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ISOLATION OF A PUTATIVE HYDROXYACYL ENZYME INTERMEDIATE OF AN EPOXIDE HYDROLASE

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Summary. A putative covalent, α-hydroxyacyl intermediate was isolated by the brief exposure of murine soluble epoxide hydrolase to its substrate. The reaction was reversed by time and blocked by competitive inhibitors. The formation of the intermediate was dependent upon the concentration of the enzyme and was increased by incubation under acidic conditions. The structure of the intermediate was supported by microchemical methods.

Epoxide hydrolases (E.C. 3.3.2.3) add water to strained 3 membered cyclic ethers known as epoxides (1). Recently several soluble epoxide hydrolases have been cloned (2-4). The sequence of these enzymes showed little overall homology to other proteins including microsomal epoxide hydrolases. However, Arand et. al. (5) noted that certain regions of the epoxide hydrolase showed high homology to a family of enzymes known as the haloalkane dehalogenases and these regions in turn were similar to the mammalian microsomal epoxide hydrolase. These dehalogenases and by inference the epoxide hydrolases belong to a diverse group of enzymes known as the α/β hydrolase fold family (6). When the X-ray structure of the substrate complex with the haloalkane dehalogenase of *Xanthobacter autotrophicus* was published (7), Lacourciere and Armstrong (8) and Arand et. al. (5) noted that the residues suggested to be catalytic in haloalkane dehalogenase were highly conserved in the soluble and microsomal epoxide hydrolases, respectively. A two step process is suggested for the hydrolysis of haloalkanes by haloalkane dehalogenase in which an acyl enzyme forms following attack on the substrate by a nucleophilic aspartic acid. Upon analogy a similar mechanism could occur with epoxide hydrolases, and low turn over experiments with $H_2{}^{18}O$ provide evidence for this hypothesis with the microsomal epoxide hydrolase (8). This

ABBREVIATIONS AND SYMBOLS:
JH, juvenile hormone; SDS PAGE, sodium dodecylsulfate polyacrylamide gel electrophoresis; BBA, *n*-butylboronic acid; BSA, bovine serum albumin; SNPG, (S,S) *trans*-3-(4-nitrophenyl) glycidol; FCO, 4-fluorochalcone oxide.

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proposed two step mechanism also predicts that one should be able to isolate a covalent intermediate between the substrate epoxide and the enzyme.

In an attempt to isolate this intermediate we selected the substrate juvenile hormone III (JH III, Fig. 1) for several reasons. Unlike many epoxides this substrate is relatively stable at low pH (9). It appears to have a high affinity for the enzyme, however JH and other trisubstituted epoxides are turned over much slower than 1, 2-disubstituted epoxides and monosubstituted epoxides (10). The hypothetical α-hydroxyacyl enzyme (Fig. 1) resulting from the attack of the nucleophilic aspartic acid should be relatively stable to base hydrolysis. Unlike most arene oxides or styrene oxide, one does not anticipate that JH will react with any functional groups on a protein unless the epoxide is activated by the enzyme. Finally, a variety of chemical standards are available for this compound from previous studies and a tritiated form is commercially available at over 10 Ci/mM. On the negative side JH III forms micelles at approximately $5 \cdot 10^{-4}$ M limiting the concentration of the enzyme that can be used.

MATERIALS AND METHODS

Enzyme preparation: The murine soluble epoxide hydrolase (3) was produced in a baculovirus expression system as described for the human enzyme (4) then purified from cell lysate by affinity chromatography to >95 percent homogeneity as determined by SDS PAGE and densitometry (11).

Enzyme labeling: In all experiments racemic C10 ^{3}H JH III from New England Nuclear was used diluted with unlabeled JH III (12) to give a $5 \cdot 10^{-3}$ M solution in ethanol. One μl of this solution was added with a Hamilton repeating dispenser to 100 μl of incubation media in a 6x50 mm glass tube held in an ice bath. In Fig. 2A the solution was mixed on a Vortex for 3 seconds at room temperature (unless indicated otherwise), 10 percent (v/v) acetic acid in water added to give the indicated pH, mixed for 3 seconds and the reaction quenched with 5 volumes of acetone at -20°C. The pH was determined on a 10 ml scale with a Corning 125 meter standardized against a pH 4 standard from Fisher Scientific. After >1 hr at -20°C the tubes were centrifuged at 7,000 g for 45 min. at -10°C. Aliquots of the supernatant were analyzed by liquid scintillation counting and TLC as described below. For TLC the acetone was shaken with dry Na_2HCO_3, dried over Na_2SO_4, and concentrated before spotting with unlabeled standards. The pellet was washed sequentially twice each with acetone and ethyl ether then resuspended in 50 μl of 1 percent aqueous acetic acid and twice reprecipitated and washed as described above. Approximately 95 percent of the ^{3}H was removed with each of the first 4 washes with organic solvent. After the first resuspension and wash the counts in the supernatant were at background levels. The experiments shown in Fig. 2B, 2C, and 2D were run in the same way except that 1 percent glacial acetic acid in acetone (v/v) was used for quenching reactions. After the final wash the pellet was redisolved in 5 percent (w/v) SDS (100 μl) and transferred to a scintillation vial in 3 sequential 100 μl water washes. All counts were corrected for differential quenching.

Microchemical analysis of α-hydroxyacyl enzyme: To evaluate the chemical nature of the ^{3}H associated with the precipitated protein, the radioactive enzyme was isolated as described above using a concentration of 25 μg of epoxide hydrolase per 100 μl of 200 mM sodium acetate buffer at pH 4.7. After the work up described above involving 3 precipitations, the pellet was dried in a Speedvac and 10 μg of unlabeled JH diol was added. Parallel reactions were run with ^{3}H JH, JH diol, and JH acid on a small scale and with these nonlabeled compounds as well as JH, JH diol, and methoprene (a related compound with a C11 methoxy

rather than an epoxide and an isopropyldienoate ester) on a larger scale to yield a variety of UV dense standards for TLC analysis. One tube of ^3H labeled protein was reacted with 100 µl of 1 M $LiAlH_4$ (Aldrich Chemical) for 30 min followed by addition of a small amount of ethyl acetate to quench the hydride and 200 µl of 3N HCl to dissolve the aluminum complexes. A second tube was reacted with 100 µl of 0.5 N NaOH in ethanol for 2 hr at 70°C (sufficient time to cleave the methoprene isopropyl ester as well as the JH methyl ester) followed by acidification with 1 N HCl. Both reactions were worked up by adding NaCl and using three sequential extractions with ethyl acetate (3·200 µl). Less than 10 percent of the original radioactivity remained in the aqueous phase and approximately 70 percent of the radioactivity was in each organic phase. The ethyl acetate fractions were dried over Na_2SO_4, the organic extract from the base reaction was divided in half, and the ethyl acetate in all three tubes removed on a Speedvac at ambient temperature. BBA (1 percent w/v in ethyl acetate, 100 µl) was added to one of the tubes from base treatment. The synthetic standards and model reactions with radioactive standards as well as the three samples from the putative hydroxyacyl enzyme intermediate were spotted on the cellulose prelayer of a Whatman Silica gel 60A fluorescent plate. The plate was developed 3 cm in 1 percent glacial acetic acid in ethyl acetate, dried under vacuum and then developed in hexane:ethyl acetate:acetic acid (70:40:1) for 20 cm. The unlabeled standards were visualized based on their quenching of ultraviolet fluorescence at 254 nm and the plate was then analyzed on a Bioscan Radioactivity Monitor. For analysis of the radiolabeled products chemically released from the putative α-hydroxyacyl enzyme of the epoxide hydrolase, the areas corresponding to the unlabeled standards were scraped into vials and the remainder of the lane scraped into vials in 1 cm sections and all samples analyzed by liquid scintillation counting.

RESULTS AND DISCUSSION

Several techniques were used to precipitate the putative covalent intermediate between ^3H JH and the epoxide hydrolase, but the acetone system was selected as a very rigorous method of removing a lipophilic substrate and product from a protein. By monitoring ^3H in the organic solvent, it was found that the successive washes with acetone and ether removed most radioactivity even from the initial pellet. However, the extensive washing procedure described above was retained for all of the experiments in this paper as evidence for covalent attachment. Even with a substrate like JH that is turned over slowly, large amounts of epoxide hydrolase will rapidly cleave all epoxide to the corresponding diol even at low temperature (Fig. 1). Thus, small amounts of enzyme were used in Fig. 2A. It was not certain that such small amounts of enzyme could be precipitated, so a BSA carrier was used at pH 4 and found to make little difference in the amount of ^3H precipitated by acetone. Surprisingly some apparent acyl enzyme could be precipitated even at pH 7.4, however it was clear from these data that the yield of radioactive protein was higher under acidic conditions. Thus, in subsequent experiments acidic acetone at -20°C was used for the first precipitation. No degradation of JH (TLC Rf 0.67) was detected upon immediate analysis of the acidic acetone, analysis after 2 hours at room temperature, after 24 hours at 0°C or following extraction of any of the BSA controls. This observation and previous work on JH stability (9) also illustrates that JH would not be expected to react with a carboxylic acid in an enzyme within seconds at low temperature unless the pK_a of the acid were reduced over that of a free aspartic acid or unless its epoxide is activated in some way. Thus a push pull mechanism for the

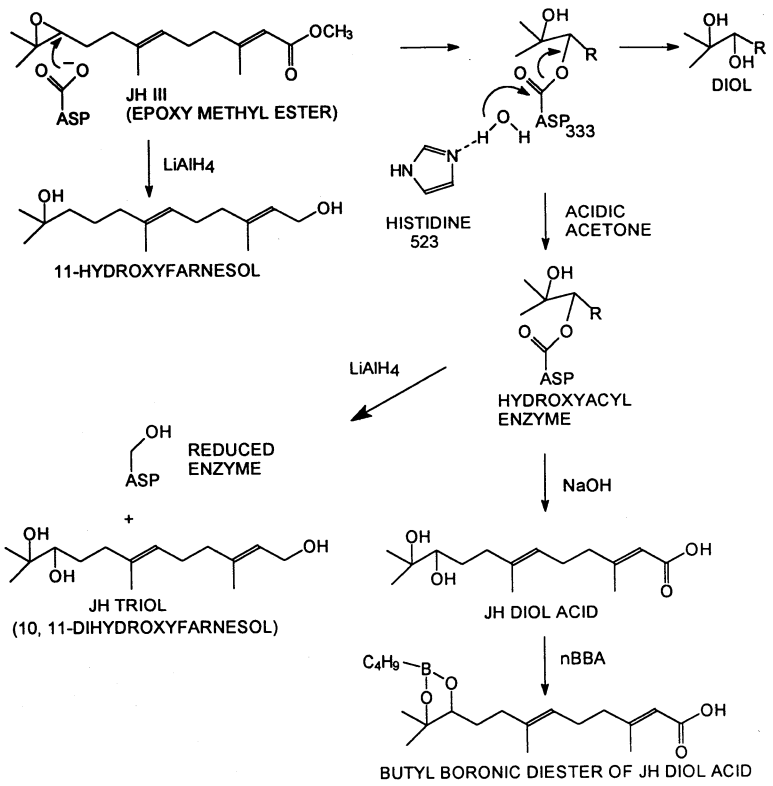

Fig. 1. The hypothetical two step catalytic mechanism of the epoxide hydrolase (top line) should involve attack of a nucleophilic ASP_{333} on the epoxide polarized by TRP_{334} and/or additional acidic groups followed by hydrolysis of the resulting α-hydroxyacyl enzyme by a HIS_{523} activated water. Treatment with acidic acetone should denature the enzyme and trap the covalent intermediate (center). Reduction or chemical hydrolysis followed by co-chromatography with synthetic standards was used to support the structure of the intermediate shown. If the substrate was simply trapped one would expect reduction to yield 11-hydroxyfarnesol which was not found. In contrast hydride treatment of the precipitated radioactivity yielded a product that co-chromatographed with the 10, 11-dihydroxyfarnesol. Base hydrolysis yielded a product co-chromatographing with JH diol acid whose structure was further supported by formation of the BBA diester.

formation of the acyl enzyme is supported indirectly. In haloalkane dehalogenase this activation is due to protonation of the substrate by two tryptophans, at least one of which is conserved in the epoxide hydrolases. The JH diol (Rf 0.3) was the only metabolite detected in all incubations with epoxide hydrolase. Its structure was supported by derivatization as the BBA diester (Rf 0.65). The soluble epoxide hydrolase has a clear pH optimum near neutrality and the catalytic activity falls off at lower pH's suggesting titration of a histidine residue. If the reaction mechanism shown at the top of Fig. 1 is correct, then one should observe increasing radioactivity bound to the epoxide hydrolase as the ability of histidine to activate water and regenerate the catalytic aspartic acid is reduced at lower pH's. The results shown in Fig. 2B support this hypothesis and indicate that acid in the acetone alone is not sufficient to trap the intermediate totally. At low pH labeling of the enzyme by JH was essentially stoichiometric. Fig. 2C demonstrates an increase in radiolabeled protein with an

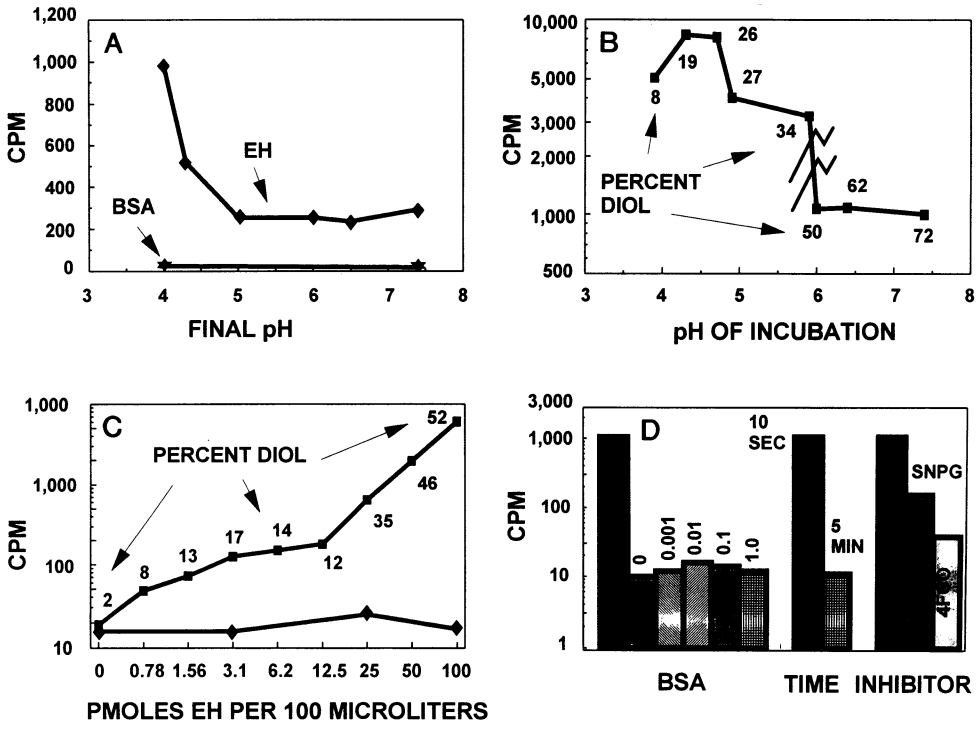

Fig. 2. Binding of ^{3}H JH III to the murine soluble epoxide hydrolase. In each case cpm on the y axis shows the total ^{3}H precipitated following extensive washes. All incubations contained $5 \cdot 10^{-5}$M substrate (ca 200 cpm/pmole) in 100 µl with precipitation of the protein and washing as described in the text. Note the logarithmic Y axis in graphs B-D. A. Effect of acetic acid on substrate binding. Three seconds after the addition of substrate, acetic acid (10 percent v/v) was added to give the indicated pH followed immediately by cold acetone. Incubations were in 90 mM sodium phosphate at pH 7.4 and included 10 pmoles epoxide hydrolase (♦) or 0.01 mg BSA (ca 167 pmole, ★). Apparently identical results were obtained at a final pH of 4 with 10 pmoles of epoxide hydrolase in the presence of 0.01 mg of BSA. B. Effect of incubation pH on substrate binding. Epoxide hydrolase (25 pmoles) was preincubated for 10 min at the indicated pH, then substrate was added and the reaction quenched with acetic acetone. The pH's were measured at 22°C. At pH's of 5.89 and below the buffer was 200 mM sodium acetate and at pH 6 and above it was 90 mM sodium phosphate with BSA included in no case. The numbers indicate the percentage of total radioactivity in the first acetone wash as diol. C. Effect of epoxide hydrolase concentrations on substrate binding. Varying amounts of epoxide hydrolase were preincubated in phosphate buffer at pH 7.4 with 0.001 mg BSA (■) or varying amounts of BSA were incubated (◆) and the reactions initiated and quenched as above. Data are averages of 2 replicates. The numbers indicate the percentage of total soluble radioactivity as diol. D. Lack of JH III binding to protein. In each case the solid bars indicate the binding of JH to 25 pmoles (3.1 µg) of epoxide hydrolase (1058±25 cpm) at pH 7.4 in 90 mM phosphate buffer. No binding above background was detected with BSA even at 1 mg per 100 µl incubation (mg BSA per tube are indicated above bar). When the enzyme was incubated for 5 min at 22°C rather than 10 sec at 0°C, all of the substrate was converted to diol and no binding was detected. The inhibitors SNPG at $5 \cdot 10^{-4}$M and FCO at $5 \cdot 10^{-5}$M reduced counts bound to 155±10 and 38±18, respectively. Due to the low substrate concentrations used, the extensive washing procedures and very short incubation times, the data in Fig. 2 should be taken as qualitative support for the two step hypothesis, but not a kinetically valid approach.

increase in enzyme concentration at pH 7.4. The lack of linearity is probably due to the problems in the technique used. As shown in Fig. 2D even very high concentrations of BSA up to 1 mg per 100 µl incubation resulted in no radioactivity detected above background. Long incubations (5 min vs 10 sec) resulted in total conversion of JH to the diol and no radioactivity bound to the protein. The binding of JH to epoxide hydrolase was reduced dramatically by preincubation of the epoxide hydrolase with the selective, competitive inhibitors S,S-3-(4'-nitrophenyl)glycidol (13) or 4-fluorochalcone oxide (14).

To further support the structure of the α-hydroxyacyl enzyme proposed in Fig. 1, labeled protein was treated in two ways. In the first case the hypothetical covalent ester was cleaved by reduction with $LiAlH_4$. The resulting radioactive product co-chromatographed with the unlabeled 10,11-dihydroxyfarnesol (Rf 0.19, Fig. 1) produced by simultaneous reduction of the synthetic JH diol. If JH were simply trapped by the enzyme one would expect it to be released upon reduction as 11-hydroxyfarnenol (Rf 0.34, Fig. 1). No radioactivity co-chromatographed with the 11-hydroxyfarnesol formed from the reduction of the epoxide substrate, and little radioactivity was found elsewhere on the TLC plate. In the second case the protein was exposed to base and the resulting product co-chromatographed with JH acid diol (Rf 0.23, Fig. 1) resulting from hydrolysis of both the C10 aspartic acid ester and the C1 enoate methyl ester. When this material was exposed to BBA, the radioactivity co-chromatographed with the corresponding diester (Rf 0.32) indicating that the product was likely a diol. Neither the basic nor the reductive conditions would be expected to cleave an ether or thioether of a denatured enzyme suggesting that the nucleophile of epoxide hydrolase is a carboxylic acid, most likely an aspartic acid consistent with other members of this branch of the α/β-hydrolase fold family (6).

With both the microsomal and soluble epoxide hydrolase, a variety of lines of evidence suggest that hydration of the epoxide occurs by an attack on one epoxide carbon by activated water resulting in inversion with retention of configuration at the other carbon (1). As early as 1980 Armstrong et. al. (15) pointed out that the available mechanistic data could not distinguish between direct attack by active water on the epoxide and a two step mechanism involving an attack by a nucleophilic acid. Workers who cloned haloalkane dehalogenase and related enzymes noted slight similarity between this prokaryotic enzyme and the mammalian epoxide hydrolase (16). However when a series of soluble mammalian epoxide hydrolases were sequenced, the relationship of the C-terminal region of this enzyme to haloalkane dehalogenase was clear (5). Since similar regions of high homology appeared in the mammalian microsomal epoxide hydrolase, it seemed that these enzymes were related and that they were members of a large family of enzymes of diverse catalytic function known as the α/β-hydrolase fold enzymes. This family of enzymes in many cases has lost much of its sequence homology, but retains similar 3 dimensional structures and spatial arrangement of a catalytic triad analogous but not homologous to the catalytic triad described for chymotrypsin (17). The publication of an X-ray structure of the substrate enzyme complex of haloalkane dehalogenase supporting a two step mechanism with a nucleophilic aspartic acid attacking a halogenated carbon strongly suggested that the epoxide hydrolase should act in the

same way. Most members of the α/β-hydrolase fold family use a nucleophilic serine (esterases and lipases) or cysteine (diene lactone hydrolase). These nucleophiles would form very stable ethers or thioethers with an epoxide, however, a nucleophilic aspartic acid would form an α-hydroxyacyl enzyme. This covalent intermediate should be able to be hydrolyzed by the attack active water on the aspartic acid carbonyl. Thus the data in this paper are consistent with the epoxide hydrolases hydrolyzing epoxides in a two step mechanism involving an α-hydroxyacyl intermediate in a fashion analogous to haloalkane dehalogenase. If this mechanism holds for other epoxide hydrolases, it could offer a simple way to radiolabel enzymes for purification.

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