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AFFINITY LABELING OF FATTY ACID SYNTHETASE FROM LACTATING RAT MAMMARY GLAND WITH S-(4-BROMO-2,3-DIOXOBUTYL)-CoA: EVIDENCE FOR A "HALF-OF-THE-SITES" CATALYTIC MECHANISM

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Summary - The classical affinity label, S-(4-bromo-2,3-dioxobutyl)-CoA, rapidly and irreversibly inhibits fatty acid synthetase from lactating rat mammary gland. The limit stoichiometry of incorporated label and the kinetics of inactivation indicate that two sites can be labeled per enzyme dimer. Strong evidence of site-site interaction (weak negative cooperativity) was observed. At relatively low concentrations, the affinity label inhibits acetyl transacylase whereas the malonyl transacylase activity is enhanced. We propose that fatty acid synthetase from lactating rat mammary gland catalyses a "half-of-the-sites" mechanism.

The overall reaction catalyzed by vertebrate fatty acid synthetase produces a long chain fatty acid, usually palmitic acid. Seven different catalytic activities are required for this synthesis: acetyl transacylase, malonyl transacylase, condensing enzyme, β-ketoacyl reductase, β-hydroxyacyl dehydrase, enoyl reductase and palmityl thioesterase (1). The vertebrate enzymes are dimers composed of large subunits (∿250,000 daltons each)(2-7). A primary objective of current research with fatty acid synthetase from vertebrate sources is to establish the relationship between the enzyme's structure and its catalytic mechanism. The present paper describes affinity labeling experiments with BDB-CoA[1] and fatty acid synthetase from rat mammary gland. BDB-CoA is a selective, potent affinity label for this

[1]The abbreviation used is: BDB-CoA, S-(4-bromo-2,3-dioxobutyl)-CoA.

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enzyme. These studies indicate that fatty acid synthetase from lactating rat mammary gland possesses a "half-of-the-sites" catalytic mechanism.

Lynen and co-workers have recently suggested that fatty acid synthetase from yeast might catalyze a half-of-the sites mechanism (8).

Materials and Methods

Materials-Fatty acid synthetase was isolated from lactating rat mammary gland by a simple procedure developed by Ahmad and Ahmad (to be published). Prior to an affinity labeling experiment, 50 µl of stock enzyme solution (∿ 30 mg per ml; specific activity ≃ 1; in 0.25 M potassium phosphate, pH 6.6, 10% glycerol, and 1 mM dithiothreitol) was mixed with 150 µl of 1 M potassium phosphate, pH 6.6, 10 mM dithiothreitol and incubated at 30° for 1 hr. The sample was then chromatographed on a 0.8 x 12 cm column of Sephadex G-25 equilibrated with 0.25 M potassium phosphate to remove dithiothreitol and glycerol. The activity of the recovered enzyme was stable at 0.5 to 0.6 units per mg over the time required for an affinity labeling experiment. (Addition of dithiothreitol reactivated the recovered enzyme to a specific activity of ∿1 after a 15 min incubation).

BDB-CoA was synthesized as described by Owens and Barden (9). CoA and malonyl-CoA were obtained from P-L Biochemicals and NADPH from Sigma. [^{3}H(G)]-Coenzyme A, [acetyl-^{3}H]-acetyl CoA, and [malonyl-1,3-^{14}C]-malonyl CoA were obtained from New England Nuclear. The 1,4-dibromo-2,3-butanedione was purchased from Aldrich. Acetyl-CoA was prepared by a published procedure (10).

Methods-The overall activity of fatty acid synthetase was assayed spectrophotometrically as described by Smith and Abraham (11), except that we used 100 µM butyryl-CoA to initiate the reaction. The catalytic activities of the acetyl transacylase and malonyl transacylase were assayed by ^{14}C-exchange methods as described by Muesing and Porter (12). All activities were assayed at 30°C.

For the affinity labeling experiments the required amount of BDB-CoA in methanol was added to a siliconized tube after which the solvent was removed under vacuum. Affinity labeling was initiated by the addition of enzyme (∿ 2 mg/ml in 0.25 M potassium phosphate, pH 6.6) and, after mixing, 50 µl aliquots were removed at various times. Each aliquot was placed in 0.1 ml of quenching solution (0.25 M potassium phosphate, pH 7.5, 100 µM butyryl-CoA and 10 mM dithiothreitol) and incubated for 1 hour prior to assay of the residual activity. The temperature was maintained at 30° C throughout this procedure.

The moles of BDB-CoA incorporated per mole of enzyme was determined following a procedure described previously (13). The enzyme was incubated with BDB-[^{3}H(G)]-CoA for 1 min and then the system was quenched (vide ante). Aliquots were removed for assay of the overall reaction and a determination of the moles of BDB-CoA bound.

Protein was assayed with the Bio Rad dye procedure, (Bio Rad Technical Bulletin #1051). Bovine γ-globulin was used as the standard.

Results and Discussion

Irreversible inhibition of the overall reaction with BDB-CoA - Fatty acid synthetase was rapidly and irreversibly inhibited by BDB-CoA; plots of

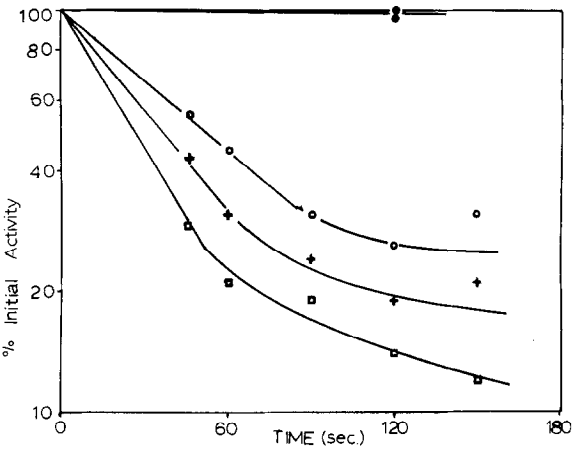


Figure 1. Inactivation of fatty acid synthetase with BDB-CoA. The enzyme was incubated with 27 (0-0), 53 (+-+) and 80 (□-□) μM BDB-CoA. In the controls (•) the same results were obtained in the absence of BDB-CoA as in the presence of pre-quenched BDB-CoA.

log (activity-remaining) versus time for several BDB-CoA concentrations are shown in Fig. 1. The data strongly suggest that more than one site is labeled by the affinity label. The kinetics of irreversible inhibition for enzymes on which the irreversible inhibitor can be bound at two interacting sites has been discussed by Rakitzis (14). The data in Fig. 1 are consistent with a two-site model that exhibits weak negative cooperativity. Considering that fatty acid synthetase is a dimer and that the two sites are most probably on separate polypeptides, the evidence of cooperativity in the data of Fig. 1 strongly suggests that this enzyme, at least in the early events of a catalytic cycle, operates by a half-of-the sites mechanism (cf 15).

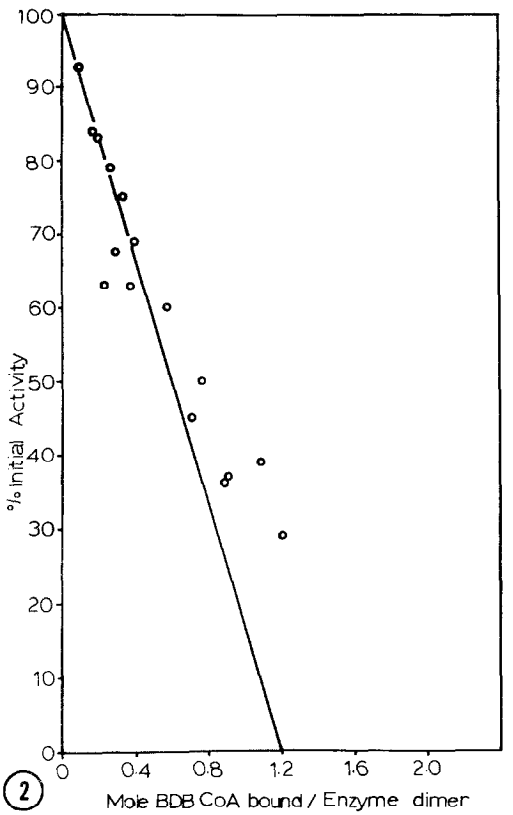
If we assume that the rate at which enzyme activity decreases is pseudo first order until 30% of the activity remains (i.e. as drawn in Fig. 1), a rough estimate of the K_I of BDB-CoA and the rate of covalent bond formation (i.e. k_3) can be obtained (16). These estimated constants are for reversible binding of the affinity label at the first site (K_I) and for the rate of covalent bond formation with only one site occupied (k_3). The estimated

values are: $K_I \simeq 8 \times 10^{-5}$ M, $k_3 \simeq 0.03$ sec^{-1}. The potency of BDB-CoA as an irreversible inhibitor is clearly due to an exceptionally high rate of covalent bond formation in the reversibly formed enzyme-inhibitor complex.

Substrate protection against irreversible inhibition of the overall reaction - When varying concentrations of acetyl-CoA, butyryl-CoA or malonyl-CoA are mixed with fatty acid synthetase prior to exposure to BDB-CoA, the amount of irreversible inhibition observed after an incubation of 1.0 min is significantly decreased. On a concentration basis, the efficiency of protection varies as follows: butyryl-CoA > acetyl-CoA > malonyl-CoA. These results indicate that BDB-CoA is a label for sites that bind CoA thiolesters. The catalytic sites for acetyl transacylase and malonyl transacylase are thus the most probable sites of attack by BDB-CoA.

Stoichiometry of BDB-CoA incorporation - A plot of (activity-remaining) versus the ratio of moles label bound per mole of enzyme was extrapolated to zero activity-remaining to obtain the limit stoichiometry of the labeled enzyme. By linear regression analysis using all of the data points, a value of 1.2 ± 0.3 was obtained for the stoichiometry. This value is consistent with the weak negative cooperativity evident in the kinetics of irreversible inhibition (Fig. 1). The experimental conditions favored labeling of the "fast" site, but since the negative cooperativity between the two sites is weak the second site was partially labeled. Note that there is a trend in the data of Fig. 2 toward a limit stoichiometry of two.

The effect of BDB-CoA incorporation on the acetyl transacylase and malonyl transacylase activities - At relatively low inhibitor concentrations the incorporation of BDB-CoA into fatty acid synthetase has opposing effects on the acetyl transacylase and malonyl transacylase activities (Fig. 3). The acetyl transacylase is inhibited; in contrast, the malonyl transacylase is activated. As expected, inhibition of the malonyl transacylase is observed at the higher concentrations of BDB-CoA (> 40 μM).



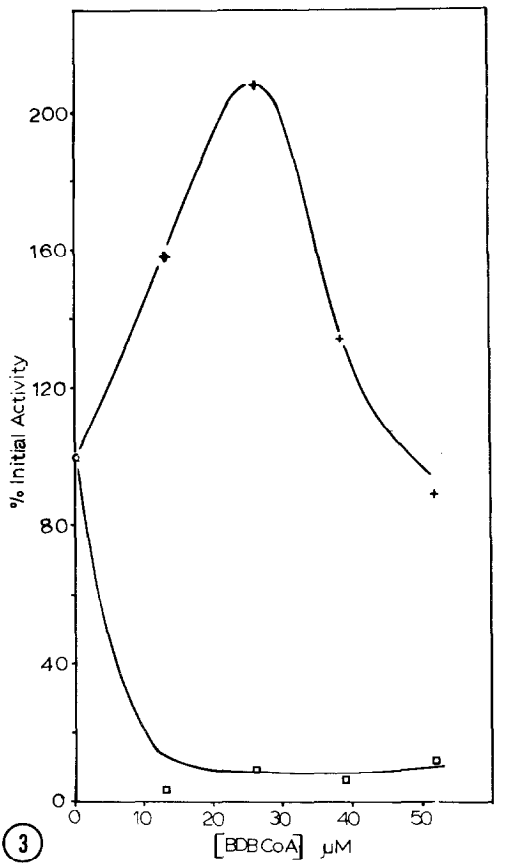


Figure 2. The stoichiometry of covalently bound BDB-CoA per enzyme dimer. The enzyme was incubated with various concentrations of BDB-[^{3}H(G)]-CoA for 1.0 min. See Methods and Materials. The plot is a composite of data from two separate experiments.

Figure 3. The effect of covalently bound BDB-CoA on the acetyl transacylase and malonyl transacylase activities. The enzyme was incubated with the indicated concentration of BDB-CoA for 1.0 min. After quenching, aliquots were removed for assay of the acetyl transacylase (□) and malonyl transacylase (+). Each measurement was corrected for the non-enzymic transacylation.

Presumably there is one transacylase site on each subunit. We conclude that the properties of each site are such that the acetyl transacylase reaction is catalyzed preferentially when the second transacylase site is not occupied. However, when the first transacylase site is occupied, or "primed", the second transacylase site undergoes a conformational change such that it preferentially catalyzes the malonyl transacylase reaction.

This switch (i.e. "flip-flop") in the preferred mode of catalysis is based on site-site cooperativity.

Concluding comments - A primary requirement of a half-of-the-sites mechanism is that the enzyme be an oligomer containing equivalent subunits (15). The currently available evidence indicates that fatty acid synthetase from vertebrate sources is composed of identical polypeptides. That is, the constituent polypeptides are equivalent in size (4,5,17,18), there is one bound pantetheine for each polypeptide (5,19), and there is one palmityl thioesterase segment for each polypeptide (20).

A second requirement for a half-of-the-sites mechanism is that there be clear evidence of site-site cooperativity. The evidence presented in this paper indicates that the affinity labeling process is characterized by a weak negative cooperativity between two transacylase sites. Furthermore, labeling at one site appears to cause a "flip-flop" in the preferred mode of catalysis at the second site, i.e. from an acetyl transacylase to a malonyl transacylase.

If the overall reaction is based on a half-of-the-sites catalytic mechanism, then a molecule of fatty acid synthetase can synthesize only one palmityl group at a time. This key issue has been resolved recently by Libertini and Smith (21). Using fatty acid synthetase from lactating rat mammary gland from which the palmityl thioesterase segments had been removed, these workers have demonstrated that only one enzyme-bound palmityl group is synthesized per molecule (dimer). This observation when combined with the results presented in this paper provides strong evidence for a half-of-the-sites catalytic mechanism for fatty acid synthetase from lactating rat mammary gland.

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