Chemistry & Biology, Vol. 10, 169–173, February, 2003, ©2003 Elsevier Science Ltd. All rights reserved. DOI 10.1016/S1074-5521(03)00023-1

Engineering of an Active Animal Fatty Acid Synthase Dimer with Only One Competent Subunit

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Summary

Animal fatty acid synthases are large polypeptides containing seven functional domains that are active only in the dimeric form. Inactivity of the monomeric form has long been attributed to the obligatory participation of domains from both subunits in catalysis of substrate loading and condensation reactions. However, we have engineered a fatty acid synthase containing one wild-type subunit and one subunit compromised by mutations in all seven functional domains that is active in fatty acid synthesis. This finding indicates that a single subunit, in the context of a dimer, is able to catalyze the entire biosynthetic pathway and suggests that, in the natural complex, each of the two subunits forms a scaffold that optimizes the conformation of the companion subunit.

Introduction

The animal fatty acid synthase (FAS) is comprised of two identical multifunctional polypeptides of 272 kDa that catalyze the entire pathway for the biosynthesis of fatty acids from malonyl-CoA. Each subunit contains three N-terminal catalytic domains, β-ketoacyl synthase, malonyl/acetyl transferase, and dehydrase, separated by a core region of approximately 600 residues from four C-terminal domains, enoyl reductase, β-ketoacyl reductase, acyl carrier protein (ACP), and thioesterase. The same basic architectural design is also employed in the modular polyketide synthases [1]. Although the FAS dimer contains two centers for palmitate synthesis [2, 3], the monomeric form of the protein is inactive in the overall FAS reaction [4–6]. For two decades, the inability of monomers to synthesize fatty acids has been rationalized by a model in which the two polypeptides are depicted lying side-by-side in a fully extended, head-to-tail configuration, such that each of the two centers for palmitate synthesis requires cooperation between catalytic domains located in the N-terminal half of one subunit with those located in the C-terminal half of the opposite subunit [7–9] (Figure 1). This model has been supported primarily by evidence indicating that the substrate loading and condensation reactions can be catalyzed by cooperation of the ACP of one subunit with the malonyl/acetyl transferase and β-ketoacyl synthase domains of the companion subunit [7, 9]. Nevertheless, recent studies employing a combination of mutant complementation in vitro and crosslinking experiments have revealed that these reactions may also be catalyzed entirely by intrasubunit cooperation between domains [10, 11]. The same in vitro mutant complementation approach has provided evidence that the β-carbon processing and chain terminating reactions involving the ACP, dehydrase, enoyl reductase, β-ketoacyl reductase, and thioesterase domains can all take place on one subunit [12]. These findings call into question long-held assumptions regarding the need for two FAS subunits to catalyze the biosynthesis of long chain fatty acids and raise the possibility that, within the context of the dimer, a single subunit may be able to catalyze the entire series of reactions in the pathway. To test this possibility, we have engineered and characterized a heterodimeric FAS in which the catalytic activity of one subunit was compromised by mutations in all seven functional domains.

Results and Discussion

A His_6-tagged FAS functionally compromised in all seven domains (7KO-FAS) was engineered by combining mutations that individually had been found to inactivate each of the functional domains (Figure 2). Residues directly involved in forming covalent acyl enzyme intermediates were replaced: the β-ketoacyl synthase active-site cysteine [13], the malonyl/acetyl transferase active-site serine [10], and the thioesterase active-site serine [14]. Posttranslational insertion of the essential phosphopantetheine prosthetic group was blocked by replacement of Ser2151 within the ACP domain [15]. Catalytic activity of the dehydrase domain was compromised by replacement of His878 [16], and binding of NADPH to the two reductase domains was compromised by replacement of glycine residues within the nucleotide binding region [12]. The purified homodimeric 7KO-FAS was completely inactive in the overall FAS assay.

Subunits from the His_6-tagged 7KO-FAS and FLAG-tagged wild-type FAS were randomized, and the heterodimers consisting of one 7KO and one wild-type subunit (7KO/wt) were isolated by sequential application to anti-FLAG and Ni-NTA affinity columns (Figures 3A and 3B).

The specific activity of the 7KO/wt heterodimer in the overall FAS reaction (316 ± 9 nmol NADPH oxidized min^{-1} mg^{-1}) was 16% of that of the parental homodimeric wild-type FAS (1970 ± 32 nmol NADPH oxidized min^{-1} mg^{-1}). Since the 7KO/wt heterodimer has only one functional ACP domain, this value is equivalent to 32% of the activity of each of the two catalytic centers in a homodimeric wild-type FAS. Palmitic acid was the major product synthesized by both the wild-type and heterodimeric FASs, accounting for >80% of the total radioactivity in the free fatty acid fraction. However, a significantly higher amount of [1-^{14}C]acetyl-CoA was formed from [2-^{14}C]malonyl-CoA by the 7KO/wt heterodimer than by the wild-type homodimer, most likely be-

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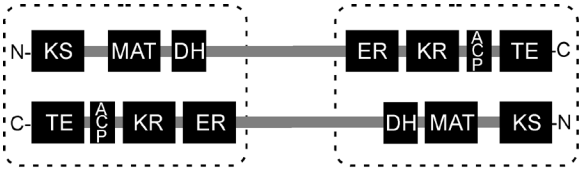

Figure 1. The Classical Textbook Model for the Homodimeric Animal FAS

The subunits lie in a fully extended, antiparallel orientation, such that two centers for fatty acid synthesis are formed at the subunit interface (enclosed in dashed boxes) through cooperation of the three N-terminal domains of one subunit with the four C-terminal domains of the opposite subunit. A central core region of approximately 600 residues has no known catalytic function and is thought to play a role in stabilization of the dimer. KS, β-ketoacyl synthase; MAT, malonyl/acetyl transferase; DH, dehydrase; ER, enoyl reductase; KR, β-ketoacyl reductase; ACP, acyl carrier protein; TE, thioesterase.

Figure 2. Location of Knockout Mutations that Compromise Functionality of Each of the Seven Domains of the FAS

cause the C161Q mutation used to compromise activity of the β-ketoacyl synthase domain inactivates the condensation step but not the malonyl decarboxylation step [13].

Three experimental approaches were utilized to determine whether subunit exchange in the isolated 7KO/wt heterodimer might have resulted in the re-formation of wild-type FAS homodimers that contributed to the activity of the heterodimer preparation.

First, a portion of the 7KO/wt heterodimer preparation was rechromatographed on a Ni-NTA column (Figure 3C). No detectable ketoreductase activity was eluted in the 15 mM imidazole zone where the homodimeric FLAG-tagged wild-type FAS would normally elute. Quantitative recovery of ketoreductase activity was achieved only on elution with 100 mM imidazole, consistent with the doubly tagged 7KO/wt heterodimer being the sole species present.

Secondly, a portion of the 7KO/wt heterodimer preparation was treated with the bifunctional crosslinking reagent dibromopropanone, and the products were analyzed by SDS-PAGE and Western blotting. This reagent is capable of crosslinking the C161 and phosphopantetheine thiols of the wild-type FAS both inter- and intrasubunit, producing three distinct species identifiable by SDS-PAGE [11]: a doubly crosslinked dimer (Figure 4, species iv), a singly crosslinked dimer (species iii), and an internally crosslinked subunit (species ii).

Since 7KO subunits lack both the C161 and phosphopantetheine thiols, the major species that can be formed by treatment of the 7KO/wild-type heterodimer with dibromopropanone should be species ii, corresponding to an internally crosslinked wild-type subunit. Thus, the pattern of crosslinking produced by this reagent provides a characteristic signature that can readily detect the presence of wild-type homodimer, should it have formed in the 7KO/wt heterodimer preparation as the result of subunit exchange. Control experiments confirmed homogeneity of the His_6-tagged 7KO homodimer (Figure 4, lane 1) and specificity of the two antibodies: His_6-tagged 7KO subunits were not detected by the anti-FLAG antibodies (lane 4), neither were FLAG-tagged wild-type subunits detected by anti-His_6-antibodies (lane 6). Treatment of the heterodimer preparation with dibromopropanone generated only species ii, corresponding to an internally crosslinked subunit (Figure 4, lane 2). This crosslinked species was recognized by anti-FLAG antibodies (Figure 4, lane 5) but not by anti-His6 antibodies (lane 7), confirming that it originated exclusively from the FLAG-tagged wild-type subunit of the heterodimer. No trace of the crosslinked dimer species iii and iv, formed characteristically from homodimeric wild-type FAS (Figure 4, lane 3), was detected in the dibromopropanone-treated heterodimer (lanes 2, 5, and 7). These results confirmed the complete absence of wild-type homodimers in the heterodimer preparation, indicating that no detectable subunit exchange had taken place.

Finally, we took advantage of an earlier observation that introduction of some of the single mutations described in Figure 2 increases the thermal lability of FAS [12]. The stability of FAS preparations can be conveniently monitored by measuring the loss of β-ketobutyryl-CoA reductase activity on exposure to elevated temperature [12]. The β-ketobutyryl-CoA reductase assay requires cooperation of the malonyl/acetyl transferase, ACP, ketoacyl reductase, dehydrase, and enoyl reductase domains to load the substrate onto the ACP, perform the β-carbon processing reactions, and transfer the butyryl product back to CoA [12]. Thus, in the case of the 7KO/wild-type heterodimer, the assay monitors the loss in conformation of the wild-type subunit. The half-lives of the FLAG-wild-type homodimer and the His_6-7KO/FLAG-wild-type heterodimers at 46.5°C were distinctly different, 13.4 and 5 min, respectively (Figure 5). If the β-ketobutyryl-CoA reductase activity in the heterodimer had been attributable to the presence of contaminating FLAG-wild-type homodimer, then the half-life should have been identical to that of the FLAG-wild-type homodimer. Thus, the catalytic activity in the 7KO/wild-type heterodimer preparation is attributable to a FAS species that is readily distinguished from wild-type homodimeric FAS by virtue of its unique thermal lability. In conclusion, the three experimental analyses provide consistent and compelling evidence that catalytic activity associated with the 7KO/wild-type heterodimer preparation does not result from contamination with wild-type homodimer.

This study directly demonstrates that, in the context of the dimer, a single subunit is capable of catalyzing the entire series of reactions that lead to the formation of palmitic acid from malonyl-CoA and underscores the need for revision of the textbook model for the FAS that traditionally has depicted the two subunits in a fully extended head-to-tail orientation such that substrate loading and condensation reactions are catalyzed only at the subunit interface. We have proposed a new model in which the substrate loading and condensation reactions can be catalyzed through interactions of the ACP domains with the malonyl/acetyl transferase and β-ketoacyl synthase domains of either subunit [12]. The results of this new study provide further validation of the model and

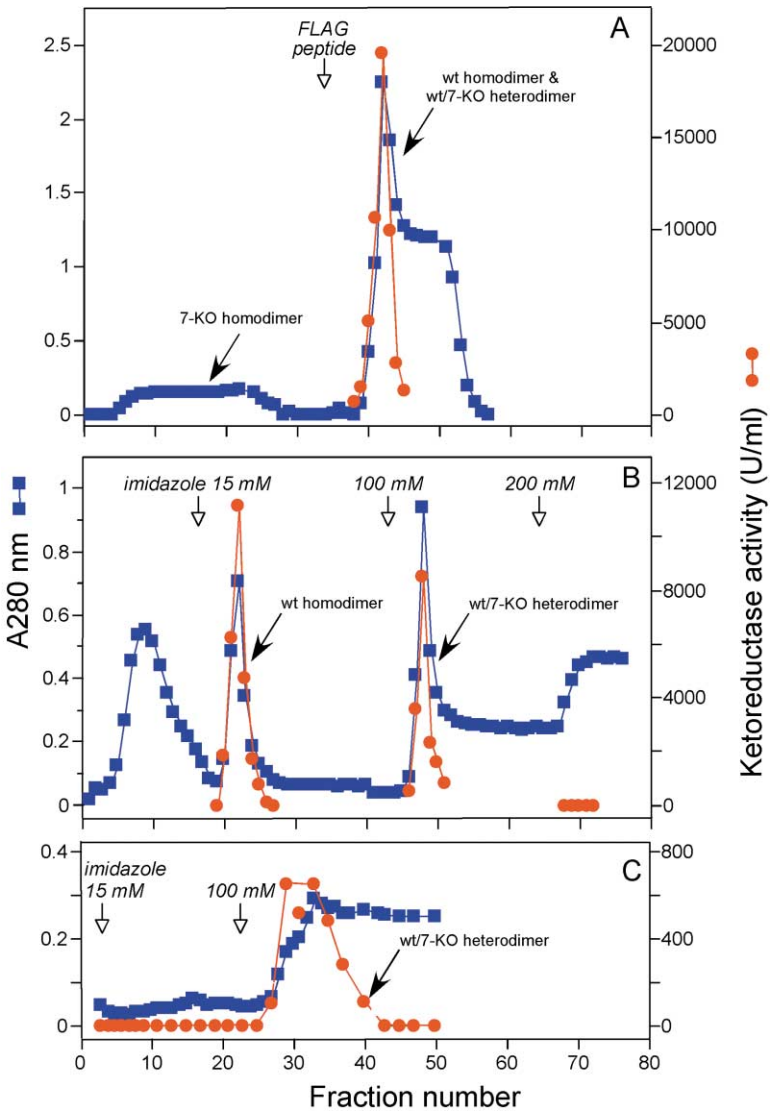

Figure 3. Isolation of 7KO/wt FAS Heterodimers

Subunits from homodimeric C terminally His_6-tagged 7KO and C terminally FLAG-tagged wild-type FAS (0.3 mg/ml each) were randomized to form a mixture of hetero- and homodimeric species and fractionated on anti-FLAG M2 agarose. Unbound His_6-7KO-FAS homodimers were eluted with 0.25 M potassium phosphate (pH 7)/10% glycerol. The bound FLAG-wild-type homodimers and His_6-7KO / FLAG-wild-type heterodimers were released with FLAG peptide, detected by assaying ketoreductase activity (A), and fractionated on a Ni-NTA column. FLAG peptide was eluted with 0.25 M potassium phosphate (pH 7)/10% glycerol, FLAG-wild-type homodimers were eluted by inclusion of 15 mM imidazole, and His_6-7KO/FLAG-wild-type heterodimers were eluted by 100 mM imidazole (B). Imidazole was removed on a 100 kDa cut-off Vivaspin concentrator (Sartorius). Three hours later, a portion of the purified heterodimer preparation was rechromatographed on Ni-NTA to determine whether subunit exchange may have caused reformation of wild-type homodimers (C).

reveal that although the FAS functions most efficiently when both inter- and intra-subunit options are available for substrate loading and condensation, the pathway is able to function at 32% efficiency when only one of the two subunits is involved (Figure 6).

This study reveals that, although a wild-type FAS polypeptide is inactive in the monomeric form, it is active in the overall biosynthetic pathway as a dimer even when paired with a subunit that is compromised in all seven functional domains. It follows, therefore, that the FAS polypeptides must assume distinctly different conformations in the monomeric and dimeric state and that, in the dimer, each of the two subunits provides a scaffold that stabilizes the other subunit in the optimal conformation for productive interactions between domains, both inter- and intra-subunit (represented as a cartoon in Figure 6).

Those regions of the polypeptide that participate directly in stabilizing the dimeric form of FAS have yet to be clearly defined. The region of approximately 600 residues between the dehydrase and enoyl reductase domains, which has not been assigned a catalytic role, is suspected to constitute a structural core that may play a role in stabilizing the dimer [17, 18]. However, there are indications that regions outside of this central core also may contribute to dimer stability. For example, recombinant FASs containing single mutations remote from the central core, in the catalytic site of the β-ketoacyl synthase (Cys161Thr, Cys161Asn, Lys326Arg, or Lys326Leu) and the nucleotide binding region of the enoyl reductase (Gly1673Tyr) and β-ketoacyl reductase (Gly1886Phe) domains, are predominantly monomeric. Many of the prokaryotic and plant counterparts that exist as separate discrete proteins are oligomeric ("type II" FAS enzymes). Whether any of the catalytic domains of the FAS dimer engage in homodimeric interactions has yet to be determined. The only domains of the animal FAS that have been isolated as individual, catalytically active recombinant proteins, the malonyl/acetyl transferase [19] and thioesterase [20] domains, are monomeric. However, a tridomain construct encompassing the β-ketoacyl synthase, malonyl/acetyl transferase,

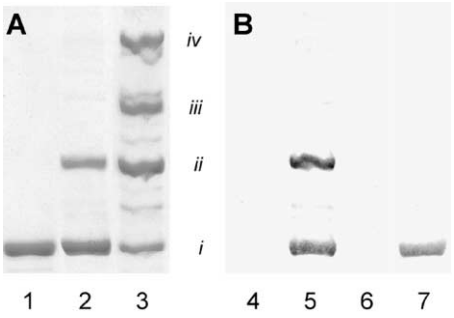

Figure 4. Analysis of FLAG-wt/His_6-7KO Heterodimers by Dibromopropanone Crosslinking

Tris(2-carboxyethyl)phosphine (1 mM) was added to the heterodimer preparation, imidazole was removed using a Microcon 100 concentrator (Amicon), and the protein was treated with a 2.5-fold molar excess of dibromopropanone [11]. Products were separated by SDS-PAGE using 3% stacking gels and 4% running gels and detected either by Pro-Blue protein staining (A) or Western blotting (B) using either mouse anti-FLAG (lanes 4 and 5) or mouse anti-His_6 (lanes 6 and 7) as primary antibodies and alkaline phosphatase-conjugated goat anti-mouse as secondary antibody. Alkaline phosphatase was detected colorimetrically. Lanes 1 and 4, His_6-7KO homodimer; lanes 2, 5, and 7, dibromopropanone-treated His_6-7KO/FLAG-wild-type heterodimers; lane 3, dibromopropanone-treated FLAG-wild-type homodimer; lane 6, FLAG-wild-type homodimer. Species i corresponds to an unreacted subunit, species ii to an internally crosslinked subunit, species iii to a singly crosslinked dimer, and species iv to a doubly crosslinked dimer.

and dehydrase domains but lacking the central core region readily dimerizes (A. Ghosal, A.K.J., and S.S., unpublished results). It seems likely then that facilitation of the conformational changes that accompany dimerization and maintenance of the integrity of the FAS dimer may depend on complex interactions involving both the catalytic domains and the central core region.

Significance

The animal fatty acid synthases (FASs) are large multifunctional polypeptide structures that play an important role in the maintenance of energy balance and also are considered paradigms for the modular polyketide synthases responsible for the biosynthesis of many clinically important antibiotics and chemotherapeutic agents. The FASs are active only in the dimeric form. The textbook model depicts the two FAS subunits as oriented in a fully extended head-to-tail arrangement, such that several of the individual catalytic steps take place at the subunit interface and involve the obligatory cooperation of domains from both subunits [18]. In this manuscript, we report the engineering and characterization of a FAS dimer that consists of one wild-type subunit paired with a "seven knockout" subunit in which each of the seven functional domains has been compromised by mutation. Contrary to prediction based on the old textbook model, the seven knockout/wild-type heterodimeric FAS is capable of synthesizing long chain fatty acids. This finding has important implications for our understanding of the structure and mechanism of action of the FASs and provides compelling evidence that the textbook model for these complexes requires revision.

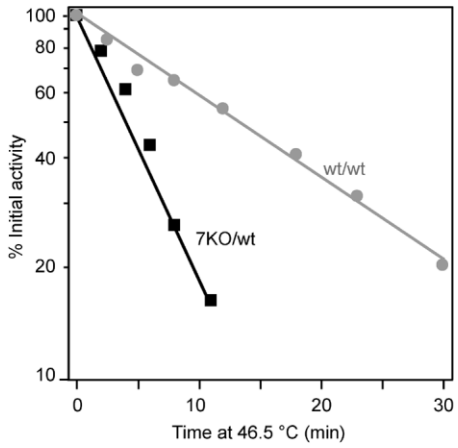

Figure 5. Comparison of the Thermal Lability of the Homodimeric Wild-Type and 7KO/wt Heterodimeric FASs

The two FAS species (100 μg/ml) were incubated in 0.25 M potassium phosphate buffer (pH 7)/1 mM EDTA/1 mM DTT/10% glycerol, and at various time intervals portions of the reaction mixture were removed for assay of β-ketobutyryl-CoA reductase activity.

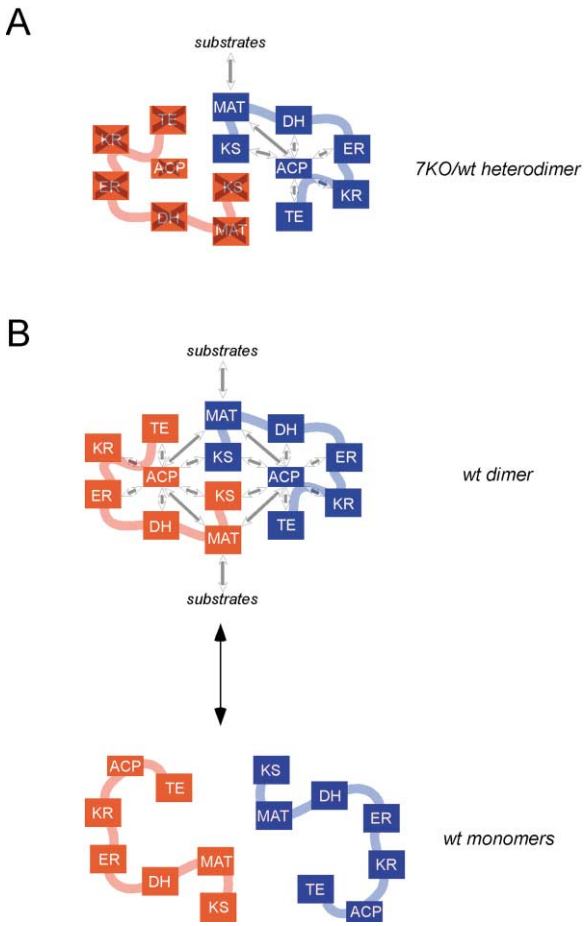

Figure 6. Cartoons Illustrating the Functional Interactions between Constituent Domains in the FAS

(A), 7KO/wild-type heterodimer; (B), wild-type homodimer. The two subunits are distinguished by red and blue coloring, and domains containing knockout mutations are marked with a dark "x". Domains engaging in functional interactions are identified by arrows. The lower panel in (B) indicates that FAS subunits assume different conformations in the monomeric and dimeric state.

A revised model is proposed in which dimerization induces conformational changes in the two subunits that promote the functional interactions between catalytic domains, both inter- and intra-subunit, that are necessary for the biosynthesis of fatty acids.

Experimental Procedures

cDNA Construction and FAS Expression

The strategy for construction of cDNAs encoding the wild-type FAS, domain-specific mutants, and incorporation of His_6 and FLAG tags has been described in detail elsewhere [11, 14–16, 21, 22]. The cDNA encoding the C terminally His_6-tagged 7KO FAS was assembled stepwise from a construct encoding the wild-type protein by replacing specific fragments with the corresponding mutated fragments, using the available restriction sites. At each step, the incorporation of the mutated fragment was confirmed by DNA sequencing. The final FAS cDNA constructs, in the context of the pFASTBAC1 vector (FB), were used to generate recombinant baculoviral stocks by the transposition method employing the BAC-to-BAC baculovirus expression system. *Sf*9 cells were then infected with the purified recombinant viruses and cultured for 48 hr at 27°C. Proteins were purified by a combination of ion exchange chromatography [21] and affinity chromatography [22]

Preparation of 7KO/wt Heterodimeric FAS

Homodimeric C terminally His_6-tagged 7KO and C terminally FLAG-tagged wild-type FAS (0.3 mg/ml each) were mixed and dissociated for 7 days at 4°C in 40 mM potassium phosphate (pH 7) (1 mM DTT, 1 mM EDTA,1% glycerol). The subunits were reassociated by adjusting the potassium phosphate to 0.25 M and the glycerol to 10% and incubating at 30°C for 45 min. The mixture (12 mg protein) was applied to a column containing 10 ml of anti-FLAG M2 Agarose (Sigma). His_6-7KO-FAS homodimers were eluted with 0.25 M potassium phosphate (pH 7)/10% glycerol. FLAG-wild-type homodimers and His_6-7KO/FLAG-wild-type heterodimers were eluted together by FLAG peptide, 1.5 mg/ml, detected by assaying ketoreductase activity with *trans*-1-decalone as substrate, and applied to a 5 ml Ni-NTA column. FLAG peptide was eluted with 0.25 M potassium phosphate (pH 7)/10% glycerol, FLAG-wild-type homodimers were eluted by inclusion of 15 mM imidazole, and His_6-7KO/FLAG-wild-type heterodimers by 100 mM imidazole. Imidazole was removed on a 100 kDa cut-off Vivaspin concentrator (Sartorius).

Enzyme Assays and Product Analysis

FAS [23], *trans*-1-decalone reductase [21], and β-ketobutyryl-CoA reductase [14] activities were measured spectrophotometrically. FAS reaction mixtures for product analysis included [2-^{14}C]malonyl-CoA, and the distribution of radioactivity in fatty acid [24] and CoA thioester [16] products was determined by hplc.

Acknowledgments

This work was supported by grant DK16073 from the NIH.

Received: December 30, 2002
Revised: January 27, 2003
Accepted: January 27, 2003

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