Reaction of chloroacetyl-CoA with rabbit fatty acid synthase

A new method to label specifically and quantify pantetheine prosthetic groups

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The substrate analogue chloroacetyl-CoA inhibits fatty acid synthase by reacting with the 'central' or pantetheine thiol and not the 'peripheral' or β-ketoacylsynthase thiol as previously reported. This was demonstrated by the isolation of [^{14}C]carboxymethylcysteamine after acid hydrolysis of enzyme labelled with chloro[^{14}C]acetyl-CoA, and by the demonstration that more than one of the partial reactions is inhibited. This reagent now represents a simple and convenient tool both for quantification of the pantetheine thiol and for labelling this site for peptide mapping and isolation.

Fatty acid synthase *Pantetheine* *Active-site labelling*

1. INTRODUCTION

Mammalian fatty acid synthase is a remarkable multifunctional protein containing up to 7 active centres on 2 subunits of M_r 250 000 [1]. There has been controversy as to whether the enzyme is a homodimer, with all active centres on each polypeptide chain, or a heterodimer, with the active centres distributed between the 2 subunits, as is the case for yeast fatty acid synthase [2,3]. This problem has been studied by limited proteolysis [4,5] and measurements of the stoichiometries of different active centres [5–10]. The controversy has been fuelled, in part, by disagreements regarding the content of the phosphopantetheine prosthetic group, with values of 0.45–1.05 phosphopantetheine groups/subunit obtained [11–13].

The use of the substrate analogue chloroacetyl-CoA, which inhibited fatty acid synthase was described [14]; it was claimed to act by transferring the chloroacetyl group to the 'peripheral' thiol in the β-ketoacylsynthase [14]. We now report that this reagent acts not by this mechanism but by alkylation of the 'central' or pantetheine thiol.

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2. MATERIALS AND METHODS

2.1. *Materials*

Chloro[^{14}C]acetic acid and iodo[^{14}C]acetamide were from Amersham International (Bucks); CoA, NADPH and acetoacetyl *N*-acetyl cysteamine from Sigma Chemical Co. (Poole, Dorset); acetyl-CoA from the Boehringer Corporation (London); malonyl-CoA and acetoacetyl-CoA from P.L. Biochemicals (Milwaukee WI); and iodoacetamide from BDH Chemicals (Poole, Dorset). Fatty acid synthase was purified and assayed as in [15]. Acetoacetyl-CoA and acetoacetyl *N*-acetylcysteamine reductases were assayed as in [16].

2.2. *Reaction of fatty acid synthase with chloroacetyl CoA*

Chloroacetyl CoA was synthesised from chloroacetic acid as in [17]. Chloro[^{14}C]acetyl CoA was synthesised by the same method using chloro-[1-^{14}C]acetic acid as the starting material. Reversed-phase HPLC using isocratic elution from a Waters μ-Bondapak C-18 column eluted with 15% methanol, 85% 50 mM KH_2PO_4 (pH 5.3) showed that $<5\%$ free CoA was present in the products of the reaction. The concentration of chloroacetyl-CoA was determined assuming $E_{260\,nm}$ =

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15 400 $M^{-1} \cdot cm^{-1}$.

Fatty acid synthase (2 mg . ml^{-1}) in 125 mM sodium phosphate, 1 mM EDTA (pH 7.0) was reacted with different concentrations of chloro[^{14}C]-acetyl CoA (54 Ci . mol^{-1}) for 5 min at room temperature. Preliminary experiments showed that labelling was effectively complete at this time. Aliquots were removed for assay of enzyme activity and to determine the stoichiometry of the labelling reaction. The stoichiometry was determined by precipitating the protein by adding several volumes of 25% (w/v) trichloroacetic acid. The solution was kept in ice for 10 min, centrifuged in an Eppendorf microfuge (14 000 × *g*; 3 min), and the pellet was washed 3 times with 1 ml 25% trichloroacetic acid to remove unbound reagent. The pellet was then dissolved in 25 μl 90% formic acid, 1 ml Fisofluor MPC was added, and the sample counted on a Nuclear Chicago Isocap 300 scintillation counter. Protein concentration was determined assuming $A_{280}^{1\%} = 10.0$ [15]. All stoichiometries are quoted assuming a subunit $M_r = 250\,000$ [15].

To determine the amino acid residues modified by chloroacetyl-CoA, 100 μg fatty acid synthase (4 mg . ml^{-1}) in 125 mM sodium phosphate, 1 mM EDTA, 0.1 mM dithiothreitol (pH 7.0) was labelled by incubation with a 50-fold molar excess of chloro[^{14}C]acetyl-CoA (54 Ci . mol^{-1}) for 5 min at room temperature. The protein was then precipitated with trichloroacetic acid, washed, and hydrolysed in 6 M HCl at 105°C for 15 h in vacuo. After removing HCl under vacuum, the residue was transferred quantitatively to a thin-layer cellulose plate (Eastman Chromagram 13255 cellulose) and subjected to electrophoresis in 10% (v/v) pyridine, 0.5% (v/v) acetic acid (pH 6.5) at 300 V for 1 h. The dried thin-layer plates were autoradiographed and the developed autoradiogram was scanned using a densitometer attachment on a Pye-Unicam SP8-100 spectrophotometer.

2.3. *Reaction of fatty acid synthase with iodo[^{14}C]-acetamide*

Fatty acid synthase (110–150 μg) was precipitated with 25% (w/v) trichloroacetic acid and washed once with water. The protein pellets were then dissolved in 50 μl 10 M urea, and reacted with iodoacetamide using the method for carboxymethylation in [18] except that iodo[^{14}C]acetamide (2 Ci . mol^{-1}) was used instead of iodoacetic acid, and that excess iodoacetamide was removed by precipitating the protein by addition of 200 μl water and 250 μl 50% (w/v) trichloroacetic acid. Protein was recovered by standing in ice for 10 min and centrifuging (14 000 × *g*; 3 min) and the pellets were washed 3 times with 25% (v/v) trichloroacetic acid. Control experiments using radioactively labelled enzyme showed that this procedure would quantitatively precipitate fatty acid synthase in the presence of urea. The labelled protein was hydrolysed and electrophoresed as described above. Under these conditions carboxymethylcysteine, derived from reaction of iodoacetamide with cysteine, is negatively charged, and carboxymethylcysteamine derived from reaction of iodoacetamide with the terminal cysteamine in the pantetheine, is neutral, and the two compounds are readily separated. After electrophoresis, the plate was dried and autoradiographed. The radioactive spots migrating as carboxymethylcysteamine were scraped from the plate, digested with 1 ml of NCS tissue solubiliser, and counted in 10 ml Fisofluor MPC.

3. RESULTS AND DISCUSSION

As reported for the rat liver enzyme [14], incubation of purified rabbit mammary fatty acid synthase with increasing concentrations of chloroacetyl-CoA leads to progressively greater inhibition of enzyme activity (fig.1). In experiments using chloro[^{14}C]acetyl-CoA complete inhibition is obtained when 0.51 ± 0.06 (mean ± SEM for 3 enzyme preparations) molecules of chloroacetyl-CoA has reacted per enzyme subunit. To examine the nature of the residue which reacts with chloroacetyl-CoA, fatty acid synthase was labelled with excess chloro[^{14}C]acetyl-CoA and subjected to acid hydrolysis and electrophoresis. All the radioactivity migrates as carboxymethylcysteamine, and not carboxymethylcysteine (fig.2). This demonstrates clearly that the chloroacetyl moiety alkylates the pantetheine thiol and is released as carboxymethylcysteamine after acid hydrolysis. In [14] the β-ketoacylsynthase thiol was identified as the site of reaction by comparison of electrophoretic mobilities of peptic peptides derived from enzyme labelled with [^{14}C]acetyl-CoA chloro[^{14}C]acetyl-CoA. Perhaps the simplest explanation of their data is that the presence of the carboxymethyl

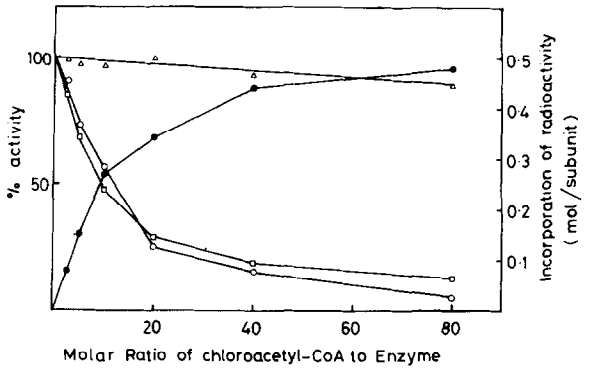

Fig.1. Inactivation of fatty acid synthase and partial activities: open symbols, activities of fatty acid synthase (circles), acetoacetyl-CoA reductase (squares) and acetoacetyl *N*-acetylcysteamine reductase (triangles) after incubation with the indicated molar ratio of chloro[^{14}C]acetyl-CoA to M_r 250 000 enzyme subunit. Also shown is the extent of modification of the enzyme as assessed by the incorporation of radioactivity.

group perturbs the peptic digestion and alters the charge of the peptides produced.

Of 4 partial reactions tested, only the β-ketoacylsynthase activity was inhibited by chloroacetyl-CoA [14]. However, of the assays used, only the β-ketoacylsynthase assay involved the endogenous pantetheine rather than an exogenous analogue. Our findings predict that all of the partial reactions would be affected when the physiological reactions using endogenous pantetheine are considered. To test this prediction, we examined the β-ketoacyl reductase activity using two different substrates. In [19], the acetoacetyl group of acetoacetyl-CoA was transferred to the pantetheine thiol before reduction, whereas acetoacetyl *N*-acetylcysteamine was reduced directly without such a transfer. Chloroacetyl-CoA should therefore inhibit reduction of the CoA ester but not the *N*-acetylcysteamine ester. Fig.1 reveals that this is indeed the case.

We propose that chloroacetyl-CoA acts by one or both of the two mechanisms shown in fig.3. Acetyl and malonyl groups are thought to be transferred to the pantetheine via an O-ester intermediate. Our data do not allow us to distinguish between direct reaction of chloroacetyl-CoA with the pantetheine thiol (left pathway) and reaction of a chloroacetyl O-ester intermediate (right pathway). However, in [14] radioactivity from chloroacetyl-

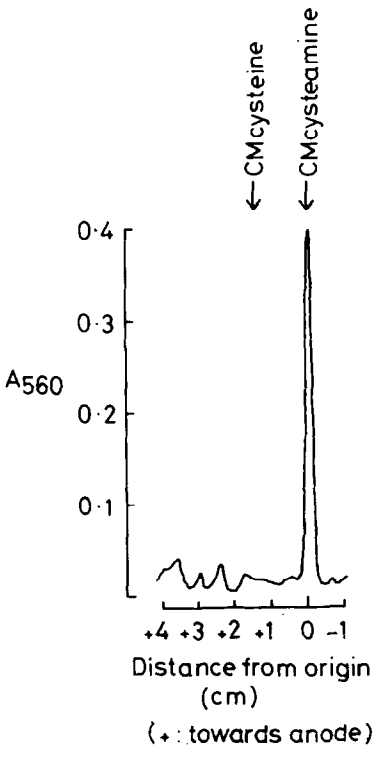

Fig.2. Nature of the residue modified by chloro[^{14}C]-acetyl-CoA. Modified enzyme was subjected to acid hydrolysis and electrophoresis as in section 2. Arrows show the migration positions of carboxymethylcysteine and carboxymethylcysteamine markers.

CoA (^{3}H[G]) was incorporated transiently into acid-stable linkage with the protein. This data would support the left pathway although not ruling out the possible simultaneous occurrence of the right pathway.

Since chloroacetyl-CoA caused complete inactivation of fatty acid synthase we investigated its utility as a method for quantification of the pantetheine thiol. The stoichiometry was determined using a large excess of chloroacetyl-CoA (table 1). We also quantified the pantetheine thiol by an independent method involving complete carboxamidomethylation of the protein using iodo[^{14}C]-acetamide in the presence of urea followed by acid hydrolysis, and isolation and counting of the [^{14}C]carboxymethylcysteamine produced. The results (table 1) are in excellent agreement with the much simpler chloroacetyl-CoA method, showing that the latter reagent does react quantitatively.

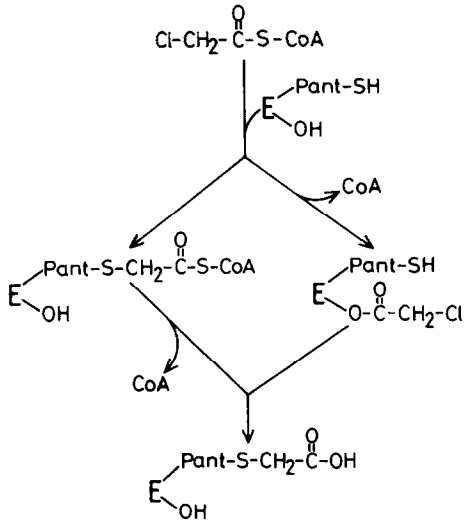

Fig.3. Proposed mechanism(s) of action of chloroacetyl-CoA. The hydroxyl indicated represents the loading site of the acyl transferase. The thiol represents the pantetheine thiol.

The stoichiometry obtained is less than that in [14]. This will be discussed more fully elsewhere, where data on the stoichiometry of other sites will be presented (in preparation).

Previous estimates of the phosphopantetheine content of fatty acid synthase have involved isolation and estimation of the β-alanine and/or taurine produced from the pantetheine after performic acid oxidation and acid hydrolysis. The phosphopantetheine has usually been first removed from the protein either by alkaline hydrolysis [12] or enzymically [13]. These procedures are lengthy and losses may occur during some of the steps. The only method that has been used to estimate the recovery of the products involves labelling of the pantetheine using injection of radioactive pantothenic acid in vivo [13].

Our method for quantification overcomes all of these problems and chloro[^{14}C]acetyl-CoA also provides a much cheaper, more rapid and convenient procedure for the specific labelling of the pantetheine thiol for peptide mapping or isolation.

Table 1

Stoichiometries of the pantetheine thiol measured using two independent radioactive procedures

	Preparation used			
Method	1	2	3	4
Chloro[^{14}C]acetyl-CoA	0.54	0.50	0.45	–
Iodo[^{14}C]acetamide	–	0.55	0.45	0.55

Stoichiometries are expressed per M_r 250 000 subunit

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