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3′,5′-CYCLIC NUCLEOTIDE PHOSPHODIESTERASE ACTIVITY OF THE SULPHATASE A OF OX LIVER

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Summary

The sulphatase A (aryl-sulphate sulphohydrolase, EC 3.1.6.1) of ox liver hydrolyses adenosine 3′,5′-monophosphate (cyclic AMP) to adenosine 5′-phosphate at an optimum pH of approx. 4.3, close to that for the hydrolysis of cerebroside sulphate, a physiological substrate for sulphatase A. The K_m is 11.6 mM for cyclic AMP. On polyacrylamide gel electrophoresis sulphatase A migrates as a single protein band which coincides with both the arylsulphatase and phosphodiesterase activities, suggesting that these are due to a single protein. Cyclic AMP competitively inhibits the arylsulphatase activity of sulphatase A, showing that both activities are associated with a single active site on the enzyme. Sulphatase A also hydrolyses guanosine 3′,5′-monophosphate, but not uridine 3′,5′-monophosphate nor adenosine 2′,3′-monophosphate.

Introduction

Sulphate esters and phosphodiesters have some structural similarity and this suggests that at least some sulphatases might show a phosphodiesterase activity. It has been shown previously [1] that a partially purified arylsulphatase from the marine snail *Charonia lampas* hydrolyses cyclic AMP to adenosine 5′-phosphate, and the present paper deals with the catalysis of this reaction by homogeneous preparations of sulphatase A (aryl-sulphate sulphohydrolase, EC 3.1.6.1) from ox liver [2].

Materials and Methods

Enzyme. Two separate samples of sulphatase A were prepared from ox liver by a slight modification of the method described previously [3]. These were stored in 0.01 M Tris-HCl, pH 7.4, at a concentration of approx. 1 mg/ml and were transported, while frozen, by air from Australia to Japan. The specific activities of the two preparations, 1 and 2, were 237 and 248 $\mu mol \cdot mg^{-1} \cdot min^{-1}$, respectively, when determined in the pH-stat with nitrocatechol sulphate as substrate [4].

Chemicals. The nucleoside 3′,5′-monophosphates and nitrocatechol sulphate were purchased from the Sigma Chemical Co. (St Louis, MO, U.S.A.). Adenosine 2′,3′-monophosphate (NH_4^+ salt) was prepared according to the synthesis of uridine 2′,3′-monophosphate [5], by Mrs. C. Machida in our Institute. Papaverine hydrochloride was kindly donated by Dr. Y. Iijima of Sankyo Co. (Tokyo, Japan).

Enzyme assays. Arylsulphatase activity was measured spectrophotometrically, at 37°C, with nitrocatechol sulphate as substrate. The reaction mixture, vol. 250 µl, contained 3 mM nitrocatechol sulphate, 0.12 M sodium acetate buffer, pH 5.0, and the enzyme. Samples (50 µl) were withdrawn from the reaction mixture at 0, 1, 3 and 5 min from the start of the reaction and immediately added to 0.5 ml 0.3 M NaOH. The absorbance of the resulting solution was read at 510 nm (ϵ_{510nm} of 4-nitrocatechol, 12 600) and from these readings v_0 was estimated graphically.

Cyclic nucleotide phosphodiesterase activity was measured by determining the amount of nucleotide 5′-phosphate formed. The reaction mixture (80 µl) contained 7.5 mM cyclic nucleotide, 0.06 M sodium acetate buffer, pH 4.3, and the enzyme. Samples (10 µl) were withdrawn from the reaction mixture at 0, 0.5, 1, 2 and 3 h from the start of the reaction and immediately spotted on thin-layer plates (unmodified cellulose: DC Plastikrolle Cellulose; Merck Ab., Darmstadt, F.R.G.). Good separation of the product from the excess substrate was obtained by development in one dimension using a solvent system prepared by dissolving 30 g $(NH_4)_2SO_4$ in 100 ml 0.2 M phosphate buffer, pH 7.0/4 ml propan-2-ol. The nucleotides were visualised under an ultraviolet lamp and marked with a soft pencil. The appropriate areas of cellulose were loosened with a spatula, quantitatively transferred by suction into a Fintip (1 ml) containing a pad of glass wool, and the nucleotides eluted by washing twice with 0.4 ml portions of 0.1 M HCl. The absorbances of the eluates at 260 nm were measured and v_0 was estimated graphically from the fraction of the substrate hydrolysed at each time, calculated as A_{260} mononucleotide/A_{260} (mononucleotide + cyclic nucleotide).

Disc gel electrophoresis. Electrophoresis was carried out at 4°C for 1.5 h at 3 mA/tube in Tris-glycine buffer, pH 8.3, on 8% polyacrylamide gels. To each gel was applied 50 µl preparation 2 (55 µg protein) together with 150 µl 66% glycerol containing Bromophenol blue. To locate the enzyme activities, the gel was cut into 0.5 mm slices using a gel slicer (Joyce Loebel Co., U.K.). Every first slice was used for the measurement of arylsulphatase activity and every second and third slices were combined for the measurement of phosphodiesterase activity. The slices of gel were suspended in 100 µl of the appropriate buf-

fer (0.25 M sodium acetate buffer, pH 5.0, for arylsulphatase activity and pH 4.3 for phosphodiesterase activity), crushed with a spatula, frozen and thawed, and then taken to 37°C.

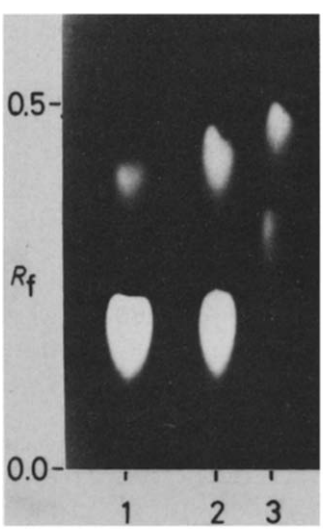
Arylsulphatase and phosphodiesterase activities were determined as described in enzyme assays, except that the reaction time after the addition of respective substrate was 1 min for arylsulphatase and 14 h for phosphodiesterase.

Protein was located by staining the gels for several hours in 15 ml 33% trichloroacetic acid, 4 ml 0.2% Coomassie brilliant blue in 50% ethanol and 41 ml methanol/acetic acid/water (3 : 1 : 6, v/v) followed by destaining in the latter methanol/acetic acid/water mixture. The relative concentration across the protein band in the gel was obtained by scanning with a soft-laser scanning densitometer SL504 (Biomed Co., U.S.A).

Results

Phosphodiesterase activity of sulphatase A. Highly purified preparations of sulphatase A hydrolysed cyclic AMP to AMP, as shown by TLC of the reaction mixtures (Fig. 1). The faster component in the hydrolysate (lane 2 in Fig. 1) was identified as AMP because of comigration with authentic AMP. This phosphodiesterase activity was evident over the pH range 3.7—6.0, with an optimum pH of about 4.3. The latter value is considerably lower than that of 5.6 for the arylsulphatase activity of sulphatase A [2].

The specific activities of the two preparations of sulphatase A with cyclic AMP as substrate were 0.0713 and 0.0795 $\mu mol \cdot mg^{-1} \cdot min^{-1}$, respectively.



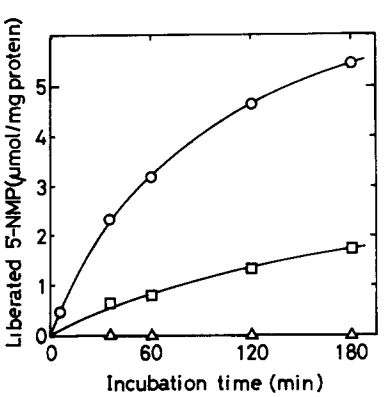


Fig. 1. Identification by TLC of the nucleotide formed by the hydrolysis of cyclic AMP by sulphatase A. The hydrolysis was for 2 h with 20 μg enzyme. 1. Hydrolysate. 2. Hydrolysate + adenosine 5′-phosphate. 3. Adenosine 3′-phosphate + adenosine 5′-phosphate.

Fig. 2. The hydrolysis of cyclic AMP (○———○), cyclic GMP (□———□) and cyclic UMP (△———△) by sulphatase A.

The ratio of cyclic nucleotide phosphodiesterase activity to arylsulphatase activity (determined spectrophotometrically) was about 1/2250 in both preparations (Table I).

Sulphatase A also hydrolysed cyclic GMP, but only at about 17—19% of the rate of hydrolysis of cyclic AMP under similar conditions (Fig. 2). Neither cyclic UMP nor adenosine 2′,3′-monophosphate was hydrolysed by sulphatase A in the pH range 4.0—7.0.

Association of phosphodiesterase and arylsulphatase activities. As shown in Fig. 3, sulphatase A gives a single protein band on gel electrophoresis at pH 8.3. The cyclic nucleotide phosphodiesterase and arylsulphatase activities were completely associated with this protein band, indicating that both were due to a single protein.

Inhibition of the enzyme activities. Papaverine, a muscle relaxant, is generally a noncompetitive inhibitor of cyclic nucleotide phosphodiesterase [6]. The latter activity of sulphatase A was inhibited by about 30% at 160 μM papaverine (Table II). On the other hand, the arylsulphatase activity of sulphatase A was only slightly affected by papaverine, 320 μM papaverine causing only about 4% inhibition (Table II).

The arylsulphatase activity was inhibited by 1 mM AMP, the reaction product of the phosphodiesterase activity, and by its analogues ADP and ATP. The inhibitory effects of these nucleotides were not as great as those of inorganic phosphate, as is shown in Table II. The inhibition of the arylsulphatase activity by ATP or by inorganic phosphate was competitive (Fig. 4) and the values of K_i were 0.031 mM and 0.018 mM, respectively. Cyclic AMP at a concentration of 1 mM had little effect on the arylsulphatase activity but at higher concentrations it was a competitive inhibitor with a K_i of 9.0 mM. The latter value is close to the K_m value for the cyclic nucleotide phosphodiesterase activity (see below). This again suggests that both activities are associated with a single active site in sulphatase A.

Kinetics of cyclic AMP hydrolysis. Double-reciprocal plots were essentially linear and gave a K_m of 11.6 mM cyclic AMP (Fig. 5) although the points at the highest substrate concentrations seemed to deviate below the line. A similar, but more obvious, nonlinearity has been noted in the hydrolysis of ascorbate

TABLE I

CYCLIC NUCLEOTIDE PHOSPHODIESTERASE ACTIVITY OF SULPHATASE A FROM OX LIVER

	Preparation 1		Preparation 2	
	v_0 ($\mu mol \cdot mg^{-1} \cdot min^{-1}$)	v_0/v_0 NCS	v_0 ($\mu mol \cdot mg^{-1} \cdot min^{-1}$)	v_0/v_0 NCS
Arylsulphatase activity towards NCS	162	1	179	1
Phosphodiesterase activity towards 3′,5′-cyclic AMP	0.0713	1/2270	0.0795	1/2250
towards 3′,5′-cyclic GMP	0.0124	1/13000	0.0152	1/11800

NCS, nitrocatechol sulphate.

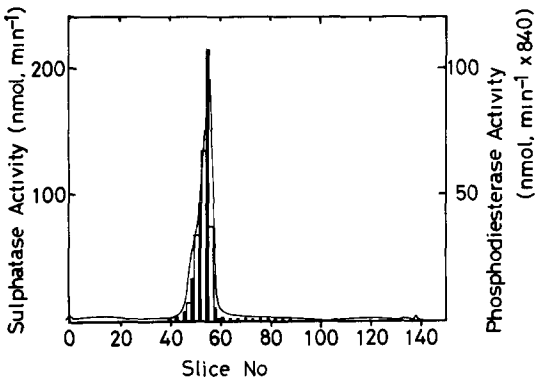


Fig. 3. Profile of the gel electrophoresis of sulphatase A. ———, relative concentration of protein; ▬▬, arylsulphatase activity with nitrocatechol sulphate as substrate, ▭▭, phosphodiesterase activity with cyclic AMP as substrate. The sulphatase activity is drawn on a scale of about 1/3300 of that of the phosphodiesterase activity.

TABLE II

INHIBITION OF THE ARYLSULPHATASE AND CYCLIC NUCLEOTIDE PHOSPHODIESTERASE ACTIVITIES OF SULPHATASE A

Addition	Conc. (mM)	Inhibition (%)	
		Arylsulphatase	Phosphodiesterase
Papaverine	0.32	<4.5	
	0.16		30.0
Adenosine	1	0	
3′,5′-cyclic AMP	1	—2.7	
3′,5′-cyclic GMP	1	0	
3′-AMP	1	10.7	
5′-AMP	1	19.6	
ADP	1	46.4	
ATP	1	58.0	88.5
NaH_2PO_4	0.1	77.6	
	0.05	58.9	

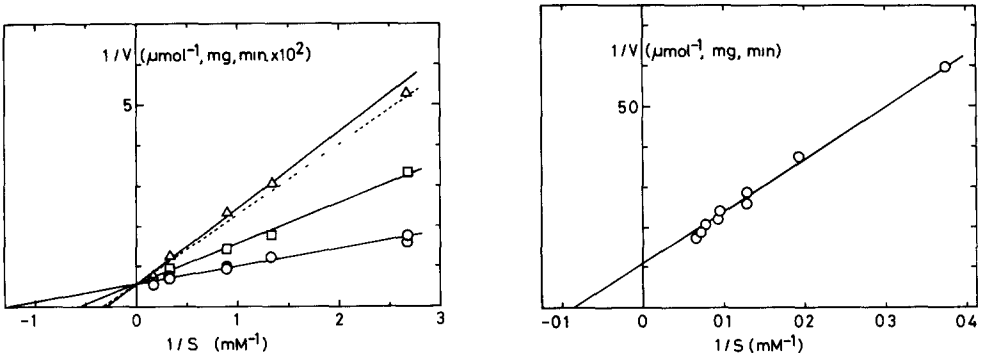


Fig. 4. The inhibitory effect of various phosphates on the hydrolysis of nitrocatechol sulphate by sulphatase A. The assay used 0.49 μg enzyme. ○———○, no inhibitor; △———△, 1 mM ATP; □———□, 11.8 mM cyclic AMP; - - - - - -, 0.05 mM NaH_2PO_4.

Fig. 5. Reciprocal plot of the hydrolysis of cyclic AMP by sulphatase A. The reaction mixture (100 μl) contained 50 mM acetate buffer, pH 4.3/23.3 μg sulphatase. The initial velocity, v_0 was estimated graphically.

2-sulphate by sulphatase A at pH 4.8 [7], where it may be due to the existence of sulphatase A as a tetramer at low pH values [8].

As is obvious in Fig. 3, the phosphodiesterase activity of sulphatase A does not follow zero-order kinetics because the reaction velocity falls significantly during the course of the reaction. It is not known whether this is a substrate-induced inactivation, similar to, but slower than, that which occurs during the arylsulphatase reaction of sulphatase A [4], or whether it is due to some other factor such as the instability of sulphatase A in acid solution [4], the accumulation of products or the disappearance of substrate. The latter seems an unlikely explanation: even with cyclic AMP as substrate only about 20% of the substrate was utilized during the reaction.

Discussion

Phosphodiesters and sulphate esters both have a single negative charge and have similar shapes because the atomic distances O-P and O-S are almost equal and the bond angles O-P-O and O-S-O are of comparable size. Because of this, and because several sulphatases are inhibited by phosphate ions [2], as well as some phosphodiesterases by sulphate ions, we predicted that some sulphatases might hydrolyse phosphodiesters, or some phosphodiesterases might hydrolyse sulphate esters. The present work shows that homogeneous preparations of sulphatase A from ox liver had, as expected, phosphodiesterase activity. Further, this enzyme showed the substrate specificity of a typical 3′,5′-cyclic nucleotide phosphodiesterase [9]: it could hydrolyse adenosine 3′,5′-monophosphate and guanosine 3′,5′-monophosphate but not uridine 3′,5′-monophosphate nor adenosine 2′,3′-monophosphate.

Crystallographic studies of guanosine 3′,5′-monophosphate [10,11], cytidine 2′,3′-monophosphate [12] and ascorbate 2-sulphate [13] have thrown some light on this specificity. The phosphate group in guanosine 3′,5′-monophosphate appears to be quite normal with respect to the length of the ester bond (O(3′)-P; 1.610 Å) and its angle (O(5′)-P-O(3′); 103.9°) [10,11]: these values are similar to the length of the sulphate ester bond (O(2)-S; 1.628 Å) and its angle (O(2)-S-O(9); 105.4°) in ascorbate 2-sulphate [13]. On the other hand, the phosphate group in cytidine 2′,3′-monophosphate is in a strained configuration in which the O(2′)-P-O(3′) bond angle of 96.2° is smaller than that of about 103° in the 3′,5′-monophosphates and, further, the five-membered phosphate ring has O(2′), C(2′), C(3′) and O(3′) in a plane while the phosphorus atom is puckered 0.47 Å towards the sugar [12]. Therefore, it appears reasonable that only the 3′,5′-monophosphates, in which the phosphate groups resemble a sulphate ester, should serve as substrates for sulphatase A. However, the inability of sulphatase A to hydrolyse uridine 3′,5′-monophosphate cannot be due to an incorrect conformation of the phosphate group because this must be very similar to that in cyclic AMP and cyclic GMP. Therefore, sulphatase A must bind the latter two compounds not only at the phosphodiester bond but also through the purine moiety. It may be pertinent that when functioning as an arylsulphatase, sulphatase A has a greater affinity for the sulphate esters of polycyclic phenols than for those of monocyclic phenols [2]: for example, there is an order of magnitude difference in the values of the K_m for phenyl

sulphate and 2-naphthyl sulphate, and a further order of magnitude difference between the latter and phenanthryl 2-sulphate.

Previous studies have shown that 3′,5′-cyclic nucleotide phosphodiesterase exists in multiple forms in a number of mammalian tissues [9,15]. The phosphodiesterase activity of sulphatase A is quite similar to that of the so-called 'high K_m' phosphodiesterase, particularly from bovine brain [16] and liver [17], in that it hydrolyses both cyclic AMP and, at a lower rate, cyclic GMP. The pH optimum of the phosphodiesterase activity of sulphatase A is, however, quite different from that of the other cyclic nucleotide phosphodiesterases in that it lies in the acid region, about pH 4.3, which is close to the optimum pH of sulphatase A for its naturally occurring substrates ascorbate 2-sulphate and cerebroside sulphate (Table III). This suggests that the phosphodiesterase activity associated with sulphatase A is different from the activity of the other phosphodiesterases so far found in mammalian tissues, a view supported by the different values of K_m and V for cyclic AMP. These are about two orders of magnitude larger and three orders of magnitude smaller, respectively, for sulphatase A than for other phosphodiesterases. However, the kintic behaviour of sulphatase A acting as a phosphodiesterase cannot necessarily be directly compared with that of the known phosphodiesterases because in the present work the assays for the phosphodiesterase activity were performed under conditions similar to those for the arylsulphatase activity: that is, unphysiologically high concentrations of cyclic AMP without the addition of Mg^{2+} or any other possible activator. No physiological role for the phosphodiesterase activity of sulphatase A can as yet be suggested and cerainly the K_m value is too high for this activity to participate in the regulation of the level of cyclic AMP in vivo.

Nevertheless, the K_m value for cyclic AMP is of the same order of magnitude as that for ascorbate 2-sulphate, a naturally occurring substrate for sulphatase A, although two orders of magnitude greater than that for cerebroside sulphate (Table III) [18], at least when the latter is measured in the presence of taurodeoxycholate and Mn^{2+} as a solubilising agent [19]. Sulphatase A functions in vivo primarily as a cerebroside sulphatase: in this situation the substrate is a one-to-one complex of cerebroside sulphate with an acidic lysosomal glycoprotein [20]. There is no evidence for any such complication in the arylsulphatase activity of sulphatase A but it is not impossible that in vivo its phos-

TABLE III

COMPARISON OF THE KINETIC PARAMETERS FOR SULPHATASE A WITH DIFFERENT SUBSTRATES

	K_m (mM)	V (μmol · min^{-1} · mg^{-1})	pH
Cerebroside sulphate *	0.060	9	4.5
Ascorbate 2-sulphate	7	90	4.8
Nitrocatechol sulphate	0.79	180	5.0
3′,5′-cyclic AMP	11.6	0.09	4.3

* In the presence of 2 mM taurodeoxycholate and 35 mM $MnCl_2$.

phodiesterase activity might be higher than has been found in vitro, because it is known that the activity of the 'high K_m' cyclic nucleotide phosphodiesterase is stimulated not only by Mg^{2+} but also by lipids [21] and an activator protein [22].

The finding that sulphatase A, like the arylsulphatase of *C. lampas* [1], shows phosphodiesterase activity suggests that this association might be a general phenomenon. The converse situation of phosphodiesterases showing arylsulphatase activity should also be considered. Perhaps pertinent, is the discovery of a 2′,3′-cyclic nucleotide 3′-phosphodiesterase (EC 3.1.4.37) in the central nervous system because no naturally occurring substrate for this enzyme has as yet been found [23].

References

1 Egami, F. and Uchida, T. (1978) Seikagaku 50, 1297—1303
2 Nicholls, R.G. and Roy, A.B. (1971) in The Enzymes, 3rd edn., (Boyer, P.D., ed.), Vol. V, pp. 21—41, Academic Press, New York
3 Nichol, L.W. and Roy, A.B. (1964) J. Biochem. (Tokyo) 55, 643—651
4 Roy, A.B. (1978) Biochim. Biophys. Acta 526, 489—506
5 Smith, M., Moffatt, J.G. and Khorana, H.G. (1958) J. Am. Chem. Soc. 80, 6204—6212
6 Weiss, B. and Levin, R.M. (1978) in Adv. Cyclic Nucl. Res. (George, W.J. and Ignarro, L.J., eds.), Vol. 9, pp. 285—303, Raven Press, New York
7 Roy, A.B. (1975) Biochim. Biophys. Acta 377, 356—363
8 Jerfy, A., Roy, A.B. and Tomkins, H.J. (1976) Biochim. Biophys. Acta 422, 335—348
9 Wells, J.N. and Hardman, J.G. (1977) in Adv. Cyclic Nucl. Res. (Greengard, P. and Robinson, G.A., eds.), Vol. 8, pp. 119—143, Raven Press, New York
10 Chwang, A.K. and Sundaralingam, M. (1974) Acta Crystallogr. 30, 1233—1240
11 Sundaralingam, M. and Abola, J. (1972) Nature New Biol. 235, 244—245
12 Coulter, C.L. and Greaves, M.L. (1970) Science 169, 1097—1098
13 McClelland, B.W. (1974) Acta Crystallogr. 30, 178—186
14 Coulter, C.L. (1969) Acta Crystallogr. 25, 2055—2065
15 Strada, S.J. and Thompson, W.J. (1978) in Adv. Cyclic Nucl. Res. (George, W.J. and Ignarro, L.J., eds.), Vol. 9, pp. 265—283, Raven Press, New York
16 Morrill, M.E., Thompson, S.T. and Stellwagen, E. (1979) J. Biol. Chem. 254, 4371—4374
17 Schröder, J. and Rickenberg, H.V. (1973) Biochim. Biophys. Acta 302, 50—63
18 Jerfy, A. and Roy, A.B. (1973) Biochim. Biophys. Acta 293, 178—190
19 Jeffrey, H.J. and Roy, A.B. (1977) Aust. J. Exp. Biol. Med. Sci. 55, 339—346
20 Fischer, G. and Jatzkewitz, H. (1977) Biochim. Biophys. Acta 481, 561—572
21 Pichard, A.-L. and Cheung, W.Y. (1977) J. Biol. Chem. 252, 4872—4875
22 Weiss, B. and Fertel, R. (1977) in Adv. Pharm. Chemother. (Garattini, S., Goldin, A., Hawking, F. and Kopin, I.G., eds.), Vol. 14, pp. 189—283, Raven Press, New York
23 Sprinkle, T.J., Zaruba, M.E. and McKhann, G.M. (1978) J. Neurochem. 30, 309—314