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SECONDARY DEUTERIUM ISOTOPE EFFECTS ON OLEFIN EPOXIDATION BY CYTOCHROME P-450

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Abstract—Secondary deuterium isotope effects have been determined for the epoxidation of *p*-phenylstyrene (1a) and *p*-methylstyrene (1b) by cytochrome P-450 of rat liver microsomes. With both substrates there is an inverse isotope effect of 7 per cent/deuterium (i.e. $k_H/k_D = 0.93$) at C_α of the olefin, but no isotope effect is observed at C_β. The epoxidation of (1a) by *m*-chloroperbenzoic acid has previously been shown to be accompanied by an inverse secondary isotope effect of 9 per cent/deuterium at C_β, with no detectable isotope effect at C_α. Thus in both the enzymatic (P-450) and non-enzymatic (peracid) epoxidation of styrene derivatives, the oxygen atom is transferred to the vinyl group in an asymmetric non-concerted fashion. However, the fact that the isotope effects for these two systems are reversed, together with previous comparisons of substituent effects on the two reactions, suggests that there is little mechanistic similarity between cytochrome P-450 enzymes and organic peracids as chemical models for these enzymes.

Cytochrome P-450-dependent mono-oxygenase enzymes initiate the metabolic clearance of many drugs and foreign substances from the body [1], and are responsible for the formation of cytotoxic, carcinogenic and mutagenic metabolites of aromatic hydrocarbons and their derivatives [2–4]. Despite considerable expenditure of effort, the exact mechanism by which P-450 enzymes activate oxygen for reaction with organic substrates has remained elusive. Although it is known that substrate, electrons, protons and O_2 are consumed in a 1:2:2:1 ratio, the difficulty in obtaining information on the nature of active oxygen and the atom-transfer process has led to considerable speculation based on putative chemical model systems. One model which has attracted considerable attention is the organic peracid [5, 6], and it has recently been suggested that a peracid or perimidic acid group is formed enzymatically at the active site of P-450 and that this is the reactive species that oxygenates the organic substrates [6]. Other proposals and models for the mechanism of cytochrome P-450 action have focused on the iron–porphyrin unit, invoking oxo derivatives of Fe (IV) or Fe (V) [7–11].

To date, most mechanistic studies of P-450 have centered on either the nature of the proteins and cofactors involved, or on the hydroxylation of aliphatic groups or aromatic rings; olefinic substrates that form stable epoxides have received relatively little attention. Because the conversion of an olefin to an oxirane involves changes in the hybridization of two carbon atoms from sp^2 toward sp^3, secondary deuterium isotope effects are well suited as an experimental probe of mechanism and transition state structure for this reaction. For example, application of this method to study the peracid epoxidation of styrene derivatives has recently revealed that this reaction occurs in two discrete C—O bond-forming steps rather than a symmetrical O-atom transfer as previously assumed [12]. Since styrenes are readily and selectively metabolized by cytochrome P-450-mediated epoxidation of the vinyl group, we have now studied the P-450 epoxidation of vinyl-deuterated styrenes as a probe of the mechanism of their enzymatic oxygenation.

EXPERIMENTAL

The deuterated styrenes used in this study (*1* in Fig. 1) were synthesized as described previously [12, 13], and were shown by nuclear magnetic resonance (n.m.r.) and mass spectroscopy to contain > 99 atom-% deuterium in the indicated positions. Microsomes for the metabolism experiments were sedimented at 105,000 *g* from a 30,000 *g* post-mitochondrial supernatant, and were supplemented with an NADPH-generating system, as described in a companion paper [13].

Enzymatic isotope effects. For determining secondary deuterium isotope effects on the enzymatic epoxidation, competition experiments were designed to give limited metabolic conversion from a

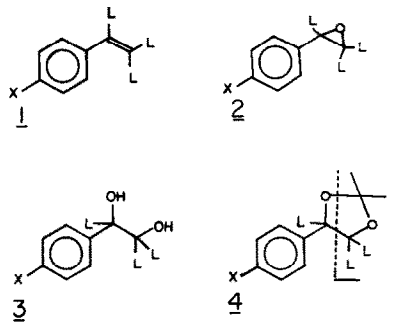

Fig. 1. Structures of styrenes, epoxide and diol metabolites, and acetonide derivatives. The X group is either C_6H_5 or CH_3, and L stands for either [^{1}H] or [^{2}H].

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large pool of mixed protio and deuterated substrates. The d_0 and d_n olefins were each weighed accurately and combined in solution to ensure mixing prior to incubation; a sample was retained for mass spectral analysis of the d_0/d_n mole fraction for comparison to the calculated ratio. Approximately 0.1 g of olefin mixture was incubated for 30 min in each run with 3 g microsomes in 30 ml buffer and NADPH-generating system in a stoppered 250-ml flask. The incubations were run under an oxygen atmosphere at 33°, and were stopped by addition of KOH followed by ether extraction.

The diol products (*3* in Fig. 1) were isolated as follows. An ether extract of the incubation was dried (Na_2SO_4) and filtered through ca. 2 g $SiO_2 \cdot 10\% H_2O$ to remove the polar phospholipids. This filtrate was concentrated and subjected to preparative thin-layer chromatography (t.l.c.) on silica eluted with ethyl acetate–hexane (60:40). Both *p*-methyl- and *p*-phenyl-styrene diols were isolated from a band of R_f 0.35 to 0.45 in yields of 3–6 mg/run, and were converted to their acetonides as described previously [14].

Mass spectra of starting olefins and acetonides of product diols were determined on an Atlas CH-5 mass spectrometer by direct probe inlet (cooled to 5–10°) using 70 eV ionization energy. The results obtained did not vary as the ionization energy was decreased, and the low inlet temperature allowed at least ten measurements of each sample to be recorded under steady state conditions in the spectrometer, which considerably improved the precision [14, 15]. For the olefins the intensity of the parent peaks was used to calculate the mole fraction of protio and deuterated olefin by a reiterative procedure which corrected for the small contributions of one compound to the ion current at the parent m/e of the other. Thus, it was absolutely necessary to have adequate mass spectral data not only for the mixtures but for the pure protio and deuterated compounds as well. As a check on the method, the mole fractions thus determined for the olefin mixtures agreed within 0.5 per cent of those calculated from the weights of the individual olefins.

For the acetonides of product diols, mole ratios of protio and deuterated forms were determined by a similar analysis of the ratio of intensities of peaks at m/e 72 and 74, formed as shown in 4 in Fig. 1. Again mass spectral data for each pure acetonide as well as for known d_0/d_n mixtures were obtained. The latter gave the same mole fraction by mass spectral analysis as by weight ratio, indicating that there was no isotope effect on the fragmentation.

RESULTS AND DISCUSSION

Cytochrome P-450 mono-oxygenases are known to effect several kinds of metabolic oxygenations [4, 5] including olefin epoxidation, aliphatic hy- oxygenases epoxidize simple olefinic substrates, we undertook a mechanistic comparison of enzymatic and peracid epoxidation in terms of secondary deuterium isotope effects.

By the measurement of secondary kinetic deuterium isotope effects, one can derive information about the hybridization changes occurring at an isotopically substituted carbon atom as a set of reactants passes from their ground states to the transition state complex [16, 17]. These effects are related to the change in vibrational energy of C—H vs C—D out-of-plane bending modes. Because the potential energy curve for an H—C (sp^2) bond is a relatively shallow parabola, the zero-point energies of the C—H and C—D reactants are relatively close. However, the potential curve for an H—C (sp^3) bond is a relatively steeper parabola, so that the zero-point energy of a C—D bond is substantially less than that of a C—H bond. Therefore, the energy of activation for an $sp^2 \rightarrow sp^3$ change in hybridization is lower for the deuterated material and the kinetic isotope effect is inverse ($k_D > k_H$).

Rat liver microsomes contain a closely coupled cytochrome P-450/epoxide hydrase enzyme system which catalyzes the net reaction *1* → *3*. The intermediate epoxides, *2*, may sometimes be trapped or isolated, but only with difficulty [4, 18–20]. Consequently, with styrene substrates one observes only styrene diols as metabolic products *in vitro* [13, 21, 22]. The secondary deuterium isotope effect on the epoxidation step can thus be determined by competition experiments using mixtures of protio and deuterio olefins and mass spectral analysis of changes in d_0/d_n mole ratios between starting olefins and diol products. Since the enzymatic hydration of both the protio- and the deutero-oxides proceeds to completion, the possibility of an isotope effect on this reaction (which is presently under investigation in our laboratory) will have no bearing on the H/D ratio observed in the diols. As shown in Table 1, the substitution of deuterium for hydrogen at C_β of the vinyl group in *p*-methyl- and *p*-phenylstyrene leads to no detectable isotope effect on their enzymatic epoxidation. Because of the nature of the mass spectral fragmentation of acetonides of styrene diols (see 4), we were unable to measure directly the isotope effect associated with deuteration at C_α. However, the results obtained with the α,β,β-d_3 derivatives clearly show that deuteration of C_α does lead to a significant secondary isotope effect. For comparison purposes, similar data for peracid epoxidation are also listed in Table 1. In all of these cases the secondary isotope effects observed are inverse, as expected for the conversion of an olefin to an oxirane.

The isotope effects observed on the P-450 epoxidations reveal several important features of the substrate oxygenation process, which in turn may be highly relevant to consideration of proposals for

Table 1. Secondary deuterium isotope effects on styrene epoxidations by microsomal cytochrome P-450 and *m*-chloroperbenzoic acid

Styrene substrate (*I*)		Epoxidizing system			
		P-450		*m*-$ClC_6H_4CO_3H$*	
X	L	Runs	k_H/k_D	Runs	k_H/k_D
C_6H_5	α-d_1			5	0.99 ± 0.03
	β,β-d_2	3	1.00 ± 0.03	5	0.82 ± 0.03
	α,β,β-d_3	4	0.93 ± 0.03	5	0.81 ± 0.03
CH_3	β,β-d_2	2	1.01 ± 0.03		
	α,β,β-d_3	2	0.93 ± 0.03		

* Data from Ref. 12.

enzyme system. This situation is analogous to the frequently observed primary deuterium isotope effects on P-450 hydroxylation and dealkylation reactions [15, 23].

The *magnitude* of an isotope effect can also give information about the detailed mechanism of a reaction [16, 17]. The maximum secondary isotope effect (k_H/k_D) expected for an $sp^2 \rightarrow sp^3$ change is 0.71. However, a value this large will only be observed if the transition state for the reaction occurs very late on the reaction coordinate and is essentially fully sp^3 hybridized like the reaction product. It is more common to observe values of k_H/k_D in the range 0.8 to 0.9, corresponding to transition state geometries somewhere between sp^2 and sp^3; some relevant examples from the literature are given in Table 2. When the reaction involves a change from sp^2 to less than fully tetrahedral geometry, as in epoxidation reactions, the transition state geometry will differ from the initial geometry even less, and the magnitude of the secondary isotope effect expected will be reduced even further. For example, in the peracetic acid epoxidation of *trans*-stilbene [23] and in the *m*-chloroperbenzoic acid epoxidation of *p*-phenylstyrene [12], the secondary isotope effects observed are both 9 per cent/deuterium. This value is close to the value of 7 per cent/deuterium observed for the enzymatic epoxidations reported in Table 1.

Perhaps the most significant and telling feature of the enzymatic isotope effects is their *inequality* at the α and β carbons of the olefinic substrates. This inequality clearly indicates that enzymatic epoxidation, like peracid epoxidation, involves two discrete C—O bond-forming steps rather than a symmetrical transfer of the oxygen atom to the olefin. A similar situation obtains in the oxidation of styrene by CrO_2Cl_2 [24]. However, the latter two chemical model reagents are clearly distinguished from the enzymatic oxygen atom-transfer reagent by the pattern of their secondary isotope effects, which is reversed from the enzymatic pattern (Table 1), and by the fact that their reaction with styrenes shows a marked dependence on substituent effects ($\rho = -1.4$ for peracid and $\rho^+ = -1.99$ for $CrO_2C)_2$) [12, 24], whereas the enzymatic epoxidation is virtually insenstive to substituent effects [13].

While it might be tempting to conclude from the reversed pattern of the enzymatic isotope effects that the enzyme attacks the substrate first at C_α and then at C_β in forming the oxirane ring, we feel that this is unlikely in view of the fact that all chemical reagents which attack styrenes in a stepwise fashion attack first at C_β because of the mechanistic advantage of resonance stabilization of intermediates. The applicability of this analogy between the chemical and enzymatic behavior of styrenes has been discussed in detail in the previous paper [13]. Assuming that the enzyme, like other chemical reagents, does attack the substrate first at C_β and then at C_α to close the oxirane ring, one is faced with the problem of explaining the lack of an isotope effect at C_β. Clearly, more information from a broader range of substrates will be needed to understand these isotope effects fully. However, the present information does lead to two straight-forward and important conclusions. The first is that the oxygenation of the vinyl side chain in styrene derivatives

Table 2. Secondary deuterium isotope effects on chemical oxidations of styrene derivatives

Substrate	Reagent	$\rho(\rho^+)$	k_H/k_D	Source of data
t-Stilbene-d_2	CH_3CO_3H		0.90	Ref. 23
	$KMnO_4$		0.82	
	OsO_4		0.88	
	Ozone		0.93	
t-Cinnamic acid	$KMnO_4$	− 1.1		*
-α-d_1			0.76	
-β-d_1			0.76	
Styrene	CrO_2Cl_2	(− 1.99)		Ref. 24
-α-d_1			0.98	
-β,β-d_2			0.88	

* D. G. Lee and J. R. Brownridge, *J. Am. chem. Soc.* **96**, 5517 (1974).

does not occur in a symmetrical concerted fashion. The second, which is also supported by substituent effect studies, is that there is little mechanistic similarity between cytochrome P-450 enzymes and organic peracids as chemical models for these enzymes.

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