Coenzyme A derivatives of bile acids—chemical synthesis, purification, and utilization in enzymic preparation of taurine conjugates

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Abstract Synthesis of the coenzyme A derivatives of bile acids by the mixed anyhydride method results in a product that is contaminated by unreacted CoASH and bile acid. These compounds can be purified by Sephadex LH-20 chromatography. In each case, the purified product is free of starting materials and exhibits an equimolar ratio of bile acid, coenzyme A, and thioester bond. Millimolar extinction coefficients were calculated for these compounds as $A_{259\ nm}$, 15.03 ± 0.58; $A_{232\ nm}$, 7.60 ± 0.17; and $A_{232\ nm}$ for the thioester bond, 4.12 ± 0.17. These CoA derivatives were hydrolyzed in strongly alkaline solution, but were stable at physiologic temperature and pH. Utilization of these compounds in the enzymic preparation of taurine conjugates of bile acids indicated 94% activity. These purified CoA derivatives may be useful in studying the enzymic conjugation of bile acids.

Supplementary key words bile acid conjugation · coenzyme A thioesters · enzymatic activity · ultraviolet extinction coefficients

The coenzyme A thioesters of bile acids have been proposed as intermediates in the enzymic conjugation of amino acids with bile acids and as the product of hepatic mitochondrial oxidation of hydroxycoprostanic acids (1–4). The study of these metabolic pathways would be facilitated if biologically active CoA derivatives of bile acids could be synthesized and if certain of their physical and chemical properties were known.

In 1968, Shah and Staple described the synthesis of choloyl CoA and trihydroxycoprostanoyl CoA (5) by a modification of the mixed anhydride method of Goldman and Vagelos (6). Shah and Staple (5) demonstrated that their products contained coenzyme A and a thioester bond and that they liberated free steroid following alkaline hydrolysis. Quantification of the stoichiometry of the CoA, steroid, and thioester bond was not reported, nor was the biologic activity of these derivatives studied. The latter point is noteworthy, since Goldman and Vagelos (6) reported that only half of their synthetic long chain fatty acyl CoA thioesters were biologically active.

Recently, Mieyal, Webster, and Siddiqui (7) reported purification of synthetic benzoyl CoA derivatives by liquid chromatography over Sephadex LH-20. We have used this technique to purify the coenzyme A thioesters of cholic, chenodeoxycholic, deoxycholic, and lithocholic acids synthesized according to Shah and Staple (5). This report shows that the purified coenzyme A derivatives conform to the predicted molecular stoichiometry and are free of unreacted starting materials. Ultraviolet extinction coefficients, stability in aqueous solution, and biologic activity in the enzymic preparation of the taurine conjugates of bile acids are demonstrated.

MATERIALS AND METHODS

Chemicals

Lithium coenzyme A was obtained from PL Laboratories, Milwaukee, Wis. Bile acids were obtained from commercial sources and recrystallized before use. Their purity (greater than 97%) was estimated by gas–liquid chromatography (8). Taurocholate, taurodeoxycholate, and taurochenodeoxycholate were obtained from commercial sources. Taurolithocholate was a gift from Dr. Leon Lack who synthesized it as previously described (9). Each of the taurine conjugates was found to be pure by thin-layer chromatography (10).

Methylene chloride, 2,4,6-trimethylpyridine, and tetrahydrofuran were obtained from commercial sources and redistilled prior to use. 3α-Hydroxysteroid dehydrogenase was obtained from Worthington Biochemicals, Freehold, N.J. Other chemicals were of reagent quality and were obtained from commercial sources.

Analytic methods

The 3α-hydroxysteroid dehydrogenase method of Talalay (11) was used to estimate the 3α-hydroxysteroid content in solutions of free, conjugated, and thioesterified cholic, deoxycholic, and chenodeoxycholic acids. Lithocholic acid was measured as the methyl ester by gas–liquid chromatography (8). In order to measure lithocholoyl CoA, the compound was hydrolyzed in 1 M sodium hydroxide at 60°C for 45 min. The hydrolysate was acidified and free lithocholic acid was extracted into ether and measured as above. Deoxycholic acid was added prior to ether extraction as an internal standard.

Reduced coenzyme A (CoASH) was estimated according to the method of Beutler, Duron, and Kelly (12).

Thioester bond was estimated by the formation of hydroxamates (2). Since extinction coefficients for choloylhydroxamate vary from laboratory to laboratory (2, 13), we synthesized the hydroxamates of deoxycholic, chenodeoxycholic and cholic acids, recrystallized them to constant melting point and utilized these synthetic compounds as reference standards in the ferric chloride reaction. Lithocholoylhydroxamate was synthesized but was not sufficiently soluble to permit its use in the spectrophotometric reaction.

Ultraviolet extinction coefficients of the CoA derivatives were measured in 0.1 M potassium phosphate buffer, pH 7.0, at 30°C. Aliquots of two preparations each of purified choloyl, deoxycholoyl and chenodeoxycholoyl CoA were extracted with ether at pH 5.0 to further minimize contaminatioin from free bile acids. The concentrations of the CoA derivatives in solution were determined by the 3α-hydroxysteroid dehydrogenase reaction and served as the basis for calculation of the extinction coefficients.

The initial rate of hydrolysis of synthetic CoA derivatives was estimated at 38°C in aqueous solutions of sodium hydroxide. Ionic strength was adjusted to 2.5 M with sodium chloride. CoASH release was measured serially at at least 5 time points during the period in which the total CoASH released was equivalent to less than 10% of the starting material. CoASH appearance was log-linear with time until greater than 12% of the starting material was hydrolyzed. Pseudo-first order rate constants were derived from these data according to $k = 0.693/t_{1/2}$, and differences in rate constants were analyzed according to Student's t test (14).

Enzymic activity of the synthetic CoA derivatives was measured at 38°C for 60 min in a medium containing 0.8–1.2 μmoles of bile acid, 80 mM taurine, 25 mM N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES) buffer, pH 8.0, final volume 2 ml. Reaction was started by addition of 0.2 mg of rat liver protein prepared as follows. The $10^5 g$ supernatant of a rat liver homogenate was fractionated by harvesting the protein that precipitated between 1.8 and 2.6 M ammonium sulfate. This fraction was chromatographed over Sephadex G150 in 25 mM HEPES buffer, pH 8.0, and portions of the eluate that catalyzed the release of CoASH from choloyl CoA were pooled and used.

The reaction was terminated by immersing the tubes in boiling water. CoASH was then determined on aliquots withdrawn from the medium following which the remainder of the incubation was extracted twice with 1 volume of n-butanol. The organic phase was dried and redissolved in ethanol. Aliquots of the ethanol were plated on activated silica gel plates and developed as previously described (10). The taurine conjugates of the appropriate bile acids were chromatographed on each plate as a reference standard. That area of the plate corresponding to the position of the standard was scraped into 3.0 ml of 43 mM sodium pyrophosphate, pH 9.5. After mixing, aliquots of the supernatant were assayed with 3α-hydroxysteroid dehydrogenase as above. Determination of the enzymic activity of lithocholoyl CoA proceeded as above except that the conjugates were estimated by measuring the lithocholic acid released following alkaline hydrolysis. In all experiments, 1 μmole of the appropriate taurine conjugate was carried through the entire procedure to correct for losses during extraction and thin-layer chromatography (average 83% recovery).

Synthesis of coenzyme A derivatives was accomplished by the method of Shah and Staple (5), except that the order of ether extraction and perchloric acid precipitation of the product was reversed, resulting in slightly greater yield of product. The acid precipitate was redissolved in approximately 2 ml of 40% methanol in water (v/v) and maintained at 5°C. This amount of solution was applied to a 2.5 × 60 cm column containing Sephadex LH-20 previously equilibrated with 40% methanol in water. Fractions of 2.2 ml were collected and analyzed for absorbance at 259 nm and for CoASH. The steroid content of the chromatographic fractions was estimated by the method of Reid and Boyd (15). Fractions possessing equimolar amounts of CoA and steroid were pooled, lyophyllized, and redissolved in sufficient water to permit a clear solution. Aliquots of the pool were frozen at −70°C until used. After several months at these conditions of

storage, there was no hydrolysis as measured by CoASH release. The overall yield from this procedure varied from 40 to 65% based on the CoASH in the starting material.

RESULTS

When the synthetic CoA derivatives were chromatographed over Sephadex LH-20, a major peak appeared (**Fig. 1**) that contained equimolar amounts of steroid and adenine. This peak represented 70–80% of the CoA and steroid present in the synthetic product prior to chromatography. While CoA to steroid and hydroxamate to steroid ratios were not significantly different when the same preparations were assayed before and after LH-20 chromatography, ($P < 0.10$), the chromatogram consistently demonstrated heterogeneity of the synthetic product with smaller peaks compatible with free bile acids, and unreacted CoASH. Rechromatography of the major peak resulted in a single peak with an equimolar steroid to CoA ratio suggesting that the CoASH and free bile acid seen on initial chromatography were not the products of hydrolysis of the thioester bond during the chromatographic procedure. On occasion, particularly when the ratio of bile acids to CoASH in the starting material exceeded 1.2, chromatography also demonstrated a second large peak that exhibited a steroid to CoA ratio of approximately two.

The compound present in the major chromatographic peak migrated as a single band on descending chromatography using unwashed Whatman 3MM filter paper developed with ethanol–ethyl acetate–0.03 M sodium acetate 2:4:3. In this system, all of the CoA derivates migrated similarly with R_f 0.73–0.84, while CoASH and free bile acids migrated with R_f 0.41–0.50 and R_f 0.89–0.99, respectively. Better separation of the CoA derivates (R_f 0.33–0.36) from bile acid (R_f 0.93–0.97) was possible when chromatograms were developed with ethanol–chloroform–ethyl acetate 1:2:1. This system, however, does not separate the CoA derivatives from CoASH (R_f 0.34–0.37). In both systems, the chromatograms of the CoA derivatives demonstrated the expected reactions when sprayed with nitroprusside reagent in the presence of methanolic sodium hydroxide, and with antimony trichloride–glacial acetic acid reagent (10).

One preparation of choloyl CoA was examined with NMR by Dr. John Meiyal of Northwestern University (7). The 270 MHz spectrum showed downfield displacement of the —CH_2—S— signal from δ 2.62 to δ 2.92 ppm, characteristic of the thioester bond. All resonances of the choloyl CoA could be accounted for by comparison to the spectra of cholic acid, CoASH, or the two combined except for a small, unidentified signal at δ 2.29 ppm. This unidentified signal accounted for less than 2% of the total integrated area.

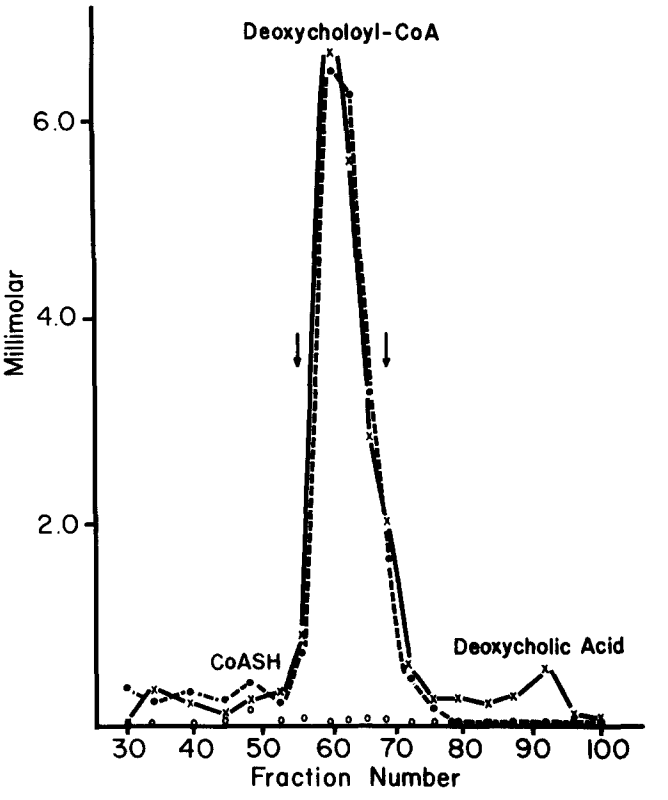

Fig. 1. Chromatographic purification of deoxycholoyl CoA. A 2 ml aliquot of crude deoxycholoyl CoA in 40% methanol–water (v/v) was placed on a 2.5 × 60 cm column containing Sephadex LH-20 previously equilibrated with the same solvent. The column was eluted at 4°C with 40% methanol–water at a flow rate of 18 ml/hr. 2.2 ml fractions were collected. The effluent was monitored for $A_{259\ nm}$ to locate the appropriate fractions. Individual fractions were assayed for $A_{259\ nm}$ (●—●), hydroxy steroid (×—×), and CoASH (○—○). See Methods for details. Fractions located between the arrows were pooled, lyophilized and utilized in further studies.

The molecular ratio of CoA to steroid was determined on four preparations each of choloyl CoA, chenodeoxycholoyl CoA, and deoxycholoyl CoA, and two preparations of lithocholoyl CoA. The ratio of steroid to thioester bond for all but the lithocholoyl CoA was also determined. These ratios (±SD) were: adenine to steroid, 1.03 ± 0.09, and thioester to steroid, 1.00 ± 0.11. CoASH contamination, estimated by the molar ratio of CoASH to steroid, was 0.01 ± 0.01.

The synthetic coenzyme A derivative gave the expected ultraviolet absorption spectrum between

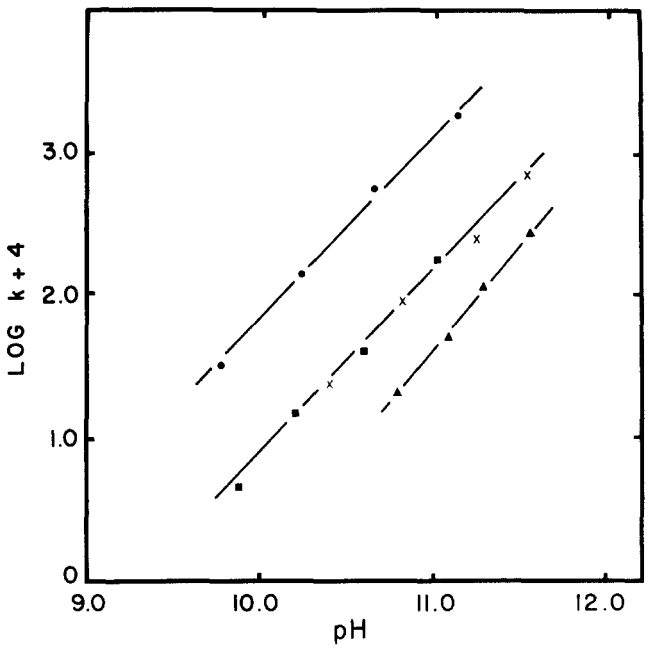

Fig. 2. The effect of pH on the rate of hydrolysis of the bile acid CoA thioesters. Samples were reacted at 38°C in aqueous solutions of NaOH at the pH values shown. Ionic strength was adjusted to 2.5 M with NaCl. (●——●) Choloyl CoA, (■——■) deoxycholoyl CoA, (×——×) chenodeoxycholoyl CoA, (▲——▲) lithocholoyl CoA. The slopes of the lines joining the points were determined by regression of the points by the least squares method. Apparent pseudo-first order rate constants were calculated as described in the text.

200 nm and 300 nm. Compared with an equimolar solution of CoASH, it exhibited a difference spectrum maximum at 232 nm, consistent with the presence of a thioester bond (16). The millimolar extinction coefficient (±SD) at 259 nm was 15.03 ± 0.58 and at 232 nm, 7.6 ± 0.17. The change in absorbance at 232 nm after alkaline hydrolysis of the thioester bond when divided by the CoASH released permitted calculation of the millimolar extinction coefficient of the thioester bond, 4.12 ± 0.17. Alkaline hydrolysis in these experiments resulted in the measured release of 0.94 ± 0.05 micromoles of CoASH per micromole of steroid.

All of the synthetic bile acid CoA derivatives were readily hydrolyzed in strongly alkaline solutions. However, while different preparations of the same bile acid CoA derivative showed similar rates of alkaline hydrolysis, the hydrolysis rates varied with the number of hydroxyl groups on the steroid moiety ($P < 10^{-4}$) (**Fig. 2**). There were no differences in the rates of hydrolysis of deoxycholoyl CoA and chenodeoxycholoyl CoA. Extrapolation of the alkaline hydrolysis data to physiologic temperature and pH indicated half-lives of approximately 185 days for choloyl CoA and 1520 days for lithocholoyl CoA. CoASH release was not detectable in acid solutions.

Biologic activity of the four bile acid CoA derivatives was greater than 94% when measured by the enzymic synthesis of taurine conjugates of the bile acids (**Table 1**). The requirement for the coenzyme A thioester in this reaction is demonstrated in experiment 1d, where equimolar amounts of cholic acid and CoASH replaced choloyl CoA. Each of the taurine conjugates formed in these experiments migrated in a single band on TLC with identical R_f to that of known taurine conjugates. With the exception of CoASH, no other products were detected.

DISCUSSIONS

Synthesis of the coenzyme A derivatives of bile acids according to the method of Shah and Staple

TABLE 1. Activity of synthetic CoA derivatives in the enzymic conjugation of bile acids with taurine

Preparation	CoA Derivative Added	CoASH Released	Taurine Conjugates Formed	CoA Derivative Conjugated
	nmoles	*nmoles*	*nmoles*	*%*
1. Choloyl CoA				
a. Complete system	954	921	1099	
b. Minus enzyme	954	1	89	
(net)			1010	105.9
c. Heated enzyme	954	23	54	(5.6)
d. Plus Cholic Acid and CoASH	0	ND	19	(2.0)
2. Deoxycholoyl CoA	1180	1029	1126	95.4
3. Chenodeoxycholoyl CoA	916	854	903	98.6
4. Lithocholoyl CoA	888	758	837	94.2

Incubations were performed as described in the text. In experiment 1c, the enzyme was heated to 80°C for 15 min prior to use. Experiment 1d included 1000 nmoles of cholic acid and 1000 nmoles of CoASH in place of choloyl CoA. Values shown are the averages of triplicate determinations. Experiments 2, 3, and 4 present the net enzymic conjugation corrected for a parallel control to which no enzyme was added.

(5) yields a product which is a mixture of the desired thioester and unreacted bile acid and CoASH. We have demonstrated that chromatography of this product over Sephadex LH-20 results in purification of the product free of unreacted starting material.

Biologic activity of our purified thioesters is greater than 94% in contrast to 51% reported by Goldman and Vagelos (6) for palmitoyl CoA and oleoyl CoA synthesized by a similar method. While the reasons for the differences in biologic activity are not clear, we have observed that when the ratio of acyl moiety to CoASH at the start of the synthesis exceeds 1.2, recommended by Shah and Staple (5), and approximates 1.5, the ratio used by Goldman and Vagelos (6), chromatography over Sephadex LH-20 demonstrates a second hydroxamate-positive fraction that contains bile acid and CoA in a ratio of 2:1. This fraction is not active in the enzymic synthesis of taurine conjugates.

The demonstration of enzymic activity of the synthetic coenzyme A derivatives confirms the hypothesis of Bremer (2) and Elliott (3) and is consistent with the demonstration of Siperstein and Murray (1) that an enzymic product compatible with choloyl CoA is the active intermediate in the enzymic conjugation of taurine with cholic acid. We have further shown that these coenzyme A thioesters are stable in solution at physiologic pH and temperature. These purified compounds may be useful, therefore, in the study of bile acid conjugation.

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