SEPARATION OF PIGEON LIVER APO- AND HOLO-FATTY ACID SYNTHETASES BY AFFINITY CHROMATOGRAPHY*,[1]

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Received April 7,1975

SUMMARY

Apo- and holo-fatty acid synthetases of pigeon liver were separated by affinity gel chromatography under conditions similar to, but not identical to, those used in separating subunits I and II of [^{14}C]pantetheine-labeled fatty acid synthetase complex [Lornitzo et al., J. Biol. Chem. 249, 1654 (1974)]. When [^{14}C]pantetheine-labeled fatty acid synthetases were separated, the enzymatically active holo form contained all of the [^{14}C] label. Incubation of the apo-pigeon liver fatty acid synthetase complex with CoA, ATP and a partially purified pigeon liver soluble enzyme system, from which fatty acid synthetase had been removed, resulted in the formation of holo-enzyme. Activation of apo-fatty acid synthetase could also be achieved by replacing the apo-(4′-phosphopantetheine-less) acyl carrier protein with holo-acyl carrier protein. It is evident, therefore, that the inactive apo-fatty acid synthetase lacks a 4′-phosphopantetheine group.

Since the discovery that E. coli holo-acyl carrier protein formation is mediated by an enzyme transferring 4′-phosphopantetheine from CoA to apo-acyl carrier protein (2), evidence for a similar or analogous process involving the 4′-phosphopantetheine (3) of fatty acid synthetase complexes has been accumulating. Tweto et al. (4) showed that [^{14}C]pantothenate turns over more rapidly in rat liver fatty acid synthetase than does [^{3}H]leucine. Furthermore, Yu and Burton (5) demonstrated that fatty acid synthetase protein synthesis commences rapidly on refeeding fasted rats, but incorporation of

*This investigation was supported in part by a grant, AM-01383, from the National Institute of Arthritis and Metabolic Diseases of the National Institutes of Health, United States Public Health Service.

[1]A preliminary report (1) of this work was presented at the 59th Annual Meeting of the American Society of Biological Chemists, Atlantic City, N.J., April 13-18, 1975. This is paper No. 4 in a series. Paper No. 3 is Ref. 13.

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[^{14}C]pantothenate does not start until 3 to 4 hours have elapsed, thus indicating the presence of inactive precursors of fatty acid synthetase. Also, Schweizer et al. (6, 7) isolated mutants of yeast that synthesize an enzymatically inactive fatty acid synthetase lacking 4'-phosphopantetheine. These results provide indirect evidence for inactive and active forms of fatty acid synthetase which bear an apo-holo relationship to one another by way of 4'-phosphopantetheine.

Recently Yu and Burton (8) demonstrated the formation of active fatty acid synthetase from an inactive precursor in the presence of CoA, ATP and a rat liver supernatant system from which fatty acid synthetase had been removed by immunoprecipitation. However, evidence for this conversion was indirect since methodology was not available for the separation of active and inactive enzyme. Hence, it became of interest to devise a method of separating the two complexes from one another in order to determine fully the relationship of inactive to active fatty acid synthetase and to estimate the quantities of these proteins in livers under different nutritional and hormonal conditions.

The inactive and active pigeon liver fatty acid synthetases copurify by the standard method of purification (9). Hence, the presence of an inactive form is evidenced only indirectly as a variation in specific activity of the overall reaction of fatty acid synthesis, which in turn is dependent on the nutritional or hormonal state of the animal (10). A method was needed, therefore, which would separate enzymatically active and inactive proteins following purification by the standard procedure. Such a method is described in this paper. In this method inactive and active fatty acid synthetases are separated from one another by means of affinity chromatography on Sepharose ε-amino caproyl pantetheine. We also report in this paper experiments on the conversion of apo-fatty acid synthetase to active or holo-enzyme.

EXPERIMENTAL

A sample of [^{14}C]pantetheine-labeled pigeon liver fatty acid synthetase was obtained from pigeons fasted 48 hours and then refed a fat-free diet for 44 hours. The pigeons were injected intravenously with [^{14}C]pantothenic acid (New England Nuclear) and then sacrificed 4 hours after injection. Liver fatty acid synthetase was purified according to the method of Hsu, Wasson

and Porter (9), except that after DEAE-cellulose chromatography a 25 to 40%, rather than a 25 to 35 %, ammonium sulfate fraction was obtained.

Sepharose ε-amino caproyl pantetheine, for affinity chromatography, was prepared as previously described (11). The substrates for the overall and partial reactions of fatty acid synthesis were obtained or prepared and the assays were carried out as previously described (12, 13). Protein was estimated by the methods of Lowry (14) and Murphy and Kies (15). Rabbit antiserum to pigeon liver fatty acid synthetase was prepared according to the method of Collins et al. (16). Ouchterlony micro-double diffusion, with rabbit antiserum and preparations of enzymatically inactive and active pigeon liver fatty acid synthetase, was carried out in 0.5% agarose.

An enzyme system containing 4′-phosphopantetheine transferase activity, which carries out the transfer of 4′-phosphopantetheine from CoA to apo-acyl carrier protein in the fatty acid synthetase complex, was prepared from the liver of a pigeon refed 12 hours following a 48-hour fast. The 100,000 g supernatant from the liver homogenate was frozen at −20° for three days, thawed at 25°, and then freed of fatty acid synthetase by passing through a 21 cm × 8.5 mm column containing 6.5 g (wet wt.) of Sepharose ε-amino caproyl pantetheine at 0° at a rate of 1 ml/20 minutes. One-ml fractions were collected. Most of the protein was eluted at the void volume with 60 mM potassium phosphate buffer, pH 7, containing 5 mM dithiothreitol. The first four fractions collected had no fatty acid synthetase activity. These were combined and the protein was precipitated between 20 and 50% saturation with ammonium sulfate. The precipitated protein was dissolved and then dialyzed 3 hours against a buffer containing 200 mM potassium phosphate, pH 7.0, 0.1 μM CoA, 1.0 mM magnesium and 5 mM dithiothreitol. This enzyme system was used to effect the conversion of apo- to holo-fatty acid synthetase.

RESULTS

DEAE-cellulose-purified [^{14}C]pantetheine-labeled pigeon liver fatty acid synthetase, 32 mg in 3.5 ml of a 0.2 M potassium phosphate, pH 7.0, 1 mM EDTA, 10 mM dithiothreitol buffer was stored frozen ten days at −20°, thawed at 25°, and then left to stand for 1 hour at room temperature to completely reassociate any dissociated enzyme. The reassociated fatty acid synthetase was adsorbed on Sepharose ε-amino caproyl pantetheine (6.5 g gel, wet wt. in a 21 cm × 8.5 mm column) at 0° at a rate of 1 ml/15-20 min-

utes. Thirty ml of 60 mM phosphate buffer, pH 7.0, containing 1 mM dithiothreitol were passed through the gel at the rate of 1 ml/15 to 20 minutes and 1 ml fractions were collected. The column was then warmed to 25° and the elution was completed with 50 ml of a 100 mM potassium phosphate, 100 mM Tris buffer, pH 8.5, containing 1 mM dithiothreitol at a rate of 1 ml/5 minutes. An aliquot of each fraction, 200 µl, was assayed for radioactivity. Assays were also carried out for the overall and partial reactions of fatty acid synthesis, Fig. 1A and B. The elution patterns for enzymatically inactive fatty acid synthetase (fractions 10 to 30) are shown in Fig. 1A and B. [^{14}C]-Pantetheine is associated with only the active or holo-fatty acid synthetase. This fraction shows overall fatty acid synthesis and β-ketoacyl synthetase activity. The other partial reactions - β-ketoacyl thioester reductase, β-hydroxyacyl thioester dehydrase, α,β-enoylacyl thioester reductase, acetyl- and malonyl-CoA transacylase and palmityl-CoA deacylase activities - are associated with both the active and inactive enzyme proteins. The inactive enzyme may be converted to active fatty acid synthetase by incubation with CoA, ATP and a 4′-phosphopantetheine transferase enzyme fraction, Table I. The inactive and active enzymes both react with rabbit antiserum and each gives a single immunoprecipitin band, Fig. 2.

DISCUSSION

The first complete separation of inactive and active pigeon liver fatty acid synthetases is demonstrated in this paper. The results reported in this paper also provide direct evidence of an apo-holo enzyme relationship of fatty acid synthetases with 4′-phosphopantetheine as the transferable prosthetic group. Such a relationship was previously implied, but not directly proven, by the results of Craig et al. (17), Tweto and Larrabee (18) and Yu and Burton (8) in studies with rat liver fatty acid synthetase. The cross reactivity between pigeon liver apo- and holo-fatty acid synthetases was anticipated from the immunological cross reactions described by Yu and Burton (5,8) and by Volpe et al. (19). The data of these workers had suggested the presence of rat liver apo- and holo-fatty acid synthetases and their cross reactivity.

The apo- and holo-fatty acid synthetases are relatively unstable on elution from the affinity column. This may be due to the low ionic strength buffer used to elute the apo-enzyme, and the pH (8.5) used to elute the holo-enzyme. We found it best, therefore, to combine the fractions corresponding

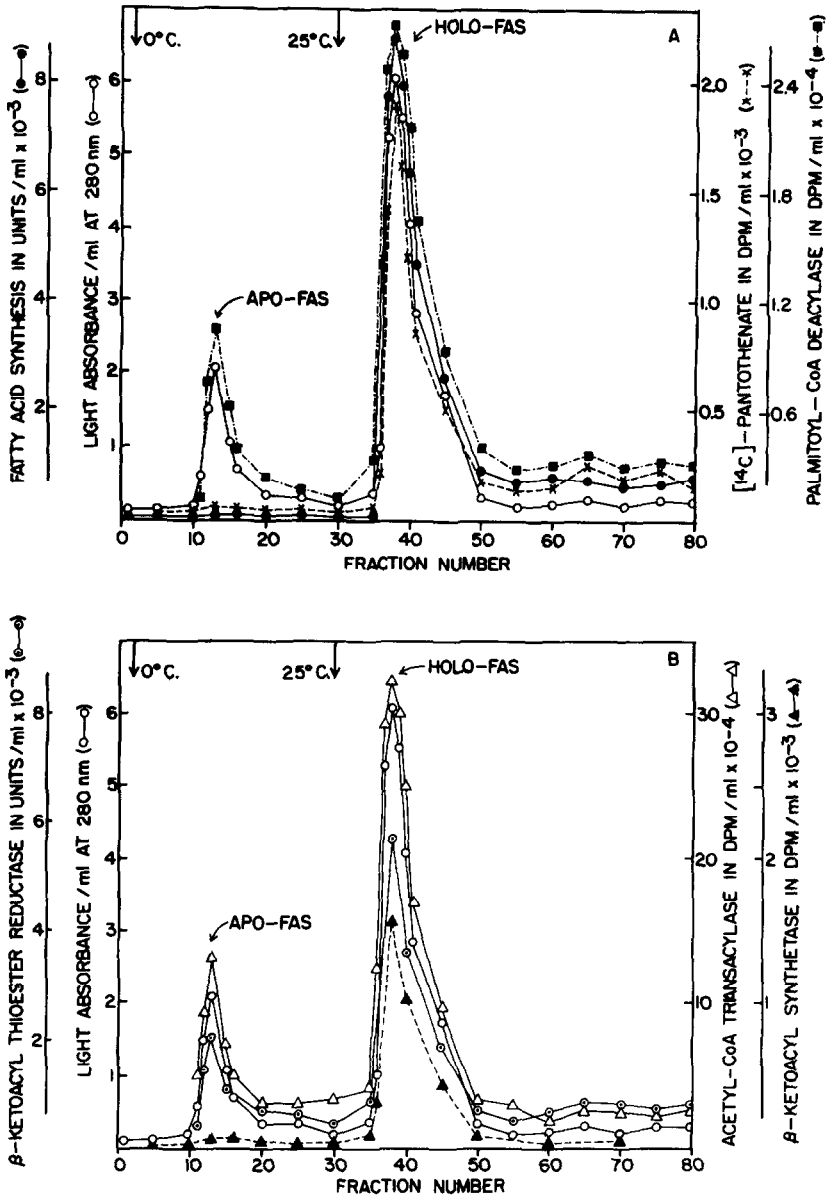

Fig. 1. Separation of apo- and holo-pigeon liver fatty acid synthetases on an affinity column. The experimental conditions used in the preparation and separation of the fatty acid synthetases are given in the Experimental and Results sections. A. o——o, Light absorption at 280 nm; ●——●, fatty acid synthetase activity, nmoles NADPH oxidized/min/ml; x---x, radioactivity of [^{14}C]pantothenate incorporated into enzyme protein, dpm/ml; ■-·-■, palmityl-CoA deacylase activity, dpm [^{14}C]palmitate/min/ml. B. o——o, Light absorption at 280 nm; ⊙——⊙, β-ketoacyl thioester reductase, nmoles NADPH oxidized/min/ml; Δ——Δ, acetyl-CoA transacylase, dpm of [^{14}C]acetyl groups transferred to pantetheine/min/ml; ▲---▲, β-ketoacyl synthetase, dpm of [^{14}C] of potassium bicarbonate incorporated into malonyl-CoA/min/ml.

TABLE I

CONVERSION OF APO- TO HOLO-FATTY ACID SYNTHETASE

Conditions	2-[^{14}C]Malonyl-CoA converted to palmitate
	dpm/ml
Complete system	11,515
Complete - CoA	2,292
Complete - ATP	1,544
Complete - ATP and CoA	387
Complete - Apo-FAS	391
Complete - Transferase	358

The complete system contained apo-fatty acid synthetase, 1.0 mg protein; a transferase preparation, 4 mg protein; ATP, 1.5 μmoles; $MgCl_2$, 1.0 μmole; CoA, 3 nmoles; potassium phosphate, pH 7, 0.2 mmole; in a total volume of 1 ml. Controls in which one or more of the above components were omitted, were also incubated. Incubations were carried out for 30 minutes at 32° and then the mixture was dialyzed for 3 hours at room temperature against 0.2 M potassium phosphate buffer, pH 7, containing 1 mM EDTA and 10 mM dithiothreitol. EDTA was used to stop the reaction by chelation with Mg^{++}. Dialysis also removed CoA, which is an inhibitor of fatty acid synthetase activity. Two changes of buffer were made during the dialysis period.

The fatty acid synthetase activity generated was assayed by the standard radiochemical method. Assay mixtures contained [2-^{14}C]malonyl-CoA, 40 nmoles (42,000 dpm); acetyl-CoA, 10 nmoles; NADPH, 100 nmoles; potassium phosphate, pH 7, 0.2 mmole; EDTA, 1 μmole; protein, 100 to 150 μg; in a total volume of 1 ml. An aliquot of the dialyzed incubation mixture, above paragraph, 100 μl, was added to start the reaction for fatty acid synthesis. Incubations were carried out at 30° for 5 minutes and then stopped by the addition of 30 μl of 60% $HClO_4$. One ml of ethanol was added to each incubation mixture and the [^{14}C]-labeled fatty acid products were extracted three times with 2 ml of petroleum ether. The extracts were combined and dried over sodium sulfate. Aliquots were assayed for radioactivity in a liquid scintillation spectrometer.

to each protein and to dialyze each against 0.2 M potassium phosphate, pH 7.0, 1 mM EDTA, and 10 mM dithiothreitol buffer, for 3 to 5 hours at room temperature as soon as possible after elution. The properties of the inactive and active enzymes treated in this way were found to be identical on DEAE-

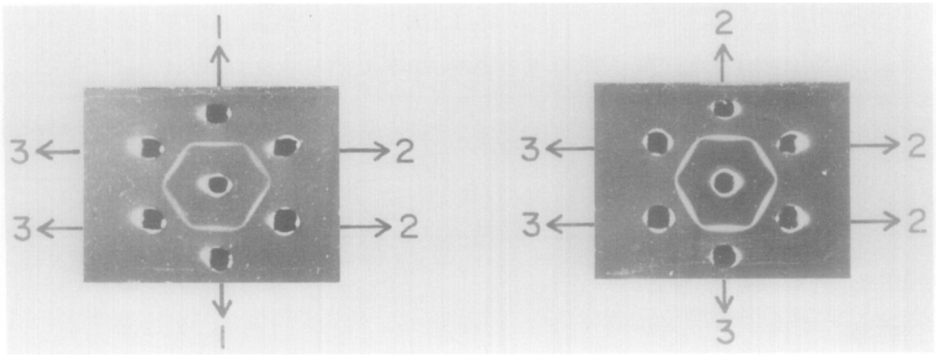

Fig. 2. Immunodiffusion of apo- and holo-fatty acid synthetases. The center wells contained fatty acid synthetase antiserum. Wells marked 1 contained fatty acid synthetase purified by the standard method (9) and then subjected to Biogel filtration. Wells marked 2 contained apo- (inactive) pigeon liver fatty acid synthetase prepared as described in the text and passed over a Biogel A-1.5 m column. Wells marked 3 contained holo- (active) pigeon liver fatty acid synthetase prepared as described in the text and passed over a Biogel A-1.5 m column.

cellulose chromatography, sucrose density gradient centrifugation, Biogel A-1.5 m filtration, and disc gel electrophoresis.

The experiment shown in Table I demonstrates that the factors needed to convert inactive to active fatty acid synthetase are CoA, ATP and an enzyme system present in the soluble portion of liver cells. In this system fatty acid synthetase is activated by the transfer of 4′-phosphopantetheine from CoA to apo-fatty acid synthetase to form holo-fatty acid synthetase.

We have also been able to demonstrate the involvement of acyl carrier protein in the conversion of inactive to active fatty acid synthetase. By using the methods previously described by us (20), we isolated a low molecular weight protein from the inactive fatty acid synthetase complex. This protein showed the physical properties of pigeon liver acyl carrier protein isolated from active fatty acid synthetase. However, this protein lacked 4′-phosphopantetheine and the ability to accept acetyl and malonyl groups from acetyl- and malonyl-CoA. When the apo-acyl carrier protein was removed from inactive fatty acid synthetase, following dissociation of the complex, and then replaced with acyl carrier protein from active fatty acid synthetase, by reassociation in the presence of acyl carrier protein-less fatty acid synthetase, a 30% recovery of fatty acid synthetase activity was obtained.

Our data suggest the possibility of an apo-holo enzyme system of control of fatty acid synthetase activity. If this is true, it should be possible to identify the nutritional or hormonal factors that control this system. It should also be possible to establish quantitative relationships between control factors and active and inactive enzyme through the separation of apo- and holo-enzymes by affinity chromatography. Studies carried out thus far have shown that the apo-enzyme is present in pigeon liver in largest amounts after a 48-hour fast. Our studies have also shown that the apo-enzyme is an intermediate in the synthesis of holo-enzyme in livers of pigeons refed a fat-free diet following 24 to 48 hours of fasting.

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