Identification of aspirinase with one of the carboxylesterases requiring a thiol group

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Aspirin-hydrolysing activity in guinea-pig liver is located mainly in the microsomal fraction. This activity was found by electrophoresis to be due to a single carboxylesterase band, out of 12 bands revealed with α -naphthyl acetate as substrate. The activity is inhibited completely and irreversibly by the carboxylesterase inhibitor bis-(4-nitrophenyl) hydrogen phosphate, and also by thiol-blocking reagents.

The hydrolysis of aspirin (acetylsalicylic acid) to salicylic acid as part of its metabolism has been recognized for a long time, but little has been done to characterize the enzyme or enzymes responsible. Liver homogenate is known to have a significant ability to hydrolyse aspirin in the guinea pig, especially when compared with serum or plasma (Vincent & Lagreu, 1949). Aspirin hydrolysis by mammalian tissues and plasma has been attributed to 'acetyl esterases', distinguishable from cholinesterases by inhibition properties (Morgan & Truitt, 1965). Pharmacokinetic studies have shown the liver and gut wall to possess significant ability to hydrolyse aspirin *in vivo* (Harris & Riegelman, 1969), the enzyme from rabbit gastric mucosa having been purified and characterized (Spenny & Nowell, 1979). Extensive work has been performed on esterases in the liver (Heymann, 1980), indicating their broad substrate specificities, including hydrolytic activity towards thioesters and some amides. Which of these enzymes, if any, is responsible for aspirin hydrolysis has not hitherto been established.

The compound bis-(4-nitrophenyl) hydrogen phosphate, which is a specific inhibitor for carboxylesterases, having no effect on other serine hydrolases such as proteinases or cholinesterases (Heymann & Krisch, 1967), was used in the present study on the characterization of the enzyme responsible for aspirin hydrolysis in guinea-pig liver.

Materials and methods

Aspirin from BDH Chemicals (Poole, Dorset, U.K.) was recrystallized from toluene/hexane (1:1, v/v). The sulphur analogue of aspirin, thioaspirin (acetyl-2-mercaptobenzoic acid), was synthesized by acetylation of 2-mercaptobenzoic acid with acidified acetic anhydride. The product obtained on addition

of water was recrystallized from toluene/hexane (1:1, v/v); it had m.p. 120–122°C, lit. 122°C (Piette *et al.*, 1977). Bis-(4-nitrophenyl) hydrogen phosphate was synthesized by the method of Moffatt & Khorana (1957), and was recrystallized from water before use. α -Naphthyl acetate and Fast Blue B diazonium salt were purchased from Sigma Chemical Co. (Poole, Dorset, U.K.). *p*-Chloromercuribenzoate was purchased from Koch-Light Laboratories (Colnbrook, Bucks., U.K.).

Cell fractions were prepared from homogenates of freshly dissected guinea-pig liver, in 0.3 M-sucrose at 4°C, by centrifugation, and a microsomal fraction was prepared from the post-mitochondrial supernatant by the Ca^{2+} precipitation method of Kamath *et al.* (1971).

Aspirin-hydrolytic and α -naphthyl acetate-hydrolytic activities were measured spectrophotometrically by the methods of Spenny (1977) and Johnston & Ashford (1980) respectively, in 0.1 M-phosphate buffer, pH 6.5, at 37°C. Hydrolysis of thioaspirin was measured spectrophotometrically by an adaptation of the Ellman assay for cholinesterase (Ellman *et al.*, 1961). Release of free thiol was measured by using the chromophore derived from 5,5'-dithiobis-(2-nitrobenzoic acid) in 0.1 M-phosphate buffer, pH 8.0, at 37°C.

Inhibition studies were performed manometrically at 37.5°C in a Warburg apparatus, with 5 mM-aspirin as substrate. Inhibitor was incubated with enzyme for 30 min before addition of substrate.

Disc electrophoresis was performed with polyacrylamide (7%) gels (100 mm $\times$ 5 mm) in a Tris/glycine buffer, pH 8.59 (Davis, 1964). Esterases were solubilized from the microsomal fraction before electrophoresis by addition of Triton X-100 to 0.5% (v/v), followed by stirring at room temperature for 30 min. The preparation was centrifuged at 100 000 g

for 60 min, and the supernatant obtained contained more than 90% of the original esterase activity with α -naphthyl acetate as substrate. Staining for thioaspirin-hydrolysing activity was performed by using the method of Chubb & Smith (1975) for staining cholinesterase activity, except that the last step, incubation in dithio-oxamate solution, was omitted, since 2-mercaptobenzoic acid forms a readily visible brown precipitate with Cu^{2+} . Esterase activity with α -naphthyl acetate as substrate was stained for with Fast Blue B diazonium salt in 0.3 M-phosphate buffer, pH 6.5.

Protein was determined by the method of Lowry *et al.* (1951), with bovine serum albumin as standard.

Results and discussion

Subcellular fractions obtained by differential centrifugation with Ca^{2+} precipitation were assayed for carboxylesterase activity with α -naphthyl acetate as substrate, and also for aspirin- and thioaspirin-hydrolysing activities. The subcellular distribution patterns shown in Fig. 1 for hydrolytic activity towards each of these esters indicate that for each compound the main activity is located in the microsomal fraction, in agreement with previous studies on subcellular localization of carboxylesterases (Heymann, 1980).

Electrophoresis of microsomal esterases solubilized with Triton X-100 reveals 12 bands (Fig. 2a), with α -naphthyl acetate as substrate. This confirms the findings obtained by Chow & Ecobichon (1973), who found 12 esterase bands in starch gels, from an extract of guinea-pig liver, staining with α -naphthyl acetate. The use of the thio analogue thioaspirin enabled hydrolytic activity

towards aspirin to be demonstrated in polyacrylamide gels. Only one band is visible in a gel of microsomal esterases solubilized with detergent. This band corresponds in relative mobility to one of the carboxylesterase bands stained with α -naphthyl acetate (Fig. 2b). Preincubation of gels in 0.1 mM-bis-(4-nitrophenyl) hydrogen phosphate before staining prevents six of the 12 carboxylesterase bands from appearing (Fig. 2c). This reflects the different susceptibility of each carboxylesterase to the inhibitor, which has been demonstrated by Brandt *et al.* (1980) for six carboxylesterases purified from rat liver. In their study 0.1 mM-bis-(4-nitrophenyl) hydrogen phosphate caused a range of inhibitions from 44% to 96% for the six enzymes, with 4-nitrophenyl acetate as substrate. The band stainable with thioaspirin is also prevented from appearing on preincubation of the gel in 0.1 mM-bis-(4-nitrophenyl) hydrogen phosphate. The carboxylesterase with the relative mobility identical with that of the thioaspirinase is one of the six bands completely inhibited by bis-(4-nitrophenyl) hydrogen phosphate. The hydrolytic activity of guinea-pig liver towards aspirin and thioaspirin is therefore due to only one of the many forms of carboxylesterase present in the liver.

Inhibition studies confirm the electrophoretic findings. With microsomal fractions pooled from four separate preparations, 0.1 mM-bis-(4-nitrophenyl) hydrogen phosphate was found to inhibit aspirinase activity by $97.8 \pm 2.3\%$ (mean $\pm$ S.D. of three assays). Two thiol-blocking reagents, HgCl_2 and *p*-chloromercuribenzoate, produced a significant inhibition of microsomal aspirinase activity: $66.1 \pm 3.6\%$ and $96.1 \pm 1.2\%$ respectively at 0.1 mM. Such a finding is not at all common among esterases. Reports have been made of esterases isolated from

Fig. 1. Subcellular distributions of ester-hydrolysing activities of guinea-pig liver

Fractions were prepared by differential centrifugation and Ca^{2+} precipitation as described in the Materials and methods section. Activities are expressed in accordance with de Duve *et al.* (1955). Fractions are numbered as follows: I, nuclear; II, mitochondrial; III, microsomal; IV, cytoplasmic.

Fig. 2. Polyacrylamide-gel electrophoresis of guinea-pig liver microsomal esterases solubilized with Triton X-100

Esterases were solubilized as described in the Materials and methods section, and electrophoresis was performed in polyacrylamide (7%) gels. Gels were stained with: (a) 5 mM- α -naphthyl acetate and Fast Blue B for non-specific esterases; (b) as (a), but with preincubation in 10 mM-bis-(4-nitrophenyl) hydrogen phosphate for 30 min, and the substrate solution also containing 10 mM-bis-(4-nitrophenyl) hydrogen phosphate; (c) 4 mM-thioaspirin/20 mM-glycine/4 mM- CuSO_4 . The arrow indicates the position of aspirin hydrolase.

Augustinsson, 1971; Arndt *et al.*, 1978). Brandt *et al.* (1980) found that HgCl_2 caused significant inhibition of six carboxylesterases purified from rat liver, as did bis-(4-nitrophenyl) hydrogen phosphate, but that *p*-chloromercuribenzoate had virtually no effect.

The present results demonstrate that the main hydrolytic activity of guinea-pig liver towards aspirin *in vitro* is due to a single carboxylesterase, which is a serine hydrolase that apparently requires a free thiol group for activity.

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mammalian liver that are inhibited both by organophosphates reacting at an active-site serine residue and by mercuric compounds (Ljungquist &