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INHIBITION OF PIGEON LIVER FATTY ACID SYNTHETASE BY SPECIFIC MODIFICATION OF LYSINE RESIDUES WITH 2,4,6-TRINITROBENZENESULPHONIC ACID

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Pigeon liver fatty acid synthetase was inactivated irreversibly by 2,4,6-trinitrobenzenesulphonic acid (TNBS). Biphasic inactivation of the enzyme was observed with the inhibitor. NADPH provided protection to the enzyme against inactivation by TNBS and the extent of protection increased with NADPH concentration indicating that the essential lysine residues are present at the NADPH binding site. The stoichiometric results with TNBS showed that 4 mol of lysine residues are modified per mole of fatty acid synthetase upon complete inactivation. The rapid reaction of two amino groups per enzyme molecule led to the loss of 60% of the enzyme activity. These approaches suggested that two lysine residues present at the active site are essential for the enzymatic activity of fatty acid synthetase.

Keywords: Fatty acid synthetase; Enoyl-CoA reductase; Active site; Chemical modification; TNBS

INTRODUCTION

Pigeon liver fatty acid synthetase (FAS) like that of other vertebrates, is a dimeric enzyme consisting of two half molecular weight multifunctional peptides of equal size which contain seven component enzyme activities required for fatty acid synthesis.[1–3] Although extensive work has been carried out on the nature of the amino acid residues present at the active site of

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chicken liver,[4] goose uropygial gland[5] and rat mammary gland[6] FAS, little information is available on the active site of pigeon liver FAS. Chemical modification of pigeon liver FAS with pyridoxal 5′-phosphate in the dark showed a reversible inhibition of overall and enoyl-CoA reductase activity.[7] 2,4,6-Trinitrobenzene sulphonic acid (TNBS) has been used as a lysine specific reagent to elucidate the role of lysine residue in various enzymes.[8-10] In the present study the reaction of TNBS with the lysine residues at the enoyl reductase domain of pigeon liver FAS is to be documented for the first time.

MATERIALS AND METHODS

Materials

TNBS, acetyl-CoA, malonyl-CoA, NADPH (Sigma), Sephadex G-50, Sephadex G-25 (Pharmacia), dithiothreitol (Calbiochem) were used. All other chemicals were of highest purity grade commercially available.

Methods

Enzyme Preparation for Assay of Overall and Reductase Activities

Pigeon liver FAS was purified and the activity was measured by disappearance of absorbance of NADPH at 340 nm on UV–VIS spectrophotometer (Shimadzu, Model UV-160A) by the method of Muesing and Porter.[11] The purified enzyme was homogeneous and showed a single band on SDS-gel electrophoresis. Assays for β-ketoacyl and enoyl reductase activities were carried out spectrophotometrically as described earlier.[12,13] The enzyme was freed from ammonium sulfate and DTT by dialysis or column centrifugation method using Sephadex G-50 according to Penefsky,[14] since these compounds interfere with the TNBS reaction. Fluorescence spectra were recorded on a luminescence spectrometer (Perkin Elmer, Model LS 50 B). Both, fluorescence and absorbance were recorded in cuvettes of 1 cm light path at 25°C.

Modification of FAS with TNBS

Inactivation was carried out in 0.2 M potassium phosphate buffer (pH 7.0), 1 mM EDTA with indicated concentration of TNBS (0.25–2 mM at the protein concentration (2 mg/ml). At specific time intervals, aliquots from the

reaction mixture were taken and assayed immediately for overall FAS and enoyl-CoA reductase activity. Controls without TNBS were run concurrently. Solution of TNBS was stored in the dark at 0°C to minimize its hydrolysis and was not used later than 36 h after having made up. Solution of NADPH was made up fresh daily. Protection experiments were performed by adding the protecting ligands 15 min before the addition of TNBS.

Measurement of Thiol Content

Thiol content of the enzyme was determined by the method of Ellman.[15] FAS (2 mg/ml) was incubated with 1 mM TNBS for 15 min. Aliquots (0.25 ml) of the reaction mixture were withdrawn and immediately passed through a Sephadex G-25 syringe column to remove excess reagent according to the method of Penefsky[14] and the eluted sample was mixed with 0.75 ml portions of 8 M urea solution in 0.2 M potassium phosphate buffer (pH 7.0) containing 0.3 mM EDTA and 20 mM 5,5′-dithiobis-(2-nitrobenzoate). The number of thiol groups in the enzyme was determined by the increase in absorbance at 412 nm using an extinction co-efficient of 13,600 $M^{-1} cm^{-1}$.

Stoichiometry of the Reaction of TNBS with FAS

Stoichiometry of the TNBS reaction with FAS was determined by incubating FAS (4 mg/ml) with 1 mM TNBS and modification was observed by the absorbance increase at 367 nm.[16] The number of moles of TNBS incorporated per mole of enzyme was determined by taking the ratio of the concentrations of the trinitrophenyl derivative of ε-amino group of lysine (ε-TNP-lysine) and the enzyme. The concentrations of ε-TNP-lysine and protein were determined by using an extinction coefficient of 11,000 $M^{-1} cm^{-1}$ at 367 nm and by the method of Lowry *et al.*[17] respectively. The molecular weight of FAS was taken as 450,000 Da.

RESULTS AND DISCUSSION

Pigeon liver FAS was inactivated by TNBS in that overall and enoyl reductase activities were lost as a function of time, without any effect on β-ketoacyl reductase activity. The time course of inactivation of overall FAS and enoyl reductase activity by TNBS is shown in Figure 1. The rate of inactivation of FAS and enoyl reductase was similar for all the concentrations of

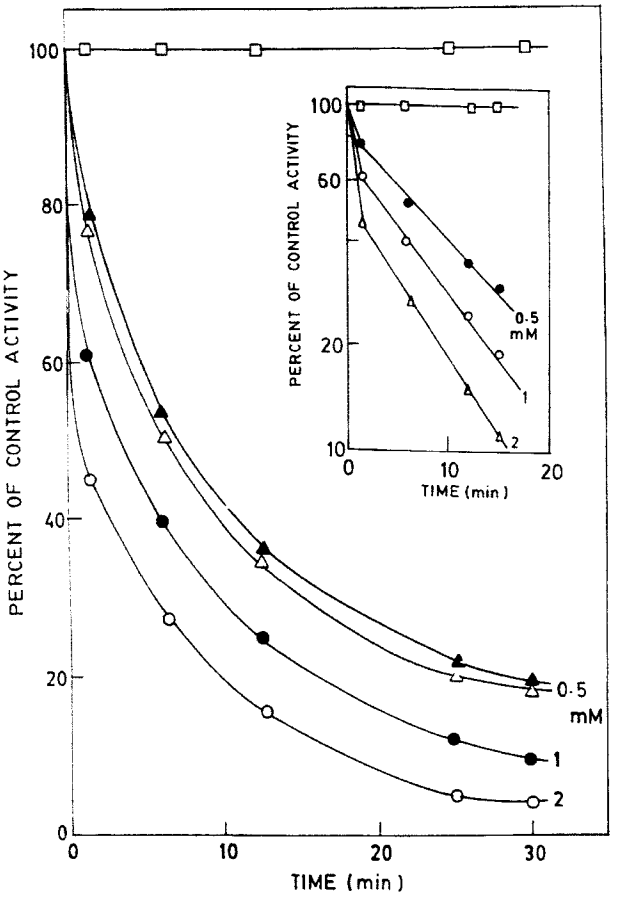

FIGURE 1 Inactivation of overall FAS and enoyl reductase activity by TNBS as a function of time. Enzyme (2 mg ml) was incubated with indicated concentrations of TNBS: (□) 0, (△) 0.5, (●) 1 and (○) 2 mM. (▲) represents inactivation of enoyl reductase at 0.5 mM of TNBS. Inset: A semilogarithmic plot of residual activity of FAS vs time.

TNBS used and hence only the result of inactivation of enoyl reductase in the presence of 0.5 mM of TNBS is shown (Figure 1). The inactivation of FAS was biphasic (Figure 1, inset) and followed pseudo-first-order kinetics with an initial rapid inactivation step followed by a slow rate of inactivation. The inactivation was irreversible since dialysis and Sephadex G-25 gel filtration of the modified enzyme could not reverse the inactivation. Incubation of the enzyme with TNBS in the presence of NADPH effectively lowered the rate of inactivation of the enzyme which increased with the increase in the concentration of NADPH (Table I). NADH and other substrates which did not show any protection. These results indicated that

TABLE I Effect of substrates on TNBS inactivation of overall FAS and enoyl-CoA reductase activities

Substrate	*Percentage activity remaining*	
	FAS	*Enoyl-CoA reductase*
None	10	8
Acetyl-CoA (1 mM)	11	—
Malonyl-CoA (1 mM)	13	—
NADH (5 mM)	12	9
NADPH (1 mM)	55	59
NADPH (5 mM)	63	68

Enzyme (2 mg/ml, specific activity 35 nmol palmitate min mg^{-1} protein) was preincubated with the indicated concentrations of substrates for 15 min prior to the addition of 1 mM TNBS. Enzyme activity was measured after 30 min of incubation as described in experimental procedures.

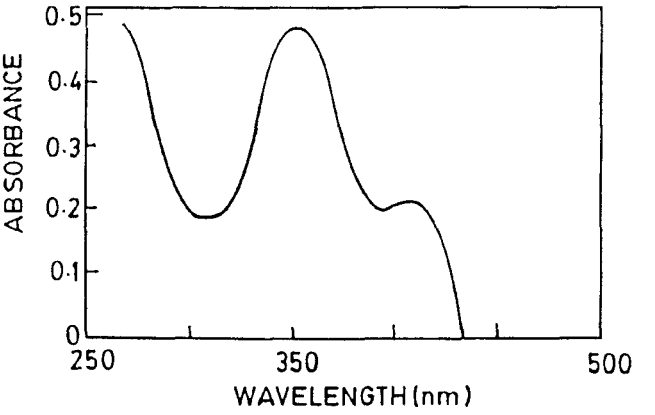

FIGURE 2 Absorption spectrum of TNBS modified FAS. The enzyme (0.5 mg/ml) was incubated with 0.5 mM TNBS in 0.2 M potassium phosphate buffer (pH 7.0) containing 1 mM EDTA for 30 min. The absorbance was determined against a blank containing an equivalent concentration of TNBS in the same buffer.

TNBS modified an essential residue (later shown to be lysine) which is present at the NADPH binding site of enoyl reductase domain.

In the modification of amino side chains, specificity is difficult to obtain since other nucleophilic side chains also react but it has been observed that TNBS at neutral pH modifies only thiol and amino groups.[18,19] The specificity of TNBS for the amino groups of the enzyme under the experimental condition was determined by comparing the thiol content of modified and unmodified enzyme. It was found that there was no change in the number of thiol groups in the 90% inactivated enzyme. By contrast, TNBS treated FAS showed a characteristic absorption spectra (Figure 2), which is specific for ε-TNP-lysine as described previously.[8]

The stoichiometry of inhibition by TNBS was determined by the absorbance increase at 367 nm. The residual overall FAS and enoyl-CoA reductase activities were determined at the same time. The percentage of residual overall activity and enoyl reductase activity was plotted as a function of number of residues modified (Figure 3). Four lysine residues per mole of the enzyme was modified with 90% loss of activity for both the FAS and enoyl reductase. Extrapolation of the plot to zero enzyme activity showed that

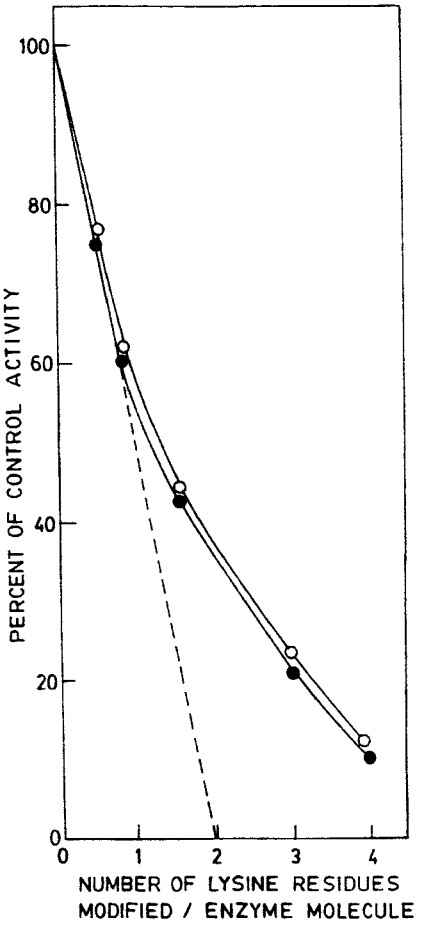

FIGURE 3 Stoichiometry of reaction of TNBS with FAS (●) and enoyl reductase (○). The enzyme (4 mg/ml) was incubated with 1 mM TNBS under the reaction conditions as described in the Materials and Methods. The amount of ε-TNP-lysine formed was determined by the absorbance increase at 367 nm. In a parallel experiment aliquots were withdrawn from the incubation mixture at identical time intervals and the residual enzyme activity was determined.

2 mol of lysine were essential for the enzyme activity. These results suggest that the inactivation of FAS by TNBS is due to the modification of one essential lysine residue per subunit present at the active site of enoyl–CoA reductase.

Present investigations have convincingly demonstrated the involvement of the lysine residue located at the active site of FAS. These results also suggested that each subunit of FAS contains one enoyl–CoA reductase domain having one lysine residue located at the NADPH binding site. Previous findings have shown that each peptide contains a covalently attached phosphopantotheine and a thioesterase domain.[20,21] All these results support the homodimer model of pigeon liver FAS. Further studies are in progress to locate the position of this critical lysine residue at the active site of FAS by isolating the reactive peptides and sequencing after modification with TNBS.

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