notes on methodology

An enzyme-coupled assay for acyl-CoA synthetase

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Summary An enzyme-coupled, colorimetric method for the assay of acyl-CoA synthetase is described. Acyl-CoA formed from fatty acid and CoA by acyl-CoA synthetase was dehydrogenated by acyl-CoA oxidase. Hydrogen peroxide produced was then converted into formaldehyde in the presence of methanol by catalase. The formaldehyde reacted with a triazole compound, 4-amino-3-hydrazino-5-mercapto-1,2,4-triazole, in an alkaline condition to form a purple dye, and the absorbance was measured spectrophotometrically.—**Ichihara, K., and Y. Shibasaki.** An enzyme-coupled assay for acyl-CoA synthetase. *J. Lipid Res.* 1991. **32:** 1709–1712.

Supplementary key words acyl-CoA oxidase • safflower seeds

Acyl-CoA synthetase or acid: CoA ligase (AMP-forming) (EC 6.2.1.3) catalyzes the condensation of long-chain fatty acid and CoA to produce acyl-CoA, which serves not only as a substrate for synthesis of cellular lipids but also as the substrate for degradation and elongation of fatty acids. In this respect, acyl-CoA synthetase plays a key role in lipid metabolism in the cell (1). In the course of an investigation of triacylglycerol biosynthesis in oil seeds, we found that it was impossible to determine true acyl-CoA synthetase activities in crude membrane preparations by measurement of acyl-CoA that accumulated at the end of incubation. This was the major reason why we sought a reliable assay procedure for acyl-CoA synthetase.

Acyl-CoA synthetase activity can be assayed by several methods (1–3), of which the measurement of incorporation of radiolabeled fatty acid into acyl-CoA is most widely used. However, crude subcellular preparations that have synthetase activity contain various enzymes that further metabolize acyl-CoA that is synthesized. The isotopic method cannot detect the synthetase activity derived from endogenous fatty acids in membrane preparations. Acyl-CoA also binds to certain proteins (4), and acyl-CoA synthetase activity would be underestimated as a result. The classical assay method involves the formation of acylhydroxamate from hydroxylamine and acyl-CoA (1). The acylhydroxamate reacts with ferric chloride to yield a purple ferric hydroxamic acid complex. However, the sensitivity of the hydroxamate method is not satisfactory and the high concentration of hydroxylamine needed for the assay has been shown to inhibit a purified acyl-CoA synthetase (1). Other methods involving either the measurement of disappearance of the CoA sulfhydryl group or the spectrophotometric measurement of the thioester formation are not specific for the acyl-CoA synthetase reaction, and many chemicals interfere with the measurement using these methods. The enzyme-coupled method reported here not only overcomes the above disadvantages but has some additional advantages.

MATERIALS AND METHODS

Acyl-CoA synthetase

Microsomes from maturing seeds of safflower (*Carthamus tinctorius*) were used as a crude preparation of acyl-CoA synthetase according to the procedure of Ichihara, Norikura, and Fujii (5). Acyl-CoA synthetase of *Pseudomonas sp.* was purchased from Toyobo, Osaka, Japan.

Assay procedure

Potassium oleate was prepared by neutralization of oleic acid in methanol with methanolic KOH and then by evaporation of the solvent under reduced pressure. The AHMT and KIO_4 solutions described below were freshly prepared prior to use or stored in a refrigerator and used within 1 day. The AHMT solution was filtered to remove insoluble materials.

The assay medium contained 0.15 M MOPS/NaOH (pH 7.6), 0.5 mM potassium oleate, 0.5 mM CoA, 4.5 mM ATP, 12 mM $MgCl_2$, 1 mM dithiothreitol, 0.55 mM Triton X-100, 1% methanol, 0.5 U acyl-CoA oxidase (from *Candida sp.*; 11.2 U/mg; Toyobo, Osaka, Japan), 1,000 U catalase (from bovine liver; 44,000 U/mg; Sigma Chemical, St. Louis, MO), and enzyme preparation in a total volume of 0.5 ml. After 15 min incubation at 30°C, the reaction was stopped by the addition of 0.5 ml 2 M KOH. To this solution was added 0.5 ml 0.6% AHMT in 0.5 M HCl. The mixture was incubated at 37°C for 10 min, and then 2 ml 0.75% KIO_4 was added. The total volume was 3.5 ml. The absorbance at 550 nm was measured at room temperature. The molar absorption coefficient, $\epsilon = 29{,}200\ M^{-1}\ cm^{-1}$ (6), of the resulting purple dye was used to calculate the amount of acyl-CoA synthesized. Control incubations were carried out in the absence of oleate or CoA; as another control, incubation was stopped at zero time. Blank values were obtained by incubation without microsomal protein. Values of absorbance were essentially the same between these control ex-

Abbreviations: AHMT, 4-amino-3-hydrazino-5-mercapto-1,2,4-triazole; DTNB, 5,5'-dithiobis(2-nitrobenzoic acid); MOPS, 3-(*N*-morpholino)propanesulfonic acid.

$$\text{Fatty acid} + \text{CoA} + \text{ATP} \xrightarrow{\text{Acyl-CoA synthetase}} \text{Acyl-CoA} + \text{AMP} + PP_i$$

$$\text{Acyl-CoA} + O_2 \xrightarrow{\text{Acyl-CoA oxidase}} \text{trans-2-Enoyl-CoA} + H_2O_2$$

$$H_2O_2 + CH_3OH \xrightarrow{\text{Catalase}} HCHO + 2H_2O$$

$$HCHO + \text{AHMT} \xrightarrow{IO_4^-,\ OH^-} \text{Triazolotetrazine dye}$$

Fig. 1. Reactions of the acyl-CoA synthetase assay.

periments and the blank, and hence control incubations were routinely carried out in the absence of microsomal protein for convenience. Data are reported as the means of triplicate assays.

Acyl-CoA synthetase activity was also determined by measurement of CoA disappearance (1). The reaction mixture was the same as that for the enzyme-coupled assay, except that dithiothreitol, acyl-CoA oxidase, catalase, and methanol were omitted. At the end of 15 min incubation, 2.5 ml of 0.5 mM DTNB in 0.1 M potassium phosphate buffer (pH 8.0) was added to the mixture. Unreacted CoA in the synthetase reaction was determined spectrophotometrically (λ = 412 nm; ϵ = 13,600 M^{-1} cm^{-1}) (7).

RESULTS

Since the isolation, in 1979, of microbial acyl-CoA oxidase from *Candida lipolytica* (8) and *C. tropicalis* (9), and of acyl-CoA synthetase from *Pseudomonas aeruginosa* (10), some acyl-CoA synthetase/acyl-CoA oxidase systems have been developed for the determination of free fatty acids, especially in serum (6, 11, 12). To assay acyl-CoA synthetase activity in crude preparations, we have used an enzymic colorimetric method in which the acyl-CoA synthetase reaction is coupled with the acyl-CoA oxidase and catalase reactions (**Fig. 1**). The acyl-CoA product of the microsomal preparation could immediately be converted into formaldehyde in the presence of excess acyl-CoA oxidase and catalase. After the enzyme reactions were stopped by the addition of KOH, a purple dye was formed from formaldehyde by the addition of AHMT and KIO_4 (6, 8, 13). The acyl-CoA synthetase activity was estimated from the absorbance of the triazolotetrazine dye at 550 nm.

Fig. 2 shows the effects of cofactors that were required for the oxidation of acyl-CoA and color development. Color development reached plateaus at 0.2 U of acyl-CoA oxidase, 200 U of catalase, and 0.2% methanol. Methanol did not denature acyl-CoA synthetase itself even at a high concentration of 4%. In most routine assays we used 0.5 U acyl-CoA oxidase, 1,000 U catalase, and 1% methanol. The acyl-CoA synthetase activity was nearly, but not completely, proportional to the concentration of microsomal protein up to 50 μg per 0.5 ml of the reaction mixture (**Fig. 3A**). The formation of acyl-CoA proceeded at a nearly linear rate with respect to incubation time, up to 15 min (Fig. 3B). Stoichiometry of the sequential reactions was confirmed with the *Pseudomonas* enzyme (**Fig. 4**). Production of the purple dye, which was shown by the increase in absorbance, was correlated with the loss of the

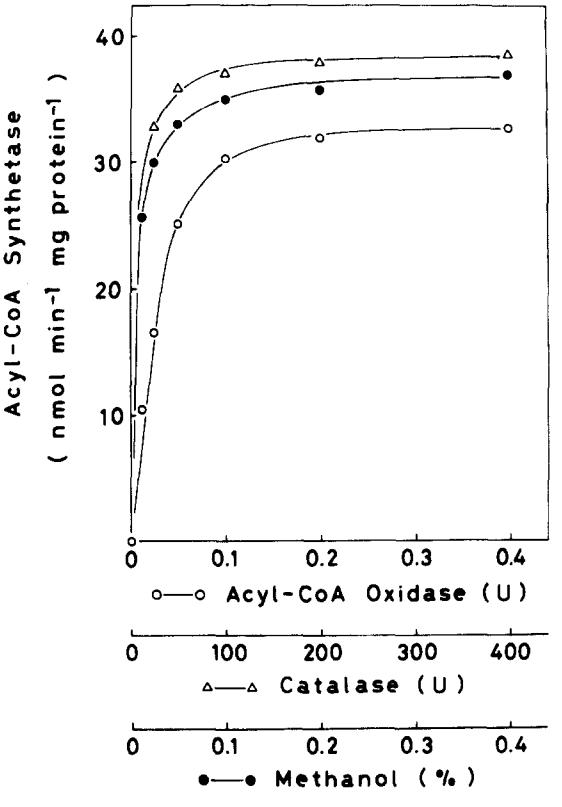

Fig. 2. Dependence of color development on the concentrations of acyl-CoA oxidase (O), catalase (△), and methanol (●).

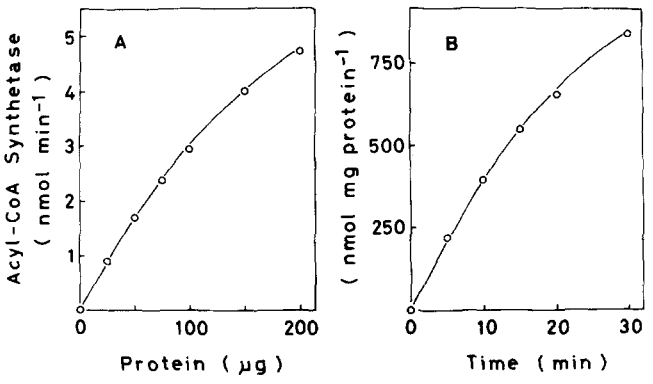

Fig. 3. Dependence of acyl-CoA synthetase activity on the concentration of safflower microsomal protein (A) and the incubation time (B).

fatty acid substrate. This indicates that the sequential coupling reactions proceeded stoichiometrically.

The synthetase activities determined by the present method were compared with those by an established endpoint assay, which involved the color development between unreacted CoA and DTNB (**Table 1**). With safflower microsomes, no significant difference in activity was found between the two different methods. With the *Pseudomonas* enzyme, the DTNB method gave a higher value than did the enzyme-coupled method. The cause of this slight difference is not known; however, the data presented here, together with the other findings, demonstrate the validity and reliability of the new method.

TABLE 1. Comparison between the enzyme-coupled method and the DTNB method

Enzyme Source	Acyl-CoA Synthetase Activity	
	Enzyme-Coupled Method	DTNB Method
	nmol min⁻¹ mg protein⁻¹	
Safflower microsomes	49.9	52.1
Pseudomonas sp.	156.6	173.0

Each value is the mean of triplicate assays.

When AHMT, methanol, and catalase were replaced by 4-aminoantipyrine (1 mM), phenol (10 mM), and peroxidase (10 U), respectively (8, 11), no synthetase activity was detected. Phenol might inhibit the synthetase reaction. In preliminary experiments, we tried other assay procedures that involved the enzymic colorimetric determination of AMP (10) or PPi. These reaction products of acyl-CoA synthesis, however, could not be accurately determined because of high backgrounds from the crude enzyme preparations.

In a separate experiment, acyl-CoA oxidase, catalase, and methanol were omitted from the incubation mixture. After 15 min incubation at 30°C, the acyl-CoA synthetase reaction was stopped by 0.02 ml 6 M HCl. To this solution were added 0.25 ml 2 M Tris/HCl (pH 7.5), acyl-CoA oxidase, catalase, and methanol. The mixture was incubated at 37°C for 10 min for the convesion of acyl-CoA to enoyl-CoA and formaldehyde. After termination of the sequential reactions with KOH, AHMT and KIO_4 were added for color development. However, the acyl-CoA synthetase activities measured by this endpoint assay were much lower (<14%) than those obtained by the enzyme-coupled procedure. This observation suggests that acyl-CoA synthesized by microsomes did not effectively serve as the substrate for exogenous acyl-CoA oxidase because the acyl-CoA was not immediately used by the oxidase.

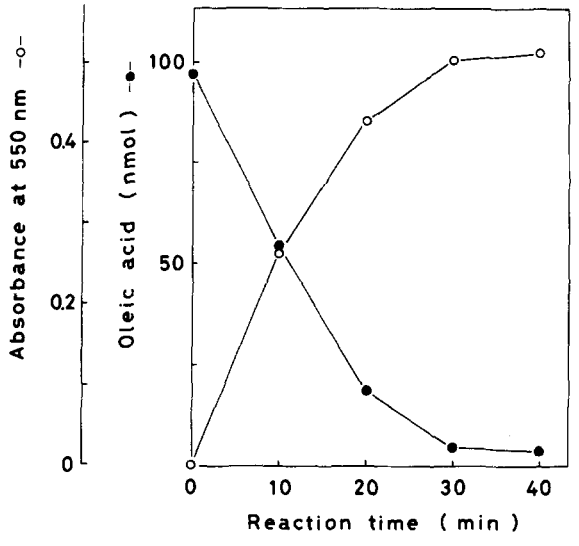

Fig. 4. Stoichiometry of the dye formation and the loss of oleic acid. The reaction mixture was the same as described in Materials and Methods, except that acyl-CoA synthetase (10 μg) was from *Pseudomonas sp.* and the concentration of potassium oleate was 0.2 mM. The loss of the fatty acid substrate was monitored by gas-liquid chromatography. The reaction was stopped by the addition of 1 ml 1 M HCl and 2 ml methanol containing 5 μg heptadecanoic acid as internal standard. Free fatty acids were then extracted with 2.5 ml chloroform. The chloroform layer was washed three times with water. The fatty acids obtained were converted into their methyl esters with 5% boron trifluoride in methanol, and analyzed by gas-liquid chromatography (15% DEGS). Only methyl oleate and heptadecanoate were detected.

DISCUSSION

Safflower acyl-CoA synthetase was successfully assayed by two independent methods, one of which was a DTNB method (Table 1). A similar DTNB method could also be used for the determination of the rat liver enzyme and gave similar values to those obtained by a radioisotopic method (14). However, the addition of sulfhydryl reagents, such as 2-mercaptoethanol and dithiothreitol, to homogenizing buffers, eluting solutions, or assay media is often essential for protection of the sulfhydryl groups of enzyme

proteins during extraction, purification, and assay. These reagents react with DTNB and hence give high backgrounds. The enzyme-coupled method can be used for both crude and purified preparations of acyl-CoA synthetase. The presence of sulfhydryl reagents does not interfere with assay. Five nanomoles of acyl-CoA gives an absorbance of approximately 0.04 and is easily detectable. The sensitivity can be improved by replacement of 2 ml 0.75% KIO_4 for color development by 1 ml 1.5% KIO_4 (the final volume is reduced from 3.5 ml to 2.5 ml). The amount of acyl-CoA synthetase to be determined could further be reduced if 1-ml cuvettes were used for the measurement of absorbance. Data obtained are reproducible and reliable; at an absorbance of 0.4, the standard deviation was less than 0.01. This procedure requires no radioactive substrates, and acyl-CoA oxidase, catalase, and other reagents are commercially available. The procedure is simple, and the assay is complete within 1 h, including the acyl-CoA synthetase reaction itself and the measurement of absorbance.

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