Fluorescence Studies of Chicken Liver Fatty Acid Synthase

SEGMENTAL FLEXIBILITY AND DISTANCE MEASUREMENTS

Zhengyu Yuan and Gordon G. Hammes

From the Department of Chemistry, Cornell University, Ithaca, New York 14853

The 4'-phosphopantetheine of chicken liver fatty acid synthase was specifically labeled with the fluorescent substrate analog coenzyme A 6-[7-nitrobenz-2-oxa-1,3-diazol-4-yl]aminohexanoate at low salt concentrations. A serine at the active site of the thioesterase was specifically labeled with the fluorescent coenzyme A. The synthetic activity is slowly restored when the dimer is raised to room temperature. The results obtained suggest that relatively large conformational changes may be part of the catalytic cycle.

Animal fatty acid synthases are multienzyme complexes that catalyze the synthesis of palmitic acid from acetyl-CoA and malonyl-CoA as a reducing agent:

Acetyl-CoA + 7 malonyl-CoA + 14 NADPH + 14 H⁺ →
palmitic acid + 8 CoA + 14 NADP⁺ + 6 H₂O

An acetyl group is first transferred from acetyl-CoA as a primer; a malonyl group is then condensed with the acetyl to form a β-ketoacyl intermediate, which is then reduced by NADPH to a β-hydroxy intermediate. This β-hydroxy intermediate is dehydrated to form a carbon-carbon double bond that is reduced by a second NADPH to give a saturated enzyme-bound fatty acid. A malonyl group is then added to the fatty acid, the process is repeated until enzyme-bound palmitate is formed. The palmitate is released from the enzyme by a thioesterase.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby solely to indicate this fact.

The abbreviations used are: NBDAH, 6-[7-nitrobenz-2-oxa-1,3-diazol-4-yl]aminohexanoic acid, NBDPAH-CoA, thioester from CoA and NBDAH; NBDFP, 6-[7-nitrobenz-2-oxa-1,3-diazol-4-yl]aminopentyl methylphosphonofluoridate; PBMPF, pyrenebutyl methylphosphonofluoridate.
the enzyme was greater than 1.6 μmol of NADPH/min/mg under standard assay conditions (Cardon and Hammes, 1982). All enzyme activity assays, chemical modifications, and physical measurements were carried out in 0.1 M potassium phosphate, pH 7.0, 1 mM EDTA, unless otherwise specified. The concentration of enzyme was determined by measurement of the absorbance at 280 nm, with an extinction coefficient of 4.82 x 10^4 M^-1 cm^-1 (Hsu and Yun, 1970). The fatty acid synthase was dissociated into monomers in 5 mM Tris/HCl, pH 8.3, 30 mM glycine, 3 mM EDTA at 1 °C (Kumar and Porter, 1971). When necessary, the enzyme was subjected to limited trypsin treatment and purified by dialysis against water, followed by gel filtration on Sephadex G-25 (Parker and Reese, 1966).

**Synthesis of NBDAH-CoA**—A thioster between NBDAH and CoA was formed via a thiophenol ester by a modification of the method of Rintzius and Schwab (1978). Various reaction mixtures were stirred continuously and purged with nitrogen. Forty milligrams of NBDAH was dissolved in 6 ml of 0.2 M thiolipin in dry tetrahydrofuran, and 6 ml of 0.2 M diisopropyl dithiocarbamidomethyl sulfide was added dropwise over a period of 15 min. The reaction was judged complete by thin-layer chromatography on silica gel (Polygram Sil). The Rf values for NBDAH-thiophenol ester and NBDAH were 0.81 and 0.58, respectively (cyclohexane/toluene (1/1, v/v)). Sixty milligrams of CoA dissolved in 2.5 ml of 1 M potassium carbonate (pH 9.8) was added dropwise in 15 min, followed by 2 ml of dimethylformamide. (The dimethylformamide dramatically increases the yield of the final product, NBDAH-CoA.) After 90 min, another 20 mg of CoA in 1.5 ml of 1.0 M potassium carbonate was added. The product, NBDAH-CoA, remained at the origin when analyzed by thin-layer chromatography, as described above. The reaction product was then purified according to Anderson and Hammes (1983), except 65% 10 mM ammonium acetate (pH 5.0) and 35% methanol was used as the eluant for the high-performance liquid chromatography. The absorbance ratio of the product, NBDAH-CoA, was determined spectrophotometrically with an extinction coefficient of 30,000 M^-1 cm^-1 (Molecular Probes). The final yield was 25% based on CoA, and the product was stored as a ~20 mM aqueous solution at -20 °C.

**Enzyme Modification**—PBMPF in isopropyl alcohol was added to ~10 μM of fatty acid synthase to the desired final concentration. Typically only a stoichiometric amount of PBMPF was needed, and the reaction mixture was incubated at room temperature for 8 min. The reaction mixture was then passed through a 3-ml Sephadex G-50 centrifuge column to remove the unreacted PBMPF (Penefsky, 1977). The amount of PBMPF covalently bound to the enzyme was determined by measurement of the absorbance, A, at 346 and 280 nm using Equation 1.

| [PBMPF]/[enzyme] | = (482/30) (A_346 - 0.016 A_280)/A_280 - 1.1 A_280 | (1) |

Here 0.016 A_280 and 1.1 A_280 were used to correct for the PBMPF absorbance at 280 nm and the enzyme absorbance at 346 nm; an extinction coefficient of 30,000 M^-1 cm^-1 at 346 nm was used for PBMPF (Foster et al., 1985).

**Inhibition of fatty acid synthase by NBDPF requires a much higher inhibitor concentration than for PBMPF. The NBDPF was dissolved in a solution of isopropyl alcohol/acetonitrile (2/1, v/v) and then added to ~10 μM enzyme at a final concentration of 55-500 μM. The amount of NBDPF covalently bound to the enzyme after 1.7 to 50 min, following passage of the reaction mixture through a centrifuge column, was calculated with the relationship

| [NBDPF]/[enzyme] = 482 A_346/(22 A_280) | (2) |

where the extinction coefficient of NBDPF at 485 nm is 22,000 M^-1 cm^-1 (Berman et al., 1985).

To modify enzyme with both NBDPF and PBMPF, the enzyme (~4 μM) was first incubated with 4 μM PBMPF at room temperature for 8 min, and then NBDPF was introduced at a final concentration of 500 μM. The reaction mixture was passed through a centrifuge column after 15 min to remove unbound inhibitors, and the amount of each inhibitor bound to the enzyme was determined spectrophotometrically with Equations 1 and 2, with a correction for the absorbance of PBMPF at 346 nm.

The modification of fatty acid synthase with NBDAH-CoA was carried out in 1 mM potassium phosphate, pH 7.4, 3 mM EDTA, and 10% glycerol. An aliquot of NBDAH-CoA stock solution was added to ~4 μM enzyme to the desired final concentration, typically 800 μM. The reaction mixture was incubated at 30 °C for 120 min and then passed through a centrifuge column to stop the reaction. The modified enzyme was incubated overnight at room temperature and passed through a centrifuge column again to remove any nonspecifically bound NBDAH. The amount of bound NBDAH was calculated as follows.

| [NBDAH]/[enzyme] = 482 A_460/(30 A_280) | (3) |

The enzyme used for fluorescence resonance energy transfer measurements usually had 1.3 molecules of NBDAH bound per enzyme molecule. The enzyme modified with both PBMPF and NBDAH-CoA was obtained by modifying the PBMPF-labeled enzyme with NBDAH-CoA as described above. The amount of NBDAH bound to the enzyme again was calculated using Equation 3.

The enzyme (~4 μM) was modified with 2 mM pyridoxal 5'-phosphate in 0.1 M potassium phosphate, pH 7.0, 1 mM EDTA, 1 mM dithiothreitol, and 10% glycerol as described by Cardon and Hammes (1983), except that NaBH_4 was dissolved in cold water to 65 mM before addition to the reaction mixture. The absorbance ratio of the pyridoxal 5'-phosphate-modified enzyme (323:280 nm) was about 0.26. The overall specific activity was ~10% of the control enzyme, which went through the same treatment in the absence of pyridoxal 5'-phosphate. The β-ketoesterase and enoyl reductase activities were >90% and ~15% of the control enzyme, respectively. To modify the enzyme with both NBDAH-CoA and pyridoxal 5'-phosphate, the enzyme was first labeled with NBDAH-CoA as described above and then modified with pyridoxal 5'-phosphate. Because NaBH_4 reduces the NBD moiety of NBDAH at room temperature, the reaction mixture was placed on ice for 2 min before addition of the NaBH_4 solution.

**Spectroscopic Measurements**—A Cary 2200 spectrophotometer was used for all absorbance measurements. Steady-state fluorescence measurements were made with a Hitachi-Perkin-Elmer MFP-44B fluorescence spectrophotometer equipped with corrected spectrum and polarization accessories. Quantum yields of fluorescent-labeled fatty acid synthase samples were made by a comparative method (Parkes and Reese, 1966):

| Q_2/Q_1 = (F_2/F_1)/(A_2/A_1) | (4) |

where Q_1 is the quantum yield of the sample, F_2 is the area under the corrected fluorescence emission spectrum, and A_2 is the absorbance at the excitation wavelength; Q_1, F_1, and A_1 are the corresponding quantities for the quantum yield standard. Quinine sulfate in 0.1 N H_2SO_4 was used as the standard, and a quantum yield of 0.70 was assumed (Scott et al., 1970). Fluorescence emission of the modified fatty acid synthase was corrected for light scattering by subtracting the apparent emission for an equal concentration of fatty acid synthase sample without the fluorescent chromophore attached. Fluorescence polarization (P) measurements were carried out as previously described (Cerione and Hammes, 1982).

**Resonance Energy Transfer Measurements**—For energy transfer determinations, the inner filter effects due to the energy acceptors were corrected for at both excitation and emission wavelengths by measurement of the enzyme absorbance with and without acceptors. The apparent energy transfer efficiency, E', was calculated from the relationship:

| E' = 1 - Q_0Q_1/Q_0 | (5) |

where Q_0 and Q_1 are the quantum yields of the enzyme with and without acceptor. The efficiency, E, is obtained by correcting E' for the stoichiometry of the bound acceptor:

| E = E' / a | (6) |

where a is the fraction of the enzyme sites labeled with acceptor. The distance between the donor and acceptor, r, was calculated from...
Fatty Acid Synthase Fluorescence Studies

Modification of Thioesterase with NBDPF and PBMPF—
The inhibition of fatty acid synthase with PBMPF has been reported (Foster et al., 1985). An active serine of the thioesterase domain was proposed as the labeling site. To directly verify that the PBMP is attached to the thioesterase domain, PBMPF-labeled enzyme was subjected to limited trypsin treatment and analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (Wakil et al., 1983; Yuan and Hammes, 1985). On the gel, fatty acid synthase separates into peptide I (M, 127,000), which contains the active cysteine and the hydroxyl acyl loading group, peptide II (M, 107,000), which contains the 4'-phosphopantetheine, and peptide III (M, 33,000), which is the thioesterase. On the polycrylamide gel, the fluorescence of PBMP was associated exclusively with the thioesterase domain.

NBDFP, which like PBMPF, has a methylphosphonofluoridate moiety, is a specific inhibitor of serine proteases (Berman et al., 1985). Aliquots of the enzyme and NBDFP reaction mixture were passed through a Sephadex G-50 centrifuge column at different times and/or different inhibitor concentrations, and the enzyme activity and inhibitor stoichiometry were determined. The results are presented in Fig. 1; the line represents the correlation expected if one NBDFP/polypeptide chain inactivates the enzyme. The good agreement of the data with the theoretical line indicates the inhibition is specific. Analysis of NBDFP-labeled enzyme by limited trypsin treatment and electrophoresis on sodium dodecyl sulfate-polyacrylamide gels showed that the NBDF fluorescence was associated with only the thioesterase fragment.

Modification of 4'-Phosphopantetheine by NBDAH-CoA—
NBDAH-CoA is a fluorescent acyl-CoA analogue with a maximum absorbance at 458 nm. The use of acyl-CoA analogues to modify chicken liver fatty acid synthase has been reported before (Cardon and Hammes, 1982, Anderson and Hammes, 1983). However, previously at least two sites of acyl binding sites were labeled. In this work, reaction conditions were found under which NBDAH-CoA specifically modifies one of the acyl binding sites, the 4'-phosphopantetheine. This specific modification was performed at a low salt concentration (1 mM potassium phosphate, 3 mM EDTA) in the presence of 10% glycerol. Incubation of the enzyme and NBDAH-CoA in 0.1 M potassium phosphate resulted in the fluorescence of NBDAH being associated with both bands I and II, after limited trypsin treatment and sodium dodecyl sulfate-polyacrylamide gel electrophoresis. When the potassium phosphate concentration is decreased to 1 mM, the reaction rate decreases by at least 60%, but the specificity of the modification increases. In order to reduce the inhibitor concentration and incubation time, which correspondingly increases the specificity of the modification, the reaction temperature

RESULTS

Modification of Thioesterase with NBDFP and PBMPF—
The inhibition of fatty acid synthase with PBMPF has been reported (Foster et al., 1985). An active serine of the thioesterase domain was proposed as the labeling site. To directly verify that the PBMP is attached to the thioesterase domain, PBMPF-labeled enzyme was subjected to limited trypsin treatment and analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (Wakil et al., 1983; Yuan and Hammes, 1985). On the gel, fatty acid synthase separates into peptide I (M, 127,000), which contains the active cysteine and the hydroxyl acyl loading group, peptide II (M, 107,000), which contains the 4'-phosphopantetheine, and peptide III (M, 33,000), which is the thioesterase. On the polycrylamide gel, the fluorescence of PBMP was associated exclusively with the thioesterase domain.

NBDFP, which like PBMPF, has a methylphosphonofluoridate moiety, is a specific inhibitor of serine proteases (Berman et al., 1985). Aliquots of the enzyme and NBDFP reaction mixture were passed through a Sephadex G-50 centrifuge column at different times and/or different inhibitor concentrations, and the enzyme activity and inhibitor stoichiometry were determined. The results are presented in Fig. 1; the line represents the correlation expected if one NBDFP/polypeptide chain inactivates the enzyme. The good agreement of the data with the theoretical line indicates the inhibition is specific. Analysis of NBDFP-labeled enzyme by limited trypsin treatment and electrophoresis on sodium dodecyl sulfate-polyacrylamide gels showed that the NBDF fluorescence was associated with only the thioesterase fragment.

Modification of 4'-Phosphopantetheine by NBDAH-CoA—
NBDAH-CoA is a fluorescent acyl-CoA analogue with a maximum absorbance at 458 nm. The use of acyl-CoA analogues to modify chicken liver fatty acid synthase has been reported before (Cardon and Hammes, 1982, Anderson and Hammes, 1983). However, previously at least two sites of acyl binding sites were labeled. In this work, reaction conditions were found under which NBDAH-CoA specifically modifies one of the acyl binding sites, the 4'-phosphopantetheine. This specific modification was performed at a low salt concentration (1 mM potassium phosphate, 3 mM EDTA) in the presence of 10% glycerol. Incubation of the enzyme and NBDAH-CoA in 0.1 M potassium phosphate resulted in the fluorescence of NBDAH being associated with both bands I and II, after limited trypsin treatment and sodium dodecyl sulfate-polyacrylamide gel electrophoresis. When the potassium phosphate concentration is decreased to 1 mM, the reaction rate decreases by at least 60%, but the specificity of the modification increases. In order to reduce the inhibitor concentration and incubation time, which correspondingly increases the specificity of the modification, the reaction temperature

RESULTS

Modification of Thioesterase with NBDFP and PBMPF—
The inhibition of fatty acid synthase with PBMPF has been reported (Foster et al., 1985). An active serine of the thioesterase domain was proposed as the labeling site. To directly verify that the PBMP is attached to the thioesterase domain, PBMPF-labeled enzyme was subjected to limited trypsin treatment and analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (Wakil et al., 1983; Yuan and Hammes, 1985). On the gel, fatty acid synthase separates into peptide I (M, 127,000), which contains the active cysteine and the hydroxyl acyl loading group, peptide II (M, 107,000), which contains the 4'-phosphopantetheine, and peptide III (M, 33,000), which is the thioesterase. On the polycrylamide gel, the fluorescence of PBMP was associated exclusively with the thioesterase domain.

NBDFP, which like PBMPF, has a methylphosphonofluoridate moiety, is a specific inhibitor of serine proteases (Berman et al., 1985). Aliquots of the enzyme and NBDFP reaction mixture were passed through a Sephadex G-50 centrifuge column at different times and/or different inhibitor concentrations, and the enzyme activity and inhibitor stoichiometry were determined. The results are presented in Fig. 1; the line represents the correlation expected if one NBDFP/polypeptide chain inactivates the enzyme. The good agreement of the data with the theoretical line indicates the inhibition is specific. Analysis of NBDFP-labeled enzyme by limited trypsin treatment and electrophoresis on sodium dodecyl sulfate-polyacrylamide gels showed that the NBDF fluorescence was associated with only the thioesterase fragment.

Modification of 4'-Phosphopantetheine by NBDAH-CoA—
NBDAH-CoA is a fluorescent acyl-CoA analogue with a maximum absorbance at 458 nm. The use of acyl-CoA analogues to modify chicken liver fatty acid synthase has been reported before (Cardon and Hammes, 1982, Anderson and Hammes, 1983). However, previously at least two sites of acyl binding sites were labeled. In this work, reaction conditions were found under which NBDAH-CoA specifically modifies one of the acyl binding sites, the 4'-phosphopantetheine. This specific modification was performed at a low salt concentration (1 mM potassium phosphate, 3 mM EDTA) in the presence of 10% glycerol. Incubation of the enzyme and NBDAH-CoA in 0.1 M potassium phosphate resulted in the fluorescence of NBDAH being associated with both bands I and II, after limited trypsin treatment and sodium dodecyl sulfate-polyacrylamide gel electrophoresis. When the potassium phosphate concentration is decreased to 1 mM, the reaction rate decreases by at least 60%, but the specificity of the modification increases. In order to reduce the inhibitor concentration and incubation time, which correspondingly increases the specificity of the modification, the reaction temperature
was raised to 30 °C. When the unbound inhibitor was removed from the enzyme by a centrifuge column, part of the NBDAH was nonspecifically bound to the enzyme, as indicated by both the large number of NBDAH molecules bound per enzyme molecule and the appearance of free NBDAH with time. The nonspecifically bound NBDAH was removed by passing the enzyme adduct through a second centrifuge column after at least 12 h of incubation at room temperature. This can be illustrated by the following example. When NBDAH-CoA-labeled enzyme was passed through the first centrifuge column, 85% of the specific activity was lost and 1.1 NBDAH was bound per enzyme polypeptide chain; after 16 h and passage through a second centrifuge column, 70% of the specific activity was lost and 0.7 NBDAH was bound per polypeptide chain. Further dissociation of bound NBDAH from the enzyme after this treatment was not observed.

The decrease in the enzyme specific activity as the amount of covalently bound NBDAH increases is shown in Fig. 2. The experimental data are in good agreement with the theoretical line shown for the case that one covalently bound NBDAH/polypeptide chain inactivates the enzyme. The modified enzyme used for energy transfer measurement had ~1.3 NBDAH molecules bound per enzyme molecule to minimize any nonspecific labeling. Modification of the enzyme with NBDAH-CoA resulted in the loss of overall activity and β-ketoacyl synthase activity to the same extent. However, both β-ketoacyl reductase and enoyl reductase retained more than 95% of their activity. To determine which acyl-binding site was modified by NBDAH-CoA, the enzyme-NBDAH adduct was subjected to limited trypsin treatment and analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. The only protein band on the gel that fluoresced was the band corresponding to peptide II, which contains 4'-phosphopantetheine (Wakil et al., 1983). Therefore, under the reaction conditions used, NBDAH-CoA specifically modifies the 4'-phosphopantetheine of fatty acid synthase.

**Time-resolved Fluorescence Measurements of PBMPF-labeled Enzyme**—A typical fluorescence decay of PMBMP-labeled enzyme is shown in Fig. 3. The data could not be described by a single lifetime but were well fit by Equation 11 with $F_1 = 0.36$, $F_2 = 0.64$, $\tau_1 = 65$ ns, and $\tau_2 = 178$ ns ($F_1 + F_2 = 1.00$). A plot of the normalized residuals, Δ, is shown at the bottom with a linear ordinate scale.

$F_2$ has been arbitrarily set equal to 1.00. A plot of the residuals is included in Fig. 3; for this fit, $\chi^2 = 2.7$. These parameters are independent of viscosity over the range 1.00 to 1.90 cP. The decay of the anisotropy is shown in Fig. 4. Again two exponential components were required to fit the data (Equation 12). The best fit parameters are $A_1 = 0.083$, $A_2 = 0.021$, $\phi_1 = 43.6$ ns, and $\phi_2 = 658$ ns, with $\chi^2 = 0.63$. The viscosity dependence of the time-dependent anisotropy was examined. However, fitting the data became difficult at high viscosities because $\phi_2$ became longer, whereas the fluorescence lifetimes remained constant. Previous work (Anderson and Hammes, 1983) suggests that $\phi_1$ is characteristic of rotation of the entire molecule, and its value was set equal to previously determined values in carrying out the data analysis. The viscosity dependence of $\phi_1$ is presented in Fig. 5.

The anisotropy decay also was measured after a 30-min trypsin treatment of the enzyme. Approximately 90% of the
Fatty Acid Synthase Fluorescence Studies

I analysis: EDTA and 0-23.175 glycerol (w/w) at room temperature. The lines were calculated with a linear least squares regression analysis; •, PBMP-fatty acid synthase; □, trypsin-treated PBMP-fatty acid synthase in 0.1 m potassium phosphate, pH 7.0, 2 mm EDTA and 0-23.1% glycerol (w/w) at room temperature.

Fl lines were calculated with a linear least squares regression. Thioesterase was released, as estimated from sodium dodecyl sulfate-polyacrylamide gel electrophoresis. More extensive trypsin action resulted in degradation of the thioesterase to two components of molecular weight 15,000 and 17,000 (Lin, 1978). The decay of the anisotropy was well fit by a single exponential decay with a rotational correlation time of 17 ns (\( \tau_2 = 0.93 \)) in the absence of glycerol. The viscosity dependence of the rotational correlation time is included in Fig. 5.

Time-resolved Fluorescence Measurements of the NBDAH-CoA-Labeled Enzyme—Although free NBDAH-CoA has a very short fluorescence lifetime (<1 ns), the enzyme-bound NBDAH has a relatively longer lifetime. The fluorescence decay was well fit by Equation 11 with \( F_1 = 0.79, F_2 = 0.21, \tau_1 = 2.8 \) ns, and \( \tau_2 = 8.3 \) ns with \( F_1 + F_2 = 1.00 \). The normalized residuals, \( \Delta \), are shown at the bottom with a linear ordinate scale.

Fluorescence anisotropy of the NBDAH-enzyme adduct decays very slowly as shown in Fig. 7. The data were fit to Equation 12, and the curve was calculated with the best fit parameters, \( A_1 = 0.25, \phi_1 = 625 \) ns, and \( A_2 = 0.95 \). The normalized residuals, \( \Delta \), are shown at the bottom with a linear ordinate scale.

The distance between PBMP on the thioesterase and the enzyme can be dissociated into single polypeptide chains. This permits further refinement of the interpretation of energy transfer measurements. The spectral properties of the enzyme-bound probes are given in Table I. The donor-acceptor pairs are summarized in Table I. The donor-acceptor pairs are summarized in Table II, along with the values of \( J \) and \( R_0 \) (calculated with Equation 8 and \( \kappa^2 = \frac{1}{3} \)).

The observed decrease in anisotropy is very small. Fluorescence Resonance Energy Transfer Measurements—The specific labeling of the 4′-phosphopantetheine and the serine at the active site of the thioesterase described above, plus the specific binding of NAPDH to the reductases, permits the distances between four specific sites on the enzyme to be determined. In addition, the \( \beta \)-ketoacyl reductase binding site can be specifically blocked by pyridoxal 5′-phosphate, and the enzyme can be dissociated into single polypeptide chains. This permits further refinement of the interpretation of energy transfer measurements. The spectral properties of the enzyme-bound probes are given in Table I. The donor-acceptor pairs are summarized in Table II, along with the values of \( J \) and \( R_0 \) (calculated with Equation 8 and \( \kappa^2 = \frac{1}{3} \)).

The distance between PBMP on the thioesterase and NBDAH on the 4′-phosphopantetheine was determined by comparison of the fluorescence of the PBMPF-labeled enzyme (356 nm excitation, 386 nm emission) with the same sample subsequently labeled with NBDAH. The energy transfer efficiency from two separate experiments was 0.688 ± 0.004 for the native enzyme and 0.920 ± 0.003 for the enzyme disso-
The binding of NADPH to the enzyme enhances the fluorescence where bound, and total ligand concentrations, respectively, and best fit parameters obtained from three experiments, with n (lower curve, A) at room temperature. The fluorescence emission was measured at 460 nm, with excitation at 340 nm. Fig. 8 shows typical titration curves for the enzyme with (lower curve) and without (upper curve) covalently bound NBDAH. The data were fit to Equations 13 and 14 with appropriate corrections for dilution. The best fit parameters obtained from three experiments, with n set equal to 4.0, were \( K = 1.77 \pm 0.21 \mu M \) and \( E = 0.267 \pm 0.016 \). (The ratio of quantum yields in the presence and absence of NBDAH is equal to the ratio of the corresponding fluorescence enhancements, \( f' \)). The distance between bound NADPH and the NBDAH on 4'-phosphopantetheine calculated with Equation 7 is 36.9 Å.

To determine further whether the observed energy transfer is due to chromophores on the same or different polypeptide chains, enzyme, with or without covalently bound NBDAH, was dissociated into monomers and titrated with NADPH in the dissociation buffer at 1 °C. The results obtained are shown in Fig. 8. The data could not be adequately fit to Equations 13 and 14 with \( n = 4.0 \). If \( n \) is assumed to be an integer, the best fit parameters obtained from three titrations were \( n = 2.0 \), \( K = 0.983 \pm 0.044 \mu M \), and \( E = 0.231 \pm 0.013 \). The corresponding distance calculated with Equation 7 is 38.1 Å. The reduced value of \( n \) is considered further below, but the simplest interpretation of the observed energy transfer efficiencies is that energy transfer occurs primarily between NADPH and NBDAH on the same polypeptide chain.

The efficiency of energy transfer between bound NADPH and NBDAH on the 4'-phosphopantetheine was also measured for the enzyme labeled with pyridoxal 5'-phosphate. The pyridoxal 5'-phosphate primarily modifies enoyl reductase, whereas β-ketoacyl reductase is unaffected (Poulose and Kolattukudy, 1980). To minimize the nonspecific binding of pyridoxal 5'-phosphate (Cardon and Hammes, 1983), the enzyme with ~80% of the enoyl reductase activity eliminated was used for energy transfer measurements. The NADPH fluorescence emission was observed at 470 nm, with excitation at 370 nm, to avoid interference due to the fluorescence of the covalently bound pyridoxamine 5'-phosphate. The NADPH titration and the data fitting were carried out as described above, with the number of NADPH binding sites, \( n \), fixed at 2.4 (20% of the enoyl reductase sites plus 2.0 β-ketoacyl reductase sites). The analysis of three sets of titrations gave \( K = 4.35 \pm 0.98 \mu M \) and \( E = 0.228 \pm 0.015 \) (Fig. 10). Since the calculated distance between β-ketoacyl reductase sites and the NBDAH on 4'-phosphopantetheine is essentially the same as that between all four reductase sites and NBDAH, the enoyl reductase sites must also be 38 Å from the bound NBDAH. Furthermore, the previous results indicate these distances are between sites on the same polypeptide chain.

The fluorescence resonance energy transfer from NADPH bound at the reductase sites to NBDP on the thioesterase was measured with both native and dissociated enzyme by the titration of the enzyme with NADPH as above. However, no significant energy transfer from NADPH to NBDP was observed. If an upper detection limit of 2% energy transfer efficiency is assumed, the distance between NADPH binding

\[
F = f_0[L_0] + f_0'[L_0] + f_0''[L_0] + f_0'''(1)[L_0] \tag{13}
\]

where \( f_0 \) is the fluorescence intensity corresponding to unit concentration of free ligand; \([L_0],[L_0'],[L_0'']\), and \( [L_0'''] \) are the free, bound, and total ligand concentrations, respectively, and \( f' \) is the ratio of the fluorescence of bound ligand to that of free ligand. If the binding can be described by a single dissociation constant, \( K \), then

\[
2[L_0] = n[E]_0 + [L_0] + K - \sqrt{[n[E]_0 + [L_0] + K]^2 - 4n[E]_0[L_0]} \tag{14}
\]
sites and the thioesterase active serine is greater than 56 Å.

Both PBMPF and NBDPF can covalently modify the active serine of the thioesterase. Since the enzyme is a homologous dimer, two thioesterase domains are present per enzyme molecule. To determine the distance between these two domains, the enzyme with 50% of the thioesterase labeled with PBMPF and 50% labeled with NBDPF was prepared. If a random labeling process is assumed, 25% of the enzyme should have one thioesterase labeled with PBMPF and the other labeled with NBDPF within the same molecule. After correction for inner filter effects, no energy transfer was observed (346 nm excitation, 396 nm emission). Again, if the limit of detection is 2% energy transfer efficiency, the distance between the two thioesterase domains is greater than 56 Å.

A summary of the calculated distances is given in Table III.

**Fatty Acid Synthase Fluorescence Studies**

**TABLE III**

Summary of distance measurements for fatty acid synthase

| Enoyl reductase        | 4'-Phosphopantetheine | Thioesterase serine |
|------------------------|------------------------|---------------------|
| β-ketoacyl reductase   | >15 Å                   | >56 Å               |
| 4'-phosphopantetheine | 38 Å                    | 48 Å                |
| Thioesterase serine    | >56 Å                   | >56 Å               |

*Chang and Hammes, 1986.

The specific modification of the thioesterase with both PBMPF and NBDPF suggests the thioesterase active site is similar to those of serine proteases. Indeed amino acid sequence data show a high degree of homology of the thioesterase with serine proteases (Pouloise et al., 1981). An explanation for the specific labeling of 4'-phosphopantetheine by NBDAH-CoA at low salt concentrations is necessarily speculative. Perhaps a conformational change at low salt concentrations causes the catalytic center of the transacylase to become relatively inaccessible, and the 20 Å arm of the 4'-

**FIG. 10.** Plot of fluorescence versus the concentration of NADPH in 400 μl of pyridoxal 5'-phosphate-labeled fatty acid synthase (upper curve, ○) or the same enzyme with covalently bound NBDAH (lower curve, △). The titration was carried out in 0.1 M potassium phosphate, 1 mM EDTA at room temperature. The curves represent the best fit to Equation 13 with covalently bound NBDAH. The best fit parameters are K = 4.35 μM, f' (○) = 3.87, and f' (△) = 4.65.

**FIG. 11.** Plot of β-ketoacyl reductase activity of dissociated fatty acid synthase versus the concentration of potassium phosphate in the assay solution. The assay was carried out in 5 mM Tris/HCl pH 8.3, 30 mM glycine, 3 mM EDTA, and the desired concentration of potassium phosphate at 1 °C. The activity of native enzyme in the same buffer with 87.5 mM potassium phosphate was taken as 100%. The line has no theoretical significance.
phosphopantetheine allows it to be more easily modified than the critical sulfhydryl. The acyl binding sites have been shown to have different accessibility to solvent (Anderson and Hammes, 1983). This is also consistent with the finding that the acyl-CoA derivative containing the hydrophobic chromophore pyrene does not distinguish between the different acyl binding sites.

The thioesterase domain is at the COOH terminus and can be readily cleaved from the enzyme by proteolysis. The active thioesterase fragment has been purified (Smith et al., 1976; Lin, 1978). The shortest rotational correlation time for covalently bound PBMP, \( \phi_1 = 44 \) ns, is considerably less than that for the enzyme itself, \( \phi_3 \approx 650 \) ns (Anderson and Hammes, 1983). The viscosity dependence of the rotational correlation time \( \phi_1 \) (Fig. 5) suggests that it characterizes segmental flexibility rather than local motion. If the segmental motion is independent of the global motion of the enzyme and is much more rapid (Lakowicz, 1984),

\[
A(t) = A_0 e^{-t/\tau_0} + (1 - e^{-t/\tau_0})
\]

where \( \alpha \) is a measure of the constraint of the segmental motion. With this model, the segmental motion is constrained to a cone over an angle \( \theta \), with \( \cos^2 \theta = (2a + 1)/3 \) (Yguerabide et al., 1970). For the thioesterase, \( \alpha = 0.178 \) and \( \theta = 45^\circ \). The viscosity dependence of the rotational correlation time for the trypsin-treated fatty acid synthase is consistent with both global and segmental rotation. For a sphere (Yguerabide et al., 1970)

\[
\phi = \eta V/kT
\]

where \( \eta \) is the viscosity, \( V \) is the hydrated volume of the sphere, \( k \) is Boltzmann's constant, and \( T \) is the absolute temperature. If \( V \) is estimated from the thioesterase molecular weight, with a specific hydration of 1.3 g of H2O/g of protein, then the calculated value of \( \phi \) is 17 ns, in good agreement with that observed. Thus for trypsin-treated enzyme, the rotational correlation time is characteristic of free rotation of the thioesterase. The larger correlation time for the intact enzyme is consistent with constrained segmental flexibility of the thioesterase. A less likely possibility is that free rotation of a larger fragment than the thioesterase occurs. The diameter of the thioesterase calculated from the hydrated volume is \( \approx 50 \) Å, which is similar in size to the lobes observed in electron microscopy of the enzyme, \( \approx 50 \) Å (Stoops et al., 1979). The terminal lobe may rotate relatively freely with respect to the rest of the enzyme. The ease with which this fragment can be proteolyzed indicates that the link between the thioesterase and the rest of the enzyme is quite exposed to solvent.

The results obtained from the studies of partial activities of the monomeric enzyme clearly show that the monomer lacks both the \( \beta \)-ketoacyl synthase and \( \beta \)-ketoacyl reductase activities. Furthermore, the fluorescent titrations of the enzyme with NADPH show that NADPH does not bind to the \( \beta \)-ketoacyl reductase sites in the monomeric enzyme. Previous results have been interpreted in terms of the monomer lacking only the \( \beta \)-ketoacyl synthase activity (Wakil et al., 1983). The addition of potassium phosphate to the monomeric enzyme at 1 °C causes rapid dimerization, as revealed by changes in light scattering, and restoration of the \( \beta \)-ketoacyl reductase activity. The \( \beta \)-ketoacyl synthase activity is restored much more slowly by rearrangements within the dimeric enzyme; this change occurs only when the dimer in potassium phosphate is raised from 1 °C to room temperature. This rearrangement also permits cross-linking of the 4′-phosphopantetheine and the critical sulfhydryl (Stoops and Wakil, 1982).

Several conditions are necessary for reliable interpretation of energy transfer measurements on proteins. First, the probes must be bound at specific sites. The evidence in support of this has already been discussed. Second, fluorescence changes accompanying energy acceptor binding cannot be due to factors other than energy transfer, i.e., to changes in the environment of the donor. This is supported by the following observations: the absorbance and wavelength dependences of the fluorescence spectra were not altered by the binding of acceptors; and the stoichiometry and binding constants for NADPH binding were not changed by acceptor binding. Of course, the possibility of acceptor-induced conformational changes interfering with energy transfer measurements cannot be strictly excluded. Finally, the uncertainty associated with the assumption that \( x^2 = \frac{1}{3} \) must be considered. This problem has been discussed extensively (cf. Stryer, 1978; Dale et al., 1979; Richter et al., 1985), and in no case to date has been shown to cause major errors. The maximum ranges in \( x^2 \) can be estimated from the fluorescence polarization of the donors and acceptors (Dale et al., 1979). From the data in Table I and the plots in the work of Dale et al. (1979), the range in \( x^2 \) is 0.3–1.7 for the PBMP-NBDHA donor-acceptor pair, 0.15–3.1 for the NADPH-NBDHA pair, and 0.3–1.8 for the PBMP-NBDP pair. This corresponds to uncertainties in the calculated distances of ±15, ±20, and ±15%, respectively. The actual uncertainties are probably less than these maximum estimates.

The distances from the NADPH binding sites to the acyl binding sites have previously been estimated to be 30–40 Å (Cardon and Hammes, 1983). In this study, the distances from one of the acyl binding sites to the NADPH binding sites were determined. The distances from the 4′-phosphopantetheine to the \( \beta \)-ketoacyl reductase and enoyl reductase sites on the same polypeptide are essentially the same, \( \approx 38 \) Å. The distance from 4′-phosphopantetheine to reductase on a different polypeptide must be \( \approx 55 \) Å since the energy transfer efficiency is <3% for this interaction. Similarly the distance between the thioesterase active site and 4′-phosphopantetheine on the same polypeptide is 48 and >55 Å between different polypeptides. This distance is greater than previously reported, 37 Å (Foster et al., 1985). However, in the previous work, the energy transfer was measured with an enzyme that was nonspecifically labeled; the specific portion of energy transfer was obtained by extrapolation. The closeness of the three catalytic sites on a single polypeptide to the 4′-phosphopantetheine is consistent with their residing in a single reaction center of the multi-enzyme complex.

The fact that the fluorescent probe is on the end of the 20 Å arm of 4′-phosphopantetheine makes a precise interpretation of the measured distances difficult. However, the dynamic anisotropy measurements indicate the arm is not rotating during the fluorescence decay; therefore, the probe is bound at one or more sites on the surface of the enzyme. The number of binding sites cannot be determined; one likely possibility is the CoA binding portion of the substrate loading site. The fluorescence decay of NBDHA covalently bound to fatty acid synthase is characterized by two lifetimes (Fig. 6). This can be attributed to many causes: e.g., multiple electronic transitions, two environments of the chromophore at a single binding site, or two different binding sites. If two environments or sites are present, their population ratio can be estimated from the amplitudes of the fluorescence decay as 0.79/0.21. The two distances cannot be uniquely determined for this case; however, the distances can be calculated for the extreme cases in which one of the sites is much closer than the other.

\[ Z. \text{ Yuan and G. G. Hammes, unpublished results.}\]
to the energy donor, PBMP or NADPH: the observed energy transfer is simply divided by the fraction at the closest site. With PMBP, the closest distances are 34 and 45 Å, whereas with NADPH they are 55 Å and underestimated ($E > 1$). However, it should be emphasized that no compelling evidence exists for more than a single binding site for the 4'-phosphopantetheine probe.

The distances measured are somewhat larger than might be anticipated for a multienzyme complex in which all of the site must be within reach of the 20-Å phosphopantetheine arm. In addition to the results in Table III, the $\beta$-ketoacyl reductase and enoyl reductase sites are >15 Å apart (Chang and Hammes, 1986). Thus, relatively large conformational changes may be part of the catalytic cycle. This is consistent with the segmental flexibility of the thioesterase domain. Electron microscopy also suggests that the four lobes of a single polypeptide can fold into different conformations (Stoops et al., 1979). However, no direct evidence for such conformational changes exists, and the finite size of the probe molecules and the uncertainty in the distances (±15%) prevents a firm conclusion from being reached with regard to this matter.

Acknowledgments—We thank Dr. A. H. Berman for providing NBDPF and PBMPF, and L. C. Bosch for assistance in preparing the enzyme.

REFERENCES
Anderson, V. E., and Hammes, G. G. (1983) Biochemistry 22, 2295-3001
Bevington, P. R. (1969) Data Reduction and Error Analysis for the Physical Sciences, McGraw-Hill, New York
Berman, H. A., Olshefski, D. F., Gilbert, M., and Decker, M. M. (1985) J. Biol. Chem. 260, 3462-3468
Chang, S.-I., and Hammes, G. G. (1986) Biochemistry 25, 4661-4668
Cardon, J. W., and Hammes, G. G. (1982) Biochemistry 21, 2863-2870
Cardon, J. W., and Hammes, G. G. (1983) J. Biol. Chem. 258, 4902-4907
Cerione, R. A., and Hammes, G. G. (1982) Biochemistry 21, 745-752
Cognot, J. A. H., and Hammes, G. G. (1983) Biochemistry 22, 3802-3807
CRC Handbook of Chemistry and Physics (1981) (Weast, R. C., ed.) 62nd ed., pp. D210-211, CRC Press, The Chemical Rubber Co., Cleveland, OH
Dale, R. E., Risinger, J., and Blumberg, W. E. (1979) Biophys. J. 26, 161-194
Dugan, R. E., and Porter, J. W. (1970) J. Biol. Chem. 245, 2051-2055
Förster, T. (1959) Discuss. Faraday Soc. 27, 7-17
Foster, R. J., Poulase, A. J., Bonsell, R. F., and Kolattukudy, P. E. (1985) J. Biol. Chem. 260, 2826-2833
Hammes, G. G. (1985) Curr. Top. Cell. Regul. 26, 311-324
Horecker, B. L., and Kornberg, A., (1948) J. Biol. Chem. 175, 385-390
Hsu, R. Y., and Wagner, B. J. (1970) Biochemistry 9, 245-251
Hsu, R. Y., and Yun, S.-L. (1970) Biochemistry 9, 239-245
Katzir, S. S., Cleland, W. W., and Porter, J. W. (1975) J. Biol. Chem. 250, 2709-2717
Kumar, S., and Porter, J. W. (1971) J. Biol. Chem. 246, 7780-7789
Kumar, S., Dorsey, J. A., Muesing, R. A., and Porter, J. W. (1970) J. Biol. Chem. 245, 4732-4744
Lakowicz, J. R. (1984) Principles of Fluorescence Spectroscopy, pp. 161-162. Plenum Press, New York
Lin, C. Y., and Smith, S. (1978) J. Biol. Chem. 253, 1954-1962
Mattuck, J. S., Tsukamoto, Y., Nickless, J., and Wakil, S. J. (1983) J. Biol. Chem. 258, 1529-1529
Murphy, J. J., Pecht, L., and Stryer, L. (1979) Proc. Natl. Acad. Sci. U.S.A. 76, 86-88
Parker, C. A., and Reese, W. T. (1966) Analyst (London) 85, 587-600
Penevsky, H. S. (1977) J. Biol. Chem. 252, 2981-2989
Poulose, A. J., Rogers, L., and Kolattukudy, P. E. (1980) Arch. Biochem. Biophys. 201, 313-321
Richter, M. L., Snyder, B., McCarty, R. E., and Hammes, G. G. (1985) Biochemistry 24, 5755-5763
Rutkowski, A., and Jaworski, J. G. (1978) Anal. Biochem. 91, 370-373
Schinkel, J. E., and Hammes, G. G. (1986) Biochemistry 25, 4066-4071
Scott, J. G., Spencer, R. D., Leonard, M. J., and Weber, G. (1970) J. Am. Chem. Soc. 92, 687-696
Siegel, R. C., and Cathou, R. E. (1981) Biochemistry 20, 199-198
Smith, S., Agredi, E., Liberti, L., and Dileepan, K. N. (1976) Proc. Natl. Acad. Sci. U.S.A. 73, 1184-1188
Stoops, J. K., and Wakil, S. J. (1982) J. Biol. Chem. 257, 3230-3235
Stoops, J. K., Ross, P., Arslanian, M. J., Aune, K. C., Wakil, S. J., and Oliver, R. M. (1979) J. Biol. Chem. 254, 7418-7428
Stryer, L. (1978) Annu. Rev. Biochem. 47, 819-846
Tsukamoto, Y., Wong, H., Mattick, J. S., and Wakil, S. J. (1983) J. Biol. Chem. 258, 15312-15322
Wakil, S. J., Stoops, J. K., and Joshi, V. C. (1983) Annu. Rev. Biochem. 52, 557-579
Yguerabide, J., Epstein, H. F., and Stryer, L. (1970) J. Mol. Biol. 51, 573-590
Yuan, Z., and Hammes, G. G. (1984) J. Biol. Chem. 259, 6748-6751
Yuan, Z., and Hammes, G. G. (1985) J. Biol. Chem. 260, 13532-13538