Heterodimers Consisting of Subunits That Have Been Independently Modified

To explore the domain interactions that are required for catalytic activity of the multifunctional, homodimeric fatty acid synthase (FAS), we have formulated a strategy that allows isolation of modified dimers containing independently mutated subunits. Either a hexahistidine or a FLAG octapeptide tag was incorporated into the FAS at either the amino terminus, within an internal noncatalytic domain, or at the carboxyl terminus. The presence of the tags had no effect on the activity of the wild-type FAS. His-tagged dimers were mixed with FLAG-tagged dimers, and the subunits were randomized to produce a mixture of His-tagged homodimers, FLAG-tagged homodimers, and doubly tagged heterodimers. The doubly tagged heterodimers could be purified to homogeneity by chromatography on an anti-FLAG immunoaffinity column followed by a metal ion chelating column. This procedure for isolation of FAS heterodimers was utilized to determine whether the two centers for fatty acid synthesis in the FAS dimer can function independently of each other. Doubly tagged heterodimers, consisting of one wild-type subunit and one subunit in which the thioesterase activity had been eliminated, either by mutation or by treatment with phenylmethylsulfonyl fluoride, have 50% of the wild-type thioesterase activity and, in the presence of substrates, accumulate a long chain fatty acyl moiety on the modified subunit, thus blocking further substrate turnover at this center. Nevertheless, the ability of the heterodimer to synthesize fatty acids is also 50% of the wild-type FAS, demonstrating that an individual center for fatty acid synthesis has the same activity when paired with either a functional or nonfunctional catalytic center.

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 (1, 2). In animals, however, six enzymes and an acyl carrier protein, which collectively are responsible for catalyzing the entire series of reactions, are integrated into a single multifunctional polypeptide chain, the FAS (3). Domain mapping studies employing limited proteolysis (4–7), active-site labeling (8–11), amino acid sequencing (12–14), and mutagenesis (15) have established that each polypeptide contains three amino-terminal catalytic domains, β-ketoacyl synthase, malonyl-CoA: acylcarboxyltransferase, and dehydrase, separated by a stretch of approximately 600 residues from four carboxyl-terminal domains, enoyl reductase, β-ketoacyl reductase, acyl carrier protein, and thioesterase (16). The monomeric form of the FAS is unable to couple the individual reactions to form a long chain fatty acid, whereas in the dimer the two subunits are juxtaposed such that two centers for palmitate synthesis are created (17, 18). The discovery that the two subunits could be cross-linked, via the 4-phosphopantetheine associated with the ACP domain of one subunit and the β-ketoacyl synthase active site cysteine of the opposite subunit, provided the first indication that some of the individual reactions probably were catalyzed across the subunit interface (19). More recently, we have used a mutant complementation strategy to explore in detail the functional interactions taking place between domains located on the same and on opposite subunits (20, 21). Utilization of this approach has facilitated confirmation of several features of the current model for the FAS, namely that the condensation reaction can be catalyzed by cooperation of the β-ketoacyl synthase and ACP domains from opposing subunits, whereas the release of the fatty acid product involves cooperation of the ACP and thioesterase domains of the same subunit (20, 21). On the other hand, the mutant complementation strategy has also revealed some unanticipated topological features of the FAS. For example, the dehydrase domain, which is located in the amino-terminal half of the polypeptide, cooperates with the ACP domain of the same subunit, although these domains are separated by more than 1100 residues (21). This finding provided the first experimental evidence indicating that the two constituent polypeptides are not simply positioned side-by-side in a fully extended conformation but are coiled such that contact is possible between functional domains distantlly located on the same polypeptide.

The question as to whether the two centers for palmitate synthesis function entirely independently or whether they are interactive has not been rigorously addressed. Two studies in the mid-1980s reported that 50% inhibition of acyl chain assembly by the FAS dimer was achieved when approximately 50% of the dimers were cross-linked by dibromopropanone, suggesting that the two centers for fatty acid synthesis function independently (17, 18). Using a different approach, Wang et al. (22) formed hybrid dimers from normal FAS and FASs modified with either iodoacetamide or chloroacetyl-CoA, reagents be-
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Uppercase letters indicate that the oligomer sequence matches cDNA sequence, while bases in lowercase are not present in cDNA and were incorporated into the oligomers in order to engineer restriction sites at the ends of amplified fragments or to generate affinity tag linkers. Restriction sites are underlined, and the location of the affinity tags has been specified under "Experimental Procedures."

**TABLE I**

**PCR primers used for amplification**

| Primer | Sequence | Location |
|--------|----------|----------|
| 5T     | 5'-atatggttacctATCCATATGATCCATACAAGCAGGGTCA | 6927–6948 |
| 5B     | 5'-atatggcctctactaccc | 7569–7596 |
| 13B3518| 5'-tcatgggccacctGGGCTTTTCTGCAGAGCCCTTTT | 3519–3542 |
| 13T3550| 5'-ataggtaccacaggCTTGAAGGATGACCTGAGCCCTGG | 3550–3573 |
| 13B3767| 5'-tcatgatcctctcatcTTGAGACTAGACATG | 3767–3790 |
| 2TT5   | 5'-actactaggtgcccgccATGAGGATGATGACCCGGTA | 81–106 |
| 5B1152 | 5'-cactagattCTCAGGGTIGTGTTGGGGAAGATGC | 1152–1176 |
| M878T  | 5'-GACCCTACCTCTGGTACGGCCTGCTGATGACGGCGGTG | 2695–2731 |
| nt.flg | 5'-ctgagccatctgacgacgccagcttccaggg | 81–106 |
| nt.flg B | 5'-ccgctctgtctcatctgctcttctgtacctgctctgct | 1152–1176 |
| id.6his B | 5'-aagctttaattcccagctactcag | 1152–1176 |
| id.6his T | 5'-aagctttaattcccagctactcag | 1152–1176 |
| ct.6his B | 5'-ACCAGTCTACCTGGTCGACGCCTGCATTGACGGCCGTG | 1152–1176 |
| ct.6his T | 5'-ACCAGTCTACCTGGTCGACGCCTGCATTGACGGCCGTG | 1152–1176 |
| ct.flg | 5'-ctgagccatctgacgacgccagcttccaggg | 81–106 |
| ct.flg T | 5'-ctgagccatctgacgacgccagcttccaggg | 81–106 |
| ct.flg | 5'-ctgagccatctgacgacgccagcttccaggg | 81–106 |
| ct.flg T | 5'-ctgagccatctgacgacgccagcttccaggg | 81–106 |

* a T/B in oligomer names indicates sense/antisense oligomer, respectively.

*b The base numbers are according to FAS DNA sequence (13).

Historical Notes—Wang et al. (22) and others have noted that the active-site cysteine (23) and the phosphopantetheine thiolos (24), respectively. Wang et al. (22) and others have noted that the active-site cysteine (23) and the phosphopantetheine thiolos (24), respectively. Wang et al. (22) and others have noted that the active-site cysteine (23) and the phosphopantetheine thiolos (24), respectively. Wang et al. (22) and others have noted that the active-site cysteine (23) and the phosphopantetheine thiolos (24), respectively. Wang et al. (22) and others have noted that the active-site cysteine (23) and the phosphopantetheine thiolos (24), respectively. Wang et al. (22) and others have noted that the active-site cysteine (23) and the phosphopantetheine thiolos (24), respectively. Wang et al. (22) and others have noted that the active-site cysteine (23) and the phosphopantetheine thiolos (24), respectively. Wang et al. (22) and others have noted that the active-site cysteine (23) and the phosphopantetheine thiolos (24), respectively. Wang et al. (22) and others have noted that the active-site cysteine (23) and the phosphopantetheine thiolos (24), respectively. Wang et al. (22) and others have noted that the active-site cysteine (23) and the phosphopantetheine thiolos (24), respectively. Wang et al. (22) and others have noted that the active-site cysteine (23) and the phosphopantetheine thiolos (24), respectively. Wang et al. (22) and others have noted that the active-site cysteine (23) and the phosphopantetheine thiolos (24), respectively. Wang et al. (22) and others have noted that the active-site cysteine (23) and the phosphopantetheine thiolos (24), respectively. Wang et al. (22) and others have noted that the active-site cysteine (23) and the phosphopantetheine thiolos (24), respectively. Wang et al. (22) and others have noted that the active-site cysteine (23) and the phosphopantetheine thiolos (24), respectively. Wang et al. (22) and others have noted that the active-site cysteine (23) and the phosphopantetheine thiolos (24), respectively. Wang et al. (22) and others have noted that the active-site cysteine (23) and the phosphopantetheine thiolos (24), respectively.
amplified fragment was cloned into pUCBM20 to generate pFAS 5B2.20; its sequence was confirmed, and the His6 or FLAG tag linkers, with a properly located stop codon, were incorporated in frame with the coding sequence using Nhel and NotI sites. The sequence of the linker region was confirmed, and an appropriate fragment from these constructs was used to replace the corresponding region of full-length FAS cDNA constructs in the modified FpFastBac 1 vector.

Interdomain tagging was accomplished by creating two adjacent AccB7I restriction sites between the Thr-1154 and Gly-1157 codons by polymerase chain reaction. This procedure resulted in the introduction of three new residues, Gly, Tyr, and Gly, encoded at the restriction site. Thus, a Gly was added as the new residue at position 1155 followed by the affinity tag, either His6 or the FLAG tag (DYKDDDDK), and then the residues Tyr-Gly followed by the residue formerly identified as Gly-1157 and the normal FAS sequence. The partial cDNA construct pFAS 13.20 (base pairs 2539–4858 in pUCBM20) was used as template together with two pairs of primers, M878T/13B3518 and 13T3550/13B3576 (Table 1). The amplified fragments were used to replace the corresponding region of pFAS13.20, the sequence was confirmed, and the His6 or FLAG-tag linkers were introduced in frame with the coding sequence between the AccB7I sites. Degeneracy in the AccB7I recognition sequence allowed us to carry out directional ligation with a single enzyme digestion and minimal changes in the original sequence. The resulting partial cDNA fragments were moved into full-length FAS cDNA by Nhel/NotI digestion and ligated into the modified FpFastBac 1 vector.

Expression and Purification of Tagged FASs—Sf9 cells were infected with purified recombinant viruses and cultured for 48 h at 27 °C, and the tagged FAS proteins were purified from the cytosols as described earlier (28); glycerol (10%, v/v) was included in all buffers used for chromatography.

Anti-FLAG M2 Immunoadfinity Chromatography—FLAG-tagged FAS was applied to a column (4.5 × 0.5 cm) of anti-FLAG M2 affinity gel equilibrated with buffer A (250 mM potassium phosphate buffer, pH 7.0, 0.2 mM DTT, 10% glycerol) at a flow rate of about 20 ml/h. The column was washed twice with 10 ml of buffer A, and the bound proteins were eluted with 8 ml of buffer A containing 1.5 mg/ml of FLAG octapeptide. The presence of FAS in the column eluate was conveniently monitored and quantitated by measuring the β-ketoacyl reductase activity associated with the protein.

Nickel-NTA-Agarose Affinity Chromatography—The His6-tagged FAS was applied to a column (4.5 × 0.5 cm) of nickel-NTA equilibrated with buffer A. The column was washed with 12 ml of buffer A containing 25 mM imidazole, pH 7.0, to remove weakly bound proteins. More strongly bound proteins were eluted with 10 ml of buffer A containing 200 mM imidazole, pH 7.0. DTT (2 mM, final concentration) was added to the eluate immediately. The presence of FAS in the column eluate was detected by monitoring the β-ketoacyl reductase activity associated with the protein.

Randomization of FAS Subunits—Typically, FLAG-tagged and His6-tagged FASs (in 25–35 mM potassium phosphate buffer, pH 7.0, 1 mM DTT, 0.1 mM EDTA, and ~1% glycerol) were aged at 4 °C for 7–9 days to allow self-dissociation into their component subunits (29). Spontaneous reassociation of the subunits was induced by adjustment of the solvent to approximately 200 mM potassium phosphate, pH 7.0, and 10% glycerol and incubation at 30 °C for 75 min.

Isolation of FAS Dimers Carrying Different Tags on Each Subunit—A randomized population of dimers consisting of FAS carrying the His6 tag on both subunits (His6-tagged homodimers), FAS carrying the FLAG tag on both subunits (FLAG-tagged homodimers), and FAS carrying the His6 tag on one subunit and the FLAG tag on the other subunit (doubly tagged heterodimers) was prepared as described above. This mixture of homo- and heterodimers was then applied to the anti-FLAG immunoaffinity column under the conditions described above. Proteins that initially bound to the column and were subsequently released with 1.5 mg/ml FLAG octapeptide in buffer A were immediately applied to the nickel-NTA-agarose column pre-equilibrated with buffer A. Weakly bound proteins were washed from the column with buffer A containing 25 mM imidazole, and more tightly bound proteins were eluted with 200 mM imidazole in buffer A.

Removal of Imidazole or FLAG Octapeptide and Concentration of FASs—In a routine preparation of heterodimers, after the anti-FLAG column, the nickel-NTA column, the FLAG column, and the DE-53 anion exchange cartridge (28). In some experiments, where the order of application to the affinity columns was reversed, the same procedure was used to remove imidazole from the FAS. In both cases, this procedure also simultaneously facilitated concentration of the protein. Prior to application of the samples to the cartridge, the ionic strength of the solvent was adjusted to the equivalent of 50 mM potassium phosphate, pH 7.0, by dilution with 1 mM DTT, 1 mM EDTA, and 10% glycerol. Typically, FAS was eluted from the cartridge in 1 ml of 250 mM potassium phosphate, pH 7.0, 1 mM DTT, 1 mM EDTA, and 10% glycerol and stored at ~70 °C.

RESULTS AND DISCUSSION

Engineering Peptide Tags into the FAS—Quantitative evaluation of the activities of engineered heterodimers previously has been hampered by uncertainty as to the exact composition of the dimer population that results from randomization of the subunits from two different homodimers. Theoretically, if the rates of dissociation and reassociation of the various mutant FASs employed are unaffected by point mutations, then the mutant subunits should reassemble randomly, yielding a population consisting of 50% heterodimers and 25% of each homodimer. Surprisingly, however, some single mutations at the active site of a particular catalytic domain have a marked effect on the rate of dissociation of the dimers, so that there is some doubt as to whether the heterodimers do in fact constitute 50% of the total dimer population (20, 21). To solve this problem, we explored the possibility of introducing affinity tags into the recombinant FAS that might facilitate isolation of a homogenous population of heterodimers. Because of initial uncertainty as to whether the introduction of peptide tags at certain locations might affect the activity of the FAS, we engineered tags at three different sites, the amino terminus, the carboxyl terminus, and at an internal location.

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is located in a region predicted to lie on the surface of the protein, and indeed the adjacent lysine residues, Lys-1152 and Lys-1159, are sites for attack by trypsin in the native dimer (7). Therefore, we reasoned that residues inserted at this site are unlikely to be disruptive to the FAS structure and are likely to be accessible to either the metal ion or the anti-FLAG antibody. In fact, subsequent analysis of the engineered tagged FASs revealed that the presence of either peptide tag had little or no effect on overall fatty acid synthesizing activity of the wild-type FAS, regardless of location (Table II).

**Affinity Chromatography of Tagged FASs**—Initially, optimum conditions for affinity chromatography were established using amino-terminally FLAG-tagged and carboxyl-terminally His$_{6}$-tagged wild-type FAS homodimers. The binding capacity of the anti-FLAG column was approximately 2 mg of FLAG-tagged FAS/ml of affinity matrix. A minimum concentration of 1 mg/ml of FLAG octapeptide was required to elute the bound protein from the column. Typically, more than 90% of the applied FAS activity was recovered from the column and the presence of the FLAG octapeptide, at least up to 1.5 mg/ml, had no effect on FAS activity. Under identical conditions, carboxyl-terminally His$_{6}$-tagged, wild-type FAS did not bind to the affinity column, and all of the applied His$_{6}$-tagged FAS protein was recovered in the initial wash. Similarly, optimum conditions were established for binding of the His$_{6}$-tagged FAS to the nickel-NTA-agarose column. In the absence of imidazole in the equilibration buffer, untagged wild-type FAS, FLAG-tagged wild-type FAS, and His$_{6}$-tagged FAS all bound to the column. However, inclusion of only 25 mM imidazole in the equilibration buffer eliminated the weak binding displayed by both the untagged and FLAG-tagged wild-type FASs. Subsequently, the His$_{6}$-tagged FAS could be eluted from the column by inclusion of 200 mM imidazole in the elution buffer. The binding capacity of the column was approximately 4 mg of His$_{6}$-tagged homodimeric FAS/ml of bed volume. Typically, the yield of His$_{6}$-tagged FAS in the fraction containing 200 mM imidazole was approximately 90%, as judged by the recovery of FAS activity.

These preliminary experiments confirmed the specificities of the two affinity matrices and demonstrated that both matrices could be utilized to isolate FASs carrying the appropriate peptide tag without cross-contamination with FAS carrying the other tag.

**Isolation of FAS Dimers Carrying Different Tags on Each Subunit**—Doubly tagged heterodimeric FAS species were generated by randomization of the subunits derived from a mixture of carboxyl-terminally FLAG-tagged and His$_{6}$-tagged homodimeric wild-type FASs. Initial attempts to facilitate the randomization process by utilizing a TSK-DEAE column, as described previously (20), were hampered by the fact that the elution properties of both the FAS monomers and dimers are influenced by the presence of the tags: DYKDDDDK in the case of the FLAG tag and hexahistidine in the case of the His$_{6}$ tag. Consequently, the FLAG-tagged and His$_{6}$-tagged monomers do not co-elute exactly but rather partially separate from each other, limiting the possibility of formation of doubly tagged heterodimers during the reassociation step. As a result, yields of doubly tagged heterodimers were typically no more than 50% of that anticipated. To circumvent this limitation, we utilized an alternative procedure for subunit randomization in which the tagged, homodimeric FASs are mixed, induced to dissociate in the cold, and subsequently allowed to spontaneously reassociate on incubation at 30 °C (see “Experimental Procedures”).

The randomized mixture of FAS dimers was subjected sequentially to affinity chromatography on the anti-FLAG and metal-chelating matrices. At each step in the isolation of the doubly tagged heterodimers, samples were retained for analysis by Western blotting (Fig. 1). The starting population of randomized FAS dimers reacted positively with both anti-FLAG and anti-His$_{6}$ monoclonal antibodies, confirming the presence of both tags in the mixture. Passage of the mixed FAS population through the anti-FLAG column resulted in the elution of pure His$_{6}$-tagged homodimers in the unbound fraction, as evidenced by the positive reaction with anti-His$_{6}$ antibodies (Fig. 1B, lane 2) and negative reaction with anti-FLAG antibodies (Fig. 1A, lane 2). The yield of His$_{6}$-tagged homodimers typically accounted for about 25–35% of the total FAS, based on the recovery of β-ketoacyl reductase activity. FLAG-tagged homodimers and doubly tagged heterodimers co-eluted together in the bound fraction, as evidenced by positive reactions with both antibodies (Fig. 1, A, lane 3, and B, lane 3). These two species were ultimately resolved from each other on passage through the metal ion affinity column. Thus, the FLAG-tagged homodimers emerged in the unbound fraction, exhibiting a positive reaction with the anti-FLAG antibodies (Fig. 1, A, lane 4) and a negative reaction with the anti-His$_{6}$ antibodies (Fig. 1B, lane 4) on Western analysis. The yield of FLAG-tagged homodimers typically accounted for about 25–30% of the total FAS, based on the recovery of β-ketoacyl reductase activity. The FAS eluting in the “bound” fraction from this column reacted positively with both antibodies, consistent with the presence of doubly tagged heterodimers (Fig. 1, A, lane 5, and B, lane 5). The yield of doubly tagged heterodimers typically accounted for about 30–45% of the total FAS, based on the recovery of β-ketoacyl reductase activity. Similar yields of the homo- and heterodimers were obtained when the carboxyl-terminally FLAG-tagged FAS was replaced with amino-terminally FLAG-tagged FAS (details not shown). Furthermore, doubly tagged heterodimers, purified from randomized mixtures of carboxyl-terminally His$_{6}$-tagged and either the carboxyl-terminally or amino-terminally FLAG-tagged FASs, had the same specific activity in the overall FAS reaction (Table III), indicating that the location of the tag in the FAS subunit does not affect either the yield or the enzyme activity of the heterodimers. In some experiments, the samples were applied first to the nickel-NTA column and then to the anti-FLAG column; essentially the same results were obtained.

**Addressing the Question of Independent or Interactive Centers for Fatty Acid Synthesis**—In an earlier study, Kyushiki and Ikai (26) compared the effect of exposure of guinea pig FAS to various concentrations of PMSF on the activity of the thioesterase domain and on the overall capacity for fatty acid synthesis. PMSF specifically inhibits the thioesterase by binding to the active-site serine residue, which forms part of a Gly-Xaa-Ser-Xaa-Gly motif, typical of serine active-site esterases (35). Kyushiki and Ikai did not find a linear relationship between the extent of inhibition of thioesterase and fatty acid synthesizing activities. Thus, at 50% inhibition of the thioesterase, when presumably, on average, one thioesterase domain per dimer was inactivated, approximately 75% of the fatty acid synthesizing activity was retained. The development of a procedure for isolating FAS heterodimers in which the two sub-
FIG. 1. Purification of doubly tagged, heterodimeric, wild-type FASs by affinity chromatography on anti-FLAG and nickel-NTA columns: monitoring by Western analysis. Protein samples (~1 µg) at each step of purification were subjected to SDS-polyacrylamide gel electrophoresis and electroblotted onto a nitrocellulose membrane. The blots were then probed with anti-FLAG M2 (A) and anti-His6 (B) monoclonal antibodies. Lane 1, initial mixture of randomized, tagged FASs; lanes 2 and 3, unbound and bound proteins from the anti-FLAG antibody column, respectively; lanes 4 and 5, proteins eluted from the nickel-NTA column by 25 mM and 200 mM imidazole, respectively.

Again, this result suggested that a solitary functional catalytic center paired with an inactive center has higher activity than do paired catalytically active centers. To provide a second test of this hypothesis, we next isolated a pure population of heterodimers in which the thioesterase activity of one subunit was compromised by mutation, rather than by treatment with PMSF or proteases. The inactive thioesterase domain was engineered by mutation of the active-site serine residue in the context of the carboxyl-terminally FLAG-tagged FAS. We had previously confirmed that the S2302A FAS lacks only thioesterase activity and that none of the other partial activities of the complex is affected (21). Subunits of the carboxyl-terminally FLAG-tagged thioesterase mutant FAS were randomized with those of a carboxyl-terminally His6-tagged wild-type FAS and the doubly tagged heterodimers isolated as described above. Again, this heterodimer preparation reacted positively with both anti-FLAG and anti-His6 antibodies (details not shown) and exhibited 48 ± 0.9% of the thioesterase activity of the wild-type FAS, confirming that this FAS consisted of authentic heterodimers in which only one of the two thioesterase domains had been compromised by mutation. As in the case of the heterodimer containing one PMSF-modified thioesterase domain, the heterodimer containing one mutated thioesterase domain exhibited 51 ± 0.6% of the fatty acid synthesizing capacity of the wild-type FAS (Table III). To verify that, in the heterodimer, the center containing the mutated thioesterase domain was indeed competent to synthesize a long chain fatty acyl moiety, we included [2-14C]malonyl-CoA in the assay system for overall FAS activity, isolated the radiolabeled acyl-FAS, and analyzed the fatty acyl chains covalently attached to the protein (Table IV). The FAS consisting of two wild-type subunits accumulated only substoichiometric levels of fatty acyl chains (0.08 mol/mmol subunit), mainly 16:0, consistent with the chain termination reaction being non-rate-limiting.

A second line of experimental evidence presented by Kyushiki and Ikai (26) in support of a negative interaction between centers involved the dissociation and reassociation of a mixture of normal FAS and FAS from which the carboxyl-terminal thioesterase domain had been removed by limited proteolysis. The resulting randomized population of FASs, presumably consisting of homodimeric normal FAS, homodimeric “thioesterase-less” FAS, and heterodimeric FAS containing one normal and one thioesterase-less subunit, had 20–30% higher fatty acid synthesizing activity than did the starting mixture.

### Table III

| Subunit composition | Specific activity |
|---------------------|------------------|
|                 | milliunits/mg |
| FLAG-tagged | His6 tagged |
| NH_2-terminal, wild-type | COOH-terminal, wild-type | 2410 ± 40 |
| COOH-terminal, wild-type | COOH-terminal, wild-type | 2260 ± 50 |
| COOH-terminal, S2302A | COOH-terminal, wild-type | 1180 ± 35 |
| NH_2-terminal, PMSF-treated | COOH-terminal, wild-type | 1130 ± 32 |

units may differ, either as the result of mutation or chemical treatment, allowed us to revisit these experiments under more carefully controlled conditions.

In the first experiment, the thioesterase domains of an amino-terminally FLAG-tagged, wild-type FAS homodimer were inactivated by treatment with PMSF; less than 3% of the original FAS activity remained. Subunits of this inhibited FAS were then randomized with those of carboxyl-terminally His6-tagged, wild-type FAS homodimers and the doubly tagged heterodimers isolated as described above. The doubly tagged heterodimer preparation reacted positively with both anti-FLAG and anti-His6 antibodies (details not shown) and exhibited 49 ± 0.5% of the thioesterase activity of the wild-type FAS, confirming that these dimers consisted of a carboxyl-terminally His6-tagged, wild-type subunit in partnership with an amino-terminally FLAG-tagged, PMSF-inactivated subunit. The fatty acid synthesizing ability of this authentic heterodimeric FAS preparation was 50% of the wild-type value (Table III), a finding consistent with independent rather than interactive centers for fatty acid synthesis.

A two-dimensional gel electrophoresis experiment by Kyushiki and Ikai (26) in support of a negative interaction between centers involved the dissociation and reassociation of a mixture of normal FAS and FAS from which the carboxyl-terminal thioesterase domain had been removed by limited proteolysis. The resulting randomized population of FASs, presumably consisting of homodimeric normal FAS, homodimeric “thioesterase-less” FAS, and heterodimeric FAS containing one normal and one thioesterase-less subunit, had 20–30% higher fatty acid synthesizing activity than did the starting mixture.

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On the other hand, the heterodimer consisting of one wild-type subunit and one subunit compromised by the active-site mutation in the thioesterase domain accumulated 0.9 mol of long chain acyl moieties (mainly 18:0) per mol of dimer, consistent with the synthesis, but nonremoval of a long chain fatty acyl moiety at one of the two centers for fatty acid synthesis. Since our earlier mutant complementation analysis had established that the thioesterase domain releases fatty acyl chains from the ACP domain immediately adjacent on the same polypeptide.
chain (21), the long chain acyl moiety must accumulate on the ACP domain of the mutated subunit. Similar results were obtained using the heterodimer in which activity of one of the thioesterase domains had been inactivated by treatment with PMSF (Table IV).

To ascertain whether cessation of fatty acid synthesis at one center, brought about by the S2302A mutation, might influence the kinetics of synthesis at the second center, a more detailed kinetic analysis was undertaken using the FASs consisting of two wild-type subunits and that consisting of a wild-type subunit paired with a S2302A mutant subunit. These experiments revealed no discernable difference between the two FASs in the plots of initial velocity as a function of acetyl-CoA, malonyl-CoA, or NADPH concentration, other than the 2-fold difference in specific activity (details not shown). Again, these findings are consistent with one center for fatty acid synthesis functioning normally while the other is compromised by an inability to catalyze the chain-terminating reaction.

Thus, regardless of whether the thioesterase activity of one of the two centers is compromised by mutation or chemical inhibition, the rate of fatty acid synthesis by the second center is unaffected. This finding is consistent with the operation of negative interaction between the two catalytic centers, as suggested previously. Although it is difficult to provide a rationale for the different findings of this study and the earlier one reported by Kyushiki and Ikai (26), we would argue that the new strategy presented in this study offers several advantages over the earlier approach. First of all, the activity of any of the catalytic domains, such as the thioesterase, now can be modified by mutation, rather by proteolysis or chemical modification, treatments that conceivably might have unknown secondary effects on the protein. Second, and perhaps most importantly, the benign, double tagging procedure permits isolation of a defined population of heterodimers and obviates the need to make assumptions regarding the exact composition of a randomized population of dimers formed from two differently treated FAS dimers (Kuyshiki and Ikai (26) found that the proteolysed core dimers dissociated more slowly than the native dimers). Although clearly our results are not consistent with the operation of a negative interaction, they should not necessarily be interpreted to indicate that the two centers for fatty acid synthesis function entirely independently of each other. Recently, we found compelling evidence indicating that, whereas the thioesterase and dehydrase domains are able to cooperate functionally with only the ACP domain of the same subunit (21), the malonyl/acyl transferase and β-ketoacyl synthase domains can cooperate with the ACP domains of either subunit (27). This unanticipated element of partial redundancy in the FAS mechanism blurs the assignment of each of the functional domains to a specific catalytic center, as depicted in earlier models for the FAS (3, 16, 36), and allows for the possibility of communication between the two centers for fatty acid synthesis. Inactivation of the thioesterase function of the FAS results in accumulation of a long chain acyl moiety on the 4′-phosphopantetheine of the ACP domain (33). Under the experimental conditions described in this report, for the heterodimer containing only one functional thioesterase, a long chain acyl moiety is present on the 4′-phosphopantetheine associated with the ACP domain immediately adjacent to the inactive thioesterase domain. Thus, what our results specifically demonstrate is that the continued presence of a long chain acyl moiety on one 4′-phosphopantetheine blocks further utilization of that ACP domain without affecting the ability of the second ACP domain to engage in fatty acid synthesis. Development of the double tagging procedure that allows isolation of FAS dimers in which only one subunit is modified, or in which the two subunits are modified by different mutations will now allow us to address other aspects of the complex interplay that occurs between functional domains of the FAS mechanism that previously had been refractory to analysis.

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Affinity Labeling of Fatty Acid Synthase
Differential Affinity Labeling of the Two Subunits of the Homodimeric Animal Fatty Acid Synthase Allows Isolation of Heterodimers Consisting of Subunits That Have Been Independently Modified

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