SEPARATION OF TWO ACTIVE FORMS (HOLO-a AND HOLO-b) OF PIGEON LIVER FATTY ACID SYNTHETASE AND THEIR INTERCONVERSION BY PHOSPHORYLATION AND DEPHOSPHORYLATION[1,2]

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Received July 1,1975

SUMMARY

Apo-, holo-a- and holo-b-pigeon liver fatty acid synthetases[4] were separated by affinity gel chromatography under conditions identical to those used for the separation of apo and holo forms [Qureshi et al. (1975) Biochem. Biophys. Res. Commun. 64, 836-844], except that an additional elution step at a high salt concentration, pH 7.0, and room temperature was included. The interconversion of the two forms of holo-fatty acid synthetase was then carried out in vitro. In the presence of Mg^{++} and a phosphatase preparation, holo-b was converted to holo-a. The reverse conversion, holo-a to holo-b, was carried out in the presence of ATP and a kinase fraction prepared from the 100,000 g supernatant of liver homogenate. These results indicate that the interconversion of holo-a- and holo-b-fatty acid synthetases occurs by phosphorylation-dephosphorylation. These results also suggest the possibility that this process may be a mechanism of short term regulation of liver fatty acid synthetase activity.

A number of previous reports from this laboratory indicated that the variation in specific activity of avian and mammalian liver fatty acid synthetases observed with varying nutritional or hormonal states might be due to the pre-

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[1]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.

[2] This is paper No. 5 in a series. Paper No. 4 is reference No. 6.

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[4]Since the completion of this report, apo-, holo-a- and holo-b-fatty acid synthetases have been isolated from rat liver fatty acid synthetase under conditions identical to those used for the pigeon liver enzyme except that the pooled eluate fractions were dialyzed immediately after elution from the column against 0.5 M phosphate, 1 mM EDTA, 10 mM dithiothreitol, pH 7.0.

sence of a co-purifying inactive protein (1, 2, 3). One such protein was separated from rat liver fatty acid synthetase by sucrose density gradient centrifugation (4). This protein was identified as a 7S component that was unrelated to the fatty acid synthetase complex.

More recently evidence has been presented (5) for the existence of an apo form of fatty acid synthetase. Confirmation of this suggestion has been achieved through the separation of pigeon liver apo- and holo-fatty acid synthetases by affinity gel chromatography (6). However, after removing the apo-fatty acid synthetase from the holo form, the specific activities for the over-all and the partial reactions in the remaining holo-fatty acid synthetase still showed a dependence on the nutritional state of the pigeon. Moreover, there also appeared to be a difference in specific activities of holo-fatty acid synthetase fractions when comparisons were made of the peak tube of protein from the affinity chromatographic column with the last tube from the peak, thereby suggesting the presence of more than one form of the holo-fatty acid synthetase.

In the present paper we describe the separation by affinity chromatography on Sepharose ε-amino caproyl pantetheine of the holo-fatty acid synthetase fraction into a high specific activity form (holo-a) and a low specific activity form (holo-b). The procedure and precautions for preparing and loading the column are the same as reported earlier (6, 7, 8) for the separation of the non-identical half-molecular weight subunits of the fatty acid synthetase complex and for the separation of apo- and holo-fatty acid synthetases. However, additional modifications for the elution of the two holo forms of fatty acid synthetase from the column are included in the procedure. Also, we report in this paper the in vitro interconversion of the two forms of holo-fatty acid synthetases by phosphorylation-dephosphorylation.

EXPERIMENTAL PROCEDURE

Pigeons were fasted for 48 hours and then refed for 6 hours. Four hours prior to sacrifice the birds were injected with ^{32}P-labeled phosphate. Liver fatty acid synthetase was then purified according to the method of Hsu, Wasson and Porter (9). Sepharose ε-amino caproyl pantetheine, used for subsequent affinity chromatography, was prepared as described previously (7). The substrates for the over-all and partial reactions of fatty acid synthesis were

obtained or prepared and the assays were carried out as reported earlier (8, 10). Protein was estimated by the methods of Lowry (11) and Murphy and Kies (12).

The ATP-dependent kinase was prepared from a pigeon fasted, refed for 48 hours and then fasted for 12 hours. The 100,000 g supernatant solution obtained from the liver homogenate was prepared according to the method of Hsu et al. (9). An ammonium sulphate precipitate of the protein of this solution (55 to 75% of saturation) was dissolved in 1 ml of 0.2 M potassium phosphate, 1 mM EDTA, 1 mM dithiothreitol, and then dialyzed 3 hours, with 2 changes, in the same buffer. This enzyme preparation was used immediately for the conversion of holo-a- to holo-b-fatty acid synthetase.

Magnesium-dependent phosphatase was prepared from the liver of a pigeon fasted for 48 hours and then refed for 12 hours. The 100,000 g supernatant solution was prepared as above. A 0 to 40% ammonium sulphate precipitated fraction was prepared and the precipitate was dissolved in the above buffer (1 ml), dialyzed against the same buffer, and then passed over a column of Sepharose ε-amino caproyl pantetheine (2 g) to remove the fatty acid synthetase as reported previously (6). This preparation was used for the conversion of holo-b- to holo-a-fatty acid synthetase.

RESULTS

DEAE-cellulose purified pigeon liver fatty acid synthetase (40 mg) obtained from 48-hour fasted, 6-hour refed birds was dissolved in 3.5 ml of a 0.2 M potassium phosphate, pH 7.0, 1 mM EDTA, 10 mM dithiothreitol buffer. The sample was frozen for 2 to 5 days at −20°, thawed at 25°, and then left to stand 1 hour at room temperature to completely reassociate any dissociated enzyme. The completely reassociated fatty acid synthetase was adsorbed on freshly prepared Sepharose ε-amino caproyl pantetheine gel[5] (6.5

[5] Sepharose ε-amino caproic acid can be stored at −20°C for an indefinite period of time. However, the affinity gel, Sepharose ε-amino caproyl pantetheine, is unstable under many conditions. Hence it is prepared fresh by coupling pantetheine with Sepharose ε-amino caproic acid over a period of 18-20 hours in the presence of 1-ethyl-3 (3-dimethyl aminopropyl)-carbodiimide HCl. The excess of the latter compound and pantetheine is removed by washing with ice cold water in a Buchner funnel under vacuum. The washed gel is quickly equilibrated with ice cold 60 mM potassium phosphate buffer, pH 7.0, in the same Buchner funnel. One gram of the affinity gel is then used to bind 5-10 mg of fatty acid synthetase protein. Because of the lability of the gel at the higher pH's (8.5) used to elute fatty acid synthetase the gel cannot be regenerated for reuse.

g wet weight in a 21 cm × 8.4 mm column) at a rate of 1 ml/15 to 20 minutes at 0°. Thirty ml of 60 mM potassium phosphate buffer, pH 7.0, containing 2 mM dithiothreitol was used to elute the apo-enzyme at a rate of 1 ml/15 minutes at 0°, Fig. 1. The buffer was then changed to 0.2 M potassium phosphate containing 2mM dithiothreitol, pH 7.0, and the elution of holo-a-fatty acid synthetase was carried out at a rate of 1 ml/4 minutes at 25°. After collecting 35 ml, the buffer was changed again to 100 mM Tris, 100 mM potassium phosphate, 2 mM dithiothreitol, pH 8.5, to elute holo-b-fatty acid synthetase. One ml eluate fractions were collected from this column. To determine the amount and specific activity of each modification of the enzyme, fractions 12 to 25 (apo), 35 to 60 (holo-a) and 70 to 90 (holo-b) were separately combined and dialyzed for 3 hours, with 2 changes of buffer, in 0.2 M potassium phosphate, 1 mM EDTA, 10 mM dithiothreitol, pH 7.0, at room temperature. Specific activities for fatty acid synthesis of each tube in fractions 35 to 60 and 70 to 90 were nearly constant. These activities were 68 to 71 and 7.4 to 8.5 nmoles palmitate formed/min/mg fatty acid synthetase protein, respectively.

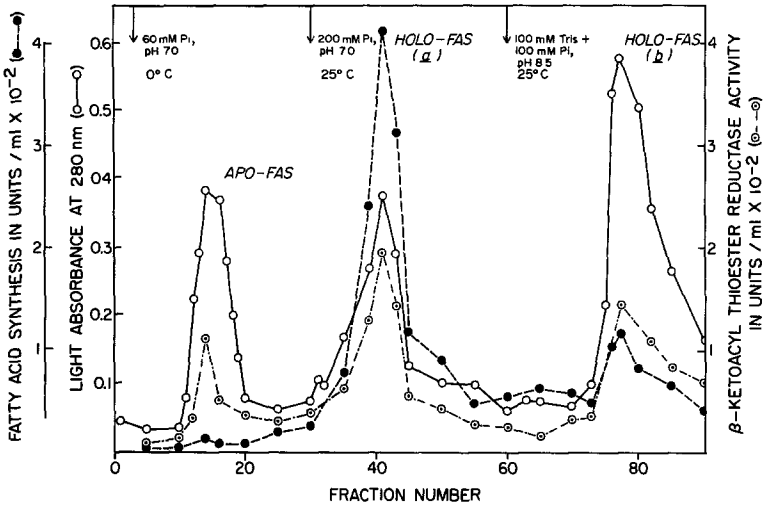

Fig. 1. Separation of apo-, high activity holo- (holo-a) and low activity holo- (holo-b) pigeon liver fatty acid synthetases. The conditions of this separation are described in the Experimental section. o——o, light absorption at 280 nm; • --- •, fatty acid synthetase activity in nmoles of NADPH oxidized/min/ml; ⊙ -·- ⊙, β-ketoacyl thioester reductase activity in nmoles of NADPH oxidized/min/ml.

The DEAE-cellulose, apo-, holo-a- and holo-b-fatty acid synthetases were shown to be homogenous by molecular filtration on Biogel A-1.5 m. Each protein had the same elution volume, thereby showing that each had the same molecular weight. Disc gel electrophoresis was carried out with samples of each fatty acid synthetase preparation. The bands for each of the complexes migrated identically, thereby indicating a similar charge behavior for each. Similar results were obtained with the dissociated enzymes. The 3 protein complexes also behaved identically on immunodiffusion.

The conversion of ^{32}P-labeled holo-b-fatty acid synthetase to the a form is reported in Table I. The amounts of holo-a- and holo-b-fatty acid synthetases were determined by affinity chromatography after incubation of the pure holo-b form with Mg^{++} ions and the phosphatase fraction. The control sample was incubated in the absence of the phosphatase fraction. The incubation of the holo-b form in the complete system resulted in a 20-fold increase in enzyme activity, and an accompanying loss of ^{32}P from the enzyme protein.

TABLE I

CONVERSION OF HOLO-b- TO HOLO-a-
PIGEON LIVER FATTY ACID SYNTHETASE

Enzyme substrate or product	Complete system −phosphatase fraction			Complete system		
	Protein in mg	Units of activity	Total cpm ^{32}P	Protein in mg	Units of activity	Total cpm ^{32}P
Holo-b-FAS	2	88.8	3300	0.62	51.2	1112
Holo-a-FAS	0	0	0	1.12	1052	370

The complete system consisted of holo-b-fatty acid synthetase (3300 cpm of ^{32}P and 2 mg of protein); $MgCl_2$, 15 mM; a phosphatase fraction containing the protein of a 0-40% $(NH_4)_2SO_4$ precipitate of supernatant solution prepared from liver of a 48-hour fasted, 12-hour refed bird and freed of fatty acid synthetase by affinity chromatography, 4 mg of protein; and potassium phosphate buffer, pH 7.0, 0.2 M. The final volume was 1 ml and the sample was incubated at 32° for 20 minutes. The sample was frozen overnight and the proteins were then separated by affinity chromatography on 1 g of gel as described under Experimental Procedure.

The conversion of the holo-a-fatty acid synthetase to the holo-b form in the presence of [γ-^{32}P]ATP and a kinase fraction is reported in Table II. The amounts of holo-a- and holo-b-fatty acid synthetases were determined by affinity chromatography after incubation of the holo-a form with the complete system or with the kinase omitted. A 15-fold decrease in fatty acid synthetase activity was obtained with the complete system. This decrease in activity was accompanied by the incorporation of radioactivity from [γ-^{32}P]ATP into protein.

DISCUSSION

A number of enzymes that occupy key positions in metabolic pathways are regulated by enzyme-catalyzed phosphorylation and dephosphorylation reactions. A comprehensive review of such enzymes was presented in a recent symposia (13). This mode of regulation is mediated by accessory enzymes acting on the regulated enzyme. Examples of these modifying enzymes are an ATP-dependent protein kinase and a Mg^{++}-dependent phosphatase. Re-

TABLE II

CONVERSION OF HOLO-a- TO HOLO-b-
PIGEON LIVER FATTY ACID SYNTHETASE

Enzyme substrate or product	Complete system –kinase fraction			Complete system		
	Protein in mg	Units of activity	Total cpm ^{32}P	Protein in mg	Units of activity	Total cpm ^{32}P
Holo-a-FAS	1.8	2272	772	0.37	434	272
Holo-b-FAS	0	0	0	1.33	141	2370

The complete system consisted of holo-a-fatty acid synthetase, 1.8 mg protein; $MgCl_2$, 1 mM; [γ-^{32}P]ATP, 5 mM and 1×10^6 cpm; a 55-75% $(NH_4)_2SO_4$ precipitate of liver supernatant protein (kinase fraction) obtained from 48-hour refed, 12-hour fasted pigeons, 4 mg of protein; and potassium phosphate buffer, pH 7.0, 0.2 M. The final volume of the incubation mixture was 1 ml. Samples were incubated at 32° for 20 minutes, frozen overnight and then the proteins were separated by affinity chromatography (1 g of Sepharose ϵ-amino caproyl pantetheine) as described under Experimental Procedure.

cently Carlson and Kim (14, 15) reported data that suggested rat liver acetyl-CoA carboxylase is regulated by a phosphorylation-dephosphorylation system. It was speculated by Rous (16) that fatty acid synthetase might also be regulated in the same way and she therefore attempted to demonstrate such a regulatory system for fatty acid synthetase in mouse liver by showing ^{32}P incorporation or turnover in the protein. She was unable to secure experimental support for this proposal in these studies. However, the animals had been fed ad libitum and were not subjected to the stresses of fasting and refeeding.

In our experiments we found that ^{32}P is incorporated into DEAE-purified fatty acid synthetase in pigeons fasted 48 hours and then refed 6 hours. Further, we found that the amount of ^{32}P is much greater in apo- and holo-b- than it is in holo-a-fatty acid synthetase. The loss of ^{32}P on incubation of holo-b-fatty acid synthetase and Mg^{++} with the protein of a 0 to 40% ammonium sulfate fraction of liver homogenate supernatant solution, with a concomitant increase in enzyme activity, is evidence for the presence of a phosphoprotein phosphatase in this fraction. It is also evidence for the loss of phosphate in the conversion of holo-b- to holo-a-fatty acid synthetase. The incorporation of ^{32}P from [γ-^{32}P]ATP, with a concomitant loss of enzyme activity, on the treatment of holo-a-fatty acid synthetase with the protein of a 55 to 75% ammonium sulfate fraction of 100,000 g supernatant solution from liver homogenate of fasted pigeons demonstrates the reverse reaction, namely the activity of a protein kinase in this fraction. It is important to note that the phosphatase fraction was obtained from 48-hour fasted and 12-hour refed birds, while the kinase fraction was obtained from 48-hour refed and 12-hour fasted birds.

The possibility of the regulation of fatty acid synthetase activity by phosphorylation-dephosphorylation is strongly suggested by the fact that the ratio of holo-a- to holo-b-fatty acid synthetase is dependent on the nutritional state of pigeons. The relative amounts of each fatty acid synthetase is dependent on the time of refeeding of 48-hour fasted pigeons. This observation, along with the data on the control of acetyl-CoA carboxylase reported by Carlson and Kim (15), is consistent with the action of a coordinate control system for the short term regulation of lipogenic enzymes. A similar coordinate control system was proposed by Lakshmanan et al. (17) for the long term regulation of fatty acid synthesis. Presumably, the short term system would also be

under the control of the ratio of insulin to glucagon.

ACKNOWLEDGEMENT

We wish to thank Dr. I. Nishigaki, Department of Pharmacology, University of Wisconsin for the generous gift of [γ-^{32}P]ATP.

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