Subunits of Fatty Acid Synthetase Complexes

ENZYMATIC ACTIVITIES AND PROPERTIES OF THE HALF-MOLECULAR WEIGHT NONIDENTICAL SUBUNITS OF PIGEON LIVER FATTY ACID SYNTHETASE*

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SUMMARY

The separation of the half-molecular weight, nonidentical subunits (I and II) of the pigeon liver fatty acid synthetase complex has been achieved on a large (20 mg) scale by affinity chromatography on Sepharose ε-aminocaproyl pantetheine. This separation requires a careful control of temperature, ionic strength, pH, and column flow rate for success. The yield of subunit II is further improved by transacetylation (with acetyl-CoA) of the dissociated fatty acid synthetase prior to affinity chromatography. The separated subunit I (reductase) contains the 4'-phosphopantetheine (A*) acyl binding site, two NADPH binding sites, and β-ketoacyl and crotonyl thioester reductases. Subunit II (transacylase) contains the B1 (hydroxyl or loading) and B2 (cysteine) acyl binding sites, and acetyl- and malonyl-CoA:pantetheine transacylases. When subunit I is mixed in equimolar quantities with subunit II, an additional NADPH binding site is found even though subunit II alone shows no NADPH binding. Both subunits contain activities for the partial reactions, β-hydroxybutyryl thioester dehydrase (crotonase) and palmityl-CoA deacylase. Subunit I has 8 sulfhydryl groups per mol whereas subunit II has 60. Reconstitution of fatty acid synthetase activity to 75% of the control level is achieved on reassociation of subunits I and II.

We previously published a preliminary communication (2) on the separation of the two nonidentical, half-molecular weight subunits of the pigeon liver fatty acid synthetase complex. In this separation we employed an affinity gel column containing ε-aminocaproyl pantetheine bound to Sepharose via the ε-amino group. (The esterified pantetheine is essential for the separation of the two nonidentical subunits.) The subunit containing β-ketoacyl thioester reductase activity (subunit I) passed through the column at pH 8.4, whereas the subunit containing acetyl-CoA:pantetheine transacylase activity (subunit II) was bound selectively to the column. Subunit II was eluted from the column with buffer at pH 10 after subunit I was collected. Final purification of each subunit was accomplished by sucrose density gradient centrifugation after reassociation of the small amount of contaminating half-molecular weight subunit with the major product of the separation. In the present paper we describe in greater detail the procedure for the preparation of the affinity column and the conditions required for larger scale separation of the subunits.

The complete separation of the two subunits has permitted the determination of the subunit location of the acetyl and malonyl binding sites reported in our previous publications (3–6). The separation of the subunits has also permitted the determination of NADPH binding properties of each subunit. Previously a determination of the NADPH binding properties of the dissociated subunits was not possible because NADPH interacts directly with the two subunits to reconstitute the fatty acid synthetase complex (7). For this reason a NADPH ligand was not chosen for the affinity column used in the separation of the subunits.

The partial reactions of fatty acid synthesis that are associated with each subunit of the pigeon liver fatty acid synthetase have also been determined. The partial reactions are assayed with the substrates acetyl-CoA, malonyl-CoA, N-acetylcysteamine S-, β-hydroxy-, α,β- and α-ε-enoxy-acyl esters, and palmitoyl-CoA. These substrates were used in the following reactions (Equations 1 to 5).

[Chemical equations are presented here, likely involving mechanisms of reductase and transacylase actions.]

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Properties of the subunits on disc gel electrophoresis, their stability for sulfhydryl groups of each of the subunits are also reported. 20 mg, and one with 40 mg of the carbodiimide. Excess pantetheine was reassociated to enzymatically active fatty acid synthetase. Analyses in dithiothreitol and P-mercaptoethanol, and their ability to precipitate of the protein with trichloroacetic acid (10). Acetyl-CoA:pantetheine transacylase and malonyl-CoA:pantetheine transacylase activities were assayed as previously described (2). Beta-Ketoacyl thioester reductase was assayed as described previously (5), except that the level of thioester substrate was 2 mM rather than 10 mM. Beta-hydroxybutyryl thioester dehydrogenase was assayed according to the spectrophotometric method of Kumar et al. (5) at 270 nm, but at a level of thioester of 7 mM and a level of protein of approximately 15 to 30 µg/ml. Crototyl thioester reductase was assayed spectrophotometrically according to the method of Kumar et al. (5), at a level of thioester of 4 mM and a concentration of protein between 15 and 50 µg/ml.

Titration of the NADPH binding sites of fatty acid synthetase and its subunits I and II was carried out by measuring the fluorescence produced in an Amino-Bowman spectrophotometer. The procedure of Dugan and Porter (11) was used, except that titrations were carried out by adding NADPH to protein (according to the method of Hsu and Wagner (12)) rather than protein to NADPH. This procedure was used because of difficulty in obtaining protein in concentrations sufficient to add in small volume increments. The titrations were carried out in low ionic strength buffer consisting of Tris-glycine-EDTA-beta-mercaptoethanol (5, 35, 1, and 1 mM, respectively). A fluorescence curve was obtained for NADPH in the absence and presence of each protein subunit and the enzyme complex. Higher protein and substrate levels than reported by Dugan and Porter (11) were used because of the relatively weak binding of NADPH to one of the sites on the separated subunit I. The values observed for total fluorescence were corrected by the method of Udenfriend (13). These data (Fig. 6) were then used to calculate, by the methods of Dugan and Porter (11) and Hsu and Wagner (12), the moles of NADPH bound per mol of protein.

Sulfhydryl estimations were carried out according to the method of Ellman (14).

RESULTS

Preparation of Affinity Gel: Coupling of Pantetheine versus Concentration of 1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide—1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide. 10 to 40 mg, was dissolved in a minimum volume (10 to 40 µl) of water and the pH of the solution was adjusted to 4.75 (pH paper) with 1 N HCl. Sepharose e-aminoacapric acid (0.3 g) was washed in a separate flask and then suspended in a solution of 0.25 ml of 0.17 M pantetheine and 0.25 ml of water. The carbodiimide solution was added to the Sepharose e-aminoacapric acid slurry. The pH of the slurry was adjusted to 4.7 with HCl over a period of 24 h, after which no further change occurred. The slurry was then stirred overnight (18 hours). Three such batches of Sepharose e-aminoacapryl pantetheine were prepared: one with 10 mg, one with 20 mg, and one with 40 mg of the carbodiimide. Excess pantetheine was removed by thoroughly washing the gel with water, and then the gel was resuspended in water. Ten microliters of 1 M KOH were added to 0.1 ml aliquots of the slurry, and each was kept at 25°C for 10 min. The aliquots were neutralized with 10 µl of 1 N HCl and the sulfhydryl content of the supernatant solution was determined by the Ellman method (14) after centrifugation to remove suspended gel particles. The amounts of pantetheine bound per g of gel were 1.4, 1.7, and 2.09 µmol, respectively. On the basis of these data, which indicate that the amounts of carbodiimide used are in the range of saturation of the carboxyl groups, large scale affinity columns were prepared with 100 mg of carbodiimide per g of gel in 100 µl water. Sepharose e-aminoacapric acid with pantetheine omitted but treated with these quantities of carbodiimide showed little or no nonspecific binding of fatty acid synthetase.

Separation of Subunits I and II on a Preparative Scale—Sepharose e-aminoacapryl pantetheine, 6.0 g, was prepared by adding 600 mg of 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide in 0.6 ml of H2O to 6 g of Sepharose e-aminoacapric acid suspended in 6 ml of 0.08 M pantetheine. The pH was adjusted to 4.7 and...
The mixture stirred for 18 hours at 25°. The gel was thoroughly washed with 1 liter of water and with 100 ml of a buffer, pH 8.4, consisting of 10 ml of 1 M Tris (free base), 10 ml of 1 M potassium phosphate, pH 7.0, 1 ml of 0.1 M EDTA, and 79 ml of distilled water. The buffer also contained 0.0025 M β-mercaptoethanol. The gel was poured into a glass-jacketed column (21 cm × 8.5 mm, inside diameter). Ice water from an ice-salt-ethanol mixture was circulated through the glass jacket of the column at a temperature of 10°. The whole assembly (column, pump, ice water reservoir, and fraction collector) was kept in a cold box at 0°. However, the box warmed up to 8° when the column was being loaded.

Twenty milligrams of fatty acid synthetase protein (2 ml) were dialyzed in 125 ml of 5 mM Tris, 35 mM glycine, 1 mM EDTA, and 2.5 mM β-mercaptoethanol for 3 hours with one or two changes of buffer. The dialysis blank was kept in ice. The fatty acid synthetase solution, diluted with an equal amount of Tris-glycine buffer, was then loaded onto the column at a rate of 4 ml per hour. Elution of subunit I (β-ketoacyl thioester reductase) was carried out at a rate of 4 ml per hour, with the pH 8.4 buffer used to wash the affinity gel. One-milliliter fractions were collected at 0°. Subunit I was completely eluted with the passage of 40 ml of buffer. The column was then removed from the cold box to allow the temperature to rise to 25°. Subunit II (acetyl- and malonyl-CoA transacylases) was removed from the column with the Tris-phosphate-EDTA-β-mercaptoethanol-ammonia buffer as previously described (2). Three-milliliter fractions were collected at a rate of 18 ml per hour, and all of subunit II was removed in approximately 30 ml of buffer. As soon as the fractions were collected they were neutralized with 1 M monobasic potassium phosphate (100 μl/ml of fraction) to pH 7.5. The separation and activities of subunits I (β-ketoacyl thioester reductase) and II (acetyl-CoA:panthetheine and malonyl-CoA:panthetheine transacylases) are shown in Fig. 1. The yield of subunit I was about 9.0 mg (70 to 90% free from subunit II) and the yield of subunit II was about 2.5 mg (70 to 90% free from subunit I) in a typical preparation. The best separations were obtained when fatty acid synthetase was stored at −60° for 6 weeks prior to use. Fatty acid synthetase stored at −20° did not yield nearly as good a separation.

Fatty acid synthetase was incubated with [1-14C]acetyl-CoA previous to the affinity column separation in order to identify the acetyl binding sites of the subunits. When this was done it was found that acetyl binding increased the yield of subunit II on affinity chromatography. Fifteen milligrams of fatty acid synthetase (33 nmol), dialyzed and dissociated as described above, were incubated with 180 nmol of [1-14C]acetyl-CoA (4.5 × 10^4 dpm) in a total volume of 3 ml at 0° for 2 min, and then loaded onto a column prepared as described above. The yield of subunit I was 7.7 mg, and the yield of subunit II, free from subunit I, was 5.0 mg.

**Sucrose Density Gradient Centrifugation**—In a typical separation, Fractions 12 to 25 containing subunit I (Fig. 1) were recombined and then dialyzed 1.5 hours at room temperature in 0.2 M potassium phosphate buffer containing 1 mM EDTA and 10 mM dithiothreitol to effect reassociation of any small amount of still unseparated subunit II with an equivalent amount of subunit I. The dialysate was then concentrated to 1.5 ml by means of an Amicon Diaflo PM-10 ultrafilter. The concentrate, in batches of 5 mg in 2 ml of buffer, was then loaded on sucrose density gradient tubes prepared from 19 ml of 57% sucrose and 19 ml of 10% sucrose in a buffer consisting of 0.1 M potassium phosphate, pH 7.0, 2.5 mM β-mercaptoethanol, and 0.5 mM EDTA. Centrifugation was carried out at 10° at 50,000 × g for 24 to 26 hours.

The same procedure was carried out with the subunit II fraction, except that dithiothreitol was used in place of β-mercaptoethanol. When studies on subunit I were to be performed, all of the previous steps in the preparation of this fraction were carried out in β-mercaptoethanol. When studies on subunit II were to be performed, all previous steps were carried out in dithiothreitol.

Fig. 2A and B shows the further purification and enzyme activities of subunits I (reductase) and II (transacylase). It should be noted (see enzyme activities) that in each centrifugation the major subunit occurred in excess of the minor subunit in the 14 S peak. In the centrifugation of subunit I there was an excess of this subunit (β-ketoacyl thioester reductase activity) over subunit II,
was cut into 0.5 cm strips and radioactivity was determined in a half of the electrophoresis paper by means of a Packard radio

pH 3.7 at 2500 volts for 90 min as reported by Chesterton et al. and then spotted on paper prior to high voltage electrophoresis at the 9 S subunits are capable of forming 14 S complexes with 0.01 N HCl, and then digested with 0.25 mg of pepsin in 0.1 ml and concentrated with an Amicon PU10 ultrafilter. The protein over subunit I in the 14 S peak, even though the amounts of I and II should be equal in the 14 S complex. This indicates that the 9 S subunits are capable of forming 14 S complexes with themselves. This phenomenon was not observed at lower protein levels (2 mg/2 ml sample). Further questions relating to this phenomenon will be presented under “Discussion.”

Analysis of [1-14C]Acetyl-bound Peptic Peptides—Dissociated fatty acid synthetase was incubated with [1-14C]acetyl-CoA and then passed through an affinity column. Seven milligrams of subunit I, obtained by this procedure, were subjected to sucrose density gradient centrifugation as described above. The fractions in the 9 S subunit I peak (tubes 18 to 22) (Fig. 2A) were combined and concentrated with an Amicon PM-10 ultrafiltrator. The protein was precipitated with perchloric acid, suspended in 0.2 ml of 0.01 N HCl, and then digested with 0.25 mg of pepsin in 0.1 ml of 0.01 N HCl (for 18 hours at 30°C). The hydrolysate was lyophilized and then spotted on paper prior to high voltage electrophoresis at pH 3.7 at 2500 volts for 90 min as reported by Chesterton et al. (9). The [1-14C]acetyl-labeled components were located on one-half of the electrophoresis paper by means of a Packard radio-active strip scanner. The other half of the electrophoresis paper was cut into 0.5 cm strips and radioactivity was determined in a Packard Tri-Carb liquid scintillation spectrometer. The plot of radioactivity versus distance from the origin on the paper strips is shown in Fig. 3A. The peaks closest to the origin correspond to the A2 peptides reported by Jacob et al. (3) and by Kumar et al. (5). The intact half of the electrophoresis paper was kept in an evacuated dessicator for 19 hours, along with a beaker containing 20 ml of performic acid solution prepared according to the method of Moore (15). The performic acid was first chilled to 0°C before the beaker was placed in the desiccator, and the latter was then evacuated. Under these conditions, 90% of the radioactivity was removed from each peak (Fig. 3A), thereby indicating the volatility of [1-14C]acetyl-CoA:pantetheine transacylase): light absorbance, 280 nm (○—○); β-ketoacyl thioreductase: light absorbance, 280 nm (Q—Q); 3H-labeled pantetheine in counts/min/ml (X—X); reduce tase activity in nanomoles of NADPH oxidized/min/ml (△—△); acetyl-CoA:pantetheine transacylase activity in nanomoles of product formed/min/ml (●—●); malonyl-CoA:pantetheine transacylase activity in nanomoles of product formed/min/ml (□—□);*, reductase activity in 0.5 mg of protein of pure subunit I (70 nmol of NADPH oxidized/min); **, acetyl-CoA:pantetheine transacylase activity in 0.5 mg of protein of subunit II (170 nmol of product formed/min); ***, malonyl-CoA:pantetheine transacylase activity in 0.5 mg of protein of subunit II (104 nmol of product formed/min).

and in the centrifugation of the subunit II there was an excess of this protein (acetyl- and malonyl-CoA transacylase activities) over subunit I in the 14 S peak, even though the amounts of I and II should be equal in the 14 S complex. This indicates that subunit II is not bound to subunit II via a thioester linkage.

Further confirmation of the presence of the 4′- phosphopantetheine group (the A2 binding site) (3) on subunit I was obtained by pepsin digestion of subunit II obtained from [14C]pantetheine-labeled fatty acid synthetase (2). Fig 3B. The [14C]labeled pantetheine peptides migrate similar to the [1-14C]acetate labeled pantetheine peptides obtained from [1-14C]acetyl-labeled enzyme on electrophoresis.

The analysis of [1-14C]acetate-bound peptic peptides of subunit II could not be carried out in a similar manner because subunit I lost [1-14C]acetate groups on elution from the affinity gel with the ammonia buffer. Instead, [1-14C]acetyl-CoA was incubated with this subunit after it was eluted from the affinity column. Pepsin digestion and high voltage electrophoresis were then carried out under the same conditions as reported for subunit I. The plot of radioactivity versus distance from the origin is shown in Fig. 3C. The major radioactive peaks correspond to the B1 peaks reported by Chesterton et al. (4). Performic acid treatment of the intact strip under exactly the same conditions as used for subunit I resulted in the retention of 98% of the radioactivity originally on the strip, indicating that the [1-14C]acetate groups are not bound to subunit II via a thioester linkage.

The above results are further evidence that the A2 binding site is on subunit I (reductase) and that the B1 (hydroxyl) binding site is on subunit II (transacylase).

The electrophoresis of the [1-14C]pantetheine-labeled peptides was carried out at a later time than the electrophoresis of the [1-14C]acetyl labeled peptides shown in Fig. 3A, C, D, and E. The procedure used for the pantetheine-labeled peptides was slightly different in that excess moisture in the electrophoresis paper was removed by blotting, after wetting the paper with buffer, whereas previously the excess moisture was simply drained off. This change in procedure may account for the difference between migration rates of the [1-14C]acetyl-labeled peptides shown in Fig. 3A, D, and E and the [1-14C]pantetheine-labeled peptides shown in Fig. 3B.
Fig. 3. Electrophoresis of [1-\(^{14}\)C]acetyl-labeled peptic peptides before and after performic acid oxidation. A, a peptic digest of [1-\(^{14}\)C]acetyl-labeled (72,000 dpm) subunit I. (Labeled subunit I was obtained from fatty acid synthetase labeled before passage through the affinity column.) Subunit I was purified by affinity chromatography and then by sucrose density gradient centrifugation. Electrophoresis was carried out as given in the text. B, a peptic digest of \(^{14}\)C]pantetheine-labeled subunit I (8,000 dpm). C, a peptic digest of subunit II. The latter was separated by affinity chromatography and then reacted with [1-\(^{14}\)C]acetyl-CoA. Transacylase protein (1 mg) was reacted with 6 nmol of [1-\(^{14}\)C]acetyl-CoA (300,000 dpm) in 1 ml of 1 mM EDTA, pH 7, at 0° for 1.5 min. The protein was then precipitated with 40 \(\mu\)l of 60\% HClO\(_4\), washed four times with 0.4 ml of ice-cold 2 n acetic acid, and suspended in 0.2 ml of 1.0 \(n\) HCl containing 0.2 mg of pepsin. The remainder of the procedure was carried out as in A. D and II were recombined in 0.1 M potassium phosphate containing 10 mM dithiothreitol, reacted with [1-\(^{14}\)C]acetyl-CoA (100,000 dpm), and then digested with pepsin. E, subunit II was reacted with [1-\(^{14}\)C]acetyl-acyl carrier protein (obtained from pigeon liver, 25,000 dpm) and then digested with pepsin. The profile of radioactivity in 1 \(\times\) 2-cm strips is given prior to performic acid treatment (A to E, \(\square\)), and after treatment (A, C, and D, \(\blacksquare\)).
Finally, an equimolar mixture of subunits I and II was dialyzed in 0.2 M potassium phosphate buffer, pH 7.0, 1 mM EDTA and, 10 mM dithiothreitol for 4 hours at room temperature, and then incubated with [1-W]acetyl-CoA. The protein was precipitated, washed, and treated with pepsin in 0.01 N HCl as before. Fig. 3D reports the electrophoretograms before and after performic acid treatment. In addition to the A1 and B1 peaks, a B2 peak appeared. The appearance of the B2 peak indicates that reassociation and reactivation (7) of the enzyme have taken place.

The location of the B2 site could not be determined by examining the acetyl-labeled subunits I and II alone. The B2 site accepts acetyl groups only when the fatty acid synthetase is in the undissociated form. Hence, the subunits could not be labeled after dissociation of the complex. We found, too, that dissociated [1-14C]acetyl-labeled fatty acid synthetase (prepared for affinity chromatography) loses acetyl groups from the B2 site either on or prior to affinity chromatography. This was not unexpected inasmuch as the B2 site would lose acetyl groups if it were located on subunit II on elution of this subunit from the gel at pH 10.

During the course of this investigation [1-14C]acetyl-labeled acyl carrier protein (obtained from the pigeon liver fatty acid synthetase complex) became available to us (16). Therefore, the following experiment was carried out to determine whether the B2 site is located on subunit II. [1-14C]Acetyl-labeled acyl carrier protein (25,000 dpm) was dialyzed 2 hours with 1 mg of subunit II in 0.2 M potassium phosphate and 5 mM dithiothreitol at 25°.

The protein was digested with pepsin and subjected to electrophoresis as described previously. Under these conditions a B2 peak, as well as a B1 peak, appeared (Fig. 3E). These results demonstrate the reversibility of the transfer of acetyl groups between the B1 and A2 sites (17) as well as the location of the B2 site on subunit II.

**Disc Gel Electrophoresis of Subunits**—Disc gels were prepared by the method of Hedrick and Smith (18). Fatty acid synthetase (freshly prepared and dialyzed 14 hours in 0.2 M phosphate buffer, containing 1 mM EDTA, and 1 mM dithiothreitol, after the final ammonium sulphate precipitation step), 100 μg of protein; fatty acid synthetase (freshly prepared and dissociated by 4 hours of dialysis and two changes of 5 mM Tris-35 mM glycine-1 mM EDTA buffer, pH 8.5, at 0°), 100 μg; subunit I, 100 μg; subunit II, 100 μg; and a mixture of the two subunits treated under conditions effecting reassociation, 100 μg of protein, were each loaded onto gels (Fig. 4A and B). The intact complex and the dissociated fatty acid synthetase each yielded two similar protein staining bands. This result indicates the interconvertibility of these components either during handling or on electrophoresis. (It was necessary to load the intact complex and the dissociated fatty acid synthetase under different buffer conditions (see legend, Fig. 4 in Ref. 7) which accounts for the slight difference in migration values of the two pairs of bands.) Subunits I and II gave single bands on electrophoresis (Fig. 4B), each of which moved ahead of the slower moving fatty acid synthetase band. The behavior of these components on disc gel indicates their homogeneity and the presence of identical or nearly identical charges on the two halves of the complex. The reconstituted complex of subunits I and II gave two bands that were similar to those produced by intact fatty acid synthetase and a third band that appeared to be a higher polymer.

**Stability of Reductase and Transacylase Activities in Subunits I and II**—Fig. 5A shows the decrease in specific activities of β-ketoacyl thioester reductase in subunit I and acetyl-CoA pantetheine transacylase in subunit II with time in 6 mM β-mercaptoethanol. Fig. 5B shows the decrease in specific activities of the same enzymes with time in 5 mM dithiothreitol. It is evident from these
results that the reductase activity is more stable in β-mercaptoethanol than it is in dithiothreitol. The reverse is true for transacylase activity.

**NADPH Binding to Subunits I and II and Reconstituted Fatty Acid Synthetase**—When 2 nmol of subunit I were titrated with NADPH the binding of more than 1 mol NADPH/mol of protein could not be accurately determined. However, when the amount of protein was increased to 5.90 nmol and the amount of ligand increased correspondingly, the apparent binding of 1.6 mol NADPH/mol of protein was attained (Fig. 6; see also “Discussion”). Subunit II, on the other hand, showed no NADPH binding. The fluorescence of NADPH was not enhanced by subunit II in the absence of subunit I. However, an equimolar mixture of subunit I and subunit II bound 3.2 mol NADPH/mol of protein as compared to 3.1 mol per mol of native fatty acid synthetase complex.

A study of the effect of removal of subunit II from subunit I on the reactivity of NADPH as a substrate for the two reductases was also carried out. The slopes for double reciprocal plots of velocity versus NADPH concentration are the same for β-ketoacyl thioester reductase in the complex and in subunit I (Fig. 7A). However, the slopes for crotonyl thioester reductase activities in subunit I and in the fatty acid synthetase complex differ significantly at low concentrations of NADPH (Fig. 7B).

**Table I**

<table>
<thead>
<tr>
<th>Reaction</th>
<th>Dissociated complex</th>
<th>Subunit I</th>
<th>Subunit II</th>
</tr>
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<tr>
<td>β-Ketoacyl thioester reductase</td>
<td>70</td>
<td>156</td>
<td>0</td>
</tr>
<tr>
<td>Acetyl transacylase</td>
<td>170</td>
<td>0</td>
<td>350</td>
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<tr>
<td>Malonyl transacylase</td>
<td>100</td>
<td>0</td>
<td>210</td>
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<td>Crotonyl thioester reductase</td>
<td>120</td>
<td>246</td>
<td>0</td>
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<tr>
<td>β-Hydroxyacyl thioester dehydrase</td>
<td>265</td>
<td>270</td>
<td>273</td>
</tr>
<tr>
<td>Palmitoyl-CoA deacylase</td>
<td>18.0</td>
<td>18.0</td>
<td>15.5</td>
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</table>

**Partial Reactions of Separated Subunits**—Table I reports the specific activities of the partial reactions obtained with purified subunits I and II and the dissociated fatty acid synthetase complex. β-Ketoacyl thioester reductase and crotonyl thioester reductase activities were found only in subunit I whereas acetyl-
CoA:pantetheine and malonyl-CoA:pantetheine transacylase activities were found only in subunit II. The other partial reactions, \( \beta \)-hydroxybutyryl thioester dehydrase (crotonase) and palmitoyl-CoA deacylase were found in both subunits. The specific activities for crotonyl thioester reductase and for the dehydrase are on the order of 100 times those expected from previous studies (5). The velocities obtained approach the same order of magnitude as that for \( \beta \)-ketoacyl thioester reductase activity. However, high specific activities are obtained only with freshly prepared and rechromatographed S-\( \beta \)-hydroxybutyryl-N-acetyllyslyeamine and S-crotonyl-N-acetylyslyeamine (see "Methods"). If either of these substrates is left at 0°C overnight, or frozen for 24 hours and then thawed, the activities obtained approach the low levels previously reported (5). Evidently the substrates either decompose or they are converted to a product, or products, which is inhibitory. The nature of this possible inhibitor is under investigation.

The specific activity obtained for \( \beta \)-ketoacyl thioester reductase was similar to that previously found (5). The specific activities for \( \beta \)-ketoacyl thioester reductase, transacylase, and palmitoyl-CoA deacylase activities in the separated subunits were those expected from the specific activities found for the dissociated fatty acid synthetase. Thus \( \beta \)-ketoacyl thioester and crotonyl thioester acyl-CoA:pantetheine and malonyl-CoA:pantetheine transacylase specific activities in the individual subunits I and II and in the complex.

**Analysis for \( \text{---SH} \) Groups**—Subunits I and II were dialyzed 2 hours in 5 mM \( \beta \)-mercaptoethanol or dithiothreitol, respectively, and then 5 hours in water (under nitrogen). Aliquots of the dialyzed protein solutions were separated from the last traces of low molecular weight thiols by precipitation with 5% trichloroacetic acid. The precipitate was dissolved in a minimum amount of 0.1 N KOH (20 \( \mu \)l) and the solution was diluted to 0.2 ml with 0.2 M potassium phosphate buffer, pH 7. Ellman's reagent was added to the buffer solution. Full color development occurred within 1 min with the trichloroacetic acid precipitated subunits and with trichloroacetic acid precipitated standard fatty acid synthetase. Similar results were obtained with unpurified fatty acid synthetase in the presence of 2.5 M guanidine (19). Only with undissociated fatty acid synthetase was there a delay in reaching maximum color development as found by Kumar et al. (19). Table II shows that most of the \( \text{---SH} \) groups are in subunit II of the fatty acid synthetase complex.

**Reconstitution of Fatty Acid Synthetase Complex from Subunits I and II**—Subunits I and II were obtained by affinity gel separation and then subjected to final purification by sucrose density gradient centrifugation. Fatty acid synthetase, used as a control, was kept for 2 days at 1°C in the buffer used for affinity gel chromatography. It was then concentrated by ultrafiltration and subjected to sucrose density gradient centrifugation.

An equimolar mixture of subunits I and II was dialyzed 4 hours in 0.2 M potassium phosphate buffer, pH 7.0, containing 1 mM EDTA and 40 mM dithiothreitol. Purified subunits I and II were also treated in the same manner. The four protein samples were assayed spectrophotometrically for fatty acid synthetase activity. The results of these assays, along with the assay of freshly prepared pigeon liver fatty acid synthetase, are shown in Table III. Control fatty acid synthetase had 75% of the specific activity of the freshly prepared enzyme, whereas the reconstituted fatty acid synthetase had 80% of the activity found for the control (Table III), or 64% of that of the freshly prepared enzyme.

**Table II**

<table>
<thead>
<tr>
<th>Preparation</th>
<th>( \text{---SH groups/mol}^{a} )</th>
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<tbody>
<tr>
<td>Fatty acid synthetase, trichloroacetic acid precipitation</td>
<td>63</td>
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<tr>
<td>Fatty acid synthetase, native</td>
<td>39-56</td>
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<tr>
<td>Fatty acid synthetase, in guanidine</td>
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<tr>
<td>Subunit I</td>
<td>8</td>
</tr>
<tr>
<td>Subunit II</td>
<td>60</td>
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*Based upon a molecular weight of 450,000 for the fatty acid synthetase complex.

**Table III**

<table>
<thead>
<tr>
<th>Protein sample</th>
<th>Protein</th>
<th>NADPH oxidation</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Freshly prepared fatty acid synthetase</td>
<td>1.0</td>
<td>1220</td>
</tr>
<tr>
<td>2. Subunit I</td>
<td>0.34</td>
<td>0</td>
</tr>
<tr>
<td>3. Subunit II</td>
<td>0.64</td>
<td>0</td>
</tr>
<tr>
<td>4. Control fatty acid synthetase</td>
<td>0.44</td>
<td>980</td>
</tr>
<tr>
<td>5. Mixture of subunits I and II</td>
<td>0.44</td>
<td>780</td>
</tr>
</tbody>
</table>

**DISCUSSION**

A number of factors are critical to the obtainment of a successful separation of subunits I and II on an affinity column. The first of these factors is the amount of carbodiimide used for forming the thioester linkage of pantetheine to Sepharose \( \epsilon \)-aminocaproic acid. If too high a concentration of carbodiimide is used (300 mg/g of gel), subunit I is not eluted at 0°C with the 0.1 M Tris-phosphate, pH 8.4, buffer. That this tight binding does not depend on pantetheine thioester is borne out by the fact that fatty acid synthetase still binds to the gel when pantetheine is omitted from the otherwise complete carbodiimide coupling reaction. When lower concentrations of carbodiimide (100 mg/g of gel) are used and pantetheine is omitted from the coupling mixture, fatty acid synthetase passes through the column in the void volume. The reason why strong, nonspecific binding of fatty acid synthetase occurs at 300 mg of carbodiimide/g of Sepharose and not at 100 mg/g is not clear.

The concentration of pantetheine is of course also a factor in determining the composition of the affinity gel at the end of the reaction period. However, pantetheine is used in large excess in this reaction. Hence, the expense and availability of this compound are the determining factors in the concentration chosen.

During the time the fatty acid synthetase is loaded onto the affinity gel and then separated into subunits, the temperature of the column must be kept near 0°C in order to maintain complete dissociation of the complex (5, 7). At the same time, the flow rate is kept low to extend the time of reaction of protein with the substrate analog. With a faster rate, resolution is poor or both subunits pass through the gel without binding.

In addition to the requirements of composition of the affinity
columns and of temperature and flow rate, there also appears to be a conformational requirement for the binding and elution of subunit II. We have already reported (2) that if fatty acid synthetase is dissociated without prior freezing, the dissociated subunits are not eluted from the Sepharose substrate analog at 0°. Likewise, if the subunits are dialyzed at 4° for too long a time (36 hours), even with full protection with dithiothreitol against oxidation, they are not eluted from the column at 0°.

It is of interest to note that when the dissociated fatty acid synthetase was treated with acetyl-CoA before loading onto the affinity gel, all of subunit I (reductase) was eluted in the first elution step, thereby leaving subunit II (transacylase) completely free of subunit I. Also, under these conditions 67% of subunit II was eluted from the column. In contrast, without prior acetyl-CoA treatment, some of subunit I remains on the column until subunit II is eluted in the second step of the procedure. Under these conditions much less of subunit II is eluted from the column (25%). We can conclude that blocking of the A2 and B2 sites with acetyl groups prevents in large measure an irreversible binding of subunit II to the column.

The formation of homodimers by the subunits, as noted on sucrose density gradient centrifugation, is a hitherto unobserved phenomenon. However, the formation of the hetero-complex is much more favored when both subunits are present under conditions favoring association of subunits. The presence of homodimers and higher polymers of subunits I and II in 0.2 M phosphate buffer was also demonstrated by gel filtration on Bio-Gel A-0.5m.

The results on acyl binding sites on the subunits confirm our previous reports (3, 4, 6) that the A2 binding site is identical to the 4'-phosphopantetheine site. In addition they confirm the presence of the A1 site on subunit I. The B1, or loading (serine) site is on subunit II. The B2 (cysteine) site is also on subunit II, as is shown by the ability of the subunit to accept an acyl group from acetyl-labeled acyl carrier protein. This transfer normally takes place only under conditions of association of the fatty acid synthetase. In the present study [14C]acetyl-l-acyl carrier protein was used to effect this transfer.

Subunits I and II have the same Rf values on disc gel electrophoresis. Therefore, these subunits have the same charge as well as the same molecular size. Theappearance of a slow moving band with the proper Rf value on disc gel electrophoresis after incubation of an equimolar mixture of subunits I and II demonstrates the reassociation of the separated subunits to the fatty acid synthetase complex. Reassociation of the subunits was also demonstrated by recovery of fatty acid synthesizing activity.

The NADPH binding studies indicate that NADPH has affinity for two sites on subunit I in the absence of subunit II. This conclusion is based on the calculation from the maximum fluorescence enhancement (Fig. 6) of 1.6 mol of NADPH bound per mol of protein. The affinity of NADPH for one of these sites is low, as binding is incomplete even at a NADPH concentration of 25 μM, which is a 5-fold excess over the protein concentration.

That the weaker binding site is associated with crotonyl thioester reductase activity is suggested by the difference in the double reciprocal plots of velocity versus concentration of crotonyl thioester substrate in the fatty acid synthetase complex and in subunit I. With the intact complex the slope decreases at low concentrations of NADPH, whereas with subunit I it remains constant. In contrast, the double reciprocal plot for β-ketoacyl thioester reductase activity is not altered by the absence of subunit II.

The third binding site does not appear until subunit II (which by itself does not bind NADPH) is present. This fact indicates that a mutual interaction of the two subunits with NADPH is required to demonstrate the binding site.

The location of crotonyl thioester and β-ketoacyl thioester reductase activities in subunit I was expected since subunit I had been shown to have two NADPH binding sites. Similarly, we expected that malonyl-CoA:pantetheine transacylase activity would be present in subunit II, along with acetyl-CoA:pantetheine transacylase activity because only subunit II has a hydroxyl or "B1 loading" site (17). Of some surprise is the existence of deacylase activity in both subunits. The appearance of deacylase activity in both subunits is consistent with the results of Kuman (20) who demonstrated that 2 eq of phenylacetylglycine fluoride per mol of complex are required to completely block deacylase (and concomitantly fatty acid synthetase) activity in pigeon liver fatty acid synthetase.

The function of two deacylase sites in a fatty acid synthetase complex is not clear. The function of the deacylase within the complex is the hydrolysis of a palmityl group from the 4'-phosphopantetheine site (3, 17). Hydrolysis of the palmityl 4'-phosphopantetheine at the completion of palmitate formation is of course a critical step in making the 4'-phosphopantetheine site ready for a new, entering acyl group. It is possible that for hydrolysis to occur the bulky palmityl and attached carboxyl thioester group must be in a specific spatial orientation with respect to a deacylase site and that two such sites are provided within the complex.

The existence of two dehydrase sites per fatty acid synthetase molecule has not previously been shown. However, the alternative possibility that there is only one dehydrase site for the fatty acid synthetase complex cannot be ruled out. If there is only one site, this activity separates equally when the fatty acid synthetase is dissociated to subunits I and II.

The partial activities of the pigeon liver fatty acid synthetase are somewhat more stable in the complex than in the separated subunits. β-Mercaptoethanol, but not dithiothreitol, partially stabilizes β-ketoacyl thioester reductase in subunit I, whereas dithiothreitol, but not β-mercaptoethanol, partially stabilizes the transacylase in subunit II. However, the stabilization of each of these subunits is of the order of less than 5 days, while the fatty acid synthetase complex stored at the same temperature in dithiothreitol is stable for several weeks. Also of interest is the difference in character of the inactivation curves of reductase in β-mercaptoethanol and dithiothreitol. Inactivation in β-mercaptoethanol appears linear with time, whereas inactivation in dithiothreitol appears exponential, although a plateau is reached at about 30% of the original activity. These differences suggest a relatively complex sequence of conformational changes leading to inactivation.

The observation that most (60 out of 68) of the thiol groups of the pigeon liver fatty acid synthetase complex are on subunit II makes the question of the role of the thiol groups in the fatty acid synthetase rather intriguing. Obviously, the thiol groups are not involved in cross-linked tertiary structure, inasmuch as reversible dissociation and reassociation take place in the presence of dithiothreitol when the thiol groups are in the reduced form (21).

The demonstration of an 80% recovery of fatty acid synthetase activity, based on the activity of a control, on reassociation of subunits I and II demonstrates that the fatty acid synthetase has been dissociated and separated under very mild conditions with a minimum of irreversible conformational change. The retention of activities by the dissociated fatty acid synthetase and the...
separated subunits for the partial reactions of fatty acid synthesis confirms this conclusion.

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F A Lornitzo, A A Qureshi and J W Porter


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