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HYDRATION OF AN ^{18}O EPOXIDE BY A CYTOSOLIC EPOXIDE HYDROLASE FROM MOUSE LIVER

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Summary

The mechanism of enzymatic epoxide hydration by a cytosolic or 100,000 g soluble mammalian liver enzyme (in contrast to the microsomal enzymes) was examined by monitoring ^{18}O distribution following chemical and enzymatic hydrations of ^{16}O or ^{18}O epoxide labeled (±) 1-(4'-ethylphenoxy)-3,7-dimethyl-6,7-epoxyoctane. Acid catalyzed hydration of the ^{18}O epoxide in ^{16}O water, and hydration of the ^{16}O epoxide in ^{18}O water, indicated that attack by water was predominantly on the tertiary carbon (C-7). Enzymatic epoxide hydration led to attack predominantly on secondary carbon (C-6). These data are consistent with water attacking as a nucleophile in the enzymatic reaction.

Mammalian epoxide hydrolases (E.C.3.3.2.3.) are important in the metabolism of epoxide containing xenobiotics and natural metabolites, some of which are toxic, mutagenic, and/or carcinogenic. Epoxide hydrolases in the microsomal (or 100,000 g) pellet of mammalian tissues have received intense investigation (1,2), and it is commonly assumed that little epoxide hydrolase activity is in the 100,000 g soluble, or cytosolic, fraction. Recent studies indicate that for many aliphatic epoxides other than styrene oxide, the initial rates of hydration are higher in the cytosolic fraction than in the microsomal fraction (3-5). The cytosolic enzyme hydrates a wide structural variety of epoxides of pharmacological and environmental interest including known mutagens (5). Some investigations have been undertaken to elucidate the mechanism of epoxide hydration by the microsomal enzymes (for references see 1,5-10), but no such investigations have been previously reported on the cytosolic enzyme. An understanding of the mechanism of hydration is crucial for a rational prediction of substrate selectivity and potential enzyme inhibitors.

The hydration of over 10 pairs of geometrical isomers of aliphatic epoxides by the cytosolic hydrolase has indicated pure trans hydration in each case (i.e., trans-epoxide yields the erythro-glycol and cis-epoxide yields the threo-glycol) (4). Such an observation is consistent with either an acid or base catalyzed mechanism of hydration (electrophilic or nucleophilic attack). However, the relative rates of hydration of mono-, di-, and trisubstituted epoxides of varying hydrophobicity (mono $\gtrsim$ cis-1,2-di > trans-1,2-di > tri > tetrasubstituted) is not consistent with the formation of a carbonium ion-like intermediate (as is observed with acid catalyzed hydration) being the rate limiting step in the reaction (4,5). The following study was designed to gain insight into the mechanism of epoxide hydration by the cytosolic fraction.

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Methods

Synthesis of substrates and standards. The terpenoid olefin (I, Fig. 1) was prepared by a Williamson ether synthesis from p-ethylphenol (Aldrich) and racemic citronellol (Givaudan) by published procedures (11-14). The olefin (I) (1 mmol) was oxidized with N-bromosuccinimide (1.1 mmol) in freshly distilled tetrahydrofuran (2.2 ml) containing ^{16}O or ^{18}O (90 mole%) water (200 µl) to the corresponding bromohydrins. The bromohydrins were cyclized in methanolic base to obtain the ^{16}O and ^{18}O labeled epoxides (II and III, respectively) which were purified by preparative thin-layer chromatography (tlc, 2 mm silica gel, Analtech, hexane ether 5 1) (7). The ^{16}O epoxide was hydrolyzed in sulfuric acid (0.05N) in 40% aqueous tetrahydrofuran with ^{16}O or ^{18}O water to yield the C-6,7 ^{16}O diol (IV) and C-7 ^{18}O diol (V). The ^{18}O epoxide was similarly hydrolyzed to yield the C-6 ^{18}O diol (VI, Fig. 1) (15-17). The structures of all compounds were supported by their nuclear magnetic resonance spectra and demonstrated one spot or peak on tlc, gas liquid chromatography (glc), and high resolution liquid chromatography (hrlc).

Enzyme preparation. The 100,000 g soluble or cytosolic liver subcellular fraction was obtained as described previously from 90-day old male, specific pathogen free, Swiss-Webster, ICR strain mice (Hilltop Laboratories, Chatsworth, CA) (3,4). The 10% w/v supernatant in pH 6.8, I=0.2M sodium phosphate buffer was passed through a Sephacryl S-200 column, and the fractions demonstrating epoxide hydrolase activity were combined and diluted to contain hydrase activity equal to a 1% w/v liver supernatant (elution volume is just ahead of the red heme band and is consistent with a molecular weight of 130,000). All operations were performed below 5°C and recovery of enzyme activity was >90%. Previous studies indicate that the diol is the sole product formed in the enzymatic reaction with the crude or partially purified epoxide hydrolase and that all epoxide hydrolase elutes from gel filtration as a single sharp peak (3-5,16,18).

Enzyme assays. Routine assay for determination of enzyme activity was performed as described earlier with a compound similar to II except for the presence of 2,3 unsaturation and ^{3}H or ^{14}C in the aromatic nucleus (4). The citronellol derivative actually used for all mechanistic studies was enzymatically hydrated at half of the rate of the unsaturated compound when ether extracts of enzyme reactions were assayed by glc with a flame ionization detector. The extent of epoxide hydration was determined in small samples (1 ml) of the enzyme incubations used for mass spectral (ms) analysis by determining the epoxide.diol n-butyl boronic diester ratios following glc on 1% OV 101 on Gas Chrom Q as described previously (4,14).

Enzymatic hydration of ^{18}O epoxide. All glassware, the sodium chloride, and the sodium sulfate were washed with ether and dried (150°C) before use. Enough ^{18}O labeled epoxide (III) in ethanol (1 ml) was added to either the enzyme fraction described above (100 ml) or the enzyme fraction containing 10% methanol (100 ml) to yield final substrate concentrations of $2x10^{-5}$ or $1x10^{-4}M$, respectively. The critical micelle concentration of the substrate is estimated to be about $10^{-5}M$. The cytosolic hydrolase has been previously shown to hydrate only the monomeric form of the substrate, although the micelle form neither enhances nor inhibits the reaction. Thus, methanol often increases the enzymatic hydration of aliphatic epoxides by solubilizing the micelle form of the substrate (4,5). The epoxide hydrolase activity had been previously titered in the fractions used with identical substrate concentrations and incubation conditions but on a smaller scale. The enzyme fractions were incubated with shaking at 37.5°C until the reactions were estimated to be 45%

complete, based on the small scale reactions. The reactions were halted by the addition of cold methanol (160 ml), small samples were removed for glc analysis, and the remaining material was extracted with petroleum ether (Nanograde, equal volume). This procedure removed most of the epoxide and essentially no diol. The aqueous phase was saturated with sodium chloride and extracted with ether petroleum ether (1:1). The residue from the organic extract was partially purified by pouring it through a 0.5 cm diameter glass column containing, sequentially, sodium sulfate (2 ml), Florisil (10 ml), and sodium sulfate (3 ml). The column was eluted with 20% ether in petroleum ether to remove nonpolar contaminants and residual epoxide, and the diol was subsequently eluted with 25 ml of ether. The organic residue following solvent evaporation was purified by hrlc. Ether in hexane (40% v/v) was delivered with a Spectra-Physics Model 740B pump at 2.4 ml/min and the effluent from the column (3x250 mm, 5 μm Spherisorb silica) was passed through a Spectra-Physics Model 230 ultraviolet (uv) monitor before being collected in small tubes. Under these conditions the column yielded about 5,000 theoretical plates and the retention time of the single uv absorbing product (7.9 min) corresponded exactly to that of authentic diol standard. The peak heights of the diol products corresponded to the enzymatic reactions being ~35 and 50% complete, respectively, based on an estimate of ~80% recovery for the preceding steps as determined by a comparative analysis using radiolabeled diol of the corresponding 2,3-unsaturated analog. The hrlc fractions corresponding to the central part of the diol peak were pooled and the concentrations of the diols estimated again based on their uv absorbance (λ_{max} 230 nm) when compared to a standard curve of authentic diol, and by glc of their n-butylboronic diesters. Nonenzymatic conversion was estimated using heat denatured protein samples incubated for equal times and similarly analyzed.

Mass spectroscopy. The epoxides and both the chemically and enzymatically produced diols were analyzed by glc/ms on a Hewlett-Packard Model 5985 glc/ms/data system in the electron impact mode. The transfer lines and jet separator were at 275°C, the source at 200°C, and the glc injector at 250°C. The ionizing voltage was 70eV. The epoxides were chromatographed on a 2 m x 2 mm i.d. glass column packed with a carbowax modified support (19) held at 180°C. The diols were chromatographed on a column prepared from the same support but coated with 3% OV-17 and held at 265°C. Helium was the carrier gas for both columns at 40 cc/min. Under these conditions the epoxides had a retention time of 2.0 min and the diols a retention time of 2.4 min.

The ^{18}O content of the intact diol was determined by monitoring the molecular ions for both the ^{16}O and ^{18}O isotopes (M/M+2). Similarly, the ^{18}O distribution at C-7 was determined by monitoring the ions m/z 59 and m/z 61 (Fig. 2). For both cases, contributions to the ^{18}O ion intensity from natural isotope abundances were subtracted. The ^{18}O content at C-6 was obtained from the difference in the atom percentage values for the molecular ion and m/z 59. At least one full spectrum was obtained for each product to confirm its structure in addition to the runs made for the isotope analysis work. The primary fragmentation of the diol generates ions with charge retention on the aromatic moiety, these are not useful for determination of isotopic composition. Alpha-cleavage ions from the diol moiety were expected to be strong. The ion at m/z 59 was intense enough to be useful, however, the corresponding fragment at m/z 235 was not only weak, but accompanied by a more intense ion at m/z 236, the latter may be the protonated form of m/z 235 but was not sufficiently characterized for isotopic studies. Prior ^{18}O distribution studies from chemically hydrated juvenile hormone I (15) were facilitated by the primary, dominant fragmentation of the diol to strong α-cleavage ions, allowing facile isotopic distribution analysis. The difference method (Table I) was adequate for determining the major site of ^{18}O in the

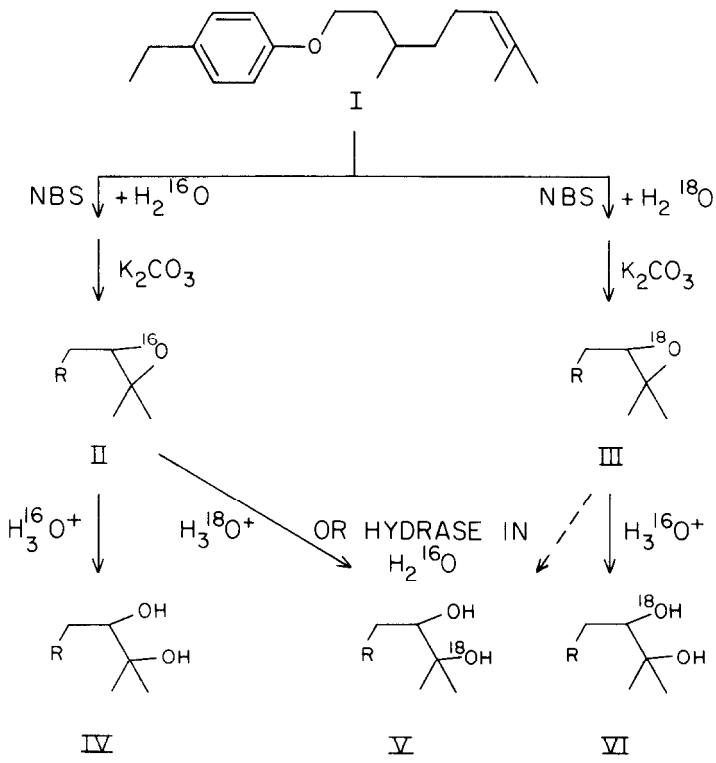

FIG. 1

Synthetic pathways for ^{16}O and ^{18}O epoxides and their subsequent hydration by aqueous acid or cytosolic epoxide hydrolase (hydrase). The isotopic distribution of ^{18}O at C-7 and C-6 indicate that acid catalyzed hydration proceeds largely by attack by water on C-7 and the enzyme catalyzed reaction largely by attack by water on C-6 (dotted line).

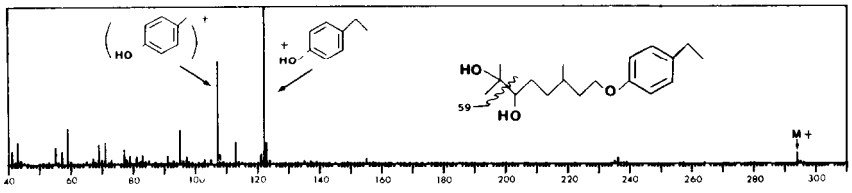

FIG. 2

Fragmentation pattern of the ^{16}O diol.

diols although the experimentally determined incorporation ratios are likely to have some variability due to the low intensity of the ions monitored.

TABLE I

^{18}O Atom Percentage Values in Epoxide Substrates, Synthetic Diol Standards, and Enzymatically Produced Diols

Compound	Source	^{18}O Molecular Ion (M^+)	^{18}O C-7 (61/61 +59)	^{18}O C-6 (M^+-61/61 +59)	% Attack by Water at C-6[a]
Epoxide II	Synthetic	0.5	-	-	-
Epoxide III	Synthetic	84.6	-	-	-
Diol IV	From epoxide II in $H_2{}^{16}O$	1.3	0.9	0.4	-
Diol V	From epoxide II in $H_2{}^{18}O$	83.5	79.6	3.9	4.7
Diol VI	From epoxide III in $H_2{}^{16}O$	85.5	4	81.5	4.7
Diol	Enzymatic +MeOH from epoxide III	84.0	77.7	6.3	99.5
Diol	Enzymatic -MeOH from epoxide III	86.0	75	11	96.3

[a]Calculated from ^{18}O atom percentage at C6 divided by atom percentage in molecular ion for synthetic diols or its reciprocal in the case of diol VI. For enzymatically produced diols, the contribution to incorporation at C-6 from non-enzymatically produced diols is 95.3% of the 3% non-enzymatic conversion. Total conversion of epoxide to diol from glc analysis was 50% in the case when 10% methanol was used as a co-solvent and 35% when it was not. The data presented is from the average of several glc/ms runs (±1-3% of the reported values).

Results and discussion

As recently illustrated by Hanzlik and Shearer (21), it is important to synthesize all of the possible products when isotope distribution is determined by ms analysis. The synthesis of diols specifically labeled with ^{18}O on C-7 (tertiary carbon) or C-6 (secondary carbon) was based on similar studies using insect juvenile hormone I. In the latter studies, the acid-catalyzed attack by water was almost exclusively at the tertiary carbon (97%) (15,21). The lower specificity of water attack at the tertiary carbon in this study (95.3% in both cases) may be due in part to a measurement error discussed above, or slightly differing substrate and hydrolysis conditions. The specificity is still quite high as could be predicted from the mechanism of electrophilic epoxide hydration (20).

Glc of the quenched reactions indicated that the reaction in the presence of methanol was slightly faster than anticipated from small scale (1 ml) reactions (estimate 45%, found 50%) while that without methanol was slightly slower (estimate 45%, found 35%). Nonenzymatic hydration by heat denatured protein accounted for approximately 3% of the total epoxide substrate added in each enzymatic assay. When estimating the percent attack by water at C-6 (Table I) nonenzymatic hydration was found to occur 95.3% by attack of water at C-7. The purity of the enzymatically produced diol following hrlc is supported by the identical estimates of purity based on uv and glc response as well as by very clean glc-ms tracings. The ms of the enzymatically produced diols were identical with that of synthetic diol V. Differences in the spectra

of diols IV and VI could be accounted for entirely based on ^{18}incorporation.

Regardless of the procedure used to estimate the percent incorporation when epoxide hydration is catalyzed by the partially purified cytosolic enzyme, the attack by water appears to be largely on the secondary C-6 carbon. Clearly the enzymatic hydration is not by a simple electrophilic (acid catalyzed) mechanism which would lead to attack by water primarily on the tertiary C-7 carbon. The data presented here are thus consistent with an enzymatically activated water molecule (7,10) or hydroxide ion attacking the less hindered carbon. The data certainly do not rule out electrophilic polarization of the epoxide by the enzyme (7) in an environment in which the water is only allowed access to the less hindered carbon or the possibility of an electrophilic mechanism with enzymatic stabilization of the incipient carbonium ion at C-6. Additional substrates need to be examined in order to determine the influence of electronic and steric factors on the regiospecificity of epoxide hydration catalyzed by the cytosolic epoxide hydrolase. Evidence to date on the mechanism of epoxide hydration by the cytosolic enzyme indicates that it may proceed in a manner similar to that observed for the microsomal enzyme, but the possible involvement of both imidazole and sulfhydryl groups at the active site as well as different substrate selectivities indicates some differences in mechanism (1,2,5-10).

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