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EVIDENCE FOR THE ESSENTIAL HISTIDINE AT THE NADPH BINDING SITE OF ENOYL-CoA REDUCTASE DOMAIN OF PIGEON LIVER FATTY ACID SYNTHETASE

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Pigeon liver fatty acid synthetase was inactivated by stoichiometric concentrations of diethylpyrocarbonate (DEP). The second order rate constant for the loss of synthetase activity was similar to the value for enoyl-CoA reductase indicating that ethoxyformylation destroys the ability of the enzyme to reduce the unsaturated acyl intermediate, without significant effect on β-ketoacyl reductase activity. NADPH provided protection to the enzyme against inactivation by DEP indicating that essential histidine residues are present at the active site. DEP-modified enzyme showed a characteristic absorption maxima at 240 nm confirming the formation of ethoxyformic histidine. The reversal of inactivation by hydroxylamine and a pK_a value of 7.0 obtained from the pH-rate profile for inactivation again confirmed the specificity of DEP for hisidine. Stoichiometric results showed that two moles of histidine residue per mole of enzyme are essential for the activity of FAS.

Keywords: Pigeon liver fatty acid synthetase; enoyl-CoA reductase; diethyl pyrocarbonate; active site; chemical modification.

INTRODUCTION

Pigeon liver fatty acid synthetase (FAS) is a dimeric enzyme consisting of two half molecular weight multifunctional peptides of equal size.[1–3] It catalyzes the synthesis of palmitic acid through successive condensation of malonyl residues with acetyl primer and the reduction of the ketoacyl intermediate from each condensation by NADPH to the corresponding saturated acyl derivative.[14] Pigeon liver FAS contains active site domains for partial reactions, such as transacylases, β-ketoacyl synthetase, β-ketoacyl reductase, β-hydroxyacyl dehydratase, enoyl-CoA

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reductase, palmityl deacylase and the prosthetic group 4′-phosphopantetheine.[5] Extensive studies performed on the structure of the active site of FAS of chicken liver, goose uropygial glands and rat lactating mammary gland, have shown that in addition to cysteinyl and phosphopantetheine SH groups present at the condensation site, hydroxyl, arginyl, histidyl and lysyl groups, are present at different active sites.[6–10] It has been reported earlier that the active site of pigeon liver FAS contains cysteine SH, pantetheine SH, hydroxyl and lysyl amino groups. The present study reports the reaction of diethylpyrocarbonate (DEP) with histidine residues at the enoyl reductase domain of this enzyme. The results implicated the presence of a histidine residue at the NADPH binding site of the enoyl-CoA reductase domain of FAS.

MATERIALS AND METHODS

Materials

Diethylpyrocarbonate, acetyl-CoA, malonyl-CoA, NADH, NADPH, 5,5′-dithiobis (2-nitrobenzoic acid), *N*-acetylhistidine, imidazole and 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.

Enzyme Preparation and Assay of Overall and Reductase Activities

Pigeon liver FAS was purified and assayed spectrophotometrically by the method of Muesing and Porter.[4] The purified enzyme was homogeneous and showed a single band on SDS-polyacrylamide gel electrophoresis. The concentrations of FAS NADPH, and CoA thioesters, and activities of β-ketoacyl and enoyl reductases were determined spectrophotometrically as described earlier.[11,12] Fatty acid synthetase activity was measured by disappearance of absorbance of NADPH at 340 nm on a UV-VIS spectrophotometer (Shimadzu, Model UV-160A).

Modification of FAS with DEP

A solution of DEP was prepared by diluting with ethanol and quantitated as ethoxyformyl imidazole following the reaction with excess of imidazole. DEP is unstable in aqueous media and decomposes into ethanol and carbon dioxide. The rate constant, K′, for the decomposition of DEP was determined by incubating the reagent under the reaction conditions in 0.2 M potassium phosphate buffer containing 1 mM EDTA and 1 mM DTT. Aliquots were withdrawn at appropriate

time intervals and added to an excess of imidazole to quantitate the formation of ethoxyformyl imidazole at 240 nm, using a molar extinction coefficient of $3.2 \times 10^3\ M^{-1}\ cm^{-1}$. The rate constant for decomposition was determined from a plot of $\log([DEP]_t/[DEP]_0)$ versus time and was used in all the calculations pertaining to DEP inactivation. The previous studies have shown that the highest stability of DEP is obtained at acidic pH, and at low temperature in potassium phosphate buffer.[12]

Since, FAS is optimally stable in high ionic strength phosphate buffer at neutral pH, the inactivation experiments were carried out by incubating the enzyme (3 μM) in the presence of 0.2 M potassium phosphate buffer (pH 7.0) containing 1 mM DTT and 1 mM EDTA with varying concentration (5–25 μM) of DEP at 0°C to minimise degradation of both reactants. At specific time intervals, aliquots of the reaction mixture were taken and assayed immediately for overall FAS, enoyl-CoA and β-ketoacyl reductase activities. Controls without DEP in the presence of ethanol were run simultaneously.

Effect of Substrates and Substrate Analogs on Inactivation of FAS by DEP

The enzyme (3 μM) was incubated with the substrate and substrate analogs for 10 min prior to the addition of DEP (15 μM). After 15 min the overall FAS and enoyl-CoA reductase activity was determined as described earlier.

Characterization of the Reaction of DEP with FAS

Reactivation with hydroxylamine was performed by incubating the enzyme (3 μM) with DEP (6 μM) at 0°C under similar reaction conditions as described above. After allowing the enzyme to react with the reagent for 30 min until 30% of synthetase activity remained, neutralised hydroxylamine was added to a final concentration of 90 mM, and the incubation was continued at ambient temperature for an additional 60 min when the enzyme activity was assayed. A control was run in parallel without the addition of hydroxylamine.

FAS (3 μM) was incubated with 6 μM DEP under the same reaction conditions and absorption spectra were recorded at time intervals 1, 5, 10 and 15 min, at 240 nm using untreated enzyme in the reference cell and treated enzyme in the sample cell. Spectra were also recorded before and after treatment of the enzyme with DEP for 15 min using a buffer blank. The thiol content of the modified enzyme and unmodified enzyme was measured according to the method of Ellman.[14]

Stoichiometry of the Reaction of DEP with FAS

The stoichiometry of reaction was determined by incubating the enzyme (3 μM) in the reaction medium with 16 μM of DEP at 0°C. At specific time intervals, aliquots

were withdrawn and diluted in 50 mM neutralised *N*-acetylhistidine to terminate the reaction and assayed for synthetase activity. Ethoxyformylation of histidine residues was monitored at 240 nm and concentrations of ethoxyformylhistidine was determined using an extinction coefficient[13] of 3.2×10^3 cm^{-1} M^{-1}.

RESULTS AND DISCUSSION

Incubation of FAS with stoichiometric concentrations of DEP resulted in a time-dependent loss of overall FAS and enoyl-CoA reductase activities. This is in contrast to chicken liver FAS which was inactivated by comparatively higher concentrations of DEP,[10] but quite similar to yeast alchohol dehydrogenase.[15] Since the inhibitor concentration was not in large excess over the enzyme the assumption of pseudo-first order kinetics is not valid and the inactivation followed a typical bimolecular kinetic process as indicated by the plot of ln[(A-P)/(B-P)] versus time (Figures 1a,b) which were linear upto 90% inactivation, where, A and B are the initial concentrations of DEP and enzyme, respectively, and P is the amount of enzyme modified at any time, t. The second order rate constant k_2 of 11 mM^{-1} min^{-1} for the inactivation of both the activites was obtained from slope, k_2(A-B) (Figures 1a,b). In the above plot time was taken as $(1-e^{-K't}/K')$ where, $K' = 0.015$ min^{-1} is the first order rate constant for the decomposition of DEP under the reaction conditions. It was found that the β-ketoacyl reductase activity was unaffected by treatment of the enzyme with DEP.

In order to ascertain the site of inhibition the effect of substrates and substrate analogs on the inactivation of enzyme by DEP was studied. Acetyl-CoA and malonyl-CoA did not show any protection, whereas NADPH or NADP provided significant protection to the enzyme against DEP inactivation (Table I). A similar extent of protection was afforded by 2′-AMP or 2′,5′-ADP but not by NAD, NADH or 5′-AMP. These results implicated the presence of a histidine residue at the binding site for the 2′-phosphate group of NADPH in the enoyl-CoA reductase domain of the FAS.

The modified enzyme showed an absorption band at 240 nm which is characteristic of ethoxyformylhistidine and the intensity increased with time (Figure 2). The involvement of essential tyrosine residues was ruled out, since the absorption at 278 nm was unchanged by ethoxyformylation (Figure 2). DEP-modified FAS having 30% residual activity regained 90% of activity when treated with 90 mM neutralized hydroxylamine in 1 h. The possibility for the involvement of an enzyme thiol groups in the reaction of DEP was excluded by the measurement of the thiol content, which showed no change between the unmodified and modified enzymes. Furthermore, studies on the pH-dependence of inactivation yielded a pK_a of 7.0.

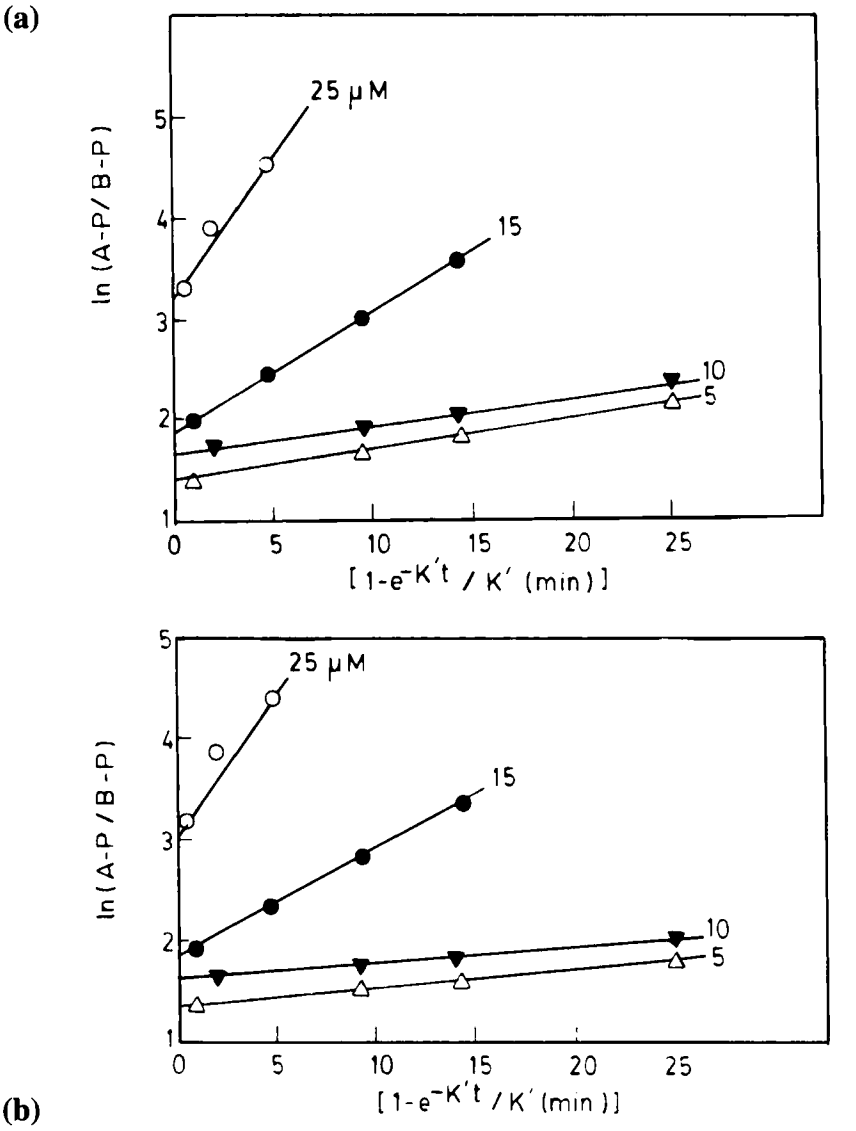

FIGURE 1 Time-dependent inactivation of overall FAS (a) and enoyl-CoA reductase (b) activity by DEP. The enzyme (3 μM) in 0.2 M potassium phosphate buffer (pH 7.0), containing 1 mM EDTA and 1 mM DTT was incubated with 5 (△), 10 (▼), 15 (•) and 25 μM (○) DEP at 0°C. Aliquots were withdrawn at the indicated time intervals and the residual activity was determined as described in "Experimental Procedures".

TABLE I Effect of substrates and substrate analogs on inactivation of FAS by DEP. The enzyme (3 μM) was incubated with the indicated substrate for 10 min prior to the addition of diethylpyrocarbonate (15 μM). After 15 min the overall FAS and enoyl-CoA reductase activity was determined as described in "Experimental Procedures".

Substrate	*Percentage of Remaining Activity*	
	FAS	*ENOYL-CoA Reductase*
None	5	6
Acetyl-CoA (1 mM)	6	–
Malonyl-CoA (1 mM)	5	–
NAD^+ (5 mM)	5	5
5′-AMP (5 mM)	6	5
2′-AMP (5 mM)	54	61
2′,5′-ADP (5 mM)	40	63
NADPH (5 mM)	50	68
$NADP^+$ (5 mM)	46	64

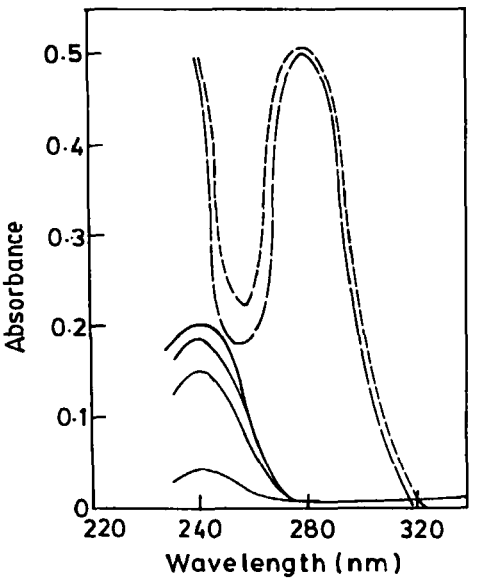

FIGURE 2 Effects of DEP on the absorption spectra of FAS. 3 μM FAS was incubated with 6 μM DEP and the absorption spectra were recorded at time intervals; 1, 5, 10, 15 min (—). Spectra were also recorded before (– –) and after (- - -) treatment of FAS with 6 μM DEP for 15 min using a buffer blank.

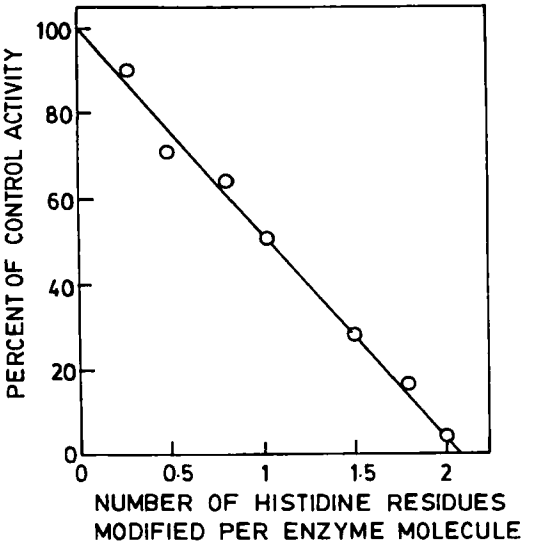

FIGURE 3 Stoichiometry of inactivation of FAS by DEP. The enzyme (3 μM) was incubated with 16 μM of DEP under the reaction conditions as described in the "Experimental Procedures". The concentration of ethoxyformyl histidine was determined by absorbance at 240 nm using an extinction coefficient of $3.2 \times 10^3\ cm^{-1}\ M^{-1}$. In a parallel experiment aliquots were withdrawn from the incubation mixture at different times and diluted in 50 mM neutralized *N*-acetylhistidine to terminate the reaction and assayed for synthetase activity.

All these results indicated that DEP inactivated FAS by specific modification of histidine residues.

The number of reactive histidine residues was determined by monitoring activity loss as a function of ethoxyformylation at 240 nm as shown in (Figure 3). A linear plot was obtained which indicated that the modification of two histidine residues resulted in a complete loss of activity. There was no change in stoichiometry even on prolonged incubation, indicating that the reaction is highly specific.

Present investigations have convincingly demonstrated the involvement of a histidine residue present at the active site of fatty acid synthetase. These results also suggested that each subunit of FAS contains one enoyl-CoA reductase domain having one histidine residue located at the NADPH binding site. Previous findings have shown that each peptide contains a covalently attached phosphopantetheine and a thioesterase domain.[16,17] All these results support the homodimer model of FAS. Further studies are in progress to locate the position of this critical histidine residue at the active site of FAS by isolating the reactive peptides and sequencing after modification with DEP.

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