Irreversible inhibition of fatty acid synthase from rat mammary gland with *S*-(4-bromo-2,3-dioxobutyl)-CoA

Effect on the partial reactions, protection by substrates and stoichiometry studies

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(Received 20 April 1982/Accepted 14 July 1982)

Fatty acid synthase from lactating rat mammary gland is rapidly and irreversibly inhibited by *S*-(4-bromo-2,3-dioxobutyl)-CoA. Of the seven partial reactions catalysed by the enzyme, the inhibition of the overall catalytic activity is closely paralleled only by inhibition of the β -oxoacyl synthase (condensing) partial reaction. Three partial reactions, β -oxoacyl reductase, β -hydroxyacyl dehydratase and enoyl reductase, are inhibited to a modest degree. The three partial reactions known to involve an acyl-CoA/CoA-binding site, acetyl acyltransferase, malonyl acyltransferase and palmitoyl thioesterase, are not inhibited by *S*-(4-bromo-2,3-dioxobutyl)-CoA. The modification process does not cause the enzyme to dissociate into catalytically incompetent monomers. Stoichiometric studies suggest that approx. 6 mol of reagent are incorporated per mol of totally inhibited enzyme (dimer). The formation of acylated enzyme from either acetyl-CoA or malonyl-CoA protects the enzyme equally well against *S*-(4-bromo-2,3-dioxobutyl)-CoA. Also, pretreatment of the enzyme with 5,5'-dithiobis-(2-nitrobenzoic acid), a thiol-specific reagent reported to block essential thiol groups in the condensing partial reaction, protects against inhibition by the reagent. On the other hand, the presence of up to 770 μ M-*S*-acetyl-CoA or dethio-CoA does not protect the enzyme from irreversible inhibition. Together, the results suggest that the primary inhibitory process is a bimolecular reaction resulting in alkylation of essential thiol groups in the condensing partial reaction; this process does not require the obligatory formation of a Michaelis–Menten complex of enzyme and reagent before the alkylation reaction.

Much has been learned about the structure and catalytic mechanism of vertebrate FAS (Bloch & Vance, 1977; Stoops *et al.*, 1977; Smith, 1980). However, many more investigations employing a wide variety of experimental approaches will be required before the detailed catalytic mechanism and the relationship between structure and function can be fully understood. One experimental approach that has not been applied extensively to FAS is

Abbreviations used: BDB-CoA, *S*-(4-bromo-2,3-dioxobutyl)-CoA; DTT, dithiothreitol; FAS, fatty acid synthase; Nbs₂, 5,5'-dithiobis-(2-nitrobenzoic acid).

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affinity labelling. Affinity labelling has become a key technique in the area of enzyme structure–function analysis (Wold, 1977). We have attempted to label acyl-CoA/CoA-binding sites on FAS from lactating rat mammary gland by affinity labelling techniques with BDB-CoA (Owens & Barden, 1978). BDB-CoA contains the α -bromoketone group, which is an established alkylating group in biological systems. The relatively large CoA moiety was expected to bestow on the reagent a high degree of binding specificity for short-chain acyl-CoA (and CoA)-binding sites. Our preliminary studies showed that brief exposure of FAS to low concentration of BDB-CoA (<60 μ M) led to rapid inactivation of the enzyme, suggesting a classical affinity labelling process (Clements *et al.*, 1979). However, the results

of more thorough studies reported here suggest that BDB-CoA directly alkylates essential enzyme thiol groups by a simple bimolecular reaction. A preliminary report of these results has been presented (Ahmad *et al.*, 1981).

Materials and methods

Materials

FAS was isolated from lactating rat mammary gland by a simple procedure (Ahmad *et al.*, 1982), and several reagents were obtained or synthesized as previously described (Barden *et al.*, 1981; Ahmad *et al.*, 1982). Cysteamine hydrochloride, pantetheine, acetyl phosphate, citrate synthase (EC 4.1.3.7) and malate dehydrogenase (EC 1.1.1.37) were obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Crotonyl chloride, Nbs₂, *trans*-decalin-1-one and β -hydroxybutyric acid were purchased from Aldrich Chemical Co. (Milwaukee, WI, U.S.A.). [G-³H]-Coenzyme A, [1-¹⁴C]palmitoyl-CoA and Ba¹⁴CO₃ were obtained from New England Nuclear Corp. (Boston, MA, U.S.A.). *S*- β -Hydroxybutyrylpantetheine and *S*-crotonyl-*N*-acetylcysteamine were prepared by the method of Kass *et al.* (1967). *S*-Acetonyl-CoA (Rubenstein & Dryer, 1980) and dethio-CoA (Grinnell & Nishimura, 1969) were synthesized by published methods.

Procedure for chemical modification

A stock solution of FAS (approx. 30 mg/ml, in 0.25 M-potassium phosphate, pH 6.6, and containing 10% glycerol and 1 mM-DTT) was stored frozen. For each experiment 50 μ l of the stock solution was diluted with 200 μ l of 0.25 M-potassium phosphate, pH 6.6, containing 0.1 mM-EDTA and 10 mM-DTT and incubated for 30–60 min at 30°C. The specific activity (1 unit equals 1 μ mol of NADPH oxidized/min per mg) of the incubated enzyme was 1.7–1.9 units, unless indicated otherwise. The incubated enzyme solution was chromatographed on a column (0.8 cm $\times$ 12 cm) of Sephadex G-50 equilibrated with 0.25 M-potassium phosphate, pH 6.6, containing 0.1 mM-EDTA to remove small solutes (particularly DTT). The specific activity of the recovered enzyme was 1.0–1.2 units, and control experiments showed that the activity was stable over the time required for the experiments described herein. (Addition of DTT plus incubation for 30 min at 37°C led to reactivation of the recovered enzyme.)

In the irreversible inhibition experiments the desired amount of BDB-CoA was added in a portion of methanol to silicone-treated tubes, after which the methanol was removed under vacuum. [Different preparations of BDB-CoA do show some variation in potency, i.e. the amount of inhibition produced under uniform conditions. The concentration of BDB-CoA solution is based on absorbance at

260 nm (CoA content). Our studies indicate that some oxidized CoA, or a CoA dimer cross-linked with 1,4-dibromobutane-2,3-dione, is present. Thus concentrations based on 260 nm absorbance measurements may overestimate the concentration of 'active' BDB-CoA by up to 15%. If the methanol and diethyl ether used in synthesis are pre-dried over anhydrous MgSO₄, the amount of dimerized CoA can be kept to a minimum.] The modification reaction was initiated by the addition of 200 μ l of the 'recovered' enzyme (0.8–1.2 mg/ml) to the tube containing BDB-CoA, followed immediately by swirling to mix the system. After incubation at 30°C for 1.0 min, 400 μ l of 0.5 M-potassium phosphate, pH 7.5, containing 10 mM-DTT, was added to quench the reaction. The residual activity was measured by one or more of the following assays for catalytic activity.

(1) The overall enzyme activity was measured spectrophotometrically by the procedure of Smith & Abraham (1975), except that 100 μ M-butyryl-CoA was used to prime the reaction and the temperature was 37°C.

(2) The acetyltransferase partial reaction was assayed by the isotope-exchange method of Muesing & Porter (1975) or by a spectrophotometric assay based on a scheme described by Wieland *et al.* (1979). In the spectrophotometric assay the cuvette contained the following, in μ mol/ml: sodium malate, 5.0; potassium phosphate, pH 7.0, 50; NAD⁺, 0.3; CoA, 0.2; DTT, 0.2; acetylpanetheine, 0.04; malate dehydrogenase, 260 units; citrate synthase, 0.45 units; and FAS, approx. 10 m-units. The reaction was started by adding FAS.

(3) The malonyltransferase partial reaction was assayed by a spectrophotometric assay based on a scheme published by Wieland *et al.* (1979). The cuvette contained the following, in μ mol/0.5 ml: potassium phosphate, pH 7.0, 150; malonyl-CoA, 0.11; pantetheine, 3.5; acetyl phosphate, 1.8; DTT, 1.0; phosphotransacetylase, 0.27 unit; FAS, approx. 10 m-units. A value of 4.5 mm $\cdot$ cm⁻¹ for ϵ_{232} was used to quantify the appearance of the thioester (Stadtman, 1957).

(4) The β -oxoacyl synthase partial reaction was assayed as described by Smith *et al.* (1976).

(5) The assay for the β -oxoreductase partial reaction was based on the procedure of Dutler *et al.* (1971), with *trans*-decalin-1-one as the substrate. The concentration of NADPH was 56 μ M and that of *trans*-decalin-1-one was 0.5 mM.

(6) The dehydratase partial reaction and the enoyl reductase partial reaction were assayed as described by Muesing & Porter (1975), except that 9 mM-*S*-hydroxybutyrylpantetheine was used as substrate for the dehydratase partial reaction.

(7) The palmitoyl thioesterase partial reaction was assayed as described by Smith *et al.* (1976).

(8) The acetyl-CoA-independent activity of FAS was assayed spectrophotometrically by 'assay d' of Kresze *et al.* (1977).

Protein was measured by the method of Lowry *et al.* (1951), with bovine serum albumin as standard and by absorbance at 280 nm. An absorbance of 0.89 was determined for a 'standard' 1.0 mg/ml solution of FAS from rat mammary gland by Libertini & Smith (1979). Agreement between the two methods was within 10%. The dye-binding method of Bradford (1976), which we used in previous studies (Clements *et al.*, 1979), was found to overestimate the amount of FAS protein by a factor of two compared with the Lowry and A_{280} assays.

For measuring radioactivity, samples were dissolved in Tritonol and then equilibrated to 4°C before scintillation counting.

Results and discussion

Partial reactions strongly inhibited by BDB-CoA

Of the seven partial reactions catalysed by FAS, only the inhibition of the β -oxoacyl synthase reaction closely parallels the loss in activity of the overall reaction (Table 1, Fig. 1). The activity of the β -oxoacyl synthase reaction is low and difficult to measure precisely with the isotope-exchange method (Smith *et al.*, 1976). Nonetheless, it is clear that the β -oxoacyl synthase reaction is strongly inhibited by chemical modification of FAS with BDB-CoA (Fig. 1). An alternative method of assessing the activity of the β -oxoacyl synthase reaction is to measure a reaction dependent on the decarboxylation of malonyl-CoA (Kresze *et al.*, 1977). As shown in Fig. 1, the acetyl-CoA-independent activity of FAS is lost almost in parallel with the loss of the overall reaction on treatment with BDB-CoA. These results indicate that a critical step in the condensation mechanism is blocked by treatment with BDB-CoA.

The condensation reaction is known to involve two thiol moieties, i.e. the enzyme-bound pantotheine thiol group and the 'peripheral' cysteine thiol group, both of which are susceptible to alkylation by thiol-specific reagents. Iodoacetamide preferentially reacts with the 'peripheral' cysteine thiol group (Oesterhelt *et al.*, 1977; Stoops & Wakil, 1981), *N*-ethylmaleimide apparently reacts with the pantotheine thiol group (Oesterhelt *et al.*, 1977) and Nbs₂ reacts with both (Stoops & Wakil, 1982). It is likely that BDB-CoA can alkylate one or both of these classes of thiol groups.

Partial reactions weakly inhibited in labelled FAS

Compared with the loss of activity for the overall reaction, the activities of the three partial reactions β -oxoacyl reductase, β -hydroxyacyl dehydratase and enoyl reductase are inhibited to a limited extent by labelling FAS with BDB-CoA (Table 1). To

Table 1. *Effect of modification with BDB-CoA on the partial reactions catalysed by FAS*

The percentage inhibition is that obtained after incubation of FAS with 60 μ M-BDB-CoA for 1.0 min. See Fig. 1 for examples of these experiments. The experimental range in the values for percentage inhibition is of the order of $\pm 3\%$.

Activity	Inhibition (%)
Overall reaction	83
Partial reactions	
Acetyltransferase	0
Malonyltransferase	0*
β -Oxoacyl synthase	76†
β -Oxoacyl reductase	24
β -Hydroxyacyl dehydratase	17
Enoyl reductase	26
Palmitoyl thioesterase	0

* Malonyl transferase is activated at the lower BDB-CoA concentrations tested, but partially inhibited by concentrations higher than 60 μ M (Clements *et al.*, 1979).

† This value is based on measurements with the 'decarboxylase' assay (see Fig. 1).

Fig. 1. *Effect of BDB-CoA on the β -oxoacyl synthase and enoyl reductase partial reactions*

FAS was incubated with the indicated concentration of BDB-CoA for 1.0 min as described in the Materials and methods section. In the first experiment, 50 μ l portions of the quenched incubation mixture were assayed for β -oxoacyl synthase activity (○) and 25 μ l portions were assayed for overall enzyme activity (x). In the second experiment, assays of the acetyl-CoA-independent activity (●) and the overall enzyme activity (□) were performed on 25 μ l portions of the quenched incubation mixture. In the third experiment, assays of the enoyl reductase activity (■) were performed on 25 μ l portions of the quenched incubation mixture.

illustrate the difference in the inactivation of the overall reaction and these partial reactions, data from experiments that tested the enoyl reductase activity are shown in Fig. 1. Other workers have observed slight inhibition of these partial reactions in FAS treated with thiol reagents (Stoops & Wakil, 1981).

Partial reactions not inhibited by BDB-CoA

Of the seven partial reactions catalysed by FAS, three are not inhibited by modification with low concentrations of BDB-CoA; these are the acetyltransferase, malonyltransferase and palmitoyl thioesterase activities (Table 1). It is noteworthy that the catalytic site for each of these partial reactions binds a CoA compound. [Palmitoyl-CoA can serve as an alternative substrate for the palmitoyl thioesterase partial reaction of FAS from rat mammary gland (Lin & Smith, 1978)]. These catalytic sites would seem to be the logical targets for BDB-CoA, if the reagent were acting as an affinity label for an acyl-CoA/CoA-binding site. However, it is clear that the chemical modification does not involve the sites for these three partial reactions.

Our recent data show that acetyltransferase is not affected by modification of FAS with BDB-CoA (Table 1). This result is in contrast with our preliminary report (Clements *et al.*, 1979) and the observation of Katiyar *et al.* (1982) with the pigeon liver enzyme. Our previous result was obtained with the isotope-exchange assay, which we subsequently found was an unreliable procedure because a linear relationship between [enzyme] and product formation was observed for only a very narrow range of [enzyme], if at all. In contrast, the spectrophotometric assay meets the tests for a proper enzyme-based assay.

The observation that palmitoyl thioesterase is not affected shows that the 'active serine' required for catalysis of this partial reaction is not derivatized by BDB-CoA. This 'active serine' does react with phenylmethanesulphonyl fluoride and di-isopropyl fluorophosphate (Kumar, 1973; Bedord *et al.*, 1978; Dileepan *et al.*, 1978).

BDB-CoA does not cause FAS to dissociate

If FAS from rat mammary gland is dissociated from the dimer to monomers, the ability to catalyse the overall reaction is lost (Smith & Abraham, 1971). Thus it is possible that BDB-CoA may produce the observed inhibitory effects by causing FAS to dissociate. A series of ultracentrifugation experiments was performed to test this point, generally by the method described by Smith & Abraham (1971). These studies showed that the enzyme species present during the chemical modification process is the dimer.

Stoichiometry of BDB-CoA incorporation

The stoichiometry of covalent incorporation of BDB-[G-³H]CoA was determined by the general procedure given in the legend to Table 2. As shown in Fig. 2, extrapolation of measured points leads to a limit stoichiometry of 6 mol of BDB-CoA per mol of FAS (dimer) for totally inhibited FAS. This stoichiometry is larger than that reported on the basis of preliminary studies (Clements *et al.*, 1979); however, in the present work the protein assay was more accurate by a factor of two.

Protection by added substrate, thiol reagent or analogue

The results summarized in Table 1 suggest that BDB-CoA acts like a simple thiol-specific reagent toward FAS. It was quite possible, in our opinion, that BDB-CoA was bound at the acyltransferase catalytic site(s) before the postulated alkylation of thiol groups essential to the condensing reaction. As a means of determining whether BDB-CoA alkylates pantetheine and/or peripheral cysteine thiol groups and whether formation of a Michaelis-Menten complex precedes the alkylation reaction, three different sets of protection experiments were performed.

Fig. 2. A plot of the extent of irreversible inhibition versus BDB-[G-³H]CoA incorporated (mol/mol of FAS). After incubation for 1.0 min with various concentrations of BDB-[G-³H]CoA and gel-filtration chromatography, assays for overall enzyme activity, protein and radioactivity were performed as described in the Materials and methods section.

Table 2. *Effect of acylation of FAS on inhibition by BDB-CoA*

In each case FAS (5.5 mg/ml; specific activity approx. 1.2 units/mg) in 0.25 M-potassium phosphate, pH 6.5, was incubated at 37°C for 5 min with $20 \times K_m$ (with respect to each substrate) of acetyl-CoA or malonyl-CoA. The enzyme was separated from reactants by chromatography on a Sephadex G-50 column in the above buffer (see the text). The stoichiometry for acyl-FAS formation was 2.15 ± 0.29 (mean $\pm$ s.d.) mol of acetate and 1.18 ± 0.09 mol of malonate incorporated/mol of FAS (dimer) under these conditions. The acyl-FAS (1.2 mg/ml) was then incubated with the indicated concentration of BDB-CoA at 37°C for 1.0 min, and quenched (see the Materials and methods section). Data are means $\pm$ s.d. for the numbers of observations shown in parentheses.

BDB-CoA (μ M)	FAS	Inhibition (%)			
		Acetyl-FAS		Malonyl-FAS	
		Protection (%)*		Protection (%)*	
10	64 $\pm$ 11 (5)	45 $\pm$ 1 (2)	30	48 $\pm$ 5 (3)	25
20	75 $\pm$ 8 (4)	60 $\pm$ 0 (2)	20	61 $\pm$ 5 (3)	19
30	84 $\pm$ 5 (5)	74 $\pm$ 0 (2)	12	74 $\pm$ 2 (2)	12

* Protection was calculated as follows:
$$\frac{(\% \text{ inhibition})_{\text{FAS}} - (\% \text{ inhibition})_{\text{Acyl-FAS}}}{(\% \text{ inhibition})_{\text{FAS}}} \times 100$$

Acetyl-CoA, butyryl-CoA and malonyl-CoA covalently modify FAS, i.e. acyl-FAS is an obligatory intermediate in the overall reaction, and thus substrate protection should occur due to the formation of acyl-FAS. As shown in Table 2, acylation of FAS does provide a partial protection against the inhibitor. The observed protection is particularly significant at the lowest concentration of BDB-CoA tested. The data in Table 2 indicate that malonyl-FAS and acetyl-FAS are protected to the same extent. This latter observation lends support to the suggestion that the thiol of enzyme-bound pantotheine is alkylated by BDB-CoA, that is, both acetyl-CoA and malonyl-CoA transfer acyl units to the thiol group of pantotheine; thus both can provide protection.

Acylation of FAS was also shown to decrease the incorporation of BDB-[G-³H]CoA. Under the conditions described in the legend to Table 2 FAS was pretreated with $20 \times K_m$ (for the respective substrates) of acetyl-CoA (440 μ M) or butyryl-CoA (60 μ M), chromatographed and then treated with 30 μ M-BDB-[G-³H]CoA. The measured incorporations were 3.3, 2.8 and 2.1 mol of reagent/mole of dimer for untreated FAS, butyryl-FAS and acetyl-FAS respectively.

Stoops & Wakil (1982) have recently reported that treating FAS with 10 μ M-Nbs₂ rapidly inhibits enzymic activity and that the inhibition is a consequence of mixed disulphide bond formation between pantotheine and 'peripheral' cysteine thiol groups. If the essential thiols in the condensing partial reaction are oxidized to disulphide bonds they will not be alkylated, and thus treatment with Nbs₂ should protect FAS against irreversible inhibition by BDB-CoA. By the procedure described in the legend to Table 2, we found that incubating FAS

(1.0 mg/ml) with 10 μ M-Nbs₂ for 1.0 min resulted in approx. 70% inhibition of the overall reaction and that activity was restored to the initial value after quenching (see the Materials and methods section) for 30 min. Pre-incubation of FAS with acetyl-CoA (0.5 mM) protected against inhibition by Nbs₂, as observed by Stoops & Wakil (1982). Under the same conditions, 60 μ M-BDB-CoA caused an irreversible inhibition of approx. 80% of the initial activity. When FAS was treated with 10 μ M-Nbs₂ for 1.0 min followed by 60 μ M-BDB-CoA for 1.0 min, the enzyme was irreversibly inhibited only to the extent of approx. 50%. Thus pre-incubation with Nbs₂ partially protects FAS against BDB-CoA.

If the inhibitory process involves the obligatory formation of a Michaelis-Menten complex before the alkylation reaction, then the presence of ligands that bind reversibly at acyl-CoA/CoA-binding sites would protect against the irreversible inhibition. S-Acetyl-CoA (Rubenstein & Dryer, 1980) and dethio-CoA (Grinnell & Nishimura, 1969) are appropriate analogues for testing this postulate since they bind at acyl-CoA/CoA-binding sites but will not activate the pantotheine or 'peripheral' cysteine thiol groups. By using the conditions cited in the legend to Table 2, a series of concentrations (0, 113, 226, 316, 450 and 770 μ M) of each analogue was tested for protection against 30 μ M-BDB-CoA. The average of the 12 measurements of 'percentage inhibition' was $77 \pm 2\%$; thus no protection was observed. Therefore the primary inhibitory process in this system appears to be the chemical modification of essential thiol groups via a simple bimolecular alkylation reaction

Concluding comments

Overall, the studies summarized in the present

paper indicate that BDB-CoA acts as a simple alkylation reagent toward catalytically essential, chemically reactive thiol groups in FAS. The protection studies show that the pantetheine and (possibly) 'peripheral' cysteine thiol groups are sites of alkylation. However, FAS is only 65–70% inhibited when 4 mol of BDB-CoA are incorporated/mol of FAS (Fig. 2); thus alkylation appears to occur also at additional, unknown sites. It is clear that BDB-CoA does not perform as a classical affinity label in this system and it thus will have limited use in identifying specific binding sites on FAS.

Recent preliminary reports show that BDB-CoA has been used as an affinity label with other enzymes known to contain a chemically reactive thiol group, i.e. pyruvate dehydrogenase (Lawlis *et al.*, 1981), FAS from pigeon liver (Katiyar *et al.*, 1982) and succinate thiokinase (Nishimura *et al.*, 1980). Convincing evidence that BDB-CoA is a classical affinity label has appeared thus far only for succinate thiokinase (Nishimura *et al.*, 1980). Our studies have shown that the utmost care must be taken to demonstrate that BDB-CoA performs as the desired binding-site-directed irreversible inhibitor, rather than as a simple alkylation reagent.

This work was supported by NIH grants CA 15196 (F.A.), RR 05690 (to Papanicolaou Cancer Research Institute) and GM 20077 (R.E.B.). R.E.B. was the recipient of an NIH Research Career Development Award (GM 00246). The authors are grateful to Dr. Soma Kumar and Dr. Stuart Smith, who each allowed us access to a manuscript before its publication.

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