Characterization of Liver Cholic Acid Coenzyme A Ligase Activity

EVIDENCE THAT SEPARATE MICROSOMAL ENZYMES ARE RESPONSIBLE FOR CHOLIC ACID AND FATTY ACID ACTIVATION

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Investigations on the cholic acid CoA ligase activity of rat liver microsomes were made possible by the development of a rapid, sensitive radiochemical assay based on the conversion of \(^{3}H\)cholic acid to \(^{3}H\)choloyl-CoA. More than 70% of the rat liver cholic acid CoA ligase activity was associated with the microsomal subcellular fraction. The dependencies of cholic acid CoA ligase activity on pH, ATP, CoA, Triton WR-1339, acetone, ethanol, magnesium, and salts were investigated. The hypothesis that the long chain fatty acid CoA ligase activity and the cholic acid CoA ligase activity are catalyzed by a single microsomal enzyme was investigated. The ATP, CoA, and cholic (palmitic) acid kinetics neither supported nor negated the hypothesis. Cholic acid was not an inhibitor of the fatty acid CoA ligase and palmitic acid was not a competitive inhibitor of the cholic acid CoA ligase. The cholic acid CoA ligase activity utilized dATP as a substrate more effectively than did the fatty acid CoA ligase activity. The cholic acid and fatty acid CoA ligase activities appeared to have different pH dependencies, differed in thermolability at 41°C, and were differentially inactivated by phospholipase C. Moreover, fatty acid CoA ligase activity was present in microsomal fractions from all rat organs tested while cholic acid CoA ligase activity was present only in liver microsomes. The data suggest that separate microsomal enzymes are responsible for the cholic acid and the fatty acid CoA ligase activities in liver.

The fate of newly synthesized bile salts is complex. Initially, bile salts are released via the bile duct into the duodenum when required. In the intestine, some of the bile salts are cleaved to bile acids (4). Bile acids and salts are absorbed in the ileum, enter the enterohepatic circulation, and are returned to the liver, where the bile acids are reconverted to bile salts by conjugation with taurine and glycine.

An important enzyme in the sequence of events is the cholic acid CoA ligase (AMP) (EC 6.2.1.7), which activates cholic acid to its coenzyme A thioester. The bile acids are transferred from their thioester derivatives to glycine or taurine to form the principal bile salts. Siperstein and Murray first demonstrated the ATP-dependent formation of choloyl-CoA in liver microsomes in 1956 (5). At about the same time, Elliott developed an assay for the cholic acid CoA ligase based on the formation of choloyl hydroxamate (6). Since these initial observations, few reports on the cholic acid CoA ligase have appeared (7-10).

In this paper, we report investigations of the cholic acid CoA ligase activity using a highly sensitive assay based on the conversion of \(^{3}H\)cholic acid to \(^{3}H\)choloyl-CoA. The cholic acid CoA ligase activity was characterized with respect to substrate kinetics, reaction dependencies, and tissue and subcellular distributions. In addition, the hypothesis that the cholic acid CoA ligase and the fatty acid CoA ligase (AMP) (EC 6.2.1.3) are dual catalytic activities of a single microsomal enzyme was tested. This question had been previously raised (9) in view of the similarity of the reactions catalyzed by the fatty acid CoA ligase and the cholic acid CoA ligase and the broad substrate specificity of the fatty acid CoA ligase (11).

EXPERIMENTAL PROCEDURES

Materials—ATP, dATP, and CoASH were purchased from P-L Biochemicals, Milwaukee, Wis. Dithiothreitol, bovine serum albumin (essentially fatty acid-free), and cholic acid (sodium salt) were obtained from Sigma Chemical Co., St. Louis, Mo. Palmitic acid was the product of the Hormel Institute, Austin, Minn. Triton WR-1339 was purchased from Rigel Chemical Co., Irvington, N. J. Bovine serum albumin (Fraction V) was obtained from Armour Pharmaceutical Co., Omaha, Neb. Crude collagenase (type 1) from Clostridium histolyticum was purchased from Worthington Biochemical Corp., Freehold, N. J. \(^{3}H\)Palmitic acid, \(^{3}H\)cholic acid, and Aquasol were products of New England Nuclear, Boston, Mass.

Highly purified phospholipase C (12) from Bacillus cereus was prepared by the procedure of Zwaal et al. (13).
All other reagents were the highest grade commercially available.

Synthesis of \([\text{H}]\text{Choloyl-CoA} - [\text{H}]\text{Choloyl-CoA} \) was synthesized from \([\text{H}]\text{cholic acid via the N-hydroxysuccinimide ester according to Al-Arif and Blecher (14). When a sample of this material was chromatographed on Whatman No. 1 in propa-}

Results

Validation of Cholic Acid CoA Ligase Assay

A sensitive assay for rat liver microsomal cholic acid CoA ligase activity was developed based on the conversion of \([\text{H}]\text{cholic acid to [H]choloyl-CoA. When a standard assay}

Assay for Fatty Acid CoA Ligase - Microsomal fatty acid CoA ligase activity was assayed by the method of Samuel and Alibaud (20) as described by Suzue and Marcel (21). The reaction mixture contained: 150 mm Tris, pH 7.4, 50 mM MgCl$_2$, 10 mm ATP, 100 mm CoA, 0.5 mm dithiothreitol, 30 mm \([\text{H}]\text{palmitic acid (100 mCi/mmol), and 1}

Characterization of Cholic Acid CoA Ligase Activity and Evidence that Cholic Acid and Fatty Acid CoA Ligase Activities Are Catalyzed by Separate Microsomal Enzymes

In the following sections, the highly sensitive assay, validated above, has been employed to characterize the cholic acid CoA ligase activity. A side by side comparison of cholic acid CoA ligase activity and of fatty acid CoA ligase activity has been performed to evaluate whether these activities are catalyzed by a single microsomal enzyme.

Substrate Dependencies of Liver Microsomal Fatty Acid and Cholic Acid CoA Ligase Activities - The substrate dependencies of the liver microsomal fatty acid and cholic acid CoA ligase activities were investigated on the same day with the same microsomal preparation. The apparent $K_m$ values for ATP derived from the double reciprocal plots shown in Fig. 9, were similar for the fatty acid and cholic acid CoA ligase activities. However, cholic acid CoA ligase activity was the same when assayed using 10 mm ATP or dATP while fatty acid CoA ligase activity was 60% lower with the deoxynucleotide. The cholic acid CoA ligase activity had an apparent $K_m$ for dATP only 50% higher than for ATP while the apparent $K_m$ for dATP increased 389% relative to ATP for the fatty acid CoA ligase activity.

The fatty acid and cholic acid CoA ligase activities had similar dependencies on CoA, with an apparent $K_m$ of about 20
FIG. 1. Linearity of liver microsomal cholic acid CoA ligase with time and protein. Activity was assayed as described under "Experimental Procedures." ○—○, time dependence using 75 μg of protein; □—□, protein dependence (10-min incubation).

TABLE I

Dependencies of cholic acid CoA ligase assay in liver microsomes

Assays were run for 10 min using the conditions described under "Experimental Procedures." Reactions employed 75 μg of liver microsomal protein.

| Assay conditions      | Cholic acid CoA ligase activity (nmol/min/mg) |
|-----------------------|-----------------------------------------------|
| Complete system       | 1.6*                                          |
| Minus microsomes      | 0                                             |
| Time zero, control    | 0                                             |
| Minus ATP             | 0.1                                           |
| Minus CoA             | 0.1                                           |
| Minus Mg²⁺            | 0.4                                           |
| Minus Mg²⁺ plus 1 mM EDTA | 0.1                                      |
| Minus dithiothreitol  | 1.9                                           |
| Minus Triton WR-1339  | 0.6                                           |

* In the complete system (see "Experimental Procedures") 5020 cpm was observed with a background (minus microsomes) of 150 cpm.

The specific activities in liver have ranged from 1 to 3 nmol/min/mg in different microsomal preparations when the complete assay system was employed.

μM (data not shown). The apparent $K_m$ for cholic acid in the cholic acid CoA ligase reaction (10 μM) was similar to the apparent $K_m$ (15 μM for palmitic acid) in the fatty acid CoA ligase reaction (see below).

pH Dependence—An investigation of the pH dependencies of the fatty acid CoA ligase and cholic acid CoA ligase activities (Fig. 3) suggested that the two activities had different pH dependencies.

Competition Experiments—The possible inhibition of fatty acid CoA ligase activity by cholic acid and of cholic acid CoA ligase activity by palmitic acid in liver microsomes was investigated. When up to 100 μM unlabeled cholic acid was added to a standard assay for the fatty acid CoA ligase activity, no inhibition of [3H]palmitoyl-CoA formation occurred (Fig. 4A). The [3H]palmitic acid dependence of the fatty acid CoA ligase activity was compared in the presence and absence of 100 μM unlabeled cholic acid. The double reciprocal plot of the data (Fig. 4B) demonstrated that 100 μM cholic acid actually stimulated the fatty acid CoA ligase activity slightly, and had very little effect on the apparent $K_m$ of 15 μM for [3H]palmitic acid.

The addition of up to 60 μM unlabeled palmitic acid to a standard assay for the cholic acid CoA ligase activity resulted in less than 10% inhibition (Fig. 4A). The [3H]cholic acid dependence of the cholic acid CoA ligase was compared in the presence and absence of 30 μM unlabeled palmitic acid. The double reciprocal plot of the data (Fig. 4B) revealed that palmitic acid did not change the apparent $K_m$ of 10 μM for [3H]cholic acid, and was not a competitive inhibitor.

FIG. 3. pH dependence of cholic acid CoA ligase and fatty acid CoA ligase activities. Assays were performed for 10 min as described under "Experimental Procedures" except that the pH of the 350 mM Tris buffer was varied. O—O, cholic acid CoA ligase assay employing 75 μg of liver microsomal protein; □—□, fatty acid CoA ligase assay employing 2 μg of protein. The data were plotted by the method of Lineweaver and Burk (22). A computer-assisted least squares analysis was used to draw all lines. Each line had a correlation coefficient of at least 0.99. O—O, ATP dependence of cholic acid CoA ligase; □—□, dATP dependence of cholic acid CoA ligase; ○—○, ATP dependence of fatty acid CoA ligase; □—□, dATP dependence of fatty acid CoA ligase.

FIG. 2. Dependence of cholic acid CoA ligase and fatty acid CoA ligase activities on ATP and dATP. Assays were performed for 10 min as described under "Experimental Procedures" in the presence of varying amounts of ATP or dATP. Cholic acid CoA ligase assays employed 75 μg of liver microsomal protein. Fatty acid CoA ligase assays employed 2 μg of protein. The data were plotted by the method of Lineweaver and Burk (22). A computer-assisted least squares analysis was used to draw all lines. Each line had a correlation coefficient of at least 0.99. O—O, ATP dependence of cholic acid CoA ligase; □—□, dATP dependence of cholic acid CoA ligase; ○—○, ATP dependence of fatty acid CoA ligase; □—□, dATP dependence of fatty acid CoA ligase.

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Effect of Phospholipase C Preincubation — The effect of a 10-min phospholipase C preincubation of rat liver microsomes on fatty acid CoA ligase and cholic acid CoA ligase activities was investigated (Fig. 5). Both microsomal activities were stable to preincubation with 0.1 unit/ml of phospholipase C, but higher levels led to the selective inhibition of the cholic acid CoA ligase activity. Preincubation of microsomes with 0.4 unit/ml of phospholipase C had no effect on fatty acid CoA ligase activity while cholic acid CoA ligase was inhibited almost 50%.

Thermolability at 41°C — Fatty acid CoA ligase and cholic acid CoA ligase activities were measured in liver microsomes which had been incubated at 41°C for various times (see Fig. 6). The semilog plot of the data revealed that both activities declined with first order kinetics until greater than 70% of the activity had been lost. The fatty acid CoA ligase activity was extremely thermolabile under these conditions, with a half-life of 3 min. Cholic acid CoA ligase activity, in the same microsomes, was considerably more stable, displaying a half-life of about 16 min.

Tissue Survey — Microsomes prepared from various rat organs were assayed for fatty acid CoA ligase and cholic acid CoA ligase activity. The results are presented in Table II. The two activities had quite different distributions. Fatty acid CoA ligase activity was found in all microsomal preparations tested, while cholic acid CoA ligase activity was detected only in liver microsomal preparations.

DISCUSSION

Investigations on the cholic acid CoA ligase have previously been hindered by inadequate methodology. The direct assay, reported here, based on the conversion of [3H]cholic acid to [3H]choloyl-CoA is over 1000 times more sensitive than the

**Table II**

| Source of microsomes | Fatty acid CoA ligase | Cholic acid CoA ligase |
|----------------------|-----------------------|------------------------|
| Liver                | 38.7                  | 3.0                    |
| Isolated fat cell    | 87.0                  | <0.01                  |
| Heart                | 19.3                  | <0.01                  |
| Skeletal muscle      | 3.5                   | <0.01                  |
| Kidney               | 3.4                   | <0.01                  |
| Brain                | 2.4                   | <0.01                  |
| Lung                 | 1.3                   | <0.01                  |
| Intestine            | 0.9                   | <0.01                  |

* Microsomes were prepared as described under "Experimental Procedures."

* Assays were performed at three different protein concentrations for 10 min as described under "Experimental Procedures."

**Fig. 6 (right).** Thermolability of liver microsomal fatty acid CoA ligase and cholic acid CoA ligase activities at 41°C. Microsomes were incubated at 20 mg/ml with shaking at 41°C. Samples taken at the indicated times were diluted as appropriate for assay at room temperature as described under "Experimental Procedures." O---O, cholic acid CoA ligase assays employed 75 µg of protein; Δ---Δ, fatty acid CoA ligase assay employed 2 µg of protein.
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hydroxamate assay and avoids the use of hydroxylamine. Further, while the hydroxamate assay required millimolar levels of bile acids, the direct assay requires only 60 μM cholic acid.

The remaining data on the characterization of the cholic acid CoA ligase activity will be discussed in relation to the hypothesis that the cholic acid and fatty acid CoA ligase activities are dual activities of a single microsomal enzyme. The subcellular distribution and the apparent Kₘ values for ATP, CoA,¹ and cholic² (palmitic) acid for the liver microsomal fatty acid and cholic acid CoA ligase activities were similar. These observations and the approximately 10-fold lower specific activity for the cholic acid CoA ligase than the fatty acid CoA ligase were consistent with either a single enzyme with two activities or two independent microsomal enzymes.

Several lines of evidence, however, suggest that the fatty acid CoA ligase and the cholic acid CoA ligase activities are separate microsomal enzymes. Cholic acid was not an inhibitor of the fatty acid CoA ligase activity and palmitic acid was not a competitive inhibitor of the cholic acid CoA ligase activity. The cholic acid and fatty acid CoA ligase activities were different in their pH dependencies, their ability to use dATP as a substrate, their thermostability at 41⁰C, and their sensitivity to phospholipase C. Moreover, the tissue survey demonstrated that cholic acid CoA ligase activity was present only in liver while the fatty acid CoA ligase activity was present in every tissue examined. The best interpretation of the available data is that separate cholic acid and fatty acid CoA ligases exist in liver microsomal preparations. The absence of cholic acid CoA ligase from all other organs examined appears reasonable physiologically since the uptake and activation of bile acids in non-hepatic tissues could lead to accumulation of bile acids which could have deleterious effects on cellular processes.

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¹ Recently a preliminary communication on the characterization of cholic acid CoA ligase in guinea pig liver microsomes appeared (10). The specific activity and apparent Kₘ values for CoA and cholic acid reported agree closely with the data presented.
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