PROPERTIES AND AMINO ACID COMPOSITION OF PURE EPOXIDE HYDRATASE

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Received 15 September 1975

1. Introduction

Rat liver epoxide hydratase [EC 4.2.1.63] which catalyses the conversion of epoxides to *trans*-dihydrodiols has been purified to apparent homogeneity as determined by three independent criteria [1]. The preparation obtained was capable of catalysing the hydration of both styrene oxide and the 4,5- (K-region)epoxide of benzo(a)pyrene [1]. Epoxides of polycyclic hydrocarbons have been implicated as the agents responsible for the cytotoxic and carcinogenic properties of such compounds (for reviews see [2–4]). A detailed knowledge of the properties of epoxide hydratase may, therefore, contribute towards an understanding of the mechanisms of cytotoxicity and carcinogenesis. We report here the results of initial investigations with the pure enzyme.

2. Materials and methods

Anhydrous hydrazine was obtained from Pierce Chem. Co., USA. Carboxypeptidases A and B were isopropylphosphate treated preparations and were obtained from Worthington Biochemical Co. Carboxypeptidase Y (from Yeast) and Carboxypeptidase P (from *Penicillium*) were kindly donated by Dr R. Hayashi from Kyoto University, Japan, and Dr E. Ichishima, Tokyo Noko University, Japan. Epoxide hydratase activity was assayed under the conditions described in detail [5], specifically in the absence of Tween 80.

2.1. *Amino acid analysis*

Samples containing 250 μg protein were dialysed against 0.2 M borate buffer, pH 9.0, free ammonia was removed by placing samples in a boiling water bath for 5 min and samples were dried in vacuo over H_2SO_4. Samples were hydrolysed under reduced pressure at 108 ± 1°C [6] in 1 ml of twice distilled 5.7 N HCl for 24 h and 144 h in evacuated sealed tubes. The hydrolysates were dried under vacuum with a rotary evaporator at 80°C. The dried residues were divided into five portions and one portion (50 μg) was applied to one column. The analysis of the hydrolysates was carried out with a Beckman amino acid analyser model 4255, equipped with a 5 cm column (for basic amino acids) and 50 cm column (for acidic and neutral amino acids) at a flow rate of 30 ml/h according to Spackmann et al. [7]. Tryptophan was analysed using 5% thioglycolic acid [8]. Tyrosine and tryptophan residues were also determined spectrophotometrically in 0.1 N NaOH according to Goodwin and Morton [9]. Cyst(e)ine and methionine residues were also determined after performic acid oxidation of the protein [10].

N-terminal analysis was performed using [^{14}C]-dinitrofluorobenzene [11] and the Edman degradation methods [12]. C-terminal analysis was performed by hydrazinolysis [13] and by carboxypeptidase

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digestion. Treatment with carboxypeptidases A and B was under standard conditions in 0.02 M $NaHCO_3$ buffer, pH 9, containing 0.1 M NaCl whilst digestion with carboxypeptidase Y (from yeast) was carried out in 0.1 M pyridinium acetate buffer pH 5.5 [14], and that with carboxypeptidase P (from *Penicillium*) was in 0.1 M pyridinium formate buffer pH 2.5 [15]. All carboxypeptidase digestions were performed at 37°C overnight.

3. Results and discussion

The absorption spectrum of the pure enzyme is shown in fig.1. The extinction maximum at 280 nm and the A_{280}/A_{260} ratio of 1.46 suggest that the preparation is largely free of Cutscum, the detergent used for solubilising the enzyme from the microsomal membrane, which has a very strong absorption with λ_{max} at 277 nm. The shoulder in the spectrum at 290 nm is indicative of the high tryptophan content as demonstrated by the amino acid analysis shown in table 1. The amounts of tryptophan and tyrosine estimated from the enzyme spectrum were even slightly lower than those estimated chemically which confirms the

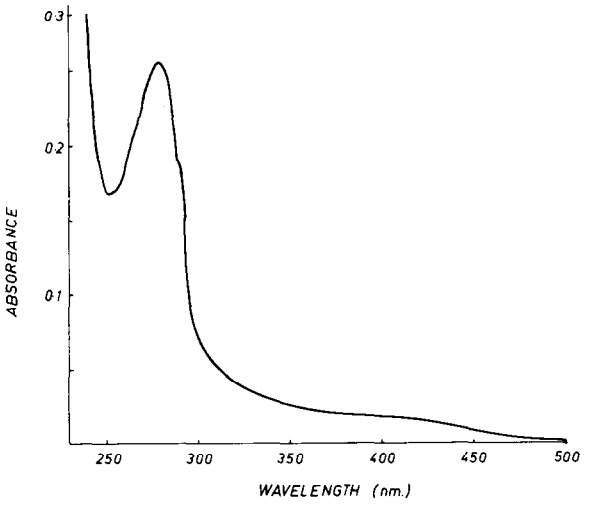

Fig.1. The absorption spectrum of epoxide hydratase. The spectrum was measured in a Beckman 25 spectrophotometer at 25°C. The enzyme was dissolved in filtered 5 mM Na-phosphate buffer pH 7.0 at a protein concentration of 0.14 mg/ml.

detergent free character of the preparation since Cutscum absorbs very strongly at the same wavelengths. The small but significant absorption between 500 nm and 300 nm accounts for the fact that enzyme solutions with a protein concentration between 2 and 3 mg/ml are a faint yellow colour.

Table 1 summarizes the results of the amino acid analysis of pure epoxide hydratase. The residues per monomer were calculated from the per cent contribution of the single amino acids to the entire protein by assuming 43 glutamate (Glx) residues based on which assumption the best fit of the resulting numbers of the single residues and the total mol. wt was obtained in that in the range of 10 000–100 000 the sum of total deviations from integers was minimal for a mol. wt of 48 300–49 000 which is in agreement with the minimum mol. wt of 49 000 as determined by SDS-gel electrophoresis [1]. The integer values are based on extrapolation to zero hydrolysis time for acid labile amino acid residues (threonine, serine, cysteine, tyrosine), on values after 144 h hydrolysis for acid resistant residues (valine, isoleucine), and on average values for the rest of the residues. As shown in table 1 there is a relatively high content of aromatic amino acids and hydrophobic amino acids. The hydrophilic amino acids lysine, histidine, arginine, aspartic acid, asparagine, threonine, serine, glutamic acid, and glutamine [16] account for only 44% of the sum of amino acid residues of the protein. This hydrophobic character could account for the aggregation of the protein in the absence of detergents previously reported [1]. The partial specific volume of the protein calculated from the amino acid analysis is 0.740–0.742 ml/g at 25°C.

No N-terminal amino acid could be detected as [^{14}C]dinitrofluorobenzene- [11] or phenylthiohydantion [12] derivative implying that the terminal residue at the end of the monomer opposite to the C-terminal does not have a free α-amino group. Free C-terminal amino acids were detected by some but not all methods, indicating the presence of a free C-terminal amino acid and allowing some conclusions with respect to its chemical nature. Digestion with carboxypeptidases A and B and hydrazinolysis did not result in detectable amounts of free amino acids. Carboxypeptidase Y digestion, however, resulted in the liberation of a considerable number of amino acids, specifically leucine (0.90), glycine (0.80),

Table 1
Amino acid analysis of epoxide hydratase[a]

Residue	Residues per monomer: Hydrolysis time 24 h	144 h	Values from extrapolation or from other methods		Integral values	Weight contribution
Lysine	32.1	32.1			32	4101
Histidine	13.5	13.3			13–14	1783–1920
NH_3	36.2	44.3	34.8[b]		(<35)	–37
Arginine	19.1	18.5			19	2967
Asparatate(Asx)	28.7	28.9			29	3338
Threonine	19.3	18.8	19.4[b]		19	1921
Serine	31.8	29.5	32.1[b]		32	2787
Glutamate(Glx)	43.0	43.0			43	5552
Proline	25.7	24.3			24–26	2331–2525
Glycine	33.5	33.6			33–34	1883–1940
Alanine	18.1	18.2			18	1279
Half-cystine	3.3	2.0	3.6[b]	4.8[d]	4–5	417–521
Valine	20.3	23.0			23[c]	2280
Methionine	12.0	10.5	8.6[f]	12.2[d]	12	1574
Isoleucine	19.6	21.8			22[c]	2489
Leucine	41.7	41.8			42	4752
Tyrosine	22.4	22.6		22.0[e]	23	3753
Phenylalanine	25.5	26.1			26	3826
Tryptophan			8.3[f]	7.1[e]	7–8	1303–1490
			Total		421–427	48 300–49 000

[a] Analysis involves ± 5% error.
[b] Subject to time dependent deviation from true value. Value obtained by extrapolation to zero hydrolysis time.
[c] Acid resistant residues, results based on the value after 144 hr hydrolysis time.
[d] Determined by performic acid oxidation.
[e] Determined spectrophotometrically.
[f] Determined with 5% thioglycolic acid.

aspartic acid (0.63), asparagine or glutamine (0.60), threonine (0.35), glutamic acid (0.60), lysine (0.42), and trace amounts of alanine, tyrosine, isoleucine and phenylalanine (numbers in brackets indicate moles of amino acid liberated per mole of monomeric protein). Carboxypeptidase P digestion liberated only threonine and asparagine or glutamine. Since hydrazinolysis would be expected to detect C-terminal threonine it may be concluded that the C-terminal amino acid of epoxide hydratase is either asparagine or glutamine.

The pH optimum of the purified enzyme was estimated in 0.125 M Tris buffer over a pH range from 8.0 to 10.0, Na-phosphate buffer I 0.1, between pH 7.0 and pH 10.0 and in 0.05 M glycine buffers between pH 9.0 and pH 10.0 with 2 mM styrene oxide as substrate. Non-enzymic hydration rates which were less than 10% over the entire pH range studied have been subtracted from all the values shown. The pH optimum in Tris buffer was at pH 8.9 and that in Na-phosphate buffer at pH 9.0. In glycine buffer the optimum pH appears to be somewhat higher at pH 9.4 (fig.2). The pH optimum of the pure enzyme estimated in Tris buffer is very similar to those obtained using quite crude epoxide hydratase preparations from guinea pig [17] and human [18] liver. Epoxide hydratase activity did not appear to depend on the ionic strength of the medium. Thus, the activity of the enzyme was not affected by variations in the Tris buffer concentrations at pH 8.9 from 0.025 M to 0.375 M.

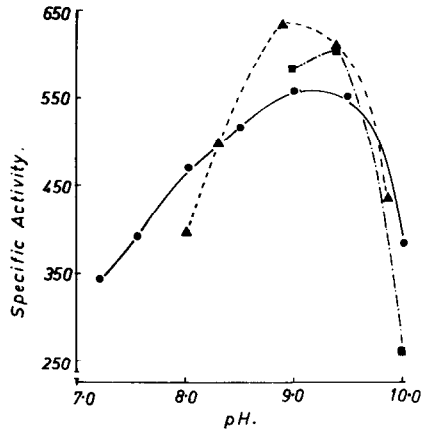

Fig.2. Activity of epoxide hydratase as a function of pH. The buffers used were (▲) 0.125 M Tris, (●) Na-phosphate I 0.1 and (■) 0.05 M glycine. Phosphate solution above pH 8.5 contained 0.005 M glycine buffer at the appropriate pH and sufficient Na_2HPO_4 solution (pH adjusted with NaOH) to produce the desired ionic strength. All assays were performed with 2 mM styrene oxide for 10 min at 37°C. Checks were made to ensure linearity of styrene glycol formation with time at several pH values.

A double reciprocal plot [19] of the variation of the initial specific rate with substrate (styrene oxide) concentration was linear throughout the range of substrate concentrations used (0.2–6 mM) at protein concentrations between 8 and 31 μg/ml. A K_M of 0.67 mM with a maximum specific rate of 800 nmole styrene glycol/min per mg protein was obtained. The maximum specific rate suggests a turnover number of 40 molecules styrene glycol produced per min per molecule of epoxide hydratase using a mol. wt of 49 000.

In conclusion the data show that the purified epoxide hydratase is relatively free from detergent as judged by the u.v. spectrum and a comparison of the spectrophotometrically and chemically determined values for tryptophan and tyrosine content. The amino acid analysis shows a relatively high content of aromatic and hydrophobic amino acids which agrees with the tendency of the enzyme to form very high molecular weight aggregates in the absence of detergent. The presence of a free N-terminal amino acid can be excluded while the C-terminal appears to be the amide form of an acidic amino acid, i.e. either asparagine or glutamine. The enzyme with styrene oxide as substrate shows an alkaline pH optimum at about pH 9 in 3 different buffers with a K_M of 0.67 mM and V_{max} of 800 nmol/min per mg protein estimated at pH 8.9 in Tris buffer resulting in a turnover number of 40 molecules styrene glycol formed per min per minimal weight molecule of enzyme.

Acknowledgement

We are grateful to Mrs J. Dent for skillful technical assistance and to the Deutsche Forschungsgemeinschaft for financial support.

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