The Malonyl/Acetyltransferase and β-Ketoacyl Synthase Domains of the Animal Fatty Acid Synthase Can Cooperate with the Acyl Carrier Protein Domain of Either Subunit†

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ABSTRACT: The active form of the animal fatty acid synthase (FAS) is a dimer of identical multifunctional polypeptides, each containing seven discrete functional domains, that cooperate to form two centers for palmitate synthesis. To assess the importance of domain cooperation across the subunit interface in the reaction mechanism, we have utilized a strategy based on complementation analysis in vitro of modified FASs carrying critical mutations in specific catalytic domains. Homodimeric FASs carrying the same mutation(s) in both subunits are unable to synthesize fatty acids. As predicted by the current head-to-tail model for the animal FAS, heterodimeric FASs formed between the acyl carrier protein (ACP) mutant and either the β-ketoacyl synthase (KS) or malonyl/acetyltransferase (MAT) are active in palmitate synthesis, confirming that the KS and MAT domains can cooperate with the ACP domain of the opposite subunit. Contrary to this model however, heterodimeric FASs formed between the KS and MAT mutants, between a MAT, ACP double mutant, and a KS mutant, and between a KS, ACP double mutant, and a MAT mutant are also active in palmitate synthesis, indicating that the MAT and KS domains can also cooperate with the ACP domain of the same subunit. The results of this study reveal an unanticipated element of redundancy in the FAS reaction mechanism in that the amino-terminal KS and MAT domains can make functional contact with the penultimate carboxy-terminal ACP domain of either subunit. A revised model for the FAS is proposed in which the substrate loading and condensation reactions can be catalyzed either by one of the two subunits or by cooperation between domains across the subunit interface.

In animals, the *de novo* synthesis of long-chain fatty acids from malonyl-CoA is catalyzed by a single protein, the fatty acid synthase (FAS)[1] that consists of two identical, 272 kDa polypeptides. Each multifunctional polypeptide contains six catalytic domains and an acyl carrier protein (ACP) arranged in the order (from the aminoterminus) β-ketoacyl synthase, malonyl/acetyltransferase,[2] dehydrase, enoyl reductase, β-ketoacyl reductase, ACP, and thioesterase. The dehydrase and enoyl reductase domains are separated by approximately 600 amino acids that have not been ascribed any catalytic function. Only the dimeric form of the protein is capable of coupling the individual reactions to effect the synthesis of a long-chain fatty acid, suggesting that some of the reactions may be catalyzed at the subunit interface. Following the discovery that the two subunits can be cross-linked via the β-ketoacyl synthase, active-site cysteine thiol of one subunit, and the 4′-phosphopantetheine thiol of the companion subunit (*1*), it has generally been accepted that the two subunits are arranged in a head-to-tail orientation, so that the amino-terminal β-ketoacyl synthase domain of one subunit is juxtaposed with the penultimate carboxy-terminal ACP domain of the other subunit. Thus, the various descriptions of a model for the animal FAS (*2*−*4*) share the common feature that the two polypeptides lie side-by-side in a fully extended, antiparallel configuration such that each of the two centers for palmitate synthesis requires cooperation between catalytic domains located in the amino-terminal half of one subunit with those located in the carboxy-terminal half of the adjacent subunit (Figure 1). Recently, we have devised an experimental approach for testing and refining this model that is based on the prediction that certain inactive mutant FASs should be able to form catalytically active species when their subunits are recombined with those of certain other inactive mutant FASs. For example, the model predicts that although homodimers formed from single active-site mutant subunits will be completely inactive in fatty acid synthesis, since both centers for acyl chain assembly are compromised by the same mutation, heterodimers formed from subunits containing different single mutations may be capable of fatty acid synthesis if the two mutations are located on domains that normally cooperate with each other across the subunit interface. The validity of this approach

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[1] Abbreviations: FAS, fatty acid synthase; ACP, acyl carrier protein; KS, ss-ketoacyl synthase; ACP, acyl carrier protein; DH, dehydrase; TE, thioesterase; MAT, malonyl/acetyltransferase; the superscript (−) following a domain abbreviation indicates that domain is functionally inactive; DTT, dithiothreitol.

[2] The systematic name for this enzyme is malonyl-CoA/acetyl-CoA: acyl carrier protein *S*-acyltransferase.

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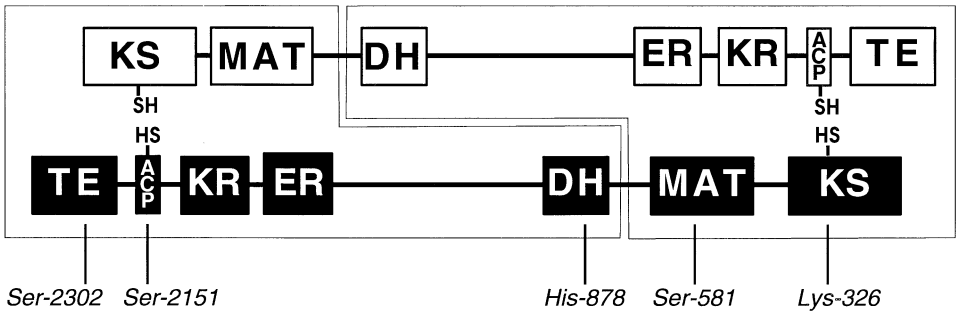

FIGURE 1: Linear domain map of the dimeric animal FAS. The subunits, one in black fill, the other in white, are oriented head-to-tail based on the demonstration that the active-site cysteine residue of the β-ketoacyl synthase of one subunit and the 4′-phosphopantetheine of the adjacent subunit can be cross-linked by dibromopropanone (*5*). Two sites for palmitate synthesis, enclosed by boxes, are formed at the subunit interface through the cooperation of domains from both subunits (*7*). The locations of catalytically essential residues targeted for mutation in this study are shown.

was initially established using two separately mutated FASs, one defective in the β-ketoacyl synthase reaction, the other lacking the 4′-phosphopantetheine that is essential for functioning of the ACP domain (*6*). These two mutants were chosen to test the experimental strategy since there is already strong evidence from the cross-linking studies (*1*), indicating that the β-ketoacyl synthase and ACP domains cooperate across the subunit interface. As predicted by the model, the respective homodimeric mutants were completely inactive in palmitate synthesis, but heterodimers formed from the two mutated polypeptides partially regained the ability to synthesize fatty acid (*6*). Subsequently, we extended this approach for mapping functional interactions within the FAS dimer to include FASs defective in the thioesterase, dehydrase, ACP, and β-ketoacyl synthase domains (*7*). The results of that study were consistent with the proposed model with one exception. The mutant FAS defective in the dehydrase domain, which is located in the amino-terminal half of the polypeptide, did not complement mutants defective in domains located in the carboxy-terminal half; instead, this mutant was complemented by a FAS mutated in the amino-terminal β-ketoacyl synthase domain. Thus, the dehydrase domain should be assigned the same complementation status as the thioesterase and ACP domains rather than the β-ketoacyl synthase domain. The implication of this result is that the β-hydroxyacyl-ACP is converted to enoyl-ACP through the action of the dehydrase domain on the same subunit (Figure 1). Since the dehydrase and ACP domains are separated by more than 1100 residues, this finding provided the first experimental evidence indicating that the two constituent polypeptides are not simply positioned side-by-side in a fully extended conformation, but are coiled such that contact is possible between functional domains distantly located on the same polypeptide. The successful engineering of a mutant FAS compromised specifically in its ability to catalyze the malonyl/acetyltransferase reaction has permitted further exploitation of the complementation strategy. In particular, we have been able to address the question as to whether this domain delivers substrates to the ACP domain located on the same, or on the opposite, subunit. The results of this study were quite unanticipated and indicate that further refinement of the FAS model is in order.

MATERIALS AND METHODS

Materials. The plasmid pUCBM20 was obtained from Boehringer-Mannheim (Indianapolis, IN), BacPAK6 DNA (*Bsu*36 I digested) and baculovirus transfer vector pBacPak9 were from Clontech (Palo Alto, CA), whereas pVL1393 transfer vector was from Invitrogen (San Diego, CA). Sequenase DNA sequencing kit and α-[^{35}S]thio-dATP were from Amersham (Arlington Heights, IL). Vent polymerase and various DNA modification enzymes were from New England Biolabs (Beverly, MA). *Escherichia coli* DH5α competent cells, pFASTBAC 1 transfer vector, Bac-to-Bac Baculovirus expression system, Grace's insect medium, and other insect cell culture medium components were obtained from Life Technologies (Grand Island, NY), oligonucleotide primers from Operon Technologies (Alameda, CA) and *Sf*9 cells from ATCC (Rockville, MD).

In Vitro Mutagenesis and Construction of cDNAs Encoding the FAS Mutants. Construction of a recombinant baculoviral transfer vector encoding the 2505-residue wild-type rat fatty acid synthase and DH$^-$ FAS mutant has been described elsewhere (*8*). Site-directed mutagenesis was carried out by the overlap polymerase chain reaction method using Vent polymerase, essentially as described by Shyamala and Ames (*9*). Partial FAS cDNA constructs pFAS74.20 (66−2544 bp in pUCBM 20), pFAS 215.20 (5541−7615 bp in pUCBM20), and pFAS 1.20 (6281−7020 bp in pUCBM20) were used as template DNA to generate mutations in the β-ketoacyl synthase, ACP, and thioesterase domains, respectively. Details of the strategy employed for the construction of these mutants have been described earlier (*6*, *7*). Briefly, in each case, the mutated DNA fragment generated by overlap polymerase chain reaction was used to replace the corresponding fragment in the parent partial cDNA construct, using the appropriate restriction sites, and the resulting DNA was cloned into *E. coli* DH5α competent cells (*10*). The sequence of the amplified region was confirmed and the appropriate fragment moved stepwise into the full-length FAS cDNA transfer vector construct. Transfer vectors, pBacPAK9 and pVL1393 were used for the β-ketoacyl synthase and ACP mutants, respectively. Recombinant baculoviral stocks were generated by cotransfecting *Sf* 9 cells with the appropriate FAS cDNA construct and BacPAK6 viral DNA (*Bsu*36 I digested) using the Lipofectin method. Purified recombinant baculovirus stocks were obtained by the plaque purification method (*11*).

The cDNA construct encoding the mutated malonyl/acetyltransferase domain, pET S581A, was obtained from Dr. V. S. Rangan (*12*), and an appropriate cDNA fragment from this construct was amplified and used to replace the

corresponding region of pFAS74.20. The sequence of the amplified region was confirmed, and the fragment transferred into the full-length FAS cDNA construct in modified pFASTBAC 1 transfer vector. To generate the FAS double mutants, MAT^-, ACP^- (S581A, S2151A), and KS^-, ACP^- (K326A, S2151A), an ACP domain cDNA fragment carrying the S2151A mutation, was substituted for the equivalent region in both the MAT^- (S581A) and KS^- (K326A) FAS cDNA constructs, in the context of the pFASTBAC 1 vector. These constructs were then used to generate recombinant baculovirus stocks by the transposition method employing the Bac-to-Bac baculovirus expression system according to the manufacturers instructions.

Expression and Purification of Mutant FASs. Sf 9 cells were infected with purified recombinant viruses, cultured for 48 h at 27 °C, and the mutant FAS proteins isolated as described previously (*8*, *13*).

Randomization of Subunits in Mixtures of Mutant FASs. The appropriate amounts of the two mutants were mixed and injected onto an anion exchange column (TSK-GEL DEAE−5PW). The column was washed with 50 mM Tris-HCl, pH 8.3, 1 mM DTT, and 1 mM EDTA to induce dissociation of the bound dimers and rebinding of the released monomers. Dimers and monomers were selectively released from the column using a potassium phosphate gradient. The extent of dissociation was estimated from the relative amounts of FAS eluted in the dimer and monomer zones. FAS monomers spontaneously reassociate following elution from the column so that these species represent dimers in which the subunits have been completely randomized. The experimental details for this procedure have been described in full previously (*6*, *7*).

Gel Filtration. A column of Superose 6 (22 × 0.9 cm), equilibrated and eluted at room temperature with 0.25 M potassium phosphate buffer, pH 7, containing 1 mM EDTA and 1 mM DTT, was used at a flow rate of 0.12 mL/min. Molecular mass standards were blue dextran ($M_r = 2 \times 10^6$), bovine thyroglobulin ($M_r = 6.69 \times 10^5$), and sweet potato β-amylase ($M_r = 2 \times 10^5$).

Preparation and Characterization of Immobilized Complementing FAS Mutants. Combinations of two mutant FASs, 0.2−0.3 mg/mL each, in 50 mM potassium phosphate buffer, pH 7, 1 mM DTT, and 1 mM EDTA were aged at 4 °C for 2 weeks to allow dissociation into monomers (*14*). The concentrations of potassium phosphate, glycerol, and DTT were increased to 0.18 M, 10%, and 5 mM, respectively, and the samples incubated at 25 °C for 40 min to promote reassociation of the subunits to dimers (*6*, *14*). The dimers were then immobilized on a column of Sepharose 4B, bed volume 4.0 mL, containing covalently bound antibodies that bind specifically to the thioesterase domain of the FAS (*15*). The FAS was recycled once through the column to ensure that saturation of all potential binding sites was achieved and the quantity of protein bound to the immunoafinity matrix was calculated by assessing the depletion of β-ketoacyl reductase activity in the eluate from the column. The binding capacity of the immunoafinity matrix was 0.18 mg of FAS/mL of gel. Enzymatic activity of the immobilized FAS was measured by introduction of substrates into the column in a continuous flow system, as described previously (*15*).

Enzyme Assays. β-Ketoacyl synthase activity was assayed by chromatographic detection of the radiolabeled β-ketobutyryl-CoA formed from [2-^{14}C]malonyl-CoA and acetyl-CoA in the absence of NADPH (*6*). Transferase activity was assayed by the chromatographic detection of radiolabeled acetyl-*S*-pantetheine formed from [1-^{14}C]acetyl-CoA and pantetheine (*12*). β-Ketoacyl reductase and enoyl reductase activities were assayed by observing spectrophotometrically the oxidation of NADPH in the presence of *trans*-1-decalone (*8*), or *S*-crotonyl *N*-acetylcysteamine (*16*), respectively. Dehydrase activity was assayed spectrophotometrically at 270 nm using *S*-D,L-β-hydroxybutyryl *N*-acetylcysteamine as substrate (*16*). Thioesterase activity was assessed by measuring the free radiolabeled palmitic acid formed from [1-^{14}C]-palmitoyl-CoA (*17*). Assay of overall FAS activity was performed spectrophotometrically (*18*). All enzyme activities were assayed at 37 °C, except the malonyl/acetyltransferase and thioesterase, which were assayed at 0 and 30 °C, respectively. Enzyme activities were directly proportional to protein concentration and time. One unit of enzyme activity corresponds to the amount of enzyme catalyzing the utilization of 1 μmol of substrate/min: for the overall FAS reaction, NADPH is the substrate used to calculate enzyme activity.

Analysis of Fatty Acids Synthesized by FAS. Reaction mixtures for the FAS assay included [2-^{14}C]malonyl-CoA (*7*). Reaction products were extracted into hexane, derivatized with phenacyl-8, and analyzed by reversed-phase high performance liquid chromatography as described previously (*19*).

RESULTS

Characterization of FAS Mutant Proteins. Five FASs, each containing a single critical mutation, in either the β-ketoacyl synthase, malonyl/acetyltransferase, dehydrase, ACP, or thioesterase domains and two FASs containing double mutations, either in the ACP and β-ketoacyl synthase domains or in the ACP and malonyl/acetyltransferase domains, were utilized in this study. The sites of the mutations were chosen based on the following considerations: Lys-326 is a residue that plays an essential role in catalysis of the β-ketoacyl synthase reaction (*6*). Ser-581 is the nucleophile at the active center of the malonyl/acetyltransferase (*12*), His-878 is an essential active-site residue for the dehydrase (*13*), Ser-2151 is the site of post-translational attachment of the 4′-phosphopantetheine prosthetic group (*20*−*22*), and Ser-2302 is the nucleophile at the active center of the thioesterase domain (*23*); all of these residues were replaced with alanine. Some of these mutants have been characterized previously, but for completeness, the properties of all of the mutants have been summarized in Table 1. All of the homodimers containing the same mutation(s) on both polypeptides were inactive in the overall FAS assay, and for the single mutants, the loss of ability to synthesize fatty acid was attributable specifically to a loss in activity of the functional domain targeted by the mutation (Table 1). Since the S2151A mutant FAS lacks the 4′-phosphopantetheine moiety, it is compromised in its ability to catalyze the β-ketoacyl synthase reaction. Other partial activities however, are assayed using model substrates that do not require prior transfer to the 4′-phosphopantetheine moiety, so these activities are not compromised by the

Table 1: Characterization of FAS Homodimeric Single and Double Mutants

	K326A[a] KS$^-$	S581A MAT$^-$	H878A[b] DH$^-$	S2151A[a] ACP$^-$	S2302A[c] TE$^-$	S581A and S2151A MAT$^-$ and ACP$^-$	K326A and S2151A KS$^-$ and ACP$^-$
				Specific Activity (% of Wild-Type FAS Activity)[d]			
fatty acid synthesis	≤0.05	≤0.15	≤0.8[e]	≤0.5	≤0.2[e]	≤0.05	≤0.05
malonyl/acetyltransferase	123 ± 6	≤0.04	121 ± 8	133 ± 7	139 ± 2	≤0.02	105 ± 1
β-ketoacyl synthase	≤0.1	≤0.03	97 ± 3	≤0.2	100 ± 3	≤0.1	0 ± 0
β-ketoacyl reductase	101 ± 7	108 ± 2	80 ± 1	97 ± 4	86 ± 2	93 ± 4	108 ± 4
dehydrase	100 ± 3	102 ± 0.1	0 ± 1	108 ± 2	100 ± 1	133 ± 4	125 ± 3
enoyl reductase	69 ± 7	98 ± 2	94 ± 3	74 ± 7	170 ± 5	90 ± 4	85 ± 8
thioesterase	133 ± 4	107 ± 2	99 ± 10	112 ± 16	2 ± 1	83 ± 9	97 ± 2
				Extent of Dissociation (% of the Eluted FAS)[f]			
monomer zone	48 ± 1	69 ± 1	62 ± 2	68 ± 1	62 ± 1	64 ± 2	96 ± 1

[a] Data taken from ref *6*. [b] Data taken from ref *8*. [c] Data taken from ref *7*. [d] The measured specific activities of the wild-type FAS (in m units/mg) were overall FAS 2050 ± 30, malonyl/acetyltransferase 1915 ± 310, β-ketoacyl synthase 125 ± 6, β-ketoacyl reductase 17 300 ± 769, dehydrase 40 ± 2, enoyl reductase 16 ±1, and thioesterase 485 ± 2. [e] Product is β-hydroxybutyryl-CoA. [f] Extent of the wild-type FAS dissociation was 62 ± 3%.

mutation in the ACP domain. The homodimeric FAS containing mutations in both the ACP and malonyl/acetyltransferase domains was compromised in both β-ketoacyl synthase and malonyl/acetyltransferase activities, whereas that containing mutations in both the ACP and β-ketoacyl synthase domains was compromised only in the condensation reaction catalyzed by the β-ketoacyl synthase domain (Table 1). In short, all of the various mutations introduced affected the catalytic properties of the FASs as we had anticipated.

Complementation Analysis of FAS Mutants. The procedure we have developed to promote rapid randomization of the subunits in mixtures of two different mutant homodimers involves, sequentially, immobilization of the dimers on a DEAE matrix, dissociation of the dimers in a high pH, low ionic strength medium, rebinding of the released monomers to the matrix, and selective elution of the monomers and undissociated dimers in a salt gradient (*6*). On elution, the monomers spontaneously reassociate. When complementation is observed between pairs of mutants, FAS activity is observed in the zone corresponding to monomers that have spontaneously reassociated, but not in the zone corresponding to dimers that have not undergone dissociation and reassociation. Provided the physical properties of the FASs are not significantly affected by the various mutations, theoretically the reassociated species eluting in the monomer zone should consist of 50% heterodimers (each subunit carrying a different mutation) and 25% of each homodimer. However, we have previously observed that some point mutations significantly affect the rate of dissociation of FAS homodimers (*6*, *7*). So, to minimize the effects of these differences on the subunit randomization process, we routinely screen new mutants to assess their susceptibility to the dissociating conditions imposed by the high pH, low ionic strength medium. This property is quantified as the percent of the FAS eluting from the DEAE matrix in the monomer zone under standard conditions. The procedure allows us to calculate the amount of each FAS mutant homodimer that is required to yield the same amount of monomer when bound to the DEAE matrix. Of the mutants employed in this study, only the KS$^-$ and ACP$^-$ double mutant exhibited a higher than normal tendency to dissociate into subunits (Table 1).

As predicted by the recently revised model (Figure 1), the mutation in the malonyl/acetyltransferase domain, which is located in the amino-terminal half of the FAS, was complemented by mutations in the thioesterase, ACP, and dehydrase domains that have been assigned to the carboxy-terminal complementation group (Table 2). The specific activities of the various combinations of randomized dimers were about 20% of that of the wild-type FAS, close to the theoretical value of 25% predicted assuming that the mutant heterodimers constitute 50% of the dimer population and are active at only one of the two centers, and assuming that the two centers function independently. Clearly then the malonyl/acetyltransferase can deliver substrate across the subunit interface to the ACP domain of the adjacent subunit. Surprisingly, however, the mutation in the malonyl/acetyltransferase domain also complemented the mutation in the β-ketoacyl synthase domain, despite the fact that these domains are located adjacent to each other in the amino-terminal half of the polypeptide. Again, the specific activity of the randomized mixture of malonyl/acetyltransferase and β-ketoacyl synthase mutants was close to that predicted for a single functional center of fatty acid synthesis. As with all other pairs of complementing mutants, FAS eluting from the DEAE matrix in the dimer zone was inactive, whereas that eluting in the monomer zone was active following spontaneous reassociation. Our previous complementation analysis had shown that the β-ketoacyl synthase mutant is also complemented by the thioesterase, ACP, and dehydrase

Table 2: Complementation Analysis of the FAS Mutant, MAT$^-$, Lacking Malonyl/Acetyltransferase Activity[a]

second mutant	specific activity (% of wild-type activity[b])	fatty acids synthesized (% of total) C14	C16	C18
KS$^-$ (K326A)	22 ± 0.6	17	75	8
DH$^-$ (H878A)	21 ± 0.5	13	83	4
ACP$^-$ (S2151A)	18 ± 0.4	12	85	3
TE$^-$ (S2302A)	22 ± 2.2	15	78	7

[a] Various combinations of mutant FASs were partially dissociated, the monomers were separated by ion exchange chromatography, allowed to reassociate spontaneously, and FAS activity was determined. Details are presented in the Materials and Methods. [b] Activity of the wild-type FAS was 2050 ± 30 m units/mg. No complementation was observed simply by mixing the pairs of mutants. FAS eluting from the column in the dimer zone had no activity, only FAS eluting in the monomer zone exhibited FAS activity when allowed to spontaneously reassociate to the dimer form. Small amounts of acetoacetyl-CoA and butyryl-CoA, amounting to less than 5% of the products, were formed by all combinations of the mutants, as well as by the wild-type FAS.

Table 3: Characterization of Heterodimers Containing Only One Active Form of the Ketoacyl Synthase, Malonyl/Acetyltransferase and ACP Domains[a]

FAS	specific activity (% of wild-type[b])	fatty acids synthesized (% of total)				
		<C14	C14	C16	C18	>C18
KS^-, ACP^- double mutant, and MAT^- single mutant	5.1 ± 0.3	2.2	7.7	84.6	5.1	0.4
MAT^-, ACP^- double mutant, and KS^- single mutant	5.2 ± 0.5	2.3	6.9	84.8	5.6	0.4
wild-type	100	1.4	8.5	82.5	6.8	0.8

[a] Subunits from combinations of double and single mutant FASs were randomized as described in Table 2 and FAS activity was determined. [b] Activity of the wild-type FAS was 2050 ± 30 m units/mg. Small amounts of acetoacetyl-CoA and butyryl-CoA, accounting for less than 5% of the total products, were also formed by both the mutant combinations and the wild-type FAS. Only FAS eluting from the DEAE matrix in the monomer zone exhibited FAS activity, following spontaneous reassociation, FAS emerging from the DEAE matrix in the dimer zone, which represents undissociated FAS, was inactive. Reaction rates were directly proportional to enzyme concentration down to the limits of detection of the assay.

mutants, members of the carboxy-terminal complementation group (*7*). Paradoxically then, the malonyl/acetyltransferase and β-ketoacyl synthase mutations both are complemented by members of the carboxy-terminal complementation group, yet complement each other. The specific activities of FAS populations formed by randomization of MAT^- subunits with those of DH^-, ACP^-, TE^-, and KS^- mutants were all close to the theoretical value of 25%, predicted assuming that the heterodimers form 50% of the FAS population. In contrast, our earlier complementation analysis of KS^- mutants (C161T, C161S, and K326A) gave appreciably lower specific activities when paired with the DH^-, ACP^-, and TE^- mutants. At present, we cannot say whether this difference results from a skewing of the heterodimer contribution to the FAS population or is related specifically to some unique property of the KS^- mutants.

The unexpected finding of the complementary nature of the MAT^- and KS^- mutants raised the possibility that the malonyl/acetyltransferase domain may be able to deliver substrates to either of the two ACPs in the dimer and that the condensation reaction may be catalyzed by the cooperation of the β-ketoacyl synthase with either of the two ACP domains. To investigate these possibilities further, we utilized two different double mutants, one containing the S2151A (ACP^-) and S581A (MAT^-) mutations, the other the S2151A (ACP^-) and K326A (KS^-) mutations (Table 1). Subunits of the ACP^- and MAT^- double mutant were randomized with those of the single mutant KS^- and the complementation status assessed. Heterodimers formed from these mutant FASs will contain only one normal catalytically competent form of each of the ACP, β-ketoacyl synthase, and malonyl/acetyltransferase domains. Again FAS activity was observed in FAS dimers that had undergone subunit randomization, but not in the mixed, undissociated dimers (Table 3). This positive complementation confirmed that the malonyl/acetyltransferase domain is capable of delivering substrates to the ACP domain of the same polypeptide (Table 3). In a similar experiment, subunits of the ACP^- and KS^- double mutant were randomized with those of the MAT^- single mutant, and again the heterodimers were found to be active in the overall FAS reaction. Since the only normal ACP and β-ketoacyl synthase domains present in the heterodimers are located on the same subunit, this finding confirmed that the condensation reaction can be catalyzed through the cooperation of ACP and β-ketoacyl synthase domains within the same subunit. In both complementation experiments involving double mutants, the level of FAS activity was about 5% of that of the wild-type FAS. If we assume that the heterodimers again make up 50% of the dimer population, then the catalytic center formed by cooperation of the ACP domain with either the malonyl/acetyltransferase or β-ketoacyl synthase of the same subunit appears to be functioning at about 20% of the rate of a normal catalytic center in the wild-type FAS.

Catalytic Activity of Immobilized FAS Mutant Heterodimers. The FAS species formed by randomization of subunits of the KS^-, ACP^-, and MAT^- mutants and those of the MAT^-, ACP^-, and KS^- mutants consisted entirely of dimers, as monitored by gel filtration on Superose 6 (details not shown, conditions specified in Materials and Methods). Thus, the introduction of the mutations did not cause the formation of any stable higher order structure. Nevertheless, to rule out the possibility that, in the presence of substrates, the complementation status of some mutants might be dependent on the establishment of transient functional contacts between dimers, we exploited a technique developed for the immobilization and assay of catalytically active FAS dimers (*15*). In this procedure, the FAS is immobilized on an immunoaffinity column containing antibodies that recognize the thioesterase domain and is assayed in a continuous flow system by pumping substrates through the column. Wild-type FAS retains about 50% activity when tethered to this immunoaffinity matrix (*15*). To ensure that the tethered FAS polypeptides are spatially separated, a crude immunoglobulin fraction that contains less than 10% specific anti-thioesterase IgG was used to construct the affinity matrix. Previously, we have found that FAS monomers immobilized in this way cannot form productive contacts with each other and are completely inactive in the overall FAS reaction (*24*). Thus, FAS dimers, either homodimers or heterodimers, formed on randomization of mutant FAS subunits, when tethered to the affinity matrix, are highly unlikely to be capable of making contacts with each other. If dimer−dimer interactions were responsible for the ability of these mutant combinations to complement each other, then the immobilized heterodimers would be unable to synthesize fatty acids. An example of the continuous flow assay is shown for the immobilized dimers formed by randomization of the ACP^- and KS^- double mutant and the MAT^- single mutant FASs (Figure 2). Clearly, the heterodimers retain FAS activity when tethered to the matrix. The extent of substrate utilization was directly proportional to the residence time of substrates on the column, which is determined by the flow rate through the column. The specific activity of the

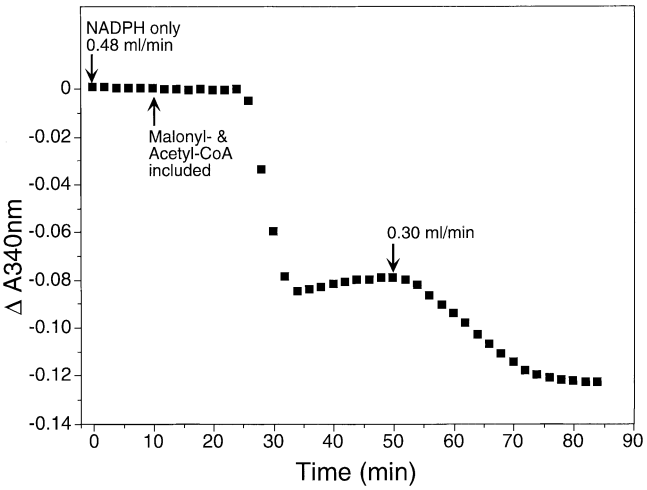

FIGURE 2: Assay of FAS activity by immobilized, complementing FAS mutants. Subunits of the ACP$^-$, KS$^-$ double mutant, and the MAT$^-$ single mutant FASs were randomized and the mixed population of homo- and heterodimers was immobilized on an immunoaffinity column as described in the Materials and Methods section. Potassium phosphate buffer (0.1 M, pH 7) containing 1 mM EDTA and 1 mM DTT was pumped through the column at the flow rate indicated. Substrates for the FAS reaction were introduced with the buffer, as indicated, and enzyme activity assessed, at room temperature, by recording the decrease in absorbance at 340 nm of the column eluate. The FAS activity was 6.1 nmol of NADPH oxidized/min at a flow rate of 0.48 mL/min, 6.0 nmol of NADPH oxidized/min at a flow rate of 0.30 mL/min.

immobilized preparation was 55% of that measured in free solution. Similar results were obtained for the heterodimers formed from the ACP$^-$ and MAT$^-$ double mutant and the KS$^-$ single mutant (details not shown). These results indicate that the complementation observed between the ACP$^-$ and KS$^-$ double mutant and the MAT$^-$ single mutant and between the ACP$^-$ and MAT$^-$ double mutant and the KS$^-$ single mutant was not dependent on the formation of higher order structures.

DISCUSSION

The results of this study argue for modification of the original model for the animal FAS. The revised model, while retaining the original concept of two subunits oriented head-to-tail with respect to each other, should also meet the additional requirement that head-to-tail contacts be possible within the individual subunits. The key experimental result supporting this proposition is the finding that mutants in the β-ketoacyl synthase and malonyl/acetyltransferase domains complement each other. This completely unexpected result prompted us to consider all alternative interpretations of the data. One possibility we have addressed is that transfer of intermediates might occur between dimers, perhaps through the transient formation of a higher order structure. Were this to be the case, then the complementation between β-ketoacyl synthase and malonyl/acetyltransferase mutants might represent complementation between dimers, rather than complementation between subunits within the dimer. This scenario seemed worthy of serious consideration since, in the related multifunctional polyketide synthases, intermediates apparently are transferred from dimer to dimer. These polyketide synthases utilize a process resembling that of fatty acid synthesis in which a primer molecule, usually acetate or propionate, is elongated by condensation with successive chain extender molecules, usually malonate or methyl malonate. However, in contrast with fatty acid biosynthesis, where complete β-carbonyl reduction following each condensation is the rule, in polyketide synthesis, the extent of β-carbonyl reduction is variable. Some polyketides are synthesized on multifunctional, or modular, proteins that are structurally related to the multifunctional fatty acid synthases. Thus the size, order, and spacing of domains in a polyketide synthase module mirrors exactly that found in a subunit of the animal FAS (*25*). One of the most extensively studied modular polyketide synthases is 6-deoxyerythronolide B synthase from *Saccharopolyspora erythraea*, which synthesizes the aglycone portion of the antibiotic erythromycin. This protein contains six FAS-like modules distributed on three polypeptides, two modules per subunit (*25*). All constituent polypeptides are homodimeric, as in the FASs (*26*, *27*). Each module catalyzes one round of chain extension, and variable β-carbonyl reduction and the six modules are positioned exactly in the order in which they are used, so that the growing acyl chain is passed sequentially through modules one to six. Consequently, the transfer of acyl chains from module two to module three, and from module four to five, necessitates a functional interaction between two dimeric polypeptides (*26*, *27*).

Evidence relating to the possibility that in the FASs, transfer of intermediates between dimers might be responsible for the unexpected mutant complementation is reviewed below.

(1) Hydrodynamic data derived for the naturally occurring FAS have revealed the presence of only two FAS species corresponding to the monomer and dimer (*14*, *18*, *28*−*30*).

(2) Active enzyme centrifugation studies indicate that the dimer is the only active form of the naturally occurring enzyme; no larger active form was detected in the presence of substrates (*30*).

(3) No stable, higher order structures, other than dimers, can be detected in any of the mixed FAS populations formed from mutant homodimers, as evidenced by gel filtration.

(4) Complementation between β-ketoacyl synthase and malonyl/acetyltransferase mutants requires prior randomization of the two types of subunit and the formation of heterodimers. Complementation is not observed when the two mutants are mixed under conditions where no subunit exchange occurs (Tables 2 and 3).

(5) If complementation between β-ketoacyl synthase and malonyl/acetyltransferase mutants necessitated even the transient formation of a higher order structure, then one would expect this interaction to be highly dependent on protein concentration. However, the FAS activity observed with randomized ACP$^-$ and KS$^-$ double mutant and the MAT$^-$ single mutant remains directly proportional to protein concentration down to the limits of detection of the assay.

(6) Finally, when potential interaction between dimers is prevented by immobilization of the heterodimers formed from the ACP$^-$ and KS$^-$ double mutant and the MAT$^-$ single mutant, complementation is still observed.

Thus, the preponderance of evidence indicates it is highly unlikely that higher order structures are formed between FAS dimers, even transiently, and that the complementation observed between the single and double mutants reflects complementation of the two mutant polypeptides in the context of the dimer, as we had originally envisioned when

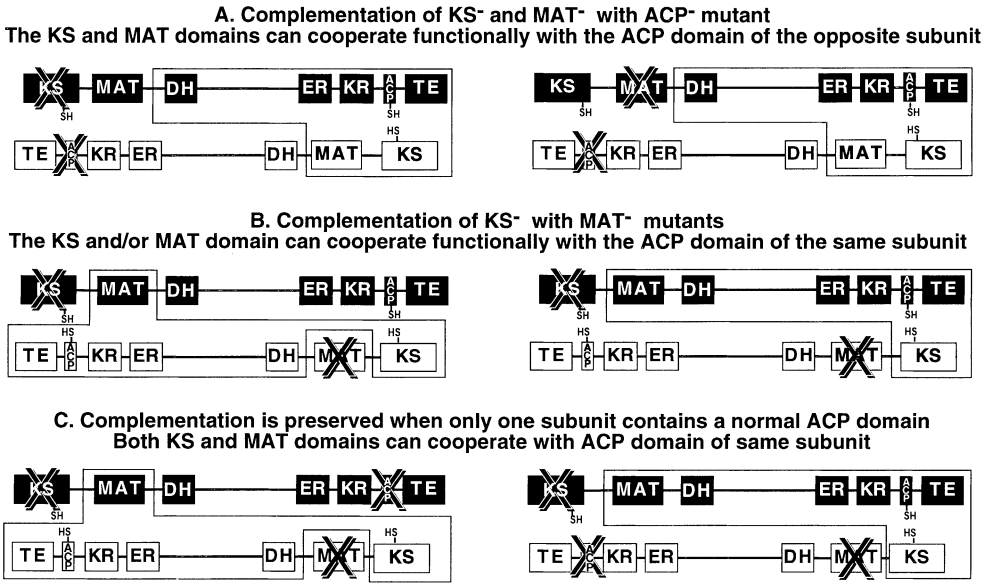

FIGURE 3: Schematic representation of the complementation data using the linear domain map format. The two subunits are distinguished by black and white fill. Domains inactivated by mutation are marked by X, and those that contribute to an active center for palmitate synthesis are enclosed by a box.

formulating the complementation strategy for mapping functional interactions between domains of the dimeric protein.

Consequently, we conclude that the complementation observed between the ACP$^-$ and KS$^-$ double mutant and the MAT$^-$ single mutant and between the ACP$^-$ and MAT$^-$ double mutant and the KS$^-$ single mutant reflects the ability of the β-ketoacyl synthase and malonyl/acetyltransferase domains to cooperate with the ACP domain on the same subunit. Since both the KS$^-$ and MAT$^-$ single mutants also complement the ACP$^-$ single mutant, it follows that the β-ketoacyl synthase and malonyl/acetyltransferase domains are able to interact functionally with either of the two ACP domains present in the FAS dimer. The results of these critical complementation analyses are summarized in schematic form in Figure 3. Panel A depicts the complementation of the ACP$^-$ single mutant by both the KS$^-$ and MAT$^-$ single mutants as predicted by the conventional model. As shown in panel B, the complementation of the KS$^-$ single mutant by the MAT$^-$ single mutant cannot be explained by the conventional model and indicates that the β-ketoacyl synthase and/or the malonyl/acetyltransferase domain must be capable of cooperating with the ACP domain of the same subunit. Finally, in panel C, the complementation of the ACP$^-$ and KS$^-$ double mutant by the MAT$^-$ single mutant and of the ACP$^-$ and MAT$^-$ double mutant by the KS$^-$ single mutant indicates that, in fact, both the β-ketoacyl synthase and/or the malonyl/acetyltransferase domains can interact with the ACP domain of the same subunit. Several important implications arise from these observations. First, the FAS has alternative mechanisms both for loading of substrate and for catalysis of the condensation reaction. Acetyl and malonyl moieties can be transferred to the 4′-phosphopantetheine through the action of the malonyl/acetyltransferase domain of the opposite subunit, according to the conventional model or by an alternative mechanism through the action of the malonyl/acetyltransferase domain of the same subunit. Second, the condensation reaction can be catalyzed through the cooperation of the ACP and β-ketoacyl synthase domains of the opposite subunits, according to the conventional model or, by an alternative mechanism, through the cooperation of the ACP and β-ketoacyl synthase domains of the same subunit. Furthermore, from the complementary nature of the KS$^-$ and MAT$^-$ single mutants, one can infer that the conventional and alternative mechanisms apparently can coexist. For example, if the active malonyl/acetyltransferase domain is cooperating with the ACP domain of the opposite subunit, in the manner of the conventional mechanism, then this ACP domain must be cooperating with the active β-ketoacyl synthase on the same subunit (Figure 3, panel B, left side). Conversely, if the active β-ketoacyl synthase domain is cooperating with the ACP domain of the opposite subunit, in the manner of the conventional mechanism, then this ACP domain must be receiving a substrate supply from the malonyl/acetyltransferase domain of the same subunit (right side, panel B). Similarly, the ability of the KS$^-$ mutant to complement the MAT$^-$ and ACP$^-$ double mutant and the MAT$^-$ mutant to complement the KS$^-$ and ACP$^-$ double mutant establishes unambiguously that the two mechanisms can indeed function together in the same FAS dimer. It is highly unlikely, therefore, that the alternative mechanisms for substrate delivery and condensation represent artifacts that result from gross perturbations in the FAS conformation brought about by the mutations. A third implication of the results is that the growing acyl chain has the possibility of transferring from one ACP to the other, via the β-ketoacyl synthase, in successive rounds of elongation. Nevertheless, this clearly is not an obligatory requirement during chain elongation, since heterodimers containing only one functional ACP domain are perfectly capable of synthesizing palmitic acid. Fourth, it is evident that if all of the substrate loading and condensation reactions were catalyzed by the alternative mechanisms and if, as we have tentatively assumed, all of the β-carbon processing reactions take place on the same subunit, then the biosynthesis of palmitic acid could take place entirely on one of the two subunits. Since FAS monomers cannot synthesize fatty acids, association with the

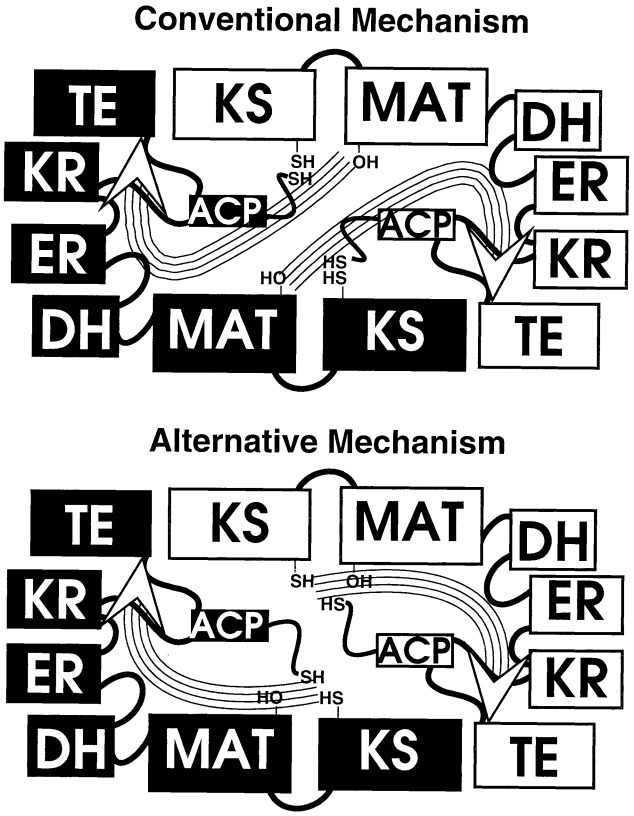

FIGURE 4: The conventional and alternative models for the animal FAS. The two-dimensional cartoon illustrates the various functional interactions between domains that contribute to the two sites for palmitate synthesis. No attempt is made to simulate a three-dimensional structure. The two subunits are distinguished by black or white fill. For clarity, the noncatalytic region extending approximately from residues 970 to 1630 has been omitted. As yet, no β-ketoacyl reductase or enoyl reductase mutants have been characterized and we have assumed tentatively that all of the β-carbon processing reactions take place by cooperation of the ACP, β-ketoacyl reductase, dehydrase, and enoyl reductase domains associated with the same subunit; this point has been verified experimentally for the ACP and dehydrase domains only (*7*). The swath of the two arrows indicates the domains that cooperate to form the two centers for palmitate synthesis according to the two models.

second subunit presumably would still be required, perhaps to stabilize the appropriate conformation of the active subunit. The construction of FAS dimers that contain catalytically competent domains on only one of the two subunits would allow testing of this possibility.

The essential elements of the conventional and alternative mechanisms for the FAS are illustrated in cartoon form as Figure 4. In the conventional mechanism, the transfer of substrates from the malonyl/acetyltransferase to ACP domains and the condensation reaction between malonyl-S-ACP and acyl-S-β-ketoacyl synthase both involve communication between the ACP domain of one subunit with the malonyl/acetyltransferase and β-ketoacyl synthase domains of the opposite subunit (top panel). In the alternative model, cooperation of the malonyl/acetyltransferase and ACP domains and/or cooperation between ACP and β-ketoacyl synthase domains take place within a single subunit (lower panel).

Despite the close attention the multifunctional FASs have received over the years, this element of redundancy had not been anticipated previously, emphasizing the unique strength of the complementation strategy as a tool for probing functional interactions between the domains of the FAS. The relative contributions of the "conventional" and "alternative" mechanisms cannot be assessed accurately at this point, owing to the element of uncertainty concerning the exact composition of the randomized subunit population. However, if we assume that the heterodimers typically represent about 50% of the total dimer population, then the alternative mechanism, in which both the interaction between malonyl/acetyltransferase and ACP domains and the interaction between ACP and β-ketoacyl synthase domains take place within a single subunit, would appear to operate with approximately 20% of the efficiency of the conventional mechanism. However, the conventional and alternative mechanisms are not necessarily mutually exclusive, so that it is entirely possible that the malonyl/acetyltransferase reaction could proceed via the alternative mechanism, but the following condensation reaction could proceed via the conventional mechanism. Indeed, this likely occurs in the heterodimer formed between the KS^- and MAT^- single mutants (see earlier discussion regarding Figure 3, panel B). In this case, where at least one of the two reactions must occur via the alternative pathway, the activity of the heterodimer is close to the theoretical value expected for a single, fully functional catalytic center for fatty acid synthesis (again, assuming that the heterodimer constitutes 50% of the total FAS species). It follows then that the alternative mechanism for one of the two reactions in question could make a significant contribution to the overall rate of fatty acid synthesis.

Why has this alternative mechanism remained undetected for so long? In fact, the cross-linking experiments with the chicken FAS that inspired the original head-to-tail model actually revealed the association of dibromopropanone with two different polypeptide species, both having slower electrophoretic mobility than the original monomer (*1*). We have confirmed this observation with the rat FAS (A. Witkowski, A. K. Joshi, and S. Smith, unpublished observations). Stoops and Wakil (*1*) concluded that the major species, accounting for about 80% of the total, resulted from double, intersubunit cross-linking of the two polypeptides via the β-ketoacyl synthase active-site cysteine of one subunit with the 4′-phosphopantetheine thiol of the other subunit. The identity of the second polypeptide species has never been established. In light of our new findings, it is conceivable that this second species might result from the cross-linking of the β-ketoacyl synthase active-site cysteine and the 4′-phosphopantetheine thiol of the same subunit. The revised model, while retaining the original concept of two subunits oriented head-to-tail with respect to each other, also meets the additional requirement that head-to-tail contacts be possible within the individual subunits. Thus, it is possible that intrasubunit cross-linking by dibromopropanone could connect the two circularized subunits rather like two links of a chain. Such a species might be expected to exhibit electrophoretic properties similar to those of the second species observed following treatment with dibromopropanone. A reexamination of the products of dibromopropanone cross-linking may provide further insight as to the preferred topological orientation of the ACP and β-ketoacyl synthase domains.

In the following paper in this issue, Khosla and coworkers, employing the same mutant complementation

strategy to the modular polyketide synthase, 6-deoxyerythronolide B synthase, report that the acyltransferase domain of module 2 can interact functionally with the ACP domain associated with module 2 of either subunit. This finding further emphasizes the functional and topological similarity between the multifunctional fatty acid and polyketide synthases.

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