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SEPARATION OF ACTIVE ENZYME COMPONENTS FROM THE FATTY ACID SYNTHETASE OF CHICKEN LIVER

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Summary

Fatty acid synthetase (EC 6.2.1.-) of chicken liver was dissociated into half-size subcomplexes and then separated into three protein fractions by the preparative disc-gel electrophoresis technique. The anodal protein (F_a) of a molecular weight of approx. 6000 contains the prosthetic group, 4′-phosphopantetheine. It binds acetyl group from acetyl-CoA and is identified as the acyl carrier protein component. The slower moving proteins (F_I and F_{II}) correspond to the subcomplexes resolved by the analytical method (Y .n, S.L. and Hsu, R.Y. (1972) J. Biol. Chem. 247, 2689—2698). Both contain acetyl transacylase and palmityl-CoA deacylase activities, but only F_I contains β-ketoacyl reductase activity. Active acetyl transacylase and palmityl-CoA deacylase components were obtained by the sucrose density centrifugation technique in a broad 3 S protein band from the F_I fraction, following dissociation at 4°C for 12 days. Slight modification of the electrophoresis conditions yields a homogeneous 1.55 S β-ketoacyl reductase component.

Introduction

The component enzymes of the dissociable fatty acid synthetase system (EC 6.2.1.-) in *Escherichia coli* have been isolated and characterized. Their availability permitted the delineation of individual steps in the reaction sequence [2]. Corresponding studies on the analogous enzymes of the highly organized multienzyme complexes of animal origin, however, were hindered by the lack of methodology to dissociate and isolate these components in active form. In a previous report, transacylase activity was detected by Plate et al. [3] in the partially resolved dissociation products of the pigeon liver complex after treatment with guanidine hydrochloride.

In the present communication, an electrophoretic technique is described for the separation of acyl carrier protein and subcomplexes from the chicken

liver synthetase. Catalytically active acetyl transacylase, palmityl-CoA deacylase and β-ketoacyl reductase components were obtained for the first time, the latter in homogeneous form.

Materials and Methods

Acetyl-CoA, malonyl-CoA, and NADPH (P-L Biochemicals) dithiothreitol, ethylenediaminetetraacetic acid (tetrasodium dihydrate) and bovine serum albumin (Calbiochem); [1-^{14}C]acetyl-CoA, [1-^{14}C]palmityl-CoA and [1-^{14}C]-pantothenate (New England Nuclear), ammonium sulfate and sucrose (special enzyme grade) (Mann), acrylamide, bisacrylamide, *N,N,N',N'*-tetramethylenediamide, ammonium persulfate (Eastman Kodak), lactate dehydrogenase and cytochrome *c* (Boehringer Mannheim) were purchased from the above designated sources. Bovine insulin was a gift of Eli Lily Company. *S*-acetoacetyl-*N*-acetyl-cysteamine was synthesized by the method of Lynen et al. [4].

Fatty acid synthetase activity was determined spectrophotometrically at 340 nm by the method of Yun and Hsu [1]. Assays for acetyl transacylase, palmityl-CoA deacylase (pH 6.8) and β-ketoacyl reductase activities were performed essentially according to Kumar et al. [5], except the latter reaction was carried out at 38° C and an NADPH concentration of 90 μM. Fatty acid synthetase concentration was determined at 279 nm [6]. Concentrations of subcomplexes and protein fragments were determined by the method of Lowry et al. [7]. Radioactivity was measured in the Beckman LS-150 liquid scintillation counter using Aquasol as the scintillation fluid. Sedimentation experiments were carried out on the Spinco Model E analytical ultracentrifuge.

Chicken liver fatty acid synthetase was purified as described previously [6]. The radioactive enzyme was prepared from livers of chickens injected with doses of [1-^{14}C]pantothenate at 4 and 24 h after refeeding [8]. The purified enzyme was precipitated at 33% ammonium sulfate saturation, centrifuged, and redissolved in a minimum volume of Tris (5 mM)/glycine (35 mM)/EDTA (1 mM), pH 8.3, buffer containing 1 mM dithiothreitol. Dissociation into half-size subcomplexes was affected by dialyzing the enzyme solution against the buffer for 40—48 h at 4° C essentially as described by Yun and Hsu [1]. Homogeneity of the purified and dissociated preparations were routinely established by velocity sedimentation.

Preparative disc-gel electrophoresis was performed on a Büchler polyprep 200 apparatus according to the procedure of Chrambach et al. [9], using a 6 cm gel column containing 5% acrylamide in the buffer system described by Yun and Hsu [1], except that the lower and upper reservoir buffers contained 1 mM dithiothreitol. After preelectrophoresis, a sample of dissociated synthetase (2—3 ml, containing 20—40 mg protein) was mixed with glycerol to give a final concentration of 10% (v/v), and carefully layered on the gel column. Electrophoresis was carried out at a constant current of 50 mA. 6-ml fractions were collected.

Results and Discussion

In a previous study [1,10], we reported the resolution of dissociated fatty acid synthetase into two subcomplex bands of approximately equal intensity

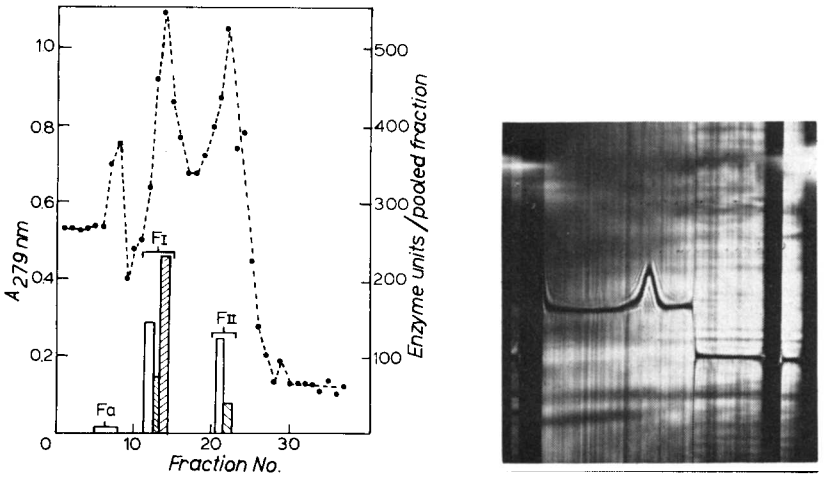

Fig. 1. Preparative disc-gel electrophoresis of dissociated fatty acid synthetase. The protein sample contained 38 mg of dissociated enzyme in a volume of 2.4 ml. Electrophoresis and enzyme assays were performed as described in Materials and Methods. Fractions 6—8 (F_a), 12—15 (F_I), and 21—23 (F_{II}) were pooled separately. F_I and F_{II} were concentrated by vacuum ultrafiltration in dialysis tubings. The acetyltransacylase □; palmityl-CoA deacylase ▨; and β-ketoacyl reductase ▩ activities of concentrated F_I and F_{II} are shown in the inset.

Fig. 2. Sedimentation pattern of concentrated F_I. Velocity sedimentation was performed at 56 000 rev./min and a bar angle of 65°. Temperature, 20° C. Sedimentation was from right to left. The picture was taken 24 min after reaching full speed.

on analytical disc-gel and acrylamide isoelectrofocusing columns. The former technique also yielded a fast anodal band, suggesting the presence of an acidic protein of low molecular weight. Further attempts were then made to isolate these protein bands on a preparative scale. Three separate protein peaks were obtained by the preparative disc-gel electrophoresis method as shown in Fig. 1. The two major peaks (F_I and F_{II}) were concentrated and examined in the analytical ultracentrifuge. F_I yielded a homogeneous 9.4 S peak (Fig. 2) indicating the presence of intact subcomplex molecules. The F_{II} fraction, however, gave three sedimenting peaks of 9 S, 6.7 S, and <3 S. Since F_{II} was initially eluted as an electrophoretically homogeneous species, it was concluded that the 9 S peak represented the intact subcomplex, which was unstable and gave rise to smaller dissociation products. The properties of these protein fragments were not further analyzed.

The enzymatic properties of concentrated F_I and F_{II} fractions are shown in the inset to Fig. 1. β-Ketoacyl reductase activity was present only in the F_I fraction, whereas acetyl transacylase and palmityl-CoA deacylase activities were found in both fractions. The ubiquitous presence of transacylase activity is unlikely to be the result of cross-contamination, since this activity was distributed approximately equally between F_I and F_{II} fractions isolated in several electrophoretic runs. The apparent paradox between this finding, which suggests the presence of transacylase component in both halves of the chicken enzyme and on the reported presence of this enzyme in only one of the two

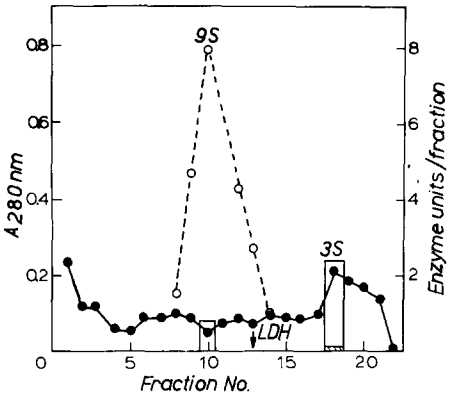

Fig. 3. Sucrose density centrifugation of aged F_I. Density gradient centrifugation was performed according to the method of Martin and Ames [14] with a Spinco SW 50L rotor in the Spinco Model L preparative centrifuge at 4°C. Gradients were made with 2.4 ml each of 25% and 10% sucrose containing 0.2 M potassium phosphate buffer, pH 7.0, and 10 mM dithiothreitol in a 5 ml Büchler linear gradient device. 0.2 ml of F_I (0.8 mg) recovered from the experiment in Fig. 2 and aged for 12 days at 4°C was layered on the sucrose gradient and centrifuged at an average speed of 39 800 rev./min for 943 min. 15-drop fractions were collected and diluted with 0.5 ml of 0.2 M potassium phosphate, pH 7.0 for protein and activity determinations. The sedimentation coefficients were estimated by comparing the distance of the protein peak (3 S) or reductase activity peak (9 S) from the meniscus against that of the lactase dehydrogenase reference (LDH) standard (7 S) [15] centrifuged simultaneously in a separate tube ●———●, 280 nm absorption; ○- - - - -○, β-ketoacyl reductase activity; □, acetyl transacylase activity; ⊠, palmityl-CoA deacylase activity.

subcomplexes of the analogous enzyme from pigeon [11] needs clarification by additional experimentation.

The homogeneous F_I fraction dissociates into smaller fragments upon aging. The sedimentation pattern of a preparation aged 10 days at 4°C showed significant reduction in the 9.4 S peak, accompanied by the appearance of a new, broad 3 S peak. The presence of these peaks were verified by the sucrose gradient experiment shown in Fig. 3. The protein peak at the bottom represents aggregation products and was not further analyzed. The intact subcomplex was not detectable by 280 nm absorption measurements, owing to its low concentration and interference by variable amounts of oxidized dithiothreitol in the samples. It was, however, located by the presence of a single β-ketoacyl reductase peak at the 9 S position which also contained acetyl transacylase activity. The palmityl-CoA deacylase activity was not detectable at this concentration. The 3 S protein peak represents dissociation products of the subcomplex including active acetyl transacylase and palmityl-CoA deacylase components which are obtained for the first time from an animal source. These results indicate, not surprisingly, that structural integrity at the subcomplex level is not required for the expression of these activities, and that active components may be readily obtained from the electrophoretically purified F_I fraction by aging. Currently, experiments are in progress to obtain preparative amounts of these components by the gel-filtration method for a study on their molecular properties.

The anodal fraction from preparative disc-gel electrophoresis (F_a in Fig. 1) was concentrated by lyophilization, and passed through a Sephadex G-25

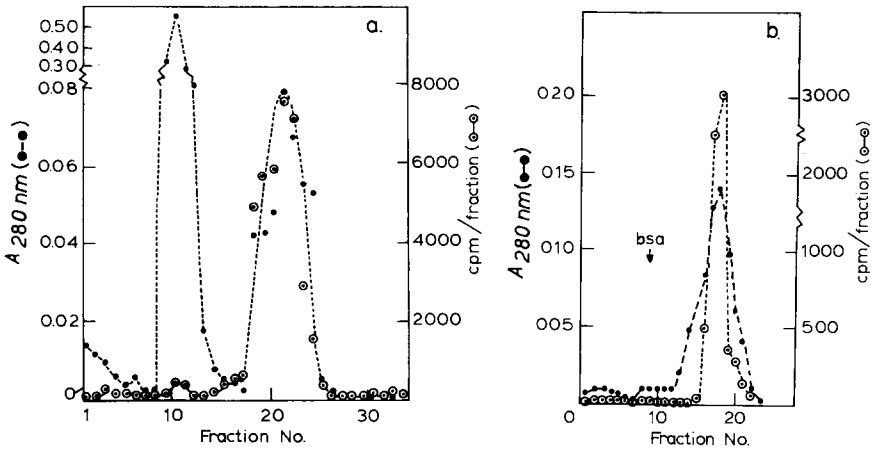

Fig. 4. (a) Binding of acetyl group by chicken liver acyl carrier protein. Purified F_a (514 μg) obtained from Sephadex G-25 column was lyophilized, and dissolved in 0.4 ml of 10 mM potassium phosphate buffer pH 7.0, containing 0.1 M KCl. Dithiothreitol (20 mM) was added. The protein solution was incubated at 30°C for 30 min and kept at 4°C overnight to effect reduction of disulfide bonds. Acetate binding was carried out by incubating the reduced protein with [1-^{14}C] acetyl-CoA (12 nmol, 1.54 × 10^5 dpm), fatty acid synthetase (20 μg), in 0.2 M potassium phosphate buffer, pH 7.0 in a volume of 1.0 ml for 5 min at 0°C. The reaction was terminated with 30 μl of 60% perchloric acid and the protein precipitate was recovered by centrifugation after the addition of 1 mg albumin as carrier. The precipitate was dissolved in 1.0 ml of potassium phosphate buffer, pH 7.0 and reprecipitated with perchloric acid as before. This procedure was repeated until all acid-soluble radioactivity (i.e. free acetyl-CoA) was removed. The labeled protein was taken up in 0.5 ml of 10 mM potassium phosphate buffer, pH 7.0 containing 1 mM dithiothreitol, and chromatographed on a Sephadex G-50 column (1 × 30 cm), equilibrated and eluted with the same buffer in 1.1 ml fractions. The elution pattern is shown in Fig. 4a. Albumin and labeled synthetase were eluted as a single peak in the void volume. ^{14}C-labeled F_a was eluted in the second peak. (b) The 4′-phospho-[1-^{14}C] pantetheine labeled protein was obtained as described in the text. After lyophilization, it was dissolved in 2.5 × Tris(5 mM)/glycine (35 mM)/EDTA (1 mM), pH 8.3 buffer containing 1 mM dithiothreitol and chromatographed on a Sephadex G-50 column (1 × 26 cm) with the same buffer. 0.85 ml fractions were collected. Arrow indicates the elution volume of bovine serum albumin (BSA).

column (0.6 × 10 cm) in the Tris/glycine/EDTA buffer. A sharp, symmetrical peak absorbing at 280 nm was obtained after the void volume, indicating the presence of a single protein of low molecular weight. This protein was concentrated as before, and tested for its ability to bind acetyl group by incubating with [1-^{14}C] acetyl-CoA in the presence of catalytic amounts of fatty acid synthetase. The reaction mixture was then freed of unbound [1-^{14}C] acetyl-CoA by repeated precipitation with perchloric acid. The protein precipitate was redissolved and rechromatographed on the Sephadex G-50 column as before (Fig. 4a). Fatty acid synthetase and carrier albumin were eluted as a single frontal peak. Nearly all radioactivity cochromatographed with the retained F_a peak, indicating the former was protein-bound as [^{14}C] acetyl-F_a. It is a small protein, eluting at the same position as bovine insulin (mol. wt 5733) [12], with a retention volume significantly larger than that of cytochrome *c* (mol. wt 12 500) [12].

Since F_a protein exhibits properties typical of the acyl carrier protein molecule [2], such as the small size, high negative charge, and the ability to bind acetate from acetyl-CoA, it was thought to be the acyl carrier protein of chicken liver. In a separate experiment, a preparation of 4′-phospho-[1-^{14}C]-

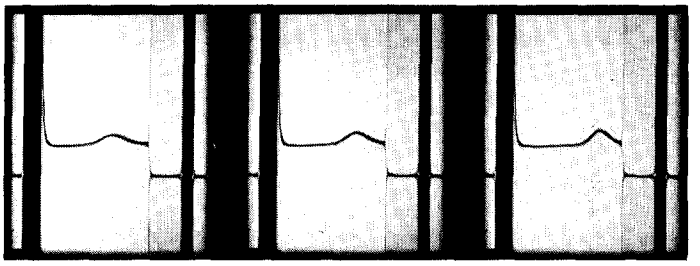

Fig. 5. Sedimentation pattern of the β-ketoacyl reductase component. The sedimentation experiment was carried out as described in Fig. 2. Sedimentation was from right to left. The pictures (from right to left) were taken at 72, 104, and 136 min after reaching full speed. This component has a sedimentation coefficient of 1.55, and catalyzed the oxidation of 29.5 nmol NADPH/min/mg protein using *S*-acetoacetyl-*N*-acetyl cysteamine as the substrate.

pantetheine-labeled fatty acid synthetase (11 mg) was subjected to preparative disc-gel electrophoresis as before. The radioactive peak appeared at the elution volume of F_a (Fig. 1). Further identification of acyl carrier protein was performed by pooling the radioactive fractions, followed by lyophilization. The lyophilized material was then subjected to Sephadex G-50 chromatography as described before. The chromatogram (Fig. 4b) showed that radioactivity was associated with a homogeneous protein of low molecular weight. The typical yield of acyl carrier protein is approx. 1.2% of purified synthetase, representing quantitative recovery on the basis of molecular weight and stoichiometry considerations [13].

While acyl carrier protein of chicken liver is readily obtained in free form by the electrophoretic technique, the analogous protein of pigeon liver is resolved as a component attached to the reductase subcomplex during affinity chromatography [10]. Notwithstanding differences in methodology, the interesting possibility of subtle species differences in the mode of attachment of this protein to the synthetase complex should be the subject of additional comparative study. The disc-gel electrophoresis technique provides a powerful tool potentially capable of resolving a number (if not all) of fatty acid synthetase components. A homogeneous β-ketoacyl reductase component (Fig. 5) was obtained immediately following the F_a fraction by electrophoresing a large amount (76 mg) of dissociated synthetase in the same buffer system, except with the omission of dithiothreitol. Moreover, when the present continuous gel system was replaced by a discontinuous system containing stacking gel, the dissociated enzyme yielded a minimum of six protein peaks. One was active in acetyl transacylation reaction, whereas two others contained palmityl-CoA deacylase activity. Work on the further refinement of this technique is currently in progress.

Acknowledgements

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