Regulation of Fatty Acid Synthetase Activity

THE 4'-PHOSPHOPANTETHEINE HYDROLASE OF RAT LIVER*

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The 4'-phosphopantetheine hydrolase of rat liver, partially purified by ammonium sulfate precipitation, catalyzes the hydrolysis of the prosthetic group 4'-phosphopantetheine from the holo-fatty acid synthetase. The two products of this reaction, 4'-phosphopantetheine and apo-fatty acid synthetase, were isolated by DEAE-cellulose chromatography and by chromatography on a Sepharose ε-aminocaproyl pantetheine column, respectively. The resultant apo-phosphopantetheine and apo-fatty acid synthetase, phosphopantetheine from the holo-fatty acid synthetase complex, have been shown to exist in E. coli (3, 4). These two enzymes were shown to catalyze the following reactions:

\[ \text{Holo-ACP + CoA} \rightleftharpoons \text{Mn}^{2+} \rightarrow 4'\text{-phosphopantetheine + apo-ACP} \]

\[ \text{Apo-ACP + CoA} \rightleftharpoons \text{Mg}^{2+} \rightarrow \text{holo-ACP} + 3',5'-\text{adenosine diphosphate} \]

Recently, Yu and Burton (5, 6) demonstrated that a crude supernatant rat liver enzyme system converted some of the inactive apoenzyme to enzyme having fatty acid synthetase activity in the presence of coenzyme A and ATP. Their data suggested the existence of an enzyme capable of carrying out the transfer of the 4'-phosphopantetheine group from coenzyme A to apo-fatty acid synthetase.

More recently a crude enzyme preparation derived from livers of fasted rats was shown by Roncari (7) to inactivate purified rat liver 4'-phosphopantetheine fatty acid synthetase by releasing its prosthetic group, thus exhibiting activity similar to that of the E. coli ACP-hydrolase.

In this paper we describe a partially purified enzyme activity designated the 4'-phosphopantetheine hydrolase, which removes the 4'-phosphopantetheine from the rat liver holoenzyme. The end products of this reaction have been identified as 4'-phosphopantetheine and apo-fatty acid synthetase. Evidence is also presented which indicates that there is only 1 mol of 4'-phosphopantetheine/mol of holo-fatty acid synthetase complex.

EXPERIMENTAL PROCEDURES

Materials—Experimental materials were obtained from the following sources: acetyl-CoA, malonyl-CoA, palmitoyl-CoA, and coenzyme A from P-L Biochemicals; [2-14C]malonyl-CoA, [1-14C]coenzyme A, and [1-14C]pantothenic acid from New England Nuclear; S-acetyl-N-acetylcysteamine, NADPH, and DEAE-cellulose from Sigma; pantetheine, reduced with sodium amalgam prior to use, and 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide-HCI, used in the preparation of the affinity column, from Sigma; and Sephadex from Pharmacia Fine Chemicals. All other reagents used were of analytical grade.

Preparation of Rat Liver Holo-fatty Acid Synthetase—Male Holtzman albino rats of approximately 200 g weight were fasted for 3 days, refed a fat-free diet for 48 h, and then killed. The livers were excised and homogenized as previously reported (10), and then the fatty acid synthetase was purified according to the method of Burton et al. (11).

Preparation of 4'-Phospho[1,14C]pantetheine Holo-Fatty Acid Synthetase—Male albino rats weighing 150 to 200 g each were fasted for 3 days and then fed a fat-free diet. Six hours after the start of refeeding, 20 μCi of [1-14C]pantothenic acid was injected intraperitoneally. Eighteen hours later another 20 μCi was injected. The rats were killed 1 h after the last injection, the livers excised, and holo-fatty acid synthetase was prepared as described above.

Preparation of Rat Liver Apo-fatty Acid Synthetase—Male Holtzman albino rats weighing approximately 200 g were fasted for 3 days, refed a fat-free diet for 3 h, and then killed. The livers were excised and the regular procedure (11) for preparation of fatty acid synthetase was followed. The 0 to 33% ammonium sulfate precipitate of the DEAE-cellulose chromatography eluate was collected by centrifugation, dissolved in 0.5 M potassium phosphate buffer, pH 7.0, containing 1 mM dithiothreitol and 1 mM EDTA, and dialyzed against the same buffer for 16 h. The dialyzed protein was assayed for overall fatty acid synthetase activity and for the two partial activities β-ketoacyl thioester reductase and palmitoyl-CoA deacylase. The apo-fatty acid synthetase activity was followed in the presence of the appropriate cofactors.

* The abbreviation used is: ACP, acyl carrier protein.
synthetase was stored at a protein concentration of 10 mg/ml in the presence of 10% glycerol at -20°C. The apo-fatty acid synthetase prepared in this way is essentially free of the holo form. The remainder of the incubation mixture was adjusted to pH 3.5 with 0.1 M potassium phosphate buffer containing 1 mM dithiothreitol, the dialyzed following solvent systems: n-butyl alcohol:acetic acid:water (5:2:3), and ethyl alcohol: n ammonium acetate, pH 7.5 (5:2). Parallel separations with authentic 4-phosphopantetheine were also carried out.

Identification of 4-Phosphopantetheine—An aliquot of the solution containing 4-phosphopantetheine was subjected to ascending paper (Whatman No. 4) chromatography at 23°C in each of the following solvent systems: n-butyl alcohol:acetic acid:water (5:2:3), and ethyl alcohol: n ammonium acetate, pH 7.5 (5:2). Parallel separations with authentic 4-phosphopantetheine were also carried out.

Amino Acid Analysis—The DEAE-cellulose fractions containing radioactivity were pooled, lyophilized, and subjected to hydrolysis with 6 N hydrochloric acid in vacuo at 110°C for 17 h. The hydrolysate was analyzed for \( \beta \)-alanine content on a Beckman model 120 amino acid analyzer equipped with a high sensitivity cuvette and a fenestrated scale expansion according to the method of Spakman et al. (16) as modified by Gerritsen et al. (17). A sample of standard 4-phosphopantetheine was treated in the same manner, and then analyzed for \( \beta \)-alanine content.

Assay for Pantothenic Acid Content—The DEAE-cellulose isolated 4-phosphopantetheine was also assayed for pantothenic acid content with Lactobacillus plantarum.

Analysis of the Protein End Product: Separation of the Protein End Product by Chromatography on a Sepharose c-Aminocaproyl Pantetheine Column—At the end of the incubation of fatty acid synthetase with hydroxide, the reaction was stopped by the addition of EDTA to a final concentration of 1 mM. The supernatant solution was partially purified through ammonium sulfate precipitation at 0 to 75% saturation. The protein eluates of the Sephadex G-50 column were determined immunochemically by a quantitative immunoprecipitation method (20, 21). The antisera to the fatty acid synthetase was prepared by twice injecting rabbits subcutaneously with DEAE-cellulose-purified fatty acid synthetase (5 mg/rabbit). The rabbits were bled 2 weeks after the last injection and the antisera purified as described by Livingston (22) except that the DEAE-
RESULTS

Requirements for Fatty Acid Synthetase Hydrolase Activity—Hydrolase activity was shown to be linearly dependent on the concentration of enzyme added (Fig. 1). Fatty acid synthetase hydrolase activity was also dependent upon magnesium ions, and to a lesser extent on dithiothreitol or mercaptoethanol (Table I).

Identification of 4'-Phosphopantetheine as a Product of the Hydrolase Reaction—The correlation between the loss in enzyme activity of 4'-phospho[14C]pantetheine-labeled fatty acid synthetase incubated with hydrolase and the radioactivity released from the enzyme is shown in Table III. When the fatty acid synthetase activity decreased to approximately one-fourth of the original value, approximately three-quarters of the original protein-bound 4'-phospho[14C]pantetheine radioactivity was released into the incubation medium.

The RF values of the radioactive non-protein end product of hydrolase action in two different chromatography systems are presented in Table IV. The product, identified by two color reagents, had RF values coincident with those of authentic 4'-phosphopantetheine.

The effect of protein concentration on the conversion of holo- to apo fatty acid synthetase. The incubation conditions and the assay for fatty acid synthetase activity were carried out as described under "Experimental Procedures."

FIG. 1. The effect of protein concentration on the conversion of holo- to apo fatty acid synthetase. The incubation conditions and the assay for fatty acid synthetase activity were carried out as described under "Experimental Procedures."

TABLE I

<table>
<thead>
<tr>
<th>Component of the incubation mixture</th>
<th>Fatty acid synthetase activity</th>
<th>Radioactivity</th>
</tr>
</thead>
<tbody>
<tr>
<td>mg MgCl₂</td>
<td>U/ml</td>
<td>cpm</td>
</tr>
<tr>
<td>0.1</td>
<td>5</td>
<td>720</td>
</tr>
<tr>
<td>0.2</td>
<td>5</td>
<td>170</td>
</tr>
</tbody>
</table>

* The initial specific activity of the fatty acid synthetase was 52 nmol of palmitate/min/mg of fatty acid synthetase protein.

TABLE II

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Enzyme activity remaining</th>
<th>Radioactivity remaining</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rat liver fatty acid synthetase</td>
<td>50%</td>
<td>100%</td>
</tr>
<tr>
<td>Pigeon liver fatty acid synthetase</td>
<td>100%</td>
<td>100%</td>
</tr>
</tbody>
</table>

* Following 60 min of incubation, the reaction mixture was heated to denature the proteins, centrifuged, and the supernatant solution was chromatographed on a DEAE-cellulose column to separate 4'-phosphopantetheine from coenzyme A. The eluted fractions containing coenzyme A were assayed for radioactivity.

TABLE III

<table>
<thead>
<tr>
<th>Incubation mixture</th>
<th>Time (h)</th>
<th>Fatty acid synthetase activity remaining</th>
<th>Radioactivity in released product (cpm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Complete system</td>
<td>0</td>
<td>8.0</td>
<td>170</td>
</tr>
<tr>
<td>Complete system</td>
<td>1</td>
<td>2.2</td>
<td>720</td>
</tr>
<tr>
<td>Complete system + hydrolase</td>
<td>1</td>
<td>8.3</td>
<td>120</td>
</tr>
</tbody>
</table>

* The complete incubation mixture contained 2 mM MgCl₂, 0.1 mM potassium phosphate, pH 7.0, containing 0.25 mM succrose and 0.01 mM 2-mercaptoethanol or 0.005 M dithiothreitol, 100 µg of purified fatty acid synthetase (FAS) protein, and 20 µg of hydrolase protein.

Identification and Quantitation of Apo-fatty Acid Synthetase as the Protein End Product of the Hydrolase Reaction—The chromatographic separation of the holo-fatty acid synthetase on a Sepharose ε-aminocaproyl pantetheine column...
at zero time and at the end of 1 h of incubation with hydrolase is shown in Fig. 3, A and B, respectively. Holo-fatty acid synthetase is presented as nanomoles of palmitic acid formed per min per mg of protein, and as units of palmitoyl-CoA deacylase activity. The apo-fatty acid synthetase activity resulting from the conversion of holo-fatty acid synthetase to apoenzyme during a 1-h incubation with hydrolase is presented as units of palmitoyl-CoA deacylase activity.

The data given in Table V are from a typical chromatographic separation of the protein end product of the hydrolase reaction. The amount of holoenzyme converted to apo-fatty acid synthetase, in terms of enzyme activity and milligrams of protein as determined by immunoassay, is therein tabulated.

The identity of apo-fatty acid synthetase, which was demonstrated by partial enzyme activities and by reactivity to antibody to the fatty acid synthetase, was confirmed by conversion to the holo-fatty acid synthetase. This conversion was effected in the presence of a fatty acid synthetase transferase activity, which transfers 4'-phosphopantetheine from coenzyme A to apo fatty acid synthetase (Table VI). The radioactivity covalently bound to protein indicates the conversion of 80% of apo- to holo-fatty acid synthetase. However, the corresponding increase in fatty acid synthetase activity was less than expected. This may be due to protein denaturation during the concentration step following elution from the Sepharose AG-1 column.

### Table IV

**Rf values of the non-protein end product of the hydrolase reaction**

Prior to the isolation of 4'-phosphopantetheine from the incubation mixture the proteins were precipitated with 5% trichloroacetic acid. The mixture was then centrifuged to remove the precipitated proteins and the supernatant solution was chromatographed on a Sepharose AG-1 column. Fractions behaving the same as authentic 4'-phosphopantetheine were then subjected to paper chromatography as described in the text.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Solvent 1</th>
<th>Solvent 2</th>
<th>Nitroprusside</th>
<th>Ammonium molybdate</th>
</tr>
</thead>
<tbody>
<tr>
<td>End product 4'-phosphopantetheine</td>
<td>0.47</td>
<td>0.53</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Authentic 4'-phosphopantetheine</td>
<td>0.47</td>
<td>0.53</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

*Solvent 1: n-butyl alcohol:acetic acid:water (5:2:3 v/v).*
*Solvent 2: ethyl alcohol:1 N ammonium acetate, pH 7.5 (5:2).*

**Fig. 2.** DEAE-cellulose chromatography of the non-protein end product of the hydrolase reaction. At the end of a 45-min incubation, the reaction mixture was treated as described under "Experimental Procedures" and the supernatant solution containing 4'-phosphopantetheine was chromatographed on a DEAE-cellulose column. The radioactivity (cpm/ml) of the hydrolase reaction product, 4'-phosphopantetheine, and absorbance at 412 nm of standard 4'-phosphopantetheine were made with the Ellman reagent (23).

**Fig. 3.** Chromatographic separation of apo- and holo-fatty acid synthetases. A, zero time incubation with hydrolase; B, 1-h incubation with hydrolase. Overall and partial activities of the fatty acid synthetase were carried out as described under "Experimental Procedures." One unit of fatty acid synthetase is equivalent to 1 nmol of palmitic acid formed/min. The 280 nm absorbing peak in Fractions 3 to 9 of A represent protein of the hydrolase preparation only inasmuch as the addition of EDTA at the zero time completely blocked the conversion of holo-fatty acid synthetase to the apoenzyme.

**Table V**

**Conversion of holo- to apo-fatty acid synthetase by the hydrolase**

The complete system consisted of holo-fatty acid synthetase, 2 mg of protein; 2 mM MgCl₂; 0.4 mg of hydrolase protein, ammonium sulfate-precipitated; and 0.1 M potassium phosphate, pH 7.0. At the end of the incubation time the reaction was stopped by the addition of EDTA and the proteins of the incubation mixture were separated by affinity chromatography. The eluted fractions were assayed for overall fatty acid synthetase and palmitoyl-CoA deacylase activities and their protein contents were determined by immunoassay.

<table>
<thead>
<tr>
<th>Conditions</th>
<th>Holo-fatty acid synthetase</th>
<th>Apo-fatty acid synthetase</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>mg protein</td>
<td>units activity</td>
</tr>
<tr>
<td>Complete system minus</td>
<td>1.64</td>
<td>69.8</td>
</tr>
<tr>
<td>hydrolase</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Complete system with</td>
<td>0.7</td>
<td>28.4</td>
</tr>
<tr>
<td>hydrolase</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*The recovery of holo-fatty acid synthetase from the affinity column was estimated to be 82%.*
*The overall fatty acid synthetase activity measured spectrophotometrically as nanomoles of palmitate per min.*
*Activity of palmitoyl-CoA deacylase.*
adex G-50 column. This step was necessary, inasmuch as the protein solution obtained from the Sephadex column was very dilute.

Quantitative Determination of the 4' Phosphopantetheine Released per Mol of Fatty Acid Synthetase by Hydrolase Action—The results of amino acid analysis for β-alanine and microbiological assay for pantetheine on the 4'-phosphopantetheine end product of the hydrolase reaction are given in Table VII. The β-alanine and pantetheine acid contents of the end product, as determined by the two methods, are in close agreement, and they show the presence of 1 mol of 4'-phosphopantetheine/mol of fatty acid synthetase complex (molecular weight of 5.4 \times 10^5).

\textbf{DISCUSSION}

Rat liver 4'-phosphopantetheine hydrolase catalyzes the hydrolysis of the prosthetic group of the rat liver holo-fatty acid synthetase to yield 4'-phosphopantetheine and a protein which has been identified as apo-fatty acid synthetase. This hydrolase has been partially purified through centrifugation and ammonium sulfate precipitation steps. The enzyme activity is stable over long periods of time when stored at -20°C under nitrogen in the presence of either β-mercaptoethanol or dithiothreitol.

The conversion of the holo- to apoenzyme by the hydrolase requires Mg^{2+} ions and either β-mercaptoethanol or dithiothreitol and this reaction is time- and protein-dependent.

The non-protein end product of the hydrolase reaction has been identified as 4'-phosphopantetheine on the basis of its \( K_p \) value on ascending paper chromatography, by its elution pattern on DEAE-cellulose column chromatography, and by amino acid analysis and microbiological assay.

The isolation of the apo-fatty acid synthetase end product from the substrate holo-fatty acid synthetase remaining at the end of the hydrolase reaction was accomplished by chromatography on a Sepharose ε-aminocaproil pantetheine column. The identity of the protein end product of the hydrolase reaction as the apoenzyme is based on its immunoreactivity to fatty acid synthetase-specific antibody and its conversion to holo-fatty acid synthetase in the presence of 4'-phosphopantetheine transferase activity. These findings eliminate the possible involvement of protease action in the deactivation of fatty acid synthetase, and they show that the observed decrease in enzyme activity is due solely to the cleavage of the prosthetic group 4'-phosphopantetheine by the hydrolase.

The average value for the amount of 4'-phosphopantetheine released by the hydrolase reaction from 1 mol of fatty acid synthetase was estimated to be 1.04 by means of the amino acid analysis and the microbiological assay. Both assays, when compared to the value obtained for the standard, showed small error. The computation was carried out on the basis of a 5.4 \times 10^5 molecular weight value for the rat liver fatty acid synthetase which was obtained earlier by sedimentation diffusion analysis (11). This finding indicates the presence of 4'-phosphopantetheine in equimolar ratio to the fatty acid synthetase.

Our results are in agreement with the value of 1.23 previously reported for the pigeon liver fatty acid synthetase (24), and confirms earlier data on the presence of two nonidentical subunits in the fatty acid synthetase complex (18). The studies discussed here indicate that the rat liver hydrolase is a phosphodiesterase which specifically catalyzes the cleavage of 4'-phosphopantetheine from rat liver holo-fatty acid synthetase. Specificity studies of this enzyme indicate that it does not catalyze the cleavage of 4'-phosphopantetheine from coenzyme A or pigeon liver holo-fatty acid synthetase. The rat and pigeon liver fatty acid synthetases are known (11) to differ in some of their properties, a fact which may account for the inability of the rat liver hydrolase to recognize the pigeon liver fatty acid synthetase.

The exact physiological function of this enzyme is unknown. The specificity of the hydrolase action lends support to the possible participation of this enzyme in regulating fatty acid synthetase activity. An efficient mode of fatty acid synthetase regulation would involve the cleavage of 4'-phosphopantetheine from rat liver holo-fatty acid synthetase. Specificity studies of this enzyme indicate that it does not catalyze the cleavage of 4'-phosphopantetheine from coenzyme A or pigeon liver holo-fatty acid synthetase. The rat and pigeon liver fatty acid synthetases are known (11) to differ in some of their properties, a fact which may account for the inability of the rat liver hydrolase to recognize the pigeon liver fatty acid synthetase.

Recent data indicate an increase in the activity of 4'-phosphopantetheine hydrolase in diabetic rats.

C. Sobby and J. W. Porter, unpublished data.
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C Sobhy


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