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CONJUGATION OF CHOLIC ACID WITH TAURINE AND GLYCINE BY RAT LIVER PEROXISOMES

Bengt Frode Kase, Kristian Prydz, Ingemar Björkhem* and Jan I. Pedersen

Institute for Nutrition Research, University of Oslo, Norway
***Karolinska Institute at Huddinge Hospital, Huddinge, Sweden**

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We have previously shown that rat liver peroxisomes catalyze conversion of 3α,7α,12α-trihydroxy-5β-cholestanoic acid into cholic acid as the CoA-ester (1). In the present work it is shown that addition of taurine or glycine to the reaction mixture, choloyl-CoA is further converted to taurocholic or glycocholic acid, respectively. The identity of these products was verified by fast atom bombardment - mass spectrometry. The peroxisomal fraction catalyzed conjugation of cholic acid with taurine (22.3 nmol x mg^{-1} x h^{-1}) or glycine (18.6 nmol x mg^{-1} x h^{-1}) at rates twice those observed with the microsomal fraction. The results indicate that conjugation of newly formed bile acids may be an important function of liver peroxisomes.

Both *in vivo* studies of peroxisomal disorders (2,3) and studies with subcellular fractions (1,4) have shown that liver peroxisomes catalyze the side chain cleavage of 3α,7α,12α-trihydroxy-5β-cholestanoic acid (THCA) with formation of choloyl-coenzyme A (CoA)(5). This final step in cholesterol catabolism is followed by conjugation with taurine or glycine (6). Conjugation of bile acids involves the intermediate formation of bile acid acyl CoA ester by the endoplasmic reticulum, and cytosolic enzymes catalyze the coupling to taurine or glycine (7-9). Controversial results with respect to subcellular localization of bile acid conjugation (7-12) led us to a reinvestigation. Here we report that the peroxisomal

Abbreviations: THCA, 3α,7α,12α-trihydroxy-5β-cholestanoic acid; T-CA, taurocholic acid; G-CA, glycocholic acid; FAB, fast atom bombardment; HPLC, high pressure liquid chromatography.

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fraction of rat liver conjugates choloyl-CoA with either taurine or glycine, indicating that the whole sequence of reactions from THCA to conjugated cholic acid may occur in this organelle.

MATERIALS AND METHODS

Chemicals. [7β-^{3}H]3α,7α,12α-Trihydroxy-5β-cholestanoic acid (200 Ci/mol) and the corresponding unlabeled compound were prepared as described (13). [^{14}C]cholic acid (50 Ci/mol) and [^{3}H]taurine (29 Ci/mmol) were from the Radiochemical Centre Amersham, England. Cholic acid and THCA were purified before use by high pressure liquid chromatography. Nycodenz and Maxidenz were from Nycomed, Oslo, Norway. Other chemicals were from Sigma Chemical Co. St. Louis,MO. All solvents were of analytical or HPLC grade.

Preparation of liver subcellular fractions. Male Wistar rats (150g body wt) given a commercial pellet diet, were used. A light mitochondrial fraction of liver homogenate was prepared as described (1,4). The pellet was washed once and layered on top of a linear Nycodenz gradient (10 - 48 % (w/v))(4). The gradient was centrifuged at 20,000 rpm (35,000 g_{av}) for 75 min in a vertical rotor (Beckman VTi 50). Fractions of 2.5 ml were collected and assayed for the peroxisomal marker enzyme, catalase (14). Protein was determined by the method of Lowry et al. (4,15).

Incubation, extraction and chromatography. All incubations were performed in duplicate. The incubation mixture contained the following in 1 ml of 0.1 M Tris-HCl buffer, pH 8.0: 7.5mM ATP, 2.6mM CoA, 7.5mM $MgCl_2$, 7.5μM FAD, 3mM NAD, 50 mM glycine or taurine, and 0.15 mg protein of the density gradient fraction with the highest catalase activity. The reaction was started by the addition of 8.9 μM THCA mixed with 200.000 cpm [^{3}H]THCA or 9.8 μM cholic acid mixed with 100.000 cpm [^{14}C]cholic acid in 5 μl ethanol. After 1 hour of incubation at 37°C, the reaction was terminated with 20 μl 6 M KOH and hydrolyzed at 50°C for 30 min. The mixture was acidified with 250 μl 2 M HCl, diluted to 5 ml with H_2O and applied on a Sep-Pak C_{18} cartridge (Waters Associates). The cartridge was washed with 5 ml H_2O before elution with 8 ml of methanol.

Half of the reaction extract was dissolved in 4 M KOH in 50 % ethanol and hydrolyzed at 120°C for 18h. After acidification, the mixture was extracted with ethylacetate (1).

Aliquots of the incubation extracts were analyzed by HPLC using a Zorbax ODS column (5.0 x 250 mm) and eluted in 20 % 30 mM trifluoroacetic acid (adjusted to pH 2.9 with triethylamine) in methanol assayed for radioactivity as described (1,4).

Identification of isolated taurocholic and glycocholic acid was performed by fast atom bombardment (FAB) mass spectrometry, using a VG 7070E double focusing instrument (VG analytical Ltd, Manchester, UK) equipped with a FAB source and an Ion Tech gun (16). The purified material (200 - 300 ng) was applied in a few microliters of methanol/water (1:1(v/v)) to the FAB target already covered with the glycerol matrix. Xenon having 7 keV energy was used for bombardment of the sample. Spectra of negative ions were recorded by scanning m/z 750-90 during 20 s for several times. The accelerating voltage was 5kV and the resolution about 1000.

RESULTS

The peroxisomal fraction of rat liver catalyzes the conversion of THCA into cholic acid when the incubation was performed as previously described (Fig. 1 A)(1,4). Addition of taurine to the reaction mixture led to an almost complete disappearance of radioactivity in the peak containing cholic acid, and the appearance of a more polar peak that eluted after 5-6 ml, was detected (T-CA in Fig. 1B). Incubation of unlabeled THCA or cholic acid with [^{3}H]taurine in 50 mM phosphate buffer (pH 8), led to formation of a tritiated product with the same elution volume as peak T-CA in Fig. 1B.

After addition of glycine to the reaction mixture with THCA, a major product peak appeared at 7 - 8 ml (G-CA in FIG. 1 C). Hydrolysis of the reaction mixture at 120°C for 18 h in 4 M KOH, to promote cleavage of bile acid conjugates, gave only trace activities corresponding to the G-CA and T-CA peaks and the activity appeared in the peak containing cholic acid.

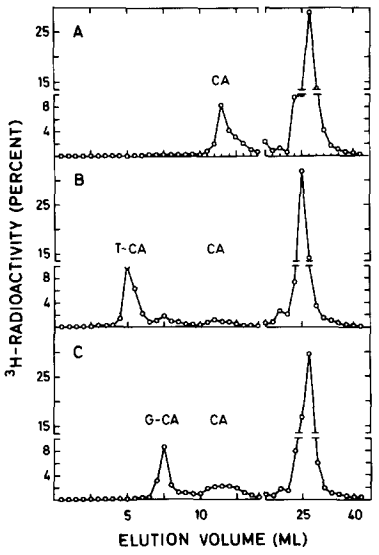

Fig. 1: Reversed phase high pressure liquid chromatograms of the extracts of incubations with 3α,7α,12α-trihydroxy-5β-cholestanoic acid (THCA) and a peroxisomal fraction of rat liver. Incubation, hydrolysis, extraction and HPLC procedures are given in Methods. The peak with elution volum 11-12 ml correspond to cholic acid (CA). Taurocholic acid (T-CA) eluted after 5-6 ml (B). Glycocholic acid (G-CA) eluted after 7-8 ml (C).

The identity of the isolated taurocholic acid was confirmed by FAB mass spectrometry (16). In addition to the glycerol peaks, only one prominent peak was seen at m/z 514, corresponding to $[Mw - 1]^-$ (Mw = molecular weight of the undissociated acid). The FAB mass spectrum of the isolated glycocholic acid also showed only one peak in addition to the glycerol peaks. In this case the peak was at m/z 464, corresponding to $[Mw - 1]^-$. The mass spectra of the isolated taurocholic acid and glycocholic acid were in accord with spectra obtained in analyses of the corresponding reference compounds under identical conditions.

In Table 1 the capacity of the peroxisomal fraction to catalyze taurocholic and glycocholic acid formation from THCA and cholic acid is shown. The extent of oxidative cleavage of the THCA side chain is only slightly increased by the presence of glycine or taurine in the reaction mixture. When the microsomal fraction was incubated with cholic acid under the same conditions as for the peroxisomal

TABLE 1: Formation of taurocholic acid (T-CA) and glycocholic acid (G-CA) from 3α,7α,12α-trihydroxy-5β-cholestanoic acid (THCA) and cholic acid by peroxisomal and microsomal fractions of rat liver

Incubation mixture	cholic acid	T-CA	G-CA	Total
	(nmol x mg^{-1} x h^{-1})			
Peroxisomes + THCA	11.1			11.1
Peroxisomes + THCA + 50 mM glycine	5.4		7.6	13.0
Peroxisomes + THCA + 50 mM taurine	2.4	11.0		13.4
Peroxisomes + cholic acid + 50 mM glycine			18.6	18.6
Peroxisomes + cholic acid + 50 mM taurine		22.3		22.3
Microsomes + cholic acid + glycine			9.0	
Microsomes + cholic acid + taurine		10.5		

The incubations were performed as in Methods.

fraction, the specific activities of conjugation were about 50 % of those observed with the peroxisomal fraction (Table 1).

DISCUSSION

The controversial results with respect to subcellular localization of bile acid conjugation (9) promoted us to a reinvestigation. In the present work, a peroxisomal fraction of rat liver was found to efficiently catalyze the coupling of choloyl-CoA with glycine and taurine. In view of the high peroxisomal contamination of the lysosomal fraction, the results are in accordance with those of Scherstén et al. (12) who reported that bile acid conjugation predominantly was localized to a lysosomal (L) fraction of human liver.

From the data given in Table 1 it is evident that the peroxisomal fraction has sufficient capacity to conjugate at least its own production of cholic acid. The peroxisomal production of taurocholic and glycocholic acid from cholic acid was about twice that of the microsomal fraction. Thus a 2 - 5 % contamination with microsomal protein can not explain the detected activities of the peroxisomal fraction.

In urine from patients with the cerebrohepatorenal syndrome of Zellweger, an inherited disease with an absence of liver peroxisomes, the percent distribution of different bile acid conjugates was found to be within normal limits (3). This finding does not necessarily mean that the peroxisomal conjugation activity is unimportant. Thus the soluble peroxisomal matrix enzymes are not deficient in livers from patients with Zellweger syndrome (17), and this may also be true for a peroxisomal choloyl-CoA:amino acid N-acyltransferase.

Peroxisomes are known to be fragile organelles that easily rupture during preparation. We may assume that at least some of the activity

previously detected in the cytosolic fraction (7-9) may be due to contamination with peroxisomal enzymes.

It is evident that the peroxisomal fraction is able not only to conjugate newly formed choloyl-CoA, but also to conjugate added cholic acid. This implies that the peroxisomal fraction catalyzes formation of choloyl-CoA. Since the choloyl-CoA synthetase probably is the rate limiting step in a coupled *in vitro* system for bile acid conjugation (9), it is unlikely that the small contamination with microsomal protein is responsible for this activity.

It is concluded that a peroxisomal fraction of rat liver has the capacity to conjugate its own production of cholic acid with either taurine or glycine. A dual localization of bile acid conjugation is probable, but the peroxisomal activity may be most important at least for newly formed bile acids.

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