Stoichiometry of substrate binding to rat liver fatty acid synthetase

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(Received 13 February 1985/29 April 1985; accepted 14 May 1985)

Two rat liver fatty acid synthetase preparations, containing 1.6 and 2.0 mol of 4'-phosphopantetheine/mol of synthetase, showed specific activity of 2006 and 2140 nmol of NADPH oxidized/min per mg of protein respectively. The two synthetase preparations could be loaded with either 3.3–4.4 mol of [1-¹⁴C]acetate or 2.9–3.7 mol of [2-¹⁴C]malonate, by incubation with either [1-¹⁴C]acetyl-CoA or [2-¹⁴C]malonyl-CoA. The 4'-phosphopantetheine site could be more than 90% saturated and the serine site about 80% saturated with malonate derived from malonyl-CoA. However, with acetyl-CoA as substrate, binding at both the 4'-phosphopantetheine and cysteine thiol sites did not reach saturation. We interpret these results to indicate that, whereas the equilibrium constant for transfer of substrates between the serine loading site and the 4'-phosphopantetheine site is close to unity, that for transfer of acetyl moieties between the 4'-phosphopantetheine and cysteine sites favours formation of the 4'-phosphopantetheine thioester. Thus, despite the apparent sub-stoichiometric binding of acetate, the results are consistent with a functionally symmetrical model for the fatty acid synthetase which permits simultaneous substrate binding at two separate active centres.

Mammalian and avian fatty acid synthetases are multifunctional proteins that contain seven active centres on two identical *M*₂₅₀₀₀₀ subunits (McCarthy & Hardie, 1983; Stoops *et al.*, 1977). Early studies (Jacob *et al.*, 1968; Chesterton *et al.*, 1968; Phillips *et al.*, 1970*a,b*) demonstrated that acetate binds to three different sites on fatty acid synthetase, i.e. the 4'-phosphopantetheine site, the cysteine site and the serine site, whereas malonate transferred from malonyl-CoA was bound only to the 4'-phosphopantetheine and serine sites. Since the animal fatty acid synthetases consist of two identical subunits, it follows that the dimer should be capable of binding a maximum of six acetate or four malonate moieties. However, published estimates of the saturation-binding stoichiometries vary widely, and none agree with the theoretical maxima. Thus values as low as 0.7 mol of malonate and 1.5 mol of acetate (Joshi *et al.*, 1970) or as high as 4 mol of acetate and 2.5–6.5 mol of malonate (Kumar & Srinivasan, 1981; Kumar, 1982) per mol of enzyme have been reported for avian fatty acid synthetases. Assessment of the significance of

published binding stoichiometries is difficult, since the 4'-phosphopantetheine content of the enzyme preparations used is seldom reported. Values of this, published independently of binding data, vary between 1 and 2 mol/mol of enzyme, depending on the laboratory and the batch of enzyme. McCarthy & Hardie (1983) have reported both binding stoichiometries and prosthetic-group content for the same batch of enzyme. They found that acetate binding varied from 2.4 to 4.0 mol/mol of rabbit mammary fatty acid synthetase, depending on the specific activity of the enzyme; non-thiol binding, i.e. at the serine loading site, varied from 1.0 to 1.8 mol/mol, again depending on the specific activity of the enzyme. The 4'-phosphopantetheine content was determined as 1 mol/mol of enzyme, independent of the specific activity; clearly this value is incompatible with a structurally and functionally symmetrical dimer model.

To evaluate unequivocally substrate-binding stoichiometries in terms of a functionally symmetrical or asymmetrical model, it is essential that experiments are performed with enzyme that consists entirely of the holo form. In this study we have used a carefully controlled assay for 4'-

Abbreviation used: Cm-, carboxymethyl-

phosphopantetheine content and have identified a batch of rat liver enzyme suitable for substrate-binding studies.

Materials and methods

Materials

[1-¹⁴C]Acetyl-CoA was synthesized enzymically as described by Hansen *et al.* (1984); [2-¹⁴C]malonyl-CoA was synthesized as described by Rutkovski & Jaworski (1978). Both [1-¹⁴C]acetyl-CoA and [2-¹⁴C]malonyl-CoA were purified by h.p.l.c. as described by Hansen *et al.* (1984). The concentration of both substrates was determined by u.v. absorption by using an absorption coefficient of $14\,500\text{ M}^{-1}\cdot\text{cm}^{-1}$ at 260 nm. [1-¹⁴C]Acetate, [2-¹⁴C]malonate and [1-¹⁴C]iodoacetamide were obtained from The Radiochemical Centre, Amersham, Bucks., U.K. Mops and dithiothreitol were from Sigma Chemical Co., St. Louis, MO, U.S.A. CoA was from P-L Chemicals, Milwaukee, WI, U.S.A. All other chemicals were of analytical grade from E. Merck, Darmstadt, West Germany.

Determination of pantetheine content by carboxamidomethylation with [1-¹⁴C]iodoacetamide, hydrolysis and determination of [¹⁴C]Cm-cysteamine

Rat liver fatty acid synthetase was purified as described by Linn (1981). Fatty acid synthetase stored in 10.0 mM-dithiothreitol/0.25 M-potassium phosphate buffer (pH 7.0)/1.0 mM-EDTA/10% (v/v) glycerol was precipitated with (NH₄)₂SO₄ and dissolved in and dialysed against 100 mM-Mops/NaOH buffer (pH 7.0)/1 mM-dithiothreitol/1.0 mM-EDTA/10% glycerol.

Just before the binding experiment, the fatty acid synthetase (10 mg/ml) in 100 mM-Mops/NaOH (pH 7.0)/1.0 mM-dithiothreitol/1 mM-EDTA/10% glycerol was diluted to 1.0 mg/ml in 100 mM-Mops/NaOH (pH 7.0)/1.0 mM-EDTA which had been previously equilibrated with N₂ gas.

A 50 µl portion of the diluted fatty acid synthetase was added to 75 µl of 0.9 M-Tris/HCl (pH 9.0)/10 M-urea, which had been equilibrated with N₂ gas. All the above steps were performed at room temperature. A 50 µl portion of this solution was transferred to an Eppendorf tube wrapped in aluminium foil, and 10 nmol of [1-¹⁴C]iodoacetamide (sp. radioactivity 53 Ci/mol) was added in 10 µl. The tube was flushed with a gentle stream of N₂, closed and incubated for 90 min at 37°C. The reaction mixture was diluted 3-fold with water and the protein precipitated with an equal volume of 50% (w/v) trichloroacetic acid. The precipitate was washed once with 10% trichloroacetic acid; then 400 µl of 6 M-HCl, containing 0.01% (v/v) 2-mercaptoethanol, was added and the tube closed.

A small hole was punched in the side of the tube just below the cap, and the tube was placed on top of another Eppendorf tube containing 400 µl of 6 M-HCl inside a glass tube containing 400 µl of 6 M-HCl/0.01% mercaptoethanol. The glass tube was evacuated and sealed, and hydrolysis was carried out for 21 h at 110°C. After hydrolysis the sample tube was removed and freeze-dried. The freeze-dried sample was dissolved in an aqueous solution of 0.1% (w/v) sodium dodecyl sulphate, 0.5% (v/v) acetic acid and 0.01% (v/v) thiodiglycol, adjusted to pH 2.8 with HCl (buffer A).

³H-labelled Cm-cysteamine and Cm-cysteine were added as internal standards (0.04 and 2.0 nmol respectively; sp. radioactivity 106 Ci/mol). A portion (200 µl) of this solution was loaded on to a stainless-steel column (25 cm × 2.4 cm) packed with 10 µm Lichrosorb RP18. The column was equilibrated with a mixture of 96% buffer A and 4% buffer B; buffer B consisted of 0.03% sodium dodecyl sulphate, 0.1% acetic acid and 75% (v/v) methanol in water. Cm-cysteamine and Cm-cysteine were eluted with a gradient of buffer B, as shown in Fig. 3.

The radioactivity in the eluate was monitored with a Berthold LB 504 HS radioactivity detector equipped with an LB 5034 splitter, which diverted 10% of the effluent through a 200 µl flow cell. Data handling was done by a Berthold LB 500 M radiochromatographic system.

The peaks containing Cm-cysteine and Cm-cysteamine were collected and the radioactivity was determined as described by Marshall & Knudsen (1977). Correction for loss of [¹⁴C]Cm-cysteine and [¹⁴C]Cm-cysteamine during the h.p.l.c. was assessed from the recovery of the ³H-labelled internal standards. The reproducibility of the method was better than ± 5% between duplicate determinations.

Substrate binding

Fatty acid synthetase (25–30 mg/ml), stored in 0.25 M-potassium phosphate buffer (pH 7.0)/1.0 mM-EDTA/1.0 mM-dithiothreitol, was preincubated at 37°C for 30 min before use. The preincubated fatty acid synthetase (350 µg) was added to 100 mM-Mops/NaOH buffer (pH 7.0)/1.0 mM-EDTA at 0°C to make a volume of 325 µl; 70 nmol of either [1-¹⁴C]acetyl-CoA (sp. radioactivity 5–17 Ci/mol) or [2-¹⁴C]malonyl-CoA (sp. radioactivity 5–15 Ci/mol) was added in 25 µl to make a total volume of 350 µl. The sample was mixed by shaking for 2 s. Two 50 µl portions were quickly removed for determination of total binding and O-ester binding. The portions for total binding were mixed at 0°C with 2.5 ml of 10% (w/v) trichloroacetic acid and left for 30 min. The precipitated enzyme was collected on a Millipore RAWP filter

(1.2 μm pore size) and washed with 3×2.5 ml of 10% trichloroacetic acid at 0°C . The filters were air-dried and transferred to a liquid-scintillation vial with 10 ml of toluene/methoxyethanol (2:1, v/v) containing 5 g of 2,5-diphenyloxazole/l and 0.3 g of 1,4-bis(5-phenyloxazol-2-yl)benzene/l. The counting efficiency was determined as 73% by using $[1\text{-}^{14}\text{C}]$ carboxamidomethyl-fatty acid synthetase as internal standard. Corrections were made for blank values, derived the same way as described above except that trichloroacetic acid was added before the substrates. For determination of performic acid-stable binding (*O*-ester binding), 50 μl portions were mixed with 200 μl of performic acid (Hirs, 1956) and incubated at 0°C for 4 h. The performic acid-oxidized protein was precipitated, at 0°C , with 2.5 ml of 10% trichloroacetic acid, washed, and radioactivity was assayed as described above. The effectiveness of performic acid oxidation in destroying thioesters was measured by monitoring the oxidation of $[2\text{-}^{14}\text{C}]$ -malonyl-CoA by using h.p.l.c. as described by Hansen *et al.* (1984). The stability of *O*-ester to the treatment was checked by overnight incubation (see the Results section). Recovery of labelled enzymes in the precipitation steps was checked with $[^{14}\text{C}]$ carboxamidomethylated enzyme.

Determination of protein concentration

The protein concentration was determined from the A_{280} by using an absorption coefficient for fatty acid synthetase of $4.80 \times 10^5 \text{ M}^{-1} \cdot \text{cm}^{-1}$ (S. Smith, unpublished work) and an M_r of 480 000 (Smith & Abraham, 1970).

Results

The specific activities of enzyme preparations 1 and 2 were respectively 2006 and 2140 nmol of NADPH oxidized/min per mg of protein at 37°C . Although the specific activity was very similar, the 4'-phosphopantetheine content was different, 1.6 ± 0.1 and 2.0 ± 0.2 (mean $\pm$ s.d. for four and six determinations respectively) mol/mol of synthetase for preparations 1 and 2 respectively. The pantetheine determination by carboxamidomethylation with $[1\text{-}^{14}\text{C}]$ iodoacetamide was very sensitive to the pH during carboxamidomethylation (Fig. 1a) and time of incubation (Fig. 1b). At pH values lower than 8.5, alkylation of the 4'-phosphopantetheine thiol group was incomplete. Furthermore, 90 min incubation was necessary for complete alkylation of this group.

The performic acid oxidation of thioesters (as monitored with malonyl-CoA) was complete within 1 h of incubation at 0°C . Non-thiol-esterified malonate was stable overnight in the performic acid reaction mixture (Fig. 2).

Fig. 1. Effect of (a) pH and (b) incubation time on carboxamidomethylation of rat liver fatty acid synthetase 4'-phosphopantetheine with $[^{14}\text{C}]$ iodoacetamide.

The fatty acid synthetase was incubated with $[1\text{-}^{14}\text{C}]$ iodoacetamide as described in the Materials and methods section. Fatty acid synthetase preparations 1 and 2 were used for studying the effect of pH and incubation time, respectively. The values are means of duplicate determinations from typical experiments, $\pm$ half the difference (shown by bars).

Cm-cysteamine and Cm-cysteine were completely separated by h.p.l.c., with a base-line separation of about 30 min (Fig. 3).

Binding of $[2\text{-}^{14}\text{C}]$ malonate and $[1\text{-}^{14}\text{C}]$ acetate as a function of substrate concentration is shown in Fig. 4. With the two substrates the enzyme is saturated at substrate concentrations above $50 \mu\text{M}$ and $100 \mu\text{M}$ respectively.

Total binding, thiol- and non-thiol-binding of $[2\text{-}^{14}\text{C}]$ malonate to enzyme preparations 1 and 2 are shown in Table 1. Total binding depends on the pantetheine content of the enzyme preparation used. $[2\text{-}^{14}\text{C}]$ Malonate binds to the 4'-phosphopantetheine thiol group only, and not to the active-site

Fig. 2. Stability of malonyl-CoA and $[2-^{14}\text{C}]$ malonate-labelled fatty acid synthetase to performic acid oxidation. $[2-^{14}\text{C}]$ Malonate-labelled fatty acid synthetase and 50 nmol of $[2-^{14}\text{C}]$ malonyl-CoA (sp. radioactivity 15 Ci/mol) was incubated with 0.5 ml of performic acid reaction mixture for the indicated time. Malonate-labelled enzyme was collected on filters and counted for radioactivity (see the Materials and methods section). The extent of malonyl-CoA oxidation was measured by h.p.l.c. Samples (20 μl) were removed at the times indicated and injected directly on the high-performance liquid chromatograph and analysed as described by Hansen *et al.* (1984). The values are means of determinations $\pm$ half the difference and are typical of at least three independent experiments. Δ , Malonyl-CoA; $\circ$, $[2-^{14}\text{C}]$ malonate-labelled enzyme.

Fig. 3. Ion-pair reverse-phase h.p.l.c. separation of ^{14}C -labelled Cm-cysteine (1) and Cm-cysteamine (2). The sample (200 μl) was injected at zero time. The percentage of buffer B in the eluent is shown by the broken line. (For experimental details see the Materials and methods section.)

cysteine thiol group; total thiol binding of $[2-^{14}\text{C}]$ malonate is therefore equal to pantetheine binding. With both enzyme preparations more than 90% of the available pantetheine was loaded with malonate. Assuming a homodimer model, i.e. two

Fig. 4. Substrate-saturation curves for binding of $[1-^{14}\text{C}]$ -acetate and $[2-^{14}\text{C}]$ malonate to rat liver fatty acid synthetase incubated with $[1-^{14}\text{C}]$ acetyl-CoA (Δ) and $[2-^{14}\text{C}]$ malonyl-CoA ($\square$).

Rat liver fatty acid synthetase (specific activity 2245 nmol of NADPH oxidized/min per mg of protein) was incubated as described in the Materials and methods section. Values are means of duplicate determinations $\pm$ half the difference (shown by bars).

serine loading sites per dimer, we calculated that the serine sites were 73% and 81% saturated by malonate in enzyme preparations 1 and 2 respectively. The malonate binding in the present experiments, 2.9–3.6 mol/mol of enzyme (Table 1), is similar to the values reported for rat liver enzyme by Stern *et al.* (1982) and Ahmad *et al.* (1982), 3.0 and 3.5 mol/mol of enzyme respectively. The present values are higher than the values reported for malonate binding to chicken liver enzyme (Kumar, 1982) and rabbit mammary-gland enzyme (McCarthy & Hardie, 1983), 2.0 and 2.2–3.1 mol/mol of enzyme respectively.

Acetate binding to the two enzyme preparations varied from 3.3 to 4.4 mol of acetate bound/mol of enzyme. The highest binding was observed with enzyme preparation 2, which contained 2 mol of 4'-phosphopantetheine/mol of enzyme (Table 2). The ratio of non-thiol binding to total binding was independent of total binding (0.35–0.39).

The ratio of thiol to non-thiol binding of acetate found in the present experiment is the same as reported for acetate binding to chicken liver enzyme (Cognet & Hammes, 1983). However, McCarthy & Hardie (1983) reported a higher proportion of binding to non-thiol sites.

Total binding of both malonate and acetate was very reproducible within experiments, with good agreement between duplicates (Tables 1 and 2). However, binding of both substrates showed a greater variation between experiments carried out on different days, indicating that the actual conformation of the enzyme is critical to obtaining maximum binding (Tables 1 and 2).

Attempts to determine free pantetheine after loading of the enzyme with acetate failed, owing to

Table 1. $[2\text{-}^{14}\text{C}]\text{Malonate}$ binding to rat liver fatty acid synthetase

Fatty acid synthetase preparations 1 and 2 (specific activities 2006 and 2140 nmol of NADPH oxidized/min per mg of protein respectively) were incubated as described in the Materials and methods section. Values are means of duplicate determinations $\pm$ half the difference, from experiments performed on different days.

Enzyme preparation	Total binding (mol/mol of enzyme) (a)	Non-thiol binding (mol/mol of enzyme) (b)	Pantetheine binding (mol/mol of enzyme) (a - b)
1	2.97 $\pm$ 0.11	1.45 $\pm$ 0.05	1.52
	2.86 $\pm$ 0.05	1.45 $\pm$ 0.05	1.41
2	3.66 $\pm$ 0.05	1.68 $\pm$ 0.02	1.98
	3.34 $\pm$ 0.03	1.58 $\pm$ 0.01	1.76
	3.44 $\pm$ 0.02	1.59 $\pm$ 0.01	1.85

Table 2. $[1\text{-}^{14}\text{C}]\text{Acetate}$ binding to rat liver fatty acid synthetase

Fatty acid synthetase preparations 1 and 2 (specific activity 2006 and 2140 nmol of NADPH oxidized/min per mg of protein respectively) were incubated as described in the Materials and methods section. Values are means of duplicate determinations $\pm$ half the difference, from experiments performed on different days.

Enzyme preparation	Total binding (mol/mol of enzyme) (a)	Non-thiol binding (mol/mol of enzyme) (b)	Thiol binding (mol/mol of enzyme) (a - b)
1	3.98 $\pm$ 0.04	1.38 $\pm$ 0.08	2.60
	3.31 $\pm$ 0.07	1.30 $\pm$ 0.08	2.01
2	4.40 $\pm$ 0.01	1.62 $\pm$ 0.08	2.87
	4.43 $\pm$ 0.01	1.74 $\pm$ 0.01	2.60
	3.98 $\pm$ 0.01	1.53 $\pm$ 0.01	2.45
	4.02 $\pm$ 0.03	1.50 $\pm$ 0.03	2.52
	4.07 $\pm$ 0.03	1.52 $\pm$ 0.01	2.55

instability of the thioester-bound acetate during carboxamidomethylation. An additional complication arose from an observed transfer of 0.5 mol of $[1\text{-}^{14}\text{C}]\text{acetate}$ from thiol to non-thiol sites during the carboxamidomethylation procedure (results not shown).

Discussion

The functionally symmetrical homodimer model for fatty acid synthetase predicts a limiting stoichiometry for substrate binding of 4 malonyl moieties or 6 acetyl moieties per dimer (Singh *et al.*, 1984). Previous attempts to confirm experimentally the functional symmetry of the enzyme have been unconvincing, since the enzyme preparations used apparently contained sub-stoichiometric amounts of the 4'-phosphopantetheine prosthetic group (Singh *et al.*, 1984; McCarthy & Hardie, 1983). Often it has not been clear whether the low values reported resulted from the presence of some apoenzyme in the preparations or were a consequence of experimental error inherent in the difficult assay of 4'-phosphopantetheine. In this study, we have increased sensitivity of the analysis by using a radiolabelling procedure rather than relying on the commonly used colorimetric estima-

tion of β -alanine and taurine. Accuracy has been improved by establishing optimum conditions for complete radiolabelling and by carefully monitoring recoveries of the ^{14}C -labelled Cm-cysteamine product with an authentic ^3H -labelled internal standard. Using this approach we were able to identify one preparation of rat liver fatty acid synthetase which contained its full complement of 4'-phosphopantetheine, i.e. 2 mol/mol of enzyme dimer. Substrate-binding experiments with this batch of enzyme indicated that the fatty acid synthetase could be loaded with close to the theoretical saturating amount of malonyl moieties (4.0 theoretical, 3.5 observed). Saturation of the 4'-phosphopantetheine site was estimated to be 93% and that of the serine loading site 81%. It is not clear whether the slightly lower saturation at the loading site is a consequence of the 'loose' binding characteristic of this site. Substrate moieties bound at this site can be removed by transfer to CoA (Stern *et al.*, 1982) and by spontaneous hydrolysis. Cognet & Hammes (1983), who have also observed sub-stoichiometric binding at the serine loading site, have suggested that losses from this site may occur even when reactions are rapidly quenched by denaturation of the enzyme. Nevertheless the high degree of saturation of the two pairs of serine

loading and 4'-phosphopantetheine sites indicates clearly that both reaction centres can be loaded simultaneously. This conclusion supports the functionally symmetric 'head-to-tail' model suggested by Singh *et al.* (1984). The maximum binding of acetyl moieties observed, 4 mol/mol of dimer, is considerably lower than the theoretical limit of 6 mol/mol of dimer. This low value cannot be attributed to losses from the 'loose' serine site, since binding at this site was similar with acetate (79% saturation) and malonate (81% saturation). Clearly it is the binding at the thiol sites that is low, amounting to only 65% saturation.

We interpret these results to indicate that, whereas the equilibrium constant for malonylation/acetylation between the serine loading site and the 4'-phosphopantetheine thiol site is probably close to unity (at maximum saturation, molar fractions of malonyl-O-enzyme and malonyl-S-4'-phosphopantetheine-enzyme are 0.47 and 0.53 respectively), the equilibrium constant for acetyl-group distribution between 4'-phosphopantetheine and cysteine thiol sites favours acetyl-4'-phosphopantetheine formation. Since acetyl binding at the three sites is sequential (serine→pantetheine→cysteine), rather than independent, it follows that complete saturation of the cysteine site will not be achieved even at high acetyl-CoA concentrations. Indeed semi-quantitative assay of acetyl-S-4'-phosphopantetheine and acetyl-S-cysteine peptic peptides, derived from acetylated pigeon liver fatty acid synthetase, indicated the former species as predominant (Joshi *et al.*, 1970). Other published data suggest that the equilibrium for transfer between 4'-phosphopantetheine and cysteine thiols may favour strongly formation of the 4'-phosphopantetheine thioester for medium- and long-chain saturated acyl moieties as well as for acetyl moieties. Thus octanoyl moieties, synthesized from β -oxo-octanoyl-enzyme and NADPH, are distributed between 4'-phosphopantetheine and cysteine thiols in the proportion 0.83:0.17 (Nixon *et al.*, 1970); long-chain saturated acyl moieties synthesized on enzyme lacking thioesterase activity are located almost exclusively on the 4'-phosphopantetheine thiol (Smith & Libertini, 1979). The presence of only a small mole fraction of the saturated acyl-enzyme as the cysteine thioester may be limiting for the subsequent step, the condensation reaction. For condensation to occur, the acyl moiety must move to the cysteine thiol, vacating the 4'-phosphopantetheine thiol for ac-

cess by a malonyl moiety. It is generally accepted that the condensation reaction is rate-limiting for the overall fatty acid synthetase reaction. Perhaps the rate-limiting step for the condensation reaction is the transfer of the participating substrates, malonyl and saturated acyl moieties, to the appropriate binding sites.

The investigation was supported in part by grant AM 16073 from the National Institutes of Health.

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