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Amino Acid Sequences of Substrate-Binding Sites in Chicken Liver Fatty Acid Synthase†

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ABSTRACT: The amino acid sequences of three essential regions of chicken liver fatty acid synthase have been determined: that around 4′-phosphopantetheine ("carrier" site), the substrate "loading" site containing serine, and a "waiting" site for the growing fatty acid containing cysteine. The amino acid sequence of the 4′-phosphopantetheine region was determined for the acetyl-, malonyl-, hydroxybutyryl-, and butyryl-enzyme with peptides obtained by hydrolysis of the enzyme with trypsin and *Staphylococcus aureus* (V8) protease. The sequence region around the essential serine was obtained for the acetyl- and malonyl-enzyme. The N-terminus of the tryptic peptide was blocked. However, the same sequence is obtained for the acetyl- and malonyl-peptide after *S. aureus* protease digestion, suggesting that the enzyme contains a single acyl transferase rather than two separate transacylases. The sequence around the cysteine was obtained by use of a radioactive iodoacetamide label. An unusual sequence of three serines adjacent to the cysteine was found. The strong similarities between peptides from different species for all three of the regions suggest that the multifunctional polypeptides from yeast and animals have evolved from the monofunctional enzymes of lower species.

Chicken liver fatty acid synthase (M_r ~500 000) is one of the most complex multienzyme complexes in the animal kingdom: It consists of two identical polypeptide chains [cf. Wakil et al. (1983)] and contains six different enzyme activities. The enzyme catalyzes the synthesis of palmitic acid according to the overall reaction

$$\text{acetyl-CoA} + 7\text{malonyl-CoA} + 14\text{NADPH} + 14\text{H}^+ \rightarrow \text{palmitic acid} + 8\text{CoA} + 14\text{NADP}^+ + 6\text{H}_2\text{O} + 7\text{CO}_2$$

The mechanism involves initiation of the fatty acid chain by transfer of an acetyl group to the enzyme from acetyl-CoA[1] (acyl transferase). The chain is lengthened by the transfer of a malonyl group to the enzyme from malonyl-CoA (acyl transferase). The condensation of acetyl and malonyl gives rise to the acetoacetyl-enzyme (β-ketoacyl synthase); this enzyme-bound intermediate is reduced to 3-hydroxybutyryl-enzyme by NADPH (β-ketoacyl reductase); dehydration gives the crotonyl-enzyme (dehydratase), which is reduced to butyryl-enzyme by NADPH (enoyl reductase). This cycle is repeated a total of 7 times, with transfer of a malonyl moiety to the enzyme initiating each cycle. After the thioester of palmitic acid is formed, the free fatty acid is released into solution (thioesterase). The overall and elementary steps in the reaction mechanism, the detailed stereochemistry, the distribution of reaction intermediates on the enzyme, and the distances between several specific sites have been explored [cf. Hammes (1985), Cox and Hammes (1983), Cognet and Hammes (1983, 1985), Anderson and Hammes (1984, 1985), Yuan and Hammes (1985, 1986), and Chang and Hammes (1986)].

The structure of the enzyme has been probed by limited proteolysis and chemical modification [cf. Wakil et al. (1983)]. The two polypeptides are arranged head-to-tail with two independent catalytic centers, each derived from two different polypeptide chains. Limited proteolysis yields three polypeptides on sodium dodecyl sulfate–polyacrylamide gels (Tsukamoto et al., 1983). Peptide I contains the acyl transferase, the serine utilized as a substrate "loading" site, and the cysteine that serves as a "waiting" site by forming a thioester with the growing saturated fatty acid chains while a malonyl moiety is loaded onto the enzyme. Peptide II contains the dehydratase, the reductases, and the 4′-phosphopantetheine to which the substrate is bound while the synthase, dehydratase, reductases, and thioesterase act on it. Peptide III is the thioesterase. The β-ketoacyl synthase and β-ketoacyl reductase enzymes require both polypeptide chains for activity,

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[1] Abbreviations: CoA, coenzyme A; NADPH, reduced nicotinamide adenine dinucleotide phosphate; HPLC, high-performance liquid chromatography; EDTA, ethylenediaminetetraacetic acid; Tris, tris(hydroxymethyl)aminomethane; TPCK, L-1-(tosylamido)-2-phenylethyl chloromethyl ketone.

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whereas the other enzymes are active with only a single polypeptide chain (Yuan & Hammes, 1986).

In this study, the amino acid sequences of three essential regions of chicken liver fatty acid synthase have been determined: (1) the 4′-phosphopantetheine site, (2) the substrate loading region containing serine, and (3) the locus containing the cysteine that serves as a waiting site. The results obtained by sequencing acetyl- and malonyl-enzymes suggest that the enzyme contains a single acyl transferase rather than two separate acetyl and malonyl transferases.

Materials and Methods

Chemicals. NADPH, acetyl-CoA, malonyl-CoA, acetoacetyl-CoA, guanidine hydrochloride, hydroxylamine, trifluoroacetic acid, Sephadex G-50–80, Sephadex G-50–300, DEAE-Sephacel, hexokinase (type V), D-glucose-6-phosphate dehydrogenase (type VII), and soybean trypsin inhibitor were from Sigma Chemical Co. Disodium 2-nitro-5-(thiosulfo)-benzoate was prepared by the method of Thannhauser et al. (1984). GF/F glass microfiber filters were from Whatman. (*S*)-[4-^{3}H]NADPH was prepared as previously described (Anderson & Hammes, 1984). [1-^{14}C]Acetyl-CoA and [2-^{14}C]malonyl-CoA were from ICN and Amersham, respectively. D-[1-^{3}H]Glucose was from New England Nuclear. L-1-(Tosylamido)-2-phenylethyl chloromethyl ketone (TPCK) treated trypsin and *Staphylococcus aureus* (V8) protease were from Worthington Biochemical Corp. and ICN ImmunoBiochemicals, respectively. The Vydac 218-TP104 C-18 (10 μm particle size, 4.6 mm × 25 cm) and Vydac 218-TP5405 C-18 (5 μm, 4.6 mm × 5 cm) HPLC columns were from the Separations Group. The Aquapore RP-300 C-8 (7 μm, 4.6 mm × 10 cm) HPLC column was from Brownlee Labs. HPLC-grade triethylamine, phosphoric acid, and 2-propanol were from Fisher Scientific. HPLC-grade acetonitrile was from Burdick & Jackson Laboratories or Fisher Scientific. All other chemicals were of high-quality commercial grades, and solutions were prepared with deionized water. High-purity water from a MilliQ water system was used for HPLC buffers.

Concentrations of acetyl-CoA, malonyl-CoA, and acetoacetyl-CoA were determined by using an extinction coefficient of 1.5×10^4 M^{-1} cm^{-1} at 260 nm (P-L Biochemical Circular OR-10). The concentration of NADPH was determined by using an extinction coefficient of 6.22×10^4 M^{-1} cm^{-1} at 340 nm (P-L Biochemical Circular OR-10).

Radioactivity was determined with a Beckman LS-1801 scintillation counter after dissolving the samples in 5 or 10 mL of aqueous counting scintillant (Amersham).

Fatty Acid Synthase. The enzyme was prepared from chicken livers and assayed as previously described (Cognet & Hammes, 1985; Chang & Hammes, 1986). The specific activity of the enzyme was greater than 1.6 μmol of NADPH/(min·mg) under the standard assay conditions. The protein concentration was determined by measurement of the absorbance at 280 nm and by use of an extinction coefficient for fatty acid synthase of 4.82×10^5 M^{-1} cm^{-1} (or 0.965 cm^2 mg^{-1}; Hsu & Yun, 1970). The 10 mM dithiothreitol and 10% glycerol (w/v) necessary for storage of the purified enzyme were removed by passage of the enzyme through a 3-mL Sephadex G-50 centrifuge column (Penefsky, 1977), preequilibrated with 0.1 M potassium or sodium phosphate and 1 mM EDTA (pH 7.0).

Labeling of the 4′-Phosphopantetheine and the Essential Serine. The time courses of the acylation of fatty acid synthase were studied by mixing 0.1 mL of enzyme (1 mg/mL) in 0.1 M potassium phosphate (pH 7.0) and 1 mM EDTA with 5 μL of 8 mM [1-^{14}C]acetyl-CoA (8 cpm/pmol) or [2-^{14}C]-malonyl-CoA (4.5 cpm/pmol) in deionized water at 23 °C. After 10-s–5-min incubations, the reaction mixture was quenched by addition of 0.1 mL of 6 M guanidine hydrochloride. The acylated enzyme was sulfonated with 5 μL of 2-nitro-5-(thiosulfo)benzoate stock (50 mM) in 1 M Na_2SO_3, pH 7.5, for 10 min, precipitated by addition of 0.2 mL of 1.3 M ice-cold perchloric acid, and stored on ice for 2 h. The binding stoichiometry of the acylation was determined as described by Cognet and Hammes (1983). To determine the amount of oxyacyl-enzyme, 22 μL of 2.0 M neutralized hydroxylamine was added to the denatured and sulfonated enzyme for 20 min. Hydroxylamine cleaves thioesters but not oxyesters (Anderson & Hammes, 1983). The oxyacyl-enzyme was then precipitated by addition of 0.2 mL of 1.3 M ice-cold perchloric acid, stored on ice for 2 h, and assayed as above.

To prepare [1-^{14}C]acetyl-enzyme for tryptic digestion, 5.7 mL of fatty acid synthase (8.8 mg/mL) in 0.1 M potassium phosphate (pH 7.0) and 1 mM EDTA was mixed with 0.45 mL of 8 mM [1-^{14}C]acetyl-CoA (6.9 cpm/pmol) in deionized water for 30 s. The reaction was quenched by adding 6.2 mL of 6 M guanidine hydrochloride in 0.1 M potassium phosphate and 1 mM EDTA, and the enzyme was sulfonated with 0.5 mL of the 2-nitro-5-(thiosulfo)benzoate stock solution for 30 min. The unreacted acetyl-CoA and 2-nitro-5-(thiosulfo)-benzoate were removed by passage of the reaction mixture through a Sephadex G-50 centrifuge column preequilibrated with 3 M guanidine hydrochloride. The amount of acylated enzyme was determined from the known specific radioactivity and concentration of the enzyme. The labeled enzyme was dialyzed against 2 L of 1% acetic acid (adjusted to pH 3.6 with ammonium acetate) containing 0.1% thiodiglycol for 3 h to remove guanidine hydrochloride. This resulted in complete precipitation of the labeled enzyme. After a change of the dialysis buffer, the labeled enzyme was dialyzed overnight. The solution was centrifuged in an Eppendorf centrifuge, Model 5414, at 14000*g* for 5 min, and the pellet was resuspended with a disposable polypropylene pellet pestle mixer (Kontes) in 6 mL of 0.1 M Tris-HCl (pH 8.0) containing 0.1% thiodiglycol.

Tryptic digestion was performed by addition of 0.25 mL of TPCK-treated trypsin (2 mg/mL) to the labeled enzyme at 37 °C. After incubation for 12 h, 0.125 mL of the trypsin was added; this was followed by an additional 12-h incubation. An 0.3-mL aliquot of the digestion mixture was centrifuged, and the supernatant was analyzed by HPLC to monitor the progress of the digestion. The digestion mixture was treated with another 0.25 mL of the trypsin for 12 h. The final digestion mixture was centrifuged in an Eppendorf centrifuge for 5 min, and the supernatant was analyzed immediately by HPLC or stored at −70 °C.

To prepare [1-^{14}C]acetyl-enzyme for *S. aureus* (V8) protease digestion, 1.4 mL of fatty acid synthase (8.8 mg/mL) in 0.1 M potassium phosphate and 1 mM EDTA (pH 7.0) was mixed with 0.11 mL of 8 mM [1-^{14}C]acetyl-CoA (6.9 cpm/pmol) in deionized water for 30 s. The rest of the procedures were carried out in a similar manner as for the tryptic digestion, except that every 8 h an 0.2-mL aliquot of V8 protease (1 mg/mL) was added to the labeled enzyme in 2.0 mL of 0.1 M Tris-HCl (pH 8.0) containing 0.1% thiodiglycol; the digestion was continued for 48 h.

Preparation of the [2-^{14}C]malonyl-enzyme for V8 protease digestion was done in a similar way as the [1-^{14}C]acetyl-enzyme.

To distinguish between the oxyacyl-peptide and the thioacyl-peptide, 0.2 M neutralized hydroxylamine (final con-

centration) was added to the trypsin digestion and the V8 protease digestion supernatant for 20–40 min.

Labeling of the Essential Cysteine. The fatty acid synthase was labeled with iodoacetamide as previously described (Yuan & Hammes, 1985); this procedure gives specific labeling of the essential cysteine. To prepare iodoacetamide-labeled enzyme for proteolytic digestion, 7.8 mL of fatty acid synthase (2.7 mg/mL) in 0.1 M potassium phosphate (pH 5.5) and 1 mM EDTA was mixed with 0.50 mL of 7.6 mM [1-^{14}C]-iodoacetamide (41 cpm/pmol) in ethanol for 20 min. The reaction was stopped by passage through two consecutive centrifuge columns equilibrated with 0.1 M potassium phosphate (pH 7.0) and 1 mM EDTA. After 40 min, the specific activity of the labeled enzyme and the binding stoichiometry for the inhibitor were determined. The labeled enzyme was denatured with 8.3 mL of 6 M guanidine hydrochloride for 10 min and dialyzed against 2 L of 1% acetic acid (pH 3.6) containing 0.1% thiodiglycol for 3 h. After a change of the dialysis buffer, the labeled enzyme was dialyzed overnight. The solution was centrifuged at 8000 rpm for 5 min, and the pellet was resuspended in 2 mL of 0.1 M Tris-HCl (pH 8.0) containing 0.1% thiodiglycol.

Tryptic digestion was performed by addition of 22 μL of TPCK-treated trypsin (10 mg/mL) to the labeled enzyme at 37 °C. After 5 h, another 22 μL of TPCK-treated trypsin was added and the reaction mixture incubated for 24 h. After centrifugation of the digestion mixture for 15 min, the supernatant was stored at −70 °C.

Preparation of the 3-Hydroxybutyryl- and Butyryl-Enzymes. The time course of the reduction of acetoacetyl fatty acid synthase by NADPH was studied by mixing 25 or 250 μg of acetoacetyl-enzyme, prepared by preincubation of 25 or 250 μg of enzyme with 15 or 150 nmol of acetoacetyl-CoA for 2 min in 0.1 M sodium phosphate (pH 7.0) and 1 mM EDTA at 23 °C, with 0.35 or 3.5 nmol of (*S*)-[4-^{3}H]NADPH (33.7 cpm/pmol). After 10-s–5-min incubations, the reaction was quenched by addition of 3 M guanidine hydrochloride for 10 min. The amount of reduced acetoacetyl-enzyme was determined as described before (Cognet & Hammes, 1983) after precipitation of the denatured labeled enzyme by addition of 0.75 M ice-cold perchloric acid. To determine the amount of thioester present on the reduced acetoacetyl-enzyme, 0.2 M neutralized hydroxylamine was added to the denatured labeled enzyme for 20 min before precipitation with the ice-cold perchloric acid. The location of the reduced intermediate on the polypeptide was determined by mixing enzyme, after limited trypsin treatment, with acetoacetyl-CoA and (*S*)-[4-^{3}H]NADPH as above, followed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (Anderson & Hammes, 1985).

For proteolytic digestion of reduced acetoacetyl-enzyme, 7.2 mL of fatty acid synthase (4.6 mg/mL) in 0.1 M sodium phosphate and 1 mM EDTA (pH 7.0) was incubated with 0.17 mL of trypsin (0.1 mg/mL, 25 units/μg) at 23 °C. After 40 min, 1 mL of 20 mM acetoacetyl-CoA was added, followed by 3 mL of 0.16 mM (*S*)-[4-^{3}H]NADPH (30 cpm/pmol) 2 min later. After 45 s, the reaction was quenched by 15 mL of 6 M guanidine hydrochloride; part of the reaction mixture was quenched with 0.1% sodium dodecyl sulfate for polyacrylamide gel electrophoresis (Anderson & Hammes, 1985). In a separate set of experiments, the acetoacetyl-enzyme was incubated with varying concentrations of (*S*)-[4-^{3}H]NADPH; the mole ratio of [NADPH]/[enzyme] was 0.3–3.0. The labeled enzyme was dialyzed against 2 L of 1% acetic acid (pH 3.6) for 3 h. After a change of dialysis buffer, the enzyme was dialyzed overnight. The solution was centrifuged at 7700*g* for 5 min, and the pellet was resuspended in 3 mL of 0.1 M Tris-HCl (pH 8.0) and homogenized by use of a stirring bar.

Tryptic digestion was carried out by addition of 33 μL of trypsin (10 mg/mL, 25 units/μg) to 3 mL of the homogenized labeled enzyme at 23 °C. After incubation for 4 h, 33 μL more of the trypsin was added, followed by an additional 33 μL after 24 h. After 24 h the digestion mixture was centrifuged for 10 min; the supernatant was collected, lyophilized, and stored at −70 °C.

V8 protease digestion was carried out in a similar manner, except that 50 mM ammonium (or sodium) acetate (pH 4.0) was used instead of 0.1 M Tris-HCl (pH 8.0).

Separation of Labeled Peptides. [1-^{14}C]Acetyl-, [2-^{14}C]-malonyl-, [1-^{14}C]iodoacetamide-, or (*S*)-[4-^{3}H]NADPH reduced acetoacetyl-labeled peptides were first separated on a Vydac 218-TP104 C-18 (10 μm, 4.6 mm × 25 cm) or Vydac 218-TP5405 C-18 (5 μm, 4.6 mm × 5 cm) column by elution at 1.0 mL/min with a 120-min linear gradient from 98% solvent A (0.1% triethylammonium phosphate; 0.1% phosphoric acid adjusted to pH 6.3 with triethylamine) to 40% solvent B (100% acetonitrile). Fractions of 1.3 or 0.6 mL were collected. The radioactive fractions were pooled and further purified on the Vydac C-18 column or Aquapore RP-300 C-8 (7 μm, 4.6 mm × 10 cm) column by elution at 1.0 mL/min with an 80-min linear gradient from 98% solvent C (0.1% trifluoroacetic acid) to 40% solvent D (0.1% trifluoroacetic acid/99.9% acetonitrile), 40% solvent E (0.1% trifluoroacetic acid/99.9% 2-propanol), or 40% solvent F (0.1% trifluoroacetic acid/9.9% H_2O/90% acetonitrile). Fractions of 1.3 or 0.6 mL were collected. If necessary, the radioactive fractions were further purified under the same conditions for confirmation of peak purity.

Analysis of Purified Peptides. The purified peptides were characterized by amino acid and sequence analyses. Amino acid analyses were performed with a Waters Pico Tag amino acid analysis system; the samples were hydrolyzed and derivatized according to the method of Bidlingmeyer et al. (1984). For an accurate amino acid analysis, a standard protein and a blank sample (100 μL of solvent C plus 100 μL of solvent F) were also hydrolyzed and derivatized at the same time. Sequence analysis was performed on an Applied Biosystems, Model 470A gas-phase sequencer with an on-line 120A PTH analyzer. A portion (15% or 60%) of the material recovered after each cycle was used for ^{14}C or ^{3}H radioactivity determinations. Amino acid analyses and automated sequence analyses were carried out by the Cornell University Biotechnology Program facility.

Results

Analysis of Acetyl- and Malonyl-Enzyme. For 10-s–5-min incubations of enzyme and acetyl- or malonyl-CoA, the total acetyl or malonyl bound to the enzyme was constant, 2.9–3.8 acetyl groups/enzyme molecule or 2.7 malonyl groups/enzyme molecule. The average fraction of oxyacetyl- and oxymalonyl-enzyme in the total acyl-labeled enzyme was 0.35 and 0.52, respectively. For peptide analysis, the acyl-labeled enzyme was prepared by incubation of either radioactive acetyl- or malonyl-CoA with fatty acid synthase for 30 s. The labeled enzyme had ~3.4 acetyl groups/enzyme molecule or 2.7 malonyl groups/enzyme molecule.

The chromatogram of the tryptic digest of the [1-^{14}C]-acetyl-labeled enzyme by reversed-phase HPLC on a Vydac 218-TP104 C-18 analytic column with solvent system A and B (see Materials and Methods) is shown in Figure 1A. Three major radioactive peaks were found, T-1, T-2, and T-3.

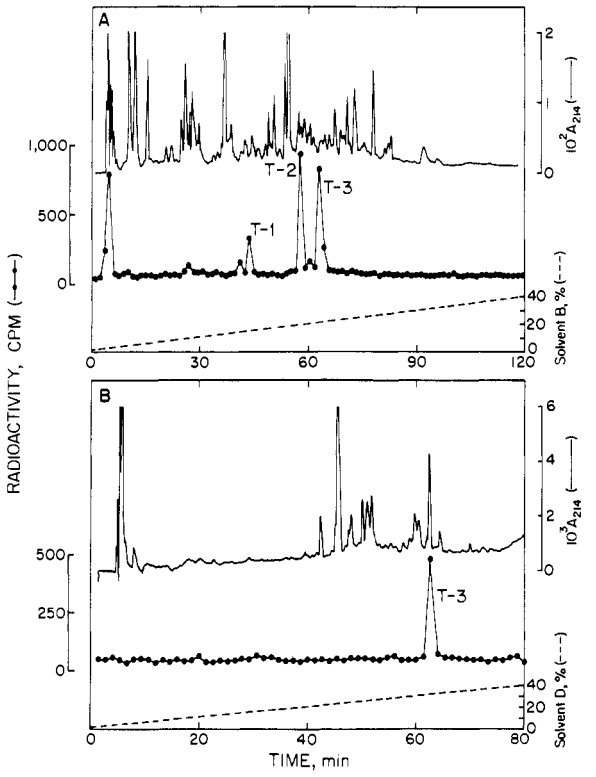

FIGURE 1: Chromatograph of trypsin digest of [1-^{14}C]acetyl-labeled chicken liver fatty acid synthase by reversed-phase HPLC. (A) About 5% of the total labeled peptides were chromatographed on a Vydac C-18 column (4.6 mm × 25 cm) by elution at 1.0 mL/min with an 120-min linear gradient from 98% solvent A to 40% solvent B as described under Materials and Methods. Fractions (1.3 mL) were collected, and 200-μL aliquots from each peak fraction were analyzed for ^{14}C radioactivity. The three major radioactive peaks are labeled T-1, T-2, and T-3. (B) The radioactive peptides T-3 from 33% of the total labeled peptides were chromatographed on a Vydac C-18 column (4.6 mm × 25 cm) by elution at 1.0 mL/min with an 80-min linear gradient from 98% solvent C to 40% solvent D as described under Materials and Methods. Fractions (1.3 mL) were collected, and 30-μL aliquots from each peak fraction were analyzed for ^{14}C radioactivity.

Treatment of the tryptic digest with 0.2 M neutralized hydroxylamine eliminates T-3 but has no effect on T-1 and T-2: The radioactivity previously associated with peak T-3 eluted at the void volume of the HPLC chromatogram. Therefore, T-3 is a thioacetyl-peptide, whereas peaks T-1 and T-2 are oxyacetyl-peptides (Anderson & Hammes, 1983). T-1 occurs only after prolonged trypsin digestion and represents a digestion product of T-2. The three peptides were further purified on the Vydac C-18 column with solvent system C and D. A typical chromatogram for the purification of T-3 is shown in Figure 1B. Sequence analysis of the three peptides indicated the N-termini of T-1 and T-2 were blocked. Amino acid analysis of the three peptides showed that T-1 does not contain arginine or lysine and could be a fragment of T-2. The sequence of peptide T-3 is presented in Table I and is in good agreement with the amino acid analysis. Since none of the amino acids are cysteine, the peptide must contain acetyl-labeled 4′-phosphopantetheine. No significant radioactivity was found in the amino acid derivatives obtained from each sequence cycle. This is probably due to hydrolysis of the phosphodiester bond between serine and 4′-phosphopantetheine during sequencing [cf. Poulose et al. (1984)], or the amount of radioactivity may be too little to detect.

To find the amino acid sequence of the essential hydroxyl group site, [1-^{14}C]acetyl-labeled fatty acid synthase was digested with *S. aureus* protease. Figure 2A shows the HPLC

Table I: Amino Acid Sequences of Acetyl-, Malonyl-, Iodoacetamide-, and Reduced Acetoacetyl-Peptides from Chicken Liver Fatty Acid Synthase

peptide	sequence
T-3, T″-3, T″-4[a]	DVSSLNAESSLADLGLDSLMGVEVR
S-2[b]	LGLDSLMGVE
S-3, S′-3[c]	SSLADLGLDSLMGVE
S-1, S′-1[c]	GILGHSVGE
T′-1[d]	ISYFYDFTGPSLTIDTACSSSLM

[a] From acetyl (T-3)- and reduced acetoacetyl (T″-3, T″-4)-enzyme peptides. [b] From acetyl-enzyme peptides. [c] From acetyl (S-1, S-3)- and malonyl (S′-1, S′-3)-enzyme peptides. [d] From iodoacetamide-modified enzyme peptides.

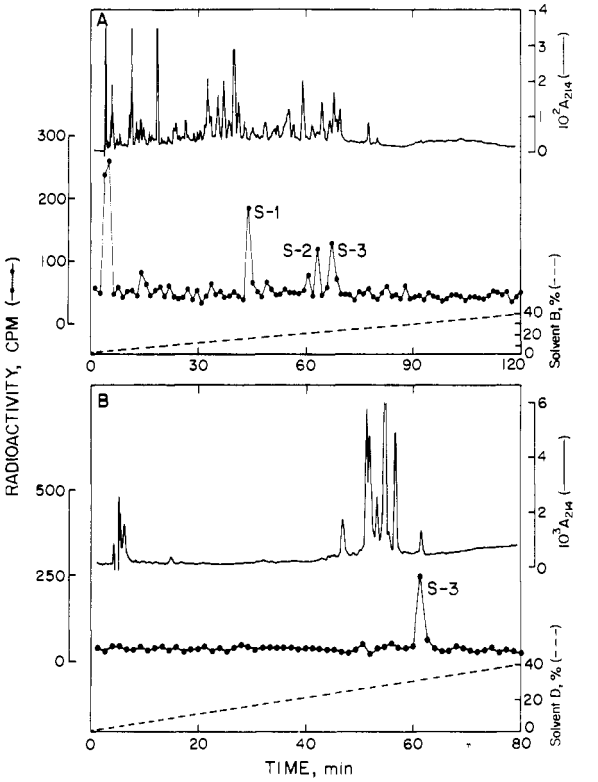

FIGURE 2: Chromatograph of *S. aureus* protease digest of [1-^{14}C]-acetyl-labeled chicken liver fatty acid synthase by reversed-phase HPLC. (A) About 45% of the total labeled peptides were separated as described in the legend to Figure 1A. Fractions (1.3 mL) were collected, and 40-μL aliquots from each peak fraction were analyzed for ^{14}C radioactivity. The three major radioactive peaks are labeled S-1, S-2, and S-3. (B) The radioactive peptides S-3 were chromatographed as described in the legend to Figure 1B. Fractions (1.3 mL) were collected, and 50-μL aliquots from each peak fraction were analyzed for ^{14}C radioactivity.

chromatogram of the *S. aureus* protease digest of the [1-^{14}C]acetyl-labeled enzyme with the same conditions as in Figure 1; three major radioactive peaks (S-1, S-2, and S-3) were found. Two radioactive peaks (S-2 and S-3) disappeared after treatment of the digest with 0.2 M neutralized hydroxylamine. This indicates that peaks S-2 and S-3 are thioacetyl-labeled peptides and that peak S-1 is the oxyacetyl-labeled peptide (Anderson & Hammes, 1984). The peptides were further purified on the Vydac C-18 column with solvent system C and D or the Aquapore RP-300 C-8 column with solvent system C and F. A typical chromatogram for the purification of S-3 is shown in Figure 2B.

The amino acid sequences of peptides S-1, S-2, and S-3 are given in Table I. The amino acid compositions of the peptides were in good agreement with the sequences. No significant radioactivity was released at any cycle from automated Edman

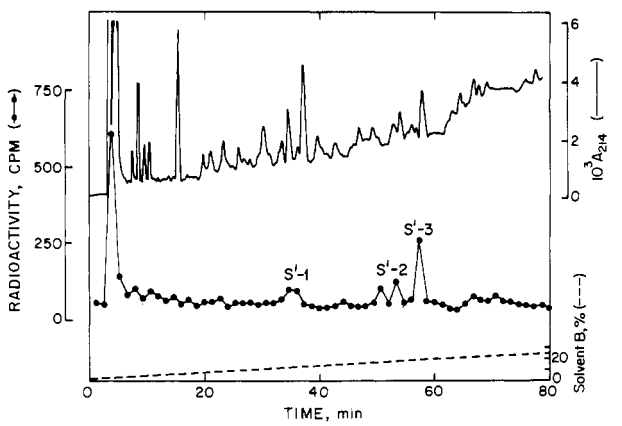

FIGURE 3: Chromatograph of *S. aureus* protease digest of [2-^{14}C]-malonyl-labeled chicken liver fatty acid synthase by reversed-phase HPLC. About 5% of the total labeled peptides were separated as described in the legend to Figure 1A. Fractions (1.3 mL) were collected, and 400-μL aliquots from each peak fraction were analyzed for ^{14}C radioactivity. The primary radioactive peaks are labeled S'-1, S'-2, and S'-3.

degradations. This indicates that the *O*-acyl bond in the acetylated serine of peptide S-1 may be unstable during sequencing [cf. Mikkelsen et al. (1985a)] or the amount of radioactivity may be too little to detect. Since the peptide S-1 was insensitive to neutralized hydroxylamine, it must be acetylated at the serine residue. The sequence of peptide S-3 shows that this peptide resulted from cleavage after Glu of peptide T-3 by *S. aureus* protease, in accordance with the known specificity of this enzyme (Drapeau, 1977). Peptide S-2 resulted from cleavage between Asp and Leu of the peptide S-3 [cf. Drapeau (1977)].

The [2-^{14}C]malonyl-labeled enzyme was prepared in a similar way as for the [1-^{14}C]acetyl-labeled enzyme. The chromatograms for the [2-^{14}C]malonyl-labeled enzyme digested with *S. aureus* protease are very similar to those of the [1-^{14}C]acetyl-labeled enzyme, except the former have shorter retention times (Figure 3). The peptides (S'-1, S'-2, and S'-3) were further purified as with the acetyl-labeled enzyme (data not shown). Purification of peptide S'-2 was not achieved even after several columns. As expected, the amino acid composition of S'-3 was identical with that of the corresponding peptide of the acetyl-enzyme, i.e., both malonyl and acetyl bound to the same peptide containing 4'-phosphopantetheine. The amino acid compositions of [2-^{14}C]malonyl-labeled peptide S'-1 and [1-^{14}C]acetyl-labeled peptide S-1 are identical. The peptide S'-1 was sequenced for nine steps with the automatic gas-phase sequencer. The sequence of the first five residues was identical with that of the acetyl-labeled S-1, but the yield after step 5 was dramatically decreased due to malonylserine [cf. Mikkelsen et al. (1985a)]. Although residues 6–9 can be only tentatively identified, the identity of the first five residues and the amino acid analyses suggest the acetyl- and malonyl-peptides are identical.

Analysis of the Iodoacetamide-Modified Enzyme. The modification of fatty acid synthase with iodoacetamide has been reported (Yuan & Hammes, 1985; Poulose et al., 1984; Stoops & Wakil, 1981; Kresze et al., 1977; Phillips et al., 1970a,b; Joshi et al., 1970). In order to have specific labeling of the essential cysteine, the labeling reaction was stopped when the specific activity of the labeled enzyme was about 65% of the control value. The stoichiometry of iodoacetamide labeling was ~0.5 iodoacetamide/fatty acid synthase (mol/mol).

The chromatogram of the tryptic digest of the [1-^{14}C]iodoacetamide-modified enzyme by reversed-phase HPLC on a Vydac 218-TP104 C-18 analytic column with solvent system

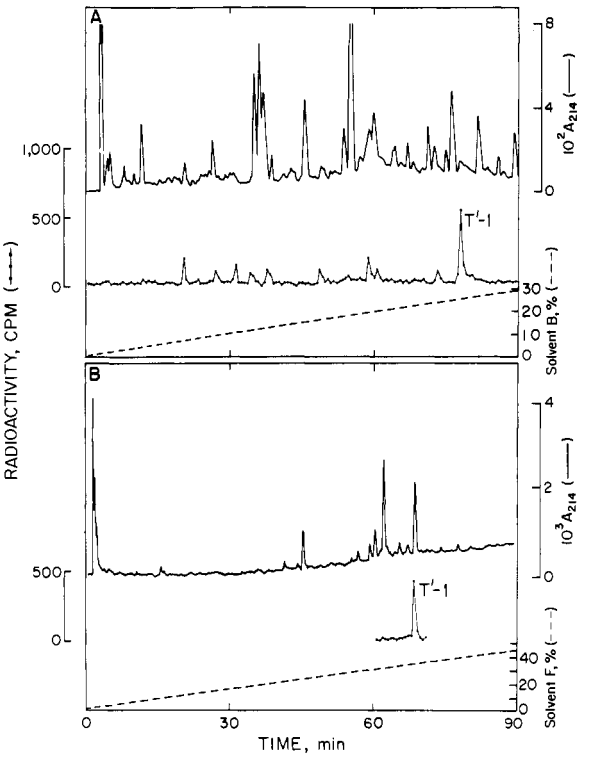

FIGURE 4: Chromatograph of trypsin digest of [2-^{14}C]iodoacetamide-modified chicken liver fatty acid synthase by reversed-phase HPLC. (A) About 45% of the total labeled peptides were separated as described in the legend to Figure 1A. Fractions (0.6 mL) were collected, and 10-μL aliquots from each peak fraction were analyzed for ^{14}C radioactivity. (B) The radioactive peptides T'-1 from 45% of the total labeled peptides were separated on an Aquapore RP-300 C-8 column (4.6 mm × 10 cm) by elution at 1.0 mL/min with an 80-min linear gradient from 98% solvent C to 40% solvent F as described under Materials and Methods. Fractions (0.6 mL) were collected, and 20-μL aliquots from each peak fraction were analyzed for ^{14}C radioactivity.

A and B is shown in Figure 4A. Only one major radioactive peak (T'-1) was found. The peptide was further purified on an Aquapore RP-300 C-8 column with solvent system C and F (Figure 4B).

The amino acid sequence of the iodoacetamide-modified peptide T'-1 is given in Table I. Monitoring of the radioactivity during sequencing suggests that the amino acid at cycle 18 is (carboxamidomethyl)cysteine. After cycle 23, the amount of recovery was too low to identify the amino acids.

3-Hydroxybutyryl- and Butyryl-Enzymes. For 10-s–2-min incubations of acetoacetyl-enzyme and (*S*)-[4-^{3}H]NADPH, the amount of reduced acetoacetyl-enzyme is ~0.5 reduced acetoacetyl/enzyme (mol/mol). When the reduced acetoacetyl-enzyme is treated with 0.2 M hydroxylamine (pH 7.0) for 20 min after being denatured in guanidine hydrochloride, more than 90% of the radioactivity is removed, indicating that essentially all of the bound substrate is a thioester. Analysis of the reduced substrate-enzyme (limited trypsin treatment) by sodium dodecyl sulfate–polyacrylamide gel electrophoresis indicated about 75% of the substrate is on peptide I and 25% on peptide II when [NADPH]/[enzyme] is ~7. Peptide I contains the essential cysteine and peptide II the 4'-phosphopantetheine (Tsukamoto et al., 1983; Yuan & Hammes, 1985).

The chromatogram of the tryptic digest of the radioactive reduced acetoacetyl-labeled enzyme obtained by reversed-phase HPLC on a Vydac C-18 column with solvent system A and B is shown in Figure 5A. The ratio [(*S*)-[4-^{3}H]-NADPH]/[enzyme] was ~7 per dimer. Reducing the ratio [NADPH]/[enzyme] decreased the ratio of peak T''-3 to T''-4;

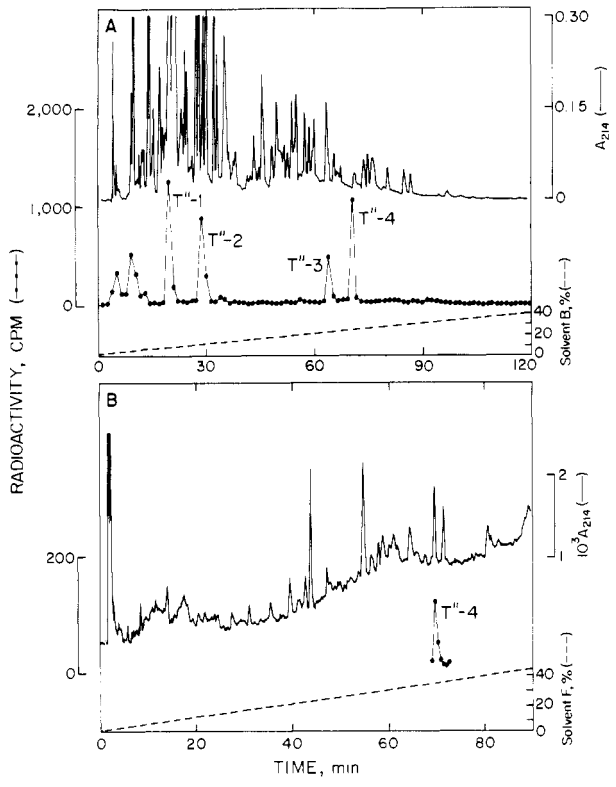

FIGURE 5: Chromatograph of trypsin digest of [2-^{3}H]-3-hydroxybutyryl- and [2-^{3}H]butyryl-modified chicken liver fatty acid synthase by reversed-phase HPLC. (A) About 20% of the total labeled peptides were chromatographed as described in the legend to Figure 1A. The major peaks in radioactivity are labeled T''-1, T''-2, T''-3, and T''-4. (B) The peptides T''-4 from about 40% of the total labeled peptides were chromatographed as described in the legend to Figure 4B. Fractions (0.6 mL) were collected, and 5-μL aliquots from each peak fraction were analyzed for ^{3}H radioactivity.

when the ratio [NADPH]/[enzyme] was 0.3, peak T''-3 is dominant (data not shown). As expected, the four major peaks (T''-1, T''-2, T''-3, and T''-4) disappeared after 0.2 M neutralized hydroxylamine was added to the digested supernatant (Anderson & Hammes, 1983; Anderson & Hammes, 1985). The peptides were further purified on an Aquapore RP-300 C-8 column with solvent system C and F. A typical chromatogram for the purification of T''-4 is shown in Figure 5B.

The sequences of peptides T''-3 and T''-4 are identical and the same as T-3, indicating these peptides contain 4'-phosphopantetheine (Table I). Again, amino acid analyses are in good agreement with the sequences; T''-3 probably contains hydroxybutyryl and T''-4 butyryl attached to the peptide. Peaks T''-1 and T''-2 did not contain significant amounts of amino acids; these are probably the 3-hydroxybutyryl- and butyryl-labeled thiols, respectively. Monitoring of the radioactivity during sequencing shows that the 3-hydroxybutyryl and butyryl groups are associated with the serine residue to which 4'-phosphopantetheine is attached. However, the recovery of the radioactivity is very low (about 50 cpm above background).

The reduced acetoacetyl-enzyme was digested with *S. aureus* protease at pH 4.0. The chromatograms of the radioactive reduced acetoacetyl-enzyme by reversed-phase HPLC showed only peaks T''-1 and T''-2 (data not shown). This indicates that the thioester bond is acid-labile. Since analysis of the reduced acetoacetyl-enzyme by sodium dodecyl sulfate–polyacrylamide gel electrophoresis showed about 75% of the substrate is on peptide I and no peptides containing cysteine are found, the cysteine thiol-substrate on the enzyme must be unstable under the conditions used for proteolysis.

Table II: Comparison of the Primary Structure of the 4'-Phosphopantetheine Site from Chicken Liver Fatty Acid Synthase with Acyl Carrier Proteins from Other Procaryotic and Eucaryotic Fatty Acid Synthases[a]

Eucaryotic fatty acid synthase	
Chicken	D V S S L N A E S S L A D L G L D S L M G V E V R
Goose[b]	D V S S L N A D S T L A D L G L D S L M G V E V R
Rabbit[c]	D L A G I N L D S S L A D L G L D S L M G V E V R
Rat[d]	D L A G I N L D S S L A D L G L D S L M G V E V R
Spinach[e]	A D V V V T A D S E F S K L G A D S L D T V E I V
Barley[f]	D G T P V T A E S K F S E L G A D S L D T V E I V
Yeast[g]	G G K S T V Q N E I L
Procaryotic fatty acid synthase	
E. coli[h]	Q E E V T D N A S F V E D L G A D S L D T V E L V
R. sph.[i]	E E K V T E T T S F I D D L G A D S L D T V E L V

[a] The sequences are aligned via the active serine (arrow). Residues identical with those in the sequence from chicken are boxed. [b] Poulose et al., 1984. [c] McCarthy et al., 1983a. [d] Witkowski et al., 1987. [e] Kuo & Ohlrogge, 1984. [f] Hoj & Svendsen, 1983. [g] Schreckenbach et al., 1977. [h] Vanaman et al., 1968. [i] Cooper et al., 1987.

DISCUSSION

The results obtained provide amino acid sequences for three essential regions of chicken liver fatty acid synthase: the 4'-phosphopantetheine site, a substrate "carrier" region; the serine site, a substrate "loading" region; and the cysteine site, a substrate "waiting" region. In addition, some interesting aspects of the stability and distribution of intermediates have been discovered.

With the acetylated enzyme, peptides were found containing the serine and the 4'-phosphopantetheine sites. A peptide containing the essential cysteine was not found. Although the isolation of a cysteine-containing peptide has been reported (Phillips et al., 1970a; Joshi et al., 1970), the evidence is not convincing: The amino acid analyses suggest the "cysteine peptide" is probably a contaminated peptide containing 4'-phosphopantetheine. Previous results have suggested that the acetyl group is not extensively bound to cysteine (Yuan & Hammes, 1985). Mikkelsen et al. (1985c) have reported that ~15% of the acetyl groups are on cysteine for the rat liver fatty acid synthase. The maximum number of acetyl groups bound per enzyme molecule is about 4 [cf. Cognet and Hammes (1983) and Mikkelsen et al. (1985c)], suggesting that steric hindrance prevents all six potential sites from being occupied simultaneously. Another possible explanation for the absence of a cysteine-containing peptide is the instability of this particular thioester. This is suggested by the experiments in which a 3-hydroxybutyryl/butyryl-enzyme was generated from acetoacetyl-enzyme and radioactive NADPH. In this case, a substantial amount of the cysteine-enzyme intermediate was generated since the majority of radioactivity was found on peptide I after sodium dodecyl sulfate–polyacrylamide gel electrophoresis (Wakil et al., 1983). However, after proteolysis with trypsin (pH 8.0) or *S. aureus* protease (pH 4.0), a cysteine peptide could not be isolated. The reason for the instability of the cysteine-substrate thioester is not known.

The amino acid sequence of the 4'-phosphopantetheine region was determined for the acetyl-enzyme, malonyl-enzyme, hydroxybutyryl-enzyme, and butyryl-enzyme with two different proteases (Table I). In Table II the sequence of the chicken peptide is compared with that of *Escherichia coli* (Vanaman et al., 1968), *Rhodopseudomonas sphaeroides* (Cooper et al., 1987), yeast (Schreckenbach et al., 1977),

Table III: Comparison of the Primary Structure of the Essential Serine Site of Acetyl/Malonyl Transferase from Chicken Liver Fatty Acid Synthase with That of Acyl Transferase and Thioesterase from Other Fatty Acid Synthases and Serine Proteases[a]

	↓
Chicken acetyl-/malonyl-transferase	G I L G H S V G E
Goat acyl-transferase[b]	D G I I G H S L G E V A
Goat medium-chain acyltransferase[c]	D G I I G H S L G E V A
Rabbit acyl-transferase[d]	S L G E V A
Yeast acetyl-transferase[e]	K G A T G H S N G L V T
Yeast malonyl/palmitoyl-transferase[e]	A T F A G H S L G E Y A
Goose thioesterase[f]	S F G A C V
Rat thioesterase[g]	V A G Y S F G A C V
Rabbit thioesterase[h]	V A G Y S Y G
Rat medium-chain thioesterase[i]	F A F F G H S F G S Y I
Duck medium-chain thioesterase[j]	F A L F G H S F G S F V
Human plasmin[k]	D S C Q G D S G G P L V
Bovine trypsin[k]	D S C Q G D S G G P V V

[a] The sequences are aligned via the active serine (arrow). Residues identical with those in the chicken acetyl/malonyl transferase are boxed. [b] Mikkelsen et al., 1985a. [c] Mikkelsen et al., 1985b. [d] McCarthy et al., 1983b. [e] Schweizer, 1986. [f] Poulose et al., 1981. [g] Witkowski et al., 1987. [h] Hardie et al., 1985. [i] Safford et al., 1987. [j] Poulose et al., 1985. [k] Dayhoff et al., 1972.

barley (Hoj & Svendsen, 1983), spinach (Kuo & Ohlrogge, 1984), rat (Witkowski et al., 1987), rabbit (McCarthy et al., 1983a), and goose (Poulose et al., 1984). The sequence homologies among the species are remarkable. Of the 25 amino acids near the 4′-phosphopantetheine (which is linked to the protein via a serine residue) for the chicken and goose, 23 are identical and another 2 differ by a single point mutation; for the chicken and rabbit, 19 are identical; for the chicken and rat, 19 are identical; for the chicken and *R. sphaeroides*, 9 are identical; for the chicken and *E. coli*, 9 are identical, but there are similarities in the immediate vicinities of the serine group, with 8 identities in 11 residues (from Asp to Glu); for chicken and barley, 11 are identical, with 7 identities in 10 residues in the immediate vicinity of the serine group. For the spinach enzyme, 9 out of 25 residues are identical, with 7 identities in 10 residues in the immediate vicinity of the serine group.

The sequence region around the essential serine (loading site) was obtained for the acetyl- and malonyl-enzyme. Unfortunately, the N-termini of the tryptic peptides are blocked. A similar result has been found with rabbit mammary (McCarthy et al., 1983b) and goat (Mikkelsen et al., 1985a) enzymes. This has been attributed to an O → N migration from an N-terminal acylated serine (McCarthy et al., 1983b). However, the peptides of interest can be obtained from the *S. aureus* protease digestions. Since the same sequence is obtained with the acetyl- and malonyl-peptides, the enzyme probably contains a single transacylase rather than two separate transacylases. The same result has been found with the rabbit mammary (McCarthy et al., 1983b) and goat (Mikkelsen et al., 1985a) enzymes. Earlier kinetic studies have suggested that acetyl- and malonyl-CoA compete for the same serine hydroxyl and the 4′-phosphopantetheine sites (Plate et al., 1970; Nixon et al., 1970; Stern et al., 1982). Table III compares the sequence of the chicken peptide with that of goat (Mikkelsen et al., 1985a,b), rabbit (McCarthy et al., 1983b), and yeast (Schweizer, 1986). Goose thioesterase (Poulose et al., 1981), rat thioesterase (Witkowski et al., 1987), rabbit thioesterase (Hardie et al., 1985), rat medium-chain thioesterase (Safford et al., 1987), duck medium-chain thioesterase (Poulose et al., 1985), and human plasmin and bovine trypsin (Dayhoff et al., 1972) sequences are included in Table III. The sequence homologies among the species again are remarkable. Of the nine amino acids adjacent to the acyl-serine site of the chicken and goat, seven are identical and another two differ by a single mutation; for chicken and yeast (acetyl transacylase and malonyl/palmitoyl transacylase), five are identical. Of the four amino acids from Ser to Glu in chicken and rabbit and goat, three are identical. A characteristic feature of the sequences appears to be that the third residue preceding the essential serine and the residue immediately after the serine are variable.

Table IV: Comparison of the Primary Structure of the Essential Cysteine Site from Chicken Liver Fatty Acid Synthase with That of Other Fatty Acid Synthases[a]

	↓
Chicken	I S Y F Y D F T G P S L T I D T A C S S S L M
Goose[b]	G P S L S I D T A C X S S L M
Yeast[c]	T P V G A C

[a] The sequences are aligned via the essential cysteine (arrow). Residues identical with those in the chicken fatty acid synthase are boxed. [b] Poulose et al., 1984. [c] Kresze et al., 1977.

The region around the essential cysteine was determined with a radioactive iodoacetamide label. The specificity of this label has been previously documented (Yuan & Hammes, 1985), and only a single radioactive peptide was found. Table IV compares the sequence of the chicken peptide with that of the goose (Poulose et al., 1984) and yeast (Kresze et al., 1977). For the chicken and goose, 13 of the 15 amino acids are identical, whereas for the chicken and yeast, 3 of the 6 amino acids are identical. The unusual sequence of three serines adjacent to the cysteine may be related to the difficulty in isolating a peptide containing the enzyme-substrate thioester.

The strong homologies between peptides from different species lend credence to the hypothesis that the multifunctional polypeptides from yeast and animals have evolved from the monofunctional enzymes of lower species [cf. Poulose et al. (1984) and Hardie and McCarthy (1986)]. Obviously, for yeast and animals, parallel gene fusion must be postulated. For the animal species a single transacylase apparently suffices, whereas in yeast separate enzymes have been reported for acetyl and malonyl transacylase activities, although the malonyl and palmitoyl transacylase activities reside on a single enzyme (Schweizer, 1986). Thus, intergene diffusion and/or gene duplication may have occurred. The similarities in amino acid sequence (and in mechanism) between the transacylases, the thioesterase, the medium-chain thioesterases, and serine proteases also merit mention [cf. Hardy and McCarthy (1986)]. The evolution of the complex fatty acid synthase enzyme complexes should be clarified as more sequence data become available.

In summary, the amino acid sequences of three essential regions of chicken liver fatty acid synthase have been determined and compared with known sequences from other species. The availability of this information should be useful for the design of genetic probes.

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