 0.7		18.2 ± 0.8	
norcamphor-5,6-d_2 (I)	13.8 ± 0.6	1.22	5.1 ± 0.7	1.16	20.7 ± 0.8	0.87
norcamphor-3,3,5,6-d_4 (II)	13.6 ± 1.2	1.24	4.7 ± 0.4	1.25	23.5 ± 0.5	0.77
d-camphor	49.6 ± 1.4				61.4 ± 0.6	

[a] The rates shown correspond to the conditions described under Materials and Methods and represent V_{max} rates.

Scheme I: Regiospecificity of Norcamphor Hydroxylation by P-450$_{cam}$[a]



[a] The relative yields of the products are 6-*exo*-hydroxynorcamphor, 47%; 5-*exo*-hydroxynorcamphor, 45%; and 3-*exo*-hydroxynorcamphor, 8%. The regiospecificity of hydroxylation changes with deuterium substitution at various positions of the norcamphor skeleton as described in the text. The regiospecificity observed with the substrate camphor is included for comparison.

shown that hydrogen peroxide production does not increase as a result of deuterium substitution, but there is an isotope-dependent switching from monooxygenase to oxidase activity. In the same study it was shown that the 5- and 6-alcohols are produced with nearly complete specificity for exo hydrogen (deuterium) abstraction. Therefore, the isotope effects reported here on the kinetics of various P-450-dependent processes should be near maximal for the conditions used. The values may be slightly diminished due to trace amounts of endo abstraction and the fact that the substrates are less than 100% isotopically pure.

Oxygen Consumption. Rates of oxygen consumption by P-450$_{cam}$ in the presence of several substrates were determined under steady-state conditions as described under Materials and Methods. Oxygen uptake was linear with time for several minutes in the reactions used to calculate absolute rates. The kinetic traces for reactions are shown in Figure 1, and the rates, as determined from the traces, are summarized in Table I. Rates of oxygen utilization in the presence of the various norcamphors are significantly slower than the observed rate when *d*-camphor is the substrate. In the absence of added substrate, oxygen consumption is less than 10% of that seen with norcamphor after 10 min, demonstrating that the vast majority of oxygen consumption is dependent on the presence of norcamphor. The deuterium isotope effects on norcamphor-dependent oxygen consumption are 1.22 and 1.24 for *exo,exo*-norcamphor-5,6-d_2 (I) and *exo,exo*-norcamphor-3,3,5,6-d_4 (II), respectively. The trace for II is not shown in the inset of Figure 1 since it was nearly identical with that of the d_2 substrate (I). The complete kinetic profile, in which the reactions reach completion, indicates that there is a significant difference in the shape of the curves. Under these conditions (increased oxidase activity for the deuteriated

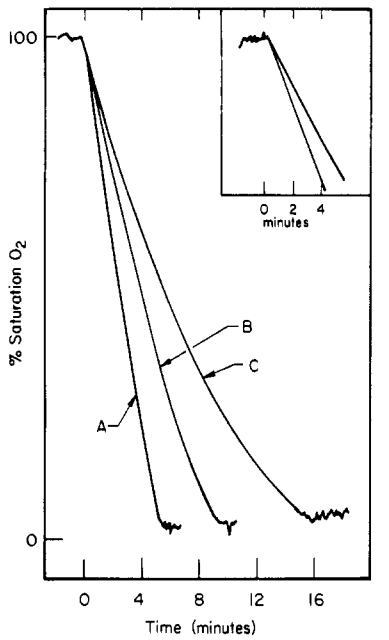


FIGURE 1: Kinetics of oxygen consumption. The symbols used are (A) norcamphor, (B) *exo,exo*-norcamphor-5,6-d_2, and (C) *exo,exo*-norcamphor-3,3,5,6-d_4. The inset shows the kinetics of the "slow" reactions, as described under Materials and Methods, which were used to obtain absolute rates of oxygen consumption.

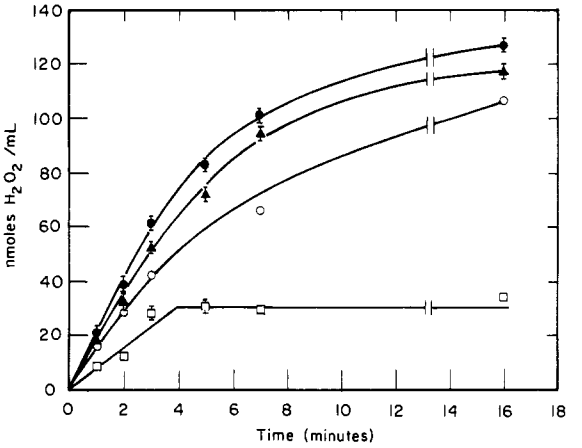


FIGURE 2: Kinetics of hydrogen peroxide formation. The rates of hydrogen peroxide formation in the presence of the various substrates were determined as described under Materials and Methods. The symbols used are (●) norcamphor, (▲) *exo,exo*-norcamphor-5,6-d_2, (○) *exo,exo*-norcamphor-3,3,5,6-d_4, and (□) camphor.

substrates) the pyridine nucleotide becomes partially limiting as the reaction proceeds. This is supported by the observation that when the initial NADH concentration is increased, the linear portion of the curve extends for a longer period of time with the deuteriated substrates.

Hydrogen Peroxide Formation. The isotope effects observed on H_2O_2 production are very similar to those seen for oxygen comsumption. The results that are depicted in Figure 2 confirm the previous observation that there is not a significant increase in the levels of H_2O_2 upon deuteriation of norcamphor

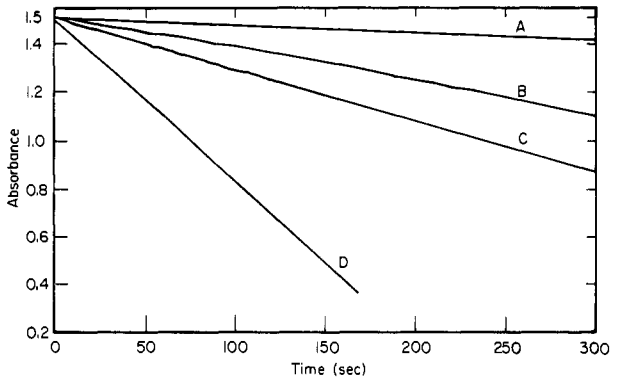


FIGURE 3: Kinetics of NADH consumption. The symbols used are (A) substrate free, (B) norcamphor, (C) *exo,exo*-norcamphor-*3,3,5,6-d*$_4$, and (D) camphor.

(Atkins & Sligar, 1987). These stoichiometry experiments suggested that there was a slight decrease in the levels of H_2O_2 with I and II when NADH was the limiting reagent. This is indicated in the present study as well. Values for the rates of H_2O_2 formation were calculated from the linear portions of the kinetic curves; for I and norcamphor the first four time points were used including 0 min. Linear regression of the values used to obtain the linear plots gave 20.1 nmol/(mL·min), r^2 = 0.998, and 17.4 nmol/(mL·min), r^2 = 0.996, for norcamphor and I, respectively. As is the case with the oxygen uptake experiments, the time course of H_2O_2 formation for II is not linear for the same range of experimental determinations, so the absolute rate is estimated from three points only. For this substrate the rate is 16.1 nmol/(mL·min), r^2 = 0.996. The slow rates of H_2O_2 evolution are summarized in Table I. Several features of these results are noteworthy. The amount of H_2O_2 present at the end of the reaction is very similar to the values reported when a catalase assay was utilized for H_2O_2 determination, when normalized to the NADH consumption (Atkins & Sligar, 1987). In this system, which is open to the atmosphere, NADH is limiting with a concentration of 900 nmol/mL. Previously we found that approximately 90 nmol of H_2O_2/570 nmol of NADH was produced. This would predict a concentration of 140 nmol of H_2O_2/mL of reaction in the present case, which is very close to the value observed. Second, the isotope effect is small but definitely greater than 1 and similar to that seen for O_2 consumption. This sharply contrasts with what is observed with NADH consumption. Lastly, it is evident that the amount of H_2O_2 obtained from norcamphor metabolism is significantly greater than that obtained when *d*-camphor is the substrate.

NADH Oxidation. There is a striking effect on the rate of NADH utilization when the substrate norcamphor is deuteriated. Results from typical NADH determinations are shown in Figure 3, and the corresponding rates and isotope effects are summarized in Table I. A slow autoxidation in the absence of substrate is apparent. This is common for P-450 turnover systems, and the values reported in Table I are corrected for this background utilization of NADH. It is clear that there is an inverse isotope effect of substantial magnitude. As is expected, the rate of camphor-induced NADH oxidation is faster than is seen with norcamphor, by a factor of approximately 3 under the conditions used here. The increased rate of NADH oxidation in the presence of deuteriated norcamphor requires substrate/P-450$_{cam}$ interaction, since incubation of either I or II with the turnover system in the absence of the cytochrome demonstrates no measurable usage of pyridine nucleotide above background. Thus, the inverse isotope effect is not the result of any trace contaminants in either deuterionorcamphor preparation which oxidize NADH. Furthermore, rates of NADH consumption are not increased when hydrogen peroxide or potassium superoxide are added to the system as shown in Table II. These results suggest that the enhanced rate of NADH utilization is dependent on norcamphor/P-450 interaction but is not due to any dissociable reactive oxygen intermediates that might oxidize NADH.

Table II: Rates of NADH Consumption by Cytochrome P-450$_{cam}$

system/substrate	nmol of NADH/(min·nmol of P-450)
complete/norcamphor	18.4 ± 0.2
complete/norcamphor + 50 μM H_2O_2	18.1 ± 0.09
complete/norcamphor + 100 μM H_2O_2	18.0 ± 0.3
complete/norcamphor + 50 μM superoxide	17.9 ± 0.2
complete/norcamphor + 100 μM superoxide	17.5 ± 0.2
−P-450/norcamphor	0.40 ± 0.03
−P-450/norcamphor-d_4	0.40 ± 0.06
complete/camphor	58.7 ± 0.8
complete/camphor + 1 mM norcamphor-d_4	56.9 ± 0.5

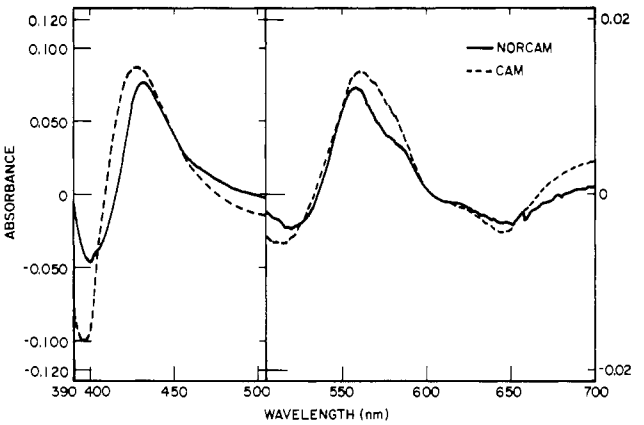


FIGURE 4: Steady-state difference spectroscopy: difference spectra of the dioxy ferrous P-450 minus ferric P-450 in the presence of norcamphor (solid line) and camphor (dashed lined). Identical reaction mixtures were placed in both sample and reference cuvettes, and NADH was added to the sample cuvette as described under Materials and Methods.

Steady-State Spectroscopy. In order to investigate the possibility that norcamphor metabolism results in a distribution of enzyme intermediates that is distinct from camphor metabolism, UV–visible difference spectra were obtained as described previously (Estabrook et al., 1971; Tyson et al., 1972). The spectra shown in Figure 4 represent the species present during steady-state turnover. These spectra are obtained by placing identical reaction mixtures in each of two cuvettes and adding NADH to the sample cuvette, followed by repetitive scanning of the 390–700-nm range. The superimposability of several consecutive spectra indicates that the system has reached steady state. It is well documented that, with several substrates and P-450 isozymes, the predominant form of P-450 present during turnover is the dioxy ferrous P-450 (Peterson et al., 1972; Bonfils et al., 1979). As shown in Figure 4, the difference spectra obtained with norcamphor as substrate are slightly different than the spectra obtained when camphor is metabolized. The λ_{max} of the Soret region has shifted from 428 to 432 nm, and a significant shoulder is present on the peak centered at 560 nm. These differences do *not* reflect the presence of a unique intermediate when norcamphor is the substrate but merely result from a contribution of the low-spin ferric enzyme with saturating levels of norcamphor. This was demonstrated by producing a (dioxy ferrous P-450) − (ferric P-450) difference spectrum in the presence of norcamphor by the photoreduction method previously described (Lipscomb

et al., 1976). The spectrum obtained by photoreduction (not shown) is identical with that observed during steady state of NADH-driven norcamphor metabolism (Figure 4). Thus, the predominant species present is the dioxy ferrous form, as is the case with camphor present, suggesting that the transfer of the second electron is largely rate determining.

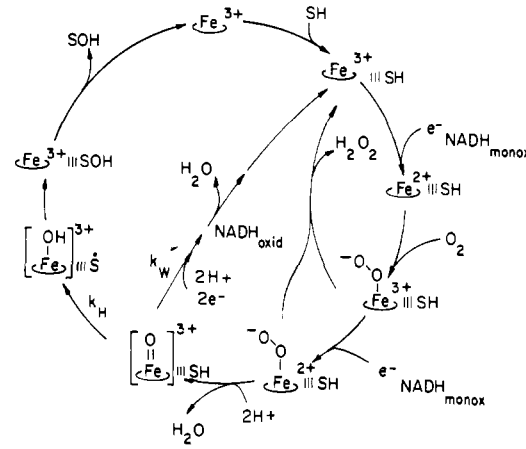
In an attempt to detect differences in concentrations of P-450 intermediates which might result from deuterium substitution of norcamphor, we have attempted isotopic difference spectroscopy during steady state. This consists of measuring the steady-state difference spectra with isotopically labeled norcamphor in the sample cuvette and undeuteriated norcamphor in the reference cuvette. If an isotope-dependent redistribution of enzyme intermediates was of significant magnitude, then it should be detected by this approach. No detectable difference in steady-state intermediates is observed when norcamphor is deuteriated, even with extensive magnification of the absorbance scale (data not shown). This lack of isotopic difference spectra has significant implication for the isotope effect on NADH consumption. Deuterium substitution of norcamphor does not result in an increase in the steady-state concentration of dioxy ferrous P-450. This is expected if steps subsequent to transfer of the second electron maintain a high commitment to catalysis, with no reversibility in the chemical steps including O–O bond scission and formation of a high-valent iron–oxo species. Therefore, the increased rate of NADH consumption associated with deuteriation of norcamphor does not result from an isotope-dependent increase in the steady-state concentration of dioxy ferrous P-450.

Discussion

The intermolecular isotope effects on O_2 consumption and H_2O_2 formation are, by themselves, not extremely informative since no single step in the reaction cycle is entirely rate determining. Furthermore, it is established for several P-450 systems that, during steady-state turnover, the most abundant form of the enzyme is the one electron reduced dioxy P-450 species (Tyson et al., 1972), which is consistent with the report that the slow step in the reaction is input of the second electron from putidaredoxin (Pederson, 1977). From these results and deuterium isotope studies it appears that hydrogen abstraction is fast relative to other chemical and physical steps, so the magnitude of the intrinsic isotope effect k_H/k_D is significantly masked. Thus, deuterium substitution of norcamphor would be expected to have a minimal effect on the observed steady-state kinetics of O_2 and H_2O_2 metabolism. Gelb et al. (1982) found that, with deuteriated camphors, the observed $^HV/^DV$ on NADH consumption was approximately 1.1–1.2. With camphor as substrate, the pyridine nucleotide reducing equivalents are quantitatively utilized for monooxygenase chemistry, so it is reasonable to speculate that the isotope effect on O_2 uptake would be 1.1–1.2 in this tightly coupled system, although this value was not experimentally determined. The $(^HV/^DV)_{O_2}$ for norcamphor metabolism is somewhat greater than this value, which may suggest that hydrogen abstraction contributes more to the overall rate of reaction in the presence of norcamphor. In general, however, little information is available from the isotope effects on O_2 and H_2O_2 metabolism, until they are considered in light of the isotope effect observed for NADH consumption.

Inverse deuterium isotope effects are generally associated with orbital rehybridization of the carbon to which the isotopic atom is bonded. Rehybridization from trigonal sp^2 to tetrahedral sp^3 configuration is kinetically more favorable when deuterium is substituted for hydrogen, thus giving rise to an inverse isotope effect (Klinman, 1978). Obviously this situation is not relevant to the hydroxylation of norcamphor at any of its sp^3 carbon centers. This, and the fact that the oxygen and hydrogen peroxide isotope effects are normal, eliminates the possibility that the NADH isotope effect is due to any novel mechanism at the chemical level, and we expect that hydroxylation of norcamphor occurs via hydrogen abstraction to afford substrate radical, followed by oxygen rebound, as suggested by several lines of evidence (Groves, 1976, 1978; White, 1986). An inverse deuterium isotope effect has been observed for both turnover and inactivation of β-lactamase by sulbactam-*6,6-d*$_2$ and the elegant interpretation offered in that study prompted us to consider the norcamphor/P-450 system in an analogous manner (Brenner & Knowles, 1981).

Scheme II: Proposed Monooxygenase and Oxidase Cycles of P-450$_{cam}$[a]



[a] The terms "$NADH_{monoox}$" and "$NADH_{oxid}$" indicate points at which pyridine nucleotide reducing equivalents are utilized for the normal monooxygenase cycle and the further reduction of oxygen to result in oxidase stoichiometry, respectively. Hydrogen peroxide may be generated from dismutation of 2 equiv of superoxide anion or directly from collapse of the two electron reduced intermediate prior to O–O bond scission. It is not the intent of this scheme to imply that this intermediate has been detected. Reduction of the hydroxylating intermediate, $[FeO]^{3+}$, may occur as two one-electron reductions. SH and SOH refer to substrate and hydroxylated product.

The isotope effects discussed here can be interpreted in terms of an isotope-dependent partitioning of enzyme intermediates, and these effects have significant mechanistic implications concerning the four-electron oxidase activity of P-450's in general. If $(^HV/^DV)_{O_2}$, $(^HV/^DV)_{H_2O_2}$, and $(^HV/^DV)_{NADH}$ were all inverse, it would suggest that product release (hydroxynorcamphor) or a step downstream from irreversible hydrogen abstraction was rate limiting. Isotope-dependent switching to afford increased oxidase activity would result in fast regeneration of substrate-bound ferric enzyme and H_2O, therefore bypassing slow product release. However, the disparity in isotope effects for H_2O_2 and O_2 metabolism vs NADH consumption requires a model in which there is a route for NADH utilization that is kinetically independent from O_2 uptake, H_2O_2 formation, and the normal NADH oxidation associated with monooxygenase chemistry. This is easily rationalized if one considers a kinetic scheme as depicted in Scheme II. Both monooxygenase and oxidase activities share a common cycle for most of their respective pathways. At some point, however, there must be an additional route that provides an additional path for utilization of NADH reducing equivalents without concomitant O_2 utilization. There are effectively two competing cycles such that the relative flux through each is affected by deuterium substitution of norcamphor. The normal kinetic isotope effect on the hydrogen

abstraction step results in slower overall flux through both cycles, as indicated by $(^{H}V/^{D}V)_{O_2}$ and $(^{H}V/^{D}V)_{H_2O_2}$, but due to the stoichiometry of the oxidase cycle, NADH is utilized at a faster rate when this oxidase pathway is more extensively traversed. This suggests that there is an isotope-dependent redistribution of internal oxy P-450 species, resulting in a higher steady-state concentration of a species that is a substrate for two-electron reduction by NADH. As depicted in Scheme II, these results are consistent with a reduction of $[FeO]^{3+}$, the hydroxylating intermediate. It is possible that a species prior to formation of $[FeO]^{3+}$ is the substrate for reduction, and deuterium substitution leads to increased levels of several intermediates. This would require reversibility in the steps preceding $[FeO]^{3+}$. The experiments utilizing isotopic difference spectroscopy indicate no such reversibility and suggest that hydrogen abstraction is the only isotopically sensitive step. It is important to emphasize that the model depicted in Scheme II does not require an inverse isotope effect on NADH consumption. Any disparity in kinetic isotope effects where each isotope effect is greater than 1 but the magnitude of the effect on NADH consumption, H_2O_2 production, and oxygen consumption is less than the magnitude of the effect on substrate hydroxylation would be consistent with this model. We have previously reported a kinetic effect on norcamphor hydroxylation $(^{H}V_{max}/^{D}V_{max})_{hydrox}$ of about 3.0–3.7. Also, Harada et al. (1984) found nearly equivalent isotope effects, 1.0–1.1, on H_2O_2 and NADH metabolism, with an isotope effect on substrate oxidation of significantly greater magnitude. This disparity observed with the liver microsomal P-450/ethoxycoumarin system might also be explained by isotope-dependent branching between monooxygenase and oxidase reactivity.

The magnitude of the observed isotope effect on NADH oxidation warrants some discussion. The NADH consumption associated with oxygen activation leading to the normal monooxygenase intermediate $[FeO]^{3+}$, ($NADH_{monoox}$), and the NADH consumption associated with the two-electron reduction of this species to form water, ($NADH_{oxid}$), cannot be individually quantitated in the turnover systems employed here. Therefore, the reported value for $^{H}V_{NADH}/^{D}V_{NADH}$ represents the "bulk effect" on the NADH utilization. If one could separate these and assumes, conservatively, that the $NADH_{monoox}$ isotope effect is similar to that for oxygen consumption and hydrogen peroxide production, i.e., is 1.1–1.3, then the actual isotope effect on $NADH_{oxid}$ is necessarily greater in magnitude than the value reported in Table I would suggest. In other words, the actual value of $^{H}NADH_{oxid}/^{D}NADH_{oxid}$ is significantly less than 0.77. The actual value cannot be determined without a knowledge of the absolute rates of $NADH_{monoox}$. This can be estimated, however, as follows:

$$^{H}NADH_{total} = {}^{H}NADH_{monoox} + {}^{H}NADH_{oxid}$$

$$^{D}NADH_{total} = {}^{D}NADH_{monoox} + {}^{D}NADH_{oxid}$$

where $^{H}NADH_{total}$ and $^{D}NADH_{total}$ correspond to the observed rates of total NADH consumption for norcamphor and fully deuteriated norcamphor, respectively, and $NADH_{monoox}$ and $NADH_{oxid}$ represent these rates for reducing equivalents used in the normal monooxygenase cycle and those used for further reduction of P-450 intermediates to afford water with an oxidase stoichiometry.

If one assumes absolute rates for $^{H}NADH_{monoox}$ and $^{D}NADH_{monoox}$ are similar to the observed rates of O_2 consumption at steady state, 16.8 and 13.6 nmol/(min·nmol), respectively, and if we utilize the experimentally observed values for $NADH_{total}$, then $^{H}NADH_{oxid} = 1.4$ and $^{D}NADH_{oxid} = 9.8$, and $^{H}NADH_{oxid}/^{D}NADH_{oxid} = 0.15$. This corresponds to a 7-fold increase in the rate of $NADH_{oxid}$ upon deuterium substitution of norcamphor. If the steps preceding formation of $[FeO]^{3+}$ are isotopically insensitive and irreversible, and if the rate constant for reduction of $[FeO]^{3+}$, k_w, is also insensitive to deuterium substitution, then this would suggest a 7-fold increase in the effective steady-state concentration of $[FeO]^{3+}$. One may have expected to observe such a large change in the concentration of this species by the isotopic difference spectroscopy experiments. The fact that no isotope-dependent spectral changes were detected would indicate that the intermediate or intermediates that are reduced to afford water constitute a very small fraction of the total P-450 population.

It is particularly interesting that isotope effects on NADH consumption in the presence of substituted camphors are normal or greater than unity. Gelb et al. (1982) found normal isotope effects with deuteriated camphor, as mentioned above, and 5,5-*gem*-difluorocamphor resulted in NADH oxidation rates that were one-third of those of camphor (Eble & Dawson, 1984). Neither of these investigations quantitated hydrogen peroxide or hydroxycamphor produced, so it is possible that any isotope-dependent switching to afford oxidase chemistry may not have been great enough to result in an inverse isotope effect as is observed with norcamphor.

We have considered the possibility that a mechanism is operative which allows for hydrogen peroxide mediated reduction of the iron–oxo intermediate to afford dioxygen and water in a fashion analogous to catalase (Jarnigan & Wang, 1958) and chloroperoxidase (Hager et al., 1972). This could result in normal isotope effects on O_2 and H_2O_2 metabolism and could conceivably lead to an inverse isotope effect on NADH consumption if hydroxynorcamphor release was rate limiting and H_2O_2-mediated breakdown of the iron–oxo species afforded the ferric substrate complex poised for the next reaction cycle. Several observations suggest that this type of scheme is not operative: (1) multiple attempts to directly observe peroxide-dependent oxygen evolution by $P\text{-}450_{cam}$ have failed to reveal any such activity in the presence and absence of a variety of substrates including norcamphor (data not shown); (2) if enzymatically generated hydrogen peroxide were reacting with the iron–oxo intermediate to afford ferric P-450, then one would expect exogenously added H_2O_2 to result in a similar rate enhancement of NADH oxidation. As can be seen in Table II, this is not the case. Addition of superoxide and hydrogen peroxide results in a slight decrease in rates of NADH consumption, presumably due to a low level of protein oxidation.

Lastly, the model presented here and the stoichiometry results reported previously (Atkins & Sligar, 1987) would suggest that, in the presence of norcamphor, P-450 catalyzes the heterolytic scission of dioxygen to afford a species that would be formally equivalent to compound I of the peroxidases, such that further two-electron reduction would afford water. If O–O scission were homolytic, producing a species formally equivalent to compound II, then one-electron reduction would afford water. Recently, it has been suggested that $P\text{-}450_{cam}$ substituted with 2,3-diacetyldeuteroheme catalyzes the homolytic cleavage of dioxygen to produce a compound II type intermediate in the presence of camphor (Kobayashi et al., 1987). Furthermore, it has been documented that various porphyrin model systems are capable of catalyzing both homolytic and heterolytic O–O bond scission and that the preferred pathway is oxidant dependent (Lee & Bruice, 1985). It is likely that P-450's are capable of performing both hete-

rolytic and homolytic O–O bond scission, and this mechanism may be isozyme and substrate dependent. The difference in degree of hydration of the heme environment in the presence of camphor vs norcamphor may be significant enough to result in differences in the mechanism of O–O bond scission.

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