Characterization of fatty acid synthase monomers restrained from reassociating by immobilization to a solid support

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The controversial question as to whether the ketoreductase activity of the animal fatty acid synthase is lost on dissociation of the homodimer has been addressed by using immobilized subunits which cannot reassociate under the conditions of assay. Ketoreductase activity, assessed with the model substrate *S*-acetoacetyl-*N*-acetylcysteamine, was identical in immobilized monomers and dimers, exhibiting normal Michaelis–Menten kinetics with K_m values in the millimolar range. When acetoacetyl-CoA was used as a substrate, however, biphasic kinetics were observed in the case of the dimer, with estimated K_m values in the micro- and milli-molar ranges, but only the high- K_m reaction was observed with the monomer. Thus when the ketoreductase activities of the monomer and dimer are assessed with acetoacetyl-CoA at concentrations sufficient to saturate only the low- K_m

reaction, it appears that the ketoreductase activity towards acetoacetyl-CoA is lost upon dissociation. Reduction of acetoacetyl-CoA via the low- K_m pathway is CoA-dependent, indicating that acetoacetyl-CoA can react with the dimer by two mechanisms: a high- K_m pathway analogous to that utilized by model substrates and a low- K_m pathway in which substrate and product are transferred between acyl-CoA and acyl-enzyme forms. The results indicate that the ketoreductase activity *per se* is unaffected by subunit dissociation and are consistent with a model in which the transfer of substrate from CoA ester to the acyl-carrier-protein domain necessitates juxtaposition of the transferase active-site serine residue of one subunit and the phosphopantetheine moiety of the adjacent subunit.

INTRODUCTION

The animal fatty acid synthase consists of two identical polypeptide chains of 272 kDa, both of which contain the seven activities required for the synthesis of palmitic acid from acetyl-CoA, malonyl-CoA and NADPH [1–3]. The reaction sequence is initiated by the transfer of acetyl and malonyl moieties to the active-site cysteine residue of the ketoacyl synthase and the 4'-phosphopantetheine of the acyl carrier protein respectively. Condensation of the acetyl and malonyl moieties results in the formation of a ketoacyl moiety on the 4'-phosphopantetheine. The acyl chain is then translocated through sites of keto reduction, dehydration and enoyl reduction and returned to the active-site cysteine residue as a saturated acyl moiety in preparation for condensation with a second malonyl moiety. Repetition of this series of reactions results ultimately in the formation of a saturated C_{16} acyl chain which is then released from the 4'-phosphopantetheine as free palmitic acid through the action of a thioesterase [4]. It is well established that the two subunits are oriented head-to-tail with the 4'-phosphopantetheine of one subunit located within 0.5 nm (5 Å) of the active-site cysteine residue of the ketoacyl synthase of the opposing subunit such that the condensation reaction requires participation of both subunits [5]. The animal fatty acid synthase can be reversibly dissociated into monomers which cannot catalyse the overall synthesis of fatty acids [6–8]. Although it is clear that the inability of the monomer to synthesize fatty acid must be attributable in part to the separation of the two critical elements of the ketoacyl synthase reaction, there is disagreement as to whether the monomer also lacks ketoreductase activity. Whereas Kumar et al. [9] found only a 30 % reduction in the ketoreductase following dissociation of the avian fatty acid synthase into its component subunits, Kashem and Hammes [8] found essentially complete inactivation and suggested that the loss in activity might be due to disruption of the NADPH-binding site in the

monomer. Similarly Dodds et al. [10] found that the ketoreductase activity associated with the mammalian fatty acid synthase was completely lost on dissociation. However, direct comparison of these studies is hampered by the fact that different substrates were used and in some cases the conditions used for assay (acetoacetyl-CoA, NADPH, phosphate and temperatures above 10 °C) promoted dimerization of the fatty acid synthase. In order to resolve this question unambiguously we have devised a novel system to study the activities associated with the fatty acid synthase monomer under conditions where reassociation is impossible. Our strategy involved the characterization of fatty acid synthase monomers which were restrained from reassociating by binding to immobilized anti-(thioesterase I) antibodies and comparison of the catalytic activities with those of similarly immobilized dimers.

EXPERIMENTAL

Materials

Acetyl-CoA, malonyl-CoA, palmitoyl-CoA and NADPH were obtained from Sigma (St. Louis, MO, U.S.A.) and CoA from Pharmacia–LKB (Piscataway, NJ, U.S.A.). *N*-Acetylcysteamine was prepared from *N,S*-diacetylcysteamine (Sigma) as described by Gerstein and Jencks [11]. Acetoacetyl-CoA and *S*-acetoacetyl-*N*-acetylcysteamine were synthesized by reaction of the appropriate thiol with diketene (Aldrich, Milwaukee, WI, U.S.A.) by the method of Moore and Jencks [12]. The concentrations of the acetoacetyl thioesters were determined from the absorbance at 310 nm of a solution in 67 mM Tris/ H_2SO_4 buffer (pH 8.1)/5 mM $MgSO_4$, a molar absorption coefficient of $9300\text{ M}^{-1}\cdot\text{cm}^{-1}$ [12] being used. $[1-^{14}C]$ Palmitoyl-CoA was obtained from Amersham (Arlington Heights, IL, U.S.A.) and iodo $[1-^{14}C]$ acetamide from du Pont–New England Nuclear (Boston, MA, U.S.A.). ATP:citrate-lyase [13] and fatty acid synthase [14] were purified from rat liver.

Preparation of immobilized fatty acid synthase dimers and monomers

CNBr-activated Sepharose 4B (Sigma) was coupled with the IgG fraction of anti-(thioesterase I) antiserum as described previously [15] with a yield of approx. 3 mg of IgG/ml of Sepharose. For immobilization of the fatty acid synthase dimer, typically 25 μ g of enzyme was mixed with 0.5 ml of IgG-Sepharose in 100 mM potassium phosphate buffer (pH 7)/1 mM EDTA (final volume 4 ml) for 30 min at 20 °C. The Sepharose was washed thoroughly to remove unbound enzyme and poured into a 1 cm $\times$ 10 cm Econo-column (Bio-Rad, Richmond, CA, U.S.A.), fitted with a flow adapter to minimize dead space. The column was placed in a water bath at 25 °C and equilibrated with 100 mM potassium phosphate buffer (pH 7)/0.1 mM dithiothreitol for at least 30 min prior to initiation of enzyme assays.

Fatty acid synthase monomers were prepared by cold-induced dissociation of the dimer (2–5 mg/ml) in 100 mM potassium phosphate buffer (pH 7)/1 mM EDTA/1 mM dithiothreitol at 4–6 °C [6]. After 7–9 days, when the enzyme was $\geq$ 90 % dissociated, the monomers were separated from the residual dimers by high-performance gel filtration on a column (9.4 mm $\times$ 250 mm) of Zorbax GF-250 (du Pont, Wilmington, DE, U.S.A.) at 0–4 °C. The isolated monomers (20–30 μ g) were immediately added to an ice-cold suspension of 0.5 ml of IgG-Sepharose in 100 mM potassium phosphate buffer (pH 7)/1 mM EDTA (final volume 4 ml) and mixed for 0–4 °C for 30 min. The Sepharose-coupled fatty acid synthase monomer preparation was washed using ice-cold buffers to minimize dimer formation from free monomers, poured into a column (1 cm $\times$ 10 cm) and equilibrated for at least 30 min with 100 mM potassium phosphate buffer (pH 7)/0.1 mM dithiothreitol at 25 °C.

Quantification of the amount of fatty acid synthase bound to the IgG-Sepharose was achieved using enzyme that had been labelled with [14 C]iodoacetamide (approx. 1 mol/mol of subunit) prior to immobilization [15]. Portions of the radio-labelled immobilized enzyme were dried under vacuum and radioactivity assayed by liquid-scintillation spectrometry.

Enzyme assays

Fatty acid synthase and ketoreductase assay systems for solution-phase enzyme contained 100 mM potassium phosphate buffer (pH 7), 1 mM dithiothreitol, 300 μ M NADPH, and either 60 μ M acetyl-CoA and 80 μ M malonyl-CoA (for overall fatty acid synthase) or 1 mM *S*-acetoacetyl-*N*-acetylcysteamine (for ketoreductase) at 25 °C. Assays for the immobilized enzyme were adapted essentially as described previously [15]. Substrates were introduced into the immobilized enzyme column via a multi-port manifold attached to a peristaltic pump. The flow rate was 0.6 ml/min.

Thioesterase activity of solution-phase and immobilized enzyme was assayed radiochemically by measuring the formation of [14 C]palmitic acid from [14 C]palmitoyl-CoA [16] in a 125 μ l assay system containing 100 mM potassium phosphate buffer (pH 7), 1 mM EDTA, 5 μ M [14 C]palmitoyl-CoA and 0.1–0.3 μ g of fatty acid synthase. BSA (8 μ g/ml) was included in assays of the free enzyme. Incubations were for 1 min at 25 °C.

The activity of acetoacetyl-CoA towards proteolytically derived transferase was assessed using pantetheine as the model acceptor [17]. The initial reaction mixture contained 100 mM potassium phosphate (pH 7), 1.7 mM pantetheine, 0.12 μ g (0.1 μ M) of isolated transferase and acetoacetyl-CoA, in a final volume of 25 μ l. The incubation was for 5–15 min at 20 °C. The

amount of CoA produced in the transferase reaction was then determined spectrophotometrically in a coupled reaction containing ATP: citrate-lyase and malate dehydrogenase, as described previously [17].

CoA-scavenging system

The effect of CoA depletion on the ketoreductase activity of fatty acid synthase dimer in solution with acetoacetyl-CoA as substrate was examined using ATP: citrate-lyase as a CoA scavenger [18]. Reaction mixtures contained 250 mM Tris/HCl buffer (pH 7.5), 5 mM dithiothreitol, 25 mM MgCl₂, 300 μ M NADPH, 50 mM potassium citrate, 7.5 mM ATP, 0–3 mM acetoacetyl-CoA, ATP: citrate-lyase (75 μ g) and fatty acid synthase dimer (5 μ g). All components except acetoacetyl-CoA and citrate were incubated for 5 min at 25 °C. Acetoacetyl-CoA was added, and the rate of decrease in A_{340} was monitored for 30–45 s. Citrate was immediately added to initiate the CoA-scavenging system, and the new rate of decrease in A_{340} was monitored for an additional 30 s. Essentially identical results were obtained when ATP: citrate-lyase was initiated with ATP instead of citrate. Control experiments with twice the normal amount of ATP: citrate-lyase showed no difference in observed rate, indicating that CoA depletion was essentially instantaneous using the amount of lyase indicated above.

RESULTS AND DISCUSSION

Immobilization of the fatty acid synthase dimer by binding to a Sepharose matrix containing antibodies that recognize specifically the thioesterase domain of the fatty acid synthase preserves the ability to synthesize fatty acids [15]. In this study we adapted the procedure so as to immobilize individual fatty acid synthase monomers which cannot catalyse the overall reaction of fatty acid synthesis. Based on the assays of the residual fatty acid synthase activity these immobilized fatty acid synthase preparations typically consisted of 88 % monomers and 12 % dimers (Table 1). When a column containing the immobilized fatty acid synthase monomers was perfused with 100 mM potassium phosphate buffer (pH 7)/0.1 mM dithiothreitol at 25 °C for up to 6 h, no increase in fatty acid synthase activity was observed. Since these conditions promote complete dimerization of solution-phase subunits with accompanying restoration of overall fatty acid synthase activity, it is clear that the tethered subunits are restrained from reassociating, presumably because of their spatial separation on the supporting matrix.

A previous study with immobilized dimers revealed that the kinetics of the overall reaction, assessed in a continuous-flow assay system, are not significantly affected by immobilization, and activity is not limited by movement of substrates through the Nernst diffusion layer surrounding the matrix particles. Nevertheless the specific activity of the immobilized dimer for the overall reaction was decreased 55–60 %, and the previous study had not addressed the question of whether the subunit tethered to the anti-thioesterase antibody might be compromised in its ability to catalyse the thioesterase reaction so that only one of the two sites for fatty acid synthesis in the dimer might be functional. Since in our studies with the immobilized monomer we would observe exclusively the activities associated with the tethered subunits, we thought it important to ascertain whether or not the conformation of these subunits was affected adversely by interaction of the antibody with the thioesterase domain. Thioesterase assays on the immobilized dimer and monomer preparations show that this activity was not compromised by association of the fatty acid synthase with the antibody. Indeed,

Table 1 Comparison of activities of the immobilized fatty acid synthase monomer with those of the immobilized and free dimer

Enzyme	Activity (nmol/min per mg of protein at 25 °C)		
	Dimer		Monomer, immobilized
	Free	Immobilized	
Fatty acid synthase	1226 ± 22 (2)	425 ± 15 (2)	53 ± 22 (6)
Thioesterase	223 ± 11 (2)	330 ± 13 (4)	412 ± 38 (6)
Ketoreductase (<i>S</i> -acetoacetyl- <i>N</i> -acetylcysteamine)	698 ± 11 (2)	693 ± 13 (2)	670 ± 33 (2)

Figure 1 Ketoreductase activity of immobilized fatty acid synthase monomers and dimers

Enzyme activity of immobilized monomers (open symbols) and dimers (filled symbols) was assayed using either acetoacetyl-CoA or *S*-acetoacetyl-*N*-acetylcysteamine as substrate. The reported activities attributable specifically to the fatty acid synthase monomers were calculated from the observed activities by correcting for the contribution of immobilized dimers in the preparation. This correction was made by first determining the fatty acid synthase activity of the immobilized enzyme preparation (attributable exclusively to dimers) and then calculating the corresponding reductase activities based on previous measurements of the activities of immobilized dimer preparations.

the thioesterase activities of the immobilized enzyme preparations was significantly greater than that of dimer in solution (Table 1). However, we believe this to be due to the extreme sensitivity of the assay to the total protein concentration in the assay medium. For example, BSA stimulates thioesterase activity presumably by binding the long-chain acyl-CoA substrates and reducing micelle formation [19]. In control experiments (not shown) we observed that anti-thioesterase IgG also stimulated thioesterase activity of the free fatty acid synthase, so it is possible that the apparently higher thioesterase activity observed with the immobilized enzyme may result from a non-specific effect of the IgG on the substrate. This uncertainty aside, since immobilization of the monomer requires all thioesterase domains be bound to the

Figure 2 Effect of CoA depletion on the reduction of acetoacetyl-CoA by solution-phase fatty acid synthase dimers

Enzyme activity of freely soluble fatty acid synthase dimers was assessed with acetoacetyl-CoA as substrate in the absence (▲), ATP omitted; (▲), citrate omitted or presence (○, ●) of an active scavenging system. The scavenging of CoA by ATP: citrate-lyase was initiated either by citrate (●) or ATP (○).

support, it is clear that interaction with the antibody does not affect the integrity of the thioesterase active site.

More important in the present study, the ketoreductase activity, assessed with the model substrate *S*-acetoacetyl-*N*-acetylcysteamine, was unaffected either by immobilization or by dissociation of the subunits (Table 1). Previous comparisons of the ketoreductase activity of the dimeric and monomeric forms of fatty acid synthase had utilized either *S*-acetoacetyl-*N*-acetylcysteamine [9] or acetoacetyl-CoA [8,10]. Unlike the model *S*-acyl-*N*-acetylcysteamine substrates, CoA thioesters can transfer their acyl moieties to the phosphopantetheine of the fatty acid synthase and thus can act as substrates for fatty acid synthesis [10,20]. We therefore carried out a detailed kinetic study of the ketoreductase associated with the immobilized dimer and monomer using both acetoacetyl-CoA and *S*-acetoacetyl-*N*-acetylcysteamine as substrates (Figure 1).

With the model compound *S*-acetoacetyl-*N*-acetylcysteamine as substrate standard Michaelis-Menten kinetics were observed and were indistinguishable between monomer and dimer. In contrast, kinetics for the reduction of acetoacetyl-CoA were quite different with immobilized monomer and dimer. Whereas Michaelis-Menten kinetics were observed for the reaction catalysed by the immobilized monomer (K_m of 0.7 mM), a non-hyperbolic substrate/velocity plot was observed for the reaction catalysed by immobilized dimer. These kinetic data suggested that acetoacetyl-CoA might be reduced via a single 'low-affinity' pathway by the monomer but via two pathways, one 'low-affinity', one 'high-affinity', by the dimer; an acetoacetyl-CoA K_m value of 15 μ M was calculated for the high-affinity pathway. To verify that the acetoacetyl moiety is an effective substrate for the transferase component of the fatty acid synthase, activity was assessed using the proteolytically derived transferase domain with pantetheine as the model acceptor [17]. At 1.7 mM pantetheine, the apparent K_m for acetoacetyl-CoA was 76 μ M

and the apparent $V_{\max}$ was $1.28 \mu\text{mol}/\text{min}$ per mg (results not shown). Thus, by comparison with previous results [17], the order of effectiveness of CoA thioesters as substrates for the transferase is: malonyl = acetyl > butyryl > acetoacetyl > hexanoyl.

If the apparently biphasic kinetics observed for acetoacetyl-CoA reduction by the fatty acid synthase dimer result from the ability of this substrate to present to the enzyme both as a covalently linked acetoacetyl-enzyme intermediate and as a 'model' substrate, then the former pathway (the 'high-affinity pathway'), but not the latter (the 'low-affinity pathway') ought to be dependent on the availability of free CoA as an acceptor for β -hydroxybutyryl moieties formed in the reaction. To test this possibility we utilized ATP:citrate-lyase as a CoA-scavenging system and studied the effect on the kinetics of acetoacetyl-CoA reduction by solution-phase fatty acid synthase dimers. When the CoA-scavenging system was inactive (either ATP or citrate omitted), non-hyperbolic kinetics were observed, similar to those observed using the immobilized dimer (Figure 2). When the CoA-scavenging system was activated, however, standard Michaelis-Menten kinetics were observed, similar to those observed with the immobilized monomers; the K_m for acetoacetyl-CoA under these conditions was found to be 1.4 mM . Thus, as predicted by the hypothesis outlined above, only the high-affinity pathway was blocked by CoA depletion. This experiment shows that acetoacetyl-CoA can be reduced via a low-affinity CoA-independent pathway as well as via the high-affinity CoA-dependent pathway that requires formation of a covalent acetoacetyl-enzyme intermediate. Furthermore, since in the CoA-scavenged system the transferase reaction is arrested once the acetoacetyl moiety is transferred to the 4'-phosphopantetheine of the fatty acid synthase, the presence of an acetoacetyl moiety at this site does not prevent direct reduction of acetoacetyl moieties via the CoA-independent pathway. In other words the two pathways of acetoacetyl-CoA reduction can function independently.

Some of the confusion in the literature surrounding the ketoreductase activity of the dissociated enzyme can now be clarified. Previous reports that ketoreductase activity is lost upon dissociation of the fatty acid synthase were based on experiments where acetoacetyl-CoA was used as substrate [8,10]. The substrate concentrations used were generally sufficient to saturate only the high-affinity pathway of acetoacetyl-CoA reduction in the dimer. The high-affinity, but not the low-affinity, pathway for acetoacetyl-CoA reduction is lost on dissociation of the fatty acid synthase dimer, giving the mistaken impression that the ketoreductase activity itself is lost. Our results indicate that

operation of the high-affinity pathway requires that there be free transfer of the substrate and product between acyl-CoA and covalently bound acyl-enzyme forms, and it is this activity that is lost in the monomer.

The results of the present study are entirely consistent with the recently refined model for the animal fatty acid synthase in which two centres for initiation, growth and termination of acyl chains are formed by the transferase, ketoacyl synthase and dehydrase of one subunit in co-operation with the acyl carrier protein, enoyl reductase, ketoreductase and thioesterase of a second subunit juxtaposed in head-to-tail orientation [21]. This model predicts that transfer of acyl moieties from CoA thioester via the transferase active-site of one subunit to the 4'-phosphopantetheine of the opposing subunit that forms part of the same centre for fatty acid synthesis will be compromised by dissociation of the enzyme, a prediction confirmed by the experimental results presented here.

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