Mapping the Functional Topology of the Animal Fatty Acid Synthase by Mutant Complementation in Vitro†

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ABSTRACT: An in vitro mutant complementation approach has been used to map the functional topology of the animal fatty acid synthase. A series of knockout mutants was engineered, each mutant compromised in one of the seven functional domains, and heterodimers generated by hybridizing all possible combinations of the mutated subunits were isolated and characterized. Heterodimers comprised of a subunit containing either a β-ketoacyl synthase or malonyl/acetyltransferase mutant, paired with a subunit containing mutations in any one of the other five domains, are active in fatty acid synthesis. Heterodimers in which both subunits carry a knockout mutation in either the dehydrase, enoyl reductase, keto reductase, or acyl carrier protein are inactive. Heterodimers comprised of a subunit containing a thioesterase mutation paired with a subunit containing a mutation in either the dehydrase, enoyl reductase, β-ketoacyl reductase, or acyl carrier protein domains exhibit very low fatty acid synthetic ability. The results are consistent with a model for the fatty acid synthase in which the substrate loading and condensation reactions are catalyzed by cooperation of an acyl carrier protein domain of one subunit with the malonyl/acetyltransferase or β-ketoacyl synthase domains, respectively, of either subunit. The β-carbon-processing reactions, responsible for the complete reduction of the β-ketoacyl moiety following each condensation step, are catalyzed by cooperation of an acyl carrier protein domain with the β-ketoacyl reductase, dehydrase, and enoyl reductase domains associated exclusively with the same subunit. The chain-terminating reaction is carried out most efficiently by cooperation of an acyl carrier protein domain with the thioesterase domain of the same subunit. These results are discussed in the context of a revised model for the fatty acid synthase.

The animal fatty acid synthase is comprised of a pair of identical 272 kDa polypeptides that contain all of the catalytic components needed to direct the entire series of reactions required for this biosynthetic pathway. Unraveling the details of how these reactions are coupled together in a single protein represents a major challenge in structural and biochemical analysis.

Domain mapping of the homodimeric animal FAS[1] employing limited proteolysis (*1*−*4*), active-site labeling (*5*−*8*), amino acid sequencing (*9*−*11*), and mutagenesis (*12*) established that each polypeptide contains three N-terminal catalytic domains, β-ketoacyl synthase (KS), malonyl/acetyltransferase (MAT), and dehydrase (DH), separated by approximately 600 residues from four C-terminal domains, enoyl reductase (ER), β-ketoacyl reductase (KR), acyl carrier protein (ACP), and thioesterase (TE) (*13*). The dual-specificity MAT is responsible for translocating acetyl and malonyl substrate moieties from the CoA ester form to the phosphopantetheine prosthetic group of the ACP domain. The KS is responsible for the translocation of acetyl (and subsequently C4:0-C14:0) moieties from the phosphopantetheine to the active-site cysteine thiol (*14*). Condensation of the chain extender malonyl moiety with the acetyl (and subsequently C4:0-C14:0) moiety is catalyzed by the KS, and the β-C processing reactions are catalyzed by sequential action of the KR, DH, and ER. Normally, when the saturated acyl chain length reaches 16 C atoms, the chain-terminating thioesterase releases the palmitoyl moiety from the phosphopantetheine thiol as the free acid.

Development of a model for the FAS was strongly influenced by two observations. First, the monomeric form of the protein is inactive in the overall FAS reaction (*15*−*17*), yet two centers for palmitate synthesis are present in the dimer (*18*, *19*). Second, the classical experiments of Stoops and Wakil (*20*) showed that the two subunits can be cross-linked by dibromopropanone via the active-site cysteine residue of the KS and the phosphopantetheine thiol of the ACP domain associated with the companion subunit. Thus, in the textbook model for the FAS, the two polypeptides are shown 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 (*13*, *21*, *22*).

To provide a rigorous test of the head-to-tail model, we introduced a mutant complementation strategy that facilitated mapping of the functional topology of the FAS dimer.

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[1] Abbreviations: FAS, fatty acid synthase; KS, β-ketoacyl synthase; ACP, acyl carrier protein; DH, dehydrase; KR, ketoacyl reductase; ER, enoyl reductase; TE, thioesterase; MAT, malonyl/acetyltransacylase. The symbol $^-$, following a domain abbreviation, indicates functionality has been compromised by mutation.

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Table 1: PCR Primers Used for Engineering Enoyl Reductase and Ketoreductase Mutants

primer[a]	sequence[b]	location[c]
ER G1672V		
FAS 67T	5′-GACTGCATGCTTGGCATGGAG	4849−4869
FAS 1672T	5′-TCACTCGGGCTCC**KYA**GGTGTGGGCCAA	5082−5109
FAS 1672B	5′-TTGGCCCACACC**TRM**GGAGCCCGAGTGA	5109−5082
FAS 67B	5′-CATCCAGCAGGATCCCATGGA	5455−5435
ER G1673Y		
FAS 1673T	5′-ACTCGGGCTCCGGT**TWT**GTGGGCCAA	5084−5109
FAS 1673B	5′-TTGGCCCAC**AWA**ACCGGAGCCCGAGT	5109−5084
ER G1675A/G1672V		
FAS 1675T	5′-ACTCGGGCTCCGGTGGTGTG**RCA**CAAGCGGCCATT	5084−5188
FAS 1675B	5′-AATGGCCGCTTG**TGY**CACACCACCGGAGCCCGAGT	5188−5084
KR G1886F		
FAS 200T	5′-CATGGCCATCTTCTTGAAGAA	5406−5427
FAS 1886T	5′-ACATCATCACTGGT**TWT**CTAGGTGGCTT	5723−5751
FAS 1886B	5′-AAAGCCACCTAG**AWA**ACCAGTGATGATT	5751−5723
KR G1888A		
FAS1888T	5′-ATCACTGGTGGCCTA**RCA**GGCTTTGGCCTGGAA	5728−5760
FAS1888B	5′-TTCCAGGCCAAAGCC**TGY**TAGGCCACCAGTGAT	5760−5728
FAS200B	5′-GTGACGGTGTCGCGTAGAGA	6331−6310

[a] T/B (top/bottom) indicate sense/antisense oligomers, respectively. [b] Bold letters indicate mutated nucleotides. [c] Residue numbering is according to the FAS cDNA sequence (*10*).

Several of our findings are incompatible with the classical model. In particular, our results revealed that functional interactions can occur between domains distantly located on the same polypeptide (*23*, *24*). Furthermore, in reexamining the products formed by treatment of the FAS with dibromopropanone, we found that this reagent is capable of cross-linking the active-site cysteine residue of the KS domain and the pantetheine thiol of the ACP domain both between and within subunits (*25*). Approximately 35% of the wild-type FAS polypeptides could be cross-linked internally by the reagent, even though the participating thiols are located 1990 residues distant on the same polypeptide. Consequently, we proposed a revised model in which head-to-tail contacts are possible both between and within subunits (*24*−*26*).

The previously reported mutant complementation analyses included FASs defective in the KS, MAT, DH, ACP, and TE domains. Assay of the fatty acid synthesizing capacity of complementary mutants was performed on mixtures containing both the active heterodimers and the inactive parental homodimers. Subsequently, we have devised a double-tagging, dual-affinity chromatographic procedure that permits isolation of heterodimers and have engineered FASs defective in their ER and KR functions. In this paper, we report the isolation and characterization of FAS heterodimers constructed from all possible combinations of subunits defective in one of the seven functional domains.

MATERIALS AND METHODS

Materials. Anti-FLAG M2 affinity chromatography gel was purchased from Eastman Kodak Co., and Ni−NTA agarose from Qiagen Inc. FLAG octapeptide (DYKDDDDK) was synthesized at the PAN Facility, Beckman Center, Stanford University Medical Center (Stanford, CA). The sources of other materials have been described in detail in previous publications (*12*, *23*, *27*, *28*).

Construction of cDNAs Encoding His_6- and FLAG-Tagged FASs and Expression of the Proteins in Sf9 Cells. Procedures for the construction and expression of full-length wild-type rat FAS, and mutants thereof, defective in the thioesterase (*23*), malonyl/acetyltransferase (*24*), ACP (*27*), and β-keto-acyl synthase (*23*) domains were described in detail previously. His_6 and FLAG tags were introduced at the N-terminus, at the C-terminus, or in the central core of the polypeptide, between residues 1154 and 1157, to facilitate isolation of the heterodimers by dual-affinity chromatography (*28*). In vitro, site-directed mutagenesis was carried out by the overlap polymerase chain reaction method (*29*) using Vent DNA polymerase. The sequences and locations of various primers used to mutate residues in the reductase domains are described in Table 1.

A partial FAS cDNA construct pFAS 305.20 (nucleotides 2539−7615 in pUCBM20) was used as the DNA template. Mutations in the ER domain were introduced using the appropriate set of mutagenic primers in combination with end primers 67T and 67B, whereas those in the KR domain were introduced using the mutagenic primers in combination with end primers 200T and 200B. The mutated partial cDNA fragment, amplified by the overlap polymerase chain reaction, was used to replace the corresponding fragment in pFAS 305.20, using standard cloning techniques (*30*). The amplified DNA region was sequenced and moved stepwise into an appropriate His_6- or FLAG-tagged, full-length, wild-type construct. The final FAS cDNA constructs, in the context of the pFASTBAC1 vector (FB), were used to generate recombinant baculovirus stocks by the transposition method, employing the BAC-to-BAC baculovirus expression system, according to the manufacturer's instructions. *Sf*9 cells were then infected with the purified recombinant viruses and cultured for 48 h at 27 °C. The tagged FAS proteins were purified from the cytosols as described previously (*31*), except that glycerol (10%, v/v) was included in all buffers used for chromatography. Purified FASs were stored at −80 °C in 0.25 M potassium phosphate buffer (pH 7) containing 1 mM dithiothreitol, 1 mM EDTA, and 10% glycerol.

Randomization of FAS Subunits and Isolation of Heterodimers. Typically, the purified homodimeric His_6- and FLAG-tagged FAS preparations were diluted approximately 10-fold, with 1 mM dithiothreitol and 1 mM EDTA in water, to give a final potassium phosphate buffer concentration of 25−35 mM and a glycerol concentration of ∼1%. The

appropriate pairs of homodimeric FASs were mixed together at equal concentrations, typically 0.3 mg/mL, and stored at 4 °C for 7−9 days to promote dissociation into the component subunits. Spontaneous reassociation of the subunits was induced by adjustment of the solvent to approximately 200 mM potassium phosphate (pH 7), 1 mM EDTA, 1 mM dithiothreitol, and 10% glycerol, and incubation for 75 min at 30 °C. The heterodimers, containing different mutations on each subunit, were then isolated by sequential affinity chromatography on an anti-FLAG antibody column, using the FLAG octapeptide in the eluting buffer, and a Ni−NTA column, using imidazole in the eluting buffer (*28*).

Thermal Lability of FASs. Homodimeric FASs (0.2 mg/mL) were incubated at 45 °C in 0.25 M potassium phosphate buffer (pH 7.0), 1 mM EDTA, and 1 mM dithiothreitol (<1% glycerol). Because heterodimeric FAS preparations were obtained at much lower protein concentrations, slightly different conditions were used to assess heat lability. FASs (0.1 mg/mL) were incubated at 47.5 °C in 0.25 M potassium phosphate buffer (pH 7.0), 1 mM EDTA, 1 mM dithiothreitol, and 10% glycerol. At various intervals, portions were removed for an assay of either crotonyl-CoA reductase or β-ketobutyryl-CoA reductase activity. Assay systems, at 37 °C, contained 0.1 M potassium phosphate buffer (pH 7), 40 μM CoA, and 290 μM NADPH; FAS was added, and 1 min later, the reactions were started by the addition of substrate, either crotonyl-CoA or β-ketobutyryl-CoA, at a final concentration of 0.1 mM. Activity was monitored spectrophotometrically by recording the disappearance of NADPH at 340 nm.

Other Enzyme Assays. Overall fatty acid synthesizing activity was measured spectrophotometrically (*32*). β-Ketoacyl reductase activity was monitored spectrophotometrically using *trans*-1-decalone as the substrate (*31*).

Fatty Acid Analysis. [2-^{14}C]Malonyl-CoA was included in the assays for overall fatty acid synthesizing activity, and the products were analyzed as phenacyl derivatives by HPLC (*33*).

RESULTS

Identification of FAS Mutants Defective in Keto Reductase and Enoyl Reductase Activities. Our strategy for engineering FASs defective in β-ketoacyl and enoyl reductase activities was directed toward identification of mutations that might disrupt each of the two NADPH binding sites. The pyridine nucleotide-binding sites of many enzymes are characterized by the presence of a glycine-rich region, typically conforming to the GxGxxG/AxxxA motif, which marks the location of a turn between a β-sheet/α-helix structure critical for nucleotide binding (*34*, *35*). The absence of side chains at the conserved positions appears to be important in facilitating nucleotide binding (*36*, *37*). The rat FAS amino acid sequence contains two glycine-rich regions, 1670-GSGGVGQAAISIA in the enoyl reductase domain and 1885-GGLGGFGLDLA in the β-ketoacyl reductase domain (*10*). Replacement of single glycine residues within the reductase domains severely reduced FAS activity (Table 2).

In all cases, the glycine replacements reduced the activity of the targeted reductase domain without compromising the activity of the other reductase domain. Since the original identification of the two reductase domains had been inferred from indirect evidence (*10*, *13*), this experimental evidence provided the first formal proof of their location in the multifunctional FAS polypeptide. Thus, these reductase mutants appeared to be suitable for complementation analysis.

Table 2: Properties of Ketoreductase and Enoylreductase Mutants

FAS type	fatty acid synthesis	ketoreductase[a]	enoylreductase[b]
wild type	2200 ± 48	16000 ± 710	720 ± 39
G1673Y	0	15900 ± 100	0
G1672V	4 ± 3	14200 ± 510	1 ± 1
G1672V/G1675T[c]	2 ± 1	16300 ± 1000	2 ± 1
G1886F	6 ± 2	44 ± 2	673 ± 73
G1888A	136 ± 3	354 ± 3	834 ± 7

[a] Assayed using *trans*-1-decalone as a substrate. [b] Assayed using crotonyl-CoA as a substrate. [c] The G1675T mutation was unintentionally introduced during the polymerase chain reaction step.

Thermal Stability of Homodimeric FAS Mutants. We had previously observed that some FASs containing single mutations that compromised the activity of only one functional domain, nevertheless, exhibited decreased stability, as measured by an increased tendency toward subunit dissociation or increased susceptibility to proteolysis (*23*). We therefore designed a simple thermal lability assay to assess the effects of mutations in the reductase domains on the overall stability of the protein. FASs were incubated at 45 °C and reductase activities assessed periodically, using either β-ketobutyryl-CoA or crotonyl-CoA as a substrate. The FAS reduces these substrates by first translocating the crotonyl or β-ketobutyryl moiety from CoA to the phosphopantetheine of the ACP domain, in a reaction catalyzed by the MAT domain (*23*). The crotonyl, or β-ketobutyryl, moiety is then reduced to a butyryl, or β-hydroxybutyryl, moiety in a reaction catalyzed by the ER, or KR, domain. The butyryl moiety can be translocated back to a CoA acceptor, in a reaction catalyzed by the MAT domain. The β-hydroxybutyryl moiety either can be translocated directly back to a CoA acceptor or can undergo further conversion to either a crotonyl or butyryl moiety, depending on whether the required DH and ER domains are catalytically active, prior to translocation back to CoA. Thus, the successful reduction of either of these substrates depends on the ability of several domains within the FAS dimer to interact functionally with each other. Consequently, by first subjecting the FASs to heat treatment, the assays can be used to assess the effect of mutations on the maintenance of the physical and functional integrity of the complex. For comparison, the stabilities of wild-type homodimers and homodimers carrying a mutation in the KS, DH, or TE domains were also determined. Because the functionality of the MAT and ACP domains is required for the assays, the effects of mutations in these domains could not be assessed by this procedure in homodimeric FASs.

The thermal lability of the wild-type FAS homodimers was unaffected by the presence of a FLAG or His_6 tag, and the half-life was essentially the same, regardless of whether activity was assessed using crotonyl-CoA or β-ketobutyryl-CoA as a substrate (Table 3).

The first ER$^-$ and KR$^-$ mutants we engineered, G1673Y and G1886F, respectively, exhibited seriously impaired

Table 3: Thermal Stability of Homodimeric FAS Mutants[a]

FAS type[b]	substrate	half-life (min)
WT[c]	4:1-CoA	22
WT	AcOAc-CoA	20
KR$^-$ (G1886F, cF)	4:1-CoA	1
KR$^-$ (G1888A, cF)	4:1-CoA	16
ER$^-$ (G1673Y, cF)	AcOAc-CoA	2
ER$^-$ (G1675T/G1672V, cF)	AcOAc-CoA	6
ER$^-$ (G1672V, cH)	AcOAc-CoA	8
KS$^-$ (C161S)	4:1-CoA	30
KS$^-$ (C161T)	4:1-CoA	2
KS$^-$ (K326A)	4:1-CoA	23
KS$^-$ (K326L)	4:1-CoA	14
KS$^-$ (K326R)	4:1-CoA	2
DH$^-$ (H878A)	4:1-CoA	13
TE$^-$ (S2302A)	4:1-CoA	23

[a] FASs (0.2 mg/mL) were preincubated at 45 °C in 0.25 M potassium phosphate buffer (pH 7), 1 mM EDTA, and dithiothreitol, and at intervals, portions were removed for an assay of either crotonyl-CoA reductase (4:1-CoA) or acetoacetyl-CoA reductase (AcOAc-CoA) activity. [b] Proteins are untagged unless otherwise stated; the location and identity of the tag are indicated by c (for C-terminal) and either F or H (for FLAG or His_6, respectively). [c] N-Terminally, internally, and C-terminally FLAG-tagged and C-terminally His_6-tagged wild-type FAS exhibited the same half-life.

stability, prompting us to seek alternative mutations. Subsequently, we engineered a KR$^-$ mutant with significantly improved stability (G1888A) and two ER$^-$ mutants with slightly improved stabilities (the double mutant G1675T/G1672V and the single mutant G1672V). Among the various KS$^-$ mutant homodimers that were examined, only the C161T and K326R homodimers exhibited markedly decreased stability; the C161T mutant had previously been found to dissociate into subunits more readily than did the wild-type FAS and to exhibit increased susceptibility to limited proteolysis by trypsin (*23*). The stability of the DH$^-$ mutant was reduced slightly, and the stability of the TE$^-$ mutant was similar to that of the wild-type FAS.

Isolation and Characterization of FAS Heterodimers. Early attempts to form heterodimeric FASs in vitro from pairs of purified mutated homodimeric FASs revealed that FAS dimers do not engage in spontaneous subunit exchange to any significant extent (*27*). Consequently, to randomize the subunits from two different FAS mutants, it is necessary first to deliberately induce dissociation of the dimers and then facilitate reassociation into a mixture of both hetero- and homodimers. The animal FAS is a cold labile protein that can be dissociated into its component subunits by storage in low ionic strength media in the refrigerator. Typically, wild-type homodimers stored at 0−4 °C in 0.05 M potassium phosphate buffer (pH 7), containing 1 mM DTT, 1 mM EDTA, and 10% glycerol, undergo more than 90% dissociation in 1 week. These monomers undergo spontaneous and complete reassociation when the phosphate concentration is adjusted to 0.25 M and the temperature increased to 30 °C for several minutes. Fatty acid synthesizing activity of the wild-type FAS is recovered fully through this dissociation−reassociation process. The subunits from 34 different pairs of FAS mutants were randomized in this way, and the heterodimers, containing a different mutation on each subunit, were separated from the parental homodimers, using the dual-affinity chromatographic procedure, and their fatty acid synthesizing abilities assessed (Table 4).

Table 4: FAS Activity of Isolated Heterodimers Consisting of Two Differently Mutated Subunits[a]

subunit A	subunit B	FAS activity[b] (% of wild-type value)
KS$^-$ (K326A, iF)	MAT$^-$ (S581A, cH)	39 ± 1.0
KS$^-$ (K326A, iF)	DH$^-$ (H878A, cH)	22 ± 0.8
KS$^-$ (K326A, cH)	ER$^-$ (G1673Y, cF)	19 ± 0.4
KS$^-$ (K326A, cH)	KR$^-$ (G1886F, cF)	21 ± 1.0
KS$^-$ (K326A, cH)	KR$^-$ (G1888A, cF)	22 ± 0.8
KS$^-$ (K326A, iF)	ACP$^-$ (S2151A, cH)	19 ± 0.7
KS$^-$ (K326A, cH)	ACP$^-$ (S2151A, nF)	16 ± 0.7
KS$^-$ (C161S, cH)	ACP$^-$ (S2151A, nF)	18 ± 0.3
KS$^-$ (C161N, cH)	ACP$^-$ (S2151A, nF)	21 ± 1.0
KS$^-$ (C161Q, cH)	ACP$^-$ (S2151A, nF)	25 ± 1.0
KS$^-$ (K326A, cH)	TE$^-$ (S2302A, cF)	23 ± 0.8
MAT$^-$ (S581A, iF)	DH$^-$ (H878A, cH)	37 ± 1.3
MAT$^-$ (S581A, cH)	ER$^-$ (G1673Y, cF)	28 ± 0.8
MAT$^-$ (S581A, iF)	ER$^-$ (G1672V, cH)	21 ± 0.9
MAT$^-$ (S581A, cH)	KR$^-$ (G1886F, cF)	19 ± 0.6
MAT$^-$ (S581A, cH)	KR$^-$ (G1888A, cF)	33 ± 0.8
MAT$^-$ (S581A, iF)	ACP$^-$ (S2151A, cH)	31 ± 0.7
MAT$^-$ (S581A, cH)	TE$^-$ (S2302A, cF)	38 ± 1.2
DH$^-$ (H878A, cH)	ER$^-$ (G1673Y, cF)	1.0 ± 0.3
DH$^-$ (H878A, cF)	ER$^-$ (G1672V, cH)	1.0 ± 0.3
DH$^-$ (H878A, cH)	KR$^-$ (G1888A, cF)	3.0 ± 0.5
DH$^-$ (H878A, cH)	ACP$^-$ (S2151A, nF)	0
DH$^-$ (H878A, cH)	TE$^-$ (S2302A, cF)	3.0 ± 0.5
ER$^-$ (G1673Y, cF)	KR$^-$ (G1888A, cH)	3.0 ± 0.5
ER$^-$ (G1672V, cH)	KR$^-$ (G1888A, cF)	3.0 ± 0.9
ER$^-$ (G1673Y, cF)	ACP$^-$ (S2151A, cH)	0.5 ± 0.1
ER$^-$ (G1672V, cH)	ACP$^-$ (S2151A, nF)	0.4 ± 0.1
ER$^-$ (G1672V/G1675T, cF)	TE$^-$ (S2302A, cH)	3.0 ± 0.4
ER$^-$ (G1672V, cH)	TE$^-$ (S2302A, cF)	1.0 ± 0.2
KR$^-$ (G1886F, cF)	ACP$^-$ (S2151A, cH)	0.6 ± 0.2
KR$^-$ (G1888A, cH)	ACP$^-$ (S2302A, nF)	3.5 ± 0.2
KR$^-$ (G1886F, cF)	TE$^-$ (S2302A, cH)	3.0 ± 0.4
KR$^-$ (G1888A, cH)	TE$^-$ (S2302A, cF)	5.0 ± 0.4
ACP$^-$ (S2151A, cH)	TE$^-$ (S2302A, cF)	3.0 ± 0.9

[a] Following the identity of the mutations, the location and type of the tags are indicated by n, i, or c (for N-terminal, internal, and C-terminal, respectively) and either F or H (for FLAG or His_6, respectively). [b] The average specific activity of wild-type FAS homodimers was 2050 ± 30 munits/mg. The specific activities of homodimers carrying the designated mutations in both subunits were less than 1% of the wild-type value, with the exception of those for G1888A (KR$^-$) and S2302 (TE$^-$), which were 6.6 ± 0.1 and 2.0 ± 1% of the wild-type values, respectively (measured under conditions optimal for wild-type FAS). The major product synthesized by all heterodimers comprised of strongly complementary mutants was palmitic acid.

Despite differences in the specific activities observed with different heterodimers, without exception, the results reveal an entirely consistent theme, and the mutants can be classified into two distinct complementation groups. Thus, knockout mutations in either the KS or MAT domain are complemented strongly by knockout mutations in any of the six other domains (heterodimers exhibit 16−39% of the wild-type activity), whereas knockout mutations in the DH, ER, KR, ACP, and TE domains either do not complement each other or exhibit only weak complementation (heterodimers exhibit 0−5% of the wild-type activity). For convenience, the former group of mutants is subsequently termed the strongly complementary group. Further experimentation was required to determine whether members of the second group were indeed truly weakly complementary, or whether alternative explanations might account for the low FAS activity detected in some heterodimers formed from these mutants.

Evaluation of Factors Influencing the Ability of Mutant FASs To Exhibit Complementation. Several different possible

explanations have been evaluated to account for the low, or absent, FAS activity observed in heterodimers constructed from some pairs of mutant FASs. (i) Low activity could result from residual FAS activity in some of the mutants. (ii) Low activity could result from weak functional interactions between dimers, rather than between subunits of the same dimer. (iii) Low activity could reflect the true ability of certain domains to cooperate functionally to a very limited degree across the subunit interface. (iv) The ability of some mutants to complement each other may have been greatly *underestimated*, perhaps as a result of the destabilizing effects of some of these mutations on FAS architecture.

In some cases, incomplete inactivation of FAS activity as a result of the mutation clearly contributed to the low activity observed in the heterodimers. Thus, residual FAS activity in the G1888A KR$^-$ mutant (6% of the wild-type activity, in the homodimeric form) could account for the ~3% activity observed for those heterodimers formed with DH$^-$, ER$^-$, and ACP$^-$ mutants. In the case of the H878A DH$^-$ mutant, β-hydroxybutyryl moieties that accumulate on the FAS, as a result of the defective dehydrase, are slowly released by transfer to a CoA acceptor, so when the activity of the H878A homodimers is assessed spectrophotometrically, the rate of NADPH oxidation (due to reduction of the β-ketobutyryl−enzyme intermediate to the β-hydroxybutyryl−enzyme intermediate) is approximately 1% of that observed with the wild-type enzyme (*12*). Consequently, a small amount of FAS activity observed with heterodimers containing the H878A mutation can be attributed to the ability of this mutant to perform the β-ketobutyryl reduction reaction.

The low FAS activity associated with other heterodimers cannot be explained entirely by the residual activity of the G1888A and H878A mutants, notably, the S2151A ACP$^-$/S2302A TE$^-$ (3% of the wild-type activity), G1888A KR$^-$/S2302A TE$^-$ (5%), G1886F KR$^-$/S2302A TE$^-$ (3%), G1672V ER$^-$/S2302A TE$^-$ (1%), G1672V/G1675T ER$^-$/S2302A TE$^-$ (3%), and H878A DH$^-$/S2302A TE$^-$ (3%). A common feature of these heterodimers is that they all contain the S2302A TE$^-$ mutation, suggesting that the thioesterase domain of one subunit has a low, but measurable, ability to release the fatty acid product either from the ACP domain of the companion subunit or from the ACP domain of another dimer.

Experiments were performed to distinguish between these two possibilities. In these experiments, the activities of FAS mutants were assessed radiochemically, by assessing the incorporation of [2-^{14}C]malonyl-CoA into long chain fatty acid, eliminating contributions to NADPH oxidation as is observed with the DH$^-$ mutant. Second, the activities of each pair of mutants were assessed immediately following mixing and after randomization of the subunits and isolation as heterodimers. Thus, activity resulting from functional interactions between homodimeric mutants would be manifest in the mixed homodimers, whereas activity resulting from mutant complementation within the dimer would be manifest only in the randomized, purified heterodimers. The activities of mixtures of all combinations of homodimers either were <0.1% of that of the wild-type FAS homodimers (TE$^-$ mixed with either ACP$^-$, ER$^-$, or DH$^-$ mutant FASs) or could be accounted for by residual FAS activity in one of the mutants (TE$^-$ mixed with KR$^-$ mutant FAS); however, activities of heterodimers derived from the same pairs of mutant homodimers were uniformly and significantly higher than the activities of the corresponding homodimeric mixtures (Table 5).

Table 5: Comparison of Fatty Acid Synthase Activity of Paired Mutants, as Mixed Homodimers and Heterodimers

FAS	% of wild-type activity[a]
TE$^-$ (S2302A, cF) homodimer	0.05 ± 0.01
ACP$^-$ (S2151A, cH) homodimer	0
KR$^-$ (G1888A, cH) homodimer	3.1 ± 0.23
ER$^-$ (G1672V, cH) homodimer	0.02 ± 0.001
DH$^-$ (H878A, cH) homodimer	0
TE$^-$ + ACP$^-$ homodimers	0.03 ± 0.001
TE$^-$/ACP$^-$ heterodimer	1.75 ± 0.67
TE$^-$ + KR$^-$ homodimers	1.56 ± 0.18
TE$^-$/KR$^-$ heterodimer	3.09 ± 0.39
TE$^-$ + ER$^-$ homodimers	0.06 ± 0.01
TE$^-$/ER$^-$ heterodimer	1.42 ± 0.04
TE$^-$ + DH$^-$ homodimers	0.06 ± 0.01
TE$^-$/DH$^-$ heterodimer	4.98 ± 0.34

[a] Activity was assayed radiochemically, by assessing the incorporation of [2-^{14}C]malonyl-CoA into long chain fatty acid. The average chain length of fatty acids synthesized by the TE$^-$/ACP$^-$, TE$^-$/KR$^-$, TE$^-$/ER$^-$, and TE$^-$/DH$^-$ heterodimers was 18.3, 17.0, 18.3, and 18.4, respectively.

This result indicates clearly that no functional interactions occur as a result of dimer−dimer contacts and the TE$^-$ mutant FAS is indeed weakly complementary to knockout mutations in the ACP and β-carbon-processing domains in the context of a heterodimer. The products formed and released by the TE$^-$/ACP$^-$, TE$^-$/ER$^-$, and TE$^-$/DH$^-$ heterodimers were mainly 18:0 (~68 mol %) and 20:0 (~26 mol %) free fatty acids. Since FAS is capable of elongating acyl chains up to 22 carbon atoms, when the activity of the chain-terminating thioesterase is compromised (*38*), this finding is consistent with the chain-terminating step being rate-limiting in these heterodimers. The TE$^-$/KR$^-$ heterodimer synthesized and released mainly 16:0 (41 mol %) and 18:0 (42 mol %), most likely because the two ACP domains release fatty acids at different stages of elongation. Thus, residual activity in the G1888A mutation allows slow assembly of fatty acids on the immediately adjacent ACP domain that can be effectively removed at the 16:0 stage. On the other hand, fatty acids assembled on the ACP domain of the subunit carrying the TE$^-$ mutation can be removed only by the relatively inefficient interaction with the active TE of the opposite subunit. The implication of these findings then is that an ACP domain that is part of a catalytic center, fully functional except for the immediately adjacent TE domain, can be serviced by the TE domain of the opposite subunit, albeit with low efficiency.

The possibility that the ability of the TE$^-$ mutant to complement ACP$^-$, DH$^-$, ER$^-$, and KR$^-$ mutations might be grossly *underestimated*, as a result of the destabilizing effects of some mutations, also has been considered. Although, with some pairs of strongly complementary mutants, the presence of a thermally unstable mutated subunit in a FAS heterodimer may have influenced FAS activity quantitatively, e.g., compare S581A MAT$^-$/G1886F KR$^-$ (19% of the wild-type activity) and S581A MAT$^-$/G1888A KR$^-$ (33% of the wild-type activity), in no case was the level of complementation eliminated or even reduced to that observed with the weakly complementary group. Even the least thermally stable reductase mutants, G1886F and

Table 6: FAS Activity of Complementary Mutants

		% wild-type activity	
subunit A	subunit B	randomized not fractionated[a]	purified heterodimers[b]
MAT$^-$ (S581A)	KS$^-$ (K326A)	22 ± 0.6	39 ± 1.0
MAT$^-$ (S581A)	TE$^-$ (S2302A)	22 ± 2	38 ± 1.2
MAT$^-$ (S581A)	DH$^-$ (H878A)	21 ± 0.5	37 ± 1.3
MAT$^-$ (S581A)	ACP$^-$ (S2151A)	18 ± 0.4	31 ± 0.7
KS$^-$ (K326A)	TE$^-$ (S2302A)	9.2 ± 1.5	23 ± 0.8
KS$^-$ (K326A)	DH$^-$ (H878A)	8.8 ± 0.2	22 ± 0.8
KS$^-$ (C161S)	ACP$^-$ (S2151A)	10 ± 0.3	18 ± 0.3
KS$^-$ (K326A)	ACP$^-$ (S2151A)	10 ± 0.3	19 ± 0.7

[a] The parental homodimeric FASs did not carry either the His_6 or FLAG tag; data for the MAT mutants were taken from ref *24*, and data for the KS mutants were taken from ref *23*. [b] Data from Table 3.

G1673Y, were able to form heterodimers with either KS$^-$ or MAT$^-$ subunits that exhibited at least 19% of the activity of the wild-type homodimer. Thus, it appears highly unlikely that the instability of certain mutated FAS subunits could be responsible for either the absence of complementation or the weak complementation observed with some pairs of mutants. This view is supported by the direct assessment of the thermal stability of some heterodimers (see Materials and Methods for details). Thus, heterodimers formed from mutants that do not complement each other (H878A DH$^-$/G1888A KR$^-$, G1672V ER$^-$/S2151A ACP$^-$, and H878A DH$^-$/S2151A ACP$^-$), from mutants that exhibit weak complementation (S2151A ACP$^-$/S2303A TE$^-$ and H878A DH$^-$/S2303A TE$^-$), and from mutants that strongly complement each other (S581A MAT$^-$/G1888A KR$^-$, S581A MAT$^-$/S2151A ACP$^-$, and S581A MAT$^-$/S2303A TE$^-$) all exhibit similar thermal stabilities ($t_{1/2}$ at 47.5 °C in the range of 18−28 min, details not shown).

Although none of the homodimeric FAS catalytic mutants, when simply mixed together and not subjected to the dissociation−reassociation procedure, generated catalytically active heterodimers, it remained a formal possibility that, perhaps as a result of destabilization of the dimers due to certain mutations, some isolated heterodimers might undergo subunit exchange, resulting in the re-formation of homodimers and lowering of FAS activity over time. In the early experiments, prior to introduction of the dual-affinity labeling procedure for isolation of heterodimers, FAS assays were performed on the randomized but unfractionated mixtures of hetero- and homodimers. In these experiments, were subunit exchange to continue following randomization, the FAS activity of the mixture would not be expected to change. When the activities of several purified heterodimer preparations were compared with those of the equivalent randomized, nontagged, hetero- and homodimer mixtures, the two procedures always gave remarkably similar results for any given pair of mutants, if one assumes that the unfractionated mixtures consisted of 50% catalytically active heterodimers and 25% of each of the two inactive homodimeric species (Table 6). Thus, for those pairs of mutants exhibiting strong complementation, the ratio of activity in purified heterodimers to that in randomized but not fractionated dimers was always in the range of 1.7−2.5. Similarly, the DH$^-$ and ACP$^-$ mutants were assessed as noncomplementary, regardless of whether the heterodimers were purified from the randomized mixture of dimers prior to an assay.

Table 7: FAS Activity of Heterodimers Consisting of One Wild-Type and One Mutated Subunit

subunit A	subunit B	FAS activity (% of wild-type value)
WT (cF)[a]	KS$^-$ (C161Q, cH)	50 ± 1.5
WT (cF)	MAT$^-$ (S581A, cH)	78 ± 1.6[b]
WT (cF)	DH$^-$ (H878A, cH)	51 ± 0.8
WT (cF)	ER$^-$ (G1672V, cH)	47 ± 1.5
WT (cH)	KR$^-$ (G1888A, cF)	48 ± 1.7
WT (cF)[a]	ACP$^-$ (S2151A, cH)	48 ± 0.6[b]
WT (cH)[a]	TE$^-$ (S2302A, cF)	53 ± 1.5

[a] cF C-terminally FLAG-tagged; cH, C-terminally His_6-tagged. [b] Data from ref *26*.

Furthermore, the activity of all isolated heterodimers remained stable, at least over a period of several hours at 20 °C, or following storage for up to 1 year at −70 °C. Thus, it is highly unlikely that the lower-than-expected activities of some complementary mutant pairs can be attributed to a skewing of the heterodimer population due to spontaneous subunit exchange.

Finally, the possibility that the thermal instability of certain mutants might result in unusual sensitivity of some heterodimers to assay temperature was investigated by performing assays at both 37 and 20 °C. Heterodimers containing the thermally unstable G1675T/G1672V ER$^-$ or the G1886F KR$^-$ mutant subunits exhibited the same activity, expressed as the percentage of wild-type activity, at the two temperatures (details not shown). This result indicated that an assay of FAS activity at 37 °C is unlikely to underestimate the activities of heterodimers containing thermally unstable mutated subunits.

In conclusion, the absence of complementation between DH$^-$, ER$^-$, and KR$^-$ mutant FASs cannot be attributed to the instability of heterodimers formed from these mutants, whereas the weak complementation observed between each of these mutants and the TE mutant clearly reflects the ability of the TE domain to access, albeit inefficiently, the ACP domain of the companion subunit.

Activity of FAS Dimers Containing One Wild-Type and One Mutated Subunit. A panel of heterodimeric FASs was engineered in which the wild-type subunit was combined with seven differently mutated subunits, each compromised in one of the functional domains. All combinations of wild-type/mutant subunits exhibited close to 50% of the homodimeric wild-type FAS activity, with the exception of the wild-type/MAT$^-$ heterodimer, which exhibited 78% of the homodimeric wild-type FAS activity (Table 7). The values for these catalytic activites are significantly higher than those for heterodimers constructed from pairs of complementary mutants, which never exceed ∼40% of the values for wild-type homodimers (Table 2). We have previously shown that wild-type/MAT$^-$ and wild-type/KS$^-$ heterodimers are able to assemble acyl chains on the phosphopantetheine moieties of both ACP domains (*26*), consistent with the concept that the ACP domains can be serviced by MAT and KS domains from either subunit (*24*). The high activity of the wild-type/MAT$^-$ heterodimer likely is a result of the high turnover number for the MAT-catalyzed reaction (*22*, *39*) so that a single catalytically active MAT is able to service two ACP domains at a rate approaching that required for the maximum capacity of the overall reaction. In the case of the five

remaining wild-type/mutant heterodimers, only the ACP domain associated with the wild-type subunit participates in fatty acid production, since the ability of the second ACP to participate in β-carbon-processing and chain-terminating reactions is compromised by the mutations in that subunit (DH^-, ER^-, KR^-, ACP^-, and TE^-). Similarly, in heterodimers comprised of two complementary mutants, only one of the two ACP domains is functional, since the functionality of the second ACP is compromised by a mutation in either the DH, ER, KR, ACP, or TE domain. The question arises then as to why activity of the wild-type/DH^-, ER^-, KR^-, ACP^-, or TE^- heterodimers is always close to 50% whereas the activity of heterodimers comprised of two complementary mutants never exceeds 40%. We propose an explanation based on the possibility that the apparent redundancy in the mechanisms for substrate delivery and condensation provides a small, but measurable, catalytic advantage to the FAS. Thus, the wild-type/DH^-, ER^-, KR^-, ACP^-, or TE^- heterodimers retain both options for substrate delivery and condensation, since the MAT and KS domains of either subunit are able to cooperate with the functional ACP. Heterodimers comprised of complementary mutants, on the other hand, lack one of the options for either substrate delivery or condensation, since either the KS or MAT domain of one subunit is compromised by mutation. For the wild-type homodimers, the availability of alternative routes for substrate delivery and condensation could contribute approximately 20% to the overall rate of fatty acid biosynthesis.

CONCLUSIONS

The results of the complementation analysis are summarized in Table 8.

Table 8: Summary of Mutant Complementation Analysis[a]

	KS^-	MAT^-	DH^-	ER^-	KR^-	ACP^-	TE^-
KS^-	0	+	+	+	+	+	+
MAT^-		0	+	+	+	+	+
DH^-			0	0	0	0	>0
ER^-				0	0	0	>0
KR^-					0	0	>0
ACP^-						0	>0
TE^-							0

[a] +, strongly complementary; 0, noncomplementary; >0, very weakly complementary.

Knockout mutations in either the KS or MAT domains complement knockouts in any of the other functional domains, whereas knockout mutations in the DH, ER, KR, and ACP domains do not complement each other. A TE knockout mutation very weakly complements knockout mutations in the DH, ER, KR, and ACP domains. Thus, the substrate loading and condensation reactions can be catalyzed by the cooperation of an ACP domain of one subunit with the MAT and KS domains, respectively, of either subunit. The β-carbon-processing reactions responsible for complete reduction of the β-ketoacyl moiety following each condensation step are catalyzed exclusively by cooperation of an ACP domain with the KR, DH, and ER domains associated with the same subunit. The chain-terminating reaction is carried out preferentially by cooperation of an ACP domain with the TE domain of the same subunit, although this reaction can also be carried out by cooperation between ACP and TE domains from different subunits, albeit much less efficiently. The conclusion that reduction of both the β-ketoacyl-ACP and enoyl-ACP moieties and hydrolysis of the palmitoyl-ACP product are predominantly intrasubunit events is consistent with the results of earlier studies in which the distances between the active sites of these enzymes and the ACP phosphopantetheine were estimated by fluorescence resonance energy transfer (*40*, *41*). In these experiments, the phosphopantetheine, thioesterase active-site serine, and pyridine nucleotide-binding domains were labeled with different fluorescent probes. No significant interpolypeptide chain energy transfer was detected between any of the probes.

This study completes the mapping of functional interactions that occur between and within subunits of the FAS dimer. Whereas the information is of value in revealing details of the FAS reaction mechanism, perhaps the most intriguing implications of this study concern the structural organization of the complex. Earlier attempts to describe a model for the animal FAS envisioned the two multifunctional polypeptides lying side by side in a fully extended antiparallel orientation (*13*, *21*, *22*). However, the cornerstone of these representations rested on an interpretation of the dibromopropanone cross-linking studies as indicating that the active-site cysteine thiol of the KS domain and the phosphopantetheine thiol of the ACP domain could be cross-linked only across the subunit interface (*20*). A recent reinvestigation of the specificity of the interaction of dibromopropanone with the animal FAS, which benefited from the availability of cysteine and phosphopantetheine mutants, has shown unequivocally that cross-linking can occur between cysteine and phosphopantetheine thiols located on the same or opposite subunits (*25*). Collectively then, results of the cross-linking studies and the mutant complementation analysis identify domains, and in some cases specific residues, that can make physical and functional contacts with each other in the context of the FAS dimer. The data are consistent with a revised model that allows for physical and functional interactions between domains on the same polypeptide, which are separated by more than 2000 residues. In particular, the KS and MAT domains of both subunits must be positioned such that they contact the ACP domains of both subunits, whereas the KR, DH, and ER domains, and to some extent the TE domain, are limited to interacting with the ACP domain of the same subunit. A revised model that incorporates these new data is presented in cartoon form as Figure 1.

This model retains the original concept of subunits oriented in a head-to-tail fashion, in the sense that the KS and ACP domains of opposite subunits make structural and functional contact. However, the new model also satisfies requirements dictated by the results of dibromopropanone cross-linking and mutant complementation studies, in that head-to-tail structural and functional interactions can also occur within each of the two subunits. Earlier electron micrographic studies indicated that the two TE domains are located at opposite poles of the FAS dimer (*42*), and this feature is retained in the revised model. Thus, the limited, but detectable, ability of the TE domains to access ACP domains of the opposite subunit implies remarkable flexibility in the ACP−TE linker region.

The multifunctional FASs and modular polyketide synthases share a number of common features, in primary

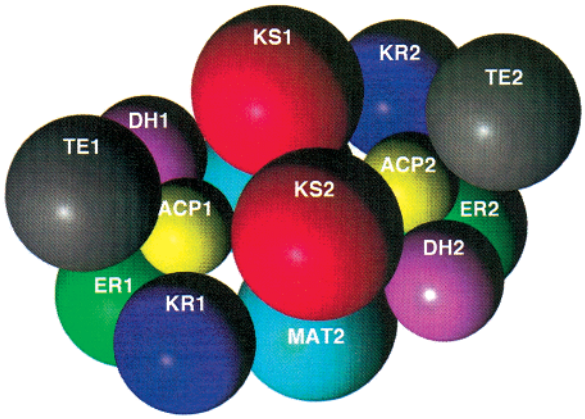

FIGURE 1: Cartoon representation of the revised model for FAS. The KS domains of subunits 1 and 2 are colored red, MAT domains light blue, DH domains pink, ER domains green, KR domains purple, ACP domains yellow, and TE domains gray. Interdomain linkers and the noncatalytic central core region, for which a function has yet to be assigned, are omitted.

structure, in the ordering of functional domains, and in the catalytic mechanisms of the constituent enzymes. Several models have been proposed for the modular polyketide synthases, characterized variously as a "parallel side by side homodimer", a "parallel double helical homodimer", and a "tetrahedral homodimer" (*43*, *44*). A common feature of these models is a head-to-head orientation of the two subunits. This characteristic, and several other features of these polyketide synthase models, is accommodated in our revised model for the animal FAS.

Thus far, the multifunctional animal fatty acid synthases have proven to be refractory to crystallographic analysis, necessitating the exploitation of novel approaches, such as mutant complementation and cross-linking, in mapping the functional organization of these proteins. We are hopeful that recent advances in methods of structural analysis will eventually facilitate more detailed resolution of the molecular architecture of these complex proteins.

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