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Fatty Acid Synthetase from Lactating Rat Mammary Gland

III. DISSOCIATION AND REASSOCIATION*

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SUMMARY

Fatty acid synthetase from lactating rat mammary gland is shown to be a cold-labile multienzyme complex. The native (13 S) form slowly dissociates into half-molecular weight (9 S) subunits on aging in the cold. Fatty acid synthetase activity observed on addition of the 9 S subunits to the assay system between 20 and 30°, is shown to result from rapid reassociation of the subunits to the 13 S form.

Dissociation of the enzyme into subunits is accompanied by a change in the number of protein sulfhydryl groups accessible to 5,5′-dithiobis(2-nitrobenzoate), but reassociation to the parent form is not dependent on the presence of a reduced thiol. The dissociation does not involve oxidation of protein sulfhydryl groups, as the total number of sulfhydryl groups titratable with 5,5′-dithiobis(2-nitrobenzoate) in the presence of 6 M urea was the same in the native enzyme and its subunits. Blocking more than two of the 28 subunit sulfhydryl groups with *p*-chloromercuribenzoate restricted the ability of the subunits to undergo heat-induced reassociation. Evidence is presented which suggests that although protein sulfhydryl groups may be involved in the dissociation-reassociation phenomenon, hydrophobic bonding is probably a more critical factor. Similar studies carried out with the fatty acid synthetase purified from rat liver indicate that this enzyme too exhibits cold lability.

Enzyme systems capable of synthesizing fatty acids via the malonyl-CoA pathway have been shown to occur in bacteria (3, 4), yeast (5), plants (6), fish (7), birds (8, 9), and mammals (10). The conversion of malonyl-CoA to fatty acids is catalyzed by a number of enzymes which in yeast and animals are bound together in the form of a multienzyme complex, the fatty acid synthetase. In plants and bacteria, however, with the exception of *Mycobacterium phlei* (11), the enzymes catalyzing the synthesis of fatty acids exist as individual units which can be separated by conventional protein fractionation procedures. The multienzyme complex represents a level of organization above that of the soluble monofunctional enzymes, providing a more efficient system through attachment of individual enzymes to one another in a specific pattern. Thus the multienzyme complexes provide models for the study of behavior of enzyme systems at a level of organization higher than is possible with the monofunctional enzymes.

Studies on the quaternary structure of the fatty acid synthetase multienzyme complexes from animals have shown them to be rather unstable (12). In a previous communication, we suggested that the mammary gland complex was, in fact, a cold-labile enzyme (13). In this publication we report a detailed study of the dissociation-reassociation phenomenon, especially in relation to the apparent enzyme activity observed with the subunit species of the enzyme.

EXPERIMENTAL PROCEDURE

Materials—Cofactors were obtained from Calbiochem or Sigma and were of the highest purity available: *p*-chloromercuri[7-^{14}C]-benzoate was purchased from CalAtomic, Los Angeles, California.

Syntheses—Acetyl-CoA and malonyl-CoA were prepared as described previously (13) and purified by chromatography on DEAE-cellulose columns (14). [1,3-$^{14}C_2$]Diethylmalonate (15) was used for synthesis of [1,3-$^{14}C_2$]malonyl-CoA. The [1,3-$^{14}C_2$]-diethylmalonate was saponified and the resulting [1,3-$^{14}C_2$]malonic acid purified by sublimation *in vacuo* (16) and converted to [1,3-$^{14}C_2$]malonyl-CoA by the method of Lynen (17). The product was purified by DEAE-cellulose chromatography (14).

Animals—Lactating rats (10 to 18 days post-partum) of the Long-Evans strain which were suckling at least six pups were used for this study. Dams were given Purina Rat Chow and water *ad libitum*.

Assay for Fatty Acid Synthetase Activity—Unless otherwise stated, assay systems consisted of 0.1 M potassium phosphate buffer, pH 6.6, 1.5×10^{-4} M NADPH, and 5×10^{-5} M acetyl-CoA. Reaction mixtures were incubated at 30° for 5 min, then malonyl-CoA (final concentration 5.4×10^{-5} M) was added to start the reaction. Activity was measured spectrophotometrically or where indicated, radiochemically, with [1,3-$^{14}C_2$]malonyl-

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CoA as substrate (13). A unit of activity is defined as the amount of enzyme catalyzing the malonyl-CoA-dependent oxidation of 1 nmole of NADPH per min at 30°.

Purification of Fatty Acid Synthetase—This was carried out as described previously (13). The fatty acid synthetase was also purified from rat liver by an identical procedure. Livers were obtained from either lactating rats or male rats which had been fasted 2 days and refed a fat-free, high carbohydrate diet for 3 days. Livers from either lactating (18) or fasted, refed rats (19) contained elevated levels of fatty acid synthetase compared with livers obtained from chow-fed, nonlactating animals. Homogeneity of the liver synthetase preparations was checked by sucrose density gradient centrifugation. The final specific activity of these preparations was usually between 800 to 1000 units per mg of protein.

Protein Determination—Protein was estimated either by the biuret method of Gornall, Bardawill, and David (20), or by the method of Lowry, *et al.* (21).

Sucrose Density Gradient Centrifugation—The procedure of Martin and Ames (22) was used except for minor changes. Samples (0.2 ml, unless otherwise indicated) were centrifuged on linear 5 to 20% sucrose density gradients containing 0.25 M potassium phosphate, pH 7.0, and 1×10^{-3} M EDTA. A Beckman SW 39.5 rotor was used. The concentration of DTT[1] in the gradient was always the same as that of the sample. Contents of the centrifuge tubes were removed either by piercing the bottom of the tubes with a 22-gauge needle and collecting fractions of 3 drops each, or by collecting from the top with a Buchler auto Densi-Flow and polystaltic pump system. The procedures are referred to in the figures as manual and automatic, respectively.

Titration of Subunit Sulfhydryl Groups with [7-^{14}C]p-CMB—Subunits of fatty acid synthetase (9 S) were prepared by aging the native enzyme (13 S) at 0° for 6 days in 0.25 M potassium phosphate, pH 7.0, containing 1×10^{-3} M EDTA and 1×10^{-3} M DTT. A portion of material was centrifuged on a sucrose density gradient at 0° to confirm that complete dissociation of the enzyme into 9 S subunits had occurred. The remaining sample was adjusted to 33% saturation with respect to ammonium sulfate and the precipitated protein was redissolved in 0.25 M potassium phosphate, pH 7.0, containing 1×10^{-3} M EDTA. Precipitation with ammonium sulfate did not modify the activity or S value of the native enzyme or its subunits. Agglutinated protein was removed by centrifugation for 10 min at 100,000 × *g*. Portions of the subunit preparation (5 mg) were incubated with various amounts of [7-^{14}C]p-CMB (0 to 0.6 μmole) for 1 hour at 0° in a final volume of 5 ml. Excess [7-^{14}C]p-CMB was removed from the samples by ultrafiltration with a Diaflow PM-30 membrane. For this purpose, samples were diluted to 10 ml with 0.25 M potassium phosphate, pH 7.0, containing 1×10^{-3} M EDTA and reduced to a volume of 2 ml in an Amicon model 12 ultrafiltration cell. The filtration procedure was repeated and portions were then taken for measurement of protein content, radioactivity, and fatty acid synthetase activity. Certain samples were selected and incubated for 15 min at 30°, then centrifuged on sucrose density gradients at 0°. A sample of subunit material not treated with p-CMB was carried through the ultrafiltration and incubation procedures as a control.

[1] The abbreviations used are: DTNB, 5,5′-dithiobis(2-nitrobenzoate); DTT, dithiothreitol; p-CMB, p-chloromercuribenzoate.

RESULTS

Effect of Temperature on Dissociation of Fatty Acid Synthetase—When samples of mammary gland fatty acid synthetase were aged at identical DTT concentrations, but at different temperatures and centrifuged on sucrose density gradients, it was found that the protein species present were different (Fig. 1). Fatty acid synthetase aged at 0° had dissociated over 90% into the 9 S subunit species, whereas that aged at 8° had dissociated only about 60%. In a separate experiment, fatty acid synthetase was aged under conditions identical with those described in Fig. 1 except that the temperature was 21°. No dissociation was observed after 6 days of storage. These experiments showed that the enzyme is cold labile in that dissociation into subunits is favored at low temperatures. The observation of enzyme activity associated with the subunit species is dealt with in detail later in this report.

Effect of Presence of DTT during Aging of Fatty Acid Synthetase and Its Subunits—When fatty acid synthetase was aged for 6 days in 0.25 M potassium phosphate, pH 7.0, containing 1×10^{-3} M EDTA and 0.01 M DTT, the loss of activity was the same whether the samples were stored at 0 or 8° (Fig. 1). The same was true with samples aged in 1×10^{-3} or 1×10^{-4} M DTT at 0 and 8°, although the loss in activity was greater at the lower thiol concentrations (70% of activity lost at 1×10^{-4} M DTT, 56% lost at 1×10^{-3} M DTT, and 38% lost at 0.01 M DTT). Evaluation of the stabilizing effect of DTT in these experiments is difficult as

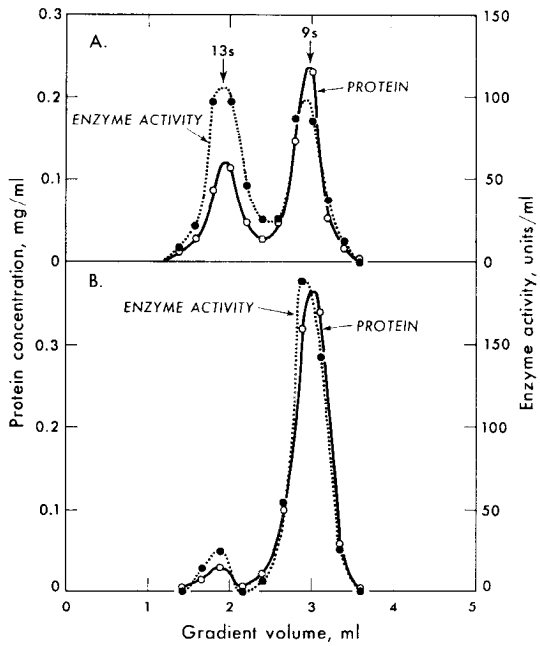

FIG. 1. Sucrose density gradient profiles of mammary gland fatty acid synthetase aged at different temperatures. Freshly prepared fatty acid synthetase (specific activity 735 units per ml, protein concentration 1.2 mg per ml) was aged for 6 days in 0.25 M potassium phosphate, pH 7.0, containing 1×10^{-3} M EDTA and 0.01 M DTT. *A*, stored at 8°; *B*, stored at 0°. The apparent activity of the two samples was measured before application to the gradient and found to be 455 and 467 units per mg in the samples stored at 1 and 8°, respectively. Samples were then centrifuged at 0° on the sucrose density gradient for 15½ hours at 35,000 rpm. Contents of the centrifuge tubes were removed by the manual procedure. O——O protein, ●····● enzyme activity.

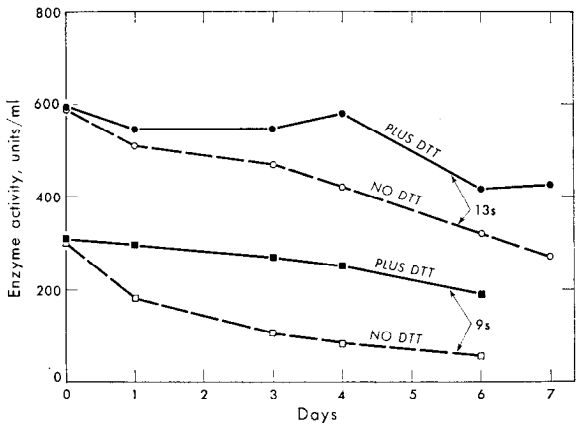

FIG. 2. The effect of the presence of DTT during aging of the 13 S and 9 S species of mammary gland fatty acid synthetase. The 9 S material was obtained by aging the native fatty acid synthetase in 0.25 M potassium phosphate, pH 7.0, containing 1×10^{-3} M EDTA and 1×10^{-3} M DTT at 0° for 1 week. Removal of DTT from samples was carried out by gel filtration on a column (27×1 cm) of Sephadex G-25 at 0° for the 9 S species and 20° for the 13 S species. The 13 S material was then stored with and without 1×10^{-3} M DTT at 21° and the 9 S material with and without 1×10^{-3} M DTT at 0°. Activity was measured spectrophotometrically at the intervals shown.

TABLE I

Effect of removal and replacement of DTT on mammary gland fatty acid synthetase activity

The 13 S species used had been aged at 20° for 1 week at a protein concentration of 6.4 mg per ml in 0.25 M potassium phosphate, pH 7.0, containing 1×10^{-3} M EDTA, with or without 0.01 M DTT. At this stage, the sedimentation coefficients were confirmed as 13 S by sucrose density gradient centrifugation at 17°. The 9 S species were produced by first aging the native fatty acid synthetase at 0° for 2 weeks at a protein concentration of 7.1 mg per ml in 0.25 M potassium phosphate, pH 7.0, containing 1×10^{-3} M EDTA and 0.01 M DTT. The sedimentation coefficient was then confirmed as 9 S by sucrose density gradient centrifugation at 0°. Aging was continued at 0° for 1 week in the presence or absence of 0.01 M DTT. In the DTT replacement experiments, the temperature was 20° for the 13 S and 0° for the 9 S species (to prevent reassociation) and the DTT concentration 0.01 M. After reactivation, the sedimentation coefficients were found to be unchanged.

Species	Fatty acid synthetase activity			
	Aged with DTT	Aged without DTT, then DTT replaced		
		0 hr	1 hr	2 hrs
	units/mg	*units/mg*		
13 S	447	301	381	413
9 S	141	19	101	120

both dissociation and inactivation occurred. To examine the effect of DTT under conditions where dissociation was not a complicating factor, experiments were performed whereby the native enzyme was stored at 21° and the subunits at 0°. Although the native enzyme stored at 21° did not dissociate into subunits, it did slowly lose enzyme activity (Fig. 2). The rate of loss of activity was more marked when the enzyme was stored in the absence of DTT than in its presence. Similarly, when the subunits of fatty acid synthetase were stored at 0° with and without DTT, enzymatic activity declined more rapidly in the absence of DTT. Thus, the presence of DTT during aging exerts a protective effect on fatty acid synthetase activity whether the enzyme is stored in the 13 or the 9 S form.

Reactivation of Fatty Acid Synthetase with DTT—To determine whether the enzyme activity lost following removal of DTT from 13 and 9 S species could be restored by replacing the DTT, the experiment described in Table I was performed. The results indicate that the loss in enzyme activity is indeed reversible. Interconversion of the high and low activity 13 S species took place at 21° without any observed change in sedimentation coefficient. Similarly, no change in sedimentation coefficient was observed on interconversion of the high and low activity forms of 9 S species. Since the sucrose density gradient technique cannot detect small changes in S values, it is possible that a small configurational change takes place on interconversion of the high and low activity forms of the enzyme.

Dissociation and Reassociation of High and Low Activity Forms of Fatty Acid Synthetase—To determine whether both the high and low activity forms of the fatty acid synthetase dissociate into "high" and "low" activity forms of the subunit species, the following experiment was performed. The high (specific activity 447 units per mg) and low (301 units per mg) activity forms of 13 S species were obtained by aging with and without DTT as described in Table I. Both forms were than stored at 0° for 12 days. As a result, the specific activities were 80 units per mg and zero for the samples stored with and without DTT, respectively. Sucrose density gradient analyses showed that both samples contained predominantly 9 S species, confirming that both the high and low activity forms of 13 S species undergo cold-induced dissociation.

We next sought evidence of whether the 9 S species formed in the presence and absence of DTT, *i.e.* the high and low activity forms, could undergo heat-induced reassociation to the high and low activity forms of the 13 S species. Details of this experiment are given in Fig. 3. It can be seen that the high and low activity forms of the 9 S species each reassociated on warming, but to different extents. The high activity form of 9 S reassociated approximately 70% to the high activity 13 S species (specific activity from the gradient, 500 units per mg), whereas the low activity form of 9 S reassociated only about 50% to a 13 S species which had no enzyme activity.

These two experiments showed that both high and low activity forms of fatty acid synthetase undergo reversible dissociation.

Evaluation of Fatty Acid Synthetase Activity Observed with Subunit Species—Fatty acid synthetase activity measured at 30° was shown with the subunit species and was not dependent on the presence of DTT in the assay medium. To investigate the possibility that the observed activity with the subunit species might result from reassociation of inactive subunits to active species during the assay, we studied the effect of temperature on enzyme activity and structural integrity of the 9 and 13 S species.

The temperature dependence of the fatty acid synthetase activity was markedly different when native enzyme or subunit species was added to the assay system (Fig. 4). Significant enzyme activity with the native enzyme was observed over the entire 0–30° temperature range. Proportionately, very little activity was observed below 20° with the subunit species and over the

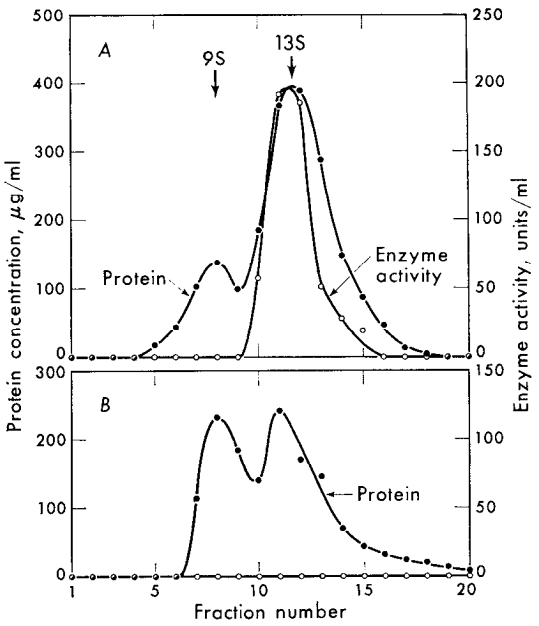

FIG. 3. Sucrose density gradient profiles of mammary gland fatty acid synthetase after aging subunit species with and without DTT and incubating at 30°. A preparation of 9 S material was obtained by aging the native enzyme in 0.25 M potassium phosphate, pH 7.0, containing 1×10^{-3} M EDTA and 1×10^{-3} M DTT at 0° for 1 week. A portion of the 9 S material was then dialyzed against 0.25 M potassium phosphate containing 1×10^{-3} M EDTA at 0° for 2 days to remove DTT. Protein in both the dialyzed and nondialyzed preparations was precipitated between 0 to 33% saturation with respect to ammonium sulfate and dissolved in 0.25 M potassium phosphate, pH 7.0, containing 1×10^{-3} M EDTA. Both preparations were incubated for 30 min at 30° and the fatty acid synthetase activity determined. The nondialyzed sample gave an apparent specific activity of 309 units per mg and the dialyzed sample, 9 units per mg. A portion of the nondialyzed sample (0.82 mg) and the dialyzed sample (0.66 mg) was centrifuged on sucrose density gradients at 17° for 9 hours at 39,500 rpm. As controls, portions of each sample were maintained at 17° for 9 hours without centrifugation and the fatty acid synthetase activities found to be 283 units per mg for the nondialyzed sample, zero for the dialyzed sample. *A*, profile for nondialyzed sample. *B*, profile for dialyzed sample. Contents of the centrifuge tubes were removed by the automatic method. ●—● protein, ○—○ enzyme activity.

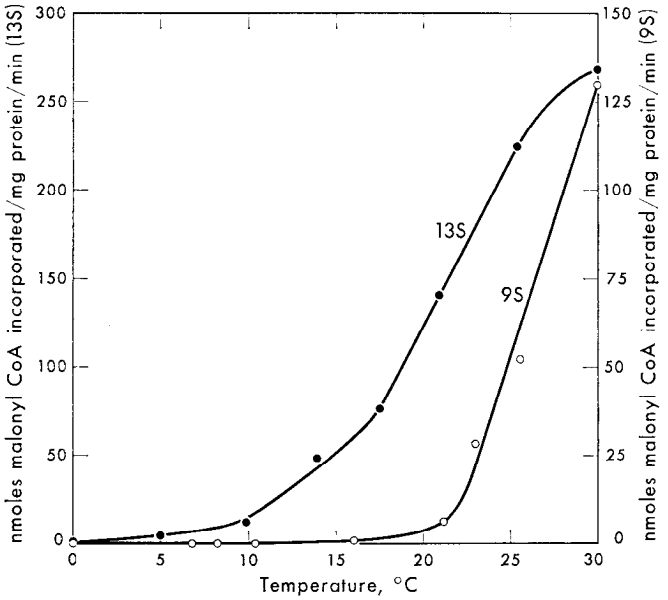

FIG. 4. Assay temperature dependence of mammary gland fatty acid synthetase activity observed with 13 and 9 S species. The procedures for obtaining 9 S material and for removal of DTT from samples were described in the legend to Fig. 2. The radiochemical assay system was used as described under "Experimental Procedure." ●—●, 13 S species; ○—○, 9 S species.

20–30° range the temperature dependence of fatty acid synthetase activity was more marked than with the native enzyme (Q_{10}[13 S] = 2.4, Q_{10}[9 S] = 11.2).

To check whether between 20 and 30°, the greater temperature dependence of the fatty acid synthetase activity observed with the subunit species compared to the native enzyme, might be caused by heat-induced reassociation of subunits during the assay, the experiment described in Fig. 5 was carried out. Subunit species were obtained by aging the fatty acid synthetase in the cold for 1 week. After removal of DTT, the samples were incubated for 3 min at various temperatures and centrifuged immediately on sucrose density gradients at 0° degrees. As shown previously (13), the 13 S species centrifuged on sucrose density gradients at 0° does not undergo dissociation on the gradient. Thus, as the cold-induced dissocation of fatty acid synthetase is essentially a very slow process, any 13 S material formed during the brief incubation at 30° would be completely preserved during the time required for centrifugation. The sample incubated at 0° contained only about 9% of the 13 S species, but those incubated at 20 and 30° contained 20 and 40%, respectively, of the 13 S species. Thus, the fatty acid synthetase activity observed with the 9 S species is demonstrable only at temperatures between 20 and 30°, precisely the same temperature range in which reassociation of the subunits takes place.

If reassociation of subunits to the active native species during the assay were responsible for the observed activity, then obviously the reassociation must take place very rapidly, in contrast to the slow cold-induced dissociation. The routine spectrophotometric assay procedure for measurement of fatty acid synthetase activity, involves incubation of the enzyme preparation with potassium phosphate buffer, acetyl-CoA, and NADPH for 5 min at 30°, then the reaction is initiated by addition of malonyl-CoA. Under these conditions, with the subunit species the reaction velocity was found to proceed maximally immediately on addition of malonyl-CoA. However, when the assays were conducted without prior equilibration of the subunit species to 30°, quite a different situation was observed (Fig. 6). In these assays where the complete reaction mixture minus enzyme was equilibrated to 30° and then the subunit species added, a definite lag period was observed before the reaction commenced. Furthermore, the lag time was dependent on the concentration of subunits, being greatest at lowest protein concentrations. After the initial time lag, reaction rates were found to be directly proportional to protein concentration. No time lag was observed when the 13 S species was added to the reaction mixture under these conditions. The time course of these reactions suggests strongly that with the subunit species enzyme activity is observed only after exposure of the protein to the reaction mixture for a specific length of time.

Thus, the effect of temperature on synthetase activity and structural integrity of the subunit species provides strong evi-

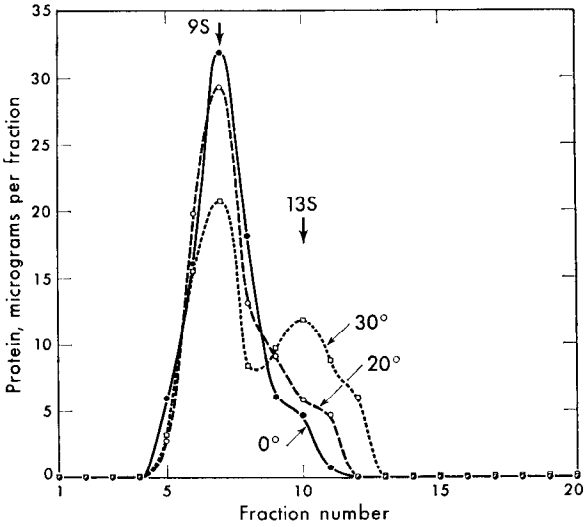

FIG. 5. Sucrose density gradient profiles of mammary gland fatty acid synthetase 9 S species incubated for 3 min at various temperatures. The procedures for obtaining 9 S material and for removal of DTT from samples was described in the legend to Fig. 2. After removal of DTT from the 9 S material (protein concentration, 0.42 mg per ml), portions were incubated for 3 min at various temperatures, cooled to 0°, and 0.2-ml portions centrifuged on the sucrose density gradients at 0° for 16.3 hours and 30,000 rpm. Contents of the centrifuge tubes were removed by the automatic method. ●—●, incubated 0°, ○- - -○, incubated 20°; □- - -□, incubated 30°. The somewhat broad protein peak in the 13 S region might indicate the presence of more than one species. We believe it is more likely caused by the use of the Buchler auto Densi-Flow system for removal of the density gradients. This procedure consistently gave broad peaks with considerable tailing, see also Fig. 3. In other experiments where the 9 S subunits were reassociated at 30° and then subjected to sucrose density gradient centrifugation without the use of the Buchler system, symmetrical peaks were observed with no evidence of more than one species in the 13 S region (*e.g.* see Fig. 8).

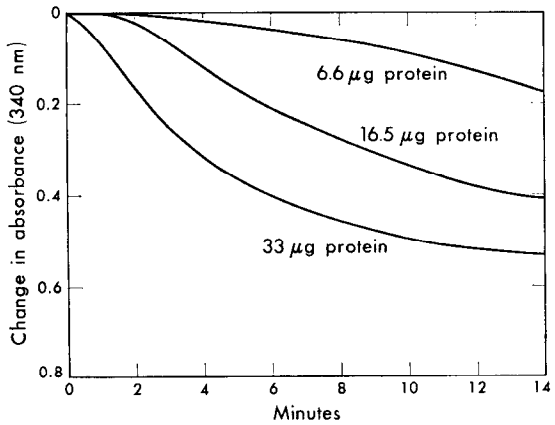

FIG. 6. The effect of subunit concentration on the time course of mammary gland fatty acid synthetase reaction. The procedures for obtaining 9 S material and removal of DTT from samples were described in the legend to Fig. 2. Assays for fatty acid synthetase activity were conducted in the absence of DTT by first incubating 0.1 M potassium phosphate, pH 6.6, 1.5×10^{-4} M NADPH, 5×10^{-5} M acetyl-CoA, and 5.4×10^{-5} M malonyl-CoA for 3 min at 30° and then starting the reaction by addition of the subunit species.

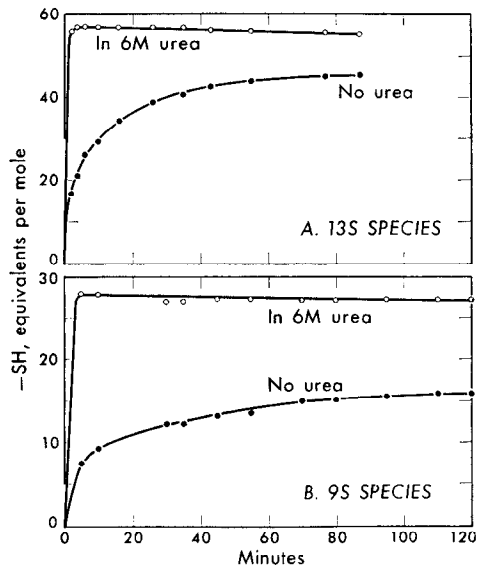

FIG. 7. Titration of sulfhydryl groups of mammary gland fatty acid synthetase with DTNB in the presence and absence of 6 M urea. The procedures for obtaining 9 S material and for removal of DTT from samples were described in the legend to Fig. 2. For the titration with DTNB, cuvettes contained in a final volume of 3 ml, fatty acid synthetase in 0.25 M potassium phosphate, pH 7.0. The reaction was started by the addition of 0.5 nmole of DTNB and the absorbance at 412 nm was recorded. No visible agglutination of protein occurred during the 2-hour reaction time. *A*, 1 nmole of 13 S species was titrated at 20°; *B*, 1 nmole of 9 S species was titrated at 0°. The number of sulfhydryl equivalents reacted was calculated with the molar extension coefficient of 13,600 for the reduction product of DTNB (23). ○—○, in 6 M urea; ●—●, no urea.

dence to suggest that the temperature of the assay medium induces transformation of the subunit species to the active species of the native 13 S form.

Titration of Sulfhydryl Groups of Native Enzyme and Subunits with DTNB—In an attempt to ascertain the possible role of protein sulfhydryl groups in the dissociation and inactivation of fatty acid synthetase, we measured the protein sulfhydryl groups accessible to DTNB (in the presence and absence of 6 M urea) in both the native enzyme and its subunits (Fig. 7). The freshly prepared native enzyme was found to contain 57 thiol eq per mole as determined by titration with DTNB in 6 M urea. This was in agreement with the value (56 half-cysteines, one 4′-phosphopantetheine) obtained by analysis of the products resulting from performic acid oxidation and hydrolysis of the enzyme (13). Of the total thiol equivalents present, 47 were accessible to DTNB in the absence of urea.

When the subunit species (formed by cold-induced dissociation of the enzyme in the presence of DTT) was titrated with DTNB, a total of 28 sulfhydryl eq per mole were detected of which 16 were accessible to DTNB in the absence of urea. Thus, dissociation of the fatty acid synthetase occurs with retention of all protein sulfhydryl groups (57 in the native enzyme, 28 in the half-molecular weight subunits). There is however, a change in the number of protein sulfhydryl groups accessible to DTNB in the absence of urea (82% of the total sulfhydryl groups in the native enzyme, 57% in the subunits).

To determine whether the protective effect of DTT against

TABLE II

Effect of [7-^{14}C]p-CMB binding on ability of 9 S subunits of mammary gland synthetase to reassociate to active 13 S species in fatty acid synthetase assay

The procedure for measuring [7-^{14}C]p-CMB binding is given under "Experimental Procedure."

[7-^{14}C]p-CMB bound to 9 S	Enzyme activity
nmoles/nmole	*units/mg*
0	295
1.1	269
1.9	198
3.5	78
7.3	0

loss of synthetase activity on aging might be caused by prevention of oxidation of protein sulfhydryl groups, we titrated the native enzyme and its subunits with DTNB at various intervals under the experimental conditions shown in Fig. 7. After 1 week of storage in the absence of DTT, the native enzyme and the subunit species showed only slight decreases in the sulfhydryl groups titratable with DTNB in the presence and absence of 6 M urea. In three separate experiments for both the native enzyme and its subunits stored without DTT for 1 week, the average change in sulfhydryl titer, either in the presence or absence of 6 M urea, was approximately 1 sulfhydryl eq per mole, that is within the experimental error of the method. Thus, the loss in activity on storage is not the result of oxidation of protein sulfhydryl groups.

Effect of p-CMB on Reassociation of Subunit Species—In order to test whether the forces holding together the two subunits of fatty acid synthetase might involve sulfhydryl groups, experiments were carried out with [7-^{14}C]p-CMB as a sulfhydryl-blocking agent. When 9 S subunits were titrated with [7-^{14}C]p-CMB, the observed activity in the fatty acid synthetase assay decreased (Table II). This observation could be caused either by (*a*) blocking of —SH groups essential for reassociation of the subunit in the assay, or (*b*) blocking of —SH groups essential for activity of the reassociated enzyme. To distinguish between these two possibilities, subunit samples titrated with various amounts of [7-^{14}C]p-CMB were incubated at 30° for 15 min and centrifuged on the sucrose density gradients at 0°. As can be seen in Fig. 8, the untreated subunits reassociated 60% to the 13 S species after incubation at 30°. Similarly, the subunit sample titrated to 1.9 eq of p-CMB per mole of enzyme reassociated 60%. Thus, the sulfhydryl groups blocked with p-CMB did not affect the ability of the subunits to undergo heat-induced reassociation. However, the [7-^{14}C]p-CMB-labeled 13 S species formed from this subunit sample, had a lower specific activity in the fatty acid synthetase assay (220 units per mg) than did the 13 S species formed by warming the untreated subunits (432 units per mg). Whereas the blocked sulfhydryl groups do not participate in the reassociation of subunits, they are required for full activity of the enzyme. Titration of more than 1.9 sulfhydryl eq per mole of 9 S species inhibited the ability of the subunits to reassociate. Thus, the sample titrated to 3.5 eq of p-CMB per mole of enzyme reassociated only 30% on warming. This would suggest that some of these sulfhydryl groups are involved in holding together the two subunit species.

Cold Lability of Rat Liver Fatty Acid Synthetase—Studies in

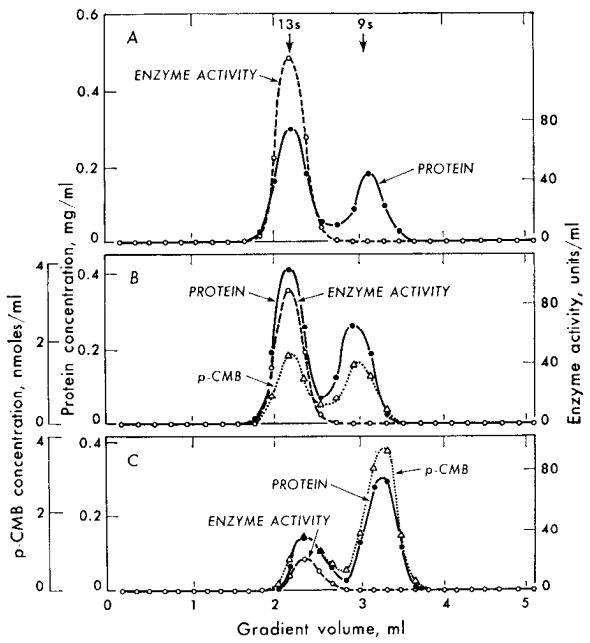

FIG. 8. The effect of p-CMB on reassociation of mammary gland fatty acid synthetase subunits. Samples were titrated to various extents with [7-^{14}C]p-CMB, incubated for 15 min at 30° and centrifuged on sucrose gradients as described under "Experimental Procedure." *A*, not treated with p-CMB; *B*, titrated to 1.9 eq of p-CMB per mole of subunit before incubation; *C*, titrated to 3.5 eq of p-CMB per mole of subunit before incubation. Contents of the centrifuge tubes were removed by the manual procedure. ●—●, protein, ○- - -○, enzyme activity; △····△, p-CMB.

Porter's laboratory have shown that the fatty acid synthetases from pigeon and rat liver readily undergo reversible dissociation (12, 24). In view of the fact that the fatty acid synthetases from rat mammary gland and rat liver are antigenically similar,[2] we were interested to determine whether the rat liver enzyme exhibited the same properties as the mammary gland enzyme in terms of its dissociation-reassociation characteristics.

The fatty acid synthetase purified from rat liver was aged at either 0 or 21° in 0.25 M potassium phosphate, pH 7.0, containing 1×10^{-3} M EDTA and either 1×10^{-3} or 1×10^{-2} M DTT. The native enzyme was found to sediment as a 14 S species on sucrose density gradients. At various intervals, portions of each sample were centrifuged on sucrose density gradients at 0° and the distribution of protein and fatty acid synthetase activity determined. Typical sucrose density gradient profiles of enzyme stored 4 days at 0 and 21° are shown in Fig. 9. Approximately 68% of the sample stored at 0 and 10% of the sample stored at 21° had dissociated into subunits at this time. The specific activity of the 14 S species remained at 1000 units per mg in the 21° sample and was 700 units per mg in the 0° sample. After 6 days, the enzyme kept at 0° had dissociated 80% into 9 S subunits and that kept at 21°, only 12%. This was true of samples stored at both 1×10^{-3} and 1×10^{-2} M DTT concentrations. As was found with the mammary gland enzyme (13), the 9 S subunit species gave enzyme activity when added to the assay medium, about 140 units per mg (Fig. 9). As no DTT was present in the assay system, the observed activity was presumably a result of

[2] S. Smith, unpublished observations.

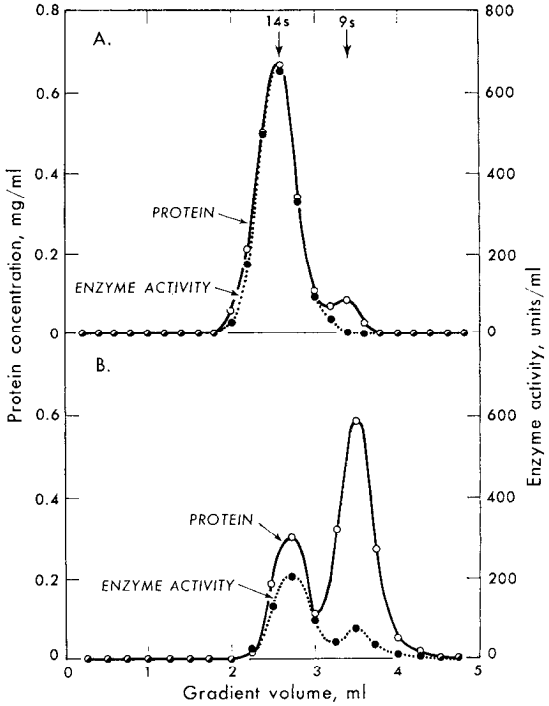

FIG. 9. Sucrose density gradient profiles of liver fatty acid synthetase stored 4 days at 21 and 0°. The initial specific activity of the preparation was 1,000 units per mg. Portions (0.1 ml) of each sample were centrifuged on 5 to 20% sucrose gradients in 0.25 M potassium phosphate, pH 7.0, containing 1×10^{-3} M EDTA and 1×10^{-3} M DTT for 16½ hours at 30,000 rpm. Contents of the centrifuge tubes were removed by the manual procedure. ○—○, protein; ●····●, enzyme activity. *A*, 21°; *B*, 0°.

heat-induced reassociation of the 9 S subunits to the active 14 S species.

Thus, the liver fatty acid synthetase resembles the mammary gland enzyme in that it dissociates in the cold into subunits which can rapidly reassociate under the conditions of enzyme assay.

DISCUSSION

The fatty acid synthetase from rat mammary gland has been shown to exist in several interconvertible forms, depending largely on conditions of temperature and thiol (DTT) concentration. A schematic representation is proposed to illustrate the interconversion of the various forms of the enzyme (Fig. 10).

The native enzyme (*13 S**) stored at 21° in the absence of a reduced thiol is slowly converted to a less active, or inactive species (*13 S*) (Fig. 2). The process is reversed by replacing the reduced thiol (Table I). When stored in the cold, in the presence of a reduced thiol, the native enzyme dissociates into a half-molecular weight species (*9 S**). This species, on addition to the fatty acid synthetase assay system, even in the absence of a thiol, gives enzyme activity. However, the observed activity is a direct result of rapid, heat-induced reassociation to the active, native 13 S* species in the assay system. The 9 S* species, when aged in the absence of a thiol is slowly converted to a form (*9 S*) which on addition to the assay medium does not give enzyme activity (Fig. 2). The conversion is reversed by replacement of the thiol at 0°. The low activity 13 S species (most likely a mixture of active 13 S* and inactive 13 S forms) when aged in the cold in the absence of a thiol, dissociates into 9 S subunits which on addition to the assay system gives no enzyme activity. When the inactive 9 S species is rewarmed in the absence of a thiol, reassociation to an inactive 13 S species occurs (Fig. 3).

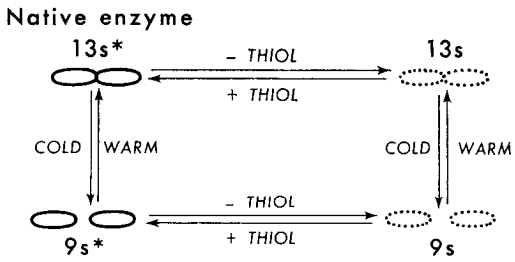

FIG. 10. Subunit interactions of rat mammary gland fatty acid synthetase.

The results suggest that the dissociation-reassociation phenomenon involving either active or inactive forms of the enzyme, is influenced largely by temperature conditions, whereas interconversion of the active and inactive forms is influenced by the presence or absence of a thiol. The fact that the 9 S* and 9 S species reassociate on warming to active and inactive dimers respectively, suggests that the active configuration of the enzyme can be protected even in the subunit form by the presence of a thiol. Thus, rewarming of this protected 9 S* form in the assay results in direct, rapid reassociation to the active 13 S* species.

A number of enzymes have been found to exhibit cold lability and a comprehensive list has been prepared by Irias, Olmsted, and Utter (25). No generalized explanation for this phenomenon has been advanced however, as several different mechanisms seem to be operative, depending on the particular enzyme. Some cold-labile enzymes appear to dissociate into subunits on exposure to the cold, *e.g.* mitochondrial ATPase (26), others actually undergo aggregation in the cold, *e.g.* glycogen phosphorylase from muscle (27), while with some enzymes the cold inactivation seems to be linked with a conformational change rather than a dissociation or aggregation process, *e.g.* 17β-hydroxysteroid dehydrogenase (28). The fatty acid synthetases from rat mammary gland and rat liver would be classified with the first group.

Butterworth *et al.* (12), as a result of their studies on dissociation and reassociation of the pigeon liver fatty acids synthetase, suggested that sulfhydryl groups are wholly or partly responsible for the noncovalent binding of the subunits to form the enzymatically active complex. More recent work (29) has in fact shown that dissociation of the pigeon liver enzyme is accompanied by the loss of 5.5 titratable sulfhydryl eq per mole of enzyme. Our studies with the rat mammary gland enzyme show that indeed reassociation of the subunits can be prevented by blocking certain sulfhydryl groups with *p*-CMB. However, one cannot rule out the possibility that insertion of the bulky mercurial reagent into the protein brings about changes in the steric configuration of the enzyme which in turn hinder the subunit interaction. Data obtained on the effect of temperature on the stability of the rat mammary gland and rat liver enzymes have led us to believe that hydrophobic bonds are probably of prime importance in the maintenance of the quaternary structure of these enzymes at physiological temperatures. Of the possible interactions usually considered responsible for maintenance of quaternary structure, two types show some temperature dependence. Hydrophobic bonds weaken as the temperature is decreased from 60–0°, whereas hydrogen bonds strengthen (30). With most proteins,

increased temperature induces unfolding of the helix and is therefore associated with decreased stability. This would suggest that hydrogen bonds are a major factor in the maintaining structure of most proteins. However, as pointed out by Scheraga, Némethy, and Steinberg (30), the simultaneous presence of hydrophobic and hydrogen bonds in a protein could lead to a modification of the α helix stability with respect to temperature. Thus, in proteins having a high degree of hydrophobic bonding, an inversion of the curve of α helix stability *versus* temperature might occur, giving rise to the phenomenon of cold lability. As pointed out in this study, the dissociation of the mammary gland fatty acid synthetase does not involve oxidation of protein sulfhydryl groups. There is, however, some evidence of a configurational change occurring on dissociation as the number of sulfhydryl groups accessible to DTNB in the absence of urea is altered. We feel that the temperature stability characteristics of the enzyme fit the idea that the degree of hydrophobic bonding is of prime importance in the maintenance of quaternary structure at warm temperatures, and that lower temperatures weaken the hydrophobic forces, causing unfolding of the helical structure and consequent dissociation of the enzyme.

It is tempting to speculate on the possible importance of dissociation-reassociation phenomena in biological systems. Perhaps changes in the cellular environment could affect the assembly or disassembly of enzyme subunits, affording a control mechanism for regulation of a particular pathway.

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