Effect of Modification of the Length and Flexibility of the Acyl Carrier Protein−Thioesterase Interdomain Linker on Functionality of the Animal Fatty Acid Synthase†

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ABSTRACT: A natural linker of approximately 20 residues connects the acyl carrier protein with the carboxy-terminal thioesterase domain of the animal fatty acid synthase. This study examines the effects of changes in the length and amino acid composition of this linker on catalytic activity, product composition, and segmental motion of the thioesterase domain. Deletion of 10 residues, almost half of the interdomain linker, had no effect on either mobility of the thioesterase domain, estimated from fluorescence polarization of a pyrenebutyl methylphosphono moiety bound covalently to the active site serine residue, or functionality of the fatty acid synthase; further shortening of the linker limited mobility of the thioesterase domain and resulted in reduced fatty acid synthase activity and an increase in product chain length from 16 to 18 and 20 carbon atoms. Surprisingly, however, even when the entire linker region was deleted, the fatty acid synthase retained 28% activity. Lengthening of the linker, by insertion of an unusually long acyl carrier protein−thioesterase linker from a modular polyketide synthase, increased mobility of the thioesterase domain without having any significant effect on catalytic properties of the complex. Interdomain linkers could also be used to tether, to the acyl carrier protein domain of the fatty acid synthase, a thioesterase active toward shorter chain length acyl thioesters generating novel short-chain fatty acid synthases. These studies reveal that although truncation of the interdomain linker partially impacts the ability of the thioesterase domain to terminate growth of the acyl chain, the overall integrity of the fatty acid synthase is quite tolerant to moderate changes in linker length and flexibility. The retention of fatty acid synthesizing activity on deletion of the entire linker region implies that the inherent flexibility of the phosphopantetheine "swinging arm" also contributes significantly to the successful docking of the long-chain acyl moiety in the thioesterase active site.

The linker regions between functional elements of multidomain and multifunctional assemblies are usually poorly conserved, unstructured flexible regions located at the surface of the protein. There are some exceptions, however, as in the case of the glucose transporter of the bacterial phosphotransferase system, where the two domains of the transmembrane subunit are linked by a highly invariant sequence (KTPGRED) that forms a hinge of precise but restricted mobility (*1*). More typically, although interdomain linkers are often enriched in particular amino acids, the actual amino acid sequence appears to be unimportant. For example, the ~16-residue flexible linker region connecting the two domains of prokaryotic initiation factor 3 is unusually rich in Lys residues, yet they can be replaced with uncharged hydrophilic amino acids without loss of function (*2*). In the cellulobiohydrolases and glucoamylases, the catalytic core is joined to the carbohydrate-binding domain by an extended O-glycosylated interdomain linker rich in Ser and Thr residues; nevertheless, these sequences can be replaced with artificial proline-rich linkers with essentially no effect on catalysis (*3*, *4*). Perhaps the most extensively studied interdomain linkers are the Ala-Pro-rich sequences of pyruvate dehydrogenase that connect the lipoyl, subunit-binding, and catalytic domains of the dihydrolipoamide acetyltransferase subunits. Substantial variations in linker sequence, length, and composition can be tolerated without serious loss of function, and NMR spectroscopy studies have confirmed that these linkers enjoy considerable conformational mobility (*5*). Nevertheless, the adoption of the *trans* configuration by Ala-Pro bonds in the linker exerts a stiffening effect that confers a degree of rigidity in the linker that may minimize the adoption of collapsed conformations, ensuring that the connected domains are held at a distance most suited for promoting functional interactions (*6*−*8*). Naturally occurring proline-rich sequences are also found in some interdomain linkers associated with fatty acid synthases, FASs, and modular polyketide synthases (*9*).

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                      ACP                                                      THIOESTERASE
              2171                       trypsin                                        2225
               ↓     helix 4                ↓                          helix 1            ↓
Rat          IREVRQLTLRKLQEMSSKAGS---DTELAAPKSKND------------TSLKQAQLNLSILLVNPEGPTL
Human        VREVRQLTLRKLQELSSKADE---ASELACPTPKEDG-----------LAQQQTQLNLRSLLVNPEGPTL
Chicken      MREIRLLTINKLRELSSKTGT---AEELKPSQVLKT------------GPGEPPKLDLNNLLVNPEGPTI
C. elegans   MKEIRTLTLNKLQQLADQGGTGRTALQVNELEMKKDGERDAELNTAEMLEQQMNQLFKMRVDVNDLDPQD
             ::*:* **:.**::::.: .      ::                     : : :*    : **  .*
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FIGURE 1: Clustal W alignment of the sequences of animal FASs proximal to the putative ACP−thioesterase boundary. Identical residues are indicated by asterisks, conservative substitutions of major groups by colons, and conservative substitutions of minor groups by periods. The annotations apply to the rat sequence, and the putative linker region lies between helix 4 of the ACP and helix 1 of the thioesterase domain.

The first interdomain linker to be studied in the animal FAS[1] was that connecting the C-terminal thioesterase domain to the adjacent ACP domain. This region was found to be highly susceptible to attack by proteases, and cleavage of the linker resulted in the loss of overall FAS activity (*10*). The solving of the three-dimensional structure of the ACP domain of the rat FAS by nuclear magnetic resonance spectroscopy revealed that the last defined structural element, helix 4, extends from residues 2179 to 2187 so that the unstructured region immediately following likely marks the beginning of the interdomain linker (*11*). This region is characterized by the absence of any notable sequence similarity among the animal FASs, two insertions in the *Caenorhabditis elegans* sequence, and the presence of a site in the rat FAS (Lys-2199) that is readily susceptible to cleavage by trypsin (Figure 1). From an examination of multiple sequence alignments, we estimated that the linker region likely extends approximately from Lys-2188 to Asn-2212 and chose to examine the effects of engineering several deletions and an insertion in this region on conformational mobility of the thioesterase domain and the catalytic integrity of the complex. Shortly after submission of the original version of this paper, a crystal structure was solved for the thioesterase domain of the human FAS (*12*). The first defined structural element in the thioesterase is a short helix LRS that corresponds to the sequence ^{2213}LSI in the rat protein and allows more precise localization of the linker−thioesterase boundary (Figure 1).

EXPERIMENTAL PROCEDURES

Cloning and Mutagenesis. The general strategies for construction of cDNAs encoding the wild-type FAS (*13*), in vitro site-directed mutagenesis using PCR (*14*−*16*), and incorporation of a His_6 tag (*17*) have been described in detail previously. The strategy used in the generation of various modified FAS constructs is summarized in Figure 2, and a complete description is available as Supporting Information.

Protein Expression and Purification. The final FAS cDNA constructs, in the context of modified pFastBac 1 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 (Invitrogen). *Sf*9 cells were then infected with the purified recombinant viruses and cultured for 36−48 h at 27 °C. The tagged FAS proteins were purified from the cytosols as described earlier (*17*).

[1] Abbreviations: FAS, fatty acid synthase; ACP, acyl carrier protein; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid).

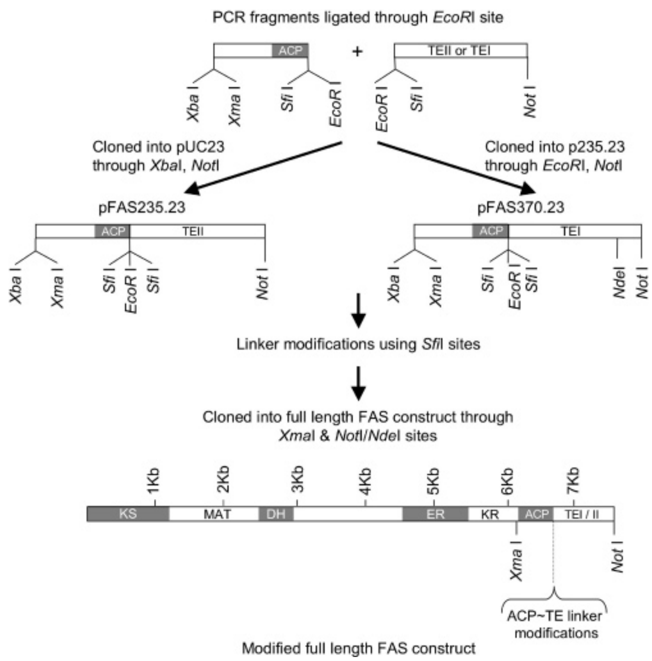

FIGURE 2: Scheme for generating various modified FAS constructs. The cartoon outlines the strategies used for modification of the linker region between ACP and the resident thioesterase domain (TEI) and generation of novel FASs in which TEI was replaced by thioesterase II (TEII), a broad-specificity enzyme normally expressed as a free-standing protein in lactating mammary glands. Restriction sites exploited in the constructions are marked. The PCR-amplified fragments of rat FAS cDNA (*Xba*I−*Eco*RI) and rat TEII (*Eco*RI−*Not*I) were ligated through the *Eco*RI site and cloned into *Xba*I and *Not*I sites of the pUC23 vector to generate the initial chimeric construct pFAS235.23. The engineered *Sfi*I−*Eco*RI sites were used to modify the linker length between the ACP and TEI or TEII domains, while *Xma*I and *Eco*RI or *Eco*RI and *Not*I sites were used to exchange PCR-amplified fragments encoding ACP and TEI. The *Xma*I−*Not*I/*Nde*I sites were used to move the appropriate fragment into a cDNA encoding full-length FAS. The nucleotide numbering refers to the full-length FAS cDNA, and the coding regions for the β-ketoacyl synthase (KS), malonyl/acetyl transferase (MAT), dehydrase (DH), enoyl reductase (ER), β-keto reductase (KR), ACP, and thioesterase (TE1 and TE2) are also shown.

Thioesterase I Construction and Purification. The engineering of an expression plasmid for the free-standing form of the full-length thioesterase (thioesterase I) domain of FAS, with the N-terminal sequence MESKN, cloning into pJLA, expression in *Escherichia coli* DH5α cells, and purification was described earlier (*18*). An additional purification step was included, involving anion-exchange chromatography on a column of TSK-DEAE-5PW. The procedure for cloning of an N-terminally truncated, C-terminally His_6-tagged form of thioesterase I (N-terminal sequence MAQASI) is available

as Supporting Information. This enzyme was purified by a combination of metal ion affinity and anion-exchange chromatography. The specific activities of the full-length and truncated forms of the free-standing thioesterase domain were 6200 ± 250 and 3165 ± 380 nmol of palmitoyl-CoA hydrolyzed min^{-1}•mg^{-1}, respectively.

Enzyme Assays. FAS was assayed spectrophotometrically at 37 °C, essentially as described earlier (*19*). Incubation mixtures contained 0.1 M potassium phosphate buffer (pH 6.6), 190 μM NADPH, 60 μM acetyl-CoA, and 110 μM malonyl-CoA.

Activities of thioesterases I and II were assayed spectrophotometrically at 412 nm by continuous detection of the free CoA formed upon hydrolysis of the acyl-CoA substrate, using dithionitrobenzoate (*20*); blank reactions were performed with no added acyl-CoA substrate. Thioesterase I activity was assayed at 30 °C: incubation mixtures contained 50 mM potassium phosphate buffer (pH 8), 20 μM palmitoyl-CoA, 50 μM DTNB, and 0.1 mg/mL bovine serum albumin. Thioesterase II activity was assayed at 37 °C: incubation mixtures contained 100 mM potassium phosphate buffer (pH 8), 100 μM decanoylpantetheine, 0.1 mM DTNB, and 0.5 mg/mL bovine serum albumin.

For assay of the β-ketoacyl synthase reaction, incubation mixtures, at 37 °C, contained 200 mM potassium phosphate buffer (pH 6.6), 60 μM acetyl-CoA, 110 μM [2-^{14}C]malonyl-CoA, and 100 μM CoASH. The condensation reaction was monitored by taking advantage of the high activity of the "malonyl/acetyl transferase" substrate-loading enzyme exhibited toward β-ketobutyryl moieties (*21*). Thus, the β-ketobutyryl moieties formed on the ACP in the condensation reaction can be off-loaded to a CoA acceptor in a non-rate-limiting manner, allowing assay by HPLC (*15*).

Activity of the β-ketoacyl reductase domain was monitored spectrophotometrically at 340 nm using either *trans*-1-decalone (*13*) or β-ketobutyryl-CoA (*16*) as substrate.

Fatty Acid Analysis. Fatty acid products were identified as phenacyl derivatives by HPLC (*22*).

Synthesis of 4-(1-Pyrenyl)butyryl-CoA. The 4-(1-pyrenyl)-butyryl-CoA was synthesized according to the published protocol (*23*). The 4-(1-pyrenyl)butyryl-CoA was purified from the aqueous phase by chromatography on a SepPac C-18 cartridge (Waters). Unreacted CoA was eluted with 10% acetonitrile in 10 mM ammonium acetate, pH 5.6, and 4-(1-pyrenyl)butyryl-CoA with 30% acetonitrile in 10 mM ammonium acetate, pH 5.6. Finally, the 4-(1-pyrenyl)butyryl-CoA solution was concentrated using a SpeedVac. The concentration of 4-(1-pyrenyl)butyryl-CoA was determined spectrophotometrically at 345 nm using an extinction coefficient of 40000 M^{-1} cm^{-1}, and the thioester concentration was measured by DTNB titration of thiols released by hydroxylaminolysis (0.2 M hydroxylamine, pH 8, for 20 min). The yield of 4-(1-pyrenyl)butyryl-CoA was 8.2 μmol from 50 μmol of CoASH, and the preparation contained at least 91% of thioester.

Labeling of FAS with Fluorescent Probes. FAS was selectively labeled in the thioesterase domain by incubation with a 1.6−2-fold excess of pyrenebutyl methylphosphonofluoridate for 10−15 min at room temperature. Unreacted reagent was removed using a desalting column (PD-10, Amersham) equilibrated with 0.2 M potassium phosphate buffer (pH 7) and 1 mM EDTA. The protein concentration was calculated after correction for the absorbance of the pyrenebutyryl moiety at 280 nm according to formula: [protein] = A_{280} − (0.67A_{346}). The extent of modification was calculated according to Berman et al. (*24*), utilizing calculated molar extinction coefficients for FAS mutants. Sixty-five to eighty-five percent of thioesterase was labeled with pyrenebutyl methylphosphonofluoridate, and the extent of modification was correlated directly with the loss of thioesterase activity.

FAS, lacking the thioesterase domain, was labeled with 4-(1-pyrenyl)butyryl moieties by incubation with 225 μM 4-(1-pyrenyl)butyryl-CoA for 90 min at 30 °C. The enzyme preparation was purified by three passes through PD-10 desalting columns. Modified FAS contained approximately two 4-(1-pyrenyl)butyryl moieties per subunit and lost 63% activity of β-ketobutyryl-CoA reductase activity.

Fluorescence Polarization. Steady-state fluorescence polarization measurements were performed according to Berman et al. (*24*), using either a Perkin-Elmer LS-54B luminescence spectrometer or a ISS PC1 photon counting spectrofluorometer, both equipped with a thermostatically controlled cuvette holder. Excitation and emission wavelengths were set to 348 and 400 nm, respectively. Excitation and emission slits were set to a 5.5 nm spectral band-pass. Labeled protein, in 0.2 M potassium phosphate buffer (pH 7) containing 1 mM EDTA, was mixed with sucrose solution made in the same buffer. FAS dimer concentration was kept at 0.25 μM, and the sucrose concentration was varied from 0% to 50% in 5% increments. Readings were made when the temperature of the optical cell and solution stabilized at 24 °C, typically after a 5 min preincubation. Polarization (P), *G* factor, and rotational correlation time (Φ) were calculated as described previously (*24*). Intensity values were corrected for a scatter control measured for buffer and sucrose solutions.

RESULTS

Effect of Linker Deletion and Replacement on Catalytic Activity of FAS. Investigation of the potential importance of the linker structure to FAS function was initiated by making a deletion of 10 residues (residues 2198−2207) from the putative ACP−thioesterase linker region (Figure 1). The deletion reduced thioesterase activity slightly but had no significant effect on FAS activity (Table 1). Since this sequence element appeared nonessential for function, we focused on determining the minimal linker length that was required for maintaining FAS functionality by expanding the deleted region toward both the C- and N-termini (Δ14, Δ18, and Δ22). Removal of these additional residues progressively decreased FAS activity to 28% of normal, in the case of the 22-residue deletion. Assays for thioesterase activity, determined with palmitoyl-CoA, a model substrate that interacts directly with the active site of the thioesterase and does not require cooperation with the adjacent ACP domain, revealed that the most C-terminal deletions (Δ14 and Δ22) decreased thioesterase activity appreciably (55% and 48% of wild-type activity, respectively) whereas further deletion of residues toward the N-terminal side of the linker had only a modest effect (Table 1, compare Δ10, 80% activity, to Δ18, 74% activity). None of the deletions had any effect on the ability of the FAS to catalyze the β-ketoacyl synthase (condensation)

Table 1: Effect of ACP−Thioesterase I Linker Length on Catalytic Activity and Rotational Motion of the Thioesterase Domain

FAS	Sequence[a]	Enzyme activity (% wt)[b]		η[c] (ns)
		FAS	Thioesterase	
wt	LRKLQEMSSKAGSDTELAAPKSKNDTSLKQAQLNLSILLV	100 ± 6	100 ± 2	88 ± 1
Δ10	LRKLQEMSSKAGSDTELAA----------QAQLNLSILLV	98 ± 1	80 ± 2	89 ± 2
Δ14	LRKLQEMSSKAGSDTELAA--------------QASILLV	77 ± 2	55 ± 1	161
Δ18	LRKLQEMSSKA------------------QAQLNLSILLV	58 ± 1	74 ± 2	151
Δ22	LRKLQEMSSKA----------------------QASILLV	28 ± 1	48 ± 3	172
Δ7i20	LRKLQEMSSKAGSDTELAAPK/ \KQAQLNLSILLV EEKAAAPAGAHSGADTGAGA	86 ± 2	98 ± 1	46

[a] A fragment of the wt FAS sequence, residues 2179−2218, is shown in the first row. [b] The specific activity of the wt FAS (100% value) was 2201 ± 128 nmol of NADPH oxidized $min^{-1}\cdot mg^{-1}$ in the FAS assay and 1104 ± 21 nmol of palmitoyl-CoA hydrolyzed $min^{-1}\cdot mg^{-1}$ in the thioesterase assay. The condensing activity of the wt FAS, 99.8 ± 2 nmol of β-ketobutyryl-CoA formed $min^{-1}\cdot mg^{-1}$, was not altered significantly in any of the mutants. [c] The active site serine (S2302) of the thioesterase domain of FAS was labeled with pyrenebutyl methylphosphonofluoridate, and the viscosity-dependent rotational correlation time (Φ) was estimated as described in the Experimental Procedures. Using the same reagent, the Φ was estimated for the free-standing thioesterase domain (24 ns), and using 4-(1-pyrenyl)butyryl-CoA, the Φ was estimated for a FAS from which the thioesterase domain had been deleted by mutagenesis (415 ns).

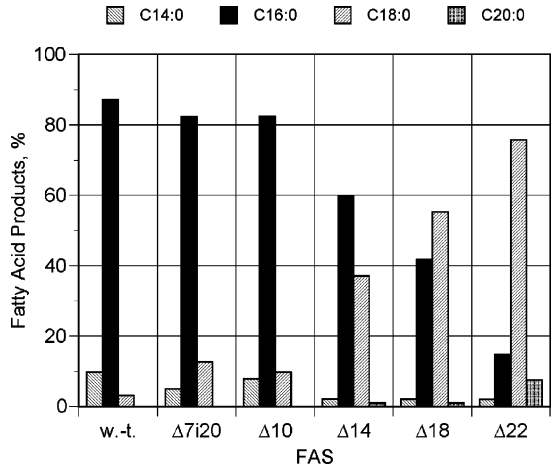

FIGURE 3: Effect of linker deletion and replacement on products formed by FAS−thioesterase I. The mole percent contribution of each fatty acid to the total is shown on the vertical axis, and the FAS constructs are identified on the horizontal axis. FAS proteins were incubated with malonyl-CoA, acetyl-CoA, and NADPH, and fatty acids were extracted and analyzed as described in the Experimental Procedures.

reaction. To determine whether lengthening of the linker region might impede the ability of the thioesterase domain to access acyl chains formed on the phosphopantetheine of the ACP domain, we replaced 7 residues of the linker with a stretch of 20 residues modeled after an unusually long, naturally occurring linker in the C-terminal module of the picromycin modular polyketide synthase found in *Streptomyces venezuelae* (*25*). Introduction of an additional 13 residues to the linker decreased FAS activity only slightly and had no effect on the catalytic activity of the thioesterase domain, as assessed using palmitoyl-CoA as a model substrate (Table 1, Δ7i20).

Effect of Linker Deletion and Replacement on Products Formed by FAS. Analysis of the fatty acid products formed by the various mutants provided further insight into the effects of linker modification on functionality of the FAS (Figure 3). Wild-type FAS synthesizes mainly palmitic acid (C16), and neither lengthening of the linker by 13 residues nor shortening by 10 residues altered this tight product specificity (Figure 3, wt, Δ7i20, and Δ10). Stearic acid (C18) is normally a minor product of the FAS, but shortening of the linker by more than 10 residues progressively increased the proportion of C18 produced at the expense of C16. Thus, C18 was the major product formed by the construct carrying the longest deletion (Δ22 produced 76% C18 and only 14% C16; Figure 3).

Effect of Linker Deletion and Replacement on Mobility of the Thioesterase Domain. Segmental flexibility of the thioesterase domain was assessed by estimating the rotational correlation time of a fluorophore bound covalently to the active site serine residue using the LS-54B instrument (Figure 4). Limiting polarization for the probe, P_0, was calculated from the *y*-intercepts of linear regression plots of $(1/P - {}^1/_3)$ vs (T/η), and its average amounted to 0.125 ± 0.011. Previously published data reported P_0 values for pyrenbutyl in the range 0.29−0.32 in the context of acetylcholinesterase and chicken FAS (*24*, *26*). However, in our hands, repeated measurements on a different instrument (ISS PC1) also consistently gave significantly lower values (average P_0 value of 0.17 ± 0.02). We have no explanation for this discrepancy.

The rotational correlation times (Φ) were calculated from the *y*-intercept and slope of the plots. Despite the fact that the mobility of the thioesterase is reduced in the covalently bound, compared to the free-standing, form (Φ = 88 and 24 ns, respectively), the thioesterase domain enjoys considerably more conformational mobility than the rest of the FAS protein (Φ = 415 ns for the FAS lacking the thioesterase domain). Deletion of 10 residues from the linker had no significant effect on mobility of the thioesterase domain, but further deletions reduced mobility considerably (Φ = 172 ns for Δ22). In contrast, lengthening of the interdomain linker significantly increased conformational mobility of the thioesterase domain (Φ = 45 ns for Δ7i20).

Replacement of the Resident Long-Chain-Specific Thioesterase I with a Covalently Linked Broad-Specificity Enzyme, Thioesterase II. The lactating mammary glands of mammals and uropygial glands of birds contain a free-standing, broad-specificity enzyme, thioesterase II, that is

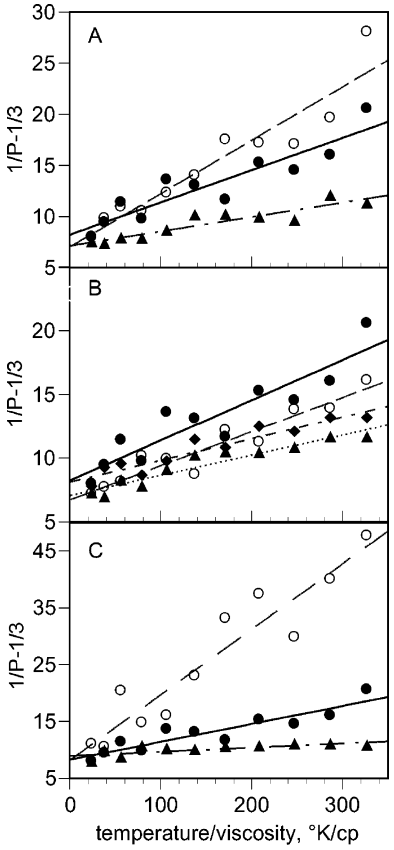

FIGURE 4: Effect of isothermal variation of viscosity on fluorescence depolarization of pyrenebutyl methylphosphono-labeled thioesterase I domain of FAS. Polarization (*P*) changes were measured by isothermal variation of viscosity effected by addition of sucrose. The following slopes were calculated for FAS constructs: wt (●, all panels), 0.032 ± 0.005; Δ22 (▲, panel A), 0.014 ± 0.002; Δ7i20 (○, panel A), 0.052 ± 0.006; Δ10 (○, panel B), 0.027 ± 0.003; Δ14 (◆, panel B), 0.017 ± 0.002; Δ18 (▲, panel B), 0.016 ± 0.002. For comparison, independently expressed PBMP-labeled thioesterase domain (○, panel C) and PBA-labeled FAS lacking the thioesterase domain (▲, panel C) are shown with slopes of 0.115 ± 0.010 and 0.007 ± 0.002, respectively. The average limiting polarization, P_0, was 0.125 ± 0.011.

capable of interrupting the elongation of acyl chains by the FAS and causing the premature release of short- and medium-chain fatty acids (*27*−*29*). Lipids containing these shorter chain length fatty acids play special roles in neonatal nutrition in mammals and in waterproofing the feathers of aquatic birds. To determine whether it was possible to engineer a novel FAS that produced short chain length fatty acids by tethering a new chain-terminating enzyme to the ACP domain, we replaced the resident thioesterase of the rat FAS (thioesterase I) with the rat mammary gland thioesterase II, using two covalent interdomain linkers of different length. Linker L1 is identical with the long linker used in the Δ7i20 construct described earlier, and the shorter linker L2 is closer in length to the natural linker found between the ACP and thioesterase I domains (Table 2). Covalent attachment of thioesterase II to the ACP domain by the two linkers produced catalytically active FASs (Table 2).

FAS−thioesterase II constructs have full keto reductase, dehydrase, enoyl reductase, and thioesterase II activities when assayed with model substrates (details not shown). These substrates do not require participation of the ACP domain. However, the β-ketoacyl synthase activity of these novel

Table 2: Catalytic Activity of FAS in Which the Resident Chain-Terminating Thioesterase I Is Replaced with Thioesterase II

linker/ thioesterase	FAS activity (nmol·min^{-1}·mg^{-1})	thioesterase activity (nmol·min^{-1}·mg^{-1})		β-ketoacyl synthase activity (nmol·min^{-1}·mg^{-1})
		C10[a]	C16[b]	
wt/TE I	1930 ± 51	19 ± 2	1129 ± 25	99.8 ± 2.2
L1/TE II[c]	133 ± 1	445 ± 11	71 ± 1	21.1 ± 4.2
L2/TE II[d]	113 ± 3	342 ± 3	95 ± 3	19.1 ± 0.9

[a] The substrate was *S*-decanoylpantetheine; the activity of thioesterase II is identical with *S*-decanoylpantetheine and decanoyl-CoA as substrates. [b] The substrate was palmitoyl-CoA. [c] Linker sequence, L1/TE2 (ACP)-KAGSDTELAAPK**EEKAAAPAGAHSGADTGAGA**-KQAQ-(TE2). Bold letters indicate the engineered amino acid sequence. [d] Linker sequence, L2/TE2 (ACP)-KAGSDTELAAPK**AELAANSA**-KQAQ(TE2).

FASs was reduced to ∼20% of that of the wild-type FAS, and the rate of acyl chain elongation, assessed from the rate of NADPH oxidation, was only ∼6% of that of the wild-type FAS. The lower activity of FAS with thioesterase II, rather than thioesterase I as the resident chain-terminating enzyme, was not due to misfolding of the protein, since removal of the thioesterase II domain by mild treatment with trypsin restored the condensation activity to normal (details not shown). It is possible that the permanently tethered thioesterase II interferes sterically with the normal juxtapostioning of the β-ketoacyl synthase and ACP domains. However, the rate of release of fatty acids by the newly resident thioesterase II [31 nmol min^{-1} (nmol of thioesterase II)$^{-1}$] is very close to the calculated V_{max} [28 nmol min^{-1} (nmol of thioesterase II)$^{-1}$] for the release of fatty acids by free-standing thioesterase II from FAS that has been compromised in its resident thioesterase I activity. Thus thioesterase II may be operating near its maximum turnover rate, and the retardation in the overall rate of elongation may result from the sequestering and relatively slow release of short chain length intermediates by the enzyme.

Specificity of these novel FASs was shifted toward shorter chain fatty acyl moieties, reflecting the inherent specificity of the new thioesterase II domain (Table 2). This altered specificity was evident in the spectrum of products formed by the novel FASs: both FASs produced mainly short chain length fatty acids (Figure 5).

DISCUSSION

Although the covalent connection between the ACP and thioesterase domains of FAS was first recognized as essential for the integrity of the FAS almost 3 decades ago, little attention has been paid to the role of interdomain linkers in the functioning of the complex. Studies in Hammes' laboratory, involving the site-specific introduction of fluorochromes at the 4′-phosphopantetheine of the ACP domain and the serine active site of the thioesterase, revealed, by fluorescence resonance energy transfer experiments, that these sites are ∼48 Å apart (*26*). Since the thioesterase-catalyzed hydrolysis of palmitoyl moieties from the 4′-phosphopantetheine necessitates direct functional interaction of these sites, it seemed likely that mobility of the 20 Å long 4′-phosphopantetheine moiety might be insufficient to bridge this gap. Indeed, dynamic anisotropy measurements indicated that the thioesterase domain has considerable segmental flexibility,

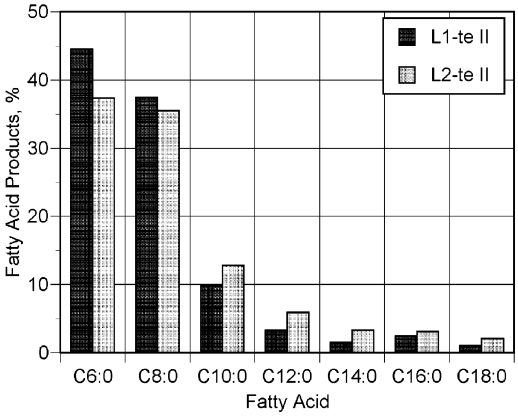

FIGURE 5: Products formed by FAS in which the resident chain-terminating thioesterase is replaced by thioesterase II. FAS chimeras were incubated with malonyl-CoA, acetyl-CoA, and NADPH, and fatty acids were extracted and analyzed as described in the Experimental Procedures.

suggesting that substantial conformational changes, facilitated by flexibility of the ACP−thioesterase linker, may accompany the catalytic process (*26*). In the present study we have assessed the importance of linker length and flexibility to catalysis of the chain-terminating reaction. The likely N-terminal boundary of the linker was estimated on the basis of the established three-dimensional structure of the ACP domain (*11*). The C-terminal boundary of the linker was more difficult to ascertain, and our estimate is based primarily on multiple sequence alignments that include known chain-terminating thioesterases, assuming that the most poorly conserved regions likely are associated with the linker (Figure 1). In addition, previous studies had shown that chain-terminating thioesterases of the free-standing variety and those covalently associated with FAS complexes share similar catalytic mechanisms (*30*−*33*) and exhibit low, but nevertheless detectable, sequence similarity (*34*). In the free-standing thioesterases, the active site serine residue is located ∼100 residues from the N-terminus, and in the rat FAS the active site serine is located 103 residues downstream from the tryptic cleavage site. On the basis of these considerations, we estimated that sequences between residue 2188 and approximately residue 2210 might possibly be considered as part of an interdomain linker. Linker regions in multifunctional proteins are commonly rich in proline residues, and prolines occur with a frequency of ∼18% in the interdomain linkers but only ∼5% in the entire FAS. Nevertheless, the ACP−thioesterase linkers of animal FASs are not particularly proline rich, and that of the *C. elegans* FAS appears to contain no prolines at all.

Our initial characterization of the FAS labeled at the thioesterase active site serine residue with pyrenebutyl methylphosphonofluoridate confirmed the earlier findings from Hammes' laboratory that this domain enjoys considerably more segmental flexibility than the FAS as a whole. Surprisingly, more than 10 residues had to be deleted from the linker region before any significant effect on functioning of the FAS was observed. With deletions of 14, 18, and 22 residues, FAS activity declined progressively and the product profile shifted from predominantly C16 to C18. The formation of longer chain length products is indicative of a reduced ability of the thioesterase to release acyl chains from the 4′-phosphopantetheine thiol, as the FAS is capable of slowly elongating acyl chains to 20 carbon atoms when the chain-terminating thioesterase is compromised (*28*).

Two of the linkers (Δ18 and Δ22) were designed with the idea of removing all of the N-terminal end of the linker, and two (Δ14 and Δ22) were designed to eliminate all of the C-terminal end of the linker, even at the possible expense of encroaching on the N-terminus of the thioesterase I domain. Indeed, the Δ14 and Δ22 FASs, in which the deletions extend to Leu-2213, have significantly reduced thioesterase activity, as assessed using palmitoyl-CoA as a model substrate. Shortly after the initial submission of our paper, the crystal structure of the thioesterase domain of the human FAS was published (*12*). The first recognizable structural element is a short α-helix that corresponds to residues 2213−2215 in the rat FAS. Thus, it seemed possible that the thioesterase activity of the FAS may have been affected to some extent by direct modification of the catalytic domain, in addition to the modifications within the linker. To examine this possibility, we constructed a new free-standing, N-terminally truncated thioesterase (N-terminal sequence MAQASI) that mimicked the sequence of the thioesterase domain associated with the Δ14 and Δ22 FAS constructs (AQASI, Table 1). Activity of the free-standing thioesterase would not be influenced by the proximity of other FAS components, allowing direct assessment of the effect of N-terminal deletions on thioesterase activity. With palmitoyl-CoA as a model substrate, the catalytic activity of the purified N-terminally truncated thioesterase was 51% of that of a free-standing thioesterase that included further upstream sequence (N-terminal sequence MESKND). Thus, part of the loss in thioesterase activity observed in FASs carrying the most C-terminally truncated linkers (Δ14 and Δ22) indeed can be attributed to a loss in catalytic activity of the thioesterase.

The 10-residue deletion had very little effect on thioesterase mobility, and the greatest reduction in mobility was seen with the longest, 22-residue deletion. However, the 14- and 18-residue deletions caused only slightly less of a reduction in mobility than did the 22-residue deletion. Insertion of a sequence that effectively lengthened the linker by 13 residues increased mobility of the thioesterase domain markedly but had little effect on either FAS activity or the product chain length.

These experiments reveal that the FAS can tolerate substantial changes in length of the ACP−thioesterase linker without incurring serious effects on integrity of the complex, consistent with the observation that this linker is poorly conserved in FASs of different species. In this regard, the ACP−thioesterase linker resembles the interdomain linker of the dihydrolipoamide acetyltransferase component of the pyruvate dehydrogenase complex, which also can be shortened appreciably without significant effect on catalytic activity (*35*). Nevertheless, in general, longer linkers that result in increased mobility of the tethered thioesterase domain are better tolerated than are shorter linkers that restrict motion of the thioesterase, and product fidelity is best maintained with linker lengths of at least 10 residues. The tolerance exhibited toward longer linkers is consistent with the observation that some naturally occurring FASs (for example, that of *C. elegans*) and modular polyketide synthases (for example, the picromycin modular polyketide

synthase of *S. venezuelae*) have relatively long ACP−thioesterase linkers. Most surprisingly, removal of what we estimate is the entire interdomain linker (Δ22) does not completely compromise functionality of the FAS; this mutant retains 28% of the activity of the wild-type FAS, although it synthesizes mainly stearic, rather than palmitic, acid. This finding suggests that, in addition to the flexibility of the interdomain linker, mobility of the 4′-phosphopantetheine, the "swinging arm", may also contribute to the successful docking of the long-chain fatty acyl moiety into the active site of the thioesterase.

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SUPPORTING INFORMATION AVAILABLE

A detailed description of the procedures used to engineer the various FAS mutants. This material is available free of charge via the Internet at http://pubs.acs.org.

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