

Facile enzymatic synthesis of fatty acyl-coenzyme A thioesters

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Summary The fatty acid:CoA ligase (acyl-CoA synthetase, EC 6.2.1.3) of rat liver microsomes was solubilized with Triton X-100 and bound to Matrex Gel Red A. Fatty acid:CoA ligase immobilized on Matrex Gel Red A was active and proved useful for the synthesis of fatty acyl-CoA thioesters. The immobilized activity was characterized by a 3-fold higher apparent K_m for ATP than the soluble activity, similar apparent K_m values for CoA and palmitate, and a shift in the pH dependence. Quantitative incorporation of fatty acid or CoA was possible. Long chain-fatty acyl CoA thioesters were purified in a single step by hydrophobic chromatography on Octyl-Sepharose. In addition to producing the thioesters of typical fatty acids (e.g., myristic, palmitic, stearic, oleic, *cis*-vaccenic, linoleic, and arachidonic), analogs such as the fluorescent molecules β -parinaroyl-CoA and palmitoyl-(1- N^6 -etheno-)CoA were easily synthesized. These procedures should be generally applicable for both the small-scale, i.e., 1 to 10 μ moles, and large-scale, i.e., 50 to 250 μ moles, scale preparation of numerous fatty acyl-CoA's and related compounds.—Merrill, A. H., Jr., S. Gidwitz, and R. M. Bell. Facile enzymatic synthesis of fatty acyl-coenzyme A thioesters. *J. Lipid Res.* 1982. **23**: 1368–1373.

Supplementary key words fatty acyl-CoA synthetase • fluorescent fatty acyl-CoA • immobilized enzymes

A variety of chemical and enzymatic methods have been developed to synthesize fatty acyl-CoA thioesters. The chemical syntheses (1–7) are relatively simple, but can yield small quantities of other products (8), (perhaps by acylation of the ribose hydroxyl or of adenine) which are not biologically active (3, 7), and are not uniformly successful with unsaturated fatty acids (8). These problems are circumvented by the greater specificity of the enzymatic methods (9–11). However, isolation of fatty acid:CoA ligase activity free of interfering enzymes and endogenous substrates and the recovery of the reaction products have been major problems associated with the reported enzymatic syntheses (11). Herein we describe an enzymatic procedure that is both easy and useful for the syntheses of a broad range of fatty acyl-CoA's and derivatives.

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EXPERIMENTAL

Chemicals

Tris, sucrose, EDTA, dithiothreitol, 5,5'-dithiobis(2-nitrobenzoic acid) and the unlabeled fatty acids were purchased from Sigma Chemical Co., St. Louis, MO. Reduced CoA (ChromatoPure), ATP, β -parinaric acid, and (1- N^6 -etheno-)CoA were obtained from P-L Biochemicals, Milwaukee, WI. Triton X-100 (scintillation grade) was the product of Research Products International Corp., Mt. Prospect, IL. [9,10-³H(N)]Palmitic acid, [1-¹⁴C]oleic acid, [1-¹⁴C]linoleic acid, and Aquasol-2 were obtained from New England Nuclear, Boston, MA. [1-¹⁴C]Arachidonic acid was provided by Dr. Marvin Siegel, Burroughs-Wellcome Corp., Research Triangle Park, NC. Matrex Gel Red A was purchased from Amicon Corp., Lexington, MA, and Octyl-Sepharose CL-4B was from Pharmacia Fine Chemicals, Piscataway, NJ. Silica Gel-H plates (250 μ m) were purchased from Analtech, Inc., Newark, DE, and Whatman No. 1 chromatographic paper was obtained from Whatman, Inc., Clifton, NJ.

Preparation of detergent-solubilized fatty acid:CoA ligase

Six adult, female rats (Charles River strain, or equivalent) were maintained on a diet of standard lab chow until 12 hr before use; during this last period they were only given water. They were killed by decapitation and the livers were quickly removed and chilled on ice. The livers were minced in 150 ml of 50 mM Tris-HCl (pH 7.4), 0.25 M sucrose, 5 mM EDTA, and 5 mM dithiothreitol, and homogenized with three to six strokes of a glass homogenizer with a motor-driven Teflon pestle. The homogenate was centrifuged for 1 hr at 105,000 *g*. The high-speed pellet was resuspended by homogenization in 100 ml of the same buffer minus sucrose and centrifuged for 1 hr at 105,000 *g*. This "washed" pellet was resuspended by homogenization in 100 ml of the buffer minus sucrose and stirred gently while 4 ml of 20% (w/v) Triton X-100 was added dropwise. After 1 hr, the insoluble material was removed by centrifugation at 105,000 *g* for 1 hr, and the resulting clear supernatant was divided into 25-ml aliquots and stored at -80°C . No detectable loss of activity was observed after 6 to 12 months of storage.

Preparation of fatty acid:CoA ligase bound to Matrex Gel Red A

One frozen aliquot of the Triton X-100-solubilized enzyme (approximately 180 mg of protein) was allowed to thaw at room temperature. To this was added 5 ml

of Matrex Gel Red A that had been washed according to the manufacturer's instructions and equilibrated with 0.1 M Tris-HCl (pH 7.4), 0.1% (w/v) Triton X-100, and 5 mM dithiothreitol. This mixture was stirred gently for 2 to 3 hr, and then poured into a small chromatographic column (1 × 10 cm). Material not bound by the beads was allowed to flow through the column. Additional proteins were removed by washing the beads with 25 ml of 0.1 M Tris-HCl (pH 8.0), 0.1% Triton X-100, and 5 mM dithiothreitol, 25 ml of this buffer containing 0.25 M NaCl, 25 ml of this buffer containing 10 mM ATP, and 25 ml of buffer alone. The immobilized fatty acid CoA:ligase activity could be stored at 4°C for several days.

Enzymatic synthesis of fatty acyl-CoA's

Reaction mixtures (typically 50 ml)³ contained 0.1 M Tris-HCl, 10 mM ATP, 30 mM MgCl₂, 0.1% Triton X-100 (w/v), 5 mM dithiothreitol, 1 mM reduced CoA, and 1 mM fatty acid, and had a final pH of 8.2. Saturated fatty acids were prepared as concentrated solutions in 0.5% Triton X-100 and 0.1 M Tris-HCl (pH 8.2) and were added dropwise to the other components of the reaction mixture to yield the final concentrations. Unsaturated fatty acids were dissolved in a minimal volume of ethanol and added to the reaction mixtures after the solutions had been equilibrated with N₂. A fine precipitate occasionally formed in the reaction mixtures; however, this precipitate (presumably fatty acids) redissolved in the course of the enzymatic reaction.

The reaction mixture was pumped through the ligase beads at room temperature using a peristaltic pump at a flow rate of 0.5 to 2.0 ml/min. The reaction mixture was continuously recycled through the column by placing the effluent tubing into the flask that contained the initial reaction solution. When radiolabeled fatty acids or CoA were used, the recycling was continued until conversion was complete (see below); otherwise, the reaction was conducted for 2 to 3 hr. When the reaction was complete, the effluent was diverted to a separate flask and collected. Products remaining in the column were washed from the beads with 3 column volumes of buffer and added to the product mixture.

Purification of the fatty acyl-CoA's

A concentrated LiCl solution (4 M) was added to the product mixture to yield a solution containing 1 M LiCl,

³ Only a portion of the beads is needed if smaller scale syntheses are conducted.

and 1 ml of Octyl-Sepharose beads (equilibrated with 1 M LiCl) per ml of initial reaction mixture was added with gentle stirring. This mixture was poured into a chromatographic column, the beads were washed by 5 to 10 column volumes of 1 M LiCl, and the fatty acyl-CoA was eluted by a decreasing gradient of LiCl (1 M to 0 M). The eluate was checked for purity by comparing the absorbances at 280, 260, 250, and 232 nm and, if needed, trace impurities were removed by precipitating the fatty acyl-CoA with 1% (v/v) perchloric acid and washing the precipitate with acetone and diethyl ether (4). The Octyl-Sepharose beads can be reused after washing with n-butanol to remove Triton X-100 and fatty acids.

Analytical methods

The fatty acyl-CoA's were analyzed for purity by several methods. Absorbances at 280, 260, 250, and 232 nm were measured at room temperature using samples in 0.1 M potassium phosphate (pH 7.0) and were compared to published values (12, 13). Concentrations were calculated from the absorbances at 260 nm ($\epsilon = 15,400 \text{ M}^{-1} \text{ cm}^{-1}$) (12) and were compared to those obtained by scintillation counting of radiolabeled fatty acyl-CoA's. Biological activity was assessed by determining the amount of monoacyl-[³H]glycerol-3-phosphate which was formed in 1.0-ml reaction mixtures containing 25 to 75 μM fatty acyl-CoA and 2.5 μg of the reconstituted glycerol-P acyltransferase which had been purified to homogeneity from *Escherichia coli* (14). Paper (Whatman No. 1) and thin-layer (Silica Gel-H) chromatograms were developed with 1-butanol-acetic acid-water 5:2:3 (v/v/v). Free fatty acids and CoA were additionally estimated by extraction (15) and reaction with 5,5'-dithiobis (2-nitrobenzoic acid) (16), respectively.

Enzymatic assays were conducted using aliquots of the ligase purified by chromatography on Matrex Gel Red A (using conditions similar to those described in reference 17) or small amounts of the beads with the bound enzyme. Each assay (100 μl) contained: 0.1 M Tris-HCl, 10 mM ATP, 5 mM dithiothreitol, 30 mM MgCl₂, 100 μM CoA, 50 μM [³H]palmitic acid, and 0.1% (w/v) Triton X-100 (final pH 8.0). After 10 min at room temperature, the [³H]palmitoyl-CoA was extracted (15) and quantitated by liquid scintillation counting. Kinetic analyses were conducted by varying the concentration of a single assay component and plotting the data according to Lineweaver and Burk (18). Tests for interfering enzymes were conducted by flowing 100 μM [³H]palmitoyl-CoA through the beads and following its conversion to other compounds by extraction (15) and paper chromatography.

RESULTS AND DISCUSSION

The procedure for synthesizing fatty acyl-CoA thioesters described under Experimental was developed by analyzing intermediate fractions in a purification of the fatty acid:CoA ligase based on a published method (17) for their utility in converting 1 mM [^3H]palmitic acid and CoA to their thioester. This analysis revealed that not only were endogenous substrates and interfering enzymatic activities, i.e., palmitoyl-CoA hydrolase (19) and palmitoyl-CoA:ethanol acyltransferase (20), removed by chromatography of the detergent extract on Matrex Gel Red A, but also, the column itself could be used as a convenient material for the desired synthesis.

Characterization of the immobilized fatty acid:CoA ligase

Approximately 63% of the detergent-solubilized ligase was bound by the beads after 2 to 3 hr (Fig. 1); of this, 15% was released by washing with buffer, salt, and ATP as described under Experimental. The Matrex Gel Red A retained 50 mg of protein and at least 28% of the initial activity, based on the amount of the activity that was eluted by 1 M NaCl and 10 mM ATP. When the beads were assayed directly, only 7.5% of the initial activity was found; hence, it appears that the immobilized ligase has diminished activity, as has been reported for other enzymes (21). Some of the ligase (approximately 0.4% of the bound activity per column volume of eluate) was leached from the column by the reaction mixture.⁴

The ligase bound to Matrex Gel Red A also differed from the Triton X-100-solubilized enzyme in having a 3-fold higher K_m for ATP, i.e., 6.0 versus 1.7 mM, and a sharper dependence of the rate on pH. Both enzyme preparations exhibited maximal activities at alkaline pH, i.e., \geq pH 8.0; however, only the solubilized enzyme had significant activity at pH 7.0, i.e., 48% of that at pH 8.0. Otherwise, the stability and kinetic properties of the immobilized enzyme were similar to those for the enzyme solubilized from rat liver microsomes. In these kinetic analyses, no activity was found if a single substrate (ATP, MgCl_2 , or fatty acid) was omitted; hence, the endogenous substrates present in microsomes (11) appear to have been removed. Nor was evidence for palmitoyl-CoA hydrolase or palmitoyl-CoA:ethanol acyltransferase activities found. When [^3H]palmitoyl-

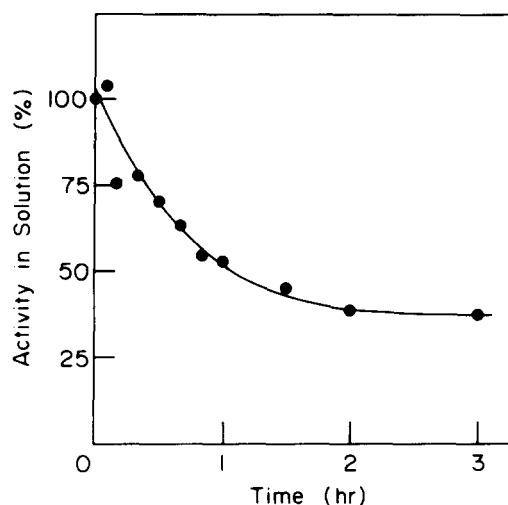


Fig. 1. Fatty acid:CoA ligase binding to Matrex Gel Red A. All procedures were conducted as described under Experimental. Activities are expressed as percent of the starting activity of the Triton X-100-solubilized enzyme. Aliquots were removed at various time intervals during the initial mixing of enzyme with the beads, centrifuged briefly, and activity remaining in solution (i.e., in the supernatant) was assayed.

CoA was recycled through the beads, 75% was recovered as the thioester and 22% as the free fatty acid, which was similar to the extent of hydrolysis that occurred in solution at this alkaline pH. When fatty acids were added in ethanol, the ethyl esters were not detected by extraction or thin-layer chromatography (20). Furthermore, both fatty acid and CoA were quantitatively utilized in synthesizing the thioesters and appeared in the final product in stoichiometric amounts (see below).

Preparation of fatty acyl-CoA's

Shown in Fig. 2 is a typical reaction progress curve. By 1 hr, 86% of the palmitic acid and CoA had been converted to their thioester, and by 2 hr complete conversion had been effected.

Separation of the fatty acyl-CoA's from other components of the reaction mixture was initially accomplished by extracting the fatty acids and Triton X-100 with chloroform and collecting the precipitated acyl-CoA. Recoveries of the desired products proved erratic, probably because of varying amounts of residual Triton X-100. Hydrophobic chromatography on Octyl-Sepharose CL-4B was found to be a more reproducible procedure with recoveries of 50 to 90% for this step (the recoveries did not appear to depend upon the type of fatty acyl-CoA). The long chain fatty acyl-CoA's were bound only in the presence of salt; hence, they could be separated from water-soluble contaminants by washing the column with 1 M LiCl and eluted free of more

⁴ If a more permanent immobilization of the ligase is desired, it was found that covalent attachment could also be effected with recovery of enzymatic activity using either cyanogen bromide-activated or epoxy-activated Sepharose beads.

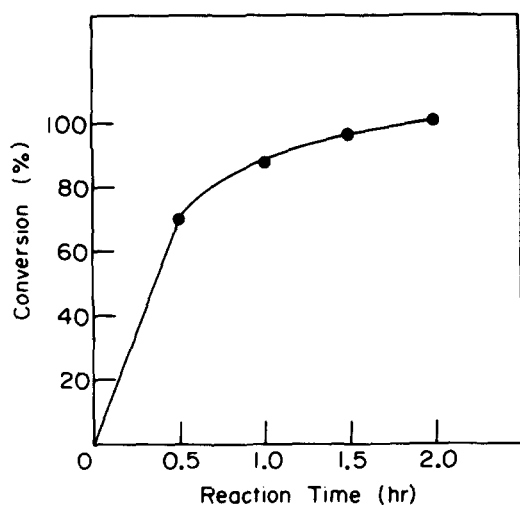


Fig. 2. Synthesis of [^3H]palmitoyl-CoA. All procedures were conducted as described under Experimental. A 50-ml reaction mixture was recycled through 5 ml of immobilized ligase at a flow rate of 2 ml/min. At the times shown, aliquots of the effluent were analyzed for percent conversion of [^3H]palmitate to its thioester.

hydrophobic contaminants i.e., fatty acids and Triton X-100, which are very tightly bound, by lowering the salt concentration (**Fig. 3B**). It was important to mix the reaction mixture with Octyl-Sepharose before pouring the column; otherwise, the upper portion of the column developed a high ratio of detergent to octyl groups, which was found to adversely effect the yield and purity.

The purity of the fatty acyl-CoA thioesters thus pre-

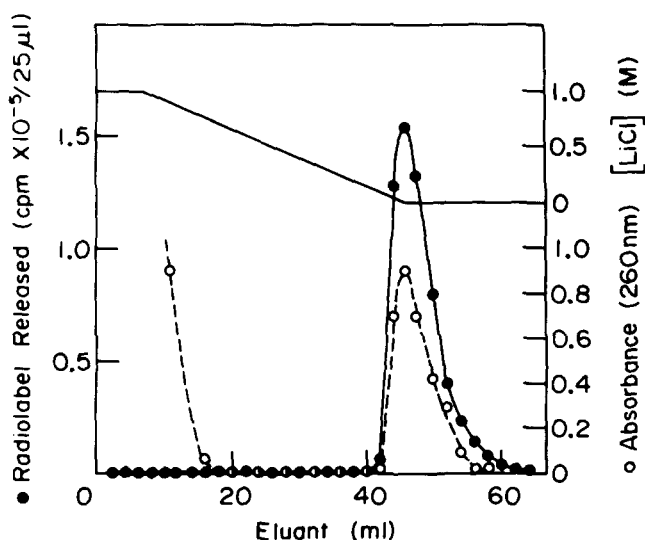


Fig. 3. Isolation of [^{14}C]oleoyl-CoA by chromatography on Octyl-Sepharose CL-4B. The product mixture (3 ml) from the synthesis of 2 μmol of [^{14}C]oleoyl-CoA (56.6 mCi/mmol) was chromatographed on 2 ml of Octyl-Sepharose CL-4B and eluted with a decreasing concentration of LiCl.

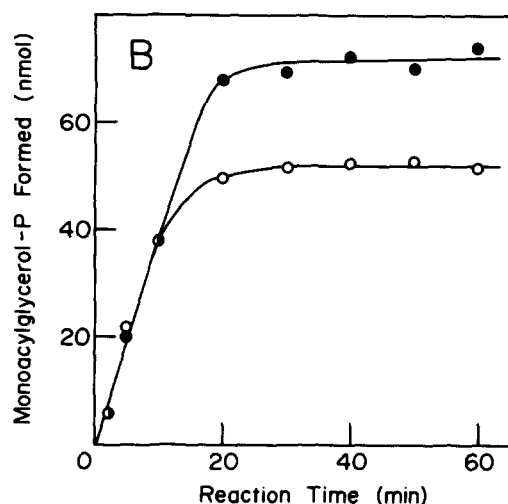
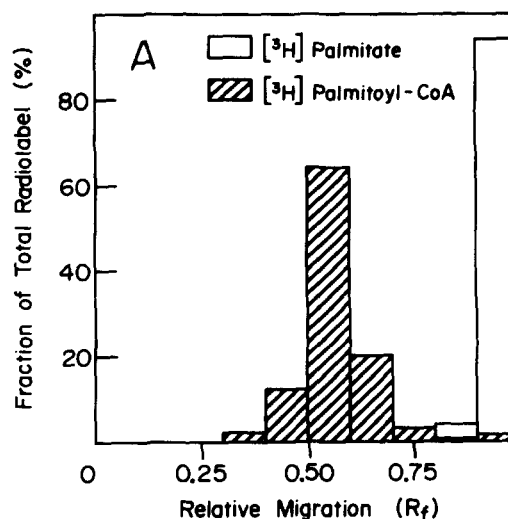


Fig. 4. Analyses of enzymatically synthesized palmitoyl-CoA. Analyses were conducted as described under Experimental. A, chromatography of [^3H]palmitic acid and [^3H]palmitoyl-CoA on Whatman No. 1 paper using 1-butanol-acetic acid-water 5:2:3 (v/v/v). B, amounts of monoacylglycerol-P formed by the purified glycerol-P acyltransferase in 1-ml reaction mixtures containing 54 (O) and 68 (\bullet) μM palmitoyl-CoA prepared by two separate enzymatic syntheses.

pared was consistently high. The ratios of absorbances at 280, 250, and 232 versus 260 nm were 0.24, 0.86, and 0.54 ± 0.01 , respectively, which were identical to those we obtained with commercially prepared samples from various suppliers. A single iodine-positive spot was observed on thin-layer and paper chromatograms with the mobilities expected for the thioesters (7). No free CoA was detected; however, a small amount (1.5%) of free fatty acid was found on paper chromatograms (**Fig. 4A**) and may have been formed during the analysis. When radiolabeled substrates were used, the concentrations based on absorbance measurements and scintillation counting agreed within experimental error.

The fatty acyl-CoA's were also fully competent for the formation of monoacylglycerol-P by the glycerol-P acyltransferase purified from *Escherichia coli*, as is illustrated in Fig. 4B for two different preparations of palmitoyl-CoA.

This procedure has been used for the preparation of myristoyl-, palmitoyl-, oleoyl-, *cis*-vacenoyl-, stearoyl-, linoleoyl-, and arachidonoyl-CoAs, in final yields of 24, 46, 46, 36, 32, 39, and 18%, respectively. These are merely representative of the acyl-CoA's that can be made, based on the broad substrate specificity reported for the microsomal enzyme (17). Additional compounds which have been prepared include two analogs, β -parinaroyl-CoA (11% yield) and palmitoyl-(1-N⁶-etheno-)CoA, (33% yield), which are composed of fluorescent fatty acyl and CoA moieties, respectively.⁵ These analogs should prove useful as probes of the interactions of acyl-CoA's with membranes and proteins, and for the biosynthesis of lipids containing fluorescent fatty acids.

Many fatty acyl-CoA's are not commercially available. A substantial number of these molecules can be prepared easily and economically using this enzymatic method. Unlike chemical syntheses that require activation of each fatty acid before coupling with CoA, one enzyme preparation can be used to directly prepare numerous acyl-CoA thioesters. Also, the mild reaction conditions and fidelity of formation of only the desired thioester among other reactive groups should allow the preparation of fatty acyl-CoA's that are not amenable to synthesis by other methods. Some fatty acids will not be substrates for the ligase and must be used in chemical syntheses, or perhaps with fatty acid:CoA ligases from other sources. Testing of a fatty acid that has not been previously demonstrated to be a substrate is relatively simple, however, especially if (1-N⁶-etheno-)CoA is employed so that the products can easily be visualized on paper chromatograms.

Use of the fatty acid:CoA ligase to prepare fatty acyl-CoA's has been reported previously (9-11); however, only one of these earlier preparations of the ligase described the removal of endogenous fatty acids (11). Binding the ligase to Matrex Gel Red A accomplished not only the removal of endogenous fatty acids and interfering enzymes in a single step (as compared to the prior use of acetone precipitation and chromatography on Sepharose 6B and hydroxylapatite) (11), but also provided a convenient material for the enzymatic synthesis. These advantages were complemented by the development of a simple method for recovering the fatty acyl-CoA's by chromatography on Octyl-Sepharose. This procedure should be generally applicable for

⁵ The fluorescent analog 16-(9-anthroyloxy) palmitic acid, however, was not converted into the corresponding CoA thioester.

the preparation of numerous fatty acyl-CoA's and related compounds. ■■

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