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THE SULPHATASE OF OX LIVER

XXII. FURTHER OBSERVATIONS ON THE CEREBROSIDE SULPHATASE ACTIVITY OF SULPHATASE A *

A.B. ROY

Department of Physical Biochemistry, John Curtin School of Medical Research, Australian National University, P.O. Box 334, Canberra City, ACT 2601 (Australia)

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Summary

Further studies have been made of the cerebroside sulphatase activity of the sulphatase A (aryl-sulphate sulphohydrolase, EC 3.1.6.1) of ox liver. It is concluded that a cerebroside sulphate-modified form of the enzyme is not produced and that the kinetics of the reaction can be explained by the utilisation of the substrate and accumulation of SO_4^{2-}. The hypothesis is advanced that this difference between the cerebroside sulphatase and arylsulphatase activities arises from non-polar binding of the cerebroside to the enzyme.

Possible reasons for the differences between these results and those of other (Stinshoff, K. and Jatzkewitz, H. (1975) Biochim. Biophys. Acta 377, 126—138) are considered.

Introduction

Although it is now clearly established that sulphatase A (aryl-sulphate sulphohydrolase, EC 3.1.6.1) undergoes a substrate-induced inactivation when functioning as an arylsulphatase [1], there are conflicting reports on its behaviour as a cerebroside sulphatase. Jerfy and Roy [2] could not isolate a cerebroside sulphate-modified form of the sulphatase A of ox liver and they concluded that the slight inactivation which took place during the reaction was caused by the vigorous stirring of the reaction mixture in the pH-stat. On the other hand, Stinshoff and Jatzkewitz [3] showed the formation of a

* Part XXI: see Ref. 1.

cerebroside sulphate-modified form of the sulphatase A of human liver and kidney. They did not isolate this substrate-modified enzyme, but they did show that it was not reactivated by SO_4^{2-}, with cerebroside sulphate as substrate. It was found that the hydrolysis of cerebroside sulphate by crude sulphatase A from human fibroblasts was not linear with time 'even over short time intervals' [4], yet more recently, the same reaction apparently under identical conditions catalysed by the purified sulphatase A of human urine, has been described as linear with time [5].

As noted in other studies [1,2], there were differences in the inactivation of sulphatase A by aryl sulphates at pH 4.5 (the optimum pH for cerebroside sulphatase activity) and pH 5.6 (the optimum pH for arylsulphatase activity), we have performed a more detailed investigation of the behaviour during the hydrolysis of cerebroside.

Methods

Preparation and assay of sulphatase A. Sulphatase A was prepared from ox liver by the method of Nichol and Roy [6]. Routine assays of arylsulphatase activity were carried out in a pH-stat (assembly PHM26-TT11-SBR2-ABU12; Radiometer, Copenhagen) at 37°C with 3 mM nitrocatechol sulphate in 0.1 M KCl (pH 5.6) and v_0 was computed by fitting an exponential curve to the data [1]. The formation of substrate-modified sulphatase A was followed by the method of Nicholls and Roy [7], except that the substrate concentration was 3 mM nitrocatechol sulphate and the activation was brought about by the addition of 3 mM K_2SO_4. An estimate of the amount of substrate-modified enzyme present was given by the ratio of v_0, the initial velocity computed as above, to v_f, the velocity given by the slope of the progress curve between 20 and 30 min after the addition of K_2SO_4.

Cerebroside sulphatase activity was determined as before [2] in the pH-stat at 37°C with 0.2 mM cerebroside sulphate (Supelco, Bellefonte, PA) in 0.035 M $MnCl_2$ containing 2 mM sodium taurodeoxycholate and 0.5 mM sodium acetate (pH 4.5). As no satisfactory method of computing v_0 in this assay is available, the velocity 1 min after the start of the reaction, v_1, was obtained by drawing the tangent to the progress curve at this point [2].

The saturated KCl in the reference electrode of the pH-stat was changed daily to prevent inactivation of sulphatase A by diffusion of HgCl into the reaction mixture [8].

Results

Inactivation by nitrocatechol sulphate

The data in Table I show that the inactivation of the enzyme in the presence of nitrocatechol sulphate occurs at both pH 4.5 and 5.6 but, as previously shown [1], the inactivation was more rapid at the latter pH. The activity remaining after 30 min was greater at pH 4.5 than at 5.6. The most striking difference was the response of the modified enzyme to SO_4^{2-}: at pH 4.5 the modified enzyme is inhibited by SO_4^{2-}, whether it was prepared at pH 4.5 or 5.6 (Table I). Conversely, the modified enzyme was activated by SO_4^{2-} at pH

TABLE I

THE EFFECT OF SO_4^{2-} ON SUBSTRATE-MODIFIED SULPHATASE A

The modified enzyme was formed by incubation with nitrocatechol sulphate at pH 5.6 or 4.5: v_0 and k^* (the apparent velocity constant for the inactivation) were computed as before [1] and v_{30} was estimated from the slope of the progress curve 30 min after the start of the reaction, immediately before the addition of K_2SO_4 and any change in pH. v_f was measured between 50 and 60 min after the start of the reaction (20—30 min after adding K_2SO_4). Enzyme concentration about 0.25 μg/ml.

First stage				Second stage		
pH	v_0 ($\mu mol \cdot l^{-1} \cdot min^{-1}$)	k^* (min^{-1})	v_{30} ($\mu mol \cdot l^{-1}$)	pH	SO_4^{2-} (μmol)	v_f ($\mu mol \cdot l^{-1} \cdot min^{-1}$)
5.6	48.3	0.24	5.9	5.6	0	8.1
				5.6	30	9.3
				4.5	0	4.9
				4.5	30	1.3
4.5	27.2	0.15	6.5	5.6	0	6.3
				5.6	30	7.3
				4.5	0	2.4
				4.5	30	1.3

5.6, whether it had been prepared at pH 4.5 or 5.6. It should be noted that an exact correspondence of the final velocities in Table I is not to be expected because the concentration of SO_4^{2-} in the latter stages of the reaction is not the same in all cases due to the different velocities in the initial stages.

Progress curves for the hydrolysis of cerebroside sulphate

A typical progress curve for the hydrolysis of cerebroside sulphate by sulphatase A at pH 4.5 in 0.035 M $MnCl_2$ and 2 mM taurodeoxycholate is shown in Fig. 1. The reaction velocity fell quite rapidly and this was due to disappearance of cerebroside sulphate and the accumulation of SO_4^{2-}: no other factor was involved, as shown by the agreement between the experimental points and the theoretical line from Eqn. 1. The latter is the integrated form of the Michaelis equation, when one of the reaction products is a non-competitive inhibitor (as SO_4^{2-} is for the hydrolysis of cerebroside sulphate [2]): p is the amount of product at time t and K_1 is the K_i for a product which is a non-competitive inhibitor

$$Vt = K_m\left[1 + \frac{s_0}{K_1}\right] \ln \frac{s_0}{s_0 - p} + \left[1 - \frac{K_m}{K_1}\right]p + \frac{1}{2K_1} \cdot p^2 \qquad (1)$$

Cerebroside, the other product of the reaction is not an inhibitor of the hydrolysis of cerebroside sulphate by the sulphatase A of ox liver under the above conditions [2].

In another experiment the concentration of cerebroside sulphate was kept approximately constant by the addition, at about 2-min intervals of 10-μl portions of a sonicated [9] suspension of cerebroside sulphate (of the same concentration as the NaOH used as titrant in the pH-stat (about 0.015 M)) and the results are also shown in Fig. 1. The experimental points agree well with the

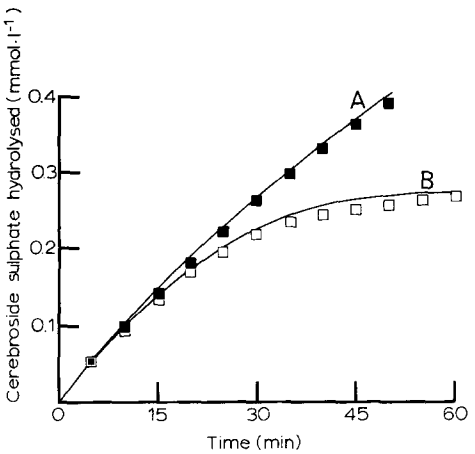

Fig. 1. Progress curves for the hydrolysis of 0.275 mM cerebroside sulphate at pH 4.5 in 0.035 M $MnCl_2$ and 0.002 M sodium taurodeoxycholate. In A the concentration of cerebroside sulphate was kept approximately constant by its addition at appropriate intervals but in B the hydrolysis was allowed to go to completion. The points are taken from the pH-stat recordings and the lines are drawn from Eqns. 2 and 1, respectively.

theoretical curve from Eqn. 2, which corresponds to Eqn. 1 with the condition that the substrate concentration is held constant:

$$Vt = \left[1 + \frac{K_m}{s_0}\right]\left[p + \frac{1}{2K_i} \cdot p^2\right] \qquad (2)$$

When the conditions were those of the standard assay, in which about 30% of the substrate is hydrolysed in 3 min, the experimental points again fitted the theoretical curve given by Eqn. 1 (Fig. 2). This is in contrast to the situation with nitrocatechol sulphate, where only about 5% of the substrate is

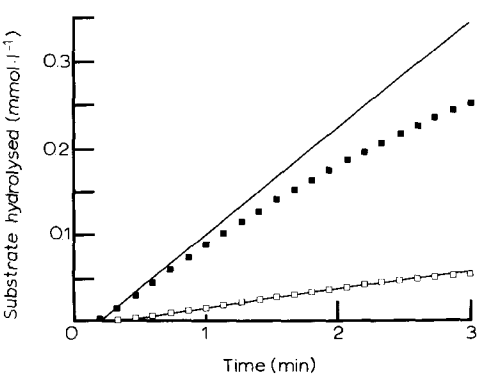

Fig. 2. Progress curves for the hydrolysis of 0.2 mM cerebroside sulphate (□) and 3 mM nitrocatechol sulphate (■) at pH 4.5 and 5.6, respectively. The points are taken from pH-stat recordings: the lines are drawn from Eqn. 1 and the corresponding expression for a competitive inhibition by sulphate. The equations are those for a simple enzyme reaction: no terms have been introduced to account for the sigmoidality in the initial stages of a pH-stat recording (see Ref. 1) hence the lines do not pass through the origin but have been adjusted to pass through the initial points: this is equivalent to an error in the zero time of about 12 s.

hydrolysed in 3 min. Any deviation from linearity in this case should be small but, because of the pronounced substrate-induced inactivation [1], the experimental points do not fit the theoretical curve for a reaction where one of the products, SO_4^{2-}, is a competitive inhibitor.

Attempted detection of cerebroside sulphate-modified sulphatase A

The results in Figs. 1 and 2 suggested that the kinetics of the hydrolysis of cerebroside sulphate by sulphatase A could be explained by the disappearance of substrate and the accumulation of SO_4^{2-}. Further evidence for this was provided by measuring the arylsulphatase activity remaining after sulphatase A had functioned as a cerebroside sulphatase. 4 μg/ml sulphatase A was incubated for 1 h at 37°C in the pH-stat in 0.035 M $MnCl_2$ and 2 mM taurodeoxycholate, with or without 0.25 mM cerebroside sulphate, after which a 0.5 or 1 ml sample of the reaction mixture was assayed for arylsulphatase activity at pH 5.6. The concentration of $MnCl_2$ and taurodeoxycholate in the latter reaction were 3.5 mM and 0.2 mM, respectively; the concentrations of cerebroside sulphate and its hydrolysis products obviously could not be controlled, but they did not exceed 0.025 mM. The results again give no indication of any cerebroside sulphate-induced inactivation of sulphatase A (Table II). It should be noted that only at pH 4.5 was there a rapid hydrolysis of cerebroside sulphate during the initial incubation.

Experiments in the absence of taurodeoxycholate

A suspension (corresponding to 0.16 mM) of cerebroside sulphate was prepared by treatment of the solid lipid in 0.01 M HCOONa, adjusted to pH 5.6 with HCOOH (see Ref. 3), for two 30-s periods at 0°C in an MSE ultrasonic power unit [9]. This suspension was used as a potential substrate for sulphatase A in the pH-stat. At 4 μg/ml enzyme, no hydrolysis was detectable at several

TABLE II

THE EFFECT OF PREINCUBATION, WITH OR WITHOUT CEREBROSIDE SULPHATE, ON THE ARYLSULPHATASE ACTIVITY OF SULPHATASE A

In the pretreatment, sulphatase A (4 μg/ml) was incubated for 1 h at the appropriate pH in 0.035 M $MnCl_2$ and 0.002 M taurodeoxycholate with or without 0.25 mM cerebroside sulphate. The reaction mixture was then taken for the assay of its arylsulphatase activity.

Pretreatment			Arylsulphatase assay	
pH	Cerebroside sulphate (mM)	v_1 (μmol · mg^{-1} · min^{-1})	v_0 (μmol · mg^{-1} · min^{-1})	v_0/v_f
—	—	—	296 ± 22	5.4 ± 0.6
—	—	—	275 ± 23 *	5.1 ± 0.5 *
4.5	0.0	—	220	4.5
4.5	0.25	7.8	262	5.0
5.0	0.0	—	295	6.0
5.0	0.25	0.5	308	6.2
5.6	0.0	—	319	6.2
5.6	0.25	0.0	344	6.1

* 0.025 mM cerebroside sulphate present in assay.

pH values between 4.5 and 5.6. Addition of the appropriate amounts of $MnCl_2$ and sodium taurodeoxycholate at pH 4.5 allowed hydrolysis to begin. It was not possible to obtain accurate values of v_1 subsequent to the addition of the taurodeoxycholate because the solubilisation of the cerebroside sulphate took several minutes, judging by the rate of clearing of the suspension.

Discussion

The fact that the substrate-modified enzyme formed at pH 4.5 was activated by SO_4^{2-} at pH 5.6 justified attempts to detect the formation of cerebroside sulphate-modified sulphatase A at pH 4.5 by subsequently following the hydrolysis of nitrocatechol sulphate at pH 5.6 (assuming that any cerebroside sulphate-modified enzyme would have the same properties as the nitrocatechol sulphate-modified enzyme, Table I).

The results with cerebroside sulphate (Figs. 1 and 2, Table II) show, in agreement with previous results [2], that there is no evidence to suggest the formation of a cerebroside sulphate-modified sulphatase A. The fall in the velocity of hydrolysis is due to the decreasing concentration of cerebroside sulphate and the increasing concentration of SO_4^{2-}, a non-competitive inhibitor.

The results appear to be at variance with those of Stinshoff and Jatzkewitz [3] with human sulphatase A. In this case, both products, SO_4^{2-} and cerebroside, are inhibitors: the former is non-competitive, but no information is available about the latter. It is likely to be competitive because, in a uni-bi reaction, the inhibition by one product is competitive and by the other, non-competitive. Assuming this to be the case, the data in Table II of Ref. 3 gives a value of 0.03 mM cerebroside for K_i. Even allowing for inhibition by both products, the fall in velocity noted by Stinshoff and Jatzkewitz [3], for example, in Fig. 1 of Ref. 3 cannot be explained. Another interpretation of their results is possible, however: the fall in velocity could be due to the utilisation of substrate and accumulation of products if the substrate concentration were lower than expected. The suggestion is not that the cerebroside sulphate was impure, but that, because the suspension of cerebroside sulphate is heterogeneous with respect to particle size [9], only a fraction of the total concentration may be available as substrate. If it is assumed that the available substrate was completely hydrolysed in the experiment shown in Fig. 1 or Ref. 3, (i.e. that the actual substrate concentration was 0.03 mM compared with a total concentration of 0.1 mM), then the shape of the progress curve is explicable by the drop in substrate concentration and accumulation of products. This is shown in Fig. 3 where the experimental points are taken from Fig. 1 of Stinshoff and Jatzkewitz [3] and the lines are drawn from Eqn. 3. This is the integrated Michaelis equation for a reaction where there is non-competitive inhibition by one product (K_1) and competitive by the other (K_2). The data in Table III of Ref. 3, which show an

$$Vt = \left(1 + \frac{s_0}{K_1}\right)\left(1 + \frac{s_0}{K_2}\right) \ln \frac{s_0}{s_0 - p} + \left[\left(1 - \frac{K_m}{K_2}\right) - \frac{K_m}{K_1}\left(1 + \frac{s_0}{K_2}\right)\right] p + \frac{1 - K_m/K_2}{2K_1} \cdot p^2 \qquad (3)$$

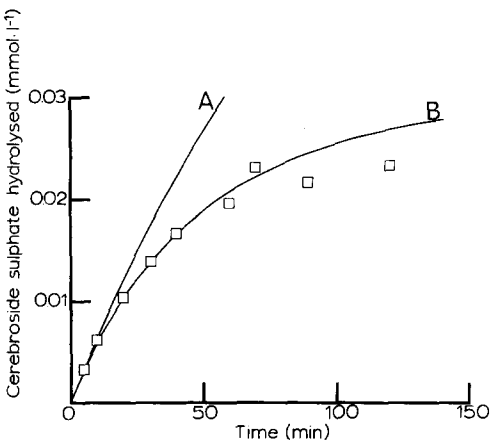

Fig. 3. Progress curves for the hydrolysis by sulphatase A from human liver of cerebroside sulphate at pH 5.3 in 0.01 M sodium formate buffer. The points are taken from Fig. 1 of Ref. 3 and the lines are drawn from Eqn. 3. In A the substrate concentration was taken to be 0.1 mM, the total concentration of cerebroside sulphate; in B to be 0.03 mM.

inactivation of sulphatase A by preincubation with cerebroside sulphate, cannot be explained in this way and no alternative can be offered. It may, therefore, be that the above suggestion is incorrect and that a cerebroside sulphate-induced inactivation does indeed occur under the conditions used by Stinshoff and Jatzkewitz [3], where the substrate is 'micellar' cerebroside sulphate [9]. This may not be the case where the substrate is incorporated into micelles of taurodeoxycholate, as in the present study and those of Porter et al. [4] and Stevens et al. [5] which are considered below. Unfortunately, it has not been possible to repeat the experiments of Stinshoff and Jatzkewitz [3] with the sulphatase A of ox liver because the latter enzyme, as reported above, will not hydrolyse cerebroside sulphate at a measurable rate in the absence of $MnCl_2$ and taurodeoxycholate. Any hydrolysis which does occur is at a rate of less than 0.2 μmol $\cdot$ mg^{-1} $\cdot$ min^{-1}, less than 2% of the rate at pH 4.5 in $MnCl_2$ and taurodeoxycholate and less than 5% of that reported for human sulphatase A under similar conditions [3].

The fall in velocity which occurs during the hydrolysis of cerebroside sulphate by the sulphatase A of human fibroblast [4] can also be accounted for by utilisation of substrate and accumulation of products. In this case, the concentration of substrate is known with certainty because it is incorporated into micelles of taurodeoxycholate [4]. If a value of 0.03 mM is again assumed for the K_i of cerebroside, Eqn. 3 gives a line which is in accord with the data of Fig. 3 in Ref. 4. The fact that hydrolysis of cerebroside sulphate by the sulphatase A of human urine is linear with time was first postulated by Stevens et al. [5]. This would be the case if the sensitivity of the assay were such that the measurements were made under conditions where there was no significant change in the concentration of substrate. This is not possible in the pH-stat assay described above but should be in the radiochemical assay described in Ref. 5.

The present results, therefore, suggest that there is no cerebroside sulphate-

induced inactivation of sulphatase A, a situation which is much more reasonable from the physiological point of view than that existing with the arylsulphatase activity. The reason for the different behaviour of the two types of substrate has not been established but an explanation can be offered. It has been suggested [1] that the inactivation occurring during the hydrolysis of arylsulphates involves the enzyme-SO_4^{2-} complex produced during the catalytic cycle. Such a complex may not be formed during the hydrolysis of cerebroside sulphate because the simplest explanation of the non-competitive inhibition of the reaction by SO_4^{2-} is that this is the first-released product, leading to the formation of an enzyme-cerebroside complex. In the arylsulphatase reaction, on the other hand, SO_4^{2-} is a competitive inhibitor, suggesting that it is the last-released product, arising from an enzyme-SO_4^{2-} complex. The difference in behaviour could arise from a non-polar interaction between the sulphatase and cerebroside sulphate or cerebroside [10]: such an interaction could not take place between the normally used arylsulphates and the enzyme. Attempts are being made to prepare suitable model compounds to allow a more detailed investigation of this point.

Finally, it should be noted that no method is yet available for computing v_0 from pH-stat recordings of the hydrolysis of cerebroside sulphate: theoretically, either Eqn. 1 or Eqn. 3 could be used, but they are too complex in practice. The method used in the assay of arylsulphatase [1] is invalid, first because there is no evidence of any cerebroside sulphate-induced inactivation and second, because, even if such an inactivation were occurring, the extent of hydrolysis of the substrate is too great to allow the simplification made in Ref. 1.

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