PURIFICATION OF RAT LIVER EPOXIDE HYDRATASE TO APPARENT HOMOGENEITY

Philip BENTLEY and Franz OESCH
Section on Biochemical Pharmacology, Institute of Pharmacology, University of Mainz, Obere Zahlbacher Strasse 67, D-65 Mainz, West Germany

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1 . Introduction

Epoxide hydratase (EC 4.2.1.63) is a microsomal enzyme which catalyses the conversion of epoxides to *trans*-dihydrodiols. Epoxides, produced by the action of microsomal monooxygenases (EC 1.14.1.1) from aromatic and olefinic compounds, are thought to be responsible for many of the harmful effects of polycyclic hydrocarbons and related compounds. Thus epoxide hydratase, together with glutathione *S*-transferases, (EC 2.5.1.18) may play an important role in the removal of carcinogenic and cytotoxic metabolites (for reviews see [1–3]). It has been reported [4,5] that dihydrodiols formed from some polycyclic hydrocarbons (benz(a)anthracene and benzo(a)pyrene) are reactivated by the microsomal monooxygenases to dihydrodiol epoxides which may represent ultimate carcinogens. If this is the case epoxide hydratase could play a critical role being responsible both for forming the proximate carcinogen and removing the ultimate carcinogen. Indeed an inverse correlation between susceptibility to carcinogenesis by polycyclic hydrocarbons and inducibility of epoxide hydratase by the same agents at different developmental stages of the same animal species has been observed [6].

A thorough knowledge of the properties of the enzymes involved in the metabolic activation and inactivation processes is essential for an understanding of the mechanisms of cytotoxicity and carcinogenesis by polycyclic hydrocarbons. Many research groups have studied the monooxygenases and several have recently reported a purification of cytochromes P_{450} and P_{448} [7–9]. However, although many inhibitors, activators, and inducers of epoxide hydratase exhibiting various degrees of specificity are known [2], pure enzyme is not available for studies on its molecular properties. Moreover, pure epoxide hydratase may provide a valuable tool for evaluating the relative importance of epoxides amongst other reactive metabolites in in vitro systems. In the present study a procedure for the purification of this hydrophobic membrane protein to homogeneity was established.

2. Materials and methods

Chemicals were analytical reagent grade where available and were purchased mainly from E. Merck, Darmstadt, West Germany. Protein standards for electrophoresis were obtained from Boehringer, Mannheim, W. Germany. Cutscum (isooctylphenoxypolyethanol) was purchased from Fisher Scientific Co., Pittsburg, Pa., USA. De 11 and P 11 celluloses were purchased from Whatman Biochemicals, Maidstone, England. Sepharose 4B was from Pharmacia, Uppsala, Sweden. Butyl-Sepharose was prepared by a modification of the method of Er-el et al. [10]. [7-^{3}H]styrene oxide was synthesized as described [11]. [^{3}H]benzo(a)pyrene 4,5-oxide was prepared by H. R. Glatt of this laboratory as described [12].

Epoxide hydratase was assayed with substrates 7-[^{3}H]styrene oxide, using conditions described in detail [13] specifically having no Tween 80 in the incubation mixture. [^{3}H]benzo(a)pyrene 4,5-oxide [14] and *p*-chlorophenyl-2,3-epoxypropylether [15]. 1 Unit of enzyme is defined as the amount producing 1 nmol of styrene glycol per min under the conditions of the styrene oxide assay used [13]. Protein determinations [16] were performed using bovine serum albumin as standard. All buffers contained 0.3 mM EDTA unless otherwise stated.

2.1. *Purification of epoxide hydratase*

50 male Sprague–Dawley rats (200–250 g) were killed by a blow on the head. The livers were removed, washed in ice cold 10 mM Na-phosphate buffer pH 7.0 containing 0.25 M sucrose and homogenised in the same buffer (3 ml/g liver) using a Braun MX 3 blender. Homogenisation and all subsequent steps were performed at 0–4°C. The homogenate was centrifuged at 10 000 *g* for 15 min and the supernatant (fraction 1) was further centrifuged for 1 h at 100 000 *g*. The resultant crude microsomal pellet was resuspended in 10 mM Na phosphate buffer pH 7.0 containing 1% Cutscum, diluted to the same volume as fraction 1 and stirred for 20 min (fraction 2). $(NH_4)_2SO_4$ (140 g/l) was added, the solution stirred for 20 min and centrifuged for 20 min at 10 000 *g* yielding 3 phases, a floating viscous brown layer, a red intermediate phase and a small pink sediment. The intermediate phase was removed by siphon and discarded. The remaining two phases were combined, dissolved in a small volume of 5 mM Na-phosphate buffer pH 7.0 and dialysed against 5 × 4 litre of the same buffer over a 36 h period. The dialysate was cleared by centrifugation (fraction 3) and applied to a DEAE-cellulose column (800 ml vol) which had previously been equilibrated with 5 mM Na-phosphate buffer pH 7.0. The epoxide hydratase was not bound to this column which was washed with the equilibration buffer until no further activity was eluted. The combined active fractions (fraction 4) were applied directly to a column of cellulose phosphate (2.5 × 36 cm) which had previously been equilibrated with 5 mM Na-phosphate buffer, pH 7.0. A fraction of the enzyme and the major part of the detergent flowed through the column. This effluent was discarded. The column was washed with 5 mM and 50 mM Na-phosphate buffer, pH 7.0, until no further protein was eluted as judged by the absorbance at 280 nm measured in a LKB Uvicord II flow cell. The hydratase was then eluted by 50 mM Na-phosphate buffer pH 7.0 containing 0.5 M NaCl. Active fractions were combined and concentrated by overnight dialysis against 50 mM Na-phosphate buffer pH 7.0 containing 1 mM EDTA and sufficient $(NH_4)_2SO_4$ to bring the total vol (both inside and outside the dialysis bag) to a concentration of 430 g/l. The hydratase, which precipitated under these conditions, was collected by centrifugation, dissolved in a small vol of 50 mM Na phosphate buffer, pH 7.0, and dialysed against 2 × 1 litre of the same buffer. The dialysate (fraction 5) was then applied to a column of butyl-Sepharose (0.9 cm × 15 cm) which had previously been equilibrated with 5 mM Na-phosphate, pH 7.0. The column was washed with the same buffer until no more protein was eluted before epoxide hydratase was eluted using 5 mM Na phosphate buffer pH 7.0 containing 0.05% Cutscum (fraction 6). The preparation was then either (a) concentrated by dialysis against $(NH_4)_2SO_4$ as described above and used directly or (b) rechromatographed on a small cellulose phosphate column (0.9 × 3 cm) followed by concentration by dialysis against $(NH_4)_2SO_4$ as described above (fraction 7). In both cases the enzyme was dialysed against 50 mM Na-phosphate buffer, pH 7.0, before use.

2.2. *Electrophoresis*

SDS-gel electrophoresis was performed in 2 mm thick slab gels with a 10% acrylamide concentration. The mol. wt was estimated by comparison of the mobility of the purified enzyme with that of standard proteins of known molecular weight using the method of Weber and Osborn [17]. Gels were stained in Coomassie blue as described [17].

3. Results and discussion

The results of a typical enzyme purification are shown in table 1. The epoxide hydratase was solubilised with Cutscum a non-ionic detergent which had proved satisfactory for solubilisation of the enzyme from guinea pig liver microsomes [18] and human liver microsomes [13]. After solubilisation and $(NH_4)_2SO_4$ precipitation the preparation was applied to a DEAE-cellulose column. The effluent from this column contained highly purified epoxide hydratase at a relatively high yield. However large amounts of activity were then lost upon the cellulose phosphate column, yet this step was essential for removal of the detergent which was a prerequisite for hydrophobic chromatography. Hydrophobic arms of different lengths and geometry were coupled to sepharose. Selective adsorption of the hydratase followed by elution with low concentrations of detergent proved most satisfactory when using *n*-butyl residues as hydrophobic arms. The detergent was then removed

Table 1
Purification of rat liver epoxide hydratase

Purification step	Volume (ml)	Total protein (mg)	Total units	Specific activity[a]	Relative purification	Yield %
10 000 *g* supernatant (fraction 1)	1660	49 800	82 557	1.67	1	100
Solubilised microsomes (fraction 2)	1660	11 454	102 107	8.91	5.3	124
Ammonium sulphate precipitate (fraction 3)	360	5184	99 270	19.15	11.5	120
DEAE-cellulose effluent (fraction 4)	500	740	69 300	93.8	56	84
Conc. cellulose phosphate effluent (fraction 5)	6	80	24 000	300	179	29
Butyl-Sepharose effluent (fraction 6)	50	21	14 500	690	415	17.7
Final preparation (fraction 7)	4.8	15.7	8100	516	310	9.8

[a]Specific activity expressed as nmol styrene glycol per mg protein per min.

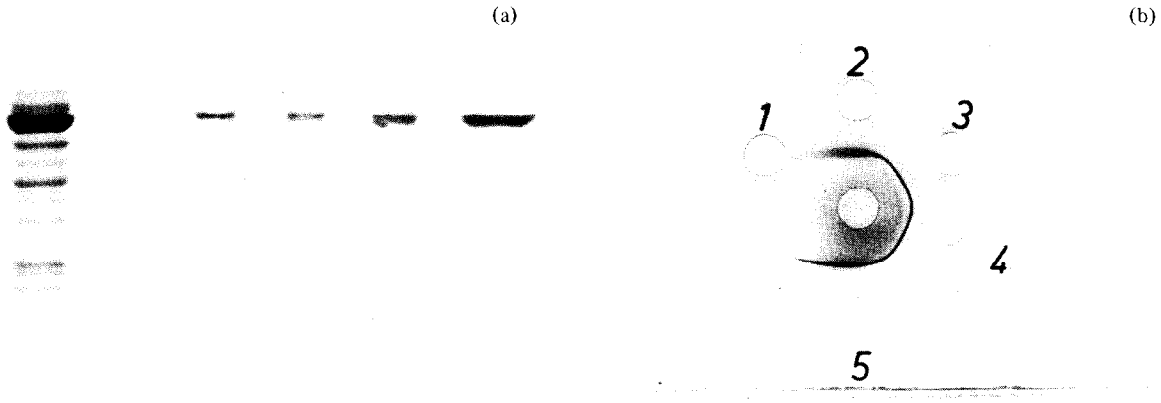

Fig.1. Criteria for the homogeneity of the purified epoxide hydratase. (a) SDS-gel electrophoresis of purified epoxide hydratase and fraction 5 obtained during purification. Electrophoresis was performed in 2-mm thick slab gels containing 10% acrylamide and 0.1% SDS. The fractions shown are from left to right cellulose phosphate effluent (fraction 5) 20 μg, and 0.5 μg, 1 μg, 2 μg, and 5 μg of pure epoxide hydratase. (b) Ouchterlony double diffusion analysis of purified epoxide hydratase. The centre well contained 7.5 μg purified epoxide hydratase. The outer wells each contained 10 μl of (1) control serum, (2) 1/8 dilution of antiserum, (3) 1/4 dilution of antiserum, (4) 1/2 dilution of antiserum, (5) undiluted antiserum.

by chromatography on a small cellulose phosphate column.

The specific activities of the final material was between 520 and 690 units per mg protein in the presence of the detergent, Cutscum, (fraction 6) and between 450 and 520 units per mg protein after removal of the detergent (fraction 7) with a final yield of 7 to 10%. The activity lost by removal of the detergent could be restored by inclusion of Cutscum in the assay mixture. Detergent to protein ratios between 0.3 and 5 mg Cutscum per mg protein gave an apparent activation of 30 to 40%.

In the presence of 0.2% SDS at protein concentrations of 2.5 mg/ml the preparation sedimented as a single symmetrical peak of $S_{20w} = 3$. SDS-gel electrophoresis in 10% polyacrylamide gels yielded a single major band as shown in fig.1A. The electrophoretic patterns of the proteins present in the phosphocellulose effluent (fraction 5 of the purification scheme) are also shown. Ouchterlony double diffusion analysis [19] of various concentrations of the enzyme and antiserum raised against the enzyme in New Zealand White Rabbits (fig.1B) showed a single precipitation line both before (data not shown) and after staining with Coomassie blue. Thus the enzyme preparation appears homogeneous by analytical ultracentrifugational, SDS-gel electrophoretical and immunological criteria.

The minimum molecular weight of epoxide hydratase was estimated by SDS-gel electrophoresis on 10% polyacrylamide gels in the presence of standards of known molecular weight. A plot of the variation of the mobility with the logarithm of the mol. wt is shown in fig.2. In separate experiments using two entirely different enzyme preparations, values for the mol. wt of 49 500 and 48 500 were obtained. This suggests that the minimum mol. wt of the epoxide hydratase is 49 000, which is of the order of magnitude suggested by the sedimentation coefficient measured in the presence of 0.2% SDS of $S_{20w} = 3$. In the absence of detergent the enzyme aggregated forming a very high mol. wt oligomer which sedimented with a sedimentation coefficient of $S_{20w} \sim 14.5$ and did not migrate into the separating gel during polyacrylamide gel electrophoresis.

The homogeneous enzyme possesses a broad specificity for epoxides widely different in structure. The specific activity expressed as nmol product per mg protein per min of the final preparation was 133 towards benzo(a)pyrene 4,5-(K-region-)oxide and 653 towards *p*-chlorophenyl-2,3-epoxypropylether.

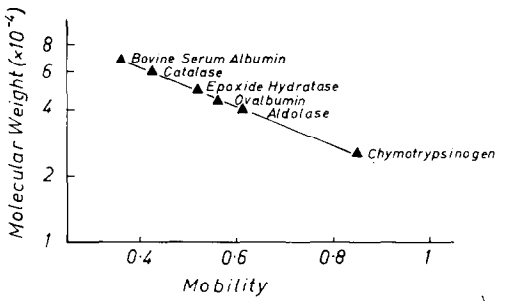

Fig.2. Determination of the minimal mol. wt by electrophoresis on SDS-polyacrylamide gels. Electrophoresis was performed in 2-mm thick slab gels containing 10% acrylamide and 0.1% SDS. Samples of 4 μg of each protein were applied to the same gel, which was dried after staining. Mobilities were measured on the dried gel. Mol. wts of standard proteins are taken from Weber and Osborn [17].

The product of the preparation is exceedingly stable. There was no detectable loss of activity (measured with styrene oxide and benzo(a)pyrene 4,5-oxide as substrates) throughout 2 months storage at 0°C (in an ice bath) in 50 mM Na-phosphate buffer pH 7.0. Similarly an enzyme solution at a concentration of 0.23 mg/ml in 10 mM Na-phosphate buffer, pH 7.0, was completely stable for 5 h at 37°C and showed less than 10% inactivation when maintained at room temperature (25°C) for 24 h.

In conclusion we have developed a method for an over 300-fold purification of epoxide hydratase from rat liver. The product is exceedingly stable and appears homogeneous by ultracentrifugational, electrophoretical and immunological criteria. The minimum mol. wt has been estimated as 49 000 by SDS-gel electrophoresis. The results from the analytical ultracentrifugation in presence of detergent are compatible with this value. The purified enzyme is capable of hydrating both alkene oxides (styrene oxide, *p*-chlorophenyl-2,3,-epoxypropylether) and arene oxides, specifically the 4,5-(K-region) epoxide of benzo(a)pyrene. The availability of this pure enzyme should, therefore, contribute to the understanding of the role of epoxides of polycyclic hydrocarbons in carcinogenesis and mutagenesis. The effect of pure epoxide hydratase upon the mutagenesis of some polycyclic hydrocarbons to bacterial tester strains is at present under investigation.

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