Interaction of Hepatic Microsomal Epoxide Hydrolase Derived from a Recombinant Baculovirus Expression System with an Azarene Oxide and an Aziridine Substrate Analogue†

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Received October 2, 1992; Revised Manuscript Received December 29, 1992

ABSTRACT: A recombinant baculovirus (vEHX) encoding rat hepatic microsomal epoxide hydrolase has been constructed. Infection of *Spodoptera frugiperda* (Sf9) cells with the recombinant virus results in the expression of the enzyme at a level estimated to be between 5% and 10% of the cellular protein. The enzyme, which can be purified in 15% yield by a simple three-step procedure involving detergent extraction, DEAE-cellulose chromatography, and removal of the detergent on hydroxylapatite, has physical and kinetic properties very close to those of the enzyme obtained from rat liver microsomes. The interaction of the enzyme with two nitrogen-containing analogues of the substrate phenanthrene 9,10-oxide (**1**) was investigated in order to delineate the contributions of the oxirane group and the hydrophobic surface of the substrate to substrate recognition. The enzyme exhibits altered kinetic properties toward 1,10-phenanthroline 5,6-oxide (**2**) in which the biphenyl group of **1** is replaced with a bipyridyl group, suggesting that hydrophobic interaction between the complementary surfaces of the substrate and active site has an influence on catalysis. The conjugate acid of the aziridine analogue of **1**, phenanthrene 9,10-imine (**3**), in which the oxirane oxygen is replaced with NH, has a pK_a of 6.1, which allows the characterization of both the neutral and protonated aziridine ($3H^+$) as substrate analogues for the enzyme. The pH dependence of the solvolysis reveals that $3H^+$ rearranges to a 65/35 mixture of 9-aminophenanthrene and 9-amino-10-hydroxy-9,10-dihydrophenanthrene 10^3-fold faster than does **3**. The neutral aziridine is a competitive inhibitor (K_i = 26 μM) of the enzyme at pH 8. The K_i increases to 120 μM at pH 6.5, suggesting that $3H^+$ is not a transition-state analogue for the enzyme-catalyzed hydration of **1**. Neither **3** nor $3H^+$ appears to be a substrate for the enzyme. The results are consistent with the notion that protonation of the oxirane oxygen is not an important aspect of transition state for the enzyme-catalyzed hydration of arene oxides.

Microsomal epoxide hydrolase (EC 3.3.2.3) catalyzes the addition of water to epoxides and arene oxides and is, as a consequence, involved in the metabolism of a large number of xenobiotic compounds bearing this functional group. The primary structures of the enzyme from three different species (rabbit, rat, and human) have been determined or deduced from cDNA clones (Heinemann & Ozols, 1984a,b; Porter et al., 1986; Jackson et al., 1987; Skoda et al., 1988; Hasset et al., 1989). Hydropathy plots of the primary structure and chemical modification suggest that the enzyme is anchored to the membrane of the endoplasmic reticulum by a hydrophobic N-terminal α-helix with the bulk of the protein exposed on the cytoplasmic side of the membrane (Craft et al., 1990). This view is certainly consistent with the ease with which the enzyme can be extracted from the microsomal membrane, its stability in aqueous solution, and its formation of high molecular weight oligomers or aggregates in the absence of detergents (Lu et al., 1975). The molecular and catalytic properties of a number of purified enzymes have been reviewed (Lu & Miwa, 1980; Seidegard & DePierre, 1983; Armstrong, 1987).

Membrane-bound proteins from eukaryotes are often difficult to isolate or to produce in their active or native form with common plasmid-based bacterial expression systems, a fact that often limits the investigation of their functional properties. Epoxide hydrolase appears to be no exception. Although one recent report suggests that efficient expression in *Escherichia coli* is possible (Bell & Kasper, 1991), attempts in our laboratory to produce useful quantities of microsomal epoxide hydrolase with several common bacterial expression systems have not yet been fruitful. For this reason an alternative expression system for this membrane-bound enzyme was sought. Among the numerous alternatives available, including yeast and COS-1 cells, in which the human enzyme has been expressed at low levels (Skoda et al., 1988; Eugster et al., 1991), a baculovirus-based expression system appeared to be the most feasible with respect to the ease of construction and the potential for producing usable quantities of protein. In addition, epoxide hydrolase was deemed a good initial candidate to test the production of microsomal detoxification enzymes in baculovirus-infected cells since the enzyme is not extensively buried in the membrane and is neither proteolytically processed nor posttranslationally modified when

† This work was supported in part by grants from the National Institutes of Health (GM30910) to R.N.A. and the U.S. Department of Agriculture (90-37153-5557) to V.N.V.

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0006-2960/93/0432-2610$04.00/0

synthesized in its native habit (DuBois et al., 1979; Craft et al., 1990).

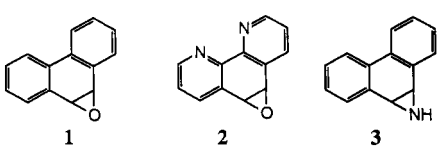
A considerable amount of information concerning the substrate specificity and mechanism of action of microsomal epoxide hydrolase has been gathered over the years. The enzyme is very specific for the oxirane functional group; it does not appear to catalyze the addition of water to other substrates. The enzyme is less sensitive to the nature of the rest of the substrate structure. The pioneering work of Jerina, Daly, and co-workers (Oesch et al., 1971) delineated many of the features of substrate structure favorable for catalysis. In general, monosubstituted and cis-disubstituted epoxides with hydrophobic substituents are preferred. Although it is generally agreed that hydrophobic substrates are favored, the extent to which the hydrophobicity of the substituents contributes to substrate recognition and catalysis has not been directly addressed.

The mechanism of the enzyme-catalyzed reaction remains an enigma. Although there is a large amount of circumstantial evidence to suggest that general base catalysis involving a histidine residue is important [see Armstrong (1987) and references therein], the extent to which other factors such as protonation of the oxirane or strain participate is not clear. Two previous investigations have concluded that protonation of the oxirane oxygen is not important in catalysis (Hanzlik et al., 1976; Bellucci et al., 1981). If the transition state for the enzyme-catalyzed reaction were to resemble a protonated oxirane, it would be anticipated that protonated aziridines (nitrogen analogues of oxiranes) might act as substrates or as potent transition-state analogue inhibitors. Indeed, this may be the case with the 5,6-iminocholestane inhibition of the cholesterol-epoxide hydrolase (Watabe et al., 1981; Nashed et al., 1985). However, aziridines in general have been variously reported to have either very little effect on catalysis (Oesch et al., 1971) or to act as poor substrates (Watabe et al., 1971) or poor competitive inhibitors (Watabe & Akamatsu, 1974; Watabe et al., 1980) of the microsomal enzyme. It must be pointed out that these investigations did not use homogeneous enzyme preparations, nor was the issue of the ionization state of the aziridine addressed experimentally.

In this paper we report the expression, purification, and preliminary characterization of rat liver microsomal epoxide hydrolase in *Spodoptera frugiperda* (Sf9) cells infected with a recombinant baculovirus. The baculovirus-based expression system when coupled to a simple three-step purification provides sufficient quantities of enzyme for mechanistic investigations. The sensitivity of the enzyme-catalyzed reaction to substitution of nitrogen for oxygen in the oxirane ring and for carbon distal to the reaction site has been used to probe the specificity of the recombinant epoxide hydrolase toward the oxirane functional group and the biphenyl ring system of the substrate phenanthrene 9,10-oxide, respectively.

EXPERIMENTAL PROCEDURES

General Materials and Methods. Phenanthrene 9,10-oxide, **1**, and 1,10-phenanthroline 5,6-oxide, **2**, were synthesized as previously described (Krishnan et al., 1977). The aziridine analogue of **1**, phenanthrene 9,10-imine, **3**, was prepared as described by Ittah et al. (1978). Racemic *trans*-9-amino-10-hydroxy-9,10-dihydrophenanthrene, **4**, was a gift from Dr. D. M. Jerina. 9-Aminophenanthrene, **5**, and 9-phenanthrol, **6**, were from Aldrich. All buffer salts, chemical reagents, solvents, detergents, enzymes, and media were of the highest quality commercially available. Sodium dodecyl sulfate (SDS)–polyacrylamide gel electrophoresis was performed as described by Laemmli (1970). Molecular weight of the native enzyme was estimated by gel filtration on 60- × 1.6-cm columns of Sepharose CL6B or Sephacryl S-500HR which had been calibrated with aldolase, catalase, thyroglobin, and ferritin. Oligonucleotides were synthesized with a Biosearch 8750 automatic DNA synthesizer. Protein samples for sequencing were electrophoretically transferred from SDS–polyacrylamide gels (run at neutral pH) to Immobilon poly(vinylidene difluoride) membranes and sequenced on an Applied Biosystems Model 477A sequencer following the procedure described by Moos et al. (1988). The concentration of purified epoxide hydrolase was measured spectrophotometrically at 280 nm using an extinction coefficient of 80 400 M^{-1} cm^{-1} calculated as described by Demchenko (1986).



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Cells and Virus. Spodoptera frugiperda (Sf9) insect cells were obtained from American Type Culture Collection (ATCC, Rockville, MD). *Autographa californica* nuclear polyhedrosis virus (AcNPV) was obtained from the laboratory of Max Summers (Texas A&M University, College Station, TX). Cells were cultured in Hink's TNM-FH medium (JRH Biosciences, Lenexa, KS) supplemented with 10% fetal calf serum (Hyclone, Logan, UT), 50 μg/mL gentamicin sulfate, and 2.5 μg/mL amphotericin B (Fungizone) in either Falconware tissue culture flasks at 28 °C or spinner flasks (Bellco, Vineland, NJ) at room temperature by the procedures of Summers and Smith (1987). Cells grown in spinner flasks contained 0.1% Pluronic F-68 (JRH Biosciences, Lenexa, KS) in addition to the above supplemented medium.

Construction of Transfer Vector. Plasmid pEH52 (Porter et al., 1986) containing the full-length cDNA encoding rat microsomal epoxide hydrolase was obtained from Professor Charles Kasper, University of Wisconsin. Baculovirus transfer vectors pVL1392 and pJVP10Z were obtained from the laboratories of Max Summers (Texas A&M University) and C. Richardson (National Research Council, Montreal), respectively. The cDNA encoding epoxide hydrolase was excised from the plasmid pEH52 by digestion with the restriction enzymes *Hin*dIII and *Pst*I to release a 1325-base-pair fragment which contained 10 base pairs of the 5′-untranslated region but lacked the last 50 base pairs of the 3′-end of the coding region. A 57-base-pair synthetic linker containing the missing 3′-portion of the coding region and the stop codons and flanked by *Hin*dIII and *Nhe*I restriction sites was ligated to the 1325-base-pair fragment, and the resulting fragment was then cloned between the *Pst*I and *Xba*I (*Nhe*I-compatible) sites of the pVL1392 vector. A representative clone, pVLPEHX, was selected and digested completely with *Bgl*II and partially with *Asp*718 to release a 1850-base-pair fragment containing the full-length coding region. The 350-base-pair fragment flanked by *Bam*HI and *Asp*718 restriction sites was isolated from the pJVP10Z vector (Vialard et al., 1990). The 1850- and 350-base-pair fragments were then ligated with the large fragment obtained from *Asp*718 digestion and dephosphorylation of pJVP10Z. Competent INVα *E. coli* was transformed with the ligation mixture and the resulting plasmid pJVPEHX was selected. The correct orientation of the gene was confirmed by sequencing the plasmid DNA (Sanger et al., 1987). A map of the transfer vector is illustrated in Figure 1.

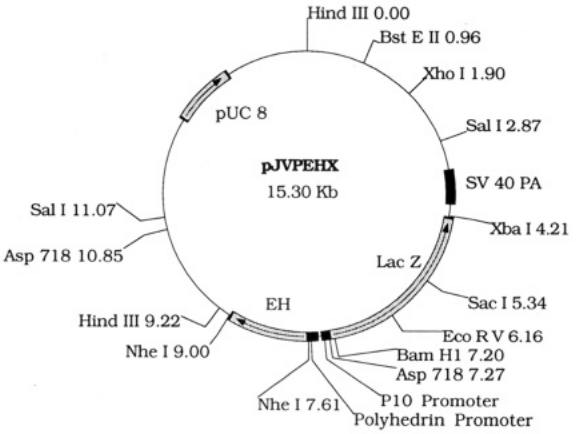


FIGURE 1: Map of the transfer vector pJVPEHX.

Construction of Recombinant Virus. A recombinant virus with epoxide hydrolase under the control of the polyhedrin promoter was generated by homologous recombination as described by Summers and Smith (1987). Briefly, 2×10^6 Sf9 cells were cotransfected with 2 μg of pJVPEHX DNA and 1 μg of wild-type AcNPV DNA. Recombinant virus was identified as blue plaques on plates containing X-gal due to the simultaneous expression of β-galactosidase. Putative recombinant viruses were plaque-purified three times before virus stocks were prepared for further analysis. The presence of the epoxide hydrolase gene in the recombinant virus was confirmed by PCR using the recombinant viral DNA and a pair of specific primers. The recombinant virus was designated vEHX.

Production and Purification of Epoxide Hydrolase. The purified recombinant virus was used to infect Sf9 cells. The infected cells were grown in cell suspension flasks for 3–4 days in medium containing bovine fetal calf serum. Cells were harvested and washed with phosphate-buffered saline and frozen as a cell pellet until needed. Roughly 5 mL of packed cells was obtained from a 1-L suspension. In a typical purification, 5 mL of packed cells were resuspended in 25 mL of 10 mM Tris buffer (pH 7.6) (buffer A) and sonicated with four 30-s bursts from a Branson sonifier 250. Lubrol PX was added to the sonicated cells to a final concentration of 0.5–1.0%, and the mixture was homogenized with 10 strokes in a 30-mL glass/Teflon homogenizer. The homogenate was centrifuged at 8000*g* in 40-mL centrifuge tubes, and the supernatant was recovered and diluted with buffer A to a final detergent concentration of 0.1%. This solution (ca. 125 mL) was loaded on a 37- × 2.6-cm DEAE-cellulose column (Whatman DE52) which had been equilibrated with buffer A containing 0.1% Lubrol PX. The column was washed with buffer A containing 0.1% Lubrol PX and 5–7-mL fractions were collected. Fractions containing the epoxide hydrolase activity were pooled and applied to a 6- × 1-cm column of hydroxylapatite which had been equilibrated with 10 mM potassium phosphate (pH 7.4) (buffer B). The column was then washed with 10 volumes of buffer B to remove the detergent, and the enzyme was eluted with 0.4 M potassium phosphate (pH 7.4). Fractions containing enzyme activity were pooled, concentrated, dialyzed against buffer B, and frozen for storage at −70 °C. The detergent Lubrol PX, which was used in most of this work, is no longer commercially available. However, we have found that Genapol C-100 from Calbiochem gives identical results.

Enzyme Assay and Kinetics. The hydration of phenanthrene 9,10-oxide was followed spectrophotometrically at 290 nm ($\Delta\epsilon$ = −5250 M^{-1} cm^{-1}) on a Perkin-Elmer λ 4B spectrophotometer essentially as described by Armstrong et al. (1980). All assays were performed at 25 °C. Assay of the enzyme during the various steps of the purification was accomplished by combining 925 μL of buffer B with 50 μL of a 2 mM solution of phenanthrene 9,10-oxide in CH_3CN, equilibrating the solution for 5 min at 25 °C, and initiating the reaction with 25 μL of an appropriate concentration of enzyme. Kinetic analysis of the enzyme was done in essentially the same manner except that the reactions were carried out in 50 mM 3-(*N*-morpholino)propanesulfonic acid (MOPS) buffer (pH 8.0) and the concentration of phenanthrene 9,-10-oxide was varied between 2 and 20 μM. Initial velocity data were analyzed by the program HYPER (Cleland, 1979). Continuous time-course assays (Armstrong et al., 1980) using an initial substrate concentration of 20 μM and analyzed with the integrated Michaelis–Menten equation, $[P]/t = -K_m \ln([S]_0/[S])/t + V_{max}$, gave equivalent results. The kinetics of hydration of 1,10-phenanthroline 5,6-oxide, **2**, were determined from initial velocity measurements in a similar manner to that described for **1** except the reactions were monitored at 313 nm ($\Delta\epsilon$ = 3750 M^{-1} cm^{-1}) and the substrate concentration was varied in the range of 100 μM–1 mM. The interaction of the enzyme with phenanthrene 9,10-imine was examined by monitoring its decomposition in the presence of the enzyme and by its ability to inhibit the hydration of **1** in the continuous assay. The K_i for **3** was obtained from plots of the apparent K_m for **1** ($K_{m,app}$) versus the concentration of **3** and the relationship $K_{m,app} = (K_m/K_i)[I] + K_m$.

Chemical Stability of Phenanthrene 9,10-Imine. The pH dependence of the stability of phenanthrene 9,10-imine, **3**, in the absence of enzyme was determined by monitoring its decay kinetics spectrophotometrically at 260 nm in the pH range of 3–9 and 25 °C. Observed first-order rate constants were obtained from semilog plots of $(A_\infty - A_t)$ vs t. Buffers used to span the pH range were sodium citrate (pH 3–6), 2-(*N*-morpholino)ethanesulfonic acid (MES) (pH 5.5–6.3), MOPS (pH 6.5–8) and 2-(cyclohexylamino)ethanesulfonic acid (CHES) (pH 9). Buffer concentrations were varied from 0.1 to 0.3 M to assure that there was no effect of the buffer on the observed rate constants for the reactions. The pH dependence of k_{obs} was analyzed with the program WAVL (Cleland, 1979). The products of the reaction were examined by reversed-phase HPLC on a Beckman Ultrasphere octadecylsilane column eluted at 0.5 mL/min with 50% 0.1 M ammonium acetate (pH 3.6) and 50% CH_3OH for 10 min, followed by a linear gradient to 100% CH_3OH in 30 min. Under these conditions, potential products of the solvolysis of **3** eluted at the following retention times: **4** (8.9 min), **5** (33.0 min), and **6** (36.3 min). The relative amounts of each product were quantified directly from the integrated peak areas using UV detection at 270 nm where the extinction coefficients for **4**, **5**, and **6** are similar. It should be noted that **4** either decomposed on or did not elute from the column in the absence of the ammonium acetate buffer. The apparent pK_a of the iminium ion ($\mathbf{3H^+}$) was determined by potentiometric titration (glass electrode) of a 2.0 mM solution of the aziridine in 6 mL of 50% (v/v) dioxane in water with a standardized solution of 25 mM HCl dispensed with a Metrohm Herisau Dosimat E535 titrater. The pH of the solution was adjusted to 12 before the titration commenced. The pK_a was obtained by fitting the titration data to the equation $pH = pK_a + \log[\alpha/(1-\alpha)]$, where α is the fraction of the aziridine in the unprotonated form.

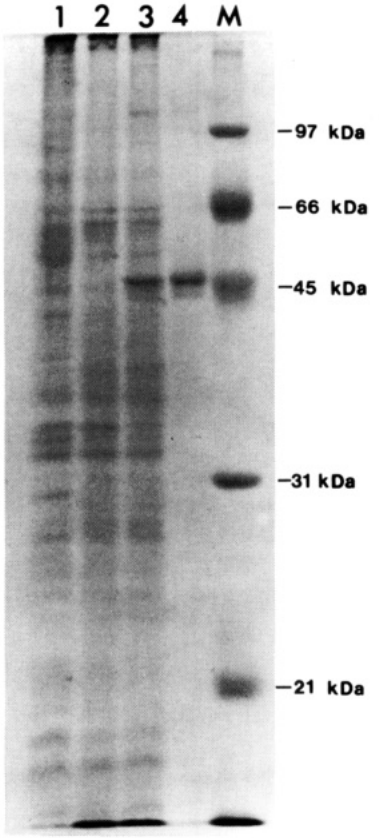


FIGURE 2: Sodium dodecyl sulfate–polyacrylamide gel electrophoresis of proteins produced by Sf9 cells infected by recombinant baculovirus. The samples in the various lanes are as follows: 1, uninfected Sf9 cells; 2, cells infected with wild-type virus; 3, cells infected with vEHX encoding epoxide hydrolase; 4, epoxide hydrolase purified from vEHX-infected cells; M, marker proteins. The purified enzyme in lane 4, which is from a typical (not the best) purification, had a turnover number of 0.6 s^{-1} and was about 90% pure after the two-step purification procedure.

RESULTS AND DISCUSSION

Construction of Recombinant Virus and Expression of Epoxide Hydrolase. The cotransfection of Sf9 cells with the transfer vector pJVPEHX and wild-type baculovirus resulted in a recombinant virus vEHX which placed the gene encoding microsomal epoxide hydrolase behind the polyhedrin promoter and simultaneously incorporated the *lacZ* gene behind the P10 promoter. The incorporation of the *lacZ* gene greatly simplified the search for recombinant virus since the expression of β-galactosidase under control of the P10 promoter could be used to monitor infection of Sf9 cells with recombinant virus in the presence of the chromogenic substrate X-gal.

Infection of Sf9 cells with purified vEHX resulted in the expression of both β-galactosidase and microsomal epoxide hydrolase. Expression levels of both enzymes were high enough that the proteins could be easily visualized on Coomassie blue-stained SDS–polyacrylamide gels of whole-cell extracts as illustrated in Figure 2. The N-terminal sequence of the 52-kDa protein produced in the vEHX-infected cells, determined on a sample electrophoretically transferred from an SDS–polyacrylamide gel similar to that shown in lane 3 of Figure 2, was found to be MWLELVLASLLGFVI, which is identical to that expected for native epoxide hydrolase. The fact that the disrupted cells exhibit a very significant epoxide hydrolase activity toward phenanthrene 9,10-oxide is the most convincing evidence that a functional enzyme is produced by the infected cells. Uninfected cells or cells infected with a recombinant virus encoding only β-galactosidase show no detectable epoxide hydrolase activity with this substrate. From the activity of the cells and the specific activity of the purified enzyme, it can be estimated that the disrupted cells typically obtained from 1 L of the cell suspension contain between 5 and 8 mg of epoxide hydrolase.[1]

Table I: Typical Purification of Microsomal Epoxide Hydrolase from vEHX-Infected Sf9 Cells

step	total protein (mg)	specific activity (μM min^{-1} mg^{-1})	total activity (μM min^{-1})	% yield
sonication	86	49.2	4230	100
solubilization	52.4	61.0	3200	76
DEAE-cellulose	3.44	494	1700	40
hydroxylapatite	1.2	524	629	15

Purification and Characterization of Recombinant Epoxide Hydrolase. The purification of the epoxide hydrolase from the infected Sf9 cells summarized in Table I is straightforward and simple, involving only two chromatographic steps. The key to the brevity of the procedure is the fact that the crude solubilized enzyme is not at all retarded in the first chromatographic step (DEAE-cellulose, 0.1% Lubrol PX), yet virtually all other proteins are retained to some extent. The net result is that the epoxide hydrolase which elutes from the DEAE column is ≥90% pure as judged by its turnover number and by SDS–polyacrylamide gel electrophoresis (Figure 2). The subsequent chromatography on hydroxylapatite simply serves to remove the detergent from the protein preparation. Enzyme prepared in this way typically has a turnover number between 0.6 and 0.8 s^{-1} when measured with 100 μM phenanthrene 9,10-oxide, which is equivalent to the turnover number found for the enzyme purified from rat liver microsomes (Lu et al., 1975; Armstrong et al., 1980). The molecular weight of the polypeptide was estimated to be 52 000 by SDS–polyacrylamide gel electrophoresis. Gel-filtration chromatography of the native enzyme on calibrated columns of Sepharose CL6B (10 mM potassium phosphate, pH 7.0) and Sephacryl S-500HR (10 mM potassium phosphate and 0.1% *n*-octyl β-glucopyranoside, pH 7.0) gave molecular masses of 340 and 309 kDa, respectively, which is consistent with the enzyme existing as a hexameric aggregate. This molecular mass is about half that previously reported (600–650 kDa) by Lu et al. (1975) and may be due to the use of deoxycholate in the previous work or a concentration dependence of the aggregation state of the protein.[2] The hydrodynamic behavior of the protein certainly bears further investigation.

As expected, the enzyme catalyzed the hydration of **1** with kinetic properties similar to those of the protein isolated from rat liver. Initial velocity kinetics and analysis of the full time course of the reaction at pH 8, using the integrated Michaelis–Menten equation, gave essentially equivalent kinetic constants as shown in Table II. It should be noted that a consistently larger turnover number (0.6–0.8 s^{-1}) was obtained at the high concentration (100 μM) of **1** used in the standard assay. This has been observed previously (Armstrong et al., 1980) and probably reflects a small amount of enzyme activation by the higher concentration of substrate.

The Substrate 1,10-Phenanthroline 5,6-Oxide. A large part of the substrate recognition process by epoxide hydrolase

[1] Preliminary results suggest that this yield can be significantly increased (4-fold) by using a bioreactor to maintain dissolved oxygen and control pH and nutrient (glucose) levels after infection of the cells with the virus (W. Bentley and M.-Y. Wang, unpublished results).

[2] Initial sedimentation equilibrium studies in the absence of detergent indicate aggregates with molecular mass ≥ 1000 kDa exist at higher concentrations (E. Eisenstein, unpublished results). The aggregation state of the protein may be concentration dependent.

Table II: Comparison of Kinetic Constants Obtained from Initial Velocity Analysis of Microsomal Epoxide Hydrolase with the Substrates Phenanthrene 9,10-Oxide, **1**, and 9,10-Phenanthroline 5,6-Oxide, **2**

substrate	k_{cat} (s^{-1})	k_{cat}/K_m (M^{-1} s^{-1})	K_m (μM)	$\Delta\epsilon$ (M^{-1} cm^{-1})	λ (nm)
1	0.50 ± 0.03 (0.42)[a]	(9.0 ± 0.7) × 10^4 (6.1×10^4)[a]	5.5 ± 1.5 (6.9)[a]	−5250	290
2	53 ± 4	(9.0 ± 0.6) × 10^4	590 ± 80	3750	313

[a] Data in parentheses are from the continuous time-course assay fit to the integrated Michaelis–Menten equation.

is thought to involve the hydrophobic surface of the substrate. In seeking to assess the importance of the hydrophobicity of the biphenyl ring system to the recognition of **1**, the enzymatic hydration of **2** was investigated. Conversion of **2** to the *trans*-dihydrodiol product is accompanied by a significant change in the UV–visible spectrum of the reaction mixture. The product exhibits different absorption maxima (251 nm, ϵ = 6200 M^{-1} cm^{-1}; 301 nm, ϵ = 11 200 M^{-1} cm^{-1}) than does **2** (255 nm, ϵ = 6500 M^{-1} cm^{-1}; 296 nm, ϵ = 12 600 M^{-1} cm^{-1}), which allows for the convenient spectrophotometric assay of the enzyme at 313 nm, $\Delta\epsilon$ = 3750 M^{-1} cm^{-1}. The kinetic results shown in Table II are striking. Although k_{cat}/K_m values for the two substrates do not differ very much, there are very marked and compensating differences in k_{cat} and K_m. The much higher K_m observed for **2** suggests that the hydrophobicity or desolvation of the biphenyl ring system of **1** is an important determinant in substrate recognition. To the extent that the values of K_m approximate the dissociation constants (K_s) for these substrates, it can be calculated that the higher hydrophobicity of **1** contributes about 2.7 kcal/mol to the partitioning of the substrate between water and the hydrophobic active site.[3] A similar observation has been made with **1** and **2** as substrates for glutathione S-transferase (Cobb et al. 1983; Boehlert & Armstrong, 1984). The fact that **2** is turned over 2 orders of magnitude more quickly than **1** may also be a manifestation of the hydrophobic nature of **1**, which would make it more prone to suffer nonproductive binding. Alternatively, the effect may be electronic inasmuch as the electronegative nitrogens would be expected to increase the susceptibility of the oxirane of **2** to nucleophilic attack.

Although phenanthrene 9,10-oxide is a good substrate for epoxide hydrolase, it does suffer from the fact that it has a relatively low solubility in water and that its conversion to the dihydrodiol product is accompanied by a small $\Delta\epsilon$. The low K_m coupled with the small $\Delta\epsilon$ of **1** makes kinetic studies of the enzyme difficult in the range of substrate concentrations near the K_m. Even though the azarene oxide **2** also has a small $\Delta\epsilon$, it is much more soluble and exhibits a much higher K_m than **1** with the microsomal enzyme, which makes it a more convenient substrate in some respects.

The Substrate Analogue Phenanthrene 9,10-Imine. Phenanthrene 9,10-imine proved to be a relatively unstable molecule near or below neutral pH. Therefore, as a prelude to examining the imine as a possible substrate analogue of phenanthrene 9,10-oxide, it was necessary to determine the solvolytic stability and the protonation state of the aziridine ring in the physiological pH range. The pH dependence of the observed rate constant (k_{obs}) for the decomposition of **3** in the pH range

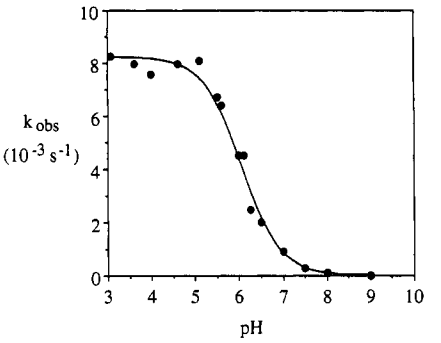


FIGURE 3: pH dependence of the decomposition of phenanthrene 9,10-imine in aqueous solution. The solid line is calculated from the fit of the experimental points to the equation log k_{obs} = log {[k_H + $k_0(K_a/H^+)$]/(1 + K_a/H^+)} using the program WAVL, where k_H = (8.2 ± 0.2) × 10^{-3} s^{-1}, k_0 = (9.6 ± 0.9) × 10^{-6} s^{-1}, pK_a = 6.06 ± 0.06, and H^+ is the hydronium ion concentration.

of 3–9 is shown in Figure 3. The kinetics of decomposition of **3** in aqueous solution in this pH range appears to be best described by the rate law given in eq 1, where k_H is the first-

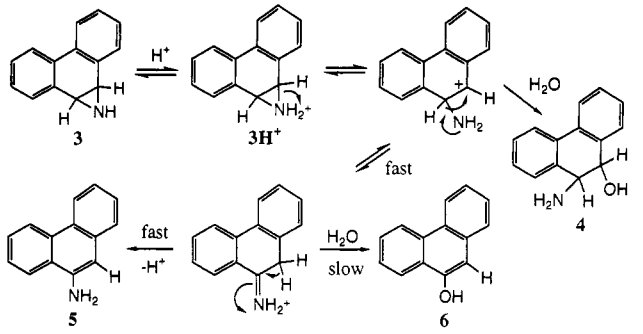
$$k_{obs} = k_H([H^+]/K_a)\alpha + k_0\alpha \quad (1)$$

order rate constant for the decomposition of the iminium ion, k_0 is the rate constant for the decomposition of the neutral imine, $[H^+]$ is the hydronium ion concentration, K_a is the acid dissociation constant of the cyclic iminium ion, and α is the fraction of the aziridine in the unprotonated form. Values for k_H, k_0, and pK_a obtained from the pH dependence of k_{obs} are (8.2 ± 0.3) × 10^{-3} s^{-1}, (9.6 ± 0.9) × 10^{-6} s^{-1}, and 6.06 ± 0.06, respectively. Although the pK_a of 6.1 for the iminium ion derived from the kinetics of decomposition of **3** may seem low, it is, in fact, a reasonable value given the expected electronic effect of the biphenyl group on the ethyleniminium ion (pK_a = 7.9) (Johnes & Arnett, 1974). Potentiometric titration of **3** in 50% (v/v) dioxane/water, a solvent system in which **3** was reasonably soluble, gave a pK_a of 5.61 ± 0.06 which is in good agreement with the kinetically determined value given the expected negative perturbation of the pK_a by the mixed solvent (Grace & Dunaway-Mariano, 1983).

The difference in stabilities of the neutral and protonated aziridine is quite pronounced. Whereas the $t_{1/2}$ of the neutral species is substantial (20 h), it is only a bit more than a minute for the aziridinium ion. Chromatographic analysis of reactions carried out at pH 4, 5, 6, 7, and 8 revealed that the major product (63–69% of the total) was 9-aminophenanthrene, **5** (Scheme I). The remainder of the product mixture consisted of the addition product, 9-amino-10-hydroxy-9,10-dihydrophenanthrene, **4** (29–35%) and a small amount of phenol, **6** (2–4%). The product composition did not vary with pH in any systematic fashion. A proposed mechanism for the rearrangement of **3**, which is analogous to the NIH shift mechanism (Daly et al., 1972) for the rearrangement of arene oxides to phenols, is shown in Scheme I. The absence of any significant variation in the product composition with pH is a convincing indication that the products arise from the same common intermediate (the carbocation) regardless of pH. The very slow rearrangement (k_0 = 9.6 × 10^{-6} s^{-1}) of the neutral aziridine at high pH is likely due to general acid catalysis by water. Although the reaction with hydroxide ion may become significant at high enough pH, **3** seemed no less stable at pH 11 than at pH 9. It is not clear if the small amount of phenol detected in the reaction mixtures is of any mechanistic significance. The formation of **6** could be due to hydrolysis of the acylic iminium ion (Scheme I) or to the elimination of

[3] This value is quite reasonable when compared to the difference in the free energies expected for partitioning of phenyl and pyridyl groups between an aqueous and a hydrocarbon environment. The difference in the free energies for partitioning of benzene and pyridine between octanol and water is 2.0 kcal/mol (Hansch & Leo, 1979).

Scheme I



ammonia from **4**. Finally, the formation of a small amount (<5%) of *cis*-amino alcohol which might be anticipated to occur by solvent capture of the carbocation from the more hindered side cannot be excluded, since this product was not specifically sought in the analysis.

Interaction of **3** *with Epoxide Hydrolase.* The aziridine, **3**, is relatively stable at pH 8 ($t_{1/2}$ = 1.9 h) and above, where the imine is largely deprotonated and its interaction with the enzyme is therefore easily investigated. Incubation of **3** (90 μM) with epoxide hydrolase (2.0 μM) at pH 8 for up to 24 h indicated that the enzyme did not catalyze the conversion of the aziridine to either the amino alcohol or 9-aminophenanthrene. The presence of enzyme had no detectable effect on the either the rate of decomposition of **3**, the final UV spectrum of the reaction mixture, or the distribution of products [**4** (37%), **5** (63%)]. The inability of enzyme to produce either product strongly suggests that it is unable to effectively protonate the aziridine. That the neutral aziridine can bind at the active site is demonstrated by the fact that **3** is a competitive inhibitor (K_i = 26 μM) of the enzyme at pH 8 with **1** as the substrate.

If the K_m for **1** is a reasonable estimate of its true dissociation constant (K_s) from the enzyme and **3** is a good structural analogue of **1**, then it would be anticipated that **3** should be a good competitive inhibitor with a $K_i \approx K_m$. The neutral aziridine is a reasonably good substrate analogue inhibitor, $K_i \approx 4K_m$, suggesting that the two assumptions are essentially correct. The modest difference between the K_i of **3** and the K_m of **1** could be due to either steric or kinetic factors. It is possible that the proton on the aziridine nitrogen interferes slightly with the binding of the analogue, in which case the fit of the oxirane in the active site must be very snug. Alternatively, it could be that the K_m of **1** underestimates the actual dissociation constant and that $K_i \approx K_s$, not K_m. This is certainly possible if the proper kinetic conditions are met. For example, the full expression of K_m for the kinetic mechanism

$$E \underset{k_{-1}}{\overset{k_1[S]}{\rightleftharpoons}} E{\cdot}S \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} E{\cdot}P \underset{k_{-3}}{\overset{k_3}{\rightleftharpoons}} E + P \qquad (2)$$

is given by

$$K_m = (k_{-1}k_3 + k_{-1}k_{-2} + k_2k_3)/k_1(k_2 + k_{-2} + k_3)$$

which, assuming the ring-opening reaction is essentially irreversible ($k_{-2} \approx 0$), reduces to

$$K_m = k_3(k_{-1} + k_2)/k_1(k_2 + k_3)$$

Given the further assumptions that substrate binding is in rapid equilibrium ($k_{-1} \gg k_2$) and that product release is rate limiting ($k_3 \ll k_2$), then the expression for K_m reduces to

$$K_m = k_3k_{-1}/k_2k_1 = (k_3/k_2)K_s$$

Thus the K_m for **1** could be significantly less than the true dissociation constant, K_s, if product release limits the rate of reaction. In this case the K_i for an unreactive substrate analogue such as **3** would more accurately reflect K_s. That the K_i of **3** is close to the observed K_m for **1** suggests product release is probably not the rate-limiting step in the hydration of **1**.

If the transition state for the enzyme-catalyzed reaction resembled a protonated oxirane, then it is certainly conceivable that the aziridinium ion $3H^+$ might be a very potent inhibitor of, or a substrate for, epoxide hydrolase. In principle, the interaction of the protonated aziridine with the enzyme can be studied at the appropriate pH. However, in practice, the lability of the $3H^+$ makes this endeavor difficult. Nonetheless, experiments were carried out at pH 6.5 to ascertain if the aziridinium ion interacted with the enzyme. This pH was chosen as a compromise between the fraction of the aziridine in the protonated form (ca. 30%) and the $t_{1/2}$ (ca. 5 min) for its decomposition. Incubation of the enzyme (1 μM) with **3** (40 μM) at pH 6.5 for up to 1 h did not accelerate the decomposition of **3**, nor did it appear to alter the ratio of the principal solvolysis products **4** and **5**. If the aziridinium ion $3H^+$ acted as a transition-state analogue, then **3** would be expected to have a much lower K_i at pH 6.5. In fact, just the opposite is true. The aziridine remained a competitive inhibitor of the enzyme versus **1** but there was a significant increase in its K_i (120 μM). It should also be noted that there was a similar increase in the K_m for **1** (38 μM) at pH 6.5. From these data it appears likely that either $3H^+$ does not bind to the enzyme or, if it does, the binding is not substantially different from that of the neutral species. The absence of any increase in the amino alcohol product indicates that the aziridinium ion certainly does not form a productive complex with the enzyme.

Some years ago Hanzlik et al. (1976) concluded on the basis of regiochemical and other arguments that catalysis by microsomal epoxide hydrolase probably involved general base catalysis and that no compelling evidence could be found for the participation of general acid catalysis. The intramolecular oxyanion trapping experiment by Bellucci et al. (1981), in which the oxyanion formed on ring opening displaces the adjacent bromide ion in the enzyme-catalyzed reaction of 3-bromo-1,2-epoxycyclohexane, is clearly consistent with the notion that protonation of the oxirane in the transition state is not crucial for catalysis. Proton transfer to the oxyanion appears to be slow relative to the trapping reaction. The failure of the aziridine analogue of **1** to behave as a substrate or a transition-state analogue inhibitor is additional, albeit negative, evidence that acid catalysis is not crucial in the enzyme-catalyzed reaction.

Conclusions. Microsomal epoxide hydrolase can be efficiently expressed and simply purified from Sf9 cells infected with a recombinant baculovirus in quantities sufficient for mechanistic investigation. The physical and kinetic properties of the enzyme so derived are similar to those of the enzyme isolated from rat liver microsomes. The kinetic parameters for the enzyme-catalyzed hydration of the model K-region arene oxide **1** are sensitive to incorporation of nitrogen into the biphenyl ring system. The enzyme is also quite sensitive to substitution of NH for O in the oxirane ring. The fact that aziridine is not a substrate for the enzyme is a strong indication that the enzyme is not capable of protonating the imine. By

implication, the protonation of oxirane substrates by the enzyme is probably not an important aspect of catalysis, a conclusion that is supported by previous investigations of an entirely different nature (Hanzlik et al., 1976; Bellucci et al., 1981). Finally, the availability of the expression system should allow the manipulation of the protein structure by site-specific or modular mutagenesis.

ACKNOWLEDGMENT

We are indebted to Professor Charles Kasper for providing the cDNA clone pEH52 and to Drs. Christopher Richardson and Max Summers for the transfer vectors pJVP10 and pVL1392. We are also grateful to Professor William Bentley and Mr. Min-Ying Wang, who have worked to optimized the expression of the enzyme in Sf9 cells, and to Professor Ed Eisenstein for his ultracentrifugation analysis of the purified enzyme. We also thank Dr. Kenneth Seamon for his help and interest.

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