Mapping of Functional Interactions between Domains of the Animal Fatty Acid Synthase by Mutant Complementation *in vitro*†

Anil K. Joshi, Andrzej Witkowski, and Stuart Smith*

Children's Hospital Oakland Research Institute, 747 Fifty-Second Street, Oakland, California 94609

Received October 28, 1996; Revised Manuscript Received December 16, 1996⊗

ABSTRACT: Polypeptides of the animal fatty acid synthase (FAS) consist of three amino-terminal catalytic domains, β-ketoacyl synthase, malonyl/acetyltransacylase, and dehydrase, separated by a 600-residue structural core from four carboxyl-terminal catalytic domains, enoyl reductase, β-ketoacyl reductase, acyl carrier protein, and thioesterase. In the active dimeric form of the protein the two identical multifunctional polypeptides are oriented head-to-tail such that two sites for palmitate synthesis are formed at the subunit interface. In order to map the functional interactions between domains of the two subunits that contribute to the two sites of synthesis, we have utilized a strategy based on complementation analysis *in vitro* of modified FASs carrying mutations in specific catalytic domains. Homodimeric mutant FASs lacking functional β-ketoacyl synthase (KS^-), dehydrase (DH^-), acyl carrier protein (ACP^-), or thioesterase (TE^-) domains, as well as heterodimers formed between ACP^- and TE^- subunits, between ACP^- and DH^- subunits, and between DH^- and TE^- subunits, were unable to synthesize fatty acids. However, heterodimers formed between KS^- and either DH^-, ACP^-, or TE^- subunits regained partial FAS activity. These data indicate that the dehydrase domain, although located in the amino-terminal half of the polypeptide, should be assigned to the complementation group located in the carboxy-terminal half that includes the acyl carrier protein and thioesterase domains. Thus, the current model for the animal FAS must be revised to reflect the finding that the two constituent polypeptides are not simply positioned side-by-side in a fully extended conformation but are coiled in a manner that allows the dehydrase domain to access the β-hydroxyacyl-ACP located more than 1100 residues distant on the same subunit.

The *de novo* synthesis of long-chain fatty acids from malonyl-CoA requires several enzyme activities that in most bacteria and plants are associated with discrete monofunctional polypeptides (Alberts & Greenspan, 1984). In animals, six enzymes and an acyl carrier protein, which collectively are responsible for catalyzing the entire sequence of 37 individual reactions, are integrated into a single polypeptide chain (Amy et al., 1989; Holzer et al., 1989; Schweizer et al., 1989). Various models for the animal FAS[1] have been proposed, all of them involving two identical antiparallel-oriented polypeptide chains that together form two centers for palmitate synthesis at the subunit interface (Chang & Hammes, 1990; Smith, 1994, Wakil, 1989). Domain mapping studies employing limited proteolysis, active-site labeling, amino acid sequencing, and mutagenesis have established that each polypeptide contains three amino-terminal catalytic domains separated by a stretch of approximately 600 residues from four carboxy-terminal domains (Figure 1). We have suggested a simple model in which the two polypeptide chains are fully extended side-by-side such that each of the two centers for palmitate synthesis comprises the three amino-terminal catalytic domains of one subunit in concert with the four carboxy-terminal domains of the adjacent subunit (Joshi & Smith, 1993b). The central core of 600 residues has not been assigned a catalytic role, and we have suggested that it may constitute a structural domain that plays a role in stabilizing the dimeric form of the protein. The advent of technology for the expression and mutagenesis of recombinant forms of the animal FAS has provided a unique opportunity to test and refine this working model. 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. Recently, we demonstrated the validity of this

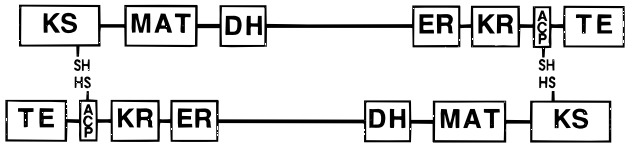

FIGURE 1: Linear domain map of the animal FAS dimer. Residues 1−406 are assigned to the ketoacyl synthase (KS), 428−815 to the malonyl/acetyltransacylase (MAT), 829−969 to the dehydrase (DH), 1630−1850 to the enoyl reductase (ER), 1870−2100 to the ketoreductase (KR), 2114−2190 to the acyl carrier protein (ACP), and 2200−2505 to the thioesterase (TE). The subunits are oriented head-to-tail based on experimental evidence indicating that the active-site cysteine of the β-ketoacyl synthase of one subunit and the 4′-phosphopantetheine of the adjacent subunit can be cross-linked by dibromopropanone (Stoops & Wakil, 1982). Adapted from Joshi and Smith (1993b).

† This work was supported by grant DK 16073 from the National Institutes of Health.

* To whom correspondence should be addressed.

⊗ Abstract published in *Advance ACS Abstracts,* February 1, 1997.

[1] Abbreviations: FAS, fatty acid synthase; ACP, acyl carrier protein; KS^-, β-ketoacyl synthase mutant; ACP^-, acyl carrier protein mutant; DH^-, dehydrase mutant; TE^-, thioesterase mutant; MAT, malonyl/acetyltransacylase.

S0006-2960(96)02696-7 CCC: $14.00

approach 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 acyl carrier protein domain (Witkowski et al., 1996). These two mutants were chosen to test the experimental strategy since there is already strong evidence indicating that the β-ketoacyl synthase and acyl carrier protein 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. We have now extended this approach for mapping functional interactions within the FAS dimer to include FASs defective in the thioesterase, dehydrase, acyl carrier protein, and β-ketoacyl synthase domains.

MATERIALS AND METHODS

Materials. TPCK-trypsin was obtained from Worthington. The sources of other materials used in this study were reported previously (Joshi & Smith, 1993a,b, Witkowski et al., 1996).

in Vitro Mutagenesis. The wild-type recombinant baculoviral transfer vector encoding the 2505-residue wild-type rat FAS was constructed as described earlier (Joshi & Smith, 1993a). Site-directed mutagenesis was carried out by utilizing the overlap polymerase chain reaction method, essentially as described by Shyamala and Ames (1991) except that amplifications were carried out with Vent rather than Taq polymerase. Details of the construction procedures for the dehydrase, H878A (Joshi & Smith, 1993a); acyl carrier protein, S2151A; and β-ketoacyl synthase, C161T and K326A (Witkowski et al., 1996), mutants have been described previously.

The thioesterase mutant, S2302A, was generated using the partial FAS cDNA construct pFAS 1.20 (6281−7020 bp in pUCBM20) as template DNA and primers FAS 67T, FAS 2303B, FAS 5B, and FAS 2303T.[2] Details of the strategy employed for the construction of mutant cDNAs has been described previously (Witkowski et al., 1996). Briefly, the DNA fragment generated by the overlap polymerase chain reaction was used to replace the corresponding fragment of the parent partial cDNA construct using appropriate restriction sites. The resulting cDNA was cloned in *E. coli* DH5α competent cells (Sambrook et al., 1989). The sequence of the amplified region was confirmed, and the fragment moved stepwise into the full-length FAS cDNA in transfer vector pVL1393. Recombinant baculovirus was generated by cotransfecting *Sf*9 cells with the appropriate FAS cDNA construct and BacPAK6 viral DNA (*Bsu*36 I digested) using the Lipofectin method according to manufacturers protocol. Purified recombinant baculoviral stocks were obtained by the plaque purification method (O'Reilly et al., 1992).

The β-ketoacyl synthase mutant C161S was generated along with C161T (Witkowski et al., 1996) by using a primer with a degenerate codon for residue 161. The amplified region of the cDNA encoding the C161S mutation was sequenced, moved into the full-length FAS cDNA construct in a modified pFASTBAC 1 transfer vector, and used to generate recombinant baculovirus stocks by the transposition method employing the Bac-To-Bac Baculovirus expression system (Life Technologies, Gaitherburg, U.S.A.) 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 (Joshi & Smith, 1993a,b). The protein purification procedure was modified by the addition of 10% v/v glycerol to all buffers used during chromatography.

Formation of Heterodimers of FAS Mutants by HPLC. In this protocol (Witkowski et al., 1996), FAS dimers immobilized to an anion exchange resin are first induced to dissociate and the free monomers allowed to rebind to the matrix. The bound dimers and monomers are then selectively released using a phosphate gradient. The extent of dissociation was estimated from the proportion of the total eluted FAS present in the monomer zone. The amount of each mutant FAS required to yield the same amount of monomer was determined by prior experimentation. The appropriate amounts of the two mutants were mixed, diluted to 2 mL with water and injected onto an anion exchange column (TSK-GEL DEAE-5PW, 0.8 × 7.5 cm, 10 μm). The column was washed for 60 min with 50 mM Tris-HCl, pH 8.3/1 mM DTT/1 mM EDTA at flow rate of 0.4 mL/min to induce dissociation of the bound dimers and rebinding of the released monomers. The column was then washed for 5 min with 50 mM potassium phosphate, pH 7/1 mM DTT/1 mM EDTA/10% glycerol and developed using a two-step gradient, from 50 to 70 mM potassium phosphate, pH 7 over 3 min, and from 70 to 200 mM potassium phosphate, pH 7 over 30 min. All gradient buffers contained 1 mM EDTA, 1 mM dithiothreitol, and 10% glycerol.

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 (Witkowski et al., 1996). Transferase activity was assayed by the chromatographic detection of radiolabeled acetyl-*S*-pantetheine formed from [1-^{14}C]acetyl-CoA and pantetheine (Rangan & Smith, 1996). β-Ketoacyl reductase and enoyl reductase activities were assayed by observing spectrophotometrically the oxidation of NADPH in the presence of *trans*-1-decalone (Joshi & Smith, 1993a) or *S*-crotonyl *N*-acetylcysteamine (Kumar et al., 1970), respectively. Dehydrase activity was assayed spectrophotometrically at 270 nm using (*S*)-D,L-β-hydroxybutyryl *N*-acetylcysteamine as a substrate (Kumar et al., 1970). Thioesterase activity was assessed by measuring the free radiolabeled palmitic acid formed from [1-^{14}C]palmitoyl-CoA (Smith, 1981). Oxidation of NADPH in the presence of various acyl-CoA substrates was monitored spectrophotometrically at 340 nm; assays contained either 0.1 mM β-ketobutyryl-CoA, 0.8 mM β-hydroxybutyryl-CoA, or 0.1 mM crotonyl-CoA in 0.1 M potassium phosphate buffer, pH 7, 0.26 mM NADPH, 0.2 mM CoASH, and 0.2 mM tris(2-carboxyethyl)phosphine. Assay of overall FAS activity was performed spectrophotometrically using up to 8 μg of protein

[2] Primers: FAS 67T, ACT CAG TGC AGA GCT CTG AGC GGC C(6766−6790); FAS 2303B, GCT CCA AAA G**C**A TAC CCA GCC ACT CGG TGG GGC C(6962−6995); FAS2303T, GGC CCC ACC GAG TGG CTG GGT AT**G** CTT TTG GAG C(6962−6995); FAS 5B, tat agc ggc cgc tag cTT CAT GGT AGG CAG GTC TAG C(7615−7595). All primers are from 5′ to 3′ direction; numbers in parenthesis correspond to rat FAS cDNA sequence (Amy et al., 1989). Outline letters indicate mutated bases, upper case letters indicate the bases matching FAS cDNA sequence, bases in lower case were incorporated into the primer to engineer the new restriction sites, underlined (gcggccgc, *Not*I; cgatcg, *Nhe*I).

Table 1: Characterization of FAS Homodimeric Mutants

	C161T[a,b] KS$^-$	C161S KS$^-$	K326A[b] KS$^-$	H878A[c] DH$^-$	S2151A[b] ACP$^-$	S2302A TE$^-$
	specific activity (% of wild-type FAS activity)[d]					
Fatty acid synthesis	≤0.5	≤0.65	≤0.5	0.8	≤0.5	≤0.2
malonyl/acetyl transferase	209 ± 28	100 ± 1	123 ± 6	121 ± 8	133 ± 7	139 ± 2
β-ketoacyl synthase	≤0.1	5.5 ± 0.2	≤0.1	97 ± 3	≤0.2	100 ± 3
β-ketoacyl reductase	84 ± 4	105 ± 1	101 ± 7	80 ± 1	97 ± 4	86 ± 2
dehydrase	77 ± 1	102 ± 2	100 ± 3	0 ± 1	108 ± 2	100 ± 1
enoyl reductase	69 ± 7	170 ± 8	59 ± 12	94 ± 3	74 ± 7	170 ± 5
thioesterase	133 ± 4	194 ± 22	90 ± 13	99 ± 10	112 ± 16	2 ± 1
	extent of dissociation (% of the eluted FAS)[e]					
monomer zone	100 ± 3	44 ± 1	48 ± 1	62 ± 2	68 ± 1	62 ± 1

[a] Mutation. [b] Data taken from Witkowski et al., 1996. [c] Data taken from Joshi & Smith, 1993b. [d] The measured specific activities of the wild-type FAS (in mU/mg) were overall FAS 2050 ± 30, malonyl/acetyl transferase 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] Extent of the wild-type FAS dissociation was 62 ± 3%.

(Smith & Abraham, 1970). All enzyme activities were assayed at 37 °C except the transferase which was assayed at 0 °C. 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 per min: for the overall FAS reaction NADPH is the substrate used to calculate enzyme activity.

Analysis of Fatty Acids Synthesized by FAS. For the radiochemical FAS assays the reaction volume was scaled down to 50 μL and [2-^{14}C]malonyl-CoA (13 Ci/mol) replaced unlabeled malonyl-CoA. Protein concentration and incubation time were adjusted to ensure that approximately 10−20% of the radiolabeled substrate was incorporated into fatty acid. Reactions were terminated by the addition of 200 μL of perchloric acid (6% v/v) and fatty acids were extracted with 0.8 mL of hexane/2-propanol (3:2, v/v); the lower phase was reextracted with hexane, and the combined upper phases evaporated in a stream of nitrogen gas. The fatty acids were derivatized with phenacyl-8 (Pierce Chemical Co.) and separated by reversed-phase high-performance liquid chromatography as described previously (Naggert et al., 1991).

RESULTS AND DISCUSSION

Characterization of FAS Mutant Proteins. All of the homodimers containing the same mutation on both polypeptides were inactive in the overall FAS assay, as anticipated. Furthermore, 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).

Surprisingly, however, some of the mutations also affected the extent of dissociation of the dimers when they were bound to the anion exchange column and exposed to the Tris-HCl pH 8.3 buffer (Table 1). The modified stability of the mutated dimers was also evident in their susceptibility to limited proteolysis by trypsin (Figure 2). C161T, the first FAS we constructed carrying a mutation at the active-site cysteine of the β-ketoacyl synthase domain, was the most severely affected. For example, under conditions where the wild-type FAS would undergo 62% dissociation into its component subunits, the C161T mutant was completely dissociated (Table 1). On exposure to trypsin, under conditions where a limited number of sites are normally cleaved generating ultimately a relatively stable core consisting of 125 and 95 kDa polypeptides, the C161T FAS mutant

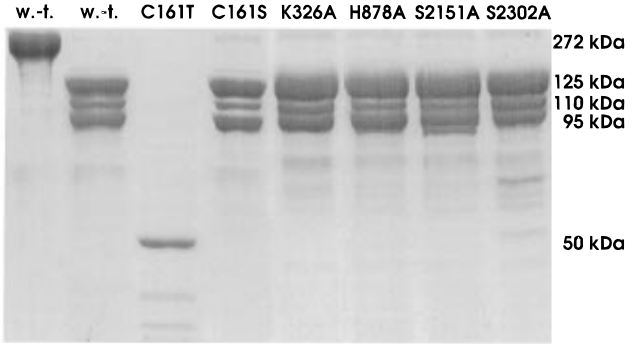

FIGURE 2: Susceptibility of wild-type and mutated FASs to limited proteolysis. FAS proteins (1 mg/mL) in 0.25 M potassium phosphate, pH 7, 1 mM EDTA, 1 mM DTT, and 10% glycerol were incubated with TPCK-trypsin (0.1 mg/mL) at 35 °C for 20 min. Reactions were stopped by addition of a sodium dodecyl sulfate−urea sample preparation buffer and analyzed on a sodium dodecyl sulfate/9% polyacrylamide gel. The major trypsin cleavage sites in the wild-type FAS dimer are following residues: 1152K, 1159K, 1276K, 1281K, 2199K and 2356R, and 2358K (Witkowski et al., 1991). The major stable product is a "nicked dimer" consisting of pairs of 125 and 95 kDa polypeptides that together contain all the activities required to synthesize a long-chain fatty acid, but lack the thioesterase domain. The 110 kDa species present in the digest is slowly converted to the 95 kDa species by removal of residues 1153−1281. The first lane shows the location of wild-type FAS that has not been treated with trypsin.

was completely degraded to small molecular mass polypeptides (Figure 2). These findings prompted us to make two changes in our strategy for complementation analysis.

First, we constructed and utilized two additional FASs, each carrying a single mutation in the β-ketoacyl synthase domain, C161S and K326A. In the latter mutant, replacement of a conserved lysine residue at position 326 with an alanine residue results specifically in a loss of β-ketoacyl synthase activity (Witkowski et al., 1996). Replacement of the active-site residue Cys-161 with Ser resulted in retention of 5% activity in the β-ketoacyl synthase assay, which measures the rate of condensation of acetyl and malonyl moieties. Apparently, substitution of the sulfur atom with the smaller oxygen atom does not completely disrupt the conformation at the active site and some of the nucleophilic character of residue 161 is preserved. Nevertheless, the rate of fatty acid synthesis by this mutant was barely detectable, presumably because the concentration of saturated intermediates available for subsequent condensations is progressively reduced at successive cycles of the reaction sequence.

Table 2: Complementation Analysis of FAS Mutants[a]

	second mutant					
first mutant	KS$^-$ (C161T)	KS$^-$ (C161S)	KS$^-$ (K326A)	DH$^-$ (H878A)	ACP$^-$ (S2151A)	TE$^-$ (S2302A)
	specific activity (% of wild-type FAS activity)[b]					
KS$^-$ (C161T)	≤0.5	nd[c]	0.2 ± 0.3	nd	2.8 ± 0.1	nd
KS$^-$ (C161S)		≤0.65	nd	10.4 ± 0.6	10.0 ± 0.3	9.5 ± 0.2
KS$^-$ (K326A)			≤0.5	8.8 ± 0.2	10.2 ± 0.3	9.2 ± 1.5
DH$^-$ (H878A)				≤0.8	0.1 ± 0.1	0.5 ± 0.1
ACP$^-$ (S2151A)					≤0.5	0.8 ± 0.4
TE$^-$ (S2302A)						≤0.2

[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 Experimental Procedures. [b] The measured specific activity of the wild-type FAS (in mU/mg) was 2050 ± 30 mU/mg. [c] Not determined.

Table 3: Analysis of Fatty Acids Synthesized by Wild Type and Various Combinations of Mutant FASs[a]

		fatty acid		
FAS type		14:0	16:0	18:0
	% of total malonyl-CoA incorporation			
KS$^-$/DH$^-$	(K326A/H878A)	8	82	10
KS$^-$/ACP$^-$	(K326A/S2151A)	7	86	7
KS$^-$/TE$^-$	(K326A/S2302A)	7	83	10
wild type		12	82	6

[a] Details are described in Experimental Procedures.

Stability of the C161S and K326A FAS mutants more closely resembled that of the wild-type protein (Table 1 and Figure 2).

Second, the extent of dissociation of each mutant FAS was determined under the conditions normally employed when the FAS is bound to the anion exchange column and exposed to the Tris-HCl pH 8.3 buffer. These data were used to determine the amount of each mutant FAS required to generate the same amount of monomer for the complementation analysis.

Complementation Analysis of FAS Mutants. As predicted by the working model, mutant FASs functionally defective in the amino-terminal β-ketoacyl synthase domain complemented FASs defective in either of the two carboxy-terminal domains, the ACP or thioesterase domains (Table 2). Additionally, mutant FASs lacking functional carboxy-terminal ACP or thioesterase domains failed to complement each other, again in accordance with the working model. On the other hand, the FAS defective in the dehydrase domain failed to complement either the ACP or thioesterase mutants but did complement the β-ketoacyl synthase mutant FAS; the working model had predicted complementation between the dehydrase and both the ACP or thioesterase mutants but no complementation with the β-ketoacyl synthase mutant. The major product synthesized from malonyl-CoA by all of the complementary mutants was palmitic acid, confirming that complementation resulted in the formation of a completely functional center for fatty acid synthesis (Table 3). The predicted and experimentally determined complementation results for the dehydrase mutant are displayed side-by-side in Figure 3, using the established domain map for the multifunctional polypeptide and assuming a head-to-tail orientation of the subunits that constitute the dimeric FAS.

Since the results with the DH$^-$ mutant were opposite to those predicted by the working model, we have considered the possibility that the results may have been influenced by some unexpected consequence of the mutations introduced into the FAS. The possibility that a false negative complementation might result from detrimental conformational changes occurring in the FAS heterodimers as the result of the juxtaposition of two particular FAS mutants was considered. Thus, we examined in detail the catalytic properties of the heterodimer/homodimer mixture formed by randomization of the H878A and S2302A mutant FASs (DH$^-$ and TE$^-$, respectively). No FAS complementation was observed with these mutants. Three spectrophotometric assays were performed using NADPH in the presence of CoA-linked substrates: β-ketobutyryl-CoA, β-hydroxybutyryl-CoA, and crotonyl-CoA. The wild-type enzyme is capable of converting each of these substrates to butyryl-CoA (Figure 4). First, the substrates are transferred, *via* the MAT active-site Ser-581, to the 4′-phosphopantetheine thiol at Ser-2151 in the ACP domain. The β-ketobutyryl moiety is successively reduced (to a β-hydroxybutyryl moiety), dehydrated (to a crotonyl moiety), and then reduced to a butyryl moiety which is released as product by transfer back to a CoA acceptor. The β-hyroxybutyryl and crotonyl moieties are transferred to the enzyme by the same mechanism, the β-carbon atoms are fully reduced and butyryl-CoA is formed as the product. The actual activity observed depends on which of the three substrates is presented since, for example, β-ketobutyryl moieties are translocated very rapidly between CoA ester and the FAS and result in two reductions, whereas β-hydroxybutyryl and crotonyl moieties are translocated relatively slowly and result in only one reduction (Dodds et al., 1981). The ability of the H878A FAS to oxidize NADPH in the presence of β-ketobutyryl-CoA is much lower than that of the wild-type FAS because in the case of the mutant the product is β-hydroxybutyryl-CoA, which is released from the enzyme very slowly (Dodds et al., 1981). When H878A (DH$^-$) and S2302A (TE$^-$) subunits were randomized, the activity in each of the three assays was as predicted based on the assumption that none of the partial activities of the original mutant homodimers were lost on randomization of the subunits (Table 4). In addition, the ketoacyl synthase and thioesterase activities of the randomized H878A/S2302A FAS also were as predicted, based on the same assumptions (Table 4). Thus, we could find no evidence that juxtaposition of the H878A (DH$^-$) and S2302A (TE$^-$) subunits in a heterodimer resulted in the loss of any of the partial activities. Furthermore, the results of the assays utilizing β-ketobutyryl-CoA, β-hyroxybutyryl-CoA, and crotonyl-CoA indicated that in this heterodimer there must be normal cooperation between the malonyl/acetyltransacylase and ACP domains and between the ACP domain and the two reductase domains. Since there is evidence that transfer of substrates between the malonyl/acetyltransacylase and ACP domains occurs across

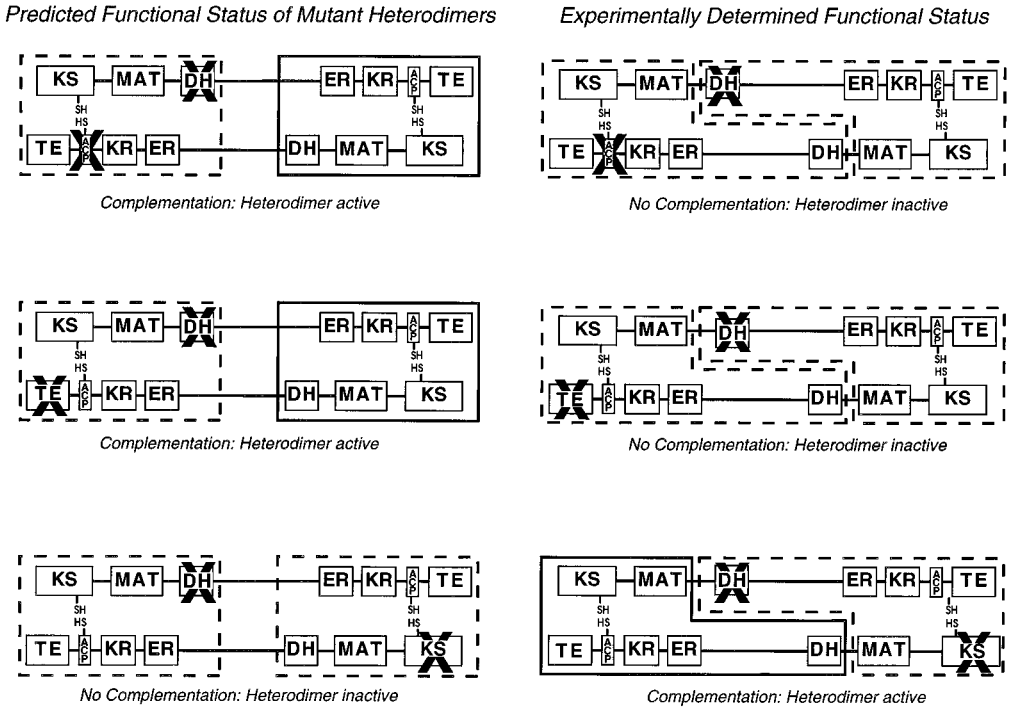

FIGURE 3: Comparison of the predicted and experimentally determined complementation properties of the mutant FAS lacking dehydrase activity, implications to the FAS model. The heterodimers formed from each pair of single-site mutants are presented in the linear domain map format (see Figure 1). The predicted functional interactions are displayed in the left side panels and the experimentally determined interactions on the right. Domains compromised by mutation are denoted by bold crosses. Domains that collectively constitute a center for fatty acid synthesis are boxed, either by a solid line, indicating a functionally active center, or by a dashed line, indicating an inactive center compromised by at least one mutation. Thus, heterodimers illustrated in the top panel were reconstituted from DH$^-$ and ACP$^-$ mutant subunits, those in the center panel from DH$^-$ and TE$^-$ mutant FASs and those in the bottom panel from DH$^-$ and KS$^-$ mutant subunits.

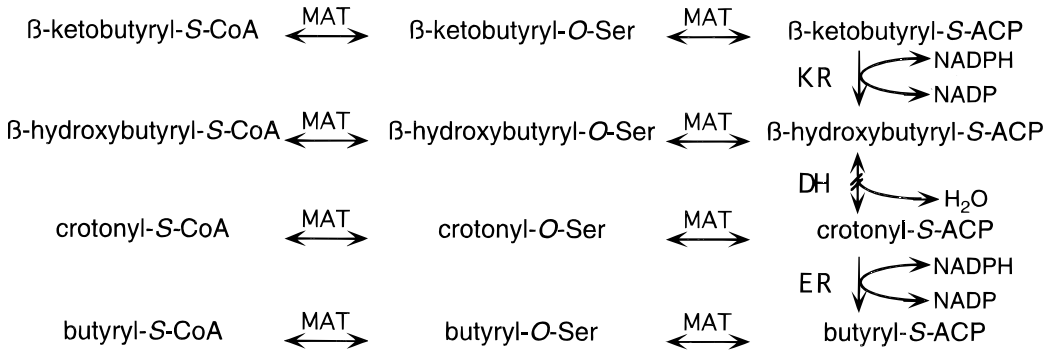

FIGURE 4: Pathway for formation of butyryl-CoA from β-ketobutyryl-CoA, β-hydroxybutyryl-CoA, and crotonyl-CoA. MAT, malonyl/acetyltransacylase; KR, β-ketoacyl reductase; DH, dehydrase; ER, enoyl reductase. The block in the dehydrase reaction caused by the H878A mutation (//) eliminates the ability of the FAS to metabolize β-hydroxybutyryl-CoA and lowers the rate of metabolism of β-ketobutyryl-CoA since the MAT has limited ability to process β-hydroxybutyryl moieties (Dodds et al., 1981).

Table 4: Catalytic Properties of Dimers Formed by Randomization of DH$^-$ and TE$^-$ Subunits

FAS type	DH$^-$, H878A	TE$^-$, S2302A	DH$^-$/TE$^-$, randomized	DH$^-$/TE$^-$, expected value
	specific activity (% of wild-type FAS activity)[a]			
β-ketobutyryl-CoA reduction	9 ± 0	103 ± 4	66 ± 1	56
β-hydroxybutyryl-CoA reduction	0 ± 1	91 ± 0	54 ± 2	46
crotonyl-CoA reduction	89 ± 3	91 ± 0	95 ± 3	90
β-ketoacyl synthase	98 ± 0	101 ± 0	101 ± 0	100
thioesterase	112 ± 4	0 ± 0	50 ± 3	56

[a] The measured specific activities of the wild-type FAS (in mU/mg) were: β-ketobutyryl-CoA reduction 5150 ± 220, β-hydroxybutyryl-CoA reduction 395 ± 2, crotonyl-CoA reduction 630 ± 20, β-ketoacyl synthase 113 ± 2, thioesterase 560 ± 60.

the subunit interface (Petithory and Smith, 1993), it seems likely that the subunits make proper functional contacts in the heterodimer. Consequently, there is every indication that in the H878A/S2302A heterodimer the only functions compromised are those which depend on the integrity of the mutagenized active sites, i.e., the dehydrase activity of one subunit and the thioesterase activity of the other subunit. The possibility that a false positive complementation might result from activation of one of the mutated domains was also investigated by examining the catalytic properties of the heterodimer/homodimer mixture formed by randomization of the H878A and C161S mutant FASs (DH$^-$ and KS$^-$,

Table 5: Catalytic Properties of Dimers Formed by Randomization of KS^- and DH^- Subunits

FAS type	KS^-, C161S	DH^-, H878A	KS^-/DH^-, randomized	KS^-/DH^-, expected value
	specific activity (% of wild-type FAS activity)[a]			
β-hydroxybutyryl-CoA reduction	75 ± 1	1 ± 2	33 ± 3	38
β-ketoacyl synthase	6 ± 1	98 ± 0	51 ± 1	52

[a] The measured specific activities of the wild-type FAS (in mU/mg) were β-hydroxybutyryl-CoA reduction 372 ± 5 and β-ketoacyl synthase 138 ± 2.

respectively). The ability of the dimers formed by randomization of the mutated subunits to metabolize β-hydroxybutyryl-CoA and catalyze the β-ketoacyl synthase reaction was as predicted, indicating that no activation of the mutated domains had occurred (Table 5). Finally, it may also be pertinent to point out that the complementation results with the DH^- subunits are internally consistent in that where complementation was anticipated, none was found; where it was not anticipated, it was found. It seems highly unlikely therefore that both findings could result from some unanticipated experimental artifact.

Consequently, the results can be interpreted to indicate quite clearly that the dehydrase domain, although located in the amino-terminal half of the polypeptide, exhibits complementation characteristics consistent with it functioning as a member of the carboxy-terminal complementation group that also includes the ACP and thioesterase domains. It can be inferred therefore that the dehydrase domain forms part of a palmitate-producing unit that includes the ACP and thioesterase domains of the same subunit and the β-ketoacyl synthase domain of the opposing subunit (Figure 3, right hand side diagrams). The catalytic elements of the FAS polypeptides located in the amino-terminal and carboxy-terminal halves are separated by more than 600 residues that appear to constitute a structural core. Thus, assignment of the dehydrase domain to the carboxy-terminal complementation group constitutes the first experimental evidence indicating that the subunits of the FAS are not positioned side-by-side in a fully extended configuration. Rather, they must be coiled in a manner that allows the dehydrase active-site histidine residue at position 878 to make functional contact with β-hydroxyacyl moieties attached to the 4′-phosphopantetheine located more than 1250 residues distant at serine-2151.

Quantitative Aspects of the Complementation Analysis. Theoretically, if the rates of dissociation and reassociation of the various FASs employed are unaffected by the point mutations, then the mutant subunits ought to reassemble randomly yielding a population consisting of 50% heterodimers and 25% of each homodimer. If the two centers for fatty acid synthesis present in the wild-type dimer function entirely independently, then a pair of complementing mutants would be expected to generate heterodimers with only one of the two centers functional. Thus, a randomized population of complementing mutant FASs ought to exhibit 25% of the normal wild-type FAS activity. None of the complementing mutants reached this level of activity. The DH^-, ACP^-, and TE^- FASs each complemented either the C161S or K326A KS^- FAS to approximately 10% of the wild-type FAS activity (Table 1). Despite the precautions taken to ensure that equal amounts of each mutant monomer were available for the reassociation process, the C161T mutant was much less effective in complementing the DH^-, ACP^-, and TE^- mutants than the C161S or K326A KS^- FASs. At present, it is not clear whether this difference results from an altered rate of reassociation of the C161T mutant subunits and a consequent distortion of the "randomized" FAS population or whether the effects of this mutation on the physical properties of the FAS also compromise the ability of other domains in the subunit to cooperate with each other functionally. The other KS^- FASs do not differ markedly from the wild-type FAS as far as dimer stability and susceptibility to digestion by trypsin is concerned and these mutants complement with DH^-, ACP^-, and TE^- mutants quite uniformly to about 10% of the wild-type activity. If, for these C161S and K326A mutations, secondary effects on physical properties of the FAS can indeed be discounted, then the data would suggest that an inactive β-ketoacyl synthase in one of the two centers for fatty acid synthesis can also affect the activity of the second center. Attempts to resolve this question by separating the active heterodimer species from the inactive homodimers are presently under way in our laboratory.

Three other domains remain to be mapped by the functional complementation strategy, the malonyl/acetyltransacylase, the enoyl reductase, and the β-ketoacyl reductase. In the case of the malonyl/acetyltransacylase, there is already independent experimental evidence indicating that this domain can transfer substrate moieties to the ACP domain of the opposite subunit (Petithory & Smith, 1993); nevertheless, there is no experimental data available that would rule out unequivocally the possibility that the malonyl/acetyltransacylase can also transfer substrates to the ACP domain of the same subunit. Therefore, in the revised model we can only tentatively assign the malonyl/acetyltransacylase to the same complementation group as the adjacent β-ketoacyl synthase domain. At present, we have no clues as to the likely functional status of the two reductase domains and they have been tentatively assigned to the complementation group that includes the two other domains located in the carboxy-terminal half of the polypeptide, the ACP, and thioesterase. We are presently constructing the additional mutant FASs that are required to complete the complementation analysis and resolve these remaining ambiguities.

ACKNOWLEDGMENT

We thank Ausaf Bari for performing the malonyl/acetyltransacylase assays.

REFERENCES

Alberts, A. W., & Greenspan, M. D. (1984) in *Fatty Acid Metabolism and Its Regulation* (Numa, S., Ed.) pp 29−58, Elsevier, Amsterdam.

Amy, C., Witkowski, A., Naggert, J., Williams, B., Randhawa, Z., & Smith, S. (1989) *Proc. Natl. Acad. Sci. U.S.A. 86*, 3114−3118.

Chang, S. I., & Hammes, G. G. (1990) *Acc. Chem. Res. 23*, 363−369.

Dodds, P. F., Guzman, M. G. F., Chalberg, S. C., Anderson, G. J., & Kumar, S. (1981) *J. Biol. Chem. 256*, 6282−6290.

Holzer, K. P., Liu, W., & Hammes, G. G. (1989) *Proc. Natl. Acad. Sci. USA 86*, 4387−4391.

Joshi, A. K., & Smith, S. (1993a) *Biochem. J. 296*, 143−149.

Joshi, A. K., & Smith, S. (1993b) *J. Biol. Chem. 268*, 22508−22513.
Kumar, S., Dorsey, J. A., Muesing, R. A., & Porter, J. W. (1970) *J. Biol. Chem. 245*, 4732−4744.
Naggert, J., Narasimhan, M. L., De Veaux, L., Cho, H., Randhawa, Z. I., Cronan, J.E., Jr., Green, B. N., & Smith, S. (1991) *J. Biol. Chem. 266*, 11044−11050.
O'Reilly, D. R., Miller, L. K., & Luckow, V. A. (1992) in *Baculovirus Expression Vectors, A Laboratory Manual*, pp 150−155, W. H. Freeman and Co., New York.
Petithory, J. R., & Smith, S. (1993) *Biochem. J. 292*, 361−364.
Rangan, V. S., & Smith, S. (1996) *J. Biol. Chem. 271*, 31749−31755.
Sambrook, J., Fritsch, E. F., & Maniatis, T. (1989) in *Molecular Cloning: a Laboratory Manual*, pp 16.66−16.67, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
Schweizer, M., Takabayashi, K., Laux, T., Beck, K.-F., & Schreglmann, R. (1989) *Nucleic Acids Res. 17*, 567−587.
Shyamala, V., & Ames, G. F.-L. (1991) *Gene 97*, 1−6.
Smith, S. (1981) *Methods Enzymol. 71C*, 181−188.
Smith, S. (1994) *FASEB J. 8*, 1248−1259.
Smith, S., & Abraham, S. (1970) *J. Biol. Chem. 245*, 3209−3217.
Stoops, J. K., & Wakil, S. J. (1982) *J. Biol. Chem. 257*, 3230−3235.
Wakil, S. J. (1989) *Biochemistry 28*, 4523−4530.
Witkowski, A., Rangan, V. S., Randhawa, Z. I., Amy, C. M., & Smith, S. (1991) *Eur. J. Biochem. 198*, 571−579.
Witkowski, A., Joshi, A. K., & Smith, S. (1996) *Biochemistry 35*, 10569−10575.

BI9626968