Participation of Covalently Linked Fatty Acyl Coenzyme A Products in the Action of Yeast Fatty Acid Synthetase*

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ABSTRACT: Palmityl peptides resulting from peptic digestion of yeast fatty acid synthetase after reaction with [^{14}C]palmityl coenzyme A (CoA) have been isolated and identified. Purification, after removal of all nonreacted palmityl-CoA, was by countercurrent distribution and thin-layer chromatography. Amino acid analyses showed that each of the isolated peptides was homogeneous. The palmityl residue is covalently bound on at least three different sites on the enzyme. Peroxidation of the palmityl peptides showed two of them to be thiol esters; one contained β-alanine and pantothenic acid, which is characteristic of the acyl carrier protein site, the other was sensitive to iodoacetamide and therefore presumably originated from the iodoacetamide-sensitive condensing enzyme. The third, nonthiol site, must be from the transacylase. The amino acid content of this peptide is identical with that for the malonyl transacylase peptide. This, together with previous information, suggests that malonyl-CoA and product acyl-CoA may react with a common transacylase. Competition experiments between acetyl-CoA and palmityl-CoA give evidence for at least a second transacylase. It is proposed that the fatty acid synthetase complex contains two transacylases, one specific for the initiation reaction (reaction with acetyl-CoA) and the other specific for elongation and termination (reaction with malonyl-CoA and palmityl-CoA).

The fatty acid synthetase from yeast resembles the mammalian and avian systems in possessing the characteristics of a stable multienzyme complex under physiological conditions. According to the results of experiments with model substrates the complex carries out at least seven distinct enzymatic reactions, each reaction occurring with the substrates and intermediates covalently bound to the complex (Lynen, 1967). Only the end products are transferred back to CoA. The main products of the synthetase are palmityl- and stearyl-CoA, and a smaller amount of fatty acyl-CoAs of shorter chain length. The reactions of fatty acid synthetase are summarized in Figure 1. The reaction cycle is repeated until the growing acyl chain is 16 or 18 carbon atoms long. According to this mechanism (Figure 1) the acyl chain of the product (palmityl or stearyl) should also be found in covalent linkage on the fatty acid synthetase, and should occupy the same thiol sites as those occupied by the acetyl residue. There has so far been no direct demonstration of the validity of this assumption. The product should also occupy a nonthiol transacylase site. If this is actually the case it would be of interest to know if this site is identical for product and substrate, since one could then make further predictions about the mechanism of action of the complex. In particular, it was hoped that an understanding of the interaction of end product with the fatty acid synthetase complex may lead to an elucidation of the problem of why the fatty acid synthetase does not synthesize chain lengths of more than 16 or 18 carbon atoms.

The isolation and identification of palmityl peptides resulting from the direct reaction of palmityl-CoA with yeast fatty acid synthetase complex are presented in this paper.

Materials and Methods

Fatty Acid Synthetase. Yeast fatty acid synthetase was purified from baker's yeast, as described by Lynen (1969). Preparative sucrose gradient centrifugation (Spinco Ti 14 rotor, Beckman-Spinco Co., Palo Alto, Calif.) was used in place of the hydroxylapatite column to remove any remaining impurities. More than 95% of the protein sedimented as a single symmetrical peak. This fraction presumably approached 100% purity. Only preparations which had a specific activity of at least 2000 in the standard spectrophotometric assay were used (Lynen, 1969). Freshly prepared enzyme, *i.e.*, not frozen or stored, was employed for all reactions.

Reagents. Palmityl-CoA was synthesized *via* the mixed anhydride by the method of Wieland and Rueff (1953), as modified by Hagen (1963), and assayed according to Seubert (1960). Acetyl-CoA was synthesized from acetic anhydride and CoA (Simon and Shemin, 1953) and assayed by the method of Buckel and Eggerer (1965). [1-^{14}C]Palmitic acid was purchased from the Radiochemical Centre, Amersham, England; [^{12}C]palmitic acid from Merck and Co., and coenzyme A from Boehringer Mannheim Corp. Silica gel G, used for all tlc, was from Merck Co.

General Methods. Protein concentration was determined by a modified Biuret reaction (Beisenherz *et al.*, 1953) or by the method of Lowry *et al.* (1951). The purification of all peptides was carried out at room temperature.

Specific loading, i.e., micromoles of [^{14}C]palmityl incorporated per micromole of enzyme, was determined on dried denatured protein, before pepsin digestion. A small amount of protein was dissolved in formic acid and samples were pipetted for measuring protein and radioactivity. Before determination of protein, by the Lowry method, the formic acid was first removed in a vacuum desiccator overnight. Radioactivity was measured by pipetting samples onto 3 × 7 cm Whatman No. 1 paper strips which were then dried under a heat lamp. The dried paper strips were inserted into scintillation vials containing 2,5-diphenyloxazole–1,4-bis[2-(4-methyl-5-phenyl-

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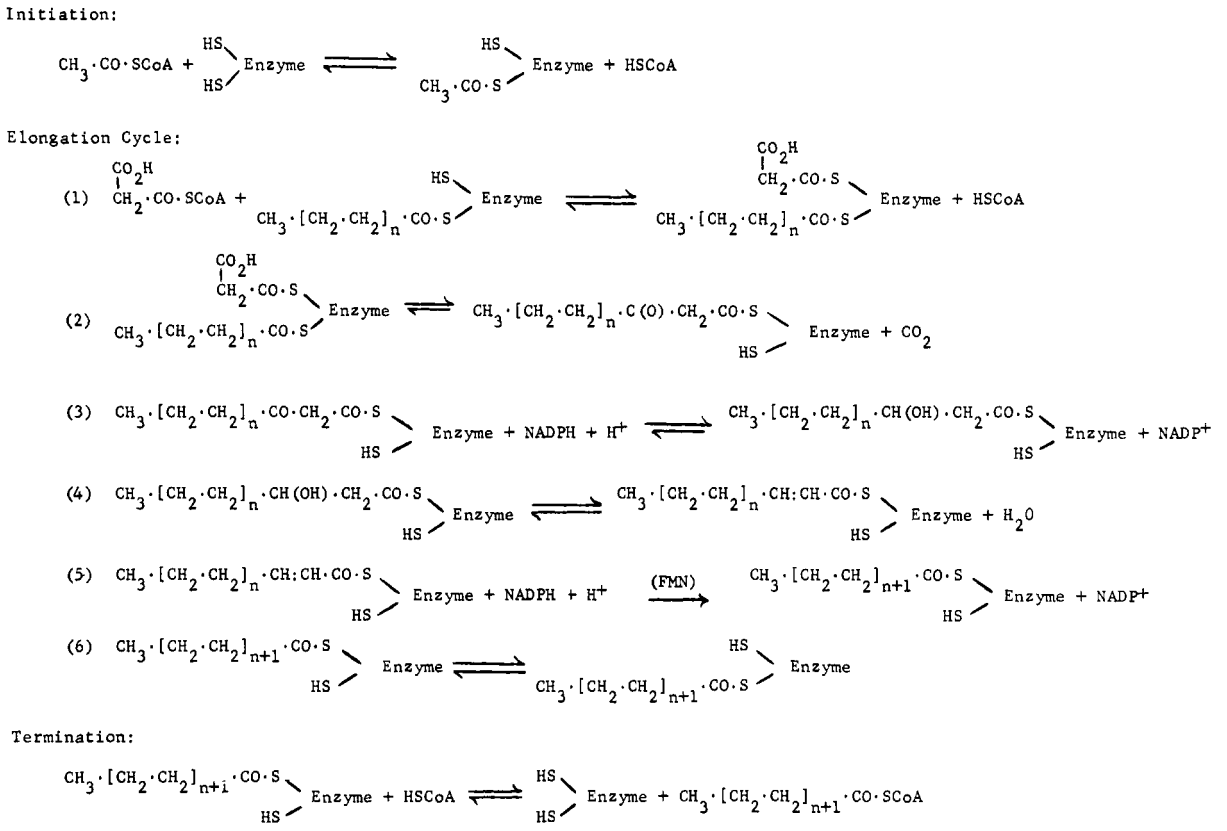


FIGURE 1: Reactions of fatty acid synthetase. The upper sulfhydryl group on the enzyme refers to the acyl carrier protein site and the lower sulfhydryl to the condensing enzyme site.

oxazolyl)]benzene–toluene solution and counted in a Packard Tri-Carb liquid scintillation spectrometer. The specific radioactivity of the starting [^{14}C]palmityl-CoA was similarly determined so that counts per minute in the protein could be directly converted to micromoles of [^{14}C]palmitate. In calculating micromoles of protein a molecular weight of 2,300,000 was used.

Results

Preparation and Isolation of Palmityl Peptides. REACTION OF FATTY ACID SYNTHETASE WITH [^{14}C]PALMITYL-CoA. About 500 mg of fatty acid synthetase was diluted to a final concentration of 5 mg/ml in 0.1 M pH 6.5 potassium phosphate buffer. [^{14}C]Palmityl-CoA (1 μCi/μmole) was added to give a final concentration of 2×10^{-4} M. The mixture was incubated at 0° for 8 min after which the reaction was stopped by the addition of cold 1 N HCl, 3 ml/100 ml of reaction mixture. The 8-min incubation at 0° was chosen in order to attain complete equilibrium and to minimize deacylase activity (see below). The reaction mixture was then brought to room temperature and sufficient 1-butanol was added to form a one-phase system (about 500 ml of butanol per 100 ml of reaction mixture).

REMOVAL OF UNREACTED [^{14}C]PALMITYL-CoA. The precipitated protein was collected by centrifugation and then homogenized in a loose-fitting glass homogenizer in 150 ml of butanol–ethanol–0.03 N aqueous HCl (1:1:1, v/v). The protein was again collected by centrifugation. Homogenization and centrifugation were repeated 20–30 times, until the radioactive counts in the supernatant became constant. The residual constant radioactivity is due to a small amount of protein which is lost in each supernatant. Figure 2 illustrates the rate at which unreacted radioactive palmityl-CoA is removed from the enzyme. About 25% of the protein is lost during this procedure. Finally the precipitated enzyme was washed twice with ether and then dried until all the ether was removed. In control experiments HCl was added to the reaction mixture at zero time. No radioactivity was found associated with the zero-time control protein after washing for an equal number of times as the incubated mixtures. This indicates that the

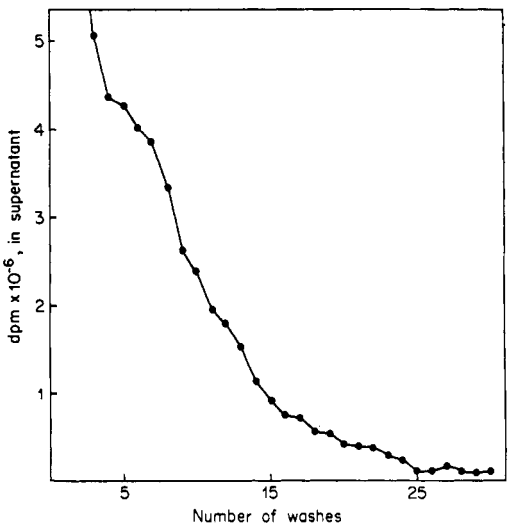


FIGURE 2: Removal of unreacted palmityl-CoA from fatty acid synthetase. Experimental conditions are given in the text.

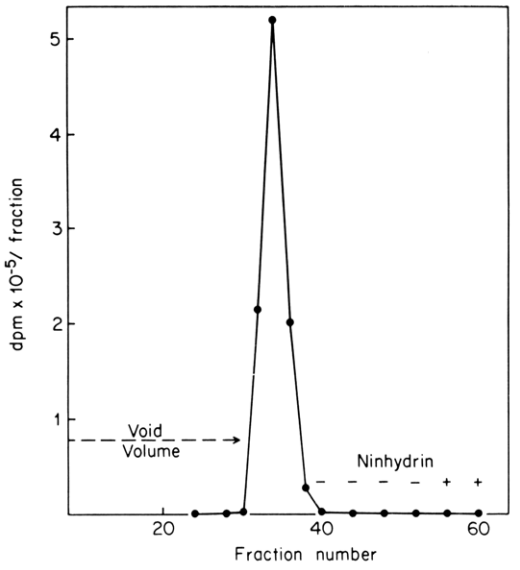


FIGURE 3: Separation of [^{14}C]palmityl peptides on the second Sephadex–butanol column (see text). The negative ninhydrin reactions in fractions 40–52 show that there is adequate separation from the nonradioactive peptides. Fractions (2 ml) were collected.

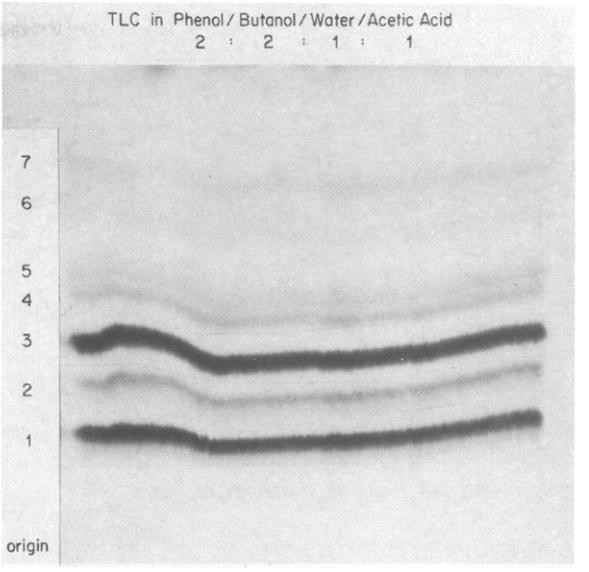


FIGURE 4: Autoradiogram of [^{14}C]palmityl peptides separated on tlc.

radioactivity associated with the incubated enzyme is bound specifically as the result of an enzymatic reaction.

DIGESTION OF THE [^{14}C]PALMITYL FATTY ACID SYNTHETASE WITH PEPSIN. The dried precipitate was suspended in 0.01 N HCl (1 g of original protein per liter). Pepsin (0.1 g/l.) was added and the reaction mixture was stirred at room temperature for 40 hr. After the first 24 hr an additional 25 mg of pepsin was added per l. of digest. After 40-hr digestion the mixture was lyophilized to dryness. To ensure complete removal of the HCl the residue was dissolved in 5–10 ml of water and lyophilized again.

SEPARATION OF PALMITYL PEPTIDES FROM THE PEPTIDE MIXTURE. To the lyophilized residue was added 2–5 ml of butanol saturated with water, and the mixture was applied to a Sephadex–butanol column. G-50 Sephadex (Pharmacia, Uppsala, Sweden) was swollen by stirring for 2 days in a large excess of butanol-saturated water, to which was added acetic acid to a final concentration of 1%. A very dilute slurry (4–5 times the final column volume) was then poured into a column, with the aid of a reservoir so that packing was continuous. A 1.5 × 150 cm column was adequate for the peptic hydrolysate from 500 mg of fatty acid synthetase. Before the sample was applied the column was equilibrated with developing medium: water-saturated butanol to which was added acetic acid to a final concentration of 1%. A pressure head equal to the height of the column bed was used, giving a flow rate of 6 ml/hr. The radioactive palmityl peptides were eluted immediately after the void volume with the butanol (Figure 3). The bulk of the nonacylated peptides was eluted from the column with water saturated with butanol. However, a small portion of the nonpalmityl peptides was eluted also with butanol saturated with water and followed closely behind the radioactive peak. To avoid any contamination by these peptides the chromatography was repeated on a second Sephadex–butanol column, similar to the first one. The recovery from each column was about 70%. The same radioactive peptide distribution on tlc (see next section) was obtained before and after column chromatography, indicating that the loss was not due to retention by the column of any specific peptide fraction.

SEPARATION OF THE COMPONENT PALMITYL PEPTIDES. The radioactive fractions from the Sephadex–butanol column were combined and lyophilized to dryness. Lyophilization of butanol saturated with water can be achieved by having a very high vacuum and a thin shell of frozen solution in the lyophilizing flask. The residue (about 2 mg from 500 mg of original fatty acid synthetase) was taken up in 0.2 ml of solvent for application to tlc plates. Methanol–water–acetic acid (5:2:1, v/v) was found to be the most efficient solvent for quantitative transfer of the palmityl peptides to tlc plates. However, the peptides must be immediately applied to the tlc plate in order to avoid methanolysis of the acyl groups. The palmityl peptides from 500 mg of starting fatty acid synthetase were applied to two 20 × 20 cm × 0.2 mm silica gel plates and developed in phenol–butanol–acetic acid–water (2:2:1:1, v/v); running time 4–5 hr. The separated bands were located by autoradiography (Figure 4). In this system palmityl-CoA remains at the origin. Lack of a radioactive band at the origin is good evidence that the washing procedure removed all noncovalently bound palmityl-CoA from the enzyme. To recover the palmityl peptides from each band a tracing was made of the autoradiogram and the tracing inverted over the thin-layer plate. Each band was marked on the silica gel by cutting through the tracings with a razor blade. The gel for each band was scraped from the plate with a narrow, blunt-ended spatula, transferred to a test tube, and eluted 3 times with methanol–water–acetic acid (5:2:1, v/v). After determination of the total radioactivity extracted from each band (about 70% recovery) the eluates were rapidly evaporated to dryness.

Test for Deacylase Activity. Fatty acid synthetase (5 mg) and 0.2 μmole of [^{14}C]palmityl-CoA (4 μCi/μmole) per ml were incubated at 0°. Aliquots of the reaction mixture, taken at 1-min intervals, were applied to tlc microplates. These were immediately developed in petroleum ether–diethyl ether–acetic acid (4:5:1, v/v). In this system palmitic acid runs with the solvent front and palmityl-CoA and acylated protein remain at the origin. With incubation times of up to 10 min there was no significant formation of palmitic acid (<0.3%) and the amount was independent of time of incubation of enzyme with palmityl-CoA.

Identification of Palmityl Peptides. According to the mechanism of fatty acid synthesis in Figure 1, there are 3 different enzymatic sites with which palmityl-CoA would be expected to react. These are on the transacylase, acyl carrier protein, and condensing enzyme, the latter two being thiol sites. In the absence of malonyl-CoA the palmityl residue could not enter the reaction sequence beyond the condensing enzyme. Since more than three peptides were separated on tlc some of these must be either peptides arising from the same site but of differing amino acid composition, or peptides arising from hitherto unrecognized sites on the fatty acid synthetase complex.

DISTINCTION BETWEEN THIOL AND NONTHIOL PALMITYL ESTERS. Thiol esters of palmityl enzyme were distinguished by the peroxidation method of Harris *et al.* (1963). Performic acid at 0° oxidizes thiol esters to the corresponding sulfonic and carboxylic acids. Palmityl peptides from a Sephadex–butanol column were lyophilized and the residue dissolved in performic acid. After incubation at 0° for 4 hr the reaction mixture was dried on a rotary evaporator; the residue was taken up in methanol–water–acetic acid, 5:2:1, and applied to a tlc plate. In control experiments formic acid was used in place of performic acid. The results of tlc in phenol–butanol–water–acetic acid (2:2:1:1, v/v), of formic and performic acid treated peptides are shown in Figure 5A. From this it is clear that the 2 slowest moving bands and the fastest band arose from thiol sites on the enzyme. The palmityl peptides remaining after peroxidation are due to reaction at nonthiol sites.

DISTINCTION BETWEEN ACYL CARRIER PROTEIN AND CONDENSING ENZYME THIOL ESTERS. Hagen (1963) showed that inactivation of fatty acid synthetase by iodoacetamide was due to reaction at the condensing enzyme only since no carboxymethylcysteamine was found in the hydrolyzed enzyme. Subsequently, Oesterhelt (1967) isolated the [^{14}C]carbamidomethyl peptide resulting from the reaction of fatty acid synthetase with [^{14}C] iodoacetamide, and showed this to be identical with the [^{14}C]acetyl peptide from the condensing enzyme site. In addition, inhibition by iodoacetamide could be diminished by protecting the enzyme with acetyl-CoA but not with malonyl-CoA (Oesterhelt, 1967). From these experiments it is clear that iodoacetamide reacts with the condensing enzyme but not with the acyl carrier protein thiol group.

Use was made of this selective reaction of iodoacetamide in differentiating between peptides arising from the active site of the condensing enzyme and from acyl carrier protein.

Experimental conditions were those used by Hagen (1963) and Oesterhelt (1967). Fatty acid synthetase (50 mg/ml) was incubated for 10 min at pH 6.5 and 0° with 0.02 M iodoacetamide. Cysteine, final concentration 0.2 M, was then added to stop the reaction of iodoacetamide with enzyme. At this time the activity of the enzyme in the overall reaction of fatty acid synthetase was less than 1%. After 10 min the treated fatty acid synthetase (10 mg/ml) was incubated with [^{14}C]palmityl CoA (0.3 μmole/ml) for 8 min at 0°. Reaction was stopped with HCl, the protein washed, and peptides prepared and purified as above. The tlc peptide map was compared to that of peptides from a control experiment in which fatty acid synthetase had been treated identically, except iodoacetamide was omitted (Figure 5B). The peptide maps are identical except for the absence of the fast-moving peptide after iodoacetamide treatment. This peptide must then be from the condensing enzyme site. It may be deduced that the two slowest moving peptides, also thiol esters, arise from the acyl carrier protein site.

VERIFICATION OF THE ACYL CARRIER PROTEIN PEPTIDE. The acyl carrier protein moiety of yeast fatty acid synthetase

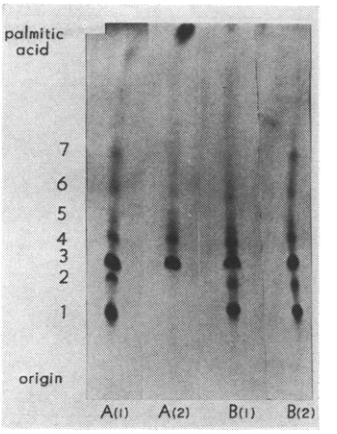


FIGURE 5: Autoradiogram of tlc plate developed in phenol–butanol–water–acetic acid (2:2:1:1, v/v). A. Effect of (1) formic acid and (2) performic acid on palmityl peptides from fatty acid synthetase. Palmitic acid, resulting from the peroxidation, has an R_F of 1. B. Effect of preincubation with iodoacetamide on the reaction of palmityl-CoA with fatty acid synthetase; (1) incubation with iodoacetamide, (2) iodoacetamide omitted.

complex is similar to the acyl carrier protein of *Escherichia coli* (Majerus *et al.*, 1965, Pugh and Wakil, 1965) in that it contains a phosphopantotheine residue at the reactive site (Lynen, 1967). Initially, an attempt was made to identify which peptides contained the phosphopantotheine by exposing the peptide mixture to snake venom phosphodiesterase.[1] A large excess of the phosphodiesterase was used, since it was expected that even if the peptide–phosphopantotheine bond were cleaved, the rate of reaction might be low. [^{14}C]Palmityl-CoA was used as a control. Tlc of [^{14}C]palmityl-CoA or [^{14}C]palmityl peptides after incubation with phosphodiesterase showed a new radioactive spot with R_F 0.07. It was assumed that this was [^{14}C]palmitylpantotheine or [^{14}C]palmitylpantotheine phosphate. The two slowest moving radioactive palmityl peptides were no longer apparent, thus indicating that they were derived from the acyl carrier protein site. In subsequent experiments, using less phosphodiesterase and longer incubation times, it was not possible to repeat these observations. It was, therefore, concluded that the phosphopantotheine bond was not cleaved but, instead, that the phosphodiesterase had bound the palmityl-CoA or palmityl peptides, thus changing their rate of migration on the tlc plate.

Although these experiments indicated that the two slower moving peptides arose from the acyl carrier protein site, the results were not conclusive. Another approach was therefore used. The two radioactive peptides, presumed to arise from acyl carrier protein, were eluted from the tlc plate, and the concentration of peptide was determined from the radioactivity (specific activity of starting palmityl-CoA was 1 μCi/μmole). The eluted peptide was then hydrolyzed and assayed microbiologically for pantothenic acid (Winnewisser, 1972[2]). The concentration of peptide calculated from the pantothenic acid content agreed exactly with the peptide concentration calculated from the radioactivity of the palmityl residue (Figure 6). In addition, amino acid analyses of the isolated

[1] The snake venom phosphodiesterase (*Crotalus terr. terr.*) was the generous gift of Dr. Jaworeck, Boehringer, Tutzing, Germany.

[2] We are most grateful to Dr. W. Winnewisser, University of Munich, for performing the microbiological assays for pantothenic acid.

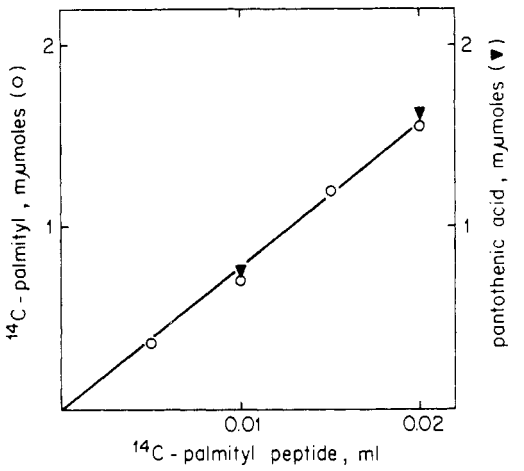


FIGURE 6: Concentration of [^{14}C]palmityl peptide calculated from disintegrations per minute compared to the concentration of pantothenic acid determined by microbiological assay.

peptide showed an equivalence of β-alanine with incorporated [^{14}C]palmitate (see below).

Competition between Acetyl and Palmityl CoA. In order to determine if the nonthiol transacylase site with which palmityl-CoA reacts is the same as that which reacts with acetyl-CoA, [^{14}C]palmityl-CoA was incubated with enzyme, together with nonradioactive acetyl-CoA. Fatty acid synthetase, 10 mg/ml, was incubated with 0.1 μmole of [^{14}C]palmityl-CoA per ml and 0.2 μmole of acetyl-CoA per ml, in 0.1 M potassium phosphate buffer (pH 6.5) for 8 min at 0°. The reaction was stopped with HCl and the precipitated protein washed, as described above, to remove all nonreacted [^{14}C]palmityl-CoA. The dried, washed precipitate was dissolved in formic acid and the specific loading determined. Aliquots of the formic acid solution of protein were peroxidized by adding 1 part of 30% aqueous H_2O_2 to 9 parts of the formic acid solution and incubating at 0° for 4 hr. The peroxidized protein was then applied to Whatman No. 1, 3 × 40 cm paper strips, and chromatographed in chloroform–methanol–water–acetic acid (60:35:8:1, v/v). Palmitic acid, liberated by the action of performic acid on thiol esters of palmitic acid with enzyme, runs with the solvent front and the protein remains at the origin. The chromatograms were run to completion (running time about 4 hr), dried, cut into 7-cm long strips and counted in 2,5-diphenyloxazole–1,4-bis[2-(4-methyl-5-phenyloxazolyl)]-benzene–toluene solution in a Packard Tri-Carb liquid scintillation spectrometer. The results given in Table I show that although there was very little effect of acetyl-CoA on the formation of thiol esters of palmitic acid with enzyme, the reaction of palmityl-CoA with nonthiol sites was about 50% inhibited by an acetyl-CoA:palmityl-CoA ratio of two.

Amino Acid Analyses of the Isolated Peptides. Peptides isolated from the peptic hydrolysate were digested in an evacuated tube at 110° in 6 N HCl for 20 hr. About 20 nmoles was used for each assay. Analyses were performed on a Beckman-Spinco Model 120B amino acid analyzer. The results of analyses of the different peptides (Figure 4) are given in Table II. Bands 6 and 7 were still contaminated with nonradioactive peptide so that precise interpretation of the amino acid analyses could not be made. However, the results were compatible with peptide 6 arising from the same transacylase site as peptides 3, 4, and 5.

TABLE I: Effect of Acetyl-CoA on the Covalent Binding of [^{14}C]Palmityl-CoA by Fatty Acid Synthetase.[a]

Binding of Palmityl Residues to:	μmoles of [^{14}C]Palmityl Incorporated/μmole of Fatty Acid Synthetase		
		+ Acetyl-CoA	% Inhibn
Nonthiol sites	1.27	0.67	47
Thiol sites	2.56	2.35	8

[a] The values, determined after equilibrium was attained, are the averages of four experiments. Reproducibility was within 5%. The two types of sites were distinguished by paper chromatography after peroxidation of the palmityl peptides. Details are given in the text.

Discussion

It is well recognized that palmityl-CoA binds strongly and nonspecifically to proteins, in most cases causing enzyme inhibition by its detergent-like action (*e.g.*, Srere, 1965, Taketa and Pogell, 1966). Yeast fatty acid synthetase is also strongly inhibited by palmityl-CoA. The inhibition is considerably diminished by serum albumin, indicating a nonspecific binding phenomenon (Lust and Lynen, 1968). Dorsey and Porter (1968) report inhibition of the fatty acid synthetase complex of pigeon liver by palmityl-CoA due to its detergent-like action. They were, however, unable to detect any covalent binding of palmityl residue to the enzyme. This discrepancy with our results is possibly due to the fact that the product of pigeon liver fatty acid synthetase is palmitic acid, whereas the product of yeast fatty acid synthetase is palmityl-CoA. On the other hand, covalently bound palmityl is very sensitive to methanolysis, especially in nonacidic solutions, and in the experiments of Dorsey and Porter the enzyme was washed free of nonspecifically bound palmityl-CoA with methanol–chloroform–water (2.5:1.25:1, v/v).

In addition to the apparently nonspecific binding, long-chain fatty acids inhibit fatty acid synthetase competitively with respect to the substrate malonyl-CoA, suggesting that there is also a specific effect of palmityl-CoA (Lust and Lynen, 1968). It was reported earlier (Numa *et al.*, 1965) that palmityl-CoA competitively inhibited acetyl-CoA carboxylase with respect to the activator, citrate. Since palmityl-CoA was a 10 times better inhibitor than free palmitic acid, and 50% inhibition was obtained with physiological concentrations, it was suggested that palmityl-CoA was involved in a negative feedback mechanism. The cellular synthesis of fatty acids would then be regulated by the effect of long-chain fatty acids on both acetyl-CoA carboxylase and the fatty acid synthetase complex. It was observed with both enzymes that the longer the chain length of the fatty acids the greater the inhibition.

The following observations presented in this paper established that in addition to the nonspecific binding, specific covalent binding of palmityl to fatty acid synthetase does occur. (1) No palmityl residues were found associated with enzyme that had been inactivated at the start of reaction. (2) After peroxidation a very specific part of the bound palmityl was released from the enzyme. (3) Following peptic digestion it was still not possible to separate palmitic acid or palmityl-

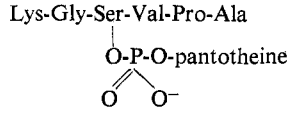
TABLE II: Amino Acid Composition of the Peptic Palmityl Peptides from Fatty Acid Synthetase.

Amino Acid	1[a]	2	3	4	5
Lysine	0.90 (1)[b]	Na[c]		Na	Na
Histidine		Na	1.01 (1)	Na	Na
Arginine		Na		Na	Na
Aspartic acid	1.08 (1)				
Threonine	1.01 (1)	0.86 (1)			
Serine	0.94 (1)	1.04 (1)	0.98 (1)	1.18 (1)	0.97 (1)
Glutamic acid	1.20 (1)	0.99 (1)	1.06 (1)	0.86 (1)	1.31 (1)
Proline					
Glycine	2.10 (2)	1.87 (2)	1.99 (2)	1.13 (1)	1.86 (2)
Alanine			0.76 (1)		
Valine	1.81 (2)	1.29 (1)			
Methionine					
Isoleucine					
Leucine			0.94 (1)	0.82 (1)	1.13 (1)
Tyrosine					
Phenylalanine					
β-Alanine	0.94 (1)	0.95 (1)			

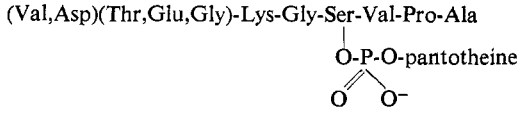
[a] The numbers refer to the peptide bands, separated by tlc, in Figure 4. [b] The measured amino acid content is given, average of duplicate analyses for peptides 1 and 3 and single analyses for peptides 2, 4, and 5, followed by the assumed number of residues in parentheses. [c] Na = not assayed.

CoA on tlc. (4) The amino acid analyses of palmityl peptides were consistent with the existence of (a) only one nonthiol binding site for palmityl and (b) two types of sulfhydryl sites, one containing β-alanine and pantothenic acid. (5) Preincubation with iodoacetamide specifically inhibited the formation of one of the palmityl peptides. (6) The amount of reaction of palmityl with the enzyme was reduced by the presence of acetyl-CoA in the incubation mixture. The conclusion that palmityl was covalently bound at three different sites on the complex is consistent with the mechanism of Lynen as outlined in Figure 1.

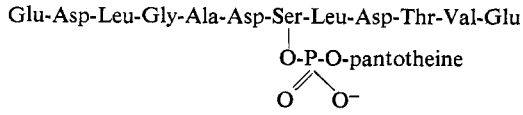
From the results given in Table II it is apparent that the two thiol peptides 1 and 2 arise from the same site. The only difference is that peptide 1 contains a valine and an aspartic acid residue which peptide 2 does not contain. The active-site peptide isolated by Oesterhelt (1967) as acetyl-acyl carrier protein had the structure



This together with the current information, gives a peptide with the structure



This differs from the acyl carrier protein of *E. coli* which around the active site has the structure (Vanaman *et al.*, 1968)



There is, however, no doubt that these peptides from the yeast fatty acid synthetase arise from the acyl carrier protein moiety of the complex since amino acid analyses showed equimolar concentrations of β-alanine; and stoichiometric quantities of pantothenic acid were found in the microbiological assay.

The nonthiol palmityl peptides, bands 3, 4, 5, and 6 (Figure 4 and Table II) have very similar amino acid composition indicating that they most probably arise from one site, *i.e.*, that of the palmityl transacylase. The main band (band 3, Figure 4), a heptapeptide, contained the amino acids: alanine, glutamic acid, 2 glycines, histidine, serine, and leucine. Bands 4 and 5 were both similar except band 4 had an alanine and a glycine less, and band 5 had only an alanine less than band 3. The structure of the palmityl transacylase peptide is therefore: Ala-Gly-(His-Ser,Leu,Glu,Gly). This is of particular interest since it is identical with the active-site peptide for the malonyl transacylase (Schweizer *et al.*, 1970). After peptic digestion of [^{14}C]malonyl enzyme Schweizer *et al.* isolated 2 homogeneous, radioactive, nonthiol peptides. One, a pentapeptide, contained the amino acids: serine, histidine, glycine, alanine, and leucine. The other, a heptapeptide, had in addition glycine and glutamic acid.

These results can be interpreted in two ways. Either the palmityl and malonyl transacylase activities of the fatty acid synthetase are carried out by one enzyme, or there is a common amino acid content at the active site of two different transacylase enzymes. The hypothesis of one transacylase being shared by malonyl and palmityl offers distinct advantages in terms of the overall enzymatic mechanism. Transacylases function in all three phases of fatty acid biosynthesis, *i.e.*, in initiation, elongation, and termination (Figure 1). Termination, *i.e.*, transfer of the palmityl residue from enzyme to CoA, must be followed by initiation, *i.e.*, transfer of acetyl from CoA to the enzyme, before the elongation cycle, which begins with the transfer of malonyl from CoA to the enzyme, can proceed. If termination were effected by the same transacylase which incorporates malonyl, the incorporation of a

malonyl residue would be momentarily blocked at termination allowing time for an acetyl residue to be transferred *via* the acyl carrier protein to the condensing enzyme.

The three following lines of evidence suggest that malonyl transacylase and palmityl transacylase may be the same enzyme. (i) It has been shown that the chain length of the product can be varied by altering the acetyl-CoA:malonyl-CoA ratio (Sumper *et al.*, 1969, Popjak, 1970, and Carey *et al.*, 1970). The higher the ratio the greater the production of shorter chain length fatty acyl-CoAs. This effect is particularly pronounced if the ratio is increased by severely restricting the malonyl-CoA concentration. If there were only one transacylase this could be readily explained as lack of competition by malonyl-CoA with the product-saturated fatty acid for the transacylase. The transacylase is therefore available to effect premature chain termination. (ii) Further evidence in support of this hypothesis is given by the inhibition studies of Lust and Lynen (1968). They have shown that C_{12}, C_{14}, C_{16}, and C_{18} saturated fatty acyl-CoAs inhibit noncompetitively with respect to acetyl-CoA but competitively with respect to malonyl-CoA. They interpreted this to be due to competition at the acyl carrier protein site, but in view of the present findings this may in fact be due to competition at the transacylase. (iii) Another indication of competition between malonyl-CoA and saturated fatty acyl-CoAs was observed by Pirson studying the initiation reaction. He found that when synthesis is initiated by substituting decanoyl-CoA for acetyl-CoA the reaction becomes inhibited when high concentrations of malonyl-CoA are used (Pirson, 1970). A similar substrate inhibition by malonyl-CoA has not been observed in the standard reaction using acetyl-CoA as initiator.

On the other hand, each of these observations could be interpreted as an effect at the acyl carrier protein site. The possibility, therefore, still remains that malonyl transacylase and palmityl transacylase are 2 different enzymes with the same amino acid sequence at their active sites.

The competition experiments using acetyl-CoA suggest that the palmityl transacylase can also react with acetyl-CoA. In the presence of acetyl-CoA the reaction of palmityl-CoA with transacylase is inhibited. Twice as much acetyl-CoA as palmityl-CoA is required to give 50% inhibition indicating that the affinity of acetyl-CoA for this site is lower than that of palmityl-CoA. If this were the only transacylase with which acetyl-CoA could react, in the reverse experiment, in which cold palmityl-CoA is used as an inhibitor of the covalent binding of [^{14}C]acetyl-CoA, it would be expected that a concentration of palmityl-CoA less than that of acetyl-CoA would give 50% inhibition. Preliminary experiments, however, indicate that this is not the case. With palmityl-CoA and acetyl-CoA in equimolar concentrations no significant inhibition was observed. It appears, therefore, that in addition to the ability of acetyl-CoA to react with the palmityl transacylase, another transacylase exists which binds specifically acetyl-CoA and not palmityl-CoA.

Recently Ziegenhorn (1970) has isolated and sequenced an acetyl peptide from fatty acid synthetase which does not contain a thiol ester bond. The peptide is therefore presumably derived from an acetyl transacylase. The amino acid content of the acetyl peptide differs from that of the malonyl and palmityl transacylase peptides indicating clearly that there is another active site with which acetyl-CoA reacts. This is analogous to the *E. coli* system which has two different transacylases, one which reacts with acetyl-CoA and one with malonyl-CoA (Williamson and Wakil, 1966).

From the above experiments, together with previous finding, it is evident that the fatty acid synthetase complex contains at least two transacylases. One of these is specific for the initiation reaction and another is active in the elongation reaction. It is still not clear whether the termination reaction uses the same transacylase as used for elongation or whether a third transacylase is involved. In either case there is no explanation of why fatty acyl-CoAs of more than 18 carbon atoms are never synthesized. In an attempt to determine why there is an absolute termination of synthesis at C_{18} a quantitative analysis was made of the reaction of fatty acid synthetase with saturated fatty acyl-CoAs from C_{10} to C_{18}. A report of this work will be presented in a subsequent communication (Ayling *et al.*, 1972).[3]

References

Beisenherz, G., Boltze, H. J., Buecher, T., Czuk, R., Garbarde, K. H., Meyer-Ahrendt, E., and Pfleiderer, G. (1953), *Z. Naturforsch 8b*, 555.

Buckel, W., and Eggerer, H. (1965), *Biochem. Z 343*, 29.

Carey, E. M., Dils, R., and Hansen, H. J. M. (1970), *Biochem. J. 117*, 633.

Dorsey, J. A., and Porter, J. W. (1968), *J. Biol. Chem. 243*, 3512.

Hagen, A. (1963), Doctoral Dissertation, University of Munich, Munich, Germany.

Harris, I., Meriwether, B. P., and Harting-Park, J. (1963), *Nature (London) 198*, 154.

Lowry, O. H., Rosebrough, N. J., Farr, A. L. and Randall, R. J. (1951), *J. Biol. Chem. 193*, 265.

Lust, G., and Lynen, F. (1968), *Eur. J. Biochem. 7*, 68.

Lynen, F. (1967), *Biochem. J. 102*, 381.

Lynen, F. (1969), *Methods Enzymol. 14*, 17.

Lynen, F., Hopper-Kessel, I., and Eggerer, H. (1964), *Biochem. Z. 340*, 95.

Majerus, P. W., Alberts, A. W., and Vagelos, P. R. (1965), *J. Biol. Chem. 240*, 4723.

Numa, S., Ringelmann, E., and Lynen, F. (1965), *Biochem. Z. 343*, 243.

Oesterhelt, D. (1967), Doctoral Dissertation, University of Munich, Munich, Germany.

Pirson, W. (1970), Doctoral Dissertation, University of Munich, Munich, Germany.

Popjak, G. (1970), British Biochemistry Past and Present, Goodwin, T. W., Ed., New York, N. Y., Academic Press, p 137.

Pugh, E. L., and Wakil, S. J. (1965), *J. Biol. Chem. 240*, 4727.

Schweizer, E., Piccinini, F., Duba, C., Günther, S., Ritter, E., and Lynen, F. (1970), *Eur. J. Biochem. 15*, 483.

Seubert, W. (1960), *Biochem. Prep. 8*, 80.

Simon, E. J., and Shemin, D. (1953), *J. Amer. Chem. Soc. 75*, 2520.

Srere, P. A. (1965), *Biochim. Biophys. Acta 106*, 445.

Sumper, M., Oesterhelt, D., Riepertinger, C., and Lynen, F. (1969), *Eur. J. Biochem. 10*, 377.

Taketa, K., and Pogell, B. M. (1966), *J. Biol. Chem. 241*, 720.

Vanaman, T. C., Wakil, S. J. and Hill, R. L. (1968), *J. Biol. Chem. 243*, 6420.

Wieland, T., and Rueff, L. (1953), *Angew. Chem. 65*, 186.

Williamson, I. P., and Wakil, S. J. (1966), *J. Biol. Chem. 241*, 2326.

[3] In preparation.

Winnewisser, W. (1972), Doctoral Dissertation, University of Munich, Munich, Germany.

Ziegenhorn, J. (1970), Doctoral Dissertation, University of Munich, Munich, Germany.

Covalent Coupling of Ribonucleic Acid to Agarose*

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ABSTRACT: A procedure for coupling RNA to a modified agarose resin is described. The amino group of $NH_2(CH_2)_5CO_2CH_3$ is coupled to the agarose by an alkaline CNBr procedure. The ester group is converted to a hydrazide by reaction with hydrazine. RNA is oxidized with IO_4^- to give a 3′-terminal aldehyde and then coupled to the resin by hydrazone formation. This coupling reaction is very slow unless negatively charged carboxyl groups on the resin surface are blocked by amide-bond formation with glycinamide as directed by a water soluble carbodiimide. The carbodiimide–glycinamide step seems to introduce some unidentified basic positively charged groups onto the resin surface, thus causing nonspecific ionic binding of RNA to the resin. The nonspecific binding accelerates the rate of hydrazone-bond formation. Excess nonspecifically bound RNA is released by raising the pH above 9. Poly(U) on the resin surface can hybridize with poly(A) in solution, and the poly(A) can be eluted in a denaturing Me_2SO solvent. With *Escherichia coli* 16S rRNA coupled to the resin, a 20-fold enrichment of rDNA from sheared denatured *E. coli* DNA of single-strand molecular weight about 7×10^4 was achieved by hybridization, followed by elution with NaOH. The procedure was not successful for enriching rDNA in a preparation of high molecular weight DNA.

In the development of techniques for the isolation of molecules which interact specifically with a given biological macromolecule, it is often useful to attach the latter to a solid support. A number of methods for attaching nucleic acids to solid supports have been developed and applied recently (Gilham, 1968, 1971; Nelidova and Kiselev, 1968; Alberts *et al.*, 1968; Litman, 1968; Alberts and Herrick, 1971; Jovin and Kornberg, 1968; Bonavida *et al.*, 1970; Poonian *et al.*, 1971). We describe here a procedure for covalent coupling of RNA molecules *via* their 3′ terminus to an agarose gel. Our final objective, the isolation of high molecular weight single strands of DNA containing rDNA genes, has not yet been achieved, but the coupling method may be useful for other applications and we accordingly describe it here.

The procedure involves the following steps: (1) coupling of $NH_2(CH_2)_5CO_2CH_3$ (ε-aminocaproic acid methyl ester) to agarose which has been activated by treatment with alkaline CNBr. The use of an ε-aminocaprolyl derivative to provide a functional group sterically separated from the agarose and therefore more accessible to reagents in solution was suggested by Cuatrecasas *et al.* (1968). The activation of a polysaccharide support by alkaline CNBr for coupling to a nucleophilic reagent was described by Axen *et al.* (1967); (2) conversion of the caprolyl ester function to a hydrazide agarose, $NH(CH_2)_5CONHNH_2$. This we call "hydrazide agarose." (4) The next step in the procedure as originally planned was the oxidation of RNA at its 3′ terminus with IO_4^- and reaction of the oxidized RNA with the hydrazide function to give a hydrazone. Low yields were observed in this step and were attributed to electrostatic repulsion between carboxyl groups on the surface of the resin and the negative polyelectrolyte RNA. Therefore step (4) was inserted into the sequence of reactions. (The coupling of RNA to polyacrylhydrazine-agar has been described by Nelidova and Kiselev (1968).) (3) In order to neutralize these putative carboxyl groups, we have treated the hydrazide agarose with glycinamide and a water-soluble carbodiimide, thus forming amide bonds to the carboxyl groups (Hoare and Koshland, 1967). The product is called "blocked hydrazide agarose." The resulting product is coupled to RNA according to step 4 above. In addition to amide-bond formation, the carbodiimide–glycinamide treatment evidently introduces some positive charges onto the resin. We find that the product is capable of binding unoxidized RNA at pH's below 7. The polynucleotide so bound is released by treatment at pH 8 or greater, leaving only oxidized RNA covalently attached by the hydrazone bond to the agarose.

Materials and Methods

Activation of Agarose. Agarose (100 ml; Sepharose 4B-200 from the Sigma Chemical Co.) was washed by filtration on a coarse sintered-glass funnel with several changes of water and suspended to a total volume of 200 ml in water. Cyanogen bromide (10 g), dissolved in 200 ml of H_2O, was added to the 200-ml suspension of agarose in H_2O. The reaction flask was immersed in an ice bath and the agarose was kept in suspension by magnetic stirring. The suspension was immediately adjusted to pH 11 and maintained at this pH for 9 min by adding 4 N NaOH. It was then washed onto a coarse sintered-

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