Inhibition of the condensing component of chicken liver fatty acid synthase by iodoacetamide and 5,5'-dithiobis-(2-nitrobenzoic acid)

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Chicken liver fatty acid synthase is inhibited by the thiol-modifying reagents 5,5'-dithiobis-(2-nitrobenzoic acid) and iodoacetamide. Total inactivation of the activity for fatty acid synthesis requires the modification of about 8 of the nearly 50 freely accessible thiol groups per molecule. The differential binding of iodo[¹⁴C]acetamide to phenylmethylsulphonyl fluoride-modified enzyme in the absence and in the presence of excess acetyl-CoA shows complete modification of one cysteine-SH site of the condensing enzyme and partial modification of the pantetheine-SH site for a total of approx. 1.4 mol of iodoacetamide bound per mol of enzyme. The reaction of the enzyme with 5,5'-dithiobis-(2-nitrobenzoic acid) generates disulphide cross-links for each molecule of the reagent added, but 95% of these cross-links are intrasubunit. Both the iodoacetamide- and 5,5'-dithiobis-(2-nitrobenzoic acid)-modified species catalyse all the component partial reactions of fatty acid synthesis except the condensation reaction. The results obtained with iodoacetamide show that in the dimeric fatty acid synthase modification of one cysteine-SH condensing site and/or one pantetheine-SH site per dimer is sufficient to affect inhibition of condensing activity and the activity for fatty acid synthesis, and are in accord with a recently proposed model for the mechanism of action of animal fatty acid synthases [Kumar (1982) *J. Theor. Biol.* **95**, 263–283].

Animal fatty acid synthases (M_r approx. 500 000) catalyse the synthesis of long-chain fatty acids, starting with acetyl-CoA, malonyl-CoA and NADPH (for review see Bloch & Vance, 1977). The biochemical steps pertaining to chain initiation by the yeast (Lynen, 1967) and avian enzymes (Phillips *et al.*, 1970) have been elucidated, and demonstrate that the process requires covalent transfer of acetyl groups to two thiol sites (cysteine-SH and pantetheine-SH) and a non-thiol site (serine-OH) and that the malonyl group transfers to a thiol site (pantetheine-SH) and a non-thiol site (serine-OH). During the elongation cycle, the intermediates remain covalently bound to the complex until the product is released (Lynen, 1967; Phillips *et al.*, 1970). The avian liver complex could be dissociated into half-molecular subunits by chemical modification (Yang *et al.*, 1967), or in low-ionic-strength medium under reducing conditions (Kumar *et al.*, 1970). The

dissociation at low ionic strength could be prevented by NADP⁺ or NADPH (Kumar & Porter, 1971). The subunits did not catalyse fatty acid synthesis or the critical condensation reaction.

On the basis of the stoichiometry of 1 mol of 4'-phosphopantetheine per approx. 500 000 g of enzyme (Smith & Abraham, 1970; Katiyar & Porter, 1977), animal fatty acid synthases were thought to have non-identical subunits. This idea appeared to find support in the genetic studies of yeast fatty acid synthase by Schweizer *et al.* (1973), who demonstrated the multifunctional nature of the non-identical subunits of the yeast enzyme, one of which contained covalently bound 4'-phosphopantetheine. However, Kumar (1973, 1975) observed a stoichiometry of 2:1 for selective inhibition of the thioesterase component of avian fatty acid synthase by PhCH₂SO₂F, indicating 2 mol of a thioesterase per mol of fatty acid synthase. A demonstrated stoichiometry of nearly 2 mol of 4'-phosphopantetheine per mol of fatty acid synthases from avian and mammalian liver (Arslanian *et al.*, 1976), goose uropygial gland (Buckner &

Abbreviations used: Nbs₂, 5,5'-dithiobis-(2-nitrobenzoic acid); PhCH₂SO₂F, phenylmethanesulphonyl fluoride.

Kolattukudy, 1976) and *Mycobacterium smegmatis* (Wood *et al.*, 1978) led to the suggestion of multifunctional homodimeric polypeptide chains of fatty acid synthases. Indirect support for homodimer structure also comes from other additional studies (Guy *et al.*, 1978; Smith & Stern, 1979; Poulouse & Kolattukudy, 1980).

Stoops & Wakil (1981) have reported that 1,3-dibromopropan-2-one inhibits chicken liver fatty acid synthase with a stoichiometry of approx. 1.5–1.8:1 and that the initial reaction leads to further cross-linking of cysteine-SH and pantetheine-SH sites of adjacent subunits. This means that, of the approx. 50 freely accessible thiol groups per dimer, the cysteine-SH site of the condensing enzyme is at least 100 times more reactive towards this reagent than is any other thiol group, including the pantetheine-SH site. On the basis of these observations, the dimer has been postulated to be functionally symmetrical, leading to the synthesis of 2 mol of fatty acid per mol of dimer in a single turnover (Wakil *et al.*, 1981). However, the studies reported in the present paper show that nearly 8 thiol groups per dimer react with two other thiol-modifying reagents, iodoacetamide and Nbs₂, at about the same rate for complete inhibition of enzyme activity. The reaction of iodo[¹⁴C]acetamide with PhCH₂SO₂F-modified fatty acid synthase in the presence and in the absence of excess acetyl-CoA shows complete modification of one cysteine residue and partial modification of a pantetheine residue for an average total of approx. 1.4 thiol groups modified per dimer. These data are consistent with a recently proposed model for the mechanism of action of animal fatty acid synthase (Kumar, 1982). A preliminary report of these findings has been published (Kumar & Varagiannis, 1982).

Materials and methods

Materials

Acetyl-CoA, malonyl-CoA, NADPH, dithiothreitol, Nbs₂ and iodoacetamide were obtained from Sigma Chemical Co. Iodo[¹⁴C]acetamide was obtained from New England Nuclear.

Preparation and assay of chicken liver fatty acid synthase

Chicken liver fatty acid synthase was prepared by the method of Srinivasan & Kumar (1976). Activity of the purified enzyme was 1600–1800 units (nmol of NADPH oxidized/min)/mg of protein at 25°C, which corresponds to an activity of approx. 3000 units if measurements were made at 37°C.

Reaction of fatty acid synthase with Nbs₂ and iodoacetamide

Before reaction with these reagents, the enzyme was freed of dithiothreitol by three repeated pre-

cipitations with (NH₄)₂SO₄ at 35% saturation. After the third precipitation, the pellet was dissolved in a minimum volume of 3 mM-EDTA/0.2 M-potassium phosphate buffer, pH 7.0, and fractionated on a Sephadex G-25 column equilibrated with the same buffer.

Reaction with Nbs₂ was carried out in 3 mM-EDTA/0.2 M-potassium phosphate buffer, pH 7.0, at 25°C at enzyme concentrations of 0.9 and 1.8 mg/ml and 1–5-fold molar excess of Nbs₂. The thiophenolate liberated was determined quantitatively by the method of Ellman (1959).

Reaction with iodoacetamide (radioactive or unlabelled) was carried out at 25°C in 3 mM-EDTA/0.2 M-potassium phosphate buffer, pH 7.0, at enzyme concentrations of 0.5 and 1.0 mg/ml. Samples were taken at different time intervals for assay of enzyme activity, and also when radioactive inhibitor was used 200 µg of the enzyme was precipitated with HClO₄, filtered, washed thoroughly with cold 10% (w/v) trichloroacetic acid and counted for radioactivity. The ratio of the mol of the inhibitor bound per mol is based on *M_r* 500000 for the dimer. PhCH₂SO₂F-modified enzyme, when needed, was prepared by treatment of the enzyme (1 mg/ml) with 1 mM-PhCH₂SO₂F. After the activity for fatty acid synthesis had reached an insignificant value, the enzyme was dialysed against 1 litre of 3 mM-EDTA/0.2 M-potassium phosphate buffer, pH 7.0, to remove excess of PhCH₂SO₂F.

Measurement of the component partial reactions of fatty acid synthesis

The reaction conditions and the methods of assay for the various component partial reactions have been described previously (Kumar *et al.*, 1970).

High-voltage-electrophoretic peptide maps of the peptic digests of fatty acid synthase containing covalently bound acetyl and malonyl groups

The following procedure was adopted for the quantification of the radioactivity bound to the peptic peptides. Enzyme (native or iodoacetamide- or Nbs₂-modified) (1 mg) in 3 mM-EDTA/0.2 M-potassium phosphate buffer, pH 7.0, at 0°C was treated with 500-fold molar excess of radiolabelled acetyl-CoA or malonyl-CoA for 30 s. The reaction was stopped with HClO₄ (5% final concn.). Since the reaction of the enzyme with Nbs₂ results in disulphide cross-links, which might alter the peptic peptide pattern, a different procedure was adopted in this case. After treatment of the Nbs₂-modified enzyme with radiolabelled acetyl-CoA, solid urea was added to a final concentration of 6 M, followed by the addition of dithiothreitol to a final concentration of 50 mM. Reduction of disulphide cross-links was allowed to proceed for 20 min at 25°C, after which the protein was precipitated with HClO₄.

The details of the experimental procedure for peptic digestion and high-voltage electrophoresis have been described previously (Kumar *et al.*, 1970).

Quantitative determination of carboxymethylcysteamine and carboxymethylcysteine

The enzyme (free of dithiothreitol) (0.7 mg/ml) was treated with 1 mM-iodo[^{14}C]acetamide for 25 min at 25°C in 3 mM-EDTA/0.2 M-potassium phosphate buffer, pH 7.0. The modification was stopped with 20 mM-dithiothreitol. The activity of the modified enzyme was 12% of the control activity. The modified enzyme was dialysed exhaustively against 10 mM- NH_4HCO_3 , pH 7.5, to remove all the unbound radioactivity. Enzyme (1.26 mg) was freeze-dried and hydrolysed with 1 ml of 6 M-HCl in an evacuated sealed tube. The labelled amino acids were separated on a Bio-Rad AG1-X8 anion-exchange resin in the acetate form with a linear gradient of 0.2 M-acetic acid–0.2 M-acetic acid/1 M-LiCl. The elution of radioactivity from the column was over 95%. Further separation and identification of the carboxymethyl derivatives was carried out by paper chromatography. The elution pattern as well as paper-chromatographic behaviour was also established with authentic carboxymethylcysteamine and carboxymethylcysteine.

Sodium dodecyl sulphate/polyacrylamide-gel electrophoresis

Gel-electrophoresis was performed in 5% acrylamide gels by the procedure of Laemmli (1970).

Results

Modification of chicken liver fatty acid synthase with Nbs_2

Inhibition of avian and yeast fatty acid synthases by thiol-modifying reagents has been previously demonstrated (Oesterhelt *et al.*, 1977; Nixon *et al.*, 1970), but the stoichiometry of this inhibition is not known. To explore this further, we have studied the reaction of the enzyme with two thiol-modifying reagents, Nbs_2 and iodoacetamide. The reaction with Nbs_2 is rapid and is complete within 1.5 min (Fig. 1a). The resulting loss of activity depends on the molar ratio of Nbs_2 to the enzyme (see Table 1). The inactivation process is reversible, since treatment of the Nbs_2 -inhibited enzyme with dithiothreitol results

Table 1. *Stoichiometry of the reaction of chicken liver fatty acid synthase with Nbs_2*

Enzyme (0.9 mg; 1.8 nmol) was incubated with Nbs_2 and assayed as indicated in the Materials and methods section.

Amount of Nbs_2 added (nmol)	Amount of thiophenolate obtained (nmol)	Stoichiometry of thiophenolate liberation (mol/mol of enzyme)	Activity remaining (% of control activity)
0.9	1.8	1.0	87
1.8	3.6	2.0	68
2.7	5.4	3.0	47
3.6	6.9	3.9	39
5.4	10.4	5.8	12
7.2	14.4	8.0	3.8

Fig. 1. Time course of (a) the reaction of chicken liver fatty acid synthase with Nbs_2 and (b) the recovery of activity when Nbs_2 -treated chicken liver fatty acid synthase is subsequently treated with dithiothreitol

(a) Time course of the reaction of fatty acid synthase (1.8 nmol) treated with a 2.5-fold molar excess of Nbs_2 in 3 mM-EDTA/0.2 M-potassium phosphate buffer, pH 7.0, at 25°C. The absorbance at 410 nm is plotted as a function of time. (b) Time course of the recovery of activity when the enzyme (0.9 mg/ml) was treated with 4-fold molar excess of Nbs_2 (final activity, 4% of the control) and was subsequently treated with 20 mM-dithiothreitol under the above experimental conditions.

in complete restoration of enzyme activity (Fig. 1b). The requirement for high concentration of dithiothreitol and the low rate of recovery of activity are indicative of the low redox potential of the oxidized thiol groups of the enzyme. A stoichiometric analysis of the loss of activity on reaction with Nbs_2 (Table 1 and Fig. 2) demonstrates that approx. 8 of the thiol groups of the enzyme need to be modified for complete inhibition of enzyme activity. Since the

Fig. 2. Plot of $(A/A_0)^{1/i}$ against the number of thiol groups per dimer of chicken liver fatty acid synthase reacting with 0–4-fold molar excess of Nbs_2 .

The enzyme (1.8 nmol) was treated with Nbs_2 as indicated in the Materials and methods section. After 5 min incubation, the absorbance was measured at 410 nm to determine the mol of thiophenolate liberated. At the same time, a sample was removed from the mixture for measurement of enzyme activity. In this Figure lines A and B have been calculated from the experimental data assuming $n = 10$ and $i = 2$ and 1 respectively.

activity of the condensing enzyme is the only partial reaction inhibited by Nbs_2 (Table 2), it appears that the reactivity of the cysteine-SH site of the condensing component is similar to that of a number of other thiol groups. Furthermore, under all conditions of this study (up to Nbs_2 /enzyme molar ratio of 4), 2 mol of the thiophenolate anion is produced per mol of Nbs_2 added (Table 1), indicating the formation of four internal disulphide bonds between the adjacent thiol groups of the enzyme for complete inhibition of activity for fatty acid synthesis.

The determination of the number of essential thiol groups was attempted according to the equation (Tsou, 1962; Norris & Brocklehurst, 1976):

$$m = n[1 - (A/A_0)^{1/i}]$$

where n , the total number of modified thiol groups per molecule, i , the number of thiol residues essential for activity, and m , the number of residues modified at any stage, are related to residual activity (A/A_0) . In Fig. 2 lines A and B have been calculated from the experimental data assuming $n = 10$ and $i = 2$ and 1 respectively. Line A, calculated by using $i = 2$, is linear and shows that the reaction of one molecule of Nbs_2 per dimer with the liberation of two molecules of thiophenolate anion is sufficient to inhibit the activity for fatty acid synthesis.

Since the internal disulphide bonds generated by using Nbs_2 can be intra- or inter-subunit, studies were undertaken to determine their position. The data in Fig. 3 show that the disulphide cross-links are mostly of intrasubunit character. At various Nbs_2 /enzyme molar ratios, a small percentage of larger-molecular-mass species (approx. 5% of the total), corresponding to the dimeric structure, can be seen. The intensity of the larger-molecular-mass band increases from 0% to about 5% with increasing Nbs_2 /enzyme molar ratios up to 5:1, but this does not correlate with the extent of inactivation of the enzyme. Stoops & Wakil (1982) report cross-linking of the cysteine-SH and pantetheine-SH sites of adjacent subunits by Nbs_2 . Since we did not observe any such cross-linking of the subunits, we wanted to establish whether cysteine-SH and pantetheine-SH sites are completely modified by Nbs_2 . This can be established from the extent of acyl transfer to the various binding sites of the dimer. The results of acetyl transfer to various sites are shown in Fig. 4. The electrophoretic peptic-peptide patterns arising from the regions of various sites have been established previously (Jacob *et al.*, 1968). On the basis of the results with the unmodified enzyme (results not shown), the data in Fig. 4 are consistent with the lack of acetyl transfer to the cysteine-SH site and significant transfer to the pantetheine-SH (30% of control) and serine-OH sites (100% of control) in the Nbs_2 -modified enzyme (Fig. 4). Similar results were obtained when the covalent transfer of malonyl

Table 2. Activities of the component reactions catalysed by the native and Nbs₂-modified chicken liver fatty acid synthase dimer

The enzyme was treated with 4-fold molar excess of Nbs₂ (see the Materials and methods section). In the condensation reaction octanoyl-CoA instead of hexanoyl-CoA was used. Specific activities are expressed as nmol of product formed/min per mg of protein. All reactions were carried out at 25°C.

Reaction	Specific activity		Activity (% of native control activity)
	Native enzyme	Nbs ₂ -modified enzyme	
Fatty acid synthesis	107	4.4	4.2
Acetyl-CoA : pantetheine transacylase	341	481	137
Malonyl-CoA : pantetheine transacylase	660	690	105
S-Acetoacetyl-N-acetylcysteamine reductase	1.38 × 10 ³	1.27 × 10 ³	92
DL-β-Hydroxybutyryl-N-acetylcysteamine dehydratase	8.8	9.8	111
Crotonyl-CoA reductase	5.1	4	78.4
Palmitoyl-CoA deacylase	37	43	116
Condensation-CO ₂ exchange	33.4	1.5	4.5

Fig. 3. Sodium dodecyl sulphate/polyacrylamide-gel electrophoresis of the native and Nbs₂-modified chicken liver fatty acid synthase

Each well contained 25 µg of the enzyme. Well (a), native enzyme; well (b), native enzyme that had reacted with a 5-fold molar excess of Nbs₂ and was then treated with 20mM-dithiothreitol in the presence of sodium dodecyl sulphate. Wells (c), (d), (e), (f) and (g) contained enzyme modified with Nbs₂ at molar ratios (Nbs₂/enzyme) of 1:1, 2:1, 3:1, 4:1 and 5:1 respectively.

group to the pantetheine-SH and serine-OH sites was estimated (results not shown). The data in Figs. 3 and 4 and Table 2 suggest that significant gross conformational changes that might exclude the acyl group from various covalent acyl-binding sites are not apparent, and that if the pantetheine-SH site(s) is

Fig. 4. High-voltage electrophoretic map of ¹⁴C-acetyl-peptides obtained by the peptic digestion of Nbs₂-modified chicken liver fatty acid synthase

The enzyme (1 mg/ml) was treated with 4-fold molar excess of Nbs₂. Other details are given in the Materials and methods section.

(are) in a disulphide linkage then such linkages must be intrasubunit.

The present studies reported on Nbs₂ modification of fatty acid synthase contrast with the data presented by Stoops & Wakil (1982). These authors report that 2mol of Nbs₂/mol is required for complete inhibition of chicken liver fatty acid synthase and that this inhibition results in the cross-linking of the condensing site of one subunit and the pantetheine-SH site of the adjacent subunit. The reason for the discrepancy between these sets of data is not apparent to us at present; however, our data are self-consistent and are supported by additional studies with iodoacetamide as the inhibitor of the condensing component.

Modification of PhCH₂SO₂F-modified chicken liver fatty acid synthase with iodoacetamide

Preliminary studies on the binding of iodoacetamide to chicken liver fatty acid synthase in the absence and in the presence of 1mM-acetyl-CoA gave a differential binding stoichiometry of approx. 1:1 for the inhibition of fatty acid synthesis (Kumar & Varagiannis, 1982). Since acetyl-CoA is hydrolysed at an appreciable rate by the enzyme, the lowering of the intrinsic acetyl-CoA concentration could decrease the protective effect of acetyl-CoA. In addition, the liberated CoA would react with iodoacetamide and thereby lower the effective concentration of iodoacetamide. The former is likely to give a higher-than-expected ratio of iodoacetamide bound in the presence of acetyl-CoA, whereas the latter effect is expected to lower that ratio. To avoid these uncertainties, we performed these experiments with PhCH₂SO₂F-modified fatty acid synthase. As shown previously, PhCH₂SO₂F inhibits the thioesterase function of fatty acid synthase without altering the catalytic properties of other component reactions (Kumar, 1975). In the modified state, the enzyme does not hydrolyse acetyl-CoA, and interference from the liberated CoA is not expected (Kumar, 1975). Fig. 5 shows the reaction of PhCH₂SO₂F-modified chicken liver fatty acid synthase with iodoacetamide. Since the PhCH₂SO₂F-modified enzyme does not catalyse fatty acid synthesis, the rate of loss of condensing

Fig. 5. Semi-logarithmic plot of the rate of loss of activity of the native or PhCH₂SO₂F-modified fatty acid synthase on treatment with iodoacetamide

Native or PhCH₂SO₂F-modified enzyme (0.5 mg/ml) was treated with 1mM-iodoacetamide in 3mM-EDTA/0.2M-potassium phosphate buffer, pH 7.0, at 25°C. Samples were taken at different time intervals to measure the activity for fatty acid synthesis of the native enzyme (□) or to determine the activity for the condensation-CO₂-exchange reaction catalysed by PhCH₂SO₂F-modified enzyme (■).

activity with time was measured and is presented as a semi-logarithmic plot. The rate of loss of activity of the native enzyme for fatty acid synthesis nearly parallels the loss of the condensing activity of the PhCH₂SO₂F-modified enzyme. These studies show that PhCH₂SO₂F modification has not altered the accessibility of iodoacetamide to the condensing site. Therefore a comparison of molar amounts of iodoacetamide bound to PhCH₂SO₂F-modified fatty acid synthase in the presence or in the absence of acetyl-CoA can give the true stoichiometry of the inhibition reaction. The results of such a study show (Table 3) that, after a 20 min incubation, 80% of the activity for fatty acid synthesis and the condensation reaction is lost (Fig. 5), and this corresponds to the differential binding of 0.85 mol of iodoacetamide per mol of dimer. After 30 min, when almost 90% of the activity is lost, this difference increases to 1.40 mol of iodoacetamide bound per mol of enzyme. These data are somewhat different from those previously published (Kumar & Varagiannis, 1982), and this shows the importance of using PhCH₂SO₂F-modified enzyme for these studies. These data also show that initial modification corresponds to a loss of fast-reacting thiol groups that are involved in fatty acid synthesis. At longer periods of time, reaction of iodoacetamide occurs at another site, which is also protected by acetyl-CoA.

Since both the cysteine-SH and pantetheine-SH sites are involved in the catalytic process, the additional site protected by acetyl-CoA is most probably the pantetheine-SH site. To obtain additional information concerning the site of iodoacetamide binding, the extent of acyl transfer to various covalent binding sites was determined in the iodoacetamide-modified species. In these studies, the concentrations of acyl-CoA esters were approx. 500-fold in excess over the enzyme to ensure

Table 3. Carboxamidomethylation of PhCH₂SO₂F-modified chicken liver fatty acid synthase dimer in the presence and in the absence of acetyl-CoA

PhCH₂SO₂F-modified dimer (0.5 mg/ml) was treated with 1.0mM-iodo[¹⁴C]acetamide in the absence or in the presence of 0.5mM-acetyl-CoA (AcCoA) in 3mM-EDTA/0.2M-potassium phosphate buffer, pH 7.0, at 25°C. Other details are given in the Materials and methods section.

Inactivation time (min)	Carboxamidomethyl groups bound (mol/mol of enzyme)			Activity of condensation reaction (% of control activity)
	AcCoA absent	AcCoA present	Difference	
10	2.74	2.35	0.39	45
20	4.63	3.78	0.85	20
30	6.85	5.45	1.40	7

Table 4. *Quantitative analysis of acetyl-binding sites and malonyl-binding sites of the native and iodoacetamide-modified chicken liver fatty acid synthase dimer*

Peptides belonging to various sites were separated on high-voltage electrophoresis and extracted before quantitative determination. In (a) below, the enzyme (0.5 mg/ml) was modified with 1 mM-iodoacetamide for 30 min under the conditions described in the Materials and methods section, and in (b) the enzyme (1 mg/ml) was modified with 1 mM-iodoacetamide for 50 min under the conditions described in the Materials and methods section.

Enzyme species	Acetyl group bound (mol/mol of enzyme)			Malonyl group bound (mol/mol of enzyme)	
	Cysteine-SH site	Pantetheine-SH site	Serine-OH site	Pantetheine-SH site	Serine-OH site
Native	0.93	1.24	1.0	1.4	1.4
Iodoacetamide-modified (a)	0	0.91	1.2	0.87	1.3
Iodoacetamide-modified (b)	0	0.8	1.28	0.84	1.45

Table 5. *Stoichiometry of iodoacetamide binding to cysteamine and cysteine residues of chicken liver fatty acid synthase*

Experimental details are given in the Materials and methods section. A 0.46 nmol portion of hydrolysed fatty acid synthase was placed on the anion-exchange column. The specific radioactivity of iodo[¹⁴C]acetamide in these experiments was 2443 d.p.m./nmol.

	Radioactivity bound		Iodoacetamide bound	
	(d.p.m.)	(% of total)	(nmol)	(mol/mol of dimer)
Cysteamine	472	6.6	0.193	0.419
Cysteine	6675	93.4	2.736	5.95

saturation of all acyl-binding sites. The radio-labelled peptides were isolated by high-voltage electrophoresis, and peptides corresponding to the region of the cysteine-SH condensing site, pantetheine-SH site and the serine-OH binding site were eluted and a quantitative determination of the bound radioactivity was made (Table 4). A 30 min incubation with iodoacetamide leads to complete loss of the transfer of acetyl group to the condensing site and a decrease of approx. 0.33 mol/mol of acetyl transfer to the pantetheine-SH-site peptides. A 50 min incubation gives a differential stoichiometry of acyl transfer to pantetheine of 0.44 mol/mol of dimer. The quantitative data do not reflect any significant decrease in the acyl transfer to the serine-OH site. Similar results are obtained when the covalent transfer of malonyl group is quantitatively determined (Table 4). The acyl transfer to the pantetheine-SH site is decreased by approx. 0.5 mol/mol whereas the extent of transfer to the serine-OH site is not appreciably altered.

The extent of the modification of cysteine-SH and pantetheine-SH sites by iodoacetamide was directly demonstrated by isolating and determining quantitatively labelled carboxymethylcysteine and carb-

oxymethylcysteamine. Since complete inactivation requires the modification of approx. 8 thiol groups per dimer (Kumar & Varagiannis, 1982), to which pantetheine modification would contribute approx. 0.4–0.5 thiol group, the distribution of label should be approx. 5.5% in carboxymethylcysteamine and approx. 94.5% in carboxymethylcysteine. This expectation is confirmed by the data in Table 5. The recovery of radioactivity in carboxymethylcysteamine and carboxymethylcysteine is 6.6% and 93.4% of the total respectively, and this corresponds to a stoichiometry of 0.4:1 and 6.0:1 for the modification of cysteamine and cysteine sites respectively. The results obtained support the results on the extent of acyl transfer to the covalent binding sites of iodoacetamide-modified enzyme (Table 4) and demonstrate that iodoacetamide modifies both the cysteine-SH and pantetheine-SH sites of fatty acid synthase.

Discussion

The results obtained in the present work with two thiol-modifying agents, Nbs₂ and iodoacetamide, contrast with the data reported by Stoops & Wakil (1981). For example, those authors report the following. (1) 1,3-Dibromopropan-2-one and Nbs₂ selectively modify only the cysteine-SH site(s) of the condensing component(s) whereas our data show that nearly 8 of the approx. 50 freely accessible thiol groups per dimer are modified at about the same rate to effect inhibition of the condensation reaction and fatty acid synthesis (Table 1). (2) Approx. 1.5–1.8 mol of 1,3-dibromopropan-2-one and 2 mol of Nbs₂ react per mol to give complete inhibition, and this has been interpreted to mean reaction at two cysteine-SH sites of the two condensing components of the dimer. Our data on the modification of PhCH₂SO₂F-modified fatty acid synthase by iodoacetamide show a stoichiometry of approx. 1.4 thiol sites from differential binding in the presence and in

the absence of excess of acetyl-CoA. In this modification, condensing activity is lost, acetyl transfer to the cysteine-SH site is not observed and covalent transfer to the pantetheine-SH site is partially diminished (Table 4). This pattern of modification is attested to by the determination of labelled carboxymethylcysteine and carboxymethylcysteamine (Table 5). (3) Stoops & Wakil (1981, 1982) postulated the cross-linking of the cysteine-SH site of the condensing component and the pantetheine-SH site of the adjacent subunit. The extent of inhibition is apparently related to the extent of cross-linking. We see very little cross-linking of subunits even when four cross-links are internally generated by reactions with Nbs₂ (Fig. 3).

On the basis of their data, Stoops & Wakil (1981, 1982) and Wakil *et al.* (1981) postulate that the subunits of dimeric fatty acid synthase are functionally identical, implying simultaneous synthesis of two fatty acyl moieties in a single turnover. 1,3-Dibromopropan-2-one has been suggested to react initially with the cysteine-SH site, followed by the reaction of the bound bromoacyl group with the pantetheine-SH site of the adjacent subunit. The value of limit stoichiometry of 1.5–1.8:1 is rationalized on the basis of the pantetheine content of the dimer and its availability to form a cross-linked dimer. However, this explanation appears to be somewhat unsatisfactory. If 1,3-dibromopropan-2-one reacts specifically and at a rapid rate with the condensing site, then the extent of inhibition of this component should reflect the modification of this site, since under the conditions of rapid inactivation the molar ratio of unmodified pantetheine will always be greater than that of the unmodified cysteine-SH site of the condensing component. For the stoichiometry of enzyme inhibition to correspond to the molar ratio of pantetheine, one would have to assume that the secondary reaction of the enzyme-bound bromoacyl group with the pantetheine is extremely rapid compared with the primary reaction. However, this remains to be demonstrated. Furthermore, these authors did not present any data for non-specific binding of the inhibitor, i.e. the binding of the inhibitor in the presence of excess acetyl-CoA. In the inhibition studies reported by Katiyar *et al.* (1982), 4 mol of 4-bromo-2,3-dioxobutyl-CoA/mol is needed to inhibit the condensing activity. However, further evidence suggests that this reagent reacts at other sites in addition to the catalytically important ones (Clements *et al.*, 1982). In view of these considerations, it is difficult to define the true stoichiometry of this inhibition. Furthermore, to prevent inactivation by these reagents, the pantetheine-SH and cysteine-SH sites on both the subunits would have to be completely saturated by acetyl-CoA. This means a limit stoichiometry of 4:1 for the covalent transfer of acetyl groups per dimer if

both the subunits are identical. Since the acetyl group is also transferred to the non-thiol site with high affinity, one would expect a minimum limit stoichiometry of at least 6 mol per mol of dimer. However, we find a limit stoichiometry of only 4:1 for the covalent transfer of acetyl group (Kumar, 1982). This limit stoichiometry has been confirmed for fatty acid synthases from other sources as well (Ahmad *et al.*, 1982; Stern *et al.*, 1982). This result is not consistent with the concept of functionally identical monomers.

The results obtained by us are consistent with a working hypothesis for the mechanism of action of animal fatty synthases that is based on the structural symmetry but induced functionally asymmetry of the dimers (Kumar, 1982). According to this model, component reactions that require the covalent transfer of substrates will elicit functional asymmetry. These reactions include overall fatty acid synthesis and the condensation reaction, and will be inhibited when 1 mol of the inhibitor is bound per mol of dimer, whereas component reactions monitored by using derivatives of *N*-acetylcysteamine or CoA, in which the model substrate is not covalently transferred to the enzyme, may require the binding of 2 mol of the inhibitor per mol of dimer for inhibition of that component reaction. Accordingly, when a component reaction is inhibited, the activity for fatty acid synthesis will decrease at a higher rate than the activity for that component reaction. A variety of evidence supports the proposed hypothesis. PhCH₂SO₂F inhibits the thioesterase function at a stoichiometric ratio of 2 mol per mol of dimer, but the activity for fatty acid synthesis is inhibited at a much higher rate than is the activity for the thioesterase function (Kumar, 1975). A report by Poulou & Kolattukudy (1980) on the inhibition of enoyl and oxoacyl reductase components by phenylglyoxal and butane-2,3-dione is consistent with the presence of reductase components on each of the subunits. But their data also show that the activity for fatty acid synthesis is lost at much higher rate than is the activity for either of the reductase components. In functionally identical subunits, one would expect the loss of fatty acid synthesis and loss of at least one of the reductase functions to follow the same time course. Additional strong evidence in favour of our hypothesis comes from the studies by Smith & Libertini (1979), who demonstrated that only 1 mol of palmitic acid is synthesized/mol in a dimer devoid of its thioesterase function. In an important recent study, Dodds *et al.* (1981) have shown that, whereas dimeric cow mammary-gland fatty acid synthase catalyses the reduction of acetoacetyl-CoA via acetoacetyl-enzyme, the subunits do not. Since the subunits have a functional pantetheine-SH site (Kumar *et al.*, 1970) and the reduction presumably takes place at this site,

functionally identical subunits should catalyse the reduction of acetoacetyl-CoA. However, according to our hypothesis, the subunits should not catalyse this reduction because it requires the covalent transfer of acetoacetyl group, which needs the participation of both the subunits present in a dimeric state.

The studies on iodoacetamide modification reported in the present paper support our working hypothesis for the mechanism of action of animal fatty acid synthases.

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References

- Ahmad, P. M., Feltman, D. S. & Ahmad, F. (1982) *Biochem. J.* **203**, 45–50
- Arslanian, M. J., Stoops, J. K., Oh, Y. H. & Wakil, S. J. (1976) *J. Biol. Chem.* **251**, 3194–3196
- Bloch, K. & Vance, D. (1977) *Annu. Rev. Biochem.* **46**, 263–298
- Buckner, J. S. & Kolattukudy, P. E. (1976) *Biochemistry* **15**, 1948–1957
- Clements, P. R., Barden, R. E., Ahmad, P. M., Chisner, M. B. & Ahmad, F. (1982) *Biochem. J.* **207**, 291–296
- Dodds, P. F., Guzman, M. G. F., Chalberg, S. C., Anderson, G. J. & Kumar, S. (1981) *J. Biol. Chem.* **256**, 6282–6290
- Ellman, G. L. (1959) *Arch. Biochem. Biophys.* **82**, 70–77
- Guy, P., Law, S. & Hardie, G. (1978) *FEBS Lett.* **94**, 33–37
- Jacob, E. J., Butterworth, P. H. W. & Porter, J. W. (1968) *Arch. Biochem. Biophys.* **124**, 392–400
- Katiyar, S. S. & Porter, J. W. (1977) *Life Sci.* **20**, 737–760
- Katiyar, S., Pan, D. & Porter, J. W. (1982) *Biochem. Biophys. Res. Commun.* **104**, 517–522
- Kumar, S. (1973) *Biochem. Biophys. Res. Commun.* **53**, 334–341
- Kumar, S. (1975) *J. Biol. Chem.* **250**, 5150–5158
- Kumar, S. (1982) *J. Theor. Biol.* **95**, 263–283
- Kumar, S. & Porter, J. W. (1971) *J. Biol. Chem.* **246**, 7780–7789
- Kumar, S. & Varagiannis, E. (1982) *Biosci. Rep.* **2**, 195–201
- Kumar, S., Dorsey, J. A., Muesing, R. A. & Porter, J. W. (1970) *J. Biol. Chem.* **245**, 4732–4745
- Laemmli, U. K. (1970) *Nature (London)* **227**, 680–685
- Lynen, F. (1967) *Biochem. J.* **102**, 381–400
- Nixon, J. E., Phillips, G. T., Abramovitz, A. S. & Porter, J. W. (1970) *Arch. Biochem. Biophys.* **13**, 372–379
- Norris, R. & Brocklehurst, K. (1976) *Biochem. J.* **159**, 245–257
- Oesterheld, D., Bauer, H., Kresze, G. B., Steber, L. & Lynen, F. (1977) *Eur. J. Biochem.* **79**, 173–180
- Phillips, G. T., Nixon, J. E., Dorsey, J. A., Butterworth, P. H. W., Chesterton, C. J. & Porter, J. W. (1970) *Arch. Biochem. Biophys.* **138**, 380–391
- Poulose, A. J. & Kolattukudy, P. E. (1980) *Arch. Biochem. Biophys.* **199**, 457–464
- Schweizer, E., Kniep, B., Castorph, H. & Holzner, U. (1973) *Eur. J. Biochem.* **39**, 353–362
- Smith, S. & Abraham, S. (1970) *J. Biol. Chem.* **245**, 3209–3217
- Smith, S. & Libertini, L. J. (1979) *Arch. Biochem. Biophys.* **196**, 88–92
- Smith, S. & Stern, A. (1979) *Arch. Biochem. Biophys.* **197**, 379–387
- Srinivasan, K. R. & Kumar, S. (1976) *J. Biol. Chem.* **251**, 5352–5360
- Stern, A., Sedgwick, B. & Smith, S. (1982) *J. Biol. Chem.* **257**, 799–803
- Stoops, J. K. & Wakil, S. J. (1981) *J. Biol. Chem.* **256**, 5128–5133
- Stoops, J. K. & Wakil, S. J. (1982) *Biochem. Biophys. Res. Commun.* **104**, 1018–1024
- Tsou, C.-L. (1962) *Sci. Sin.* **11**, 1535–1558
- Wakil, S. J., Stoops, J. K. & Mattick, J. S. (1981) *Cardiovasc. Res. Cent. Bull.* **20**, 1–23
- Wood, W. I., Peterson, D. O. & Bloch, K. (1978) *J. Biol. Chem.* **253**, 2650–2658
- Yang, P. C., Butterworth, P. H. W., Bock, R. M. & Porter, J. W. (1967) *J. Biol. Chem.* **242**, 3501–3507