Sequential treatment of the chicken liver fatty acid synthetase by trypsin and subtilisin cleaved the \( M_2 \) 267,000 subunit to \( 6-8 \) polypeptides, ranging in molecular weights from 15,000 to 94,000. Fractionation of the digest by ammonium sulfate and chromatography on a Frocien Red HESB affinity column permitted the isolation of a polypeptide \( (M_2 = 94,000) \) containing the \( \beta \)-ketoacyl reductase activity but no other partial activities normally associated with synthetase. The specific activity of the \( \beta \)-ketoacyl reductase increased 2 to 3 times in this fraction, an increase that is within the expected range based on relative molecular weight. The kinetic parameters of this fraction towards NADPH and N-acetyl-9-acetoacetyl cysteamine were essentially the same as the \( \beta \)-ketoacyl domain of the intact synthetase. However, the purified fragment did not catalyze the reduction of acetoacetyl-S-CoA derivative, a substrate that is readily reduced by the intact synthetase. Fluorescence measurements with etheno-NADP* indicate the binding of about 1 mol of NADP*/94,000 daltons, a value which is in agreement with the results obtained from fluorescence measurements with NADPH and the binding of a radiolabeled photoaffinity analog of NADP*.

Phenylglyoxal inhibits the \( \beta \)-ketoacyl reductase activity of either the intact synthetase or the isolated fragment, suggesting the involvement of an essential arginine at or near the active site.

Another fragment \( (M, 36,000) \) containing \( \beta \)-ketoacyl reductase activity was isolated from the synthetase after kallikrein/subtilisin double digestion. Previous mapping studies had shown that this fragment lies adjacent to the COOH-terminal thioesterase domain and overlaps the tryptic \( M, 94,000 \) peptide by approximately 21 daltons. This fragment, but not the \( M, 94,000 \) fragment, was found to contain the phosphopantetheine prosthetic group, indicating that the acyl carrier protein moiety is located in the 15,000-dalton segment that separates the \( \beta \)-ketoacyl reductase from the thioesterase domain.

In the preceding paper (1), we demonstrated that the fatty acid synthetase consists of a large "core" region of \( M, 230,000 \), which carries out all of the central reactions for acyl chain assembly, and a carboxyl-terminal domain of \( M, 33,000 \), which contains the thioesterase or product-releasing function. Systematic mapping studies of the fatty acid synthetase subunit using a variety of proteases showed that the 230-kDa region consists of two large domains of 127- and 107-kDa, which may be further subdivided into a number of discrete regions by the action of individual proteolytic enzymes (2). These mapping studies, therefore, provide a framework for the dissection and localization of various active centers within the parent polypeptide, so as to gain a deeper insight into the properties of each individual enzyme and the functional organization of the system. In this report, we describe the isolation of two related fragments, produced by proteolytic digestion of the chicken fatty acid synthetase, which retain the \( \beta \)-ketoacyl reductase activity. One of the fragments also contains the \( 5'- \)phosphopantetheine prosthetic group of the acyl carrier protein moiety. The physical and enzymatic properties of these peptides will be discussed, along with their location on the synthetase subunit.

**EXPERIMENTAL PROCEDURES**

**Materials**. - Trypsin-inhibitor, etheno-NADP*, N-acetyl-S-acetoacetyl cysteamine, 36,000 daltons, or phenylglyoxal from Sigma; NADP+ from BioRad; trypsin from Worthington; molecular gel retardation assay kit from Pharmacia; 150 M N-acetyl-9-acetoacetyl cysteamine from Eastman; phenylglyoxal from Aldrich; 36,000 daltons, or phenylglyoxal from Eastman; phenylglyoxal from Aldrich; 36,000 daltons, or phenylglyoxal from Eastman; phenylglyoxal from Aldrich; and ['\( ^{14} \)]C-NADP* from New England Nuclear; 4-fluoro-3-nitrophenylfluoride from Calbiochem-Behring Corporation; N,N-diethylforamide from Bausch & Lomb; 3-A molecule filters from Fishburn Chemical, Aminco-Chromatography, and 3-A molecular filters from Fisher; and 3-A molecular filters from Scientific Products.

**Enzyme Assays and Preparation of Chicken Liver Fatty Acid Synthetase**. - Assays of the fatty acid synthetase described in this paper were performed as previously reported (1, 1.5), except that the \( \beta \)-ketoacyl reductase assay was modified so that the reaction mixture contained 50 \( \mu \)l of homogenate in 5 \( \mu \)mol of Tris, pH 7.5, 7 \( \mu \)mol of NADPH, and 740 \( \mu \)M N-acetyl-9-acetoacetyl cysteamine. The reaction was started by addition of the enzymes and followed spectrophotometrically by measuring absorbance at 340 nm.

**Chicken Liver Fatty Acid Synthetase**. - A chicken liver fatty acid synthetase was purified as described previously (1), and had specific activity of 1000 mg of NADPH oxidized per mg. One mg of fatty acid synthetase was prepared according to procedure described earlier (4). One mg of fatty acid synthetase was prepared according to procedure described earlier (4).

**Isolation of the 230-kDa and 36-kDa Fragment**. - In a typical experiment the mg of fatty acid synthetase was prepared over time at 34°C against a buffer [buffer (1)] containing 50 mg of homogenate, 1 mg of fatty acid synthetase, and 1 ml of homogenate. The enzyme solution was added to the cold for 20 min. The solution was incubated at room temperature for 30 min and then added to the mixture, and the reaction was incubated at room temperature for 30 min and then added to the mixture. The reaction was incubated at room temperature for 30 min and then added to the mixture. The reaction mixture was diluted with cold saline and collected by centrifugation at 10,000 rpm for 30 min. Subsequent operations were carried out at 4°C.

**Conclusion**. - The additional ammonium sulfate (150 mg) was added slowly to the protein mixture with constant stirring. After 30 min, the precipitation was removed by centrifugation and dissolved in buffer A. This fraction contained 10 to 20% of the total protein and 7 to 15% of the reductase activity and was entirely dispersed. The supernatant fluid was concentrated by ammonium sulfate (150 mg) was added, and the mixture was centrifuged at 10,000 rpm for 30 min. The supernatant fluid was centrifuged at 10,000 rpm for 30 min.
Isolation of β-Ketoacyl Reductase Activity

For the isolation of an active fragment of the synthetase bearing β-ketoacyl reductase activity, the native enzyme was digested with a combination of trypsin and subtilisin. These proteases were chosen because it was found that their action did not seriously damage the ketoreductase activity (Table I), and also because these proteases dissect the protein into a number of defined and discrete regions. The proteolytic mapping studies presented earlier (2) showed that the following peptides, from NH₂ to carboxyl terminus, are produced from synthetase by trypsin/subtilisin double digestion: 60, 45, 23, 94, 15, and 33 kDa.

Fractionation of these digestion products was carried out by ammonium sulfate precipitation followed by matrix gel red chromatography (see “Experimental Procedures” for details), an affinity step specifically designed to bind those peptides containing NADPH binding sites. The data presented in Table I summarize the results obtained from a typical preparation. The final fraction contained 20% of the original activity with a 2.2-fold increase in specific activity. The Sephadex gel filtration step appears to be necessary, not for the removal of contaminating proteins, but to fully recover the enzyme activity, which is lost due to the presence of inhibitors leached from the matrix gel red column during NADPH elution.

Enzymatic Characterization of the 94-kDa β-Ketoacyl Reductase Fragment—Survey of the 94-kDa fragment for all the partial activities associated with the fatty acid synthetase showed that only the β-ketoacyl reductase activity is present. Since this achievement represents the first successful isolation and purification of a fragment of fatty acid synthetase that contains the β-ketoacyl reductase activity, a detailed

| Table I |
| --- |
| Purification of β-ketoacyl reductase from chicken liver fatty acid synthetase |
| | Step | Total protein | Specific activity | Total units | Yield |
| | | mg | | nmol/min/mg | % |
| Dialyzed synthetase | 210 | 2600 | 5.5 x 10⁴ | 100b |
| Tryptic digest | 210 | 2200 | 4.6 x 10⁵ | 84 |
| Tryptic/subtilisin digest | 210 | 2100 | 4.4 x 10⁵ | 80 |
| Ammonium sulfate fraction (21-36 g/100 ml) | 160 | 2940 | 6 x 10⁵ | 11 |
| Matrix Gel Red A | 20 | 3000 | 6 x 10⁵ | 11 |
| Sephadex G-100 | 20 | 5500 | 1.1 x 10⁵ | 20 |
| *Expressed as nmol of NADPH oxidized/min/mg of protein at 25 °C. Total activity as nmol/min. 
| aArbitrarily taken as 100. |

RESULTS AND DISCUSSION

For the isolation of an active fragment of the synthetase bearing β-ketoacyl reductase activity, the native enzyme was digested with a combination of trypsin and subtilisin. These proteases were chosen because it was found that their action did not seriously damage the ketoreductase activity (Table I), and also because these proteases dissect the protein into a number of defined and discrete regions. The proteolytic mapping studies presented earlier (2) showed that the following peptides, from NH₂ to carboxyl terminus, are produced from synthetase by trypsin/subtilisin double digestion: 60, 45, 23, 94, 15, and 33 kDa.

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Sodium dodecyl sulfate-polyacrylamide gel analysis of the major fractions is presented in Fig. 1. These results show that the purified β-ketoacyl reductase fraction consists primarily of a peptide with a mass of about 94 kDa. The inclusion or omission of β-mercaptoethanol had no effect on the migration of the 94-kDa protein in sodium dodecyl sulfate-polyacrylamide gels, indicating that intermolecular disulfide bonds are not present. Gel filtration on Sephacryl S-200, in the presence of standards, gave molecular weight values of about 100,000 suggesting that the monomer form of the polypeptide is the active species. This 94-kDa polypeptide is, in fact, identical to the 94-kDa tryptic fragment defined by the earlier mapping studies (2). This result indicates that subtilisin digestion would not be required to generate this fragment. However, we found that in the absence of subtilisin digestion the tryptic fragments could not be dissociated, thereby preventing purification of the β-ketoacyl reductase fragments.

Enzymatic Characterization of the 94-kDa β-Ketoacyl Reductase Fragment—Survey of the 94-kDa fragment for all the partial activities associated with the fatty acid synthetase showed that only the β-ketoacyl reductase activity is present. Since this achievement represents the first successful isolation and purification of a fragment of fatty acid synthetase that contains the β-ketoacyl reductase activity, a detailed

FIG. 1. Display of sodium dodecyl sulfate-polyacrylamide gel electrophoresis of the proteins derived during purification of the β-ketoacyl reductase activity. A polycrylamide gel gradient (6 to 15%) was employed and 10 μg of protein from each fraction used. The lanes contain the following: A, undigested synthetase; B, tryptic digest; C, tryptic/subtilisin double digest; D, matrix gel red flow-through; E, matrix gel red bound fraction; HS and LS, standard proteins (2).
for fatty acid synthetase and purified β-ketoacyl reductase. The constants for NADPH (varied between concentrations of 2.3 and 300 μM) were derived with the substrate N-acetyl-S-acetoacetyl cysteamine maintained at concentrations of 0.24 μM. The constants for N-acetyl-S-acetoacetyl cysteamine (varied between concentrations of 0.18 and 0.480 μM) and acetoacetyl-S-CoA (varied between concentrations of 0.18 and 4.8 μM) were derived with NADPH maintained at concentrations of 75 μM.

| Substrates                        | Synthetase | Reductase |
|----------------------------------|------------|-----------|
|                                  | $V_{max}$  | $K_m$     | $V_{max}$ | $K_m$ |
| NADPH                            | 3.9        | 4.1 $\times$ 10^{-4} | 3.5 | 4.8 $\times$ 10^{-4} |
| N-acetyl-S-acetoacetyl cysteamine | 5.0        | 2.6 $\times$ 10^{-3} | 5.8 | 6.6 $\times$ 10^{-3} |
| Acetoacetyl-S-CoA                 | 4.7        | 5 $\times$ 10^{-4} | Inactive |

![Graph A](image1.png)

**Fig. 2.** Protein fluorescence scan of native fatty acid synthetase and purified 94-kDa β-ketoacyl reductase fragment. A, the protein fluorescence of intact fatty acid synthetase (10.8 mg/ml) was determined in the absence (open symbols) and presence (closed symbols) of NADPH (22 μM). Emission wavelength scans were performed with the excitation wavelength held constant at 280 nm, while the excitation wavelength scans were performed with the emission wavelength held constant at 335 nm. B, the protein fluorescence of the purified 94-kDa β-ketoacyl reductase fragment (0.05 mg/ml) in the absence (open symbols) and presence (closed symbols) of NADPH (2.76 μM).

characterization of this component enzyme was undertaken.

**Kinetic Parameters**—The kinetic parameters of the β-ketoacyl reductase reaction were examined with the use of the model substrates N-acetyl-S-acetoacetyl cysteamine and acetoacetyl-CoA. The native fatty acid synthetase catalyzed the reduction of both substrates by NADPH; however, the isolated reductase preparation was active only with the cysteamine derivative. The kinetic constants for both the intact synthetase and the purified reductase fragment were determined by the usual Michaelis-Menten type studies and the values for each substrate are summarized in Table II. The apparent Michaelis constant ($K_m$) and the maximum velocity ($V_{max}$) for NADPH were the same for both preparations. However, in the case of N-acetyl-S-acetoacetyl cysteamine, the apparent $K_m$ increased by 2.5-fold, while $V_{max}$ remained unchanged. These changes were even more dramatic in the case of the substrate acetoacetyl-CoA. While acetoacetyl-CoA was the better substrate for the intact fatty acid synthetase, it did not function at all as a reducible substrate for the 94-kDa reductase over a very wide range of concentrations (Table II). The reason for the inactivity of the isolated reductase towards this substrate is not known at this time and may be due to conformational changes which sterically hinder the bulky CoA derivative from binding to the catalytic center. It may also be due to the appearance of or the presence of net negative charges near the active site of the isolated reductase which repulse the negatively charged CoA derivative. These and other possibilities are currently under active investigation.

**NADPH Binding to the β-Ketoacyl Reductase**—The number and type of NADPH binding sites on the 94-kDa reductase fragment were assessed and compared with those of the native enzyme using fluorescence measurements. As shown in Fig. 2, A and B, NADPH quenches protein fluorescence of both the intact synthetase and the 94-kDa fragment. The changes in protein fluorescence ($\Delta F$) at 335 nm over a wide range of NADPH concentrations were measured in both cases. Reciprocal plots of these values gave straight lines which on extrapolation to the ordinate yield the values for maximum changes in fluorescence ($\Delta F_{max}$). These values were 4.18 for the synthetase and 8.3 for the β-ketoacyl reductase fragment. Using these values, Scatchard plots were generated (7, 8) for both synthetase and reductase (Fig. 3, A and B). From these analyses, $K_D$ values for NADPH of 4.7 and 1.5 μM were derived.
for the intact synthetase and the isolated reductase fragment, respectively. Also, the linear Scatchard plots shown in Fig. 3, A and B implicated the involvement of one type of binding site on the enzymes. However, one cannot deduce from these plots a true stoichiometry of NADPH binding because of the vast excess of NADPH used in these experiments.

An attempt to estimate the stoichiometry of the binding of NADP⁺ to the β-ketoacyl reductase was made employing the highly fluorescent probe etheno-NADP⁺ and monitoring its fluorescence at stoichiometric concentrations of enzyme to ligand. The fluorescence spectra of the synthetase and 94-kDa reductase, as well as that of etheno-NADP⁺ after excitation at 310 nm in the presence or absence of synthetase and reductase, are shown in Fig. 4, A and B. It is apparent that there was quenching of ligand fluorescence by both enzymes, indicating interaction between the enzymes and etheno-NADP⁺. No such fluorescence quenching was obtained when bovine serum albumin was used (Fig. 4C) suggesting that the interactions noted in these experiments are due to specific binding of the etheno-NADP⁺ to the intact synthetase or 94-kDa reductase and not due to general protein-ligand interaction.

Based upon these findings, the ligand fluorescence quenching at 400 nm was measured after increasing the concentration of etheno-NADP⁺ from 1.2 to 65.7 nM, while maintaining the concentration of purified β-ketoacyl reductase at 30 nM. A complementary experiment was also performed with etheno-NADP⁺ maintained at 30 nM, while the β-ketoacyl reductase concentration was increased from 1.4 to 104 nM. The quenching values obtained from both experiments were then processed according to Azzi (7) and Sreekrishna et al. (9) and plotted, as shown in Fig. 5. A straight line was obtained, which on extrapolation intercepted the abscissa at a point corresponding to 22 nM etheno-NADP⁺ bound to 30 nM of β-ketoacyl reductase or an equivalent of 0.73 mol of etheno-NADP⁺ bound per mol of β-ketoacyl reductase (based on M, 94,000).

Binding of β-Ketoacyl Reductase to a Photoaffinity Analog of NADP⁺—The binding of NADP⁺ to the β-ketoacyl reductase was further investigated using the photoaffinity analogue of NADP⁺ described by Chen and Guillory (5, 6). Photolysis of a mixture containing the reductase and arylazido-β-alanyl NADP⁺ resulted in a rapid inhibition of the enzyme as shown in Fig. 6. Most of the activity was abolished by this treatment and could not be regenerated by dialysis or treatment with thiols. Photolysis was essential for loss of activity since similar samples kept in the dark did not lose more than 10 to 20% of activity after incubation for at least 4 h. Thus, the reaction of the analog of NADP⁺ with the reductase is specific and dependent on time of photolysis.

The binding of the photoaffinity analog to the purified 94-kDa β-ketoacyl reductase fragment is covalent as evidenced by the association of the radioactivity with the 94-kDa polypeptide and no other protein band was labeled with this analog. This finding clearly shows that the 94-kDa fragment has indeed the β-ketoacyl reductase site.

The stoichiometry of inhibition of the reductase by the photoaffinity analog of NADP⁺ was determined by a Scatchard analysis as shown in Fig. 8. The plot indicated that the binding of 0.88 mol of arylazido-β-alanyl NADP⁺ was required for complete inactivation of the β-ketoacyl reductase. This value is in good agreement with that obtained by fluorescence quenching of the etheno-NADP⁺ by the reductase (Fig. 5).
The presence, therefore, of one NADPH binding site on the enzyme, implicating the involvement of fatty acid synthetase and purified reductase fraction was carried out a sequential digestion with trypsin and subtilisin represents a rather large attempt to more closely define the actual location of the \( \alpha \)-ketoacyl reductase activity of either preparation, suggesting the enzyme with NADPH prior to treatment with phenylglyoxal protected the enzyme from inhibition suggesting that the enzyme, is either not present or has been destroyed by proteolysis. At this stage, these alternatives cannot be distinguished, as the enoyl reductase activity is in fact absent from the unfractionated synthetase after treatment with proteases.

*Inhibition Studies on the Purified Reductase*—The effect of various alkylating agents on the \( \beta \)-ketoacyl reductase activity of fatty acid synthetase and purified reductase fraction was investigated. Iodoacetamide, \( N \)-ethylmaleimide as well as phenylmethanesulfonyl fluoride had no effect on the \( \beta \)-ketoacyl reductase activity of either preparation, suggesting the noninvolvement of an active thiol or hydroxyl group at the active site. We also found, in agreement with the results of Poulose and Kolattukudy (11), that phenylglyoxal specifically inhibits the \( \beta \)-ketoacyl reductase activity of the fatty acid synthetase without affecting any of the other partial activities of this multifunctional enzyme, implicating the involvement of an arginyl group in the reaction (11-13). Preincubation of the enzyme with NADPH prior to treatment with phenylglyoxal protected the enzyme from inhibition suggesting that the two reagents share a common binding site on the protein.

*Mapping of the \( \beta \)-Ketoacyl Reductase Site and the Isolation of an Active 36-kDa Fragment*—The 94-kDa \( \beta \)-ketoacyl reductase fragment isolated from fatty acid synthetase by digestion with trypsin and subtilisin represents a rather large fraction of the synthetase polypeptide. The previous proteolytic mapping studies (2) indicated that this fragment is located about 15 kDa from the NH\(_2\)-terminal boundary of the terminal thioesterase domain, and in fact represents the bulk of the designated 107-kDa "domain II" hemisphere of the 230-kDa core region of the synthetase. Our concurrent proteolytic mapping program suggested that this 94-kDa region could be subdivided by the action of kallikrein into three fragments of approximate size 14, 56, and 36 kDa (2). Therefore, in an attempt to more closely define the actual location of the \( \beta \)-ketoacyl reductase site, we carried out a sequential digestion of the intact fatty acid synthetase with kallikrein and subtilisin (again using subtilisin cleavage as an aid for dissociation of the complex). Purification of the \( \beta \)-ketoacyl reductase

[FIG. 6. Inhibition of 94-kDa reductase fragments by arylazido-\( \beta \)-alanyl NADP\(^+\) at various times of photolysis. Purified \( \beta \)-ketoacyl reductase (0.65 mg/ml or 6.8 \( \mu \)M) was treated with arylazido-\( \beta \)-alanyl NADP\(^+\) (100 \( \mu \)M) in 50 mM Hepes, 1 mM EDTA, pH 7.2, and photolyzed as described under "Experimental Procedures." Samples were removed at various times of photolysis and assayed for reductase activity. Control sample containing the same mixture was wrapped with aluminum foil and treated in the same manner as the experimental sample. The \( \beta \)-keto reductase activity lost initially 10 to 20% of activity but maintained the same level for at least 4 h.]

![Graph showing inhibition of 94-kDa reductase fragments by arylazido-\( \beta \)-alanyl NADP\(^+\) at various times of photolysis.](http://www.jbc.org/)

[FIG. 7. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of purified reductase labeled with \( [3-\text{H}] \)arylazido-\( \beta \)-alanyl NADP\(^+\) (PA-NADP) in 50 mM Hepes, pH 7.2, containing 1 mM EDTA and photolyzed for 90 min at 4 °C. The reductase lost over 90% of its original activity. Protein (50 \( \mu \)g) was then loaded on sodium dodecyl sulfate-polyacrylamide gels (5%) and electrophoresis performed. After staining and destaining, the gel was sliced into 0.5-cm segments and assayed for radioactivity in a liquid scintillation spectrometer (10). A, Coomassie blue R-250 stained gel; B, plot of radioactivity content of the slices.]

[FIG. 8. Scatchard analysis of the inhibition of the purified 94-kDa \( \beta \)-ketoacyl reductase fragment (\( \beta \)KR) by arylazido-\( \beta \)-alanyl NADP\(^+\) (PA-NADP). Purified reductase (0.64 mg/ml, and specific activity of 3000 nmol of NADPH oxidized/min/mg) was treated with \( [3-\text{H}] \)-arylazido-\( \beta \)-alanyl NADP\(^+\) (100 \( \mu \)M) in 50 mM Hepes, pH 7.2, and 1 mM EDTA. Aliquots were removed after 3, 10, 15, 25, and 40 min of photolysis and assayed for \( \beta \)-ketoacyl reductase and protein-bound radioactivity. Control sample was treated in the same manner except that it was wrapped with aluminum foil during photolysis. Control sample after 40 min of treatment had reductase specific activity of 2500 nmol of NADPH oxidized/min/mg, whereas, experimental sample had specific activity of 200 nmol of NADPH/min/mg.]
activity from this digest was carried out using the same strategy employed previously i.e. matrix gel red affinity chromatography, with similar results. The data in Table III summarize the results of a typical preparation of a 36-kDa β-ketoacyl reductase fragment. Electrophoretic analysis of the various fractions on sodium dodecyl sulfate-polyacrylamide gels (Fig. 9) showed that the fraction eluted from the matrix gel red column contained the 36-kDa fragment in a homogeneous state. This fraction contained the β-ketoacyl reductase activity, although the apparent increase in specific activity was only 1.6 times that of the total synthetase digest (Table III). Again, it was found that this value could be substantially improved (1.5-2-fold) by subsequent gel filtration on Sephadex G-100 or even simple dialysis, in the same manner that was observed earlier with the 94-kDa fragment.

These results demonstrate that the β-ketoacyl reductase activity is localized within the 36-kDa fragment derived from the COOH-terminal end of the 230-kDa core region (1, 2). This fragment was one of those predicted, solely on the basis of the proteolytic map (2), to be related to the 94-kDa reductase fragment generated by tryptic cleavage, which demonstrates the value of the map itself as a guide to the controlled dissection of the synthetase complex. The 94- and 36-kDa fragments are actually overlapping peptides which share approximately 21 kDa of sequence in common. Therefore, the site of β-ketoacyl reductase activity must lie within this restricted segment of the synthetase polypeptide, as illustrated in Fig. 10.

The Relationship of the Acyl Carrier Peptide to the β-Ketoacyl Reductase Fragments—A number of experiments being carried out at this time to determine the position of other functional sites within the synthetase complex (1, 14) had indicated that the acyl carrier peptide might be located adjacent to the thioesterase terminus and in the vicinity of the segment shown to contain β-ketoacyl reductase activity. Therefore, the preparation of the two reductase fragments was repeated using fatty acid synthetase labeled with [14C]phosphopantetheine.

The results obtained during the purification of the 36-kDa reductase fragment from pantetheine-labeled synthetase are presented in Table III. The radioactivity was found to copurify with the 36-kDa fragment, such that, after affinity chromatography, its specific radioactivity increased to about 5 times that of the original preparation, which agrees well with the theoretical value of 7 derived from molecular weight considerations. [14C]-Labeled 36-kDa preparation (50 μg having a specific radioactivity of 4850 cpm/mg) was subjected to electrophoresis on sodium dodecyl sulfate-polyacrylamide disc gels, stained with Coomassie blue R-250, and destained. The gels were sliced into 5-mm pieces and assayed for radioactivity in a manner similar to that described in Fig. 7. Greater than

### Table III

| Step                        | Total protein (mg) | Specific radioactivity (cpm/mg) | Total radioactivity (cpm) | Increase of radioactivity (nmol/min/mg) | Increase of β-ketoacyl reductase | β-Keto reductase activity (pmol/min/mg) |
|-----------------------------|-------------------|--------------------------------|--------------------------|----------------------------------------|----------------------------------|----------------------------------------|
| Native synthetase*          | 58.4              | 1,020                          | 60,000                   | 1.5                                     | 3,400                            | 1.6                                     |
| Kallikrein/subtilisin-treated synthetase | 58.4            | 1,020                          | 60,000                   | 1.5                                     | 2,700                            | 1                                       |
| 30-50% ammonium sulfate fraction | 27.2            | 1,750                          | 47,600                   | 1.7                                     | 4,190                            | 1.6                                     |
| Matrix gel red eluate       | 1.2               | 4,850                          | 5,820                    | 4.8                                     | 4,220                            | 1.6                                     |

* Fatty acid synthetase was labeled in vivo with [14C]phosphopantetheine as described under "Experimental Procedures."

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis analysis of matrix gel red eluent showed substantial purification of the 36-kDa fragment when compared to the 30-50% ammonium sulfate fraction (Fig. 9, compare lanes D and E); however, specific activity was not substantially increased, presumably because of inhibition by procion red HE3B dye which may have been stripped from the column by NADPH (see text).

### FIG. 9
Display of the sodium dodecyl sulfate-polyacrylamide gel electrophoresis of the 36-kDa β-ketoacyl reductase at various purification steps. Polyaclamide gel gradient (5 to 15%) was employed and 10 μg of proteins from each fraction were used. The lanes contain the following: A, native synthetase; B, kallikrein digest; C, kallikrein/subtilisin digest; D, ammonium sulfate fraction (30 to 50% saturation); E, matrix gel red eluate; HS and F, molecular weight standard proteins (cf. Fig. 1); and G, dialyzed matrix gel red fraction.

### FIG. 10
Schematic presentation of the domains of the synthetase subunit and the relative location of the β-ketoacyl reductase (βKR), acyl carrier protein (ACP), and thioesterase (TE) on the subunit protein. 85% of the input radioactivity was recovered in the fraction containing the 36-kDa fragment, indicating that the labeled pantetheine was an integral component, and not merely loosely associated with this protein fraction. On the other hand, when the [14C]phosphopantetheine-labeled synthetase was subjected to trypsin/subtilisin double...
digestion as before, the 94-kDa fraction was found to contain 86% of the reductase activity, but less than 10% of the radioactivity. The bulk of the radioactive pantetheine (>80%) was associated with smaller peptides of 12 kDa or less. These results, taken together with the proteolytic map (Fig. 10), imply that the acyl carrier protein moiety is located within the 15-kDa segment that separates the tryptic 94-kDa segment and the thioesterase domain. This fragment is included within the kallikrein 36-kDa segment, and lies directly adjacent to the 21-kDa segment wherein the \( \beta \)-keto reductase activity is situated (Fig. 10). More direct confirmation of this assignment of the acyl carrier protein site is presented in a following paper (14), along with the mapping of other active centers within the fatty acid synthetase subunit.

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