4'-Phosphopantetheine, a Cofactor Bound to Guanosine Triphosphate-dependent Acyl Coenzyme A Synthetase*

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SUMMARY

A small molecular weight cofactor has been isolated from purified preparations of the guanosine triphosphate-specific acyl coenzyme A synthetase of rat liver mitochondria. The synthetase enzyme becomes inactive on removal of the cofactor, and its characteristic activity is restored by adding back the cofactor to the complete system containing CoA-SH.

In its active form, the cofactor possesses sulfhydryl groups, organic phosphate, and pantothenic acid in a ratio of 1:1:1.

4'-Phosphopantetheine can replace the cofactor in all enzymic assays and shares with the cofactor all chemical and spectral characteristics.

The isolation of a guanosine triphosphate-dependent acyl coenzyme A synthetase from liver mitochondria has been previously reported (1, 2). Coenzyme A was an obligatory participant in this reaction at all stages of enzyme purification (Equation 1)

\[
\text{GTP} + \text{RCOOH} + \text{CoA} \rightarrow \text{RCoA} + \text{GDP} + \text{Pi} \tag{1}
\]

The enzyme, purified from liver mitochondria of Holtzman strain rats (2), could be dissociated into an enzymically inactive protein and a small molecular weight cofactor containing pantothenic (3). Enzyme activity was restored by recombining the protein and cofactor in the usual synthetase assay system which routinely contained CoA-SH. The cofactor could be obtained from liver mitochondria of Wistar strain rats in an oxidized or reduced state depending on the kind of extraction procedure employed. Only the reduced cofactor restored synthetase activity. The purification and identification of the GTP-acyl-CoA synthetase cofactor are presented in this paper.

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METHODS

Enzyme Isolation

Rat liver mitochondria were prepared by the method of Schneider (4) in 0.25 M sucrose. Soluble fractions were obtained by using two different procedures.

Procedure A—This is the same as the procedure previously described (2), where mitochondrial pellets (800 mg) were suspended in 15 ml of 0.5% aqueous Triton X-100 and subjected to sonication (20 kc) for 5 min at a temperature between 0° and 4°.

Procedure B—This method differed in that mitochondrial pellets were lyophilized before treatment by sonic oscillation. The subsequent steps of isolation and purification were the same. The supernatant obtained after centrifugation at 80,000 × g for 30 min was dialyzed overnight against 1 liter of 0.1 M KCl (Fraction I). Fraction I was adjusted (with stirring at 0°) to pH 3.4 with 0.1 M HCl, and then readjusted to pH 7.0 without delay. The clear supernatant was defined as Fraction II. To this fraction, calcium phosphate gel (2 mg, dry weight, per mg of protein) was added. The inactive adsorbed protein was removed by centrifugation. Further material precipitated on adding 50 g of solid ammonium sulfate per 100 ml of supernatant. The final supernatant obtained after another centrifugation was dialyzed overnight against 1 liter of 0.1 M KCl (Fraction III).

Enzyme Assays

GTP-specific acyl-CoA synthetase activity was followed by using either short or long chain fatty acids as substrate. With short chain fatty acids (octanoate), sulfhydryl disappearance (5) or inorganic phosphate release or both were determined (2). With long chain fatty acids (oleate), the activity was followed by measuring acyl-CoA formation with acyl-CoA dehydrogenase according to the method of Tubbs and Garland (6). The assay incubation mixtures contained 0.4 M Tris-HCl (pH 7.4), 5 mM KBH₄ (omitted in the system with oleate), 5 mM MgCl₂, 2.5 mM GTP, 1 mM CoA-SH, and either 25 mM potassium octanoate or 2.5 mM sodium oleate. GTP was omitted in the blanks. Final volume was 0.3 ml; temperature of reaction was 38°; and time of incubation was 5 to 10 min.

Protein concentration was determined by the biuret (7) or the phenol reaction (8). GTP and CoA were obtained from

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Three enzyme fractions were prepared from liver mitochondria of Holtzman and Wistar strain rats according to the procedures indicated. Specific activities of the GTP-dependent acyl-CoA synthetase are expressed in nanomoles of acyl CoA per min per mg of protein. The substrate was oleate. Between 0.1 and 1.0 mg of protein was added to the 0.3-ml incubation mixture. (See "Methods" for assay conditions.)

Table I

| Fraction | I | II | III |
|----------|---|----|-----|
| Holtzman (Procedure A) | 5.5 | 36 | 42 |
| Wistar (Procedure B) | 1.2 | 5 | 34 |

RESULTS

The GTP-specific acyl-CoA synthetase, active both with short and long chain fatty acids, has been obtained from liver mitochondria of both Holtzman and Wistar strain rats of either sex (Table I). The specific enzyme activity was much higher in crude Fractions I and II recovered from Holtzman strain rats than in those from the Wistar strain. The specific activity of Fraction I from liver mitochondria of the Wistar strain was of the same order as that reported by Van Tol, De Jong, and Hulsman (13). Only in Fraction III did the specific activities approach the same value (Table I). With Procedure A (sonic oscillation without lyophilization), active enzyme fractions could not be prepared from liver mitochondria of Wistar strain rats. Only when these mitochondria were lyophilized prior to sonic oscillation and extraction could active fractions be obtained (Tables I and II). With either Procedure A or B both ATP-specific fatty acyl-CoA synthetase and succinyl-CoA synthetase were present in Fractions I and II, but not in Fraction III (2).

In order to obtain a further purification, Fraction III was lyophilized and dissolved in a small volume of water. Then, 6 mg of protein were passed through a Sephadex G-75 column (1 x 50 cm) previously equilibrated with 0.4 M KCl-0.1 M acetate buffer (pH 6). Elution was carried out with the same medium at the flow rate of 4 ml per hour. Fractions of 2 ml were collected and the optical density at 280 nm was measured in each sample. The whole operation was performed at 5°. From the column, two components were separated. The first which emerged between 24 and 30 ml, was a protein (apoenzyme); the second (between 40 and 50 ml) was a low molecular weight cofactor (3). On the average, 2 mg of protein (apoenzyme) were recovered. When GTP-dependent acyl-CoA synthetase ac-

Table II

| Source of mitochondria, isolation procedure, and additions | GTP-octanoate | GTP-oleate |
|----------------------------------------------------------|---------------|------------|
| Holtzman, Procedure A                                    | 0             | 0          |
| Protein                                                  | 0             | 0          |
| Protein + cofactor                                       | 82            | 70         |
| Wistar, Procedure B                                      |               |            |
| Protein                                                  | 0             | 0          |
| Cofactor                                                 | 0             | 0          |
| Protein + cofactor                                       | 75            | 105        |
| Protein + reduced Cofactor I                             | 120           | 117        |
| Wistar, Procedure A                                      |               |            |
| Protein                                                  | 0             | 0          |
| Cofactor                                                 | 0             | 0          |
| Protein + cofactor                                       | 110           | 108        |
| * Prepared from Wistar strain liver mitochondria by Procedure B

Activity of the GTP-dependent acyl-CoA synthetase eluted from the Sephadex G-75 column is expressed in nanomoles per min per mg of protein. In the GTP-octanoate system, change in sulfhydryl concentration was measured, and in the GTP-oleate system, change in acyl-CoA concentration was measured. The assay incubation conditions are described under "Methods." The concentration of the protein component emerging from the G-75 column was 0.1 to 0.2 mg per ml. Range of protein added to the 0.3-nl system was between 10 and 20 μg. Equal volumes of the apoenzyme and the second component (cofactor) were added to the incubation mixture where indicated. Cofactor A (active) and Cofactor I (inactive) are specified in the text. Cofactor A was added in the amount of 1 to 2 nmols, as measured by -SH analysis. A corresponding amount of Cofactor I was used before and after reduction with sodium amalgam.

The cofactor was stable for many months at -20° or 0°. The apoenzyme, however, lost 50% of its initial activity after 24 hours at 0° or -20°. Ammonium sulfate and albumin did not enhance the stability of the protein.

GTP-dependent acyl-CoA synthetase activity was assayed with the first or second component alone no activity was detected (Table II). However, a high enzymic activity with GTP and octanoate and with GTP and oleate was obtained in the system containing both the protein and the cofactor (Table II). These results were obtained with a 1:1 volume mixture of the two components emerging from the column of Sephadex G-75.

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GTP-dependent acyl-CoA synthetase activity was not detected in fractions prepared from liver mitochondria of Wistar strain rats by using Procedure A. The activity was absent both in crude extracts (Fraction I) and in subsequent Fractions II and III. Nevertheless the inactive Fraction III could be dissociated on a Sephadex G-75 column into a protein and a cofactor of low molecular weight, as described before. This cofactor as isolated was inactive in all systems tested. It is designated in Table II as Cofactor I. Nevertheless the protein isolated here showed a high synthetase activity when recombined with an active cofactor, Cofactor A (prepared by Procedure B from Wistar liver mitochondria).

Sulfhydryl groups were present in Cofactor A but they were completely absent in Cofactor I (Table III). After reduction with sodium amalgam, Cofactor I became positive to the sulfhydryl group reagent (Table III). Furthermore, after reduction

1 The abbreviation used is: ACP, acyl carrier protein.
TABLE III
Composition of cofactor preparations

All values are expressed in nanomoles. Alkaline phosphatase (Boehringer) treatment was performed by incubating the cofactors for 3 hours at 37° under the following conditions: 50 μmoles of Tris-HCl (pH 8.0) and 100 μg of alkaline phosphatase in a total volume of 0.5 ml. Orthophosphate was determined as previously reported (2). Organic phosphate was calculated by the difference between total phosphate and orthophosphate. Sulfhydryl groups (5, 12) and pantothenate (10) were assayed as described under “Methods.”

| Cofactor                | Sulfhydryl | Organic phosphate | Pantothenate |
|------------------------|------------|-------------------|--------------|
| Cofactor A              | 18a        | 26                | 0.48         |
| Cofactor A (treated with alkaline phosphatase) | 20a | 0 | 4.00 |
| Cofactor I              | 0          | 31                |              |
| Cofactor I (after reduction with sodium amalgam) | 27a | 42 | 0.94 |
| Cofactor A’             | 46.6*      | 45.8              | 1.06         |
| Cofactor A’ (after treatment with alkaline phosphatase) | 45.0 | 0 | 47.30 |

* Values obtained after complete reduction with sodium amalgam.

Cofactor A’ could reactivate the dissociated protein as effectively as Cofactor A (Table II, last experiment).

The absorption spectra of Cofactors A (active) and I (inactive) were different (Fig. 1). Cofactor A possessed a peak at 223 μm and a broad shoulder between 260 and 270 μm. Cofactor I, however, showed an adsorption peak at 225 μm and a second peak at 255 μm (Fig. 1, Curve I). After reduction with sodium amalgam the spectrum of Cofactor I shifted to become the same as that of Cofactor A.

TABLE IV
Cofactor A’, acyl carrier protein, and 4’-phosphopantetheine in enzyme reactivation

The assay incubation conditions were those described under “Methods.” The substrate was oleate. The enzyme was extracted from liver mitochondria of Wistar strain rats by Procedure B. Specific enzyme activity is expressed as nanomoles of acyl-CoA per min per mg of protein. The amount of protein added to the 0.5-ml incubation system was 10 to 20 μg (<1.0 nmole). The concentrations of cofactor A’, ACP, and 4’-phosphopantetheine (determined by sulfhydryl analysis) greatly exceeded the apo-enzyme concentration.

| Additions                      | Specific enzyme activity |
|-------------------------------|--------------------------|
| Protein                       | 0                        |
| +Cofactor A’ (7 μM)           | 68                       |
| +ACP (8 μM)                   | 0                        |
| +Cofactor A’ + ACP (8 μM)     | 12                       |
| +4’-Phosphopantetheine (7.5 μM) | 86                      |

At this stage of purification the cofactors contained organic phosphate and pantothenate (Table III). The biuret reaction was positive. The L. plantarum which was used for the microbiological analysis of pantothenate was equally sensitive to pantothenate and pantetheine. The assay was insensitive to pantetheine, 4’-phosphopantetheine, and 4’-phosphopantethine. A marked increase in L. plantarum growth was observed after treatment of the cofactor with alkaline phosphatase (Table III). This indicated the presence of 4’-phosphopantetheine in the cofactor. On the basis of these data, the cofactor appeared to be similar to the acyl carrier protein recently isolated from E. coli (14, 15). Nevertheless preparations of cofactor A were inactive in the assay for ACP employing enzymes from E. coli. Furthermore, as shown in Table IV, pure ACP could not substitute for cofactor A in the GTP-dependent acyl-CoA synthetase system. Indeed ACP inhibited the enzymic reaction when all components were present (Table IV). In similar experiments reduced dephospho Coenzyme A (15), glutathione, and cysteine could not replace 4’-phosphopantetheine.

Cofactor A (1.5 μmoles) was purified further by passing it through a column of Bio-Gel (4 x 20 cm) (Bio-Rad spherical polyacrylamide Bio-Gel P2). Elution was carried out with water at a flow rate of 2 ml/5 min. Fractions of 2 ml were collected and the optical density at 220 μm was measured in each sample. By this method four components were separated. The first, emerging between 80 and 100 ml, was inactive and was positive in the biuret reaction. The second, collected between 150 and 180 ml, was negative in the biuret reaction. This second component (Cofactor A’) reactivated the apoenzyme as isolated on the Sephadex G-75 column (Table IV). Cofactor A’ contained sulfhydryl groups and organic phosphate in a ratio of 1:1 (Table III). Organic phosphate was released as inorganic orthophosphate by alkaline phosphatase (10). After the latter treatment, Cofactor A’ possessed sulfhydryl groups and pantetheine in a ratio of 1:1. The samples of the cofactor were analyzed for ACP activity by Dr. Gerald Weeks in the laboratory of Professor S. J. Wakil, Department of Biochemistry, Duke University. The authors greatly appreciate this crucial evaluation.
Similarly, the maximum rate of enzyme activity was obtained when 4'-P-pantetheine and apoenzyme were admixed in a molar ratio of 1:1:1. This ratio would be expected for 4'-P-pantetheine. The reduced cofactor A' and 4'-P-pantetheine display identical spectra with an absorption maximum at 214 μm and the same molar extinction coefficient at this wavelength. The maximum at 214 μm probably reflects the presence of —SH groups, although a maximum of 220 μm is more typically observed.

Cofactor A' and 4'-P-pantetheine behave identically in restoring the catalytic activity of the apoenzyme. The oxidized form of the isolated cofactor and 4'-phosphopantetheine are inert in this system.

4'-P-pantetheine is bound to the apoenzyme in a molecular ratio of 1:1, presumably by weak secondary bonds since gel filtration alone effected the separation of the cofactor from protein. This property of Sephadex was previously utilized by Kissiuk (10) to remove a folate acid coenzyme from an enzyme system of E. coli which catalyzed the synthesis of methionine.

Dialysis of this enzyme preparation was much less effective.

The holoenzyme is much more stable than the apoenzyme as prepared here. In previous studies it was shown that the enzyme also contains bound lecithin which is necessary for full enzyme activity (20). In this earlier study 4'-P-pantetheine probably was not removed with lecithin from the lyophilized enzyme by acetone-water mixtures since purified egg lecithin alone could restore the activity of the lipid-depleted enzyme.

Examination of the GTP-specific synthetase would indicate that either an enzyme-bound acyl-S-4'-P-pantetheine is not an intermediate in acyl-CoA formation, or that an acyl intermediate is not as reactive with hydroxylamine as acyl-AMP. 4'-P-pantetheine could conceivably function as an allosteric effector. The inert 4'-phosphopantetheine may be inadequate since its molecular size is twice that of 4'-P-pantetheine. Coenzyme A is reported to be necessary for the full activity of ATP-specific acyl-CoA synthetase if the enzyme is assayed as the partial reaction below (24):

\[
\text{ATP} + \text{RCOOH} \rightleftharpoons \text{RCO-AMP} + \text{PP}_1
\]

\[
\text{RCO-AMP} + \text{NH}_2\text{OH} \rightarrow \text{RCO-NHOH} + \text{AMP}
\]

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Palmityl-AMP + PP_i = palmitate + ATP (4)

Similarly there is evidence to suggest that CoA or its derivatives may influence partial reactions of the succinyl-CoA synthetase system. The enzyme isolated from mitochondria of animal tissues is GTP-specific (25), and is ATP-specific in preparations from E. coli and spinach (26). These systems are formally identical with the GTP-acyl-CoA synthetase:

GTP + succinate + CoA-SH ⇌

succinyl-S-CoA + GDP + Pi (5)

With the E. coli enzyme CoA and desulfo-CoA greatly stimulate the formation of ATP from synthetic succinyl phosphate and ADP (27), as well as the reverse process (28). CoA accelerates the rate of (E. coli) ATP-ADP exchange (29); the enzyme (histidyl) phosphate-orthophosphate exchange in the E. coli enzyme (30, 31) and in the GTP enzyme from heart (32); the GTP (ITP)-orthophosphate exchange (32, 33) and the related (histidyl) phosphate-orthophosphate exchange in the E. coli enzyme CoA and desulfo-CoA greatly stimulate the formation of ATP from synthetic succinyl phosphate and ADP (27), as well as the reverse process (28). CoA accelerates the rate of (E. coli) ATP-ADP exchange (29); the enzyme (histidyl) phosphate-orthophosphate exchange in the E. coli enzyme (30, 31) and in the GTP enzyme from heart (32); the GTP (ITP)-orthophosphate exchange (32, 33) and the related GTP (ITP) arsenolysis (25, 34). Grinnell and Nishimura (28) have suggested that CoA and dephospho-CoA do function as allosteric effectors in the synthesis of succinyl phosphate. It is not unlikely that CoA (and certain of its derivatives) may play the same role in the other partial reactions (35).

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