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SOLUBILIZATION, PURIFICATION, AND PROPERTIES OF A HEPATIC EPOXIDE HYDRASE*

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SUMMARY

A microsomal epoxide hydrase, which catalyzes the hydration of styrene oxide to form styrene glycol, has been purified from guinea pig liver. Solubilization from microsomes with cutscum and further purification by $(NH_4)_2SO_4$ precipitation, Sephadex G-25 chromatography, and calcium phosphate gel adsorption and desorption resulted in an overall 40-fold increase in specific activity. The solubilized enzyme was very unstable but stability was recovered after the last purification step. The K_m and v_{max} of the purified enzyme for styrene oxide are $5.3 \cdot 10^{-4}$ M and 2.70μmoles product per mg nitrogen per 5 min, respectively. No cofactors are required. The enzyme is not inhibited by the product styrene glycol or by EDTA and is slightly inhibited by sulfhydryl reagents. The pH optimum is 8–9. The purified preparation catalyzes the enzymatic hydration of a variety of epoxides.

INTRODUCTION

A key enzyme in the metabolism and detoxification of aromatic and olefinic compounds in higher organisms is a hepatic epoxide hydrase which converts intermediate oxidation products, the arene oxides and epoxides, to *trans*-dihydrodiols[1–3]. In the case of aromatic metabolism, the dihydrodiols are then further metabolized to catechols and their conjugates. Hepatic epoxide hydrase catalyzes the hydration of a variety of epoxides such as cyclohexene oxide[2], indene oxide[2,3], chlordene oxide[4], and styrene oxide[2], in addition to arene oxides such as benzene oxide[2], naphthalene oxide[1], and indane oxide[5]. Purification of this enzyme required a convenient assay for the determination of specific activity. Such an assay was developed, based on the conversion of [^{3}H]styrene oxide to [^{3}H]styrene glycol[6]. Epoxide hydrase activity was demonstrated to be almost exclusively associated with liver microsomes in a variety of species and to be elevated by pretreatment of rats with 3-methylcholanthrene or phenobarbital[6]. Epoxide hydrase activity has now been solubilized and purified 40-fold from guinea pig liver and some of the characteristics of this important enzyme have been determined.

* A preliminary report of this work has appeared: *Federation Proc.*, 29 (1970) 423.

METHODS

Assay of epoxide hydrase activity

Epoxide hydrase activity was determined using the radiometric assay based on conversion of [^{3}H]styrene oxide to [^{3}H]styrene glycol[6]. At each step in purification, the reaction rate was ascertained to be linear with respect to time for at least 10 min. Standard incubation mixtures contained Tris buffer (pH 9.0), 50 μmoles; [7-^{3}H]-styrene oxide, 0.8 μmole, 62 800 disint./min; and enzyme preparation in a final volume of 0.4 ml. After 5 min incubation at 37°, unreacted substrate was removed by two extractions with petroleum ether, followed by extraction of product into ethyl acetate for scintillation spectrometry as described[6].

Solubilization and purification of guinea pig microsomal epoxide hydrase

Livers of male guinea pigs (250–300 g) were homogenized in 0.25 M sucrose. Microsomal pellets were prepared by centrifugation of a 8500 $\times$ *g* (15 min) supernatant at 100 000 $\times$ *g* for 1 h followed by resuspension in sufficient 0.01 M phosphate buffer (pH 7.8) to restore the original volume of the 8500 $\times$ *g* supernatant. This microsomal preparation had a protein concentration of 17.7 $\pm$ 0.9 mg protein/ml. 10 ml of a chilled 5% (v/v) aqueous solution of cutscum (isooctylphenoxypolyethanol) was added dropwise in three portions to 30 ml of stirred microsomal preparation in ice. The microsomal preparation was stirred for 5 min between each portion of added cutscum and then for an additional 15 min after the last portion. The preparation was centrifuged at 100 000 $\times$ *g* for 1 h and an upper layer, corresponding to approximately one-tenth of total volume and containing a fluffy material (with lower specific activity in respect to epoxide hydrase) was removed by aspiration. The remainder of the supernatant was filtered through glass wool.

Increasing the $(NH_4)_2SO_4$ concentration from 0 to 24% saturation caused precipitation of all the active protein. Centrifugation of the mixture at 35 000 $\times$ *g* for 15 min resulted in 10% (v/v) of the precipitate settling to the bottom of the tube while the remainder rose to the surface; only the latter portion was active. This active precipitate was resuspended in a small volume of 0.01 M phosphate buffer (pH 7.8) and desalted by Sephadex G-25 chromatography at 0–5° on a column equilibrated and eluted with 0.01 M phosphate buffer (pH 7.8). Epoxide hydrase activity appeared immediately after the void volume.

The final purification step consisted of gel adsorption and desorption. Calcium phosphate gel (22 mg/ml) was prepared as described by KEILIN AND HARTREE[7], adjusted to pH 7.4 with 25% acetic acid, washed 8 times with distilled water and aged for 2 weeks. A chilled suspension of calcium phosphate gel was then added in 0.5-ml portions to 5 ml of the Sephadex G-25 eluate containing 9.9 mg protein per ml. After each addition, the mixture was stirred for 10 min at 0–2° and then centrifuged at 1000 $\times$ *g* for 5 min. After assay of an aliquot, the material was resuspended and another 0.5-ml portion of gel added. No epoxide hydrase activity remained in the supernatant after addition of 4 ml of gel. The adsorbed proteins were desorbed with increasing buffer concentrations, beginning with 0.025 M phosphate buffer. Epoxide hydrase activity desorbed with 2 ml of 0.2 and 0.4 M phosphate buffer (pH 7.8).

Percent nitrogen was determined on particulate preparations by the method of Kjeldahl and on solubilized preparations by the biuret reaction[8]. All water was boiled

for 30 min before preparing solutions for the latter reaction, in which 0.1 ml of protein solution containing less than 2.8 mg protein was added to 0.9 ml of biuret reagent. Bovine serum albumin served as a standard. The biuret protein/Kjeldahl nitrogen ratio was 6.5 ± 0.5.

RESULTS AND DISCUSSION

Solubilization of epoxide hydrase

Epoxide hydrase in liver homogenates has been shown to be exclusively present in microsomal particles[6]. Solubilization of epoxide hydrase activity from microsomal suspension, however, could not be effected with deoxycholate as reported for squalene oxidocyclase[9]. Instead there was almost complete loss of activity. The same result was obtained during attempts to prepare active acetone powders. Use of guanidine or hypotonic buffer preserved the activity, but failed to release the enzyme from the microsomes. Finally, solubilization without loss of enzyme activity was affected under optimal conditions with a neutral detergent, cutscum. The "solubilized" preparation contained 206% of the epoxide hydrase activity originally present in the homogenate. No activity was detected in the 100 000 × *g* (1 h) sediment.

Purification and stabilization of epoxide hydrase

The upper portion of the solubilized preparation after centrifugation at 100 000 × *g* for 60 min contained a fluffy biuret-positive material which had a lower specific activity than the remainder of the supernatant. After removal of this layer, the specific activity of the "solubilized" epoxide hydrase was 6-fold greater than in liver microsomes. The solubilized epoxide hydrase preparation was very labile especially in solutions of low ionic strength. Stability was low in both Tris or phosphate buffer and was not increased by the presence of $1 \cdot 10^{-5}$ M mercaptoethanol. Higher concentrations of sulfhydryl compounds prevented assay of hydrase activity using [^{3}H]-styrene oxide because of the high control values which obtained with boiled enzyme.

The next steps in purification, namely $(NH_4)_2SO_4$ precipitation, Sephadex G-25 chromatography and calcium phosphate gel adsorption and desorption were of

TABLE I

PURIFICATION OF AN EPOXIDE HYDRASE FROM GUINEA PIG LIVER

Fraction	*Protein recovery (%)*	*Specific activity**	*Activity recovery (%)*	*Purification factor*
Liver homogenate	100	102	100	1
Microsomes	20.4	334	66.7	3.3
Supernatant after cutscum treatment and centrifugation at 100 000 × *g* for 1 h	16.9	1242	206.2	12.2
Supernatant after removal of top layer and filtration	10.0	1918	188.0	18.8
$(NH_4)_2SO_4$ precipitate after Sephadex G-25 chromatography	2.5	2837	69.4	27.8
Calcium phosphate gel adsorption and desorption	0.5	4210	20.6	41.3

* Specific activity expressed as nmoles styrene glycol per mg N per 5 min.

necessity carried out in as short a time as possible. After calcium phosphate gel desorption, an overall purification of 40-fold with a recovery of 20% of the original epoxide hydrase present in liver homogenates had been affected (Table I). The final preparation lost approx. 30% of its activity within 36 h at $-15°$ or at 0–5°. The remaining epoxide hydrase activity was remarkable stable, and lost less than 10% of its activity during storage at $-15°$ for 3 months, during preincubation at 37° for 40 min, and during six cycles of freezing and thawing. All studies reported in this paper were performed with this stable preparation of epoxide hydrase, which had a specific activity of 2.7 ± 0.2 μmoles styrene glycol formed per mg nitrogen per 5 min.

Comparison of "protein concentration" as estimated by the biuret reaction with values calculated from the $A_{280\ m\mu}/A_{260\ m\mu}$ absorption ratio[10] revealed that solubilizing cutscum (λ_{max} = 277 mμ, shoulder at 284 mμ) had remained associated with the preparation throughout purification. The cutscum could not be removed by dialysis nor by Sephadex G-25 chromatography. Sephadex G-200 chromatography (0.01 M phosphate buffer (pH 7.8)) removed at least 90% of the cutscum. Protein and hydrase activity appeared slightly after the void volume contaminated with approx. 10% of the cutscum. The remaining cutscum slowly eluted from the column. Epoxide hydrase activity appeared to be slightly retarded with respect to total protein in this column.

The Sephadex G-200 eluent was subjected to polyacrylamide disc gel electrophoresis (acrylamide concentration, 3.75, 5 or 7%; pH 9.5, staining with aniline black or Coomassie blue). One protein band which moved into the separating gel was detected, in addition to protein which did not migrate from the stacking gel. No hydrase activity could be detected by assay of unstained slices of gel.

Subsequent to our preliminary report, a procedure for solubilizing and purifying epoxide hydrase from rabbit liver microsomes was described[11]. The assay of epoxide hydrase employed 1-octene oxide as substrate, while solubilization was effected with deoxycholate. A final purification factor from liver microsomes of 3 with only a 5% recovery of enzyme activity was reported. This method is clearly much less satisfactory than ours, which results in a 12-fold purification and 30% recovery of activity from liver microsomes.

Properties of epoxide hydrase

No cofactor or metal ion requirement could be demonstrated for epoxide hydrase activity even after repeated dialysis or Sephadex G-25 or G-200 chromatography. EDTA ($2 \cdot 10^{-3}$ to $5 \cdot 10^{-3}$ M) did not inhibit the enzyme. Sulfhydryl reagents had little effect on enzyme activity. Mersalyl ($9 \cdot 10^{-5}$ M) caused 13% inhibition, while *N*-ethylmaleimide ($9 \cdot 10^{-4}$ M) had no effect. These inhibitors were incubated with enzyme for 5 min before addition of substrate.

The purified preparation of epoxide hydrase had a pH optimum at pH 9 for enzymatic hydration of styrene oxide (Fig. 1). Nonenzymatic hydration of this substrate was significant (>5%) only below pH 6.5. All values in Fig. 1 are corrected for nonenzymatic hydrations. A Lineweaver–Burk analysis of product formation with the purified preparation using $2 \cdot 10^{-4}$–$5 \cdot 10^{-4}$ M [^{3}H]styrene oxide shows an apparent K_m of $5.26 \cdot 10^{-4}$ M and an apparent v_{max} of 2.70 μmoles product per mg nitrogen per 5 min (Fig. 2). The product, styrene glycol, did not inhibit the enzyme, even at twice ($4 \cdot 10^{-3}$ M) substrate concentration.

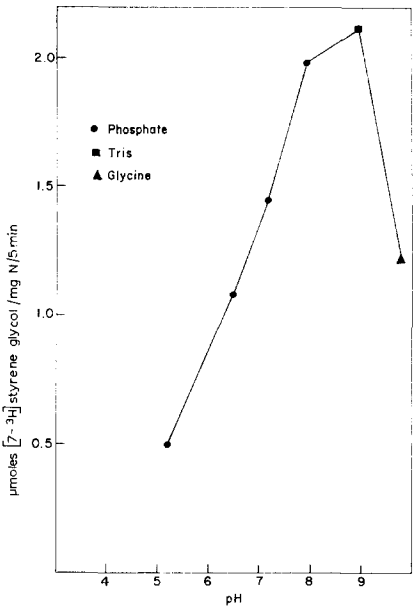

Fig. 1. Epoxide hydrase activity with [7-^{3}H]styrene oxide in a purified epoxide hydrase preparation from guinea pig liver as a function of pH.

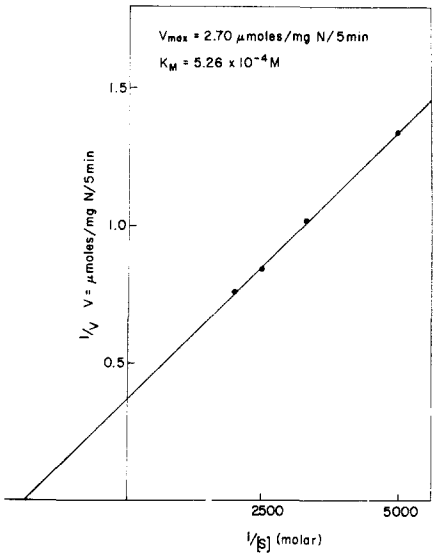

Fig. 2. Double reciprocal plot of velocity *vs.* concentration of [7-^{3}H]styrene oxide for a purified epoxide hydrase preparation from guinea pig liver.

The purified epoxide hydrase showed enhanced hydrase activity with a variety of epoxides. The following purification factors with different epoxide substrates were obtained using the stable epoxide hydrase preparation that was purified 28-fold based on [7-^{3}H]styrene oxide as substrate: [4,5-$^3H_2$]Cyclohexene oxide, 29; *p*-chlorophenyl-2,3-epoxypropyl ether, 28; naphthalene oxide, 27; and benzene oxide, 4. Details of the results obtained with these and other epoxides will be the subject of a subsequent paper[12].

CONCLUSIONS

The solubilization and purification of epoxide hydrase activity from guinea pig microsomes has provided a stable preparation for further studies on this important enzyme in drug metabolism. No cofactor or metal ion requirement could be demonstrated. Although the purification of epoxide hydrase was carried out using an assay based on [^{3}H]styrene oxide[6], subsequent studies revealed that the purified enzyme preparation shows enhanced activity with other epoxides, including cyclohexene oxide, *p*-chlorophenyl-2,3-epoxypropylether, bisnorsqualene 1,2-oxide, phenanthrene oxide, 1-octene oxide, benzene oxide, and naphthalene oxide*[,12]. Purification factors were, however, significantly different with certain epoxides, such as benzene oxide suggesting the presence of more than one epoxide hydrase in liver

* Contrary to a statement in ref. 11, which misinterprets published data[1,2], the nonenzymatic hydration of naphthalene oxide has not been demonstrated under any conditions.

microsomes. The differential stability of the final epoxide hydrase preparation also suggests the presence of isozymes of this enzyme. This point is under investigation using various substrates and inhibitors.

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