The Catalytic Mechanism of Microsomal Epoxide Hydrolase Involves an Ester Intermediate

Gerard M. Lacourciere and Richard N. Armstrong*

Department of Chemistry and Biochemistry University of Maryland, College Park, Maryland 20742

Received August 6, 1993

Microsomal epoxide hydrolase (MEH) catalyzes the trans-antiperiplanar addition of water to epoxides and arene oxides to give vicinal diol products.[1] The reaction occurs with inversion of configuration at the oxirane carbon to which the addition takes place, involves no metal ions, and does not appear to involve general acid catalysis.[2] The commonly accepted mechanism for MEH involves a direct, general-base-assisted attack of water on the oxirane (Scheme IA).[1b,2a,3,4] Some time ago, one of us proposed an alternative mechanism that involved a covalent ester intermediate (Scheme IB) and that was fully consistent with all of the available evidence in the literature but had no positive experimental support.[5] Definitive evidence to distinguish the mechanisms proposed in Scheme I for epoxide hydrolase can, in principle, be obtained from single-turnover experiments in isotopically enriched water.[1b]

In this paper we demonstrate that the oxygen atom incorporated into the product, though ultimately derived from water, is proximally derived from the enzyme by way of an ester intermediate (Scheme IB). Single-turnover experiments conducted in $H_2{}^{18}O$ result in *no* incorporation of ^{18}O into the product, while enzyme covalently labeled with ^{18}O incorporates ^{18}O into the product in single-turnover reactions conducted in $H_2{}^{16}O$. We also note, as have others, that there is a limited but rather striking sequence similarity between mammalian epoxide hydrolase and a bacterial haloalkane dehalogenase (HAD).[6] Recent high-resolution crystallographic evidence clearly supports a covalent ester intermediate in the catalytic mechanism of HAD.[7] The isotope exchange results together with the sequence identities with the active site of haloalkane dehalogenase strongly support a catalytic mechanism involving an ester intermediate and provide a tentative identification of the carboxylate side chain of Asp226 as the active site nucleophile in microsomal epoxide hydrolase.

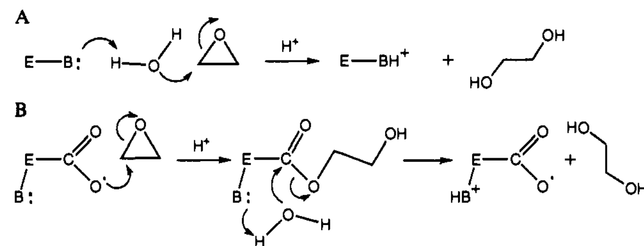
Epoxide hydrolase-catalyzed[2d] hydration of phenanthrene 9,10-oxide in $H_2{}^{18}O$ under single-turnover conditions[8] of enzyme in excess gave product *trans*-9,10-dihydro-9,10-dihydroxyphenanthrene, which contained very little ^{18}O (Table I), a result which is difficult to reconcile with the accepted general-base mechanism in Scheme IA. Multiple-turnover reactions (substrate in excess) show the expected appearance of ^{18}O in the product as the isotope is incorporated into the enzyme. These results are obviously consistent with the alternative mechanism of Scheme IB.

Table I. Isotopic Composition of *trans*-9,10-Dihydro-9,10-dihydroxyphenanthrene Obtained from the Enzymatic Hydration of Phenanthrene 9,10-Oxide under Single- and Multiple-Turnover Conditions

enzyme (μM)	substrate (μM)	av no. of turnovers [S]/[E]	^{18}O in solvent[a] (mol)	^{18}O in product[b] (mol)
		$E\text{-}^{16}O/H_2{}^{18}O$		
140	20.0	0.14	0.930	0.08
1.4	100.0	71.0	0.971	0.94
82.0	14.0	0.17	0.917	0.12
1.3	100.0	77.0	0.971	0.96
		$E\text{-}^{18}O/H_2{}^{16}O$[c]		
88.0	22.0	0.25	0.002	0.52
0.9	100.0	110.0	0.002	0.002
59.0	15.0	0.25	0.002	0.64
0.6	100.0	170.0	0.002	0.002

[a] The isotopic composition of the solvent was estimated from the isotopic content of the water used in the preparation of the sample corrected for any dilution factors. [b] The isotopic composition of the product was determined by electron impact mass spectrometry by direct introduction into a VG 7070F mass spectrometer at an ionization voltage of 70 ev. The ^{18}O content of the products was estimated by comparison of the intensities of the *m/e* peaks centered at 212 and 214 amu. [c] Enzyme was labeled with approximately 1 mol of ^{18}O by a single-turnover reaction in $H_2{}^{18}O$.[10]

Scheme I



Inasmuch as carboxylate oxygen atoms do not exchange with solvent at significant rates under physiological conditions,[9] it was possible to label the enzyme with a single ^{18}O in a single-turnover reaction in $H_2{}^{18}O$, isolate the labeled enzyme, and observe transfer of the ^{18}O to the product in a single-turnover reaction carried out in $H_2{}^{16}O$ (Table I). The labeling reaction was carried out with a different, more water-soluble, substrate, 1,10-phenanthroline 5,6-oxide,[2d] to prevent isotopic contamination of the final product.[10] The isotope dilution of about 50% seen in the product is exactly that expected from positional isotope exchange with an unlabeled carboxylate oxygen. The observation of label transfer and the extent of isotopic dilution are consistent with the covalent involvement of a protein carboxyl group in the hydration reaction.

The evidence for an ester intermediate is bolstered by the observation that parts of the sequence of MEH are homologous to the active site of a haloalkane dehalogenase from the bacterium *Xanthobacter autotrophicus* GJ10[6] and to a region of a related

* To whom correspondence should be addressed. Phone: (301) 405-1812. FAX: (301) 405-7956.

(1) (a) Oesch, F.; Kaubisch, N.; Jerina, D. M.; Daly, J. W. *Biochemistry* **1971**, *10*, 4858. (b) Armstrong, R. N. *CRC Crit. Rev. Biochem.* **1987**, *22*, 39.

(2) (a) Lu, A. Y. H.; Ryan, D.; Jerina, D. M.; Daly, J. W.; Levin, W. *J. Biol. Chem.* **1975**, *250*, 8283. (b) Hanzlik, R. P.; Edelman, M.; Michaely, W. J.; Scott, G. *J. Am. Chem. Soc.* **1976**, *98*, 1952. (c) Hanzlik, R. P.; Heideman, S.; Smith, D. *Biochem. Biophys. Res. Commun.* **1978**, *82*, 310. (d) Lacourciere, G. M.; Vakharia, V. N.; Tan, C. P.; Morris, D. I.; Edwards, G. H.; Moos, M.; Armstrong, R. N. *Biochemistry* **1993**, *32*, 2610.

(3) (a) BuBois, G. C.; Appella, E.; Levin, W.; Lu, A. Y. H.; Jerina, D. M. *J. Biol. Chem.* **1978**, *253*, 2932. (b) Dansette, P. M.; Makedonska, V. B.; Jerina, D. M. *Arch. Biochem. Biophys.* **1978**, *187*, 290.

(4) Bell, P. A.; Kasper, C. B. *J. Biol. Chem.* **1993**, *268*, 14011.

(5) Armstrong, R. N.; Levin, W.; Jerina, D. M. *J. Biol. Chem.* **1980**, *255*, 4698.

(6) Janssen, D. B.; Pries, F.; van der Ploeg, J.; Kazemier, B.; Terpstra, P.; Witholt, B. *J. Bacteriol.* **1989**, *171*, 6791.

(7) (a) Franken, S. M.; Rozeboom, H. J.; Kalk, K. H.; Dijkstra, B. W. *EMBO J.* **1991**, *10*, 1297. (b) Verschueren, K. H. G.; Seljee, F.; Rozeboom, H. J.; Kalk, K. H.; Dijkstra, B. W. *Nature* **1993**, *363*, 693.

(8) For a typical single-turnover experiment, 30 nmol of epoxide hydrolase was concentrated to a volume of 125 μL (240 μM enzyme) in an Amicon centricon-10 membrane, diluted with 125 μL of $H_2{}^{18}O$ (97.1% ^{18}O) containing 10 mM potassium phosphate (pH 7.4), and concentrated again to 125 μL. This process was repeated three times to give a solvent isotopic enrichment of >90% ^{18}O. Small portions of enzyme were withdrawn to quantify the enzyme concentration and for multiple-turnover reactions. Single-turnover reactions were initiated by rapid mixing of 1–2 μL of a solution of phenanthrene 9,10-oxide in CH_3CN to 200 μL of a 80–140 μM solution of enzyme and incubated at room temperature for 15 min. The product was adsorbed on a Sep-Pak C-18 reversed-phase cartridge (Millipore), washed with water, eluted with CH_3OH, and concentrated to ca. 10 μL for mass spectroscopic analysis.

(9) Murphy, R. C.; Clay, K. L. *Methods Enzymol.* **1990**, *193*, 338.

(10) The labeling reaction was carried out by transferring 50 nmol of enzyme into $H_2{}^{18}O$ as above. A 200-μL portion of 110 μM enzyme in $H_2{}^{18}O$ (94.3% ^{18}O) was incubated with 110 μM 1,10-phenanthroline 5,6-oxide ([S]/[E] = 1) for 15 min at room temperature. The solution was then dialyzed against 1.5 L of 10 mM potassium phosphate (pH 7.4) for 20 h at 4 °C. Single- and multiple-turnover reactions were then conducted in $H_2{}^{16}O$ as described above[8] with phenanthrene 9,10 oxide as substrate.

0002-7863/93/1515-10466$04.00/0 © 1993 American Chemical Society

```
 Oxyanion hole          Nucleophile & Oxyanion hole    Charge Relay General Base
         *                                 **                *            *
145 LMVHGWPG   211 KLMTRLGFQKFYIQGGDWGSLICTNMAQMVPNHVK  352 DDLL   428 RGGHFAA   MEH
    | :|| |         |: || :    :   |||::    :    | : |      | ||         :|||:
 51 LCLHGEPT   108 ALIERLDLRNITLVVQDWGGFLGLTLPMADPSRFK  260 DKLL   286 DAGHFVQ   HAD
    | ||| |         :: :| :    || :|:||  |  : :  |                      :|||
 30 LMLHGFPQ    89 CVMRHLGFERFHLVGHDRGGRTGHRMALDHPEAVL               269 PGGHFFV   HAcD
```

Figure 1. Sequence alignments of sections of rat liver microsomal epoxide hydrolase (MEH), haloalkane dehalogenase (HAD), and haloacetate dehalogenase (HAcD). The putative active-site residues of epoxide hydrolase deduced from the sequence alignments and the three-dimensional structure of the haloalkane dehalogenase[7] are identified by asterisks, and their possible functions are noted above the sequence. Homologous regions were identified using the Lawrence algorithm and Dayhoff cost matrix[12] imbedded in the EuGene program package (Lark Sequencing Technologies). Only parts of the homologous regions are shown. Sequence identities and similarities are indicated by | and :, respectively.

enzyme, haloacetate dehalogenase from *Moraxella* sp.,[11] as shown in Figure 1. The most striking sequence similarity (22.7% identity over 44 residues) is found in the region bounded by residues 208–251 of MEH and 106–149 of HAD. This region includes identities of Asp226 with Asp124 of HAD, the residue which has been recently shown by X-ray crystallography to act as the nucleophile in the dehalogenation of 1,2-dichloroethane and of the adjacent Trp227 of MEH with Trp125, the amide NH of which appears to help form an oxyanion hole which assists in the hydrolysis of the ester intermediate of HAD.[7b] A second amide NH (Trp150), which appears at the same position as Glu56 of HAD, is also probably part of the oxyanion hole. This position is located in a His-Gly-X-Pro sequence which forms a *cis*-proline turn in the HAD structure and is part of a homologous 31-residue segment that is 25.1% identical with the sequence of HAD.

It has been known for some time that a histidine residue,[3a] recently identified as His431,[4] is involved in the catalytic mechanism of epoxide hydrolase. The fact that His431 of MEH is located in a region that bears a clear resemblance to the sequence around His289 of HAD, the residue which acts as a general base in the hydrolysis of the ester in the second half-reaction of HAD, suggests that it serves an analogous function in epoxide hydrolase. Finally, we note that Asp260 of HAD, which is postulated to serve as part of a charge relay by assisting in deprotonation of the general base (His289), also appears to have a counterpart (Asp352) in the epoxide hydrolase sequence. When taken together, the sequence identities between MEH, HAD, and the related haloacetate dehalogenase make a compelling case for a close mechanistic and structural relationship between these enzymes.

In summary, we have demonstrated, by isotope labeling experiments, that the catalytic mechanism of MEH involves an ester intermediate. A novel feature of this mechanism, as compared to other hydrolytic enzymes, is the transfer of an oxygen atom from the enzyme to the product. Sequence identities with bacterial haloalkane dehalogenase provide tentative identification of several active-site residues of epoxide hydrolase which are postulated to serve specific functions in catalysis. Epoxide hydrolase and the haloalkane dehalogenases represent a previously unappreciated class of C–X bond hydrolases which are proposed here to be mechanistically and structurally related.

Acknowledgment. This work was supported by a grant from the National Institutes of Health (GM49878) and by the General Research Board of the University of Maryland. We thank Noel Whittaker at the National Institutes of Health for determining the isotope ratios.

(11) Kawasaki, H.; Tsuda, K.; Matsusita, I; Tonomura, K. *PIR* **1991**, Accession No. JQ0931.

(12) (a) Lawrence, C. B.; Goldman, D. A. *Computer Applications Biosciences* **1988**, *4*, 25. (b) Dayhoff, M. O. *Atlas of Protein Sequence and Structure*; National Biomedical Research Foundation: Washington, DC, 1978; Vol. 5.