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ROLE OF CYSTEINE AND 4'-PHOSPHOPANTETHEINE IN THE INACTIVATION OF PIGEON LIVER FATTY ACID SYNTHETASE BY S-(4-BROMO-2,3-DIOXOBUTYL)-COENZYME A

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S-(4-bromo-2,3-dioxobutyl)-CoA has been used as an inhibitor of fatty acid synthetase from pigeon liver. This affinity label selectively and irreversibly inhibits the acetyl transacylase and β-ketoacyl synthetase reactions of this multienzyme complex. Binding studies with [^{3}H]-labeled bromodioxobutyl-CoA have established that four mol of the inhibitor are bound per mol of the enzyme complex, and that the radioactivity of this compound is covalently bound to cysteine and 4'-phosphopantetheine moieties. Other partial reactions of fatty acid synthesis are unaffected by bromodioxobutyl-CoA.

Pigeon liver fatty acid synthetase is a multienzyme complex with a molecular weight of approximately 500,000, which contains seven different enzyme activities. These are acetyl- and malonyl-CoA transacylases, condensing enzyme, β-ketoacyl reductase, β-hydroxyacyl dehydrase, enoylacyl reductase, and palmitoyl-CoA thioesterase. These activities are required for the synthesis of long-chain fatty acids, primarily palmitate, from acetyl- and malonyl-CoA and NADPH.

It is now well recognized that fatty acid synthetases from yeast and animal tissues consist of two multifunctional polypeptide chains (1). It is also known that a critical amino acid (or acids) at the active site of an enzyme will react with active site-directed reagents with the concurrent loss of activity. Hence, one of our objectives is the identification of the essential amino acid (or acids) at each of the catalytic sites of the seven component activities of the fatty acid synthetase complex. These studies will lead to a determination of the functional and structural similarities of the subunits of the enzyme complex.

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In the present communication we report the inactivation of pigeon liver fatty acid synthetase by the affinity label S-(4-bromo-2,3-dioxobutyl)-CoA (BDB-CoA). The inhibition is irreversible, and the overall loss of enzyme activity results from the inhibition of condensing and acetyl-CoA transacylase activities. These studies indicate that four mol of BDB-CoA are bound per mol of the enzyme. No other partial enzyme activities are inhibited. This is the first report on the binding of BDB-CoA to an essential cysteine and 4'-phosphopantetheine of a fatty acid synthetase.

MATERIALS AND METHODS

Acetyl- and malonyl-CoA and coenzyme A were obtained from P-L Biochemicals, NADPH from Sigma, and dithiothreitol from Calbiochem. Other reagents were obtained as follows: acrylamide, bisacrylamide, sodium dodecyl sulfate and Coomassie brilliant blue from Bio-Rad Laboratories; [^{3}H(G)]-coenzyme A, [1-^{14}C]acetyl-CoA, [2-^{14}C]malonyl-CoA, [1-^{14}C]palmitoyl-CoA and aquasol from New England Nuclear; 1,4-dibromo-2,3-butanedione from Aldrich, and Sephadex G-25 from Pharmacia Fine Chemicals.

Preparation and assay of pigeon liver fatty acid synthetase. Pigeon liver fatty acid synthetase was purified and assayed spectrophotometrically by the method of Muesing *et al.* (2).

S-(4-bromo-2,3-dioxobutyl)-CoA. The synthesis of BDB-CoA was carried out with freshly crystallized 1,4-dibromo-2,3-butanedione by the method of Owens and Barden (3). Radioactive BDB-CoA was prepared from [^{3}H(G)]coenzyme A by the same procedure. BDB-CoA was prepared fresh for each inhibition experiment.

Inhibition of fatty acid synthetase by BDB-CoA. Three to four mg of fatty acid synthetase (10 mg/ml) were passed through a 0.4 x 30 cm column of Sephadex G-25, previously equilibrated with 0.2 M potassium phosphate buffer, pH 7.0, and 1 mM EDTA, to remove dithiothreitol. The enzyme was then incubated at 30°C with the desired concentration of BDB-CoA in 0.2 M potassium phosphate buffer, pH 7.0. Aliquots from the reaction mixture were removed at various time intervals and the reaction was stopped by adding dithiothreitol to a final concentration of 10 mM. The residual fatty acid synthetase activity was measured 60 min later.

Binding studies. The stoichiometry of the binding of inhibitor to enzyme was determined by incubating dithiothreitol-free fatty acid synthetase (1 mg/ml) with varying concentrations (5 to 50 μM) BDB-[^{3}H(G)]-CoA for 60 s. The reaction was stopped by the addition of diothiothreitol to a final concentration of 10 mM and residual enzyme activity was measured after 60 min of incubation with dithiothreitol. The remaining enzyme was precipitated with cold 15% trichloroacetic acid (TCA) and washed free of unbound radioactivity. It was then assayed for protein and bound radioactivity.

Gel electrophoresis. Tris-glycine sodium docecyl sulfate polyacrylamide gel electrophoresis was carried out by the method of Knowland (4) on 8% gels. Gels were stained for 45 min in 0.25% Coomassie brilliant blue in acetic acid: methanol:water (10:45:45) and destained in an acetic acid:ethanol:water (10:25:65) mixture for two to four days.

High voltage electrophoresis. Unmodified and BDB-CoA-modified fatty acid synthetase were labeled with [^{14}C]acetate and then digested with pepsin by a previously published procedure (5). The resultant peptic peptides were subjected to high voltage electrophoresis at 2500 V for 1.5 h in acetic acid:pyridine: water (10:1:289) buffer at pH 6.5.

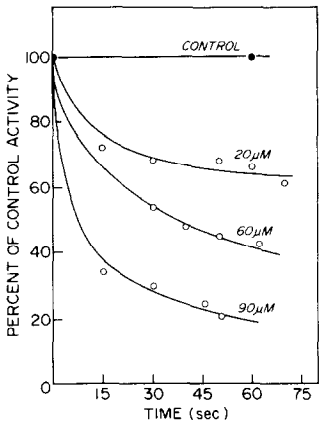

Fig. 1. Rate of inactivation of pigeon liver fatty acid synthetase (300 μg/ml) by different concentrations of BDB-CoA. Fatty acid synthetase (specific activity 140 to 160 nmol palmitate min^{-1} mg^{-1}) was incubated with 20 μM, 60 μM, and 90 μM BDB-CoA. At different time intervals aliquots of the incubation mixture were removed, the reaction was stopped by the addition of dithiothreitol, and the activity for fatty acid synthetsis was measured. Details of the experimental conditions are described in the section on Materials and Methods.

Protein and radioactivity measurements. The determination of protein for binding studies was carried out by the method of Bradford (6), with the fatty acid synthetase as the standard for the calibration plot. The concentration of fatty acid synthetase in fractions from Sephadex G-25 columns was determined from the absorption measurements at 280 nm ($A^{1\%}_{1cm}$ = 11.6). Radioactivity was measured with a Packard Model 3320 liquid scintillation spectrometer.

RESULTS AND DISCUSSION

The loss of activity of fatty acid synthetase when pigeon liver enzyme is incubated with BDB-CoA is shown in Fig. 1. The inactivation is rapid and irreversible, inasmuch as prolonged dialysis of the incubation mixture did not reactivate the enzyme. The inhibition of the fatty acid synthetase is due to covalent binding of BDB-CoA to the enzyme as shown by SDS gel electrophoresis (data not shown). The gel electrophoresis patterns of unmodified and BDB-[^{3}H(G)]CoA-modified fatty acid synthetases were identical. All of the radioactivity of BDB-[^{3}H(G)]CoA was present in the half-molecular weight subunits of the fatty acid synthetase.

The stoichiometry of inhibition by BDB-[^{3}H(G)]CoA was determined by a Scatchard analysis, Fig. 2. The plot of mol of BDB-CoA bound per mol of enzyme as a function of loss of activity for fatty acid synthesis was linear at the lowest binding ratios. Extrapolation of this plot indicated that the binding of 3.5 mol of BDB-CoA (range 3.25 to 4) would result in the complete inactivation of the fatty acid synthetase. This finding is at variance with the stoichiometry of binding reported (7) for fatty acid synthetase from lactating rat mammary gland where a value of 1.2 was reported, and it renders the earlier suggestion of a "half-of-the-sites" catalytic mechanism for the fatty acid synthetase as doubtful.

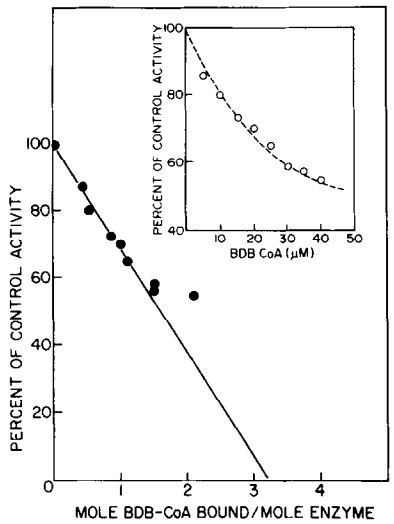

Fig. 2. Scatchard analysis of the inhibition of pigeon liver fatty acid synthetase by BDB-CoA. Pigeon liver fatty acid synthetase (10 mg/ml, specific activity 140 to 160 nmol palmitate min^{-1} mg^{-1}) was passed through a Sephadex G-25 column that had been equilibrated with 0.2 M potassium phosphate buffer, pH 7.0, and 1 mM EDTA at 30°C to remove dithiothreitol. Two hundred μg of enzyme were incubated with different concentrations of BDB-[^{3}H(G)]CoA (specific radioactivity 2500 dpm/nmol) in the range of 5 to 40 μM for 1 min. The reaction was stopped by the addition of dithiothreitol. Enzyme activity, protein concentration and bound radioactivity were then determined as indicated in the section on Materials and Methods.

The mechanism of inhibition by BDB-CoA was elucidated by identifying the sites which react with BDB-CoA. The presence of dithiothreitol in the reaction mixture protected the enzyme against inactivation by BDB-CoA and suggested that the inhibitor reacts with a thiol group (or groups). The existence of two critical thiol sites, namely a cysteine-SH at the condensing site and a pantetheine-SH on the fatty acid synthetase, is well known (5). These thiols are essential for the overall activity of the enzyme. We therefore concluded that BDB-CoA binds to one or both of these thiol sites with the resultant loss of overall synthetase activity. The identification of the inhibitor binding sites was established by high voltage paper electrophoresis of the peptic peptides obtained from [1-^{14}C]acetyl enzyme and [1-^{14}C]acetyl-labeled BDG-CoA modified enzyme, Fig. 3. High voltage electrophoresis (Fig. 3A) showed that the [1-^{14}C]-acetyl group binds to previously identified sites, namely the pantetheine-SH site (A_1 and A_2), cysteine-SH site (B_2) and hydroxyl site (B_1). On the other hand, peptide maps of BDB-CoA-modified fatty acid synthetase labeled with a [1-^{14}C]acetyl group showed that radioactivity associated with the hydroxyl site is unaltered (Fig. 3B), whereas less than 10% of the radioactivity is associated with the pantetheine-SH- and cysteine-SH-containing peptides. Since BDB-CoA-modified enzyme was 90% inhibited and possessed only 10% of the overall activity, the binding of 10% of the radioactivity to pantetheine-SH and cys-

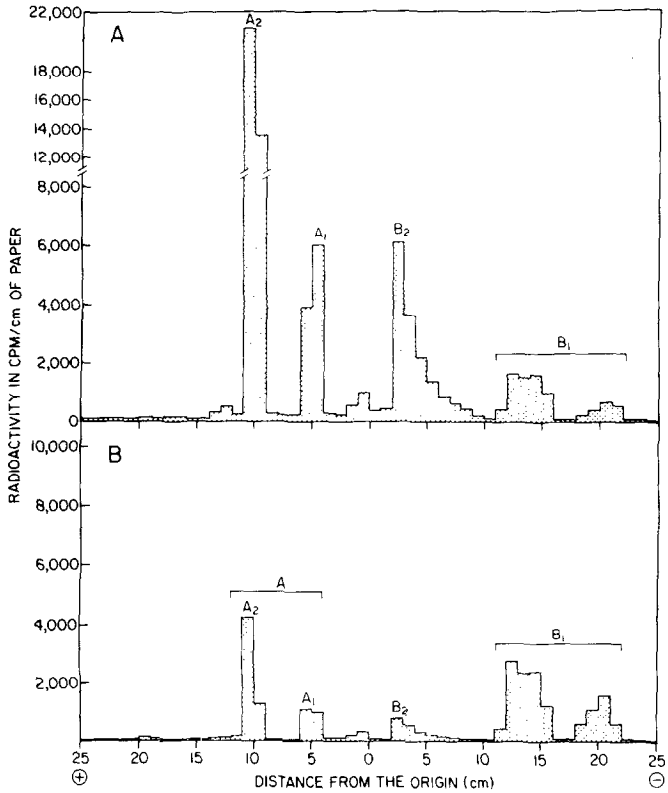

Fig. 3. High voltage electrophoretograms of the peptic digest of (A) [1-^{14}C]acetyl enzyme and (B) BDB-CoA-modified enzyme that was subsequently labeled with [1-^{14}C]acetyl groups. Pigeon liver fatty acid synthetase preparation (unmodified, 2 mg, specific activity 130 to 160 nmol palmitate min^{-1} mg^{-1}) and BDB-CoA (modified, 2.0 mg) in 0.2 M potassium phosphate, 1 mM EDTA, pH 7.0, were separately treated with a ten fold molar excess of [1-^{14}C]acetyl-CoA (specific radioactivity 25,000 dpm/nmol) for 1 min at 4°C. The reaction was stopped with 60% perchloric acid. The precipitated protein was washed with cold 0.2 N acetic acid until free of unbound radioactivity. The precipitated proteins were suspended in 0.01 N HCl and treated with pepsin (0.25 mg pepsin/mg enzyme) for 18 h at 30°C. High voltage electrophoresis of peptic peptides was carried out at 2500 V for 1.5 h on Whatman 3 mm paper.

teine-SH sites demonstrates that BDB-CoA is bound to these two critical thiol sites.

A logical corollary to the binding of BDB-CoA to these essential thiols suggests that the component activity for the condensing enzyme should also be inhibited. We have found that the BDB-CoA-modified enzyme does not catalyze the condensation-CO_2 exchange reaction and the acetyl-CoA transacylase reaction (data not shown). The binding of four mol of BDB-CoA per mol of fatty acid synthetase for the loss of complete activity indicates the presence of two cysteine-SH condensing sites and two 4'-phosphopantetheine-SH sites on the pigeon liver fatty acid synthetase. This finding is consistent with the presence of two condensing sites per mol and two 4'-phosphopantetheine sites per mol of rat liver fatty acid synthetase (8). This study provides the first report on the

inhibition of condensing enzyme activity and on the mechanism of inhibition of the fatty acid synthetase by BDB-CoA.

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