Purification and characterization of cholyl-CoA :taurine *N*-acetyltransferase from the liver of domestic fowl (*Gallus gallus*)

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The enzymological basis for the ability of mammalian liver to conjugate bile acids with both glycine and taurine, and for non-mammalian liver to make only taurine conjugates, was investigated. The taurine-conjugating enzyme has been purified 1200-fold from the liver of domestic fowl and its properties compared with those of the glycine/taurine-conjugating enzyme from bovine liver [Czuba & Vessey (1980) *J. Biol. Chem.* 255, 5296–5299]. The enzyme from both species followed a Ping Pong mechanism. The enzymes were also similar with respect to their affinity for taurine, although the enzyme from domestic fowl would not bind glycine. The affinity of both for cholyl-CoA was quite similar, too, and both enzymes were inhibited reversibly by *p*-mercuribenzoate. The enzymes, however, were quite different in size. The enzyme from domestic fowl had a mol.wt. of 63 000–65 000 by both gel filtration and sodium dodecyl sulphate/polyacrylamide-gel electrophoresis. This is approx. 15 000 mol.wt. units larger than the enzyme from bovine liver, and suggests a loss of genome over the course of evolution as the basis for the altered specificity at the amino-acid binding site.

Higher vertebrates synthesize bile salts in the liver and use them both to aid in the elimination of substances that the kidney cannot, and to aid in the digestion and absorption of fats. The final step in the synthesis of bile salts is the conjugation of the side-chain carboxy group with glycine or taurine. The reaction involves a preliminary 'activation' of the carboxy function through the formation of a thioester with CoA. This thioester then reacts with glycine or taurine in a reaction catalysed by a bile acid-CoA:glycine/taurine *N*-acetyltransferase. In vertebrates other than eutherian mammals, only taurine-conjugated bile acids are found (Haslewood, 1967). Glycine conjugates cannot be made even in systems *in vitro* (Vessey, 1978). However, most eutherian mammals make both glycine and taurine conjugates. Previous studies have shown that a single enzyme in mammals catalyses the synthesis of both the glycine and the taurine conjugates (Killenberg & Jordan, 1978; Vessey, 1979). Thus the evolution of eutherian mammals was accompanied by a fundamental alteration in the bile-acid-conjugating enzyme. In the present paper, we have begun to study this alteration by isolating the

taurine *N*-acetyltransferase from a non-mammal and comparing its properties with those of the glycine/taurine *N*-acetyltransferase from bovine liver (Vessey, 1979; Czuba & Vessey, 1980).

Materials and methods

Materials

Coenzyme A (lithium salt), 3-(3-dimethylaminopropyl)-1-ethylcarbodi-imide, and cholic acid were obtained from Sigma Chemical Co., St. Louis, MO, U.S.A. The cholic acid was further purified by treatment with activated charcoal and Celite to remove bile pigments, and then recrystallized from 70% ethanol. Cholyl-CoA was synthesized by the procedure of Shah & Staple (1968). Sephacryl S-200 and AH-Sepharose 4B were purchased from Pharmacia, Piscataway, NJ, U.S.A. The DEAE-cellulose (DE-52) was obtained from Whatman, Clifton, NJ, U.S.A.

Cholyl-AH-Sepharose 4B was prepared by incubating cholic acid with AH-Sepharose 4B in the presence of excess 3-(3-dimethylaminopropyl)-1-ethylcarbodi-imide for 20 h at 24°C. The gel was collected by filtration and washed consecutively with: twice-distilled water; 0.5 M-NaCl; 50 mM-potassium phosphate, pH 8, containing 0.5 M-NaCl;

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50 mM-potassium phosphate, pH 4.5, containing 0.5 M-NaCl; and finally with 50 mM-potassium phosphate, pH 8.

Purification

Frozen chicken livers were homogenized in 4 vol. of 0.25 M-sucrose. The resulting whole homogenate was filtered through cheesecloth and then centrifuged at 11000 *g* for 15 min in a Beckman type 30 rotor. The low-speed supernatant was again filtered through cheesecloth and then centrifuged at 100000 *g* for 60 min in a type 30 rotor. The 100000 *g* supernatant was subsequently fractionated with solid $(\text{NH}_4)_2\text{SO}_4$. The fraction precipitating between 30 and 45%-satd. $(\text{NH}_4)_2\text{SO}_4$ was dissolved in 25 mM-potassium phosphate, pH 8, and dialysed against two changes of 25 mM-phosphate, pH 8, containing 75 mM-NaCl. About 20 ml of the dialysed 30–45%-satd. $(\text{NH}_4)_2\text{SO}_4$ fraction was applied to a DEAE-cellulose column (2.5 cm int. diam. $\times$ 45 cm) previously equilibrated in 50 mM-Tris, pH 8.38 (5°C), containing 10 mM-NaCl. The *N*-acyltransferase activity was eluted from the column with a linear gradient of 50 mM- to 250 mM-NaCl. The fractions containing enzymic activity were pooled and concentrated approx. 100-fold on an Amicon ultrafiltration cell fitted with an Amicon PM-30 membrane. The protein that precipitated during ultrafiltration was removed by low-speed centrifugation. The DEAE-cellulose concentrate was then loaded on to a medium-sized Sephacryl S-200 column (1.5 cm int. diam. $\times$ 45 cm), which was equilibrated and eluted with 50 mM-potassium phosphate, pH 8, containing 250 mM-NaCl. The active fractions were pooled and concentrated 10-fold by ultrafiltration, as previously described. The total Sephacryl S-200 concentrate was loaded on to a choly-AH-Sepharose column (1.4 cm int. diam. $\times$ 1.5 cm) previously equilibrated in 50 mM-potassium phosphate, pH 8. The column was washed with equilibration buffer and then the same buffer containing 250 mM-NaCl and 2 mM-cholate. The *N*-acyltransferase activity was eluted with 50 mM-potassium phosphate, pH 8, containing 250 mM-NaCl and 4 mM-cholate. The active fractions were pooled and again concentrated 10-fold. The total enzyme concentrate was loaded on to a large Sephacryl S-200 column (2.5 cm int. diam. $\times$ 90 cm), which was equilibrated and eluted with 50 mM-potassium phosphate, pH 8, containing 0.5 M-NaCl. The *N*-acetyltransferase fractions were combined and stored at 0°C.

Enzyme assay

N-Acyltransferase activity was measured at 30°C by following the disappearance of the choly-CoA thioester absorption at 232 nm. The standard assay contained 100 mM-potassium phosphate (pH 8),

Fig. 1. Double-reciprocal plot of rates of reaction as a function of the concentration of taurine (a) or choly-CoA (b) at different fixed concentrations of the respective alternative substrate

(a) Initial rates of reaction were determined for various concentrations of taurine at choly-CoA concentrations of: 42 μM (○); 21 μM (□); 6.3 μM (△); 4.2 μM (●). (b) Initial rates of reaction were determined for various concentrations of choly-CoA at taurine concentrations of: 25 mM (○); 5 mM (□); 2.5 mM (△); 1.5 mM (●). Rates (*v*) are expressed as μmol of taurocholate synthesized/min per mg of of protein.

Table 1. Purification of cholyl-CoA : taurine *N*-acyltransferase from chicken liver

Step	Total protein (mg)	Total activity (μmol/min)	Specific activity (μmol/min per mg)
Supernatant (100000 g)	8928	169.6	0.019
30–45%-satd.-(NH ₄) ₂ SO ₄ fraction (dialysed)	1350	116	0.086
DEAE-Cellulose fractions	134	76	0.568
Medium Sephacryl S-200 fractions	41.5	56	1.35
Cholyl-Sepharose affinity column	6.0	Partial inhibition by cholate	
Large Sephacryl S-200 fractions	1.3	30.8	23.0

50mM-taurine, 80μM-cholyl-CoA and water to a final volume of 1ml. Protein concentration was determined by the method of Lowry *et al.* (1951). The contribution of thiolase activity to rates was corrected for.

Electrophoresis

Sodium dodecyl sulphate/polyacrylamide-gel electrophoresis was carried out on 7.5% gels as previously described (Vessey, 1979). The gels were stained by the method of Blakesley & Boezi (1977).

Results and discussion

The scheme for the purification of cholyl CoA:taurine *N*-acetyltransferase from chicken livers is shown in Table 1. The final preparation of enzyme had a specific activity of 23μmol of product/min per mg of protein and was enriched over 1200-fold relative to the soluble cell fraction. The enzyme preparation was not completely homogeneous, as judged by sodium dodecyl sulphate/polyacrylamide-gel electrophoresis. The impurities could be removed on a decyl-Agarose column (see below), although this was not routinely done.

The results of a kinetic analysis are shown in Fig. 1. The double-reciprocal plots of 1/*v* against 1/[S] yield a series of parallel lines when the concentration of the second substrate is changed. This is indicative of a Ping Pong mechanism. The glycine/taurine *N*-acyltransferase enzyme, purified from bovine liver (Czuba & Vessey, 1980), also gave parallel double-reciprocal plots. Extended kinetic analysis of the bovine enzyme had revealed that it followed a Ping Pong Bi Bi mechanism. A key

feature of the mechanism was that the cholyl-enzyme intermediate was unstable and would hydrolyse to form cholic acid and free enzyme. The net result was that, in the absence of taurine, the enzyme catalysed a slow hydrolysis of cholyl-CoA. This thiolase activity has also been found with the purified enzyme from chicken liver. The reaction rate for the chicken enzyme is approx. 1–2% of the maximum taurine conjugation rate. Thus the taurine enzyme from chicken liver also catalyses the half-reaction and appears to be mechanistically quite similar to the glycine/taurine enzyme from bovine liver.

The molecular weight of the enzyme from bovine liver has been determined as 47000 by gel filtration and 50900 by sodium dodecyl sulphate/polyacrylamide-gel electrophoresis (Vessey, 1979). However, gel-filtration analysis of the purified enzyme from chicken liver on a Sephacryl S-200 column yielded a mol.wt. of 63000. Sodium dodecyl sulphate/polyacrylamide-gel electrophoresis of the enzyme purified according to the scheme in Table 1 revealed several bands, including a major band at mol.wt. 65000. The contaminating bands can be removed by binding the enzyme to decyl-Agarose and eluting the enzyme activity with 8mM-cholate. This preparation has a major band with an apparent mol.wt. of 65000. Thus the enzyme from chicken has approximately the same molecular weight under both denaturing and non-denaturing conditions, and is therefore probably a monomer. A mol.wt. of 63000–65000 for the taurine enzyme from the chicken means that it is larger than the glycine/taurine enzyme from bovine liver by approx. 15000mol.wt. units, or approx. 125 amino-acid residues. Thus the expanded substrate specificity can be attributed to considerable differences in struc-

ture. It is possible that the mammalian enzyme evolved from the non-mammalian enzyme by a mere loss of genome coding for an *N*- or *C*-terminal sequence of 125 amino acids. We have not yet sequenced any portion of these enzyme forms to confirm this hypothesis.

For a Ping Pong mechanism, the Michaelis constants for the substrates can be obtained from replots of the data in Fig. 1 (Segal, 1975). This analysis gives a K_m for taurine of 4 mM and a K_m for cholyl-CoA of 0.009 mM. It is interesting that these values are close to the values of 6.5 mM and 0.007 mM respectively for taurine conjugation by the bovine enzyme. Another similarity exists between the enzyme from the two species. The enzyme from chicken is reversibly inactivated by *p*-mercuribenzoate, as was the bovine enzyme (Czuba & Vessey, 1980). In fact, considering the differences in size, the enzyme forms are strikingly similar. The only major difference in properties is that the bovine enzyme can accommodate glycine, although it still has a

quite narrow specificity, accepting neither serine, glutamine, ornithine, alanine nor β -alanine (Czuba & Vessey, 1980). The enzyme from domestic fowl did not conjugate cholyl-CoA with glycine or with β -alanine, even in concentrations as high as 0.1 M.

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