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INACTIVATION OF ENOYL-CoA REDUCTASE IN PIGEON LIVER FATTY ACID SYNTHETASE BY PYRIDOXAL 5′-PHOSPHATE: EVIDENCE FOR THE PRESENCE OF ONE LYSINE RESIDUE AT THE ACTIVE SITE

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Pigeon liver fatty acid synthetase (FAS) was rapidly inactivated by pyridoxal 5′-phosphate (PLP). Assays of the partial activities of the PLP-treated synthetase showed that only the enoyl-CoA reductase was decreased significantly. The inactivation of both the overall activity and enoyl-CoA reductase activity of FAS by PLP could be reversed by dialysis or dilution but not by reduction with sodium borohydride. Malonyl-CoA and acetyl-CoA did not protect the enzyme, whereas NADPH provided 68% protection against PLP-inactivation indicating that PLP modified lysine residues present at or near the co-enzyme binding site. PLP-treated enzyme after reduction with sodium borohydride exhibited fluorescence with a maximum at 397 nm (irradiation at 325 nm). Stoichiometric analysis showed that modification of four lysine residues per enzyme molecule resulted in complete inactivation of the overall and enoyl-CoA reductase activities of FAS. NADPH prevented the inactivation by protecting two of these lysine residues from modification, suggesting the presence of two essential lysine residues per enzyme molecule. These results are consistent with the hypothesis that each subunit of the enzyme contains an enoyl-CoA reductase domain in which a lysine residue, at or near the active site, interacts with NADPH.

Keywords: Fatty acid synthetase; Enoyl-CoA reductase; Pyridoxal 5′-phosphate; Active site; Chemical modification

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INTRODUCTION

FAS from pigeon liver, like those from other vertebrates, is a dimeric enzyme consisting of two identical subunits which contain seven catalytic activities required for fatty acid synthesis.[1-3] Extensive studies have been performed in recent years on the active site structure of FAS from chicken liver,[4] goose uropygial gland[5] and rat lactating mammary gland.[6] Little is known yet about the active site structure of pigeon liver FAS. It is known that the reaction of PLP with an ε-amino group of lysine results in the formation of a protonated Schiff base which absorbs[7,8] in the region of 410–435 nm. It has been reported earlier that PLP inactivates FAS in a reversible manner by modifying a lysine residue at the enoyl-CoA reductase domain of the enzyme.[9] Here additional evidence is provided that PLP inhibits fatty acid synthetase by specifically modifying one lysine residue per subunit of enzyme present at or near the NADPH binding site of enoyl-CoA reductase.

MATERIALS AND METHODS

Materials

Pyridoxal 5′-phosphate, acetyl-CoA, malonyl-CoA, NADPH, NADH, buffer components (Sigma); Sephadex G-50 (Pharmacia); dithiothreitol (Calbiochem); were purchased from the above designated sources. All other chemicals were of the highest purity grade commercially available.

Methods

Purification of Pigeon Liver FAS and Assay of Overall and Component Activities

Pigeon liver FAS was purified and assayed spectrophotometrically by the method of Muesing and Porter.[10] Homogeneity of the enzyme was examined by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) using the method of Laemmli;[11] it exhibited a single band corresponding to a molecular weight of 450 000. Assays for β-ketoacyl and enoyl reductase activities were carried out spectrophotometrically as described earlier.[12,13] FAS activity was measured by disappearance of absorbance of NADPH at 340 nm. Absorbance was recorded by a UV–vis

spectrophotometer (Shimadzu Model UV-160A) and fluorescence spectra were recorded by a luminescence spectrometer (Perkin Elmer, Model LS 50B). Both the fluorescence and the absorbance were recorded in cuvettes of 1.0 cm light path at 25°C.

Modification of FAS with Pyridoxal 5'-Phosphate

Inactivation of FAS was carried out at 25°C in 0.2 M potassium phosphate buffer (pH 7) containing 1 mM EDTA and 1 mM dithiothreitol at a protein concentration of 1.68 mg/ml with various concentrations (0.25–5 mM) of PLP. The incubation mixtures were protected from light with aluminium foil. At specific time intervals, aliquots of the reaction mixture were removed and assayed immediately for overall FAS activity and enoyl-CoA reductase activity. Controls, in duplicate, without PLP were run concurrently. For reversal of inactivation by PLP the mixture was diluted 80-fold, and aliquots were withdrawn at different time intervals and the activity of the enzyme determined. Protection experiments were performed by adding the protecting agent and incubating for 15 min prior to the addition of PLP.

Reduction of FAS–PLP Complex with Sodium Borohydride

FAS (2 mg/ml) was incubated with 1 mM PLP in 0.2 M potassium phosphate buffer (pH 7) containing 1 mM EDTA and 1 mM DTT at 25°C for 15 min. The reduction of the Schiff base was carried out by adding a drop of octyl alcohol followed by the addition of sodium borohydride solution (final concentration 10 mM). The reaction mixture was kept at 0°C for 15 min. The excess PLP was removed using a G-50 Sephadex column according to the method of Penefsky.[14]

Stoichiometry of Reaction of PLP with FAS

FAS (2 mg/ml) was incubated with 1 mM PLP for 15 min at 25°C in the presence and absence of 5 mM NADPH and subsequently reduced with sodium borohydride. After reduction the reaction mixture was passed through a G-50 Sephadex column[14] to remove the excess reagent. The protein concentration was determined by the method of Lowry *et al.*[15] The number of moles of PLP incorporated was determined by taking the ratio of the concentration of phosphopyridoxyllysine and enzyme using an extinction coefficient of 9700 $M^{-1} cm^{-1}$ at 325 nm.[16] The molecular weight of FAS was taken as 450 000 Da.

RESULTS AND DISCUSSION

The reaction with PLP resulted in a concentration and time-dependent loss of overall FAS and enoyl reductase activities. Both the activities rapidly decreased up to about 8 min and subsequently the rate of inactivation slowed down; equilibrium was reached after 10 min. The time course and concentration dependence of inactivation as well as the extent of inactivation were quite similar for both enoyl-CoA reductase and overall activities of FAS. Earlier studies had shown that enoyl-CoA reductase activity of FAS from other animal sources such as chicken liver, goose uropygial gland and rat mammary glands were also specifically inhibited by PLP.[4–6] In contrast, modification of yeast FAS by PLP did not inhibit enoyl-CoA reductase but led to the inactivation of β-ketoacyl reductase.[17] Previous studies using PLP as a modifying reagent on FAS obtained from other sources and on other enzymes have shown that the formation of a non-covalent complex between the protein and PLP precedes the covalent reaction, both being reversible steps.[18] To establish whether this mechanism is followed when PLP reacts with pigeon liver FAS, the data were subjected to kinetic and equilibrium analyses as described below.[18] In this model, the residual activity is due to the equilibrium between catalytically inactive Schiff base and a noncovalent enzyme–PLP complex, which dissociates readily to give the active enzyme in the reaction mixture according to the following equation of Chen and Engel (Eq. (1)):

$$E + \text{PLP} \underset{k_2}{\overset{k_1}{\longleftrightarrow}} E.\text{PLP} \underset{k_4}{\overset{k_3}{\longleftrightarrow}} E\text{–PLP} \qquad (1)$$

where $E.\text{PLP}$ is the noncovalent complex and E–PLP is the Schiff base between the enzyme and the reactant. The inactivation of overall and enoyl reductase activity followed pseudo first-order kinetics as shown by a plot of log (residual activity) *vs* time (Figure 1). A plot of the observed pseudo first-order rate constants against the concentrations of PLP gave a hyperbola, and a double reciprocal plot of pseudo first-order rate constants and PLP concentrations yielded a straight line which did not pass through the origin (Figure 1, insets (A) and (B)). These results are consistent with the concept that a kinetically significant enzyme–PLP complex is formed prior to the inactivation. The values for the rate constant ($k_3 = 0.59\ \text{min}^{-1}$) and dissociation constant ($k_1 = k_2/k_1 = 0.59\ \text{mM}^{-1}$) obtained for both overall FAS and enoyl reductase activity suggested that the inactivation of FAS is due to modification of lysine residues of the enoyl-CoA reductase domain. It was

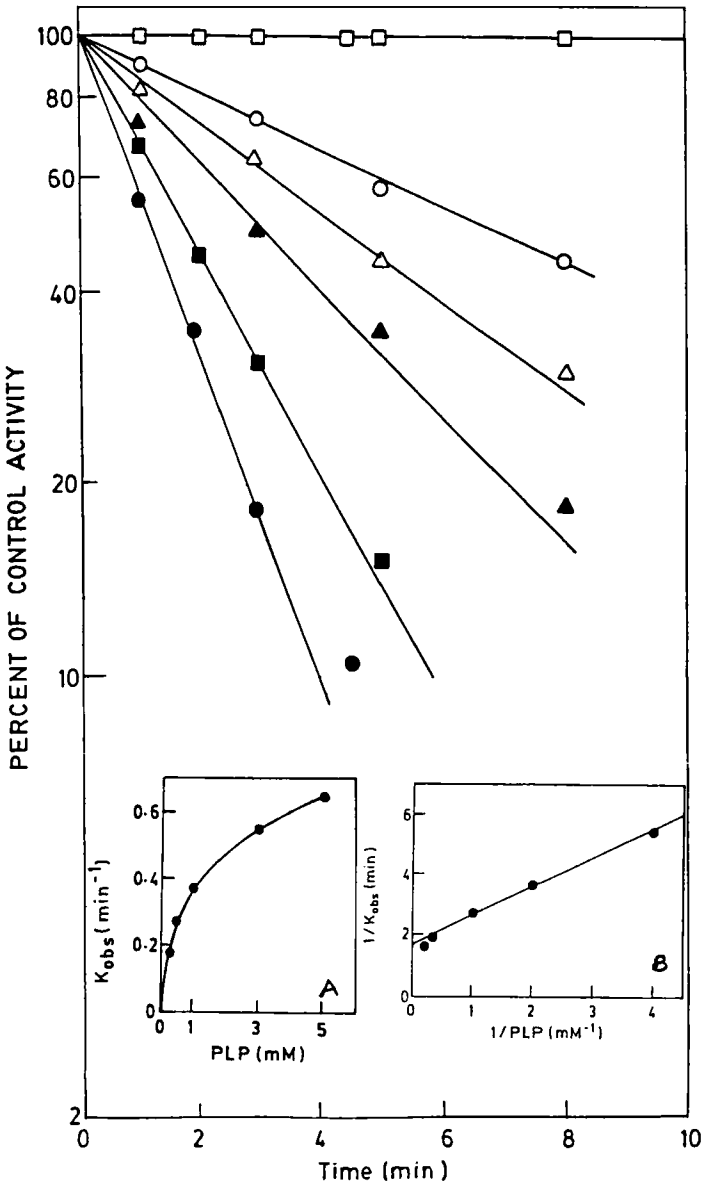

FIGURE 1 Kinetics of inactivation of overall FAS activity by PLP. The enzyme (1.68 mg/ml) was preincubated in 0.2 M potassium phosphate buffer (pH 7.0) containing 1 mM EDTA and 1 mM DTT with 0 (□), 0.25 (○), 0.5 (△), 1 (▲), 3 (■), and 5 mM (●) PLP at 25°C. Aliquots were withdrawn at the indicated time intervals and assayed for the residual activity as described in the "Experimental Procedures." Inset (A): a plot of observed pseudo first-order rate constant *vs* initial PLP concentrations, from which a second-order rate constant was obtained. Inset (B): Double-reciprocal plot of the pseudo first-order rate constants for inactivation of FAS against PLP concentrations.

found that the treatment of enzyme with apparently saturating concentrations of PLP could not produce complete inactivation. A small amount of overall activity and enoyl-CoA reductase activity was always left over as previously observed in the reaction of other enzymes with PLP.[19–21] To determine whether this residual activity was due to unreacted native enzyme or partially active modified enzyme, the data were subjected to equilibrium analysis as described by Chen and Engel.[18] According to a proposed reaction scheme (Eq. (1)), the total enzyme concentration (e) is the sum of the concentrations of free enzyme and the enzyme complexes, as described by Chen and Engel[22] (Eq. (2)),

$$e = E\left\{1 + \frac{k_1}{k_2}\left(\frac{1+k_3}{k_4}\right)[\text{PLP}]\right\} \tag{2}$$

and the fractional residual activity can be given by

$$R = \frac{1 + (k_1/k_2)[\text{PLP}]}{1 + (k_1/k_2)\cdot[\text{PLP}]\cdot(1 + k_3/k_4)}. \tag{3}$$

Assuming that the covalent complex is totally inactive and the noncovalent complex dissociates rapidly on dilution in the assay mixture, a rearrangement of Eq. (3) gives

$$1/[\text{PLP}] = \frac{k_1 k_3}{k_2 k_4} 1/(1-R) - (1 + k_3/k_4)k_1/k_2, \tag{4}$$

where R is the fractional residual activity. Thus, a plot of $1/[\text{PLP}]$ versus $1/(1-R)$ should be linear with an intercept of $-(1+k_3/k_4)(k_1/k_2)$ on the $1/[\text{PLP}]$ ordinate and a slope of (k_1k_3/k_2k_4).

The abscissa intercept represents $1+(k_4/k_3)$, the reciprocal of the maximum inactivation seen with an infinite concentration of PLP. The plot of $1/\text{PLP}$ versus $1/(1-R)$ yielded a straight line (Figure 2). From the abscissa intercept of 1.057 it was found that 5% of the FAS activity remained at the saturating concentration of PLP. Nonlinear regression analysis of Eq. (3) gave k_1 ($0.955\,\text{min}^{-1}$), k_2 ($1.31\,\text{min}^{-1}$), k_4 ($0.058\,\text{min}^{-1}$) and another estimate of k_1 (1.37 mM). The slight discrepancy observed in k_1 from kinetic and equilibrium analyses may be due to the inherent differences in these two approaches. The value of $k_2 = (k_3/k_4)$ was calculated to be 10.12 mM, showing thereby that the equilibrium lies far towards Schiff's base formation. The values obtained were almost identical for overall and enoyl-CoA reductase activity.

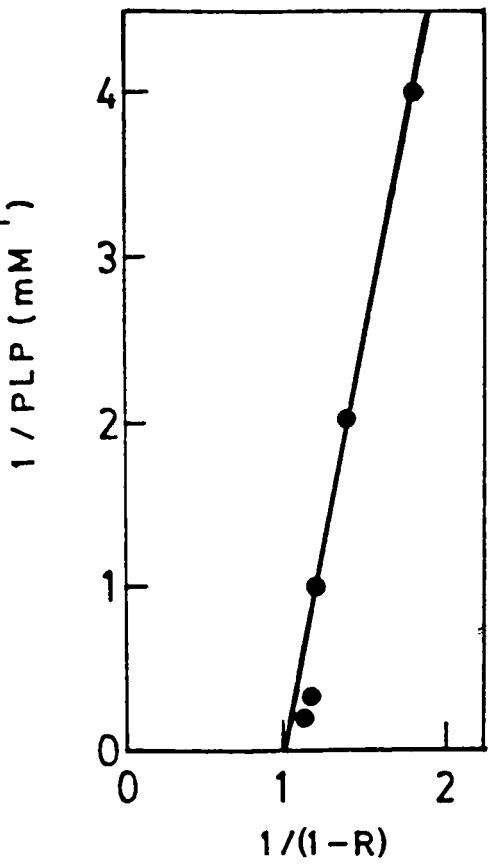

FIGURE 2 A double-reciprocal plot of fractional inactivation of overall FAS activity against PLP concentrations. *R*, fractional residual activity.

These results demonstrated that the residual activity is due to the breakdown of a catalytically inactive Schiff base to free enzyme through an intermediate noncovalent enzyme . PLP complex.

Similar kinetic and equilibrium analysis on goose uropygial gland FAS have shown higher values for the rate constants, dissociation constant and equilibrium constant suggesting that lysine residues are more susceptible towards modification by PLP.[5]

Inhibition of both the overall activity and enoyl-CoA-reductase activity by PLP could readily be reversed by dilution. On 80-fold dilution of the reaction mixture, 95% of the initial activity was recovered in 60 min. However, reduction with sodium borohydride after PLP treatment rendered the inactivation irreversible. According to the model of Chen and Engel, the

reactivation of the PLP-modified enzyme by dilution of the modification medium should be a pseudo first-order process with a constant corresponding to k_4. To calculate this rate constant from the data given in Figure 3, a plot of $\ln[(A_e-A_0)/(A_e-A_t)]$ *vs* time was obtained where A_e and A_0 are the activities at the maximal level of reactivation and at zero-time following dilution, and A_t is the activity at any time t. A straight line was obtained which gave the value of k_4 ($0.065\,min^{-1}$) (Figure 4). The calculation of the equilibrium constant k_3/k_4 using the value of k_3 obtained from kinetic analysis yielded a value of 9 mM. All these values are in agreement with those derived from equilibrium analysis. This value of k_4 can be combined with k_3 (obtained from kinetic analysis) to predict the residual activity remaining at the saturating concentration of PLP. It was calculated to be 9% which is quite close to the value obtained from the plot of 1/PLP versus $1/(1-R)$ as well as that determined from nonlinear regression analysis. These results suggested that PLP inactivated the FAS by forming a Schiff base with the ε-amino group of a lysine located at or near the active site of the enoyl-CoA reductase. In order to ascertain whether the lysine present at the active site is being modified by PLP, protection experiments were performed using substrates. Acetyl-CoA and malonyl-CoA did not provide any significant protection (Table I), while 5 mM NADPH provided 68% protection. Therefore it is clearly established that the residue modified is present at or near the NADPH binding site.

Treatment of the enzyme with PLP followed by reduction with sodium borohydride gave a fluorescence spectrum with a maximum at 397 nm after UV irridiation at 325 nm (Figure 5). This result also indicated that an ε-amino group of lysine residue is modified in the PLP reaction.

The stoichiometry of inactivation by PLP was determined by spectral analysis. Absorbance at 325 nm after reduction of the enzyme–PLP complex with sodium borohydride was taken. Since NADPH protected the enzyme from inactivation, the number of nonessential lysines modified was determined by incubating the enzyme under identical conditions in the presence of NADPH. The number of lysine residues modified in the presence of NADPH was subtracted from the corresponding number of lysines modified in the absence of NADPH to give the number of essential lysine residues modified.

Results showed that four lysine were modified per mole of FAS and only two are essential (Table II). Similar results for chicken liver FAS have been reported by Chang and Hammes[23] who found that a site whose modification by PLP led to specific inactivation of the enoyl-CoA reductase activity and a site that was not associated with enzymatic activity.

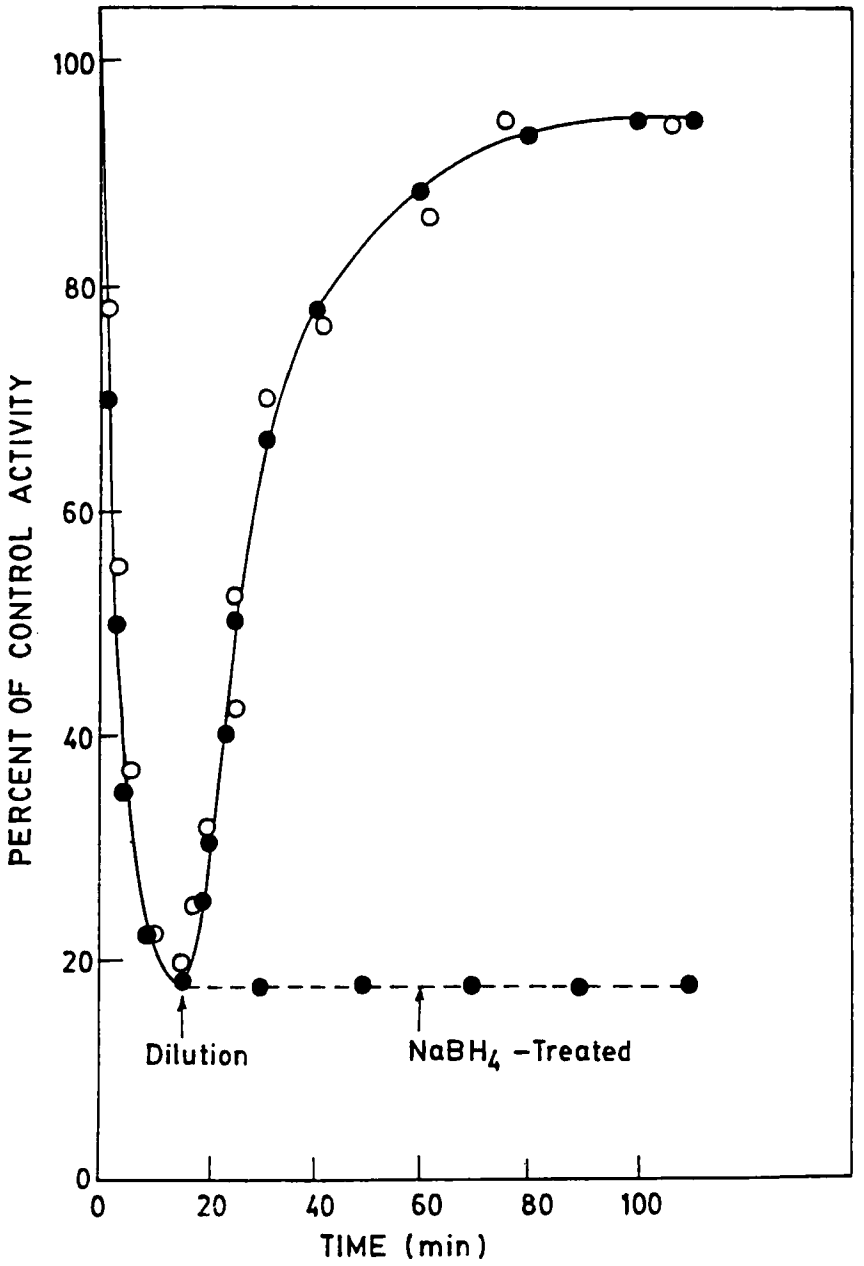

FIGURE 3 Time course of reversal of inhibition of overall FAS (●) and enoyl-CoA reductase (○) activities upon dilution of the PLP-treated FAS. The enzyme (4 mg/ml) after incubation with 1 mM PLP at 25°C for 15 min was subjected to dilution (80-fold) or sodium borohydride (10 mM) treatment prior to dilution as described in "Experimental Procedures."

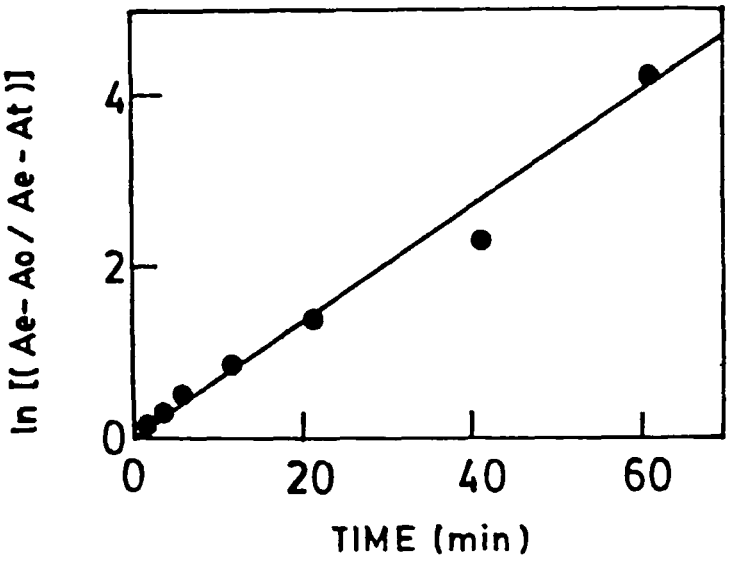

FIGURE 4 First-order plot of the reactivation of overall FAS by dilution of the PLP-treated enzyme. A_e and A_0 represent enzymic activity at the maximal level of reactivation and at zero-time respectively. A_t, activity at any time t.

TABLE I *Protection by substrates against inactivation of overall FAS and Enoyl-CoA reductase activities.* The enzyme (1.68 mg/ml, specific activity 35 nmoles palmitate per min per mg of protein) was incubated with the protecting ligands for 15 min prior to the addition of 1 mM PLP. After the incubation, overall FAS and enoyl-CoA reductase activities were measured immediately as described under "Experimental Procedures"

Protecting ligand	*Percent of activity remaining*	
	FAS	*Enoyl-CoA reductase*
None	18	20
Acetyl-CoA (1 mM)	18	—
Malonyl-CoA (1 mM)	19	—
NADH (5 mM)	19	19
NADPH (1 mM)	74	81
NADPH (5 mM)	85	95

The organisation of fatty acid synthetase varies from species to species. In most procaryotes, fatty acid synthesis is carried out by seven separable monofunctional enzymes and acyl-carrier protein.[24] However, in eucaryotes, FAS exists as a multienzyme complex. Yeast fatty acid synthetase is a complex of two different polypeptide chains whereas animal FAS, is a dimer consisting of two identical polypeptide chains having active sites for all enzyme activities. The present evidence that two lysine residues per mole

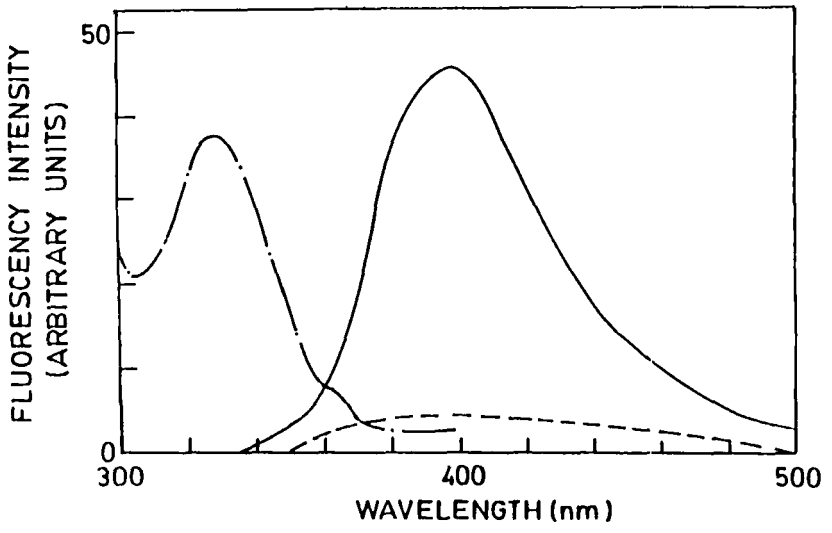

FIGURE 5 Fluorescence emission spectrum on excitation at 325 nm (—) and fluorescence excitation spectrum ($\lambda_{em} = 397$ nm) (– – –) of the PLP-$NaBH_4$-reduced enzyme and the emission spectrum of pure enzyme (----) on excitation at 325 nm. FAS (0.5 mg/ml) was incubated with 1 mM PLP under the reaction conditions for 15 min before reduction with $NaBH_4$. For details, see "Experimental Procedures."

TABLE II *Incorporation of PLP into pigeon liver FAS after sodium borohydride reduction.* Pigeon liver FAS (2 mg/ml) was incubated with 1 mM of PLP. The Schiff base was reduced with sodium borohydride and the amount of pyridoxyllysine was determined as described in "Experimental Procedures"

Condition	*Percent of control activity*		*Mol of Pyridoxyllysine per Mol of enzyme*[a]
	FAS	*Enoyl-CoA reductase*	
None	100	100	
Inactivated enzyme	18	20	4.35 ± 0.43
NADPH-protected enzyme	85	95	2.25 ± 0.7

[a] Average (± SD) of five determinations.

of pigeon liver FAS are essential for the enoyl-CoA reductase clearly establishes that there are two enoyl reductase sites per mole of enzyme.

It is also consistent with the enzyme structure that FAS consists of two identical peptides each containing the active sites for all of the steps involved in the fatty acid synthesis.

Acknowledgement

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