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α-Secondary Isotope Effects in the Lipoxygenase Reaction

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ABSTRACT: Isotope effects for the oxidation of [5,6,8,9,11,12,14,15-^{3}H]arachidonic acid catalyzed by soybean lipoxygenase and by 5-lipoxygenase were measured. This labeling pattern represents substitution at each of the vinylic hydrogens of the substrate. The observed isotope effect for soybean lipoxygenase was 1.16 ± 0.02 and for 5-lipoxygenase was 1.11 ± 0.05. These isotope effects are inconsistent with any change in hybridization (sp^2 to sp^3) at the vinylic carbons prior to or during the rate-determining step and are concluded to be most consistent with the formation of a carbanion-like intermediate or transition state. In contrast, the oxidation of arachidonic acid by Ce(IV), which is thought to proceed via a cation radical intermediate, exhibited at most a small isotope effect (1.02 ± 0.01). The reduction potential for the cation radical formed from arachidonic acid in this reaction is estimated to be 2.7 V vs NHE by comparison of the rates of oxidation of arachidonic acid and cyclohexene by Ce(IV). This is similar to the potential for the cation radical of 2-butene. No isotope effect (1.00 ± 0.03) was observed in the 5-lipoxygenase reaction for conversion of the initially formed product 5-hydroperoxyeicosatetraenoic acid to the epoxide leukotriene A_4. From this it is concluded that there is little carbon–oxygen bond formation prior to or during the rate-determining step for epoxide formation.

The kinetics and mechanism of soybean lipoxygenase have been extensively studied with the expectation that the results will be generalizable to other enzymes of this class, and the available information has been collected in several recent reviews (Schewe et al., 1986; Kühn et al., 1986; Lands, 1984; Veldink et al., 1984). The enzyme is a monomer with a single iron per active site, which is neither a heme nor an iron–sulfur center. The iron is considered to be the oxidant that oxidizes the 1,4-diene of a polyunsaturated fatty acid to a pentadienyl radical intermediate. The pentadienyl radical is then trapped by oxygen to give a lipid hydroperoxide as the final product. For example, when the substrate is arachidonic acid, as in this study, the product is 15-HPETE.[1] A pentadienyl radical

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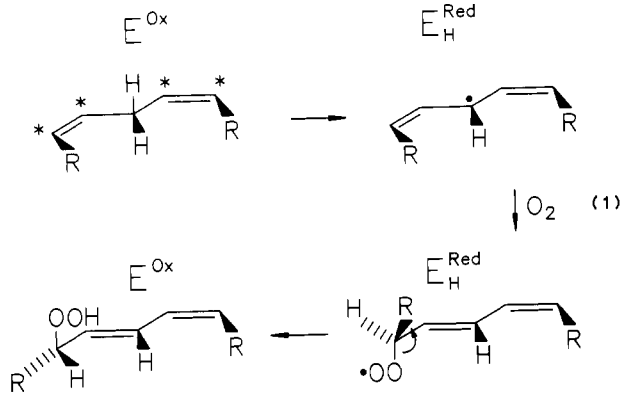
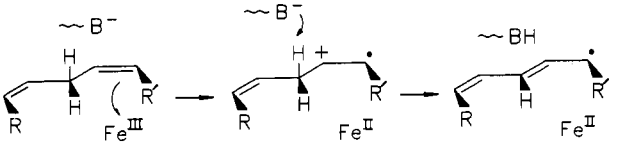
intermediate has not been directly observed but has been implied from the variation in product distributions with oxygen concentration (Corey & Nagata, 1987; Egmond et al., 1976). Loss of the methylene hydrogen of the 1,4-diene is considered to be the rate-limiting step in the formation of the pentadienyl radical since there is a large primary isotope effect on this step (Egmond et al., 1973).

The mechanism by which lipoxygenase catalyzes the formation of this pentadienyl radical has not yet been established. Energetically, abstraction of a hydrogen atom (eq 1) would



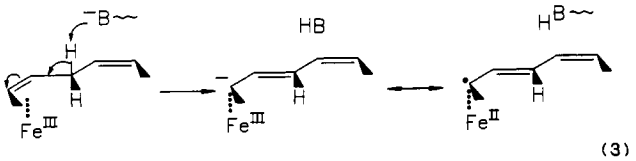
(1)

be attractive since the relatively stable pentadienyl radical would be formed directly. The anti-stereochemistry of hydrogen abstraction vs oxygen addition (Egmond et al., 1972; Corey et al., 1980) would seem to require rotation of an intermediate peroxy radical as shown. Alternatives to a hydrogen abstraction mechanism may be more in accord with the chemistry of model olefin oxidation reactions, however. Outer-sphere oxidations by metals such as Ce(IV) and Fe(III)–phenanthroline complexes have been shown to proceed via radical cation intermediates, i.e., via sequential loss of an electron and a proton (eq 2). The cation radical thus formed



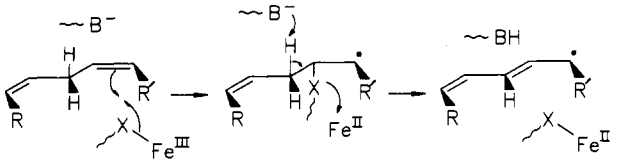
(2)

would be expected to be a high-energy intermediate (Miller et al., 1972; Fukuzumi & Kochi, 1982), and Corey and Nagata (1987) have argued that coordination of the iron to the substrate prior to transfer of the proton and electron would avoid formation of such a high-energy intermediate (eq 3). The



(3)

mechanism of epoxidation of olefins by cytochrome P-450 has also been the subject of intense investigation and may be relevant to the mechanism of lipoxygenase. In addition to a mechanism involving cation radical intermediates as in eq 2, a mechanism involving homolytic addition of an iron ligand to the double bond has been proposed (Castellino & Ortiz de Montellano, 1987, and references cited therein). In the case of cytochrome P-450 this ligand is the ferryl oxygen, while a similar mechanism for lipoxygenase can be written as in eq 4 without specifying the ligand.



(4)

One technique to provide information concerning the details of changes at different sites on the substrate through the course of the lipoxygenase reaction is to examine the effects of isotopic substitution. We therefore measured isotope effects associated with the oxidation of [5,6,8,9,11,12,13,15-^{3}H]arachidonic acid in which each hydrogen at a double bond was substituted by tritium (position of labeling indicated by asterisks in eq 1). The substrate was fully labeled so that each position in every molecule was substituted. In addition to oxidation by soybean lipoxygenase, which gives 15-HPETE as product, we examined oxidation by the 5-lipoxygenase from rat neutrophils, which gives initially 5-HPETE and ultimately LTA_4 as products. The observed isotope effects for the lipoxygenases are contrasted with those for outer-sphere oxidations by Ce(IV) and those for epoxidations of olefins by cytochrome P-450 (Hanzlik & Shearer, 1978). The results are discussed in terms of the reactions in eq 1–4.

Materials and Methods

Materials. Ceric ammonium nitrate was from Alfa Products, and acetonitrile was of HPLC grade from Baker. [1-^{14}C]Arachidonic acid, 50 mCi/mmol, and [5,6,8,9,11,12,14,15-^{3}H]arachidonic acid, 240 Ci/mmol, were from NEN Research Products. The specific activity of the [^{3}H]arachidonate was 104% of theory, and the position of labeling was confirmed by ^{3}H NMR by the manufacturer. The ^{3}H-labeled substrate was diluted with 1 equiv of unlabeled arachidonic acid (NuChK Prep, Inc.), and both the ^{3}H- and ^{14}C-labeled substrates were purified by HPLC on a Nucleosil 100 silica 50 column (5-μm particles, 0.45 × 25 cm, Alltech Associates) with 1% *i*-PrOH and 0.1% HOAc in hexane as eluant. Fractions were evaporated under Ar and redissolved in EtOH at 0.1 (^{14}C) or 0.2 mCi/mL (^{3}H).

15-HPETE was prepared by incubating a suspension of 10 mg of unlabeled arachidonic acid in 1 mL of 0.1 M borate buffer, pH 9.5, at 25 °C. Soybean lipoxygenase was added in three aliquots of 3 units each at 10-min intervals. The reaction mixture was extracted with 15% *i*-PrOH and 0.1% HOAc in hexane, acidified to pH 5 with HCl, and reextracted to remove the 15-HPETE. After evaporation of the solvent under Ar, the peroxide was dissolved in EtOH at a concentration of 10 mM.

Soybean lipoxygenase, L1 isozyme, was purified on DEAE-Sephadex to a specific activity of 100 units/mg (Axelrod et al., 1981; enzyme units as defined in this reference). 5-Lipoxygenase was partially purified from a lysate of rat peritoneal neutrophils (Skoog et al., 1986). Lysate, 122 mg of protein, was applied at 4 °C to a 2-mL column of ATP–agarose (agarose–hexane–ATP linked to C_8 of adenine, Pharmacia) equilibrated in buffer containing 0.1 M HEPES, 1 mM EDTA, and 5 mM dithiothreitol at pH 7.0. The column was eluted with 2 mL of buffer, 15 mL of 0.5 M NaCl in buffer, 5 mL of buffer, and 11 mL of 20 mM ATP (neutralized to pH 7) in buffer. The lipoxygenase was collected

[1] Abbreviations: HPLC, high-pressure liquid chromatography; HEPES, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; 15-HPETE, 15(*S*)-hydroperoxy-5(*Z*),8(*Z*),11(*Z*),13(*E*)-eicosatetraenoic acid; 5-HPETE, 5(*S*)-hydroperoxy-6(*E*),8(*Z*),11(*Z*),14(*Z*)-eicosatetraenoic acid; LTA_4, (5*S*)-*trans*-5,6-oxido-7(*E*),9(*E*),11(*Z*)14(*Z*)-eicosatetraenoic acid; k_{cat}, enzyme molecular activity (turnover number); $^3(V/K)$, the ^{3}H isotope effect on k_{cat}/K_m.

in the last 10 mL at a protein concentration of 0.065 mg/mL. It was stable indefinitely at −78 °C, could be frozen and thawed repeatedly, and was stable for at least 6 h at 0 °C. The enzyme had a native molecular weight of 100 000 on sucrose density gradients (6.2 S) and 76 000 by sodium dodecyl sulfate–polyacrylamide gel electrophoresis. The enzyme was identified on polyacrylamide gels by immunoblotting with a monoclonal antibody prepared against the rat 5-lipoxygenase and was 10–20% pure by this criterion. The membrane-containing fractions in the neutrophil lysate eluted in the initial buffer wash and were separated from soluble proteins by centrifugation for 30 min at 300000*g*. The pellet was resuspended once in 0.1 M HEPES and 1 mM EDTA buffer, pH 7.0, by sonication for 30 s, pelleted once more, resuspended at 0.88 mg of protein/mL (8-fold purification), and stored at −78 °C. The partially purified 5-lipoxygenase required the membrane fraction for activity.

Enzyme Assays. Soybean lipoxygenase was assayed at 37 °C in 0.5 mL of 0.1 M borate buffer, pH 9.5, containing 0.2 μCi of [^{14}C]arachidonic acid and 0.8 μCi of [^{3}H]arachidonic acid so that the substrate concentration was 8 μM. In some assays 1 μM 15-HPETE was added. The enzyme concentration was varied from 0.003 to 1.3 unit/mL, and the assay was quenched after 5 min with 0.6 mL of 20% CH_3CN, 0.2% H_3PO_4, and 0.1 mg/mL arachidonic acid in water. 5-Lipoxygenase was assayed at the same substrate concentrations at 37 °C in 0.5 mL of 0.1 M HEPES buffer, pH 7.0, containing 1 mM EDTA, 2 mM ATP, and 2 mM $CaCl_2$. The enzyme concentration was varied from 0.2 to 6 μg/mL, and the purified membrane fraction was present at 17 μg of protein/mL. The reaction was quenched at 5 min as for the soybean enzyme.

Oxidation by Ce(IV). Ceric ammonium nitrate, 10 μL of a 0.2 M solution in acetonitrile, was added to 100 μL of 40 μM arachidonic acid, 2 μCi/mL ^{14}C-labeled and 8 μCi/mL ^{3}H-labeled, in acetonitrile at 25 °C. The reaction was quenched with 1 mL of 2% HOAc/20% CH_3CN and analyzed immediately by chromatography (the quench slows but does not stop the reaction). Oxidation of 0.25 mM cyclohexene by 0.1 mM ceric ammonium nitrate in acetonitrile at 25 °C was followed spectrophotometrically by the change in absorbance for Ce(IV) at 330 nm.

Product Analyses. Reaction mixtures were analyzed by HPLC on Radial Pak C_{18} Columns (5-μm particle size, 0.8 × 10 cm, Waters Associates; Skoog et al., 1986). Elution buffer A was 40% CH_3CN plus 0.02% H_3PO_4 in water, and buffer B was 0.02% H_3PO_4 in CH_3CN. For analysis of 5-lipoxygenase reactions, stepwise elution profiles at a flow rate of 2 mL/min were used with solvent compositions at 0, 8, 15, and 20 min of 18, 39, 42, and 100% B, respectively. Similarly, solvent compositions for analysis of soybean lipoxygenase reactions at 0, 2, 5, and 11 min were 33.3, 50, 70, and 100% solvent B at a flow rate of 1 mL/min. The column effluent was mixed with Tru-Count scintillation fluid (flow rate 3 mL/min, Tru-Count Laboratory Supply Co.) and monitored for radioactivity in a Flo-One HP detector with a 1-mL flow cell (Radiomatic Instruments and Chemical Co.). The detector was used to detect peaks with a resolution of 0.1 min. Fractions were collected from the detector at 1-min intervals into 12.5 mL of scintillation fluid for soybean lipoxygenase and at 20-s intervals into 4 mL of scintillant for 5-lipoxygenase. Fractions were collected by hand since there was some chromatographic separation of ^{3}H and ^{14}C species, and it was important to prevent any sample loss. Radioactivity was measured in a Beckman LS-330 scintillation counter at a gain of 420 with preset windows. Internal standards were used to establish counting efficiencies at each different HPLC solvent composition throughout the chromatogram. Care was taken to wash column and injector between each chromatogram so that carryover of radioactivity from one analysis to the next was less than 0.5%. Samples were counted for 20 min each. Since counting all of the samples in an experiment required several days, counting efficiencies were redetermined every 8 h. The results, however, were insensitive to the small observed variations in counting efficiency with time or HPLC solvent composition. Observed counts in each fraction were corrected to dpm of ^{3}H and ^{14}C, and values of percent conversion for [^{3}H]- and [^{14}C]arachidonic acid to products were then calculated from the dpm in the substrate peaks relative to total dpm. Calculation of the results in this manner avoided problems that might arise from slight chromatographic separation of ^{3}H and ^{14}C species.

The analysis of reaction mixtures from the oxidation of arachidonic acid by Ce(IV) was similar to that for soybean lipoxygenase. The chromatography system used did not resolve all products, and all products were not identified. This did not affect the calculation of the isotope effect, however, since only the relative amount of total products vs unreacted substrate is required.

Calculations. The k_{cat}/K_m isotope effect for oxidation of arachidonic acid was calculated from the expression $^{3}(V/K) = \ln(^{14}f)/\ln(^{3}f)$ (Melander & Saunders, 1987). The parameters ^{14}f and ^{3}f are the fractions of unreacted [^{14}C]- and [^{3}H]arachidonic acid, respectively. If S is the amount of unreacted arachidonic acid at any time and S_0 and S_∞ are the amounts at the beginning of the reaction and after at least 10 half-lives, respectively, then $f = (S - S_\infty)/(S_0 - S_\infty)$. The amount of arachidonic acid at the beginning of the reaction as percent of total counts was >99.3%. The 0.7% or less of remaining counts arose from impurities but primarily from random noise in the base line. Although the arachidonic acid used in these experiments had been rigorously purified, a small fraction, 0.3–0.6%, did not react with either soybean or 5-lipoxygenase. This small amount of material represents S_∞ and was most likely an isomer of arachidonic acid, present at the start of the reaction or formed as a minor product of the reaction.

Results

The data for soybean lipoxygenase in the oxidation of [1-^{14}C]- vs [5,6,8,9,11,12,14,15-^{3}H]arachidonic acid are presented in Table I. The results are shown for two independent determinations using two different preparations of enzyme and two different lots of [^{3}H]arachidonic acid. The observed isotope effect was constant, $^{3}(V/K) = 1.16 \pm 0.02$, under these conditions within acceptable precision. Addition of 1 μM 15-HPETE to some assays had no effect on the isotope effect (data not shown). One possible anomalous source of the observed isotope effect in this reaction would be nonspecific labeling leading to a fraction of ^{3}H on the methylene carbon next to the double bond that is oxidized, i.e., in the 13-position. A large primary isotope effect is expected for substitution at this position (Egmond et al., 1973). The results are inconsistent with this possibility, however. An isotope effect of 1.16 measured at early reaction times would require levels of ^{3}H at the methylene carbon near 16%, and this is not observed by ^{3}H NMR (unpublished observations by the supplier). In addition, calculated isotope effects that arise due to impurities such as this will vary greatly from early to late times in the reaction, whereas the observed effects for soybean lipoxygenase were constant from 5% to 95% reaction. A second

Table I: Lipoxygenase Secondary Isotope Effects

soybean lipoxygenase[a]			5-lipoxygenase		
% conversion[b]			% conversion[b]		
^{14}C-labeled	^{3}H-labeled	isotope effect	^{14}C-labeled	^{3}H-labeled	isotope effect
5.46	4.65	1.179	7.68	6.46	1.196
13.37	11.59	1.165	15.39	14.76	1.046
28.39	24.87	1.168	31.63	29.25	1.099
58.67	52.79	1.177	44.04	40.33	1.125
94.44	91.29	1.183	78.40	75.66	1.084
		av 1.174 ± 0.007			av 1.11 ± 0.05
5.95	5.17	1.157			
23.04	20.30	1.154			
29.07	25.52	1.165			
83.64	79.87	1.130			
96.43	94.84	1.124			
99.10	98.41	1.136			
		av 1.144 ± 0.015			

[a] The data for soybean lipoxygenase represents two separate experiments. [b] "% conversion" represents the percentage of arachidonic acid converted to products. The total amount of products formed relative to the amount of unreacted substrate was determined chromatographically, and the isotope effect was calculated from the percent conversion of ^{3}H-labeled and ^{14}C-labeled substrate as described (Materials and Methods).

source of an anomalous isotope effect would be an isotope effect on the recovery of products vs substrate during chromatography. The total $^{14}C/^{3}H$ ratio for substrate plus products was constant ±0.5% despite the wide variation in the percent conversion, however, and the observed small variation was random with respect to the extent of reaction. By these criteria then, the observed isotope effect appears to be real.

Also in Table I are the data for oxidation of arachidonic acid by the 5-lipoxygenase from rat neutrophils. The data are less precise than those for the soybean enzyme because the larger number of products (Skoog et al., 1986) necessitated collecting more fractions of smaller volume (i.e., fewer total counts) to achieve the desired resolution in the chromatographic analysis of the reaction mixture. A significant isotope effect of $^{3}(V/K) = 1.11 \pm 0.05$ was observed nonetheless. The 5-lipoxygenase, in addition to oxidizing arachidonic acid to 5-HPETE, also converts 5-HPETE to LTA_4 (Rouzer et al, 1986). Free 5-HPETE is a poor substrate for the lipoxygenase, however. Early in the reaction the preferred course for LTA_4 formation is from arachidonic acid. Arachidonic acid is oxidized to 5-HPETE that, still bound to the enzyme, then partitions between dissociation from the enzyme and conversion to LTA_4 (Wiseman et al., 1987; Puustinen et al., 1987). The isotope effect for formation of LTA_4, therefore, is an isotope effect on this partitioning and is the $^{14}C/^{3}H$ ratio for the LTA_4 compared to that for the total product when these are measured early in the reaction. LTA_4 itself is unstable, so that the product actually measured is not LTA_4 but its hydrolysis products (Wiseman et al., 1987). The results in Table II demonstrate that there is no isotope effect for the partitioning of 5-HPETE from the enzyme vs its conversion to LTA_4. Note that the LTA_4 was formed at a constant 16% of products early in the reaction (8–44% turnover of arachidonic acid) when the conversion of 5-HPETE free in solution was negligible. The amount of LTA_4 as percent of products increased at longer reaction times when formation of LTA_4 from free 5-HPETE became significant. Within experimental error, ±5%, there is no isotope effect on this reaction either.

The oxidation of arachidonic acid by ceric ammonium nitrate in acetonitrile was studied as a model for metal-catalyzed oxidation of olefins. The secondary isotope effect was 1.02 ± 0.01 (Table III). There were numerous products from this reaction, and no attempt was made to isolate and identify them. The oxidation of arachidonic acid by Ce(IV) was first order in arachidonic acid for at least 2 half-lives and the rate constant was 0.0012 s^{-1} at 0.02 M Ce(IV). The calculated second-order rate constant is 0.06 M^{-1} s^{-1}. For comparison, the rate constant for oxidation of cyclohexene by Ce(IV) was 400 M^{-1} s^{-1}.

Table II: Secondary Isotope Effect on Synthesis of LTA_4

arachidonate, % conversion	LTA_4, % of products	$[^{14}C]/[^{3}H]$ ratio for LTA/$[^{14}C]/[^{3}H]$ ratio for total products
8	16	0.950
15	16	1.066
32	17	1.009
44	18	0.975
		av 1.00 ± 0.04[a]
78	22	0.997
92	33	1.051
95	42	0.978
96	44	0.981
99	40	0.971
		av 1.00 ± 0.03[a]

[a] Early in the reaction (low percent conversion of arachidonate) LTA_4 is formed primarily directly from arachidonic acid without dissociation of the intermediate 5-HPETE from the enzyme. At later times conversion of free 5-HPETE to LTA_4 becomes significant.

Table III: Secondary Isotope Effects in the Oxidation of Arachidonic Acid by Ce(IV)[a]

% conversion		
^{14}C-labeled	^{3}H-labeled	isotope effect
6.15	6.12	1.005
10.41	10.17	1.025
14.68	14.54	1.010
70.88	70.30	1.016
97.53	97.17	1.038
		av 1.02 ± 0.01

[a] Oxidation of arachidonic acid by ceric ammonium nitrate in acetonitrile at 25 °C. Calculation of results is as described for Table I.

Discussion

The secondary isotope effects measured in this work are a function of all the steps in the reaction up to and including the step that is rate-determining for k_{cat}/K_m. For soybean lipoxygenase this step is the loss of a methylene hydrogen of the 1,4-diene system, and this step is equivalent in each of the possible mechanisms considered here to formation of a pentadienyl radical (Egmond et al., 1973; Corey & Nagata, 1987). The isotope effect for [5,6,8,9,11,12,14,15-^{3}H]arachidonic acid as a substrate for soybean lipoxygenase was 1.16 ± 0.02. This seems to be a general result for the lipoxygenase reaction since a similar effect was observed for 5-lipoxygenase, although with lower precision, 1.11 ± 0.05. The observed isotope effect must be interpreted with caution at this stage since it is not known whether it represents a large effect for a single hydrogen or a smaller cumulative effect over several hydrogens. The observation of a normal isotope effect (i.e., $k_H > k_T$) was surprising, however. Thus, α-secondary isotope effects are expected to be dominated by effects due to changes in hybridization in this reaction (Melander & Saunders, 1987). The only obvious changes in hybridization at the vinylic positions in the lipoxygenase reaction are from sp^2 to sp^3, and the isotope effect in this case would be inverse ($k_T > k_H$) rather than normal. For example, trapping of the pentadienyl radical by O_2 as shown in eq 1 would involve rehybridization from sp^2 to sp^3 at the single carbon undergoing carbon–oxygen bond

formation. The observed isotope effect is therefore inconsistent with a concerted mechanism in which a pentadienyl radical is trapped by O_2 as it is formed, but rather indicates that a pentadienyl radical is formed as an intermediate that is subsequently trapped with oxygen. In the mechanism of eq 1, none of the labeled positions undergo any other changes that would be expected to yield an isotope effect so that this mechanism is unlikely.

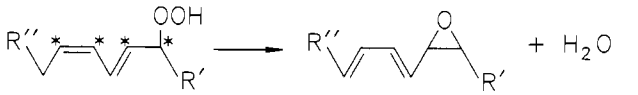
The observed isotope effect is also inconsistent with a mechanism involving homolytic addition to the double bond (eq 4). The mechanism of eq 4 requires an addition with rehybridization from sp^2 to sp^3 followed by an elimination with rehybridization from sp^3 to sp^2. Depending on the extent of rehybridization in the second step, either no isotope effect at all or a full inverse isotope effect would be expected for the overall reaction (Knier & Jencks, 1980). This is different from the observed normal isotope effect. The results for lipoxygenase contrast in this respect with those for the reaction of cytochrome P-450 with olefins. In this reaction an inverse isotope effect has been observed, consistent with substantial bond formation to the olefin at or before the rate-limiting step (Hanzlick & Shearer, 1978).

A relevant alternative source of secondary isotope effects would be an inductive effect (Melander & Saunders, 1987). These effects are generally small but detectable, especially in cases of multiple isotopic substitutions as here. The heavier isotope acts as a more electron-donating substituent in reactions involving formation of carbanionic species and would lead to a normal isotope effect for formation of a carbanionic intermediate (eq 3). The effect expected for the radical cation mechanism of eq 2 is not as clearly predictable from precedent in the literature, however, so that the oxidation of arachidonic acid by Ce(IV) was examined as a relevant model reaction. This reaction is predicted to proceed via a radical cation intermediate (Fukuzumi & Kochi, 1982), and no significant isotope effect, 1.02 ± 0.01, was observed. Similarly small inductive effects at cationic carbon centers have been observed, for example, in the epoxidation of olefins by peracids (Hanzlik & Shearer, 1975) and in the formation of charge-transfer complexes (Martens et al., 1979). The observed isotope effects, therefore, appear to be most compatible with a mechanism involving a carbanionic intermediate such as that of eq 3. The negative charge in a carbanionic intermediate (eq 3) could be distributed over as many as four isotopically labeled centers, and the observed isotope effect could therefore be as small as 1.04 per tritium. This small effect is in the correct direction and of the magnitude expected. The data, therefore, appear to support the hypothesis of Corey and Nagata (1987) of a carbanionic intermediate (chelated to iron) in the lipoxygenase reaction.

The above arguments assume that the observed isotope effect derives from chemical changes in the substrate that occur after binding to the enzyme. An isotope effect simply on binding of substrate to enzyme should perhaps also be considered. For example, separation of [^{3}H]- from [^{14}C]arachidonic acid is observed in reverse-phase HPLC systems. The origin of this effect is not well-defined but presumably derives from partitioning of the fatty acid between solvents of different hydrophobicities. The separation between peak maxima was 1 fraction at a total elution volume of 25 fractions in the system used in this work. This represents an isotope effect of 1.04, with the ^{14}C species binding more tightly. The isotope effect for HPLC represents the product of 500 or more theoretical plates, however, while binding to the enzyme represents only a single theoretical plate. Unless the enzyme shows exquisite sensitivity to isotopic substitution, therefore, an effect on binding similar to the effect in this (or other) chromatographic system is not likely to be observable. Further, NMR studies with a deuterated substrate have indicated that the substrate is relatively mobile when bound to soybean lipoxygenase (Viswanathan & Cushley, 1981). This would be inconsistent with such a high sensitivity to the small steric difference between hydrogen and tritium.

The rate of reaction of outer-sphere oxidants such as Ce(IV) with unsaturated hydrocarbons is correlated with the reduction potential of the cation radical that is formed in the reaction (Baciocchi et al., 1980; Fukuzumi & Kochi, 1982). By comparison of the rates of oxidation of arachidonic acid and cyclohexene by Ce(IV), it is possible to estimate the reduction potential for a radical cation from arachidonic acid in aqueous solution to be 2.7 V vs NHE (Fukuzumi & Kochi, 1982; assuming similar correlations for Ce(IV) and Fe(III) as oxidants). This reduction potential is close to the value of 2.75 V for 2-butene, as would be predicted (Miller et al., 1972). This potential represents a significant barrier to formation of a radical cation as an intermediate in the lipoxygenase reaction (eq 2) and reinforces the conclusions made on the basis of isotope effects that this mechanism is unlikely.

No isotope effect was observed in the formation of LTA_4 from 5-HPETE (1.00 ± 0.03). The reaction for formation of LTA_4 is shown in eq 5 (position of labeling indicated by



(5)

asterisks). Two of the isotopically labeled carbons undergo rehybridization in this reaction, and the calculated isotope effect for a productlike transition state or intermediate is 1.16 (Hanzlik & Westkaemper, 1980). The absence of an observed isotope effect therefore implies that there is very little carbon–oxygen bond formation preceding or concomitant with the rate-determining step, which is loss of the methylene hydrogen (Maas et al., 1982).

As pointed out above, the interpretation of the isotope effects for a multiply labeled substrate must be regarded as tentative. The observation of an effect, however, gives confidence that future studies with specifically labeled substrates will provide more detailed information about isotope-sensitive steps at individual carbons.

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Mechanism-Based Inactivation of L-Methionine γ-Lyase by L-2-Amino-4-chloro-4-pentenoate†

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ABSTRACT: L-2-Amino-4-chloro-4-pentenoic acid (L-ACP), an antibacterial amino acid produced by *Amanita pseudoporphyria* Hongo [Moriguchi, M., Hara, Y., & Hatanaka, S.-I. (1987) *J. Antibiot. 15*, 904–906], time dependently and irreversibly inactivates L-methionine γ-lyase. The inactivation obeys biphasic pseudo-first-order kinetics and is carried out completely with a minimum molar ratio ([L-ACP]/[enzyme tetramer]) of 5. During the incubation of enzyme, 4.4–5.0 mol of chloride ions is formed per mole of tetramer enzyme. The tetrameric enzyme is labeled with 4 mol of DL-[2-^{14}C]ACP/mol. We have isolated ^{14}C-labeled acetopyruvate and pyridoxamine 5′-phosphate from the [^{14}C]ACP-modified enzyme. The enzyme fully inactivated shows λ_{max} at 460 and 495 nm, which probably is derived from a conjugated pyridoximine paraquinoid. We have proposed a mechanism which involves enzymatic dehalogenation from C_4 of ACP to form a reactive allene. The allene is attacked by a nucleophilic amino acid residue at the active site. Analysis results of the thiol content of enzyme suggest that a cysteine residue is a possible nucleophilic residue covalently bound to the inactivator.

Mechanism-based enzyme inactivators are inherently inactive but are unmasked by the catalysis of the targeted enzyme itself to generate a reactive intermediate to inactivate the enzyme irreversibly. Thus, they show much higher selectivity toward the targeted enzyme than conventional affinity labeling agents (Walsh, 1982). Various functional groups, which can be activated by rearrangement or elimination, have been used for mechanism-based enzyme inactivators: e.g., acetylenes and olefins. The activated intermediates, generally electrophiles, react with a nucleophile at the active site as a Michael acceptor to inactivate the enzyme (Walsh, 1982).

L-2-Amino-4-chloro-4-pentenoic acid (L-ACP)[1] was isolated from fruit bodies of *Amanita pseudoporphilia* Hongo (Hatanaka et al., 1974), and it inhibits growth of various bacterial strains, e.g., *Bacillus subtilis*, *Escherichia coli*, and *Pseudo-*

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[1] Abbreviations: ACP, 2-amino-4-chloro-4-pentenoate; pyridoxal-P, pyridoxal 5′-phosphate; pyridoxamine-P, pyridoxamine 5′-phosphate; TFA, trifluoroacetic acid; HPLC, high-performance liquid chromatography; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); TNB, 5-thio-2-nitrobenzoate.

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