Fatty Acid Synthase: In Vitro Complementation of Inactive Mutants†

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ABSTRACT: The animal fatty acid synthase is a dimer of identical, multifunctional 272 kDa subunits oriented antiparallel such that two centers for fatty acid synthesis are formed at the subunit interface. In order to clarify the interdomain and intersubunit communications necessary for the operation of the two centers, we have explored the possibility of reassembling catalytically-active fatty acid synthase heterodimers from pairs of inactive dimers carrying mutations in different functional domains. To this end, rat fatty acid synthase mutants, defective in either the β-ketoacyl synthase, C161T or K326A (KS^- FAS), or the acyl carrier protein, S2151A (ACP^- FAS), domains, were engineered by site-directed mutagenesis, expressed in insect *Sf*9 cells using a baculovirus expression system, and purified. A novel procedure was devised to facilitate rapid production and isolation of a population of mixed mutant dimers that had undergone randomization of its constituent subunits. Homodimeric mutants (KS^- FAS/KS^- FAS and ACP^- FAS/ACP^- FAS) and KS^- FAS heterodimers consisting of paired C161T and K326A mutant subunits were unable to synthesize fatty acids, confirming the essential nature of residues C161, K326, and S2151A. However, KS^- FAS/ACP^- FAS heterodimers regained partial activity. Formation of these heterodimers necessitated prior dissociation and reassociation of the homodimers, indicating that the rate of spontaneous exchange of subunits in the dimer is negligible. The formation of catalytically-active heterodimers from pairs of inactive, complementary homodimers affords a useful method for testing the validity of the current model for the multifunctional complex.

In animals, biosynthesis 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, multifunctional, 272 kDa polypeptides [for recent reviews, see Chang and Hammes (1990), Smith (1994), and Wakil (1989)]. Each polypeptide contains seven functional domains arranged in the following order (from amino- to carboxy-terminus): β-ketoacyl synthase, malonyl/acetyl transferase, dehydrase, enoyl reductase, β-ketoacyl reductase, acyl carrier protein (ACP)[1] and thioesterase (Amy et al., 1989; Holzer et al., 1989, Schweizer et al., 1989). The dehydrase and enoyl reductase catalytic domains appear to be separated by several hundred amino acids that may constitute a structural core for stabilization of the dimeric form of the protein (Smith, 1994). According to the current model for the FAS, the two subunits are oriented in an antiparallel configuration such that two centers for acyl chain assembly and release are formed at the subunit interface (Smith et al., 1985; Stoops & Wakil, 1981). Consequently, although all seven functional domains are present in each individual subunit, only the dimeric form of the protein is capable of coupling the individual reactions to effect the biosynthesis of palmitate. Although the location of individual domains in the linear polypeptide has been established, their spatial arrangement relative to each other in the folded dimer is uncertain—with the exception of the ACP and β-ketoacyl synthase domains (Stoops & Wakil, 1981, Stoops & Wakil, 1982)—and details of the inter- and intrasubunit communication among the seven components of the FAS have not yet been fully elucidated. The recent development of a system that allows the expression of recombinant full-length and catalytically-active, FAS (Joshi & Smith, 1993a,b) presents a unique opportunity to address these questions by *in vitro* complementation analysis (Figure 1). Similar strategies have been used to study domain interaction in other enzymes that have an active site at the subunit interface (Distefano et al., 1990; Frimpong & Rodwell, 1994; Greene et al., 1993; Larimer et al., 1987; Wente & Schachman, 1987).

The proposed model predicts that 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. However, 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 on an intersubunit basis. In the example shown, reconstitution of the FAS dimer from one subunit defective in the ACP domain (ACP^- FAS) and another subunit defective in the β-ketoacyl synthase domain (KS^- FAS) should yield an active heterodimer (ACP^- FAS/KS^- FAS) since one center for acyl-chain synthesis will contain both mutations and the other will be comprised of normal catalytic domains.

To evaluate the mutant complementation strategy as a tool in identifying domains that cooperate functionally in an intersubunit fashion, we have engineered mutations in two domains that are known to communicate across the subunit interface, namely, the β-ketoacyl synthase and ACP domains (Stoops & Wakil, 1981, 1982). Conditions that promote exchange of FAS subunits have been investigated in order

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[1] Abbreviations: FAS, fatty acid synthase; ACP, acyl carrier protein; KS^- FAS, β-ketoacyl synthase FAS mutant; ACP^- FAS, acyl carrier protein FAS mutant.

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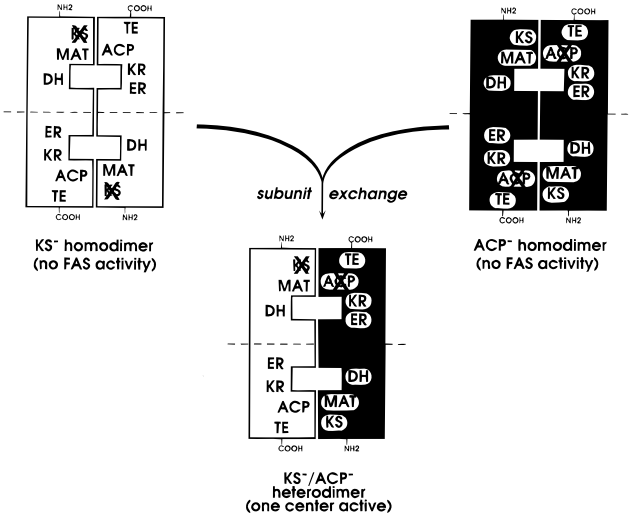

FIGURE 1: Complementation of single active-site mutants in the head-to-tail, two-center model. The wild-type FAS contains two centers for fatty acid synthesis (separated by the dashed line in the cartoon). Homodimers containing the same single active-site mutation, in either β-ketoacyl synthase (KS^-) or ACP (ACP^-), will be compromised at both centers for acyl chain synthesis and will be inactive in fatty acid synthesis. KS^- FAS/ACP^- FAS heterodimers reconstituted from these inactive homodimers will be active since one center will contain both mutations and the other, none. If the two centers function independently, the specific activity of the heterodimer formed from complementary mutants will be half that of the wild-type. KS denotes β-ketoacyl synthase, MAT malonyl/acetyl transferase, DH dehydrase, ER enoyl reductase, KR β-ketoacyl reductase, and TE thioesterase.

to acquire a population of mutant heterodimers that can be used to test the complementation strategy. An interpretation of a previous attempt to reassemble catalytically active heterodimers from the subunits separately modified by treatment with either iodoacetamide or chloroacetyl-CoA was hampered by the lack of complete specificity of the reagents, the presence of residual FAS activity in the chemically modified homodimers, and the incomplete recovery of FAS activity during the dissociation and reassociation procedure (Wang et al., 1984). The strategy described in this report circumvents these difficulties.

MATERIALS AND METHODS

Materials. The plasmid pUCBM20 was obtained from Boehringer-Mannheim (Indianapolis, IN), BacPAK6 DNA (*Bsu*36I-digested) and baculovirus transfer vector BacPAK9 were from Clontech (Palo Alto, CA), and the 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). Oligonucleotide primers were obtained from Operon Technologies (Alameda, CA), *Sf*9 cells were from ATCC (Rockville, MD), and competent *E. coli* DH5α cells, Grace's insect medium, and other insect cell culture medium components were from Life Technologies (Grand Island, NY).

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 FAS has been described elsewhere (Joshi & Smith, 1993a). Site-directed mutagenesis was carried out by the overlap PCR method, essentially as described by Shyamala and Ames (1991) except that amplifications were carried out with Vent rather than Taq polymerase. Partial FAS cDNA constructs pFAS74.20 (66−2544 bp in pUCBM 20) and pFAS 215.20 (5541−7615 bp in pUCBM20) were used as template DNA to generate mutations in the β-ketoacyl synthase and ACP, respectively. The primers used for various amplification reactions are summarized in Table 1.

The strategy employed for the construction of each mutant cDNA is described in Figure 2. In each case, the mutated DNA fragment generated by overlap PCR was used to replace the corresponding fragment of the parent partial cDNA construct using the appropriate restriction sites and cloned into competent *E. coli* DH5α cells (Sambrook et al., 1989). The amplified region was sequenced by the dideoxy method and moved stepwise into the full-length FAS cDNA transfer-vector construct. Transfer vectors, pBacPAK9 and pVL1393, were used for constructing the β-ketoacyl synthase FAS and ACP FAS mutants, respectively. Recombinant baculoviral stocks were generated by cotransfecting *Sf*9 cells with the appropriate FAS cDNA construct and BacPAK6 viral DNA (*Bsu*36I-digested) using the Lipofectin method, according to the manufacturer's protocol. Purified recombinant baculovirus stocks were obtained by the plaque purification method (O'Reilly et al., 1992).

*Expression and Purification of Mutant FASs. Sf*9 cells were infected with purified recombinant viruses and cultured for 48 h at 27 °C, and the mutant FAS proteins were 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.

Enzyme Assays. β-Ketoacyl synthase activity was assayed by measurement of the condensation rate in the absence of NADPH. Reactions were carried out in 100 mM potassium phosphate, pH 6.6, using 67 μM [2-^{14}C]malonyl-CoA, 61 μM acetyl-CoA, and 95 μM of freshly prepared CoA for 2 or 4 min. The reaction was stopped by the addition of perchloric acid (5% final concentration), and the tubes were kept on ice for 15−20 min and then centrifuged. The contents were brought to pH 5−6 with a mixture of 3M KOH and 0.5 M KH_2PO_4, centrifuged, and diluted 4 times with the starting buffer and then analyzed on reverse-phase HPLC at 35 °C, as described previously (Joshi & Smith, 1993b).

Transferase activity was assayed in 50 mM potassium phosphate buffer (pH 6.8), using [1-^{14}C]acetyl-CoA (40 μM) as acyl donor and pantetheine (2.5 mM) as acceptor; the amount of [1-^{14}C]acetylpantetheine product was measured by liquid scintillation spectrometry after separation by thin layer chromatography.

β-Ketoreductase was assayed spectrophotometrically at 340 nm: assay systems contained 0.05 M potassium phosphate buffer (pH 7), 0.18 mM NADPH, enzyme, and 10 mM *trans*-1-decalone as substrate.

Dehydrase activity was assayed spectrophotometrically at 270 nm using *S*-D,L-β-hydroxybutyryl-*N*-acetylcysteamine as substrate and enoyl reductase at 340 nm using *S*-crotonyl-*N*-acetylcysteamine as substrate (Kumar et al., 1970).

Thioesterase activity was assessed radiochemically by extracting and assaying the [^{14}C]palmitic acid formed from [1-^{14}C]palmitoyl-CoA during an incubation of 3 min duration (Smith, 1981): assay systems contained in a final volume

Table 1: Oligonucleotides Used To Generate Mutations in β-Ketoacyl Synthase and ACP Domains[a]

code	sequence	location[b]
	β-Ketoacyl Synthase Domain: Mutation: C161A/T	
Pa FAS 330T	5′-GTCAGCTATGAAGCTATTGTG	334−354
Pb FAS 161T	5′-CATTGCCCTGGACACAGCC**VV**CTCCTCTAGCCTACTGGC	543−581
Pc FAS 1039B	5′-AAGGGCTCTGGCGGAAAG	1039−1082
Pd FAS 161B	5′-GCCAGTAGGCTAGAGGAG**BB**GGCTGTGTCCAGGGCAATG	543−581
	β-Ketoacyl Synthase Domain: Mutation: K326A/R/L	
Pa FAS 330T	5′-GTCAGCTATGAAGCTATTGTG	334−354
Pb FAS 326T	5′-ATTGGCTCCACC**GCC**TCCAACATGGG	1045−1070
Pc FAS 1152B	5′-cactagaattcTTCAGGGTTGGGGTTGTGGAAATGC (*Eco*RI)	1152−1176
Pd FAS 326B	5′-CCCATGTTGGA**GGC**GGTGGAGCCAAT	1045−1070
	Acyl Carrier Domain: Mutation: S2151A	
Pa FAS 1T	5′-GCATTATCTTGGAAGCGATG	6263−6282
Pb FAS 2151T	5′-GCAGACCTCGGCCTGGAC**G**CGCTCATGGGTGTGGAA	6515−6552
Pc FAS 1B	5′-atcatctagaGGAGCACATCTCGAAGGCTAC (*Xba*I)	7000−7020
Pd FAS 2151B	5′-CACTTCCACACCCATGAGCG**C**GTCCAGGCCGAGGTCTGC	6515−6552

[a] Letter T or B in the primer names indicates sense or antisense primer, respectively. Upper case letters indicate the primer sequence matching FAS cDNA sequence, while bases in lower case are not present in the cDNA. These bases were incorporated into the primers to engineer restriction sites to facilitate cloning of PCR fragments and are not incorporated into the final construct. Pa, Pb, Pc, and Pd correspond to labels used in Figure 2. [b] The base numbers are according to the rat FAS cDNA sequence (Amy et al., 1989).

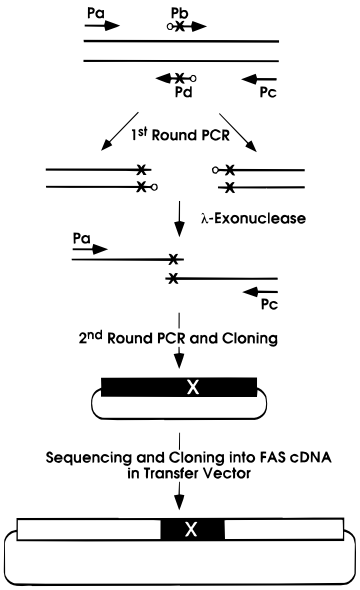

FIGURE 2: Strategy for construction of FAS mutants. Two distinct sets of amplifications were carried out for the first round of PCRs using Pa/Pd and Pb/Pc primer pairs. The mutagenic primers Pb and Pd were phosphorylated before use, since the λ-exonuclease preferentially cleaves the strand with 5′-phosphate. The location of the mutation is indicated by (X) and the 5′-phosphate by (○), and the filled box denotes a mutated fragment.

of 0.1 mL, 25 mM potassium phosphate buffer (pH 8), 20 μg/ml BSA, 10 μM [1-^{14}C]palmitoyl-CoA (20 nCi), and enzyme.

Assay of overall FAS activity was performed spectrophotometrically as described previously (Smith & Abraham, 1970). All enzyme activities were assayed at 37 °C except the transferase that was assayed at 0 °C. One unit of enzyme activity corresponds to the amount of enzyme catalyzing the utilization of 1 μmol substrate per minute.

Formation of Heterodimers of FAS Mutants by HPLC. Equal amounts of two FAS mutants, usually equivalent to between 5 and 10 units of β-ketoreductase activity, were mixed, diluted with 3−5-fold excess of water, and injected into a 10 mL loop connected to a high-performance anion exchange column (TSK-GEL DEAE-5PW, 0.8 × 7.5 cm, 10 μm). The column was washed with low ionic strength buffer as indicated under Results and Discussion, followed by a 5 min wash with 50 mM potassium phosphate, pH 7, containing 10% glycerol. The 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, was employed to separate monomer from the dimer. All buffers contained 1 mM EDTA and 1 mM dithiothreitol, and gradient buffers additionally contained 10% glycerol. Column fractions were left at room temperature for at least 1 h prior to the assays of FAS activities. Alternatively, two to three fractions were pooled, supplemented with potassium phosphate to the final concentration of 0.25 M, and protein was concentrated to 0.3 mg/mL using Microcon-50 (Amicon) before FAS assays were performed.

RESULTS AND DISCUSSION

Characterization of FAS Mutants Deficient in ACP and β-Ketoacyl Synthase Functions. Two types of mutants were designed to test the complementation strategy, one lacking the 4′-phosphopantetheine prosthetic group in the ACP domain (termed ACP$^-$ FAS), the other carrying a critical mutation in the β-ketoacyl synthase domain (KS$^-$). In the ACP$^-$ FAS, the Ser-2151, which normally carries the 4′-phosphopantetheine prosthetic group in phosphodiester linkage, was changed to Ala. As anticipated, the S2151A mutant homodimer lacked the ability to synthesize fatty acids, and of the six partial activities, only the β-ketoacyl synthase activity was eliminated (Table 2).

Verification that the S2151A substitution blocked the posttranslational phosphopantetheinylation was achieved by analysis of the product formed by incubation of the trypsinized mutant FAS with [1-^{14}C]acetyl-CoA. FAS that has been subjected to limited trypsinization consists of two major polypeptides of molecular masses 125 and 95 kDa and retains an ability to assemble fatty acyl chains *de novo* (Libertini & Smith, 1979; Mattick et al., 1983; McCarthy & Hardie,

Table 2: Enzymatic Specific Activities (% of Wild-Type FAS Activity) of FAS Mutants[a]

mutation domain	C161T β-ketoacyl synthase	K326A β-ketoacyl synthase	S2151A acyl carrier protein
fatty acid synthesis	$\leq$0.5	$\leq$0.5	$\leq$0.5
malonyl/acetyl transferase	209 $\pm$ 28	123 $\pm$ 6	133 $\pm$ 7
β-ketoacyl synthase	$\leq$0.1	$\leq$0.1	$\leq$0.2
β-ketoacyl reductase	84 $\pm$ 4	101 $\pm$ 7	97 $\pm$ 4
dehydrase	77 $\pm$ 1	100 $\pm$ 3	108 $\pm$ 2
enoyl reductase	69 $\pm$ 7	59 $\pm$ 12	74 $\pm$ 7
thioesterase	133 $\pm$ 4	90 $\pm$ 13	112 $\pm$ 16

[a] Specific activities determined for wild-type enzyme were as follows: fatty acid synthesis, 2049 $\pm$ 28; malonyl/acetyl transferase, 1915 $\pm$ 310; β-ketoacyl synthase, 124 $\pm$ 6; β-ketoacyl reductase, 17 300 $\pm$ 769; dehydrase, 39.8 $\pm$ 1.2; enoyl reductase, 15.6 $\pm$ 0.6; and thioesterase, 484 $\pm$ 1.2 milliunits/mg.

1983; Smith & Stern, 1979). The 125 kDa polypeptide, representing the N-terminal polypeptide, contains two binding sites for acetyl moieties: Cys-161, the active site of the β-ketoacyl synthase domain, and Ser-581, the active site of the transferase domain. The 95 kDa polypeptide, representing the C-terminal polypeptide starting at the residue 1281 (Witkowski et al., 1991), contains only a single acetyl binding site, the 4′-phosphopantetheine at S2151. When the radiolabeled, trypsinized wild-type and ACP$^-$ FAS were subjected to SDS−polyacrylamide gel electrophoresis, autoradiographic analysis of the gel revealed that whereas both the 125 and 95 kDa polypeptides derived from the wild-type FAS were radiolabeled, only the 125 kDa polypeptide was labeled in the case of the ACP$^-$ FAS (data not shown). This experiment verified that the 4′-phosphopantetheine was absent from the 95 kDa polypeptide in the ACP$^-$ FAS.

Two different mutants were engineered for KS$^-$ FAS. In the first one, Cys-161, which carries the saturated acyl moiety during condensation with the malonyl moiety, was changed to Thr; in the second, Lys-326 was changed to Ala. This lysine residue is universally conserved in β-ketoacyl synthases associated with polyketide synthases and all type II β-ketoacyl synthase I enzymes.

The C161T mutant homodimer lacked β-ketoacyl synthase activity and, consequently, overall FAS activity. Surprisingly, the transferase activity associated with the C161T mutant homodimer was double that of the wild type; we cannot offer any likely explanation at this time.

Similarly, the K326A homodimer also lacked β-ketoacyl synthase activity and overall FAS activity (Table 2), supporting our prediction that the universally conserved lysine residue in the β-ketoacyl synthase domain might be required for catalytic activity. We are presently attempting to define a specific role for the conserved lysine through a detailed characterization of the properties of the available FAS mutants.

Assessment of the Rate of Spontaneous Exchange among FAS Subunits. The animal FASs are cold-labile proteins that slowly dissociate into their component subunits at low temperature in low ionic strength buffers (Smith & Abraham, 1971). Relatively rapid reassociation of the subunits is promoted by increased temperature and higher ionic strength. Thus, when the cold-dissociated protein is assayed at low temperature, typically 10−12 °C, no FAS activity is observed unless it has previously been warmed in high ionic strength medium (Smith & Abraham, 1971). Although this dissociation−reassociation process has been well documented, the question as to whether the dimers can undergo spontaneous subunit exchange has not been addressed, owing to the lack of an appropriate detection system. According to the rationale outlined earlier, exchange of subunits between pairs of mutant homodimers, one defective in the ACP domain, the other in the β-ketoacyl synthase domain, should yield heterodimers in which one of the two centers for fatty acid synthesis is fully functional (Figure 1). Nevertheless, when these mutant FAS homodimers were mixed and maintained at 20 °C in 0.25 M potassium phosphate buffer, pH 7, no appreciable FAS activity appeared, even after 6 days. Similarly, mutant FASs that were mixed and maintained at 0 °C in either 0.05 or 0.25 M potassium phosphate buffer, pH 7, exhibited no FAS activity when assayed at 12 °C (details not shown). These results indicate that the FAS dimers engage in minimal subunit exchange, either at 0 °C or at 20° C, in low ionic strength or high ionic strength media. However, when the cold-stored mixture of mutant proteins was preincubated at 30 °C for 10 min prior to assay at 37 °C, these proteins were found to develop an ability to catalyze the overall FAS reaction that increased steadily on storage (Figure 3).

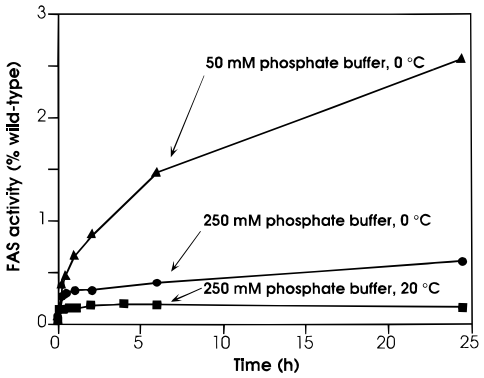

FIGURE 3: Assessment of the rate of subunit exchange in FAS by complementation of inactive mutants. Equal amounts of C161T and S2151A FAS mutants were mixed and stored in potassium phosphate buffer, pH 7, containing 1 mM EDTA and 1 mM dithiothreitol as indicated. At intervals, portions of each sample were removed and preincubated in 0.2 M potassium phosphate buffer, pH 7, 1 mM EDTA, 10 mM dithiothreitol, and 10% glycerol (final concentrations) at 30 °C for 30 min prior to radiochemical assay of FAS activity. Activities are expressed as a percentage of the activity measured for the wild-type FAS.

This acquisition of FAS activity was assisted by storage conditions that promote dissociation of the protein, viz., low ionic strength and low temperature. Since no FAS activity was observed without subjecting the preparations to the preincubation procedure, it is clear that only FAS species that had dissociated on storage and reassociated during the preincubation procedure contributed to the observed activity. Presumably because of their large size, the FAS subunits make multiple contacts with each other in the dimeric state, practically eliminating the possibility of an exchange of subunit partners.

Development of a Method for the Rapid Randomization of FAS Subunits. Critical evaluation of the current model for the FAS using the mutant complementation approach necessitates that one can identify a population of heterodimers, formed by the mixing of two different mutant homodimer species, that has undergone complete randomization of its constituent subunits. The process of dissociating

the mixed homodimers by exposure to low ionic strength solvent at low temperature, then reassociating the subunits prior to assay, can obviously provide qualitative data to address this problem. Nevertheless, the approach is hampered by the long period of time required for execution, especially when the objective is to ensure near complete dissociation of the preparation prior to reassociation and formation of heterodimers. We therefore sought an alternative approach that would facilitate the rapid production and identification of a dimer population that had undergone randomization of its component subunits.

During purification of the FAS, we have observed that, on some brands of anion-exchange matrices, the FAS elutes in two discrete zones (Joshi & Smith, 1993a; Smith & Abraham, 1975). We have hypothesized that the two zones result from dissociation of the FAS into its component subunits on exposure to the low ionic strength loading buffer and selective desorption of the bound dimers and monomers during subsequent salt gradient elution. The possibility was investigated that this system could be used to facilitate the rapid production and identification of a population of FAS dimers that had undergone randomization of its component subunits.

Previous work had revealed that when exposure to the low ionic strength buffer was minimized, most of the FAS was recovered from the anion-exchange column in the early eluting zone, suggesting that this zone corresponded to FAS that was eluted as dimers, whereas the late eluting zone corresponded to FAS eluted as monomers (Joshi & Smith, 1993a). Further evidence in support of this conclusion was obtained by ion exchange chromatography of FAS that had been cross-linked by treatment with dibromopropanone. This bifunctional reagent cross-links the two FAS subunits by forming a covalent bridge between the ACP 4′-phosphopantetheine thiol of one subunit and the β-ketoacyl synthase active-site cysteine of the adjacent subunit (Stoops & Wakil, 1982). The dibromopropanone-treated FAS, which is restrained from dissociating by the intersubunit cross-link, eluted exclusively in the early zone consistent with the identification of this zone as containing FAS dimers (details not shown).

A systematic study was undertaken to investigate conditions that might influence the fraction of FAS recovered in the monomer zone. When FAS was loaded (0.1−1 mg) onto the column and development with the phosphate gradient was initiated immediately, only 6% of the FAS eluted as monomers. However, when the column containing bound FAS dimers was washed with 50 mM Tris-HCl buffer, pH 8.3, the buffer that reportedly induces rapid dissociation of FAS (Kumar et al., 1972), the fraction of protein eluting in the late zone increased, accounting for more than 60% of the eluted protein after 30−45 min. These observations indicate that the FAS initially binds to the column in the dimer form but dissociates following exposure to the Tris-HCl buffer, releasing monomers that subsequently bind to the column more tightly than do the dimers. In the subsequent experiments described in this paper, we routinely washed the column with 50 mM Tris-HCl buffer (pH 8.3) for 30−60 min at a flow rate of 0.4 mL/min, prior to development with the phosphate gradient.

A typical anion-exchange chromatogram of wild-type FAS subjected to this procedure is presented as Figure 4. Two enzyme assay systems were routinely employed in characterization of the elution profile for the FAS: (1) the assay system for overall FAS activity that requires that the protein be present in a dimeric form; (2) the β-ketoacyl reductase assay that is independent of the oligomeric state of the protein and is unaffected by any of the mutations introduced to the FAS in this study. In this experiment, approximately equal amounts of protein were eluted in two zones, and the specific activities found in the FAS and β-ketoacyl reductase assays were identical for both zones, confirming that monomers eluting in the late zone had completely reassociated into active dimers.

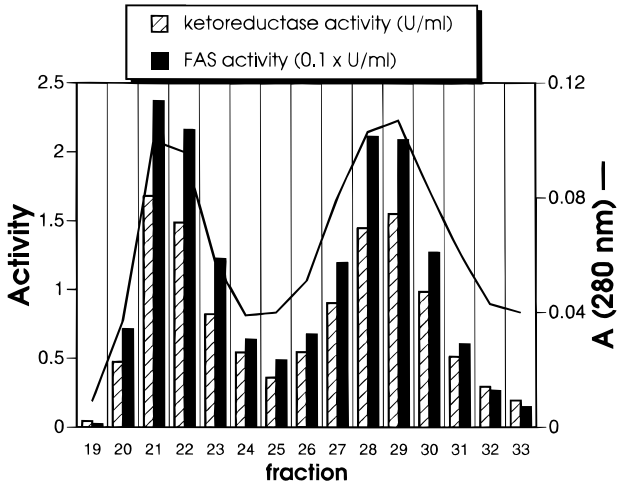

FIGURE 4: Dissociation of wild-type FAS dimers and separation of monomers by anion-exchange chromatography. FAS dimers (0.34 mg) were bound to an anion-exchange column and dissociated in pH 8.3 Tris-HCl buffer for 30 min, and the resulting mixture of dimers and monomers was separated with the potassium phosphate gradient. Fractions (0.5 min) were analyzed for absorbance at 280 nm, β-ketoreductase activity, and fatty acid synthesis activity (see Materials and Methods for details). Fifty-four percent of both activities was recovered from the column.

These preliminary experiments revealed that dissociation of the FAS prebound to an anion-exchange system offers several advantages over simple dissociation of the enzyme in solution. First of all, dissociation is much more rapid in the case of the bound FAS, possibly because the dimer is stressed by interaction with the column matrix. We have found that not all DEAE ion exchangers promote dissociation of FAS, suggesting that the precise geometry of the matrix surface may be critical. Perhaps with some DEAE matrices the dimers are bound via interactions between the exchanger and both subunits, whereas with others, such as the TSK-GEL DEAE-5PW, only one subunit of the dimer can interact strongly with the ion exchanger. Second, wild-type FAS that has undergone dissociation and reassociation by this procedure regains complete FAS activity. Finally, the late-eluting FAS zone represents a population of subunits that is released from the column exclusively as monomers, and, thus, it provides a randomized pool of subunits to be further spontaneously reassociated into the dimers.

Complementation of FAS Mutants. The ACP$^-$ FAS homodimer was mixed with either of the KS$^-$ FAS, C161T or K326A, homodimer and loaded onto the anion-exchange column. Dissociation of the dimers and rebinding of the released monomers were induced by washing with 50 mM Tris-HCl buffer (pH 8.3) for 60 min; then the FAS dimers and monomers were eluted with a phosphate gradient. The distribution of FAS protein in the chromatographic profile

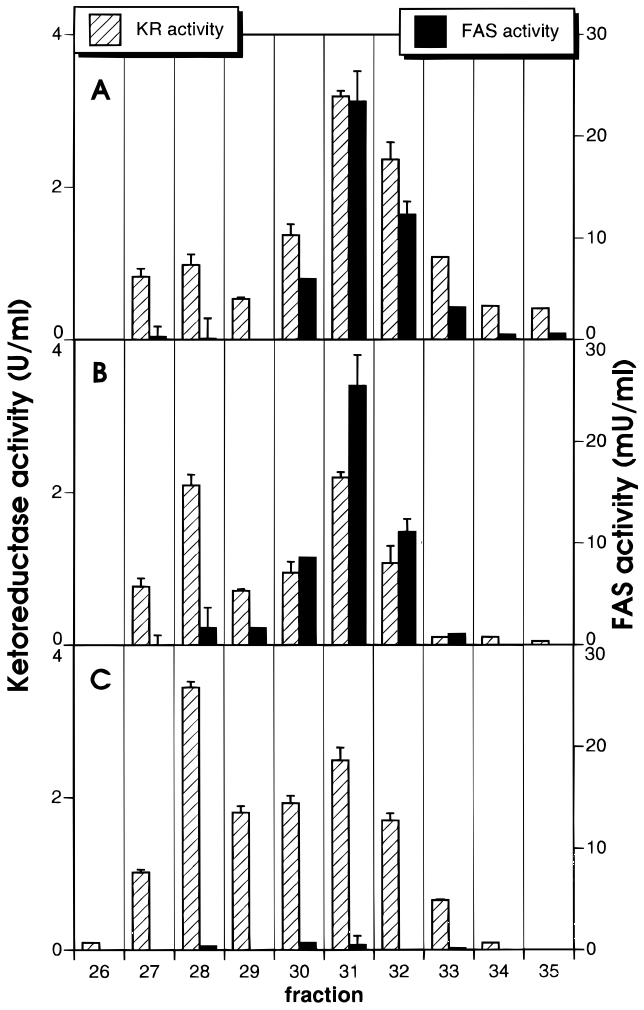

FIGURE 5: Complementation of the centers for fatty acid synthesis among two KS$^-$ FAS (C161T and K326A) and ACP$^-$ FAS (S2151A) mutants. A pair of mutants was dissociated on the anion-exchange column and eluted with the potassium phosphate gradient, and fractions (1 min) were analyzed for ketoreductase activity and fatty acid synthesis activity as described under Materials and Methods. (A) C161T and S2151A FAS dissociated for 60 min (55.7% of β-ketoacyl reductase activity was recovered); (B) K326A and S2151A FAS dissociated for 60 min (52.4% β-ketoacyl reductase activity was recovered); (C) C161T and K326A FAS dissociated for 30 min (64.8% of β-ketoacyl reductase activity was recovered).

was assessed from measurement of β-ketoreductase activity and the extent of complementation from measurement of FAS activity. In the case of both the C161T/S2151A and K326A/S2151A mixed FAS mutants, FAS eluting in the early zone (fractions 27−29) was unable to effect the biosynthesis of fatty acids, indicating that this fraction contained only KS$^-$ and ACP$^-$ FAS homodimer species, that had undergone no subunit exchange (Figure 5A,B).

In contrast, FAS eluting in the late zone (fractions 30−33), that had been reconstituted from dissociated subunits, exhibited FAS activity. This result confirmed the earlier conclusion, based on analysis of the cold-aged and reassociated enzyme, that the KS$^-$ FAS and ACP$^-$ FAS mutants complement one other. In a control experiment, the C161T and K326A mutants of β-ketoacyl synthase failed to do so (Figure 5C). The FAS:ketoreductase activity ratio appeared lower in the leading and trailing edges of the late-eluting "monomer" zone. At least two explanations for this observation are possible. First, subunit reassociation may be incomplete in those fractions containing FAS at low concentration. Second, there may be some heterogeneity in the FAS species eluting through the late zone. Indeed, fractions from the leading edge of the late zone are almost certainly contaminated by inactive FAS eluting primarily in the earlier undissociated FAS zone. To minimize these effects on the measurement of FAS activity, we routinely combined two or three fractions from the center of the late-eluting FAS zone (see Materials and Methods) and reassayed the pooled fractions 2 h later. FAS activity in these samples was not increased by the extended incubation time or concentration procedure, indicating that the activities measured in the peak fractions of the late-eluting zone reflect those of fully reassociated FAS species. Analysis of the fatty acids synthesized by the active FAS formed from the complementary mutants showed that, as with the wild-type enzyme, palmitic acid was the major product (data not shown). The results of these experiments validate the mutant complementation strategy as a valuable tool for identifying FAS domains that cooperate functionally across the subunit interface in fatty acid synthesis. Consequently, in order to extend application of this approach, we are presently engineering additional FAS mutants, each compromised of one of the consituent catalytic domains.

In theory, the complementation strategy can also be exploited to address the question as to whether the two centers for fatty acid synthesis function independently. A key requirement for this application is that the composition of the reconstituted dimer population must be known with certainty. At the outset, we had anticipated that mutating a single active-site residue would have little or no effect on physical interactions between subunits. Thus, the reconstituted dimer fraction was expected to consist of 50% heterodimers and 25% of each homodimer. However, although both KS$^-$ FAS mutants were able to complement the ACP$^-$ FAS mutant, we found that the C161T mutant dissociates more readily than other mutants (under the standard conditions, 100%, 45%, 63%, and 62% monomer was produced from C161T, K326A, S2151A, and wild-type FAS, respectively). Second, the specific activity of the FAS in the reconstituted dimers was lower with the C161T/S2151A combination (95 $\pm$ 15 milliunits/mg, $n = 2$) than with the K326A/S2151A combination (172 $\pm$ 20 milliunits/mg, $n = 8$) even if the amounts of mutant homodimers were adjusted to give an equal composition of both mutant subunits in the monomer zone. However, even in the case of the K326A/S2151A combination, the specific activity of the reconstituted FAS species was less than the value of 0.5 unit/mg that was predicted assuming that the heterodimers constituted 50% of the population and that one of the two centers for fatty acid synthesis was functional. Additionally, it seems reasonable to assume that similarly to the dissociation, some differences will exist in the rate of association of homo- and heterodimers. In this case, the catalytically-active heterodimers could constitute less than 50% of the total dimer population, and the specific activity would be reduced. Another possible explanation is that the two centers for fatty acid synthesis are not entirely independent but interact in a positive manner so that a dimer with only one intact center might have less than 50% of the activity of a dimer with two functional centers. However, because of the uncertainty regarding the precise composition of the subunit population

of partially active heterodimers, it would be premature to draw this conclusion.

The effect of a single active-site mutation on the stability of the FAS dimer was unanticipated, and as yet it is unclear whether the integrity of the β-ketoacyl synthase domain is uniquely critical in this regard. The problem could be solved either by identifying specific amino acid replacements that eliminate catalytic activity without compromising the stability of the dimer or by physically separating identifiable heterodimers from the parent homodimers. We are presently pursuing both strategies.

In summary, this study describes a novel approach for promoting the rapid randomization of subunits in FAS dimers that can be used effectively in application of the mutant complementation strategy to test the current model for the multifunctional animal FAS. Application of the domain complementation strategy has revealed that the constituent subunits of the animal FAS do not engage in significant spontaneous subunit exchange. Finally, we have identified a lysine residue in the β-ketoacyl synthase domain that appears essential for catalytic activity. Characterization of this mutant should provide further insight into the mechanism of catalysis of the β-ketoacyl synthase reaction.

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