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Fluorescent Properties of Pyrene Bound at Specific Acylation Sites of Chicken Liver Fatty Acid Synthase†

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ABSTRACT: The covalent modification of chicken liver fatty acid synthase by 4-(1-pyrenyl)butyryl-CoA (PBA-CoA), a fluorescent analogue of acetyl- and malonyl-CoA, has been studied. The binding isotherms and the kinetics of inactivation suggest 2 mol of PBA-CoA/mol of enzyme is specifically incorporated into the enzyme. Two classes of binding sites have been identified by determining the fluorescence lifetimes of enzyme-bound pyrene, by the quenching of enzyme-bound pyrene fluorescence with iodide, and by neutral hydroxaminolysis of both the native and denatured PBA-CoA-modified enzymes. Hydroxaminolysis of the denatured enzyme indicates that 4-(1-pyrenyl)butyric acid is esterified to both hydroxyl and thiol groups. The portion esterified to the hydroxyl is readily removed from the native enzyme by treatment with neutral hydroxylamine, indicating that the oxygen ester is unstable to hydroxylamine in the native enzyme. Iodide and acrylamide quenching of the enzyme-bound pyrene fluorescence shows that solvent access to both classes of pyrene binding sites is restricted. Iodide preferentially quenches one class of sites in the native enzyme, but these sites are not differentiated in the monomeric or denatured enzyme. The steady-state anisotropy, 0.083, indicates the enzyme-bound pyrene has considerable rotational freedom. The dynamic anisotropy can be characterized solely by a viscosity-dependent rotational correlation time of 610 ns, which is ascribed to the rotational motion of the dimeric enzyme.

Animal fatty acid synthases are multienzyme complexes that catalyze the synthesis of palmitate from acetyl-CoA,[1] malonyl-CoA, and NADPH. The reaction proceeds through a cycle of seven condensations of an enzyme-bound malonyl moiety with the growing enzyme-bound saturated fatty acid chain. Each condensation is followed by reduction of the β-keto intermediate, dehydration of the alcohol, and reduction of the carbon–carbon double bond formed. The acylation sites on the enzyme complex have been postulated to be 4′-phosphopantetheine, a cysteine, and a serine or threonine (Jacob et al., 1968; Chesterton et al., 1968; Phillips et al., 1970a,b). The cysteine and 4′-phosphopantetheine sites have been proposed as common sites for acetyl and malonyl residues in all eukaryotic fatty acid synthases (Stoops & Wakil, 1981). The hydroxyl site is probably a loading site, and the 4′-phosphopantetheine serves to transfer the intermediate states of the growing fatty acid chain to the various catalytic sites of the enyzme.

The nature of the acylation reaction has been previously investigated with a fluorescent CoA derivative (Cardon & Hammes, 1982, 1983) and with acetyl-CoA (Cognet & Hammes, 1983). The fluorescent CoA derivative previously used, NBD-CoA,[1] was selected because the relatively small size of the fluorophore confers binding properties on the CoA derivative which appear to be similar to those of substrates,

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[1] Abbreviations: PBA, 4-(1-pyrenyl)butanoic acid; CoA, coenzyme A; EDTA, ethylenediaminetetraacetic acid; NBD, 7-chloro-4-nitro-2,1,3-benzoxadiazole.

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and its absorption properties make it a good acceptor for resonance energy transfer from NADPH. However, the short lifetime of the fluorescence did not permit the characterization of the binding sites through dynamic fluorescent measurements. This work utilizes a pyrenyl-CoA derivative, which has a long fluorescence lifetime, to study the acetylation process further. Two different classes of acyl binding sites on the enzyme have been identified. Their accessibility to solvent has been determined by dynamic quenching, and the motions of enzyme-bound pyrene have been studied through dynamic anisotropy measurements.

Materials and Methods

Chemicals. The NADPH, acetyl-CoA, malonyl-CoA, and CoA were from Sigma. The dicyclohexylcarbodiimide was from Eastman, the PBA and *N*-pyrenylmaleimide were from Molecular Probes, and the [^{3}H]acetyl-CoA was from New England Nuclear.

Enzyme Preparation and Assay. Fatty acid synthase was prepared as described by Cardon & Hammes (1982). The activity at 25 °C was determined in 0.1 M potassium phosphate, pH 7.0, 1 mM EDTA, 100 μM NADPH, 100 μM acetyl-CoA, and 200 μM malonyl-CoA by following the decrease in NADPH at 340 nm and using an extinction coefficient of 6220 M^{-1} cm^{-1} (Horecker & Kornberg, 1948). The specific activity of the fatty acid synthase was 1.5–1.6 units/mg (1 unit is 1 μmol of NADPH oxidized per min at 25 °C). Fatty acid synthase concentrations were determined spectrophotometrically at 279 nm by using an extinction coefficient of 4.82×10^5 M^{-1} cm^{-1} (Hsu & Yun, 1970).

The fatty acid synthase was dissociated into monomers by the method of Kumar & Porter (1971). The buffer was changed by passing the enzyme through a 3-mL Sephadex G-50 centrifuge column equilibrated with 5 mM Tris–glycine, pH 8.3, and 1 mM EDTA. The eluate was cooled to 4 °C for 6 h. At this time the enzyme should be over 70% monomers (Yun & Hsu, 1972). All fluorescence measurements with the dissociated enzyme were performed at 4 °C.

Synthesis of PBA-CoA. PBA was esterified to CoA via a thiophenol ester by using the method of Rutkowski & Jaworski (1978). Forty milligrams of PBA (139 μmol) was suspended in 2 mL of ethyl acetate and dried under a nitrogen stream. The dry PBA was dissolved in 1.5 mL of 0.26 M thiophenol, and 2 mL of 0.1 M dicyclohexylcarbodiimide was added dropwise over a period of 30 min. After 3 h the reaction was judged complete by thin-layer chromatography analysis on cellulose (Eastman no. 6064). Elution with cyclohexane yielded a single fluorescent product, R_f 0.9, while a PBA standard remained at the origin. Twenty micromoles of CoA, dissolved in 0.3 mL of 1.0 M potassium carbonate (pH 9.2) and 0.3 mL of tetrahydrofuran, was added dropwise over 15 min to the PBA thiophenol ester. The heterogeneous reaction mixture was stirred continuously and kept under nitrogen. After 90 min, an additional 20 μmol of CoA was added. After 90 more min, the reaction was terminated by the addition of 250 μL of acetic acid and 500 μL of water. The solution then was extracted 4 times with 2 mL of toluene. The PBA-CoA remained in the aqueous phase and was purified by high-performance liquid chromatography on a semipreparative C_{18} column. The sample was eluted with a mixture of 10 mM ammonium acetate (pH 4.5) and acetonitrile. The major impurity, CoA, eluted in the void volume, and the PBA-CoA was eluted with 35% acetonitrile. The fraction containing PBA-CoA was collected, lyophilized, dissolved in distilled water, and stored at a concentration of 1–2 mM at 4 °C. The yield, 11 μmol of PBA-CoA, was 27.5% based on CoA. The concentration of PBA-CoA was determined spectrophotometrically at 345 nm, the absorption maximum, by using an extinction coefficient of 40 000 M^{-1} cm^{-1} (Knopp & Weber, 1969). Sulfhydryl analysis with 5,5′-dithiobis(2-nitrobenzoic acid) (Ellman, 1959) after base hydrolysis (0.1 N KOH, 15 min) or hydroxylaminolysis (0.2 M NH_2OH, pH 8.0, 30 min) quantitatively agreed with the concentration determined spectrophotometrically. No significant hydrolysis of the PBA-CoA could be detected after 2 months of storage.

Labeling of Enzyme with PBA-CoA and N-Pyrenylmaleimide. The fatty acid synthase was equilibrated with 0.1 M potassium phosphate, pH 7.0, and 1 mM EDTA by passing it through a 3-mL Sephadex G-50 centrifuge column (Penefsky, 1977). The enzyme was added to PBA-CoA samples diluted with the same buffer to give the desired final concentrations, 0.2–4 μM enzyme and 0–100 μM PBA-CoA. (PBA-CoA concentrations over 500 μM denature the enzyme.) This mixture was then incubated for a specified time at 35 °C. The labeling reaction was quenched by passing the reaction mixture through a 3-mL Sephadex G-50 centrifuge column which removed all of the unreacted PBA-CoA and product CoA. The number of pyrene molecules covalently bound to the enzyme was determined by measuring the absorbance, *a*, at 279, 347, and 370 nm and using the following relationship:

$$\text{(bound PBA)/(enzyme)} = \frac{482(a_{347} - a_{370})}{40(a_{279} - a_{370} - 0.67a_{347})} \quad (1)$$

The numerator represents the absorbance of the pyrene and the denominator that of the protein. The absorbance at 370 is a small background correction, and $0.67a_{347}$ is the absorbance of pyrene at 279 nm. The ratio 482/40 is that of the molar extinction coefficients of enzyme and pyrene at their absorption maxima of 279 and 347 nm, respectively. For low enzyme concentrations or low binding stoichiometries, the ratio of the pyrene fluorescence (excitation 345 nm, emission 390 nm) to the enzyme fluorescence (excitation 280 nm, emission 330 nm) was used to determine the labeling stoichiometry. When less than 2 mol of pyrene/mol of fatty acid synthase was bound, this fluorescence ratio was proportional to the amount of pyrene incorporated into the enzyme. The proportionality constant was determined in each experiment with a standard sample for which the stoichiometry was determined spectrophotometrically.

The enzyme was labeled with *N*-pyrenylmaleimide by incubating 110 μM *N*-pyrenylmaleimide (introduced by addition of a concentrated *N*,*N*-dimethylformamide solution) with 2 μM enzyme in 0.1 M potassium phosphate, pH 7.0, and 1 mM EDTA for 15 min at 25 °C.

The PBA-enzyme, either in 0.1 M potassium phosphate, pH 7.0, or 6 M guanidine hydrochloride (Gdn·HCl)–25 mM potassium phosphate, pH 7.0, was reacted with freshly neutralized hydroxylamine by diluting a 4 M NH_2OH solution to give a final concentration of either 0.1 or 0.5 M. The reactions were quenched by diluting a 10-μL aliquot into 90 μL of either 0.1 M potassium phosphate (pH 7.0) or 6 M Gdn·HCl and immediately passing the solution through a 1-mL Sephadex G-50 centrifuge column to remove the free PBA and hydroxylamine.

Time-Resolved Fluorescence. An ORTEC 9200 nanosecond fluorescence spectrophotometer described previously (Matsumoto & Hammes, 1975) was used for the dynamic fluorescence measurements. The pyrene-labeled enzyme was excited with light from a spark gap flash lamp that was vertically polarized and filtered through a 340-nm Ditric band-

pass filter. The fluorescence emission was passed through a polarizer oriented either vertically or horizontally and a 390-nm Ditric band-pass filter before entering the photomultiplier. A background and scattering correction was made by treating a second enzyme sample in an identical manner, except that the addition of PBA-CoA was omitted. The time-resolved fluorescence, $F(t)$, and anisotropy, $A(t)$, were calculated as

$$F(t) = V(t) - V_s(t) + 2[H(t) - H_s(t)]$$

$$A(t) = [V(t) - V_s(t) - [H(t) - H_s(t)]]/F(t)$$

where V and H are the spectra collected with the emission polarizer oriented vertically and horizontally, respectively, and the subscript s indicates the spectra collected with the unlabeled sample [cf. Waskiewicz & Hammes (1982) for a more detailed description of the experimental procedure]. The data were fit to the equations

$$F(t) = F_1 e^{-t/\tau_1} + F_2 e^{-t/\tau_2} \quad (2)$$

$$A(t) = A_0 e^{-t/\phi} \quad (3)$$

where the τ_i's are fluorescence lifetimes, ϕ is a rotational correlation time, and F_1, F_2, and A_0 are amplitude parameters; $F_1 + F_2$ is arbitrarily set equal to 1. The data were fit by a weighted nonlinear least-squares analysis in which the lamp pulse was convoluted by a numerical procedure (Munro et al., 1979). The lamp pulse was obtained from the light scattering of a 0.1% Ludox solution.

Steady-State Fluorescence Measurements. Steady-state fluorescence measurements were made on a Hitachi Perkin-Elmer MPF-44B fluorometer. The pyrene fluorescence was excited at 345 nm and the emission observed at 390 nm. Polarization measurements were carried out as previously described (Waskiewicz & Hammes, 1982).

The quenching of the steady-state fluorescence by KI, acrylamide, or CsCl was monitored by adding small aliquots of quencher to the sample solution and observing the decrease in fluorescence, with appropriate corrections for dilution. Alternatively a small fixed amount of PBA-labeled enzyme was diluted into buffer solutions containing different concentrations of quencher and the fluorescence determined. Both methods gave comparable results. The quenching data were analyzed by Stern–Volmer plots where either F_0/F or τ_0/τ is plotted vs. the concentration of the quenching species. Here F and τ are the steady-state fluorescence and the lifetimes, respectively, and the subscript 0 denotes the absence of quencher. The second-order rate constant, k_q, for the interaction between PBA and the quenching species was obtained from the slope of the line (determined by least-squares analysis) divided by τ_0 [$F_0/F = \tau_0/\tau = 1 + k_q\tau_0$(quencher); Lehrer, 1971].

Results

Binding of PBA-CoA to Enzyme. The PBA portion of PBA-CoA is covalently attached to fatty acid synthase since it is found in the eluate of a Sephadex G-50 centrifuge column, even after the enzyme has been denatured in 6 M Gdn·HCl–25 mM potassium phosphate, pH 7.0, prior to elution through the column. Figure 1 shows the incorporation of pyrenylbutyric acid into the enzyme as a function of the PBA-CoA concentration after reaction times of 45, 75, and 150 min in 0.1 M potassium phosphate, pH 7.0, and 1 mM EDTA at 35 °C. The data at a given reaction time can be quantitatively described as a hyperbolic binding isotherm plus a straight line proportional to the concentration of PBA-CoA. The hyperbolic

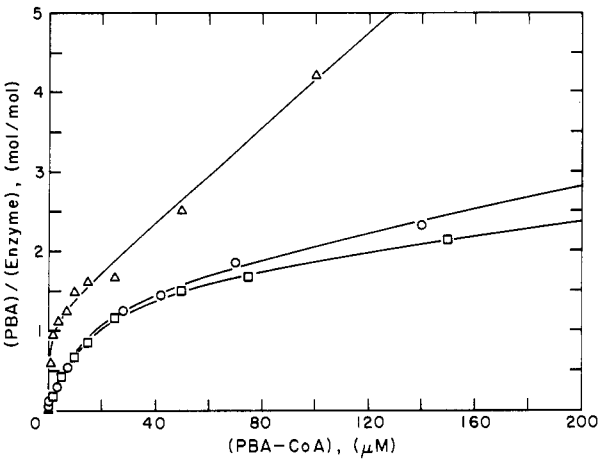

FIGURE 1: Plots of the amount of PBA covalently incorporated into fatty acid synthase, (PBA)/(enzyme), (mol/mol), vs. the concentration of PBA-CoA. The reaction was carried out with 0.25 μM enzyme in 0.1 M potassium phosphate, pH 7.0, and 1 mM EDTA, at 35 °C for 45 (□), 75 (○), and 150 (△) min. The lines represent the best fit to (PBA)/(enzyme) = n(PBA-CoA)/[(PBA-CoA) + K_1] + K_2 (PBA-CoA) where n, K_1, and K_2 are constants.

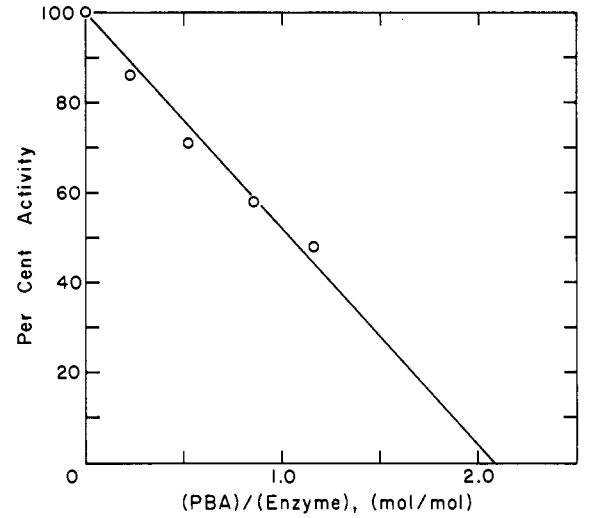

FIGURE 2: Plot of the percent activity vs. the amount of PBA covalently incorporated into fatty acid synthase, (PBA)/(enzyme), (mol/mol). 3.0 μM enzyme and 25 μM PBA-CoA were incubated in 0.1 M potassium phosphate, pH 7.0, and 1.0 mM EDTA at 35 °C. The line was obtained by a linear regression analysis with the intercept fixed at 100%.

isotherm approaches a limiting stoichiometry of ~2 PBA/enzyme. The specificity of labeling was assessed by determining the dependence of the overall enzyme activity under standard assay conditions as a function of the extent of labeling. The results are summarized in Figure 2 and also suggest that two specific binding sites must be modified for complete inactivation. The initial rate of inhibition of activity, v, as a function of the concentration of PBA-CoA is shown in Figure 3. These data were fit to the equation

$$v = \frac{k}{1 + K/(\text{PBA-CoA})} \quad (4)$$

by a nonlinear least-squares analysis. The curve in Figure 3 has been calculated with eq 4 and the best-fit parameters $k = 4.5 \times 10^{-4}$ s^{-1} and $K = 6.1$ μM. Some representative time courses of inactivation are presented in the insert of Figure 3. Clearly at low concentrations of PBA-CoA, an equilibrium between modified and unmodified enzyme is reached. Both acetyl-CoA and malonyl-CoA were found to be competitive

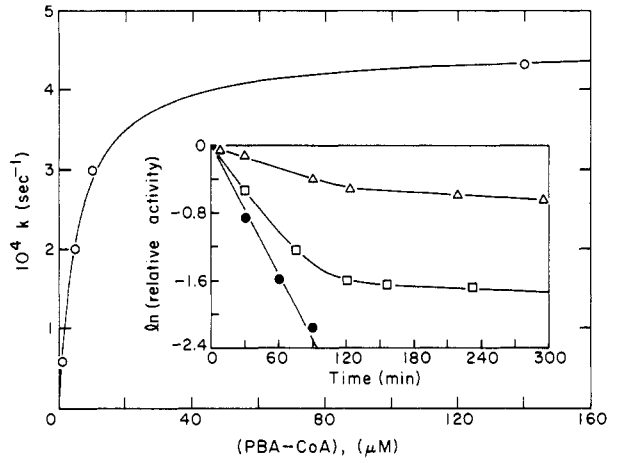

FIGURE 3: Plots of the rate constant for the initial rate of loss of fatty acid synthase activity, k, vs. the concentration of PBA-CoA in 0.1 M potassium phosphate, pH 7.0, and 1.0 mM EDTA at 35 °C. The enzyme concentration was 3.0 μM. The insert shows typical plots of the logarithm of relative activity vs. time with PBA-CoA concentrations of 1.0 (△), 10 (□), and 140 (●) μM.

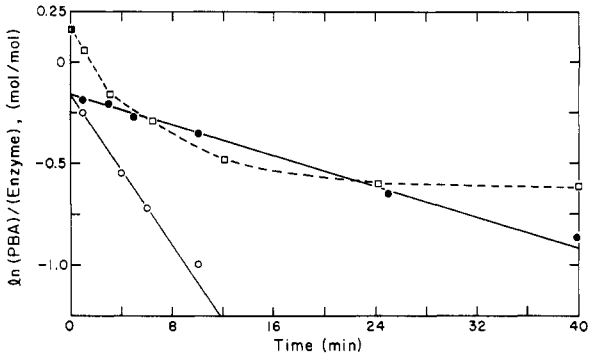

FIGURE 4: Plots of the logarithm of the amount of PBA covalently bound to enzyme, (PBA)/(enzyme), (mol/mol), vs. time. The PBA-modified enzyme was treated with 0.1 (●) and 0.5 (○, □) M NH_2OH in 0.1 M potassium phosphate, pH 7.0 (●, ○), or 6 M Gdn·HCl and 25 mM potassium phosphate, pH 7.0 (□) at 25 °C. The solid lines were calculated by linear regression analysis excluding the initial point; the dashed line has no theoretical significance.

inhibitors of the rate of inactivation of the enzyme by PBA-CoA with inhibition constants of 4 μM and 10 μM, respectively. Since both acetyl-CoA and malonyl-CoA covalently modify the enzyme, these inhibition constants cannot be interpreted in thermodynamic terms. In addition PBA-CoA is a competitive inhibitor of malonyl-CoA in the standard assay for overall activity. The competitive inhibition constant is 5 μM. This can be regarded as a thermodynamic constant since the enzyme is exposed to PBA-CoA for a maximum of 2 min; during this time interval no significant covalent modification of the enzyme has occurred. To further demonstrate the specificity of the incorporation of PBA, the extent of acetylation of the enzyme by [^{3}H]acetyl-CoA in the presence and absence of PBA was determined. If 0.2 μM enzyme is incubated with 45 μM [^{3}H]acetyl-CoA for 30 s in 0.1 M potassium phosphate, pH 7.0, and 1 mM EDTA at 25 °C, 2.6 mol of [^{3}H]acetyl/mol of enzyme is found. If the enzyme contains 1.2 mol of PBA/mol of enzyme, only 1.8 mol of [^{3}H]acetyl/mol of enzyme is present.

Thus PBA-CoA appears to bind specifically to fatty acid synthase at the same sites as acetyl-CoA and malonyl-CoA with a dissociation constant of ~5 μM, and the incorporation of 2 PBA/enzyme is sufficient to inactivate the enzyme. Further characterization of the PBA–fatty acid synthase adduct was carried out with the modified enzyme obtained by incubation of ≤3 μM enzyme with 25 μM PBA-CoA for 45 min at 35 °C, unless otherwise indicated. The modified enzyme had a stoichiometry of 1.14–1.20 PBA/enzyme (mol/mol).

To determine if the PBA was bound to fatty acid synthase by thio ester bonds, PBA-modified enzyme was treated with neutralized hydroxylamine in 0.1 M potassium phosphate, pH 7.0, and 1 mM EDTA at 25 °C. Neutral hydroxylamine cleaves thio esters and other unstable esters (e.g., 2,4-dinitrophenyl esters), but not typical oxy ester bonds. The time courses shown in Figure 4 demonstrate that hydroxylaminolysis proceeds at two markedly different rates. A fast reaction occurs within mixing (≤100 s), and a slower reaction occurs with a pseudo-first-order rate constant of 2.8 × 10^{-4} s^{-1} and 1.4 × 10^{-3} s^{-1} at hydroxylamine concentrations of 0.1 and 0.5 M, respectively. After 50 min, over 95% of the PBA is removed in the presence of 0.5 M hydroxylamine. If the PBA-modified enzyme is denatured with 6 M Gdn·HCl in 25 mM potassium phosphate, pH 7.0, and treated with 0.5 M hydroxylamine as above, 55% of the PBA is removed with a pseudo-first-order rate constant of 1.5 × 10^{-3} s^{-1}, and the remaining 45% is not removed after 90 min (see Figure 4).

The results described above suggest two classes of PBA binding sites exist on the native enzyme which differ in their ease of hydroxylaminolysis; in the denatured enzyme two classes of sites also exist, one being sensitive to treatment with hydroxylamine and the other insensitive. The correspondence between the results obtained with the native and denatured enzyme was explored by removing the rapidly reacting class of PBA binding sites on the native enzyme through reaction of the native PBA-modified enzyme with 0.1 M neutralized hydroxylamine for 10 min in 0.1 M potassium phosphate, pH 7.0. This resulted in removal of 52% of the bound PBA. The enzyme was then denatured with 6 M Gdn-HCl in 25 mM potassium phosphate, pH 7.0, and treated with 0.5 M neutralized hydroxylamine for 15 min. The treatment removed 87% of the remaining bound PBA. Since the PBA-enzyme after denaturation has fewer oxy ester bonds when the PBA-native enzyme is exposed to hydroxylamine prior to denaturation, the rapidly removed PBA in the native enzyme appears to be bound as an unstable oxy ester and the more slowly removed PBA as a thio ester.

Dynamic Fluorescence Measurements. The fluorescence lifetimes of PBA and of derivatives of PBA are summarized in Table I. A typical fluorescence decay curve of PBA-enzyme is shown in Figure 5. While the fluorescence decay of PBA can be represented by a single exponential decay, some of its derivatives required two exponential decays. In the case of PBA-CoA, apparently two conformations exist, one of which has its fluorescence greatly quenched. Surprisingly the PBA-enzyme has two lifetimes in Gdn·HCl; the short lifetime component vanishes after treatment with 0.5 M neutralized hydroxylamine. Conversely, the relative amplitude of the short lifetime component is increased if the native PBA-enzyme complex is treated with 0.1 M neutralized NH_2OH for 10 min and then denatured by passing the reaction mixture through a 3-mL Sephadex G-50 centrifuge column equilibrated with 6 M Gdn·HCl and 25 mM potassium phosphate, pH 7.0 (Table I, second to last entry).

The time-resolved anisotropy of PBA-enzyme also was measured, and a typical decay curve is shown in Figure 6. The decay parameters under a variety of conditions are summarized in Table II. Because the rotational correlation times are long relative to the fluorescence lifetime and the total anisotropy is not large, the uncertainty in the correlation times is about

Table I: Fluorescent Lifetimes of PBA at 25 °C

fluorescent species	conditions	F_1	τ_1 (ns)	F_2	τ_2 (ns)
PBA	0.1 M potassium phosphate, pH 7.0	1.0	120		
PBA	6 M Gdn·HCl, 25 mM potassium phosphate, pH 7.0	1.0	109		
PBA	cyclohexane	1.0	20.2		
PBA-CoA	0.1 M potassium phosphate, pH 7.0	0.983	22.7	0.017	126
PBA-enzyme	0.1 M potassium phosphate, pH 7.0	1.0	141		
PBA-enzyme	6 M Gdn·HCl, 25 mM potassium phosphate, pH 7.0	0.75	30.0	0.25	121
PBA-enzyme	as above after 15-min treatment with 0.5 M NH_2OH	1.0	106		
PBA-enzyme	after 10-min treatment with 0.1 M NH_2OH in 0.1 M potassium phosphate, pH 7.0, followed by denaturation in 6 M Gdn·HCl, 25 mM potassium phosphate, pH 7.0	0.86	38.6	0.14	92.6
PBA-enzyme	62 mM KI, 0.1 M potassium phosphate, pH 7.0	0.78	44	0.22	139

Table II: Rotational Correlation Times for Derivatives of Fatty Acid Synthase[a]

fluorescent species	A_0	ϕ (ns)
1.18 PBA/enzyme	0.10	610
1.14 PBA/enzyme (10% glycerol)	0.091	1200
1.14 PBA/enzyme (20% glycerol)	0.096	1700
3.8 *N*-pyrenylmaleimide/enzyme	0.19	605
1.24 PBA/enzyme (monomer)	0.095	540 (285)[b]

[a] Conditions: 0.1 M potassium phosphate, pH 7.0, 25 °C, except for the last entry which is in 5 mM Tris–glycine, pH 8.3 at 4 °C. [b] Corrected to 25 °C assuming $\phi \propto \eta/T$ where η is the viscosity and T is the absolute temperature.

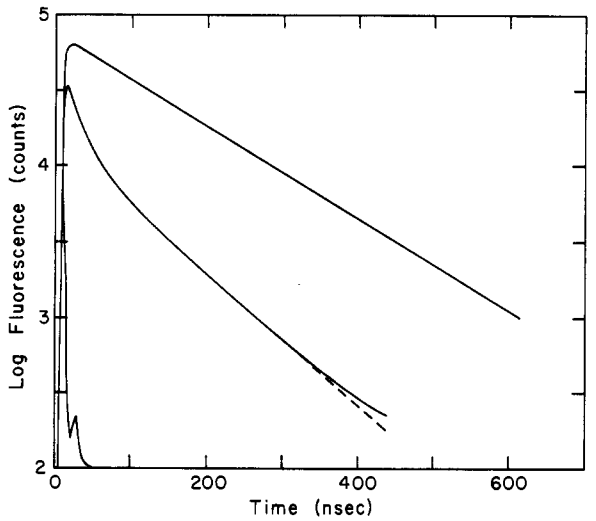

FIGURE 5: Plots of the logarithm of the relative fluorescence of PBA-labeled fatty acid synthase (1.2 mol of PBA/mol of enzyme) vs. time in 0.1 M potassium phosphate, pH 7.0, at 25 °C (upper solid line) and in the same medium plus 70 mM KI (middle solid line). The lowest solid line is the lamp pulse augmented by 10^2 counts. The upper lines were fit to eq 2; the calculated lines using the best-fit parameters correspond to the lines shown except for the deviation indicated by the dashed line. The best fit fluorescence lifetime was 147 ns for the upper line and 44 and 118 ns with relative amplitudes of 0.79 and 0.21 for the middle line.

±10%. Increasing the viscosity with glycerol dramatically increases ϕ, with no significant changes in A_0. (The viscosity is increased by factors of 1.31 and 1.76 in 10 and 20% glycerol, respectively.) This indicates that the overall rotation of the molecule is being measured. When the enzyme is dissociated into monomers (last entry of Table II), the rotational correlation time, corrected to 25 °C, is halved as anticipated. Essentially the same rotational correlation time is obtained when the enzyme is randomly labeled with *N*-pyrenylmaleimide, although the anisotropy at zero time, A_0, is greatly increased. The steady-state anisotropy, A_s, calculated from the relationship

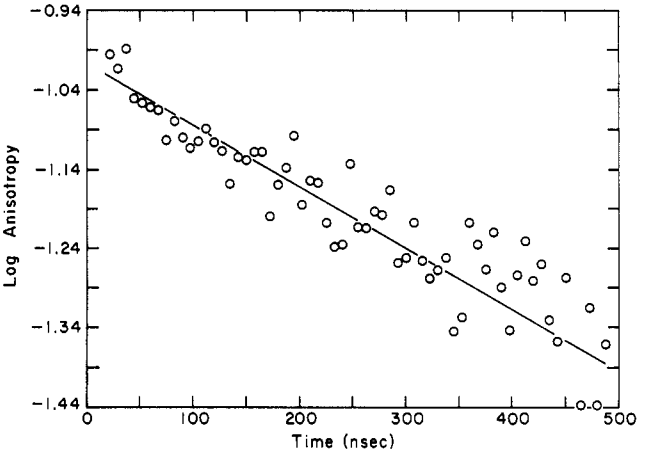

FIGURE 6: Plot of the anisotropy of fatty acid synthase covalently labeled with PBA (1.2 mol of PBA/mol of enzyme) vs. time in 0.1 M potassium phosphate, pH 7.0, and 1.0 mM EDTA at 25 °C. The line is the best fit according to eq 3 with $A_0 = 0.10$ and $\phi = 610$ ns.

$$A_s = F\tau A_0\phi/(\phi + \tau)$$

is 0.081 for the first entry in Table II, which is in good agreement with the measured value of 0.083.

Quenching of PBA-Enzyme Fluorescence. The quenching of the steady-state fluorescence of PBA-enzyme by acrylamide, KI, and CsCl was studied in 0.1 M potassium phosphate, pH 7.0, at 25 °C. These three reagents quenched free PBA with second-order rate constants of 3.1×10^9 M^{-1} s^{-1}, 1.4×10^9 M^{-1} s^{-1}, and 5.3×10^7 M^{-1} s^{-1}, respectively. The quenching of PBA-enzyme by these reagents is shown in Figure 7 as a plot of the ratio of the fluorescence in the absence of quencher to the fluorescence in the presence of quencher, F_0/F, vs. the quencher concentration. The CsCl does not quench the fluorescence appreciably (up to 625 mM), and the second-order rate constant calculated for acrylamide is 2.2×10^7 M^{-1} s^{-1}. The quenching by KI is nonlinear, but the initial slope gives a second-order rate constant of 3.0×10^8 M^{-1} s^{-1}. These results were not appreciably altered when 90% of the PBA was removed by treatment of the enzyme for 15 min with 0.5 M

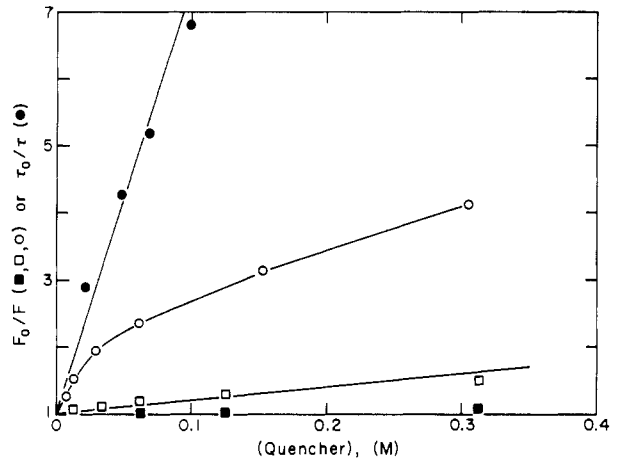

FIGURE 7: Plots of the ratio of the fluorescence in the absence of quencher (F_0) to the fluorescence (F) or the fluorescence lifetime in the absence of quencher (τ_0) to the fluorescence lifetime (τ) vs. the concentration of KI (●, ○), acrylamide (□), and CsCl (■). The PBA-modified enzyme (1.18 mol of PBA/mol of enzyme) was mixed with the quencher in 0.1 M potassium phosphate, pH 7.0, and 1.0 mM EDTA at 25 °C.

neutralized hydroxylamine. The quenching by KI was still nonlinear, and the initial slope gave a second-order rate constant of 1.9×10^8 M^{-1} s^{-1}. The quenching rate constant for acrylamide was 6.0×10^6 M^{-1} s^{-1}. When the enzyme is dissociated into monomers, the quenching plots are linear with quenching rate constants of 4.4×10^7 M^{-1} s^{-1} and 6.0×10^6 M^{-1} s^{-1} for KI and acrylamide, respectively, at 4 °C.

The fluorescence quenching was further characterized by determining the fluorescence lifetimes in the presence of KI and acrylamide. In both cases, two fluorescence lifetimes are observed (cf. the last entry in Table I and Figure 5). The longer lifetime is independent of the concentration of quencher. The variation of the shorter lifetime is included in Figure 7 where τ_0/τ is plotted vs. the concentration of quencher. The quenching rate constants derived from these data are 3.5×10^8 M^{-1} s^{-1} and 4.3×10^7 M^{-1} s^{-1} for KI and acrylamide, respectively. If the enzyme is treated with either 0.1 or 0.5 M hydroxylamine for 15 min, and the fluorescence lifetimes are determined in the presence of 70 mM KI, two exponential decays were observed with similar lifetimes and amplitudes to those observed before treatment with hydroxylamine. As a final control the quenching of fluorescence was examined after the enzyme was denatured in 6 M Gdn·HCl and 25 mM potassium phosphate, pH 7.0. Plots of F_0/F vs. the concentration of the quenching species are linear. The quenching rate constants calculated are 2.8×10^8 M^{-1} s^{-1} and 4.4×10^8 M^{-1} s^{-1} for KI and acrylamide, respectively.

Discussion

The transfer of acyl groups from acyl-CoA's to fatty acid synthase has been studied with radioactive acetyl-CoA, malonyl-CoA (Jacob et al., 1968; Chesterton et al., 1968; Phillips et al., 1970a,b), and fluorescent acyl groups (Cardon & Hammes, 1982, 1983). The work with acetyl- and malonyl-CoA identified a phosphopantetheine sulfhydryl, a cysteine sulfhydryl, and a serine or threonine hydroxyl as specific sites for acylation. The acyl groups were suggested to be originally transferred to a hydroxyl group and then intramolecularly to the sulfhydryl groups. This study is consistent with this mechanism and provides evidence that the oxy ester is destabilized in the native enzyme so that it is rapidly cleaved by neutralized hydroxylamine, and that intramolecular transfer of acyl groups occurs. Since all of the PBA is removed from the native PBA-modified enzyme by hydroxylamine, the portion found bound as an oxygen ester in the denatured PBA-modified enzyme (hydroxylamine insensitive) must be removed. Similar behavior has been observed with the acetylated enzyme (Cognet & Hammes, 1983). Furthermore, the analysis of the time-resolved fluorescence of the PBA-modified enzyme indicates that the PBA oxygen ester has a longer fluorescence lifetime than the PBA thio ester and is cleaved more rapidly by hydroxylamine. This indicates that the two esters are in different local environments and that the oxygen ester is less stable kinetically than the thio ester in the native enzyme.

Dynamic fluorescence quenching has been used to determine the solvent accessibility and fluorescent properties of different fluorophores in proteins (cf. Eftink & Ghiron, 1981; Ross et al., 1981). Both KI and acrylamide are good quenchers of PBA itself. The hyperbolic Stern–Volmer plot observed for the quenching of enzyme-bound PBA by KI indicates the presence of at least two classes of PBA with different accessibilities to KI. These classes are distinguished when the time-resolved fluorescence is determined in the presence of KI. Two distinct fluorescent lifetimes are observed: the quenching of one is characterized by a rate constant that is a factor of 4 smaller than that found for free PBA, while the other is not dynamically quenched by 100 mM KI. This indicates one class of PBA is partially shielded from solvent, while the other class is not accessible at all, e.g., in a hydrophobic pocket. Acrylamide is a much poorer quencher of enzyme-bound PBA than KI, although it is a more effective quencher of free PBA. The 140-fold decrease in the quenching rate constant indicates both PBA sites on the enzyme are not easily accessible to solvent. Perhaps the quenching by I^- is due to an enzyme-bound I^- in the vicinity of a PBA binding site. This interpretation is consistent with the fact that the quenching rate constant decreases when the enzyme is dissociated into monomers. The Stern–Volmer plot for the monomeric enzyme is linear. The native structure clearly is required for the two classes of PBA sites to be differentiated and for the specific quenching of one of the classes by I^-. As expected, dynamic quenching is very rapid when the enzyme is denatured.

Although the time-resolved fluorescence of the denatured PBA-modified enzyme during hydroxyaminolysis clearly differentiates the reactivity of the two classes of PBA binding sites to hydroxylamine, this difference was not reflected in the time-resolved fluorescence of the native PBA-modified enzyme in the presence of KI. The quenching of fluorescence by KI indicates two classes of sites, with respect to accessibility to KI, exist during hydroxaminolysis. This can be most readily explained by postulating the occurrence of an intramolecular transfer of PBA following hydroxaminolysis in the native enzyme that is fast relative to the time required for measurement of the time-resolved fluorescence (~1 h). Such a transfer apparently cannot occur in the denatured enzyme. Thus these results demonstrate that intramolecular transfer of PBA between oxygen and sulfur sites occurs and requires the native enzyme structure.

Fatty acid synthase is a dimer of apparently identical subunits (Poulose et al., 1980; Stoops & Wakil, 1981). Therefore, the presence of two different types of binding sites suggests a minimum binding stoichiometry of 4 PBA/enzyme (mol/mol). Since the incorporation of two PBA moieties completely inactivates the enzyme, the specific binding of 1 PBA/polypeptide apparently prevents the addition of a second PBA. The simplest interpretation of this observation is that binding at the sulfhydryl and oxygen sites is essentially exclusive. This

steric hindrance was not observed with NBD-CoA. Furthermore, a very stable oxy ester (hydroxylamine insensitive) was found after overnight incubation of the enzyme with NBD-CoA that specifically inactivated the enzyme (Cardon & Hammes, 1983).

The fluorescence anisotropy of enzyme-bound PBA is relatively small, and it decays with a rotational correlation time characteristic of the entire molecule. This suggests that a rapid ($\phi < 10^{-9}$ s), but restricted, rotation of the probe may occur on the enzyme surface (cf. Wahl, 1975). The lipoic acid of the α-ketoglutarate dehydrogenase complex has been found to move on the enzyme surface with a time constant of a few hundred nanoseconds or less (Waskiewicz & Hammes, 1982). The rotational correlation time observed for the native enzyme is about a factor of 2 greater than that anticipated for a spherical molecule (cf. Yguerabide et al., 1970). This suggests the structure of the native enzyme is quite asymmetric. The rotational correlation time of the monomeric enzyme is halved, as expected, but the asymmetric structure is apparently preserved. Unfortunately the anisotropy of the denatured PBA-enzyme was too low to permit dynamic anisotropy measurements.

In summary, this work has shown that two classes of PBA binding sites exist on the enzyme that are essential for catalytic activity. One appears to involve formation of a relatively unstable oxy ester and the other formation of a thio ester. These sites differ in their accessibility to solvent. Dynamic anisotropy measurements suggest a rapid restricted motion of the PBA on the enzyme and an asymmetric structure of the enzyme.

Registry No. PBA-CoA, 81923-89-9; PBA, 3443-45-6; CoA, 85-61-0; iodide, 20461-54-5; acrylamide, 79-06-1; pyrene, 129-00-0; fatty acid synthase, 9045-77-6.

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