Bile Salt Activation of Cerebroside Sulphate Sulphohydrolase

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(Received 29 January 1980)

The cholate and taurodeoxycholate activations of cerebroside sulphate sulphohydrolase (cerebroside-3-sulphate 3-sulphohydrolase, EC 3.1.6.8) activity of arylsulphatase A (aryl-sulphate sulphohydrolase, EC 3.1.6.1) were compared. Taurodeoxycholate caused a sharp peak of response at a concentration of 1.25 mg/ml (type-I activation). Cholate also showed type-I activation but, in addition, it evoked a second, higher, response plateau at concentrations between 5 and 10 mg/ml (type-II activation). At the pH of the reaction, cholate is converted largely to the sparingly soluble free acid, so at the high concentrations associated with type-II activation, copious precipitates were formed. It was found that the precipitated material was essential for the type-II activation. Type-I activation appears to involve bile salt interaction with substrate, while type-II activation appears to involve enzyme interaction with solid-phase cholic acid. The putative mutant arylsulphatase A in an unusual form of metachromatic leukodystrophy hydrolysed cerebroside sulphate only in the presence of high levels of cholate. The type-II activation may thus be simulating a physiological desulphation reaction.

The desulphation of cerebroside sulphate and related 3-O-sulphogalactolipids as a physiological role of arylsulphatase A (aryl-sulphate sulphohydrolase, EC 3.1.6.1) was deduced from the absence of arylsulphatase A in metachromatic leukodystrophy, a genetically transmitted disorder in which cerebroside sulphate accumulates in neural tissue (Dulaney & Moser, 1977). Mehl & Jatzkewitz (1964) first showed that cerebroside sulphate sulphohydrolase (cerebroside-3-sulphate 3-sulphohydrolase, EC 3.1.6.8) activity was associated with arylsulphatase A and that a supplemental factor was necessary for this hydrolysis. Recent studies indicated that this cerebroside sulphatase factor is a low-molecular-weight protein, which probably acts by dispersing the lipoidal substrate (Fischer & Jatzkewitz, 1978).

The factor requirement can be circumvented by bile salts. We utilized sodium taurodeoxycholate to demonstrate cerebroside sulphate sulphohydrolase activity in fibroblast extracts (Porter *et al.*, 1972). Percy *et al.* (1972) carried out similar studies utilizing sodium cholate. Jerfy & Roy (1973) studied the sodium taurodeoxycholate stimulation of beef liver arylsulphatase A and suggested the involvement of mixed micelles with the substrate. A

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comparison of the effects of cholate and taurodeoxycholate is reported in the present paper.

Experimental

Enzyme

Arylsulphatase A (specific activity 200–3500 units/mg of protein) was purified from human urine (Stevens *et al.*, 1975). One enzyme unit catalyses the hydrolysis of 1 μ mol of nitrocatechol sulphate/h. The enzyme was diluted to approx. 4 units/ml in 50 mM-Tris/HCl buffer, pH 7.4, containing 1 mg of bovine serum albumin/ml.

Substrate

Cerebroside [35 S]sulphate was prepared as described previously (Fluharty *et al.*, 1974), and was diluted with bovine brain sulphatide (Supelco, Bellafonte, PA, U.S.A.).

Bile salts

Sodium taurodeoxycholate and sodium cholate, obtained from Sigma Chemical Co., St. Louis, MO, U.S.A., showed traces of contaminants by t.l.c. Samples of sodium taurodeoxycholate from Calbiochem, La Jolla, CA, U.S.A. and sodium cholate from Maybridge Chemical Co., Tintagel, Cornwall,

U.K., were free of contaminants and showed identical enzyme activations. The sodium salts of deoxycholic acid and taurocholic acid were from Calbiochem, and cholic acid was from Sigma.

Enzyme assay

The cerebroside sulphate sulphohydrolase assay of Porter *et al.* (1972) was modified in order to utilize the Eppendorf 3200 Micro Centrifuge System (Brinkman Instruments, Westbury, NY, U.S.A.). A 25 μ l portion of substrate/buffer mixture composed of 5 μ l of sodium taurodeoxycholate (10 mg/ml), 5 μ l of 1M-sodium acetate buffer (pH 4.5), 5 μ l of 0.2M-MnCl₂ and 10 μ l of 1mM-cerebroside [³⁵S]-sulphate (approx. 1.4×10^5 c.p.m./ μ mol) was added to 25 μ l of enzyme solution in a 1.5ml plastic Eppendorf Micro Centrifuge tube. In studies with cholate, each component was dispensed individually in the following order with mixing after each addition: bile salt, buffer, MnCl₂, wait 10min, substrate, then enzyme. Reaction was at 37°C for 2h and was stopped with 1ml of chloroform/methanol (2:1, v/v) followed by 0.2ml of 0.12M-NaCl containing 4mM-Na₂SO₄. Tubes were capped, mixed vigorously and centrifuged for 30s. The bulk of the upper phase, containing inorganic [³⁵S]-sulphate and traces of substrate, was transferred to a fresh centrifuge tube without disturbing the interface and was washed with 0.4ml of chloroform/methanol/0.12M-NaCl (86:14:1, by vol.). A 0.2ml sample of the washed upper phase, corresponding to one-half of the reaction product, was counted for radioactivity by liquid scintillation.

Results and Discussion

Taurodeoxycholate and cholate activation

Typical responses of cerebroside sulphate sulphohydrolase to increasing concentrations of sodium taurodeoxycholate and sodium cholate are shown in Fig. 1. With taurodeoxycholate, there was one mode of activation (type-I) with a sharp maximum of activity at 1.25 mg of bile salt/ml (2.5mM). With cholate, a similar sharp maximum occurred at about the same concentration, but, as the concentration was increased, a slight decrease in activity was followed by a second maximum, indicative of a different mode of activation (type-II). The maximum activity of the type-II activation was typically 2–3-fold higher than that for the type-I activation and showed a plateau over a broad concentration range (5–10mg/ml). The cholate type-I activation maximum and transitional decrease is shown in detail in the insert in Fig. 1.

The basis of type-I activation is probably the dispersal of substrate as proposed by Jerfy & Roy (1973). Jeffrey & Roy (1977) showed that cerebroside sulphate exists in aqueous media as extremely

Fig. 1. The effect of various concentrations of sodium taurodeoxycholate (■) and sodium cholate (●) on cerebroside sulphate sulphohydrolase activity

The insert shows in detail the type-I maximum and the transitional decrease with cholate. The activity after removal of cholic acid solids is also shown (○).

large aggregates that apparently preclude productive interaction with arylsulphatase A. Dispersal of sulphatide apparently occurs through the formation of mixed micelles with micellar bile salt. Little or no activation occurs below the critical micellar concentration (Jerfy & Roy, 1973). Below this concentration, monomeric bile salt may be incorporated into sulpholipid aggregates, but there is no substrate dispersal. In the case of the first cholate maximum a mixed micellar complex of cholate ions and cholic acid would provide the vehicle for sulphatide dispersal. No significance can be attached to the juxtaposition of type-I maximal concentrations for the taurodeoxycholate and cholate systems; the relative amounts of ionic cholate and its free acid vary with pH over the range studied, whereas taurodeoxycholate remains fully ionized (Small, 1971).

Type-I activation must, however, involve more than substrate dispersal. Synthetic detergents effectively disperse cerebroside sulphate, yet fail to increase enzyme activity. The sharp decrease in rate when bile salt concentrations exceed the optimum also implies the intervention of additional factors. One unique property of bile salt micelles provides a possible explanation (Small, 1971). Most detergents show a sharp transition at the critical micellar concentration from molecular solution to a micellar phase with high aggregation numbers (40–100). Bile salts initially form primary micelles of low aggregation numbers (2–8), which have restricted hydrophobic space, but relatively low net charge. Primary bile salt micelles could thus promote maximal

cerebroside sulphate dispersal with minimal potential for interfering with enzyme-substrate interaction. At higher bile salt concentrations, primary micelles coalesce to form larger secondary micelles. Cerebroside sulphate in the larger aggregates is apparently unavailable for enzyme hydrolysis. Enzyme structural specificity, higher charge density, or surface dilution are possible contributing factors.

Cholic acid precipitate

In addition to the bimodal activation, there were several unique features to the activation by cholate. At the pH of the reaction, cholate (pK_a approx. 5) was converted largely to its sparingly soluble free acid. At concentrations for type-I activation, the precipitate was not obvious on casual examination. At the high concentrations associated with type-II activation, however, copious cottage cheese-like precipitates formed on addition of pH 4.5 buffer to the sodium cholate solution. The pK_a of taurodeoxycholic acid is near 2 and addition of buffer yielded clear solutions. Subsequent addition of cerebroside sulphate did result in some opalescence, but never in a distinct solid phase.

To determine whether the solid cholic acid was functional, reactions in its presence and absence were compared. Precipitate was formed, the tubes were centrifuged briefly and the supernatant fluid was used to make up the reaction mixtures. This yielded a bile-salt-concentration profile with a single peak at the concentration of the type-I activation (see Fig. 1). The contribution of the solid phase, determined by difference between centrifuged and uncentrifuged pairs, showed a continuous increase in activity through the type-I region with a plateau above

5 mg/ml. Thus, solid cholic acid appeared to be essential for the type-II activation.

Reactions in the presence of precipitated cholic acid were characterized by poor reproducibility. The type-II cholate data shown in Fig. 2 were selected to illustrate the type of variability encountered. Replicate determinations often yielded disparate results, which appeared to follow an all-or-none pattern. The magnitude of the maximal response and the general profile were similar in different trials, but a number of trials were usually required to validate the results.

The visual appearance of the cholic acid precipitate varied from tube to tube. It was reasoned that well-dispersed precipitates, prepared in batches, and dispensed into individual reaction tubes might provide more uniformity. Finely dispersed precipitates were achieved in three ways: (1) by suspending commercial cholic acid in buffer by mechanical mixing or sonic oscillation; (2) by subjecting freshly-prepared precipitates to sonic oscillation, and (3) by mixing buffer and cholate solutions heated at 55°C and allowing a precipitate to form on cooling at 37°C. The first two preparations were essentially inactive (Table 1). The third preparation was two-thirds as effective as the usual precipitate, but became ineffective within 24 h. Activity of the standard precipitate was found to be most effective after a brief (10–15 min) period of aging. This material also became ineffective within 24 h. A semi-stable surface configuration of freshly precipitated cholic acid appears critical for the type-II activation process.

Addition of either substrate or enzyme to the buffer before cholate resulted in decreased activity. Formation of precipitate in the presence of these components presumably restricted their availability by entrapment.

Effect of bile salts on substrate and enzyme

To compare the effect of cholate and taurodeoxycholate on cerebroside sulphate, the sedi-

Fig. 2. The effect of varying the concentration of cerebroside sulphate on the activation of cerebroside sulphate sulphonylhydrolase by sodium taurodeoxycholate at 1.25 mg/ml (■) and sodium cholate at 5 mg/ml (●). Only those points showing activity significantly greater than zero were used in constructing the cholate plot, because of the all-or-none character of this activation.

Table 1. Activation of cerebroside sulphate sulphonylhydrolase by different preparations of cholic acid precipitate

Cholic acid and treatment	Cerebroside sulphate activity ($\mu\text{mol/h per mg of protein}$)
Standard preparation	
No treatment	590
Sonic oscillation	3.6
Aged 24 h	0
Precipitated by cooling	
Fresh	380
Aged 24 h	3.5
Commercial cholic acid	
Suspended	0
Sonic oscillation	0

mentability of the radioactive substrate was examined. Centrifugation of a reaction mixture lacking bile salt resulted in complete sedimentation of the substrate, confirming the occurrence of large aggregates (Jeffrey & Roy, 1977). When taurodeoxycholate was included at the optimal concentration for type-I activation, up to 50% of the substrate was recovered in the supernatant. When cholate was included at type-II-activation concentrations, recovery was similar. Both bile salts appeared to disaggregate the sulpholipid to a similar extent, and there appeared to be no specific interaction between cerebroside sulphate and solid cholic acid. It is presumed that a type-I substrate dispersal is also essential for type-II activation.

A similar series of experiments was carried out to examine the effect of bile salts on enzyme. The amount of enzyme in the supernatant fluid was monitored by its nitro catechol sulphate reaction. With taurodeoxycholate, 75–80% of the enzyme could be accounted for in the supernatant fluid, similar to the recovery in the absence of the bile salt. With solid cholic acid present, only 35–55% resisted centrifugation. It thus appears that type-II activation may be mediated through an enzyme–solid interaction. The partial nature of the enzyme precipitation with cholic acid suggests an equilibrium between free and bound forms, but alternative explanations have not been ruled out. However, the amount of enzyme sedimenting with the cholic acid did not vary with cholate concentration and was the same under type-I and type-II activation conditions.

Other reaction parameters

In the presence of either taurodeoxycholate or cholate, cerebroside sulphate hydrolysis was linear for at least 3 h and was proportional to enzyme concentration, provided substrate hydrolysis did not exceed 50%. A plot of rate versus substrate concentration followed a simple saturation curve for taurodeoxycholate activation (Fig. 2). The apparent K_m was 0.1–0.3 mM, although this is probably not a true measure of enzyme–substrate interaction. The rate–substrate relationship for cholate at the type-I-activation optimum was similar to that with taurodeoxycholate. It appeared to be more complex under type-II conditions, a higher order interaction being suggested by a sigmoid curve.

The taurodeoxycholate and cholate activations yielded different pH profiles (Fig. 3). As with fibroblast extracts (Porter *et al.*, 1972) purified enzyme gave an extremely sharp pH optimum with taurodeoxycholate. This, however, occurred at a slightly higher pH (4.6). The profile for cholate under both type-I and type-II activation conditions was broad, extending from pH 4.6 to pH 5.0. Sulphatide hydrolysis and cholic acid precipitation increased in parallel below pH 5.5 and the plateau

Fig. 3. The effect of pH on relative cerebroside sulphate sulphonylhydrolase activity with sodium taurodeoxycholate at 1.25 mg/ml (■) and with sodium cholate at 1.25 mg/ml (●) or 5 mg/ml (▲)

Sodium acetate (100 mM) was adjusted to the indicated pH with acetic acid (100 mM).

occurred when titration of the bile acid presumably was complete. The type-II cholate activation showed maximal activity at a buffer concentration of 0.1 M.

The type-I systems were stimulated by Mn^{2+} with an optimum at approx. 20–30 mM, but no additional effects were evident in type-II activation.

Bile salt specificity

Deoxycholate (with a pK_a near that of cholate) gave a slight stimulation at concentrations below the solubility limit of the free acid. However, deoxycholic acid is considerably less soluble than cholic acid and apparently never provides enough primary micelles for maximal type-I activation. At higher concentrations there was no further activation despite a copious precipitate. This suggests that the structural requirements for type-II activation are stringent. Taurocholate, with a pK_a similar to that of taurodeoxycholate, was ineffective as an activator except for a slight effect at the highest concentration tested, 10 mM. The failure to elicit activation with taurocholate can be attributed to its unusually high critical micellar concentration.

A variety of synthetic detergents were inactive over the concentration range investigated with the bile salts.

Type-II cholate activation of a mutant form of arylsulphatase A

Fibroblast extracts from a patient with an unusual case of metachromatic leukodystrophy had decreased, but clearly measurable, arylsulphatase A activity toward synthetic substrates, yet no cere-

Table 2. *Activity of fibroblast arylsulphatase A toward different substrates*

Fibroblast extracts (Porter *et al.*, 1972) were chromatographed on DEAE-cellulose micro columns; the arylsulphatase A fractions were pooled, concentrated and assayed against nitrocatechol sulphate, methylumbelliferone sulphate (Stevens, 1974) and cerebroside sulphate with taurodeoxycholate and cholate.

Source of fibroblast extract	Substrate Bile salt concn. (mg/ml)	Activity (nmol/h per mg of protein)				
		Nitro- catechol sulphate	Methyl- umbelliferone sulphate	Cerebroside sulphate +		
				Taurode- oxycholate 1.25	Cholate	
					1.25	2.5
Unusual metachromatic leukodystrophy		290	12	1.4	1.8	0.6
Control		1890	73	54	156	87
					26	214

broside sulphate sulphohydrolase activity could be elicited by taurodeoxycholate. Growing fibroblasts were capable of significant sulphatide hydrolysis (Kihara, 1975). Cholate was found to promote sulphatide hydrolysis at about the activity expected from the synthetic substrate activities, but only under type-II activation conditions (Table 2). Thus, the type-II cerebroside sulphatase activation may reflect a physiologically significant property of the enzyme. The interaction of the mutant enzyme with cholic acid might favour a configuration that is required for hydrolysis of cerebroside sulphate but not for that of synthetic substrate. Apparently, this configuration does not occur spontaneously in enzyme extracted from this patient, but does occur in intact cells. It is suggested that the critical cholic acid surface mimics some aspect of the intralysosomal environment.

Relationship to biological activation factors

The cerebroside sulphatase factor of Mehl & Jatzkewitz (1964) acts in a manner analogous to type-I activation. This low-molecular-weight protein forms a 1:1 complex with cerebroside sulphate, making it available for enzyme cleavage (Fischer & Jatzkewitz, 1978).

In conclusion, the unimodal activation of cerebroside sulphate sulphohydrolase by taurodeoxycholate and the bimodal activation of the reaction by cholate is best explained by invoking two distinct types of activation processes. Type-I activation, facilitated by solutions of either bile salt, appears to be mediated by an activator-substrate interaction. Type-II activation, requiring the presence of precipi-

tated cholic acid, appears to involve an interaction of enzyme with the solid-phase activator and may reflect a physiological attribute of the enzyme.

This work was supported by NIH Grants NS-11665, NS-9479 and HD-4612.

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