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Catalytic Residues Are Shared between Two Pseudosubunits of the Dehydratase Domain of the Animal Fatty Acid Synthase

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SUMMARY

Expression, characterization, and mutagenesis of a series of N-terminal fragments of an animal fatty acid synthase, containing the β-ketoacyl synthase, acyl transferase, and dehydratase domains, demonstrate that the dehydratase domain consists of two pseudosubunits, derived from contiguous regions of the same polypeptide, in which a single active site is formed by the cooperation of the catalytic histidine 878 residue of the first pseudosubunit with aspartate 1032 of the second pseudosubunit. Mutagenesis and modeling studies revealed an essential role for glutamine 1036 in anchoring the position of the catalytic aspartate. These findings establish that sequence elements previously assigned to a central structural core region of the type I fatty acid synthases and some modular polyketide synthase counterparts play an essential catalytic role as part of the dehydratase domain.

INTRODUCTION

In most prokaryotes, plastids, and mitochondria, the enzymes required for the de novo biosynthesis of fatty acids exist as discrete individual proteins (type II fatty acid synthases [FASs]), but, in the cytosol of animals, they are integrated into a single multifunctional polypeptide (type I FAS) containing ~2500 residues that functions as a homodimer. Recent biochemical [1, 2], electron microscopic [3], and crystallographic [4] studies have revealed that the two subunits of the animal FAS are coiled in a head-to-head orientation that has been characterized as a "body" consisting of the two dehydratase domains flanked by dimeric enoyl reductase and β-ketoacyl synthase domains, with protruding pairs of "arms and legs" consisting of monomeric β-ketoacyl reductase and malonyl/acetyl transferase domains, respectively. Neither electron microscopy nor X-ray crystallographic studies have been able to locate the position of the acyl carrier protein (ACP) and thioesterase domains, presumably because of the inherent mobility of these structures. Nevertheless, based on their location at the C terminus of the FAS polypeptide, it seems likely that they are positioned adjacent to the β-ketoacyl reductase domains, at the distal end of the two arms. The thioesterase [5, 6], malonyl/acetyl transferase [7], and ACP [8] domains of the animal FAS have been expressed as discrete functional proteins, and their precise location in the multifunctional polypeptide has been determined. However, none of the β-carbon-processing enzymes responsible for the stepwise reduction of the β-ketoacyl moiety produced after each condensation reaction has been expressed as a freestanding, catalytically active domain, and their precise location in the FAS polypeptide remains to be confirmed. In particular, the role of a ~600 residue "core" region near the center of the polypeptide remains in doubt, because it is unclear whether the region plays a structural or catalytic role.

The freestanding dehydratases associated with type II FASs are small, homodimeric proteins, and each subunit contains 150–170 residues. Previous domain-mapping exercises had estimated that the dehydratase domain of the animal FAS was likely of similar size and was located between residues 809 and 971 [9]. Although expression of residues 1–971 yielded a dimeric protein that contained catalytically active β-ketoacyl synthase and malonyl/acetyl transferase domains, this construct lacked dehydratase activity, suggesting that additional sequences beyond residue 971 might be required for activity [1]. This conclusion was supported by the assertion that two subunits of the type II dehydratase of *Escherichia coli* (FabA) could be fitted into a 4.5 Å resolution electron density map of the porcine FAS and suggested that the dehydratase might be twice as large as originally anticipated, thus accounting for at least part of the previously unassigned core region [4]. However, since the derived model relies on a fitted, rather than real, structure, and since the 4.5 Å resolution electron density map has not yet been published, rigorous testing of the hypothesis with biochemical experiments is in order. With this goal in mind, we engineered and characterized a series of constructs, and mutants thereof, encompassing up to 1168 residues from the N terminus of the rat FAS to identify residues in the megasynthase that are required for dehydratase activity.

RESULTS

Since a construct representing residues 1–971 of the rat FAS exhibited β-ketoacyl synthase and malonyl/acetyl

transferase activity, but lacked dehydratase activity [1], we designed and engineered three constructs, based primarily on analysis of a secondary structure prediction by using the Phyre Server at Imperial College London (Figure S1, see the Supplemental Data available with this article online). The C termini were chosen so as to avoid disruption of putative secondary structural elements. Constructs encompassing residues 1–1017, 1–1154, and 1–1168 of the FAS were expressed by using the *Sf*9/baculoviral host/vector system, and the proteins were purified to homogeneity. The 1017 residue construct consisted of monomers, dimers, and higher-molecular mass oligomers, as had the original 971 residue construct. However, the two longer constructs consisted exclusively of dimers and monomers (Figure 1). All three constructs exhibited similar β-ketoacyl synthase and malonyl/acetyl transferase activities, but only the constructs containing at least the first 1154 residues of the FAS exhibited dehydratase activity (Table 1). A longer construct encompassing the first 1168 residues exhibited similar catalytic properties to the 1154 residue construct (Table 1). Activities of the malonyl/acetyl transferase and dehydratase are independent of oligomeric status, but that of the β-ketoacyl synthase is restricted to the dimeric form of FAS [9]. Thus, the similar β-ketoacyl synthase activities observed for the constructs of different lengths are consistent with the comparable dimer content of the various preparations.

These results confirmed that the dehydratase catalytic region extended considerably farther than had been initially anticipated and revealed that elements between residues 1017 and 1154 were required for activity. The observation by Maier et al. [4] that the overall double "hot-dog" fold of the putative dehydratase region appeared to closely resemble that of the prokaryotic, homodimeric dehydratases FabA and FabZ suggested that the type I and II dehydratases likely shared similar structural and catalytic features. In the homodimeric type II dehydratases, two active centers are formed at the subunit interface, and each one involves the cooperation of a histidine and an acidic residue from opposite subunits [10, 11]. An essential catalytic histidine residue was previously identified by mutational analysis as residue 878 in the rat FAS. Replacement of this residue with alanine in both subunits specifically compromises dehydratase activity, and the homodimeric mutant FAS is incompetent in fatty acid synthesis [12]. Thus, if the type I dehydratase domain consists of two contiguous "pseudosubunits," resembling the type II dimeric dehydratases, since the pseudosubunits are not identical in sequence, it seemed likely that a single active site must be located at the interface of the two pseudosubunits, and that the second catalytic residue, aspartate or glutamate, would be located in the second of the two pseudosubunits. Sequence alignment of the putative dehydratase regions of 17 animal FASs confirmed the positional conservation of the catalytic histidine residue in the first half of the region (Figure S2) and revealed two positionally conserved acidic residues in the second half of the region between residues 1017 and 1154: Asp1032 and Glu1116 in the rat FAS. In type II dehydratases, the catalytic acidic residue is usually followed by a glutamine residue at position +4 (Figure S1). Since, in 15/17 animal FAS sequences the residue at position +4 following the positionally conserved aspartate is a glutamine (chicken and porcine FASs have a histidine at this position), but in all 17 sequences the residue following the positionally conserved glutamate is phenylalanine, we considered Asp1032 to be the better candidate for a catalytic role, and we mutated this residue, as well as Gln1036, in the context of the tridomain construct consisting of residues 1–1168.

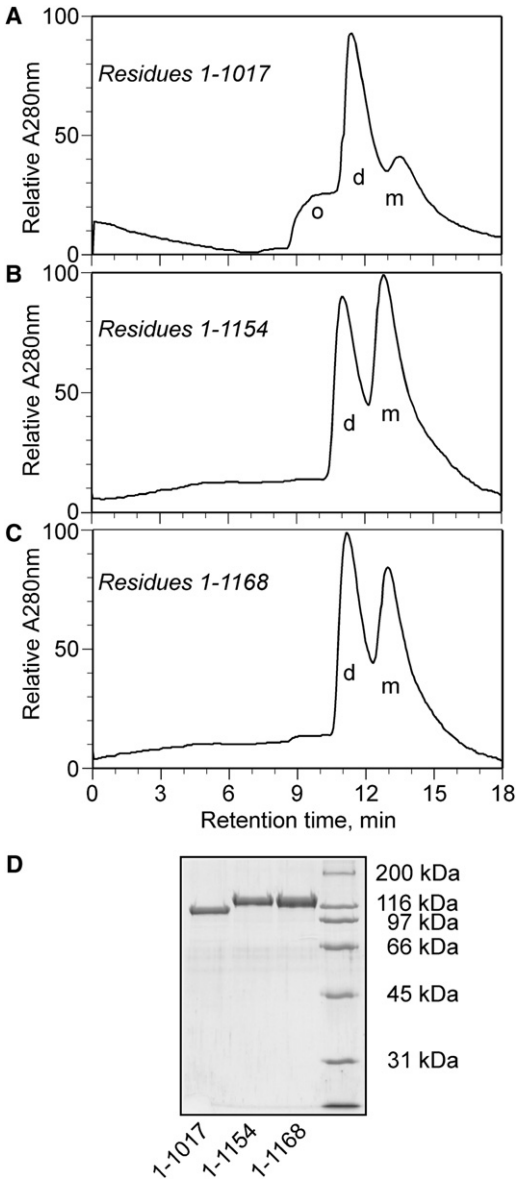

Figure 1. Molecular Masses and Oligomeric Status of FAS Tridomain Constructs

(A–D) Purified proteins representing residues (A) 1–1017, (B) 1–1154, and (C) 1–1168 were analyzed by gel filtration and (D) SDS-PAGE. M, monomers; d, dimers, o; higher oligomers.

Table 1. Catalytic Activities of Tridomain Constructs

Constructs	KS (mol Product Formed. mol Enzyme^{-1}. min^{-1})	MAT (mol Product Formed. mol Enzyme^{-1}. min^{-1})	DH (mol Product Formed. mol Enzyme^{-1}. min^{-1})
1–971[a]	45.7 ± 0.9	285 ± 8	0
1–1017	52.8 ± 0.7	561 ± 40	0
1–1154	49.3 ± 2.1	564 ± 30	163 ± 28
1–1168	54.8 ± 0.5	539 ± 42	204 ± 25
1–1168 (D1032E)	48.2 ± 0.3	566 ± 19	0
1–1168 (D1032A)	44.4 ± 0.9	514 ± 16	0
1–1168 (Q1036H)	50.3 ± 1.5	527 ± 23	187 ± 5
1–1168 (Q1036A)	51.7 ± 0.4	428 ± 19	11 ± 2
1–1168 (Q1036T)	55.3 ± 1.8	479 ± 17	3 ± 0.3

Catalytic activity of the dehydratase was assayed by measuring the reverse hydration reaction, because both the equilibrium and rate constant of the reaction greatly favor formation of the β-hydroxy rather than enoyl moieties [15]; thus, *S*-2-octenoyl pantetheine (275 μM) was the substrate. Values represent means of at least two assays ± SD.
[a] Taken from [1].

Initial characterization of the mutants, by using *S*-2-octenoyl pantetheine as the substrate, revealed that replacement of Asp1032 with either glutamine or alanine completely abolished dehydratase activity without affecting the activity of either of the two other catalytic domains (Table 1). Similarly, replacement of Gln1036 with either alanine or threonine drastically reduced dehydratase activity, but had no effect on β-ketoacyl synthase or malonyl/acetyl transferase activity (Table 1). In contrast, dehydratase activity was preserved when Gln1036 was replaced with histidine, the residue found at this position in two natural FAS sequences (Table 1). Several mutants that exhibited compromised dehydratase activity (Asp1032Ala, Gln1036Ala, and Gln1036Thr) were also found to contain significant amounts of higher-molecular mass oligomers; however, the dimeric species isolated from these preparations also exhibited compromised dehydratase activity (details not shown).

A detailed kinetic study of the dehydratase-catalyzed reaction was performed by using the tridomain construct consisting of residues 1–1168 with both 2-enoyl pantetheine and 2-enoyl-ACP substrates. The ACP-linked 2-octenoyl and 2-hexenoyl thioesters were clearly better substrates than their pantetheine counterparts, and the dehydratase exhibited a chain-length preference of 8- > 6- > 4-carbon atoms (Figure 2A).

Estimation of kinetic parameters for the dehydratase reaction was hampered by the inherently high absorbance of the substrate at 270 nm, the wavelength used to monitor hydration of the enoyl moiety. This was particularly true for all of the pantetheine-linked substrates and crotonyl-ACP, all of which appear to have K_M values in the millimolar range. Nevertheless, reliable estimates of K_M and V_{max} were possible with the ACP-linked 2-octenoyl and 2-hexenoyl thioesters, and these substrates were also used for analysis of the Gln1036His mutant of the tridomain construct encompassing residues 1–1168 (Figure 2B). For comparison, a full-length FAS construct that contained mutations in the thioesterase, malonyl/acetyl transferase, and ACP domains was also included (Figure 2C). These mutations prevented any possible hydrolysis of the 2-enoyl-ACP thioesters or translocation of the 2-enoyl moieties from the freestanding ACP to the FAS. Thus, activity observed with this full-length FAS could result only from a direct interaction between the dehydratase domain and the 2-enoyl moiety linked to the freestanding ACP (Figures 2B and 2C). Although, again, the high absorbance of the enoyl moieties limited the substrate concentrations that could be used in the assays, almost 50% saturation could be attained in most cases. Activity of the tridomain construct containing the wild-type sequence was similar to that of the full-length FAS for substrates of all chain lengths, but activity of the tridomain Gln1036His mutant was consistently lower (Figure 2; Table 2). With all three proteins, the substrate preference was C8 > C6 > C4.

DISCUSSION

In prokaryotes, two dehydratase isoforms, FabA and FabZ, catalyze the dehydration of 3-hydroxyacyl-ACP to *trans*-2-enoyl-ACP; the FabA isoform also carries out the isomerization of *trans*-2- to *cis*-3-decenoyl-ACP, an essential step in the biosynthesis of unsaturated fatty acids [13]. The subunits of both enzymes adopt a mixed α+β "hot-dog" fold, in which a multistranded, antiparallel β sheet, the "bun," envelopes an α-helical "sausage" [10, 11]. These enzymes are homodimeric and have two active centers positioned at the subunit interface, and each center is formed by the juxtapositioning of a histidine from one subunit with an acidic residue from the other subunit. Thus, the monomeric forms of the enzymes are inactive [14]. The new crystallographic [4] and biochemical (Figure 2; Tables 1 and 2) evidence reveals that the dehydratase domains of the animal type I FASs consist of two nonidentical pseudosubunits, or subdomains, that are formed

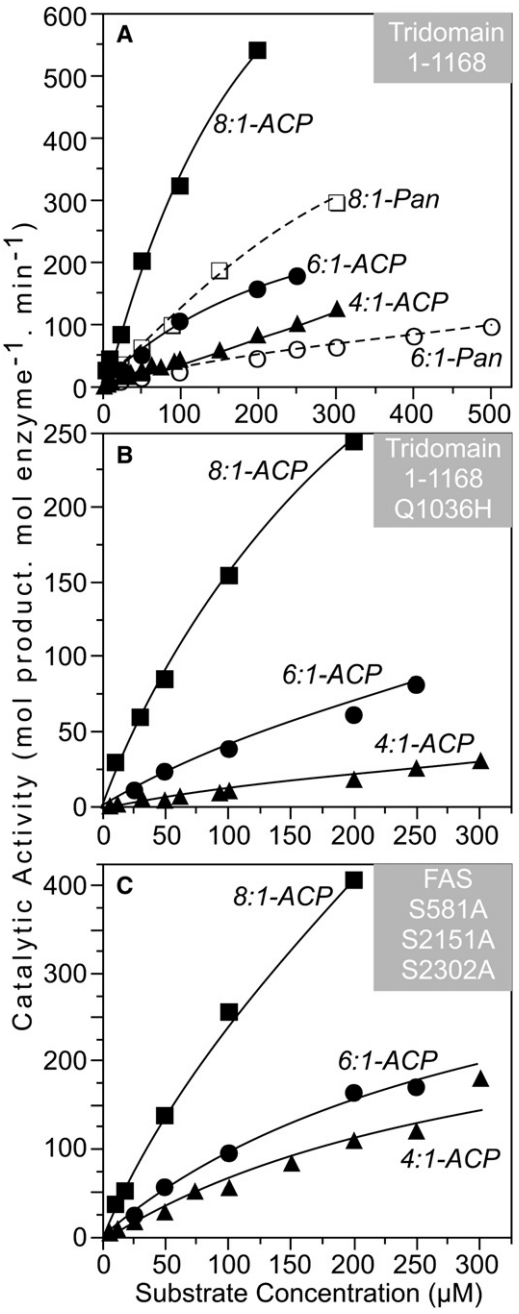

Figure 2. Comparison of Dehydratase Activities of the WT Tridomain, the Q1036H Tridomain, and Full-Length FAS

(A–C) The enzyme preparations used were: the (A) tridomain construct consisting of residues 1–1168; the (B) Gln1036His mutant of the 1–1168 construct; and a (C) full-length FAS construct. The full-length FAS construct contained knockout mutations in the malonyl/acetyl transferase (Ser581Ala), ACP (Ser2151Ala), and thioesterase (Ser2302Ala) domains that blocked any possible hydrolysis of the 2-enoyl-ACP thioesters or transfer of the 2-enoyl moieties from the freestanding ACP to the FAS. Substrates are identified by the number of carbon atoms and double bonds in the fatty acyl moiety and the type of thioester, "Pan" (pantetheine) or ACP.

by contiguous regions of the same polypeptide. The active-site histidine, His878, is located on the first pseudosubunit, and the acidic residue, Asp1032, is located on the second (Figure 3). Thus, the pseudodimeric dehydratases contain only a single catalytic center. Replacement of either the single active-site histidine [12] or the single active-site acidic residue (Table 1) eliminates catalytic activity. Since the tridomain constructs containing FAS residues 1–1154 and 1–1168 retain full dehydratase activity in their monomeric form, cooperation between the two subdomains must occur intra-, and not inter-, FAS subunit. These findings now provide a solid foundation for revision of the domain map for the animal FAS by assigning to the dehydratase domain a portion of the region formerly referred to as the central core (Figure 3). This reassignment almost certainly also applies to those modular polyketide synthases that contain dehydratase domains.

Previous experiments [1] and those reported here reveal that, although constructs containing incomplete dehydratase domains (1–971 and 1–1017) will form dimers and monomers, they also tend to form significant amounts of higher oligomers. Only those constructs containing both dehydratase pseudosubunits (1–1154 and 1–1168) fail to form the higher-oligomeric structures (Figure 1). These data suggest that the two pseudosubunits of the dehydratase domain may contribute in part to the proper dimerization of the FAS subunits. This inference is consistent with the interpretation by Nenad Ban and colleagues [4], based on analysis of the 4.5 Å electron density map of the porcine FAS, that substantial intersubunit contacts occur in the putative dehydratase region.

The role of the adjacent, unassigned region of the central core remains in doubt. The results of mutagenesis [15] and modeling [16] experiments suggest that this region may be required for proper functioning of the β-ketoacyl reductase domain, even though the two sequence elements are not contiguous and are split by the intervening enoylreductase domain. Further experimentation is required to test this hypothesis.

In the crystal structures of the *E. coli* FabA and *Pseudomonas aeruginosa* FabZ, the active-site histidine is hydrogen bonded through Nδ to a backbone carbonyl oxygen, and a catalytic water molecule is held in place by hydrogen bonds to a backbone amide at the N terminus of the central helix and to an acidic residue, Asp84 in FabA and Glu63 in FabZ [10, 11]. The catalytic acidic residue is anchored in position by hydrogen bonds to both the side chain and backbone of a glutamine residue, four residues distant on the helix (Gln88 in FabA; Gln67 in FabZ). In the proposed reaction mechanism for the type II dehydratases [10, 11], the active-site histidine residue abstracts a proton from the substrate, C2, while the acidic residue acts as a proton donor to the hydroxyl leaving from C3. Thus, the water molecule located in the active site likely represents the product of the dehydration reaction. The retention of a similar arrangement of catalytic residues at the active center of the type I animal dehydratases indicates that these enzymes likely employ the same catalytic mechanism (Figure 4E). In the naturally occurring type II

Table 2. Kinetic Properties of Dehydratase with Freestanding ACP-Linked Substrates

	8:1			6:1		
Construct	K_M (μM)	k_{cat} (min^{-1})	k_{cat}/K_M	K_M (μM)	k_{cat} (min^{-1})	k_{cat}/K_M
1–1168	332 ± 19	1430 ± 54	4.3	308 ± 22	394 ± 17	1.3
1–1168 Q1036H	290 ± 3	599 ± 4	2.0	631 ± 21	275 ± 5	0.4
Full-length FAS	330 ± 7	1080 ± 14	3.2	306 ± 8	394 ± 4	1.3

Kinetic parameters, derived from the data shown in Figure 2, represent averaged values from Lineweaver-Burk and nonlinear regression plots ± SD (Trinity Software, Inc.). The full-length FAS contained knockout mutations in the MAT (Ser581Ala), ACP (Ser2151Ala), and thioesterase (Ser2302Ala) domains that blocked any possible hydrolysis of the 2-enoyl-ACP thioesters or transfer of the 2-enoyl moieties from the freestanding ACP to the FAS.

dehydratases that utilize either a glutamate or aspartate in the active site, these residues can be swapped (aspartate for glutamate in FabA and glutamate for aspartate in FabZ), with retention of activity [11]. Nevertheless, although the codons for glutamate and aspartate can be switched by a single nucleotide change, an aspartate is universally conserved as the catalytic residue in the dehydratase domains of all known type I FASs (Figure S2), and replacement with glutamate results in loss of activity (Table 1). In contrast, whereas the residue responsible for anchoring the acidic residue appears to universally be a glutamine in the type II dehydratases, 2 of the 17 type I dehydratases that have been sequenced apparently employ a histidine residue in this role, and the others employ a glutamine (Figure S2). Indeed, replacement of the glutamine with histidine in the dehydratase domain of the rat FAS preserves catalytic activity, indicating that the imidazole side chain is also able to form a hydrogen bond with, and thus anchor, the backbone carbonyl of the active-site aspartate (Table 1). The adoption of these alternative catalytic residues during evolution presumably has been facilitated by the similarity in the codons for glutamine and histidine, which can be switched by a single nucleotide change. Although the dehydratases associated with the type I animal FASs have low sequence similarity with their type II counterparts (Figure S1), the electron density distribution within the domain [4] as well as modeling studies (Figure 4; Figure S1) indicate that they exhibit remarkably similar overall folds. Thus, the modeled structure of the rat FAS dehydratase domain (Figure 4C) resembles the crystal structure of the type II FabZ dimer (Figure 4A), with two long helices, each nestled in a multistranded, antiparallel β sheet. However, in the type I dehydratase, the two pseudosubunits are nonidentical and form only a single catalytic center by juxtaposition of the active-site histidine (His878) of the first pseudosubunit with the catalytic aspartate (Asp1032) and glutamine (Gln1036) of the second pseudosubunit (Figure 4).

The overall similarity of type II and animal type I dehydratases suggests that, in the gene fusion events that led to the formation of multifunctional polypeptides, two copies of the freestanding dehydratases were recruited into a single polypeptide chain, preserving their double hot-dog character. However, in the context of the megasynthase complex, it is possible that only one of the two active sites formed by a pseudodimeric dehydratase was capable of accessing a reaction chamber for fatty acid production, and the superfluous active site was subsequently lost through mutation.

The fungal FASs represent a remarkably different architectural option for the design of a type I megasynthase [17]. These FASs are 2.6 MDa, barrel-shaped dodecamers constructed from two different polypeptides. Recently solved structures of two fungal FASs reveal that the dehydratase domains of these $\alpha_6\beta_6$ complexes are also composed of two hot-dog-fold subdomains located proximally on the β subunit [18, 19] (Figure 4B). However, in this enzyme, a third subdomain, also resembling a truncated hot-dog fold, is inserted between the catalytic double hot-dog structure and appears to stabilize interactions

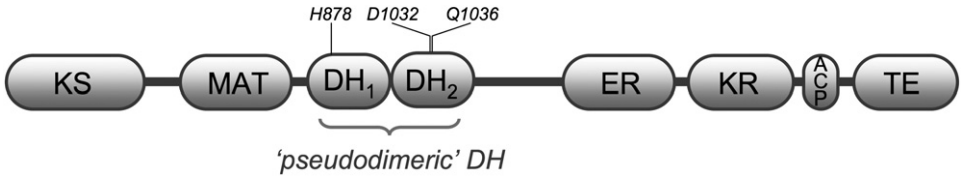

Figure 3. Revised Domain Map for the Animal FAS

The pseudodimeric dehydratase domain spans approximately residues 824–1154 of the rat FAS. The active site is formed by the juxtaposition of conserved histidine and aspartate residues from different pseudosubunits. The positionally conserved glutamine at residue 1036 is replaced by a histidine residue in some FASs. The role of the region between the second dehydratase pseudosubunit and the enoyl reductase domain has yet to be assigned; integrity of this region appears to be important for the functioning of the β-ketoacyl reductase [15], and it has been suggested that, in both FASs and the structurally related modular polyketide synthases, it may constitute a second subdomain of the β-ketoacyl reductase [16]. Estimated locations for other domains are: β-ketoacyl synthase, 1–408; malonyl/acetyltransferase, 488–809; enoylreductase, 1527–1850; β-ketoacyl reductase, 1880–2105; ACP, 2114–2202; and thioesterase, 2210–end.

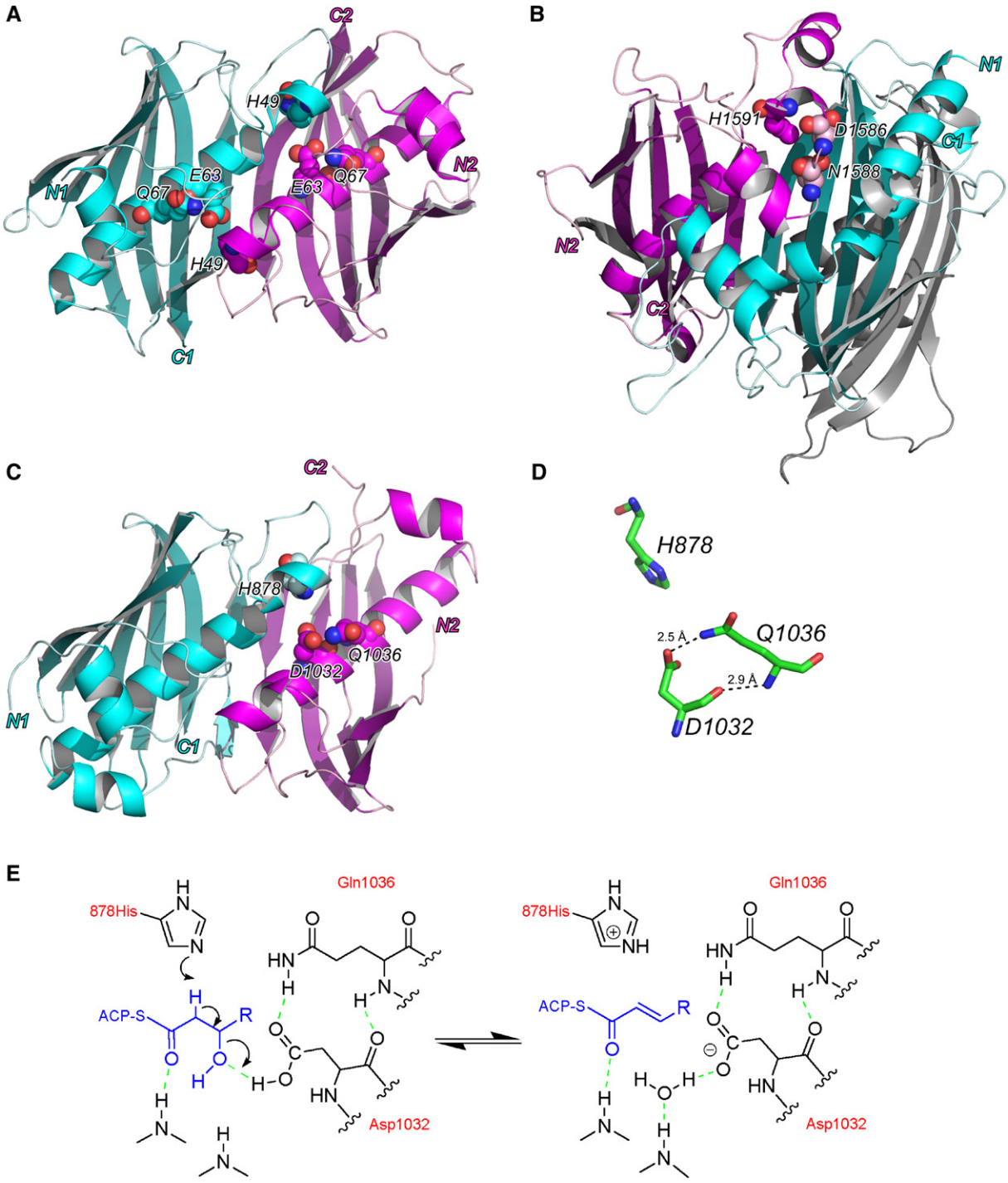

Figure 4. Structures of Type I and II Dehydratases

(A) Crystal structure of type II dehydratase, FabZ (PDB code: 1U1Z), from *Pseudomonas aeruginosa* [11].

(B) Crystal structure of the dehydratase domain of the type I *Thermomyces lanuginosus* FAS (PDB code: 2UVA) [18]; the third hot dog domain, inserted between the two pseudosubunits, that stabilizes interactions between the catalytic core and the wall of the FAS barrel is shown in gray.

(C) Model of the dehydratase domain of the type I rat FAS, derived in silico by using homology modeling. In all three cartoons, the two subunits, or pseudosubunits, are distinguished by cyan (first) and magenta (second) coloring, and their N and C termini are labeled N1, C1 and N2, C2, respectively. Catalytic residues are labeled and shown as spheres. Carbon atoms are colored according to the ribbon color: oxygen atoms are orange, and nitrogen atoms are blue.

between the dehydratase and the rest of the FAS. The structures of the fungal dehydratases resemble more closely those of the eukaryotic 2-enoyl-CoA hydratases involved in fatty acid oxidation, rather than those of the prokaryotic FabZ and FabA dehydratases involved in fatty acid synthesis. These 2-enoyl-CoA hydratases are characterized by the presence of two hot-dog subdomains and a single active center. Based on this similarity, it appears that the dehydratases associated with the fungal type I FASs also contain only one catalytic site in the pseudodimer; but, in contrast to the prokaryotic type II dehydratase and the dehydratase associated with the animal type I FASs, all three catalytic residues (His1591, Asp1586, and Asn1588 in *Thermomyces lanuginosus*) appear to reside on the same pseudosubunit.

Kinetic analyses revealed that the dehydratase activity is dependent on substrate chain length (C4 < C6 < C8). A similar preference was previously noted for the β-ketoacyl synthase [20] and is consistent with the observation that the early condensation and β-carbon-processing steps are slower than the analogous reactions with longer chain-length intermediates [21].

The type II prokaryotic dehydratases exhibit a marked preference for ACP-linked over N-acetylcysteamine thioester substrates, and K_M values are 100-fold lower and turnover rates six times greater with the ACP than with N-acetylcysteamine thioesters [22]. The necessity for specific protein-protein interactions to facilitate efficient docking of the ACP-linked substrates with the catalytic components is self evident in the case of type II systems. In a type I FAS system, however, the presence of the ACP at a high concentration in the vicinity of the various catalytic sites is assured by virtue of its covalent linkage to the megasynthase. The tridomain constructs provided a unique opportunity to assess the contribution of the ACP protein moiety to substrate binding in a type I FAS by comparison of the dehydratase activities observed with 2-enoyl thioester moieties linked to pantetheine and freestanding ACP. Clearly, freestanding ACP-linked 2-enoyl thioesters are better substrates for the dehydratase-catalyzed reaction than are the analogous pantetheine-linked substrates, indicating that the protein portion of the 2-enoyl-ACP thioesters contributes significantly to substrate recognition by the dehydratase. This view is supported by structural studies with other type I megasynthase systems that implicate specific regions of the ACP protein domains in the interaction with the β-ketoacyl synthase of fungal FASs [18, 19] and with cognate partners in modular polyketide synthases [23].

Interestingly, the kinetic parameters derived with freestanding 2-enoyl-ACP substrates were essentially the same for the tridomain construct (residues 1–1168) and the full-length FAS, which retained the endogenous, but inactivated, ACP domains (Table 2). Thus, the presence of a resident ACP domain does not appear to impede access to the dehydratase domain by a surrogate, freestanding 2-enoyl-ACP. Intact, functional animal FASs are also accessible to freestanding type II thioesterases that can gain access to the ACP and release intermediate chain-length acyl moieties as novel fatty acid products [5]. The open, flexible architectural design of the animal FAS that permits access by exogenous proteins contrasts markedly with the closed-chamber design of the fungal FASs that allows transit only of the small-molecular mass substrates and products.

SIGNIFICANCE

Domain maps of the animal fatty acid synthases (FASs) and the structurally related modular polyketide synthases have traditionally assigned ~170 residues to the dehydratase domain, approximately the same size as the subunits of their freestanding type II counterparts. However, the discovery that two subunits of a type II dehydratase could be fit into a 4.5 Å resolution electron density map of an animal FAS raised the possibility that these dehydratases consist of two nonidentical subdomains, or pseudosubunits, derived from adjacent regions of the same polypeptide [4]. The new study confirms this hypothesis by establishing that a region of ~340 residues is required for dehydratase activity and reveals that residues from both subdomains, a histidine and aspartate, cooperate to form a single active site at the subdomain interface. Mutagenesis and structural modeling studies indicate that the type I dehydratases associated with animal FAS exhibit a similar double hot-dog fold to the homodimeric type II dehydratases and utilize essentially the same catalytic mechanism. These findings provide compelling evidence that part of the region of the animal FASs and the modular polyketide synthases, previously characterized as the "central core," now can be assigned a specific role in catalysis of the dehydratase reaction.

EXPERIMENTAL PROCEDURES

Engineering of Tridomain Constructs

Rat FAS DNA was amplified by PCR and was cloned into acceptor cDNA by following standard protocols. Details of the primers and templates employed are available in Tables S1 and S2.

Expression and Purification of Tridomain Proteins

The final cDNA constructs, in the context of the pFASTBAC 1 vector, were used to generate recombinant baculovirus stocks by the transposition method employing the BAC-to-BAC baculovirus expression system (Invitrogen). P2 or older viral stocks were used to infect insect cells. Fluorescence flow cytometry was adopted to measure the titer of viral stocks [24], Insect *Sf*9 cells cultured in Ex-Cell 420 insect serum-free medium (SAFC Biosciences, Lenexa, KS, USA) supplemented

(D) Active-site region of the rat dehydratase domain showing both side chain and backbone hydrogen-bonding between aspartate and glutamine residues. Similar hydrogen bonding occurs in the *P. aeruginosa* dehydratase between the catalytic glutamate and glutamine residues. The figure was drawn with MacPyMol.
(E) Mechanism for the dehydratase reaction.

with 2% fetal bovine serum were transfected with the recombinant virus at optimal MOI and cultured at 27°C for 66–72 hr, if not indicated otherwise. Cells were homogenized at 4°C, by using a Dounce homogenizer (Wheaton, 25 strokes with a tight-fitting "pestle B") in 4 volumes/g of 50 mM potassium phosphate, 250 mM sucrose, 5 mM 2-mercaptoethanol (pH 7.0), containing a mixture of protease inhibitors [1]. The tagged proteins were purified from the cytosol initially by Ni-NTA affinity chromatography in 0.2 M potassium phosphate (pH 7), 10% glycerol, and 2 mM 2-mercaptoethanol. Proteins eluted with 250 mM imidazole were dialyzed against 50 mM potassium phosphate (pH 7), 1 mM EDTA, 1 mM dithiothreitol, and 10% glycerol, then applied to a HiTrap Q column (GE Healthcare). The column was developed with a 30 min gradient of 50–250 mM potassium phosphate (pH 7) containing 1 mM EDTA, 1 mM dithiothreitol, and 10% glycerol. Fractions containing the tridomain dimer were concentrated; dialyzed against 250 mM potassium phosphate (pH 7), 1 mM EDTA, 1 mM dithiothreitol, and 10% glycerol; flash frozen; and stored at −80°C.

Preparation of 2-Enoyl-ACPs

The plasmid encoding the ACP fragment of the rat cytosolic FAS (residues 2114–2202) was obtained from Dr. Matthew Crump, and the protein was expressed and purified in the apo-form, essentially as described earlier [25]. Purified apo-ACP was incubated with 2.5- to 5-fold molar excess of acyl-2-en-CoA in the presence of purified human phosphopantetheinyl transferase [26] at 37°C in 20 mM Bis-Tris (pH 6.8) containing 150 mM NaCl, 10% glycerol, and 12 mM $MgCl_2$. The progress of the reaction was monitored by MALDI mass spectrometry, and incubation was continued until all apo-ACP was converted to acyl-2-en-ACP. Unreacted acyl-2-en-CoA was removed by anion-exchange chromatography. The concentration of acyl-2-en-ACP was determined from the absorbance at 263 nm (enoyl content) and by back titration with 5,5′-dithio-bis(2-nitrobenzoic acid) of the free ACP thiol released by mild alkaline hydrolysis (thioester content).

Preparation of 2-Hexenoyl- and 2-Octenoyl-CoA

Acyl-2-en-CoA thioesters were synthesized from the corresponding free acids, 99% *trans*-2-hexenoic acid (Aldrich), and 85% *trans*-2-octenoic acid (Aldrich), by using the mixed anhydride method, as described previously [20, 27]. Thioesters were purified on a reverse-phase C18 SpheriSorb, 4.6 × 250, 5 μ, 300 Å (SS OD52, Waters) HPLC column developed in 20 mM ammonium acetate (pH 5.5), buffer A, and acetonitrile, buffer B, gradients: (a) the gradient for 2-hexenoyl-CoA was 100%–90% A over 25 min, to 75% A over 15 min, to 10% A over 5 min, and 5 min at 10% A (retention time was 35.5 min); (b) the gradient for 2-octenoyl-CoA was 90% A over 5 min, to 55% A over 35 min, to 10% A over 5 min, and 10 min at 10% A (retention time was 21 min). The molecular masses of the synthesized CoA thioesters were confirmed by MALDI-TOF mass spectrometry.

Preparation of *S*-2-Hexenoyl- and *S*-2-Octenoyl-Pantetheine

S-acyl-2-en pantetheinyl thioesters were synthesized from mixed anhydrides as described previously [20, 27]. Thioesters were purified on a reverse-phase C18 SpheriSorb HPLC column (4.6 × 250, 5 μ, 300 Å, SS OD52, Waters) developed by using a gradient formed from 2 mM TFA in water (solvent A) and 2 mM TFA in acetonitrile (solvent B). *S*-2-hexenoyl-pantetheine: 90% A for 5 min, to 60% A over 30 min, to 10% A over 5 min, and 5 min at 10% A; retention time was 31 min. *S*-2-octenoyl-pantetheine: 85% A for 5 min, to 55% A over 30 min, to 10% A over 5 min, and 5 min at 10% A; retention time was 34 min. The molecular masses of the synthesized thioesters were confirmed by MALDI-TOF mass spectrometry.

Assay of Dehydratase Activity

Dehydratase activity was measured in a hydration assay, by using enoyl-*S*-pantetheine or enoyl-ACP as substrates, as the reaction equilibrium is more favorable in the reverse direction [15]. Reactions were monitored spectrophotometrically at 270 nm, in 0.1 M potassium phosphate buffer (pH 7.0) at 37°C for 1 min.

Assay of Acetyl Transferase Activity

Transferase activity was monitored by using [1-^{14}C]acetyl-CoA as the acyl donor and pantetheine as a model acceptor. Reactions mixtures (20 μl) containing 50 mM potassium phosphate buffer (pH 7.0), 100 μM substrate, 3 mM acceptor, and 5 ng tridomain constructs were incubated for 5 min at 0°C, and the reactions were stopped by the addition of 5 μl 2% perchloric acid. Quenched reaction mixtures were left at 0°C for 15 min, neutralized with 1.5 M KOH and 0.5 M MES (to pH 5–6), kept at 0°C for an additional 10 min, and then centrifuged at 14,000 rpm for 5 min at 4°C. Portions (4 μl) of the supernatants were spotted onto a cellulose TLC sheet and air dried. TLC sheets were developed in butanol:acetic acid: water (5:2:3), air dried, exposed to a phosphor screen for 16–20 hr, and analyzed with a phosphoimage analyzer. Activities were determined by comparing spot volumes corresponding to the [1-^{14}C]acetyl-pantetheine product against [1-^{14}C]acetyl-CoA standards of known radioactive content.

Assay of β-Ketoacyl Synthase Activity

The acyl transferase activity of the β-ketoacyl synthase domain that is responsible for the translocation of saturated acyl moieties from the phosphopantetheine thiol to the active-site Cys161 was assessed with model substrates, by monitoring the shuttling of decanoyl moieties from pantetheine and CoA thiols [20].

Gel Filtration Studies

The oligomeric status of tridomain constructs was ascertained by gel filtration on a TSK-Gel G3000SWXL column (TOSOHAAS Bioseparation Specialists, PA, USA), operated at room temperature, in 0.2 M potassium phosphate buffer (pH 7.0) containing 10% glycerol and 1 mM dithiothreitol; the flow rate was 0.2 ml/min.

Modeling of the Dehydratase Domain

According to Maier et al. [4], two subunits of a type II dehydratase (FabA) can be fitted into the putative dehydratase region of a 4.5 Å resolution electron density map of the porcine FAS. We used the crystal structure of the FabZ dehydratase of *Helicobacter pylori* (2GLL) as the template for model building, since this enzyme, in common with the FAS, lacks isomerase activity. The process was performed separately for N-terminal and C-terminal subdomains. Low sequence homology between the dehydratase domain of rat FAS and the type II counterparts, and the presence of a linker region between the two subdomains of the type I enzyme, necessitated manual alignment and gapping of the primary sequences. The sequences were aligned based on the location of active-site residues, and a single-residue gap was introduced into the FabZ sequence to align the FPG motif preceding helix 3 (Figure S1). The intersubdomain linker was assumed to follow the region corresponding to the final FabZ structural element in the first subunit. This region is proline rich and is followed by a sequence strongly predicted by Phyre (Imperial College, London) to be an amphipathic helix that is not found in the template. In all, 38 residues from this putative linker region were excluded from the model construction (Figure S1). The alignments were submitted to Swiss Model in the alignment mode [28, 29]. The returned models, with a final total energy of −2232 kJ/mol for 147 amino acids of the N-terminal part and −1734 kJ/mol for 121 amino acids of the C-terminal part, were directly fitted into the FabZ dimer by using Spdb-Viewer. A Ramachandran plot for the final model localized 80.0% in the most favored regions and 0.4% (1 residue) in the disallowed regions. Attempts to refine the modeling process by using secondary structure predictions (Phyre) to optimize the sequence alignment resulted in a lower quality model.

Supplemental Data

Supplemental Data include two figures and two tables and are available online at http://www.chembiol.com/cgi/content/full/14/12/1377/DC1/.

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REFERENCES

1. Witkowski, A., Ghosal, A., Joshi, A.K., Witkowska, H.E., Asturias, F.J., and Smith, S. (2004). Head-to-head coiled arrangement of the subunits of the animal fatty acid synthase. Chem. Biol. *11*, 1667–1676.
2. Joshi, A.K., Rangan, V.S., Witkowski, A., and Smith, S. (2003). Engineering of an active animal fatty acid synthase dimer with only one competent subunit. Chem. Biol. *10*, 169–173.
3. Asturias, F.J., Chadick, J.Z., Cheung, I.K., Stark, H., Witkowski, A., Joshi, A.K., and Smith, S. (2005). Structure and molecular organization of mammalian fatty acid synthase. Nat. Struct. Mol. Biol. *12*, 225–232.
4. Maier, T., Jenni, S., and Ban, N. (2006). Architecture of mammalian fatty acid synthase at 4.5 Å resolution. Science *311*, 1258–1262.
5. Naggert, J., Witkowski, A., Wessa, B., and Smith, S. (1991). Expression in *Escherichia coli*, purification and characterization of two mammalian thioesterases involved in fatty acid synthesis. Biochem. J. *273*, 787–790.
6. Pazirandeh, M., Chirala, S.S., and Wakil, S.J. (1991). Site-directed mutagenesis studies on the recombinant thioesterase domain of chicken fatty acid synthase expressed in *Escherichia coli*. J. Biol. Chem. *266*, 20946–20952.
7. Rangan, V.S., Serre, L., Witkowska, H.E., Bari, A., and Smith, S. (1997). Characterization of the malonyl-/acetyltransacylase domain of the multifunctional animal fatty acid synthase by expression in *Escherichia coli* and refolding *in vitro*. Protein Eng. *10*, 561–566.
8. Tropf, S., Revill, W.P., Bibb, M.J., Hopwood, D.A., and Schweizer, M. (1998). Heterologously expressed acyl carrier protein domain of rat fatty acid synthase functions in *Escherichia coli* fatty acid synthase and *Streptomyces coelicolor* polyketide synthase systems. Chem. Biol. *5*, 135–146.
9. Smith, S., Witkowski, A., and Joshi, A.K. (2003). Structural and functional organization of the animal fatty acid synthase. Prog. Lipid Res. *42*, 289–317.
10. Leesong, M., Henderson, B.S., Gillig, J.R., Schwab, J.M., and Smith, J.L. (1996). Structure of a dehydratase-isomerase from the bacterial pathway for biosynthesis of unsaturated fatty acids: two catalytic activities in one active site. Structure *4*, 253–264.
11. Kimber, M.S., Martin, F., Lu, Y., Houston, S., Vedadi, M., Dharamsi, A., Fiebig, K.M., Schmid, M., and Rock, C.O. (2004). The structure of (3R)-hydroxyacyl-acyl carrier protein dehydratase (FabZ) from *Pseudomonas aeruginosa*. J. Biol. Chem. *279*, 52593–52602.
12. Joshi, A.K., and Smith, S. (1993). Construction, expression and characterization of a mutated animal fatty acid synthase deficient in the dehydrase function. J. Biol. Chem. *268*, 22508–22513.
13. Heath, R.J., and Rock, C.O. (1996). Roles of the FabA and FabZ β-hydroxyacyl-acyl carrier protein dehydratases in *Escherichia coli* fatty acid biosynthesis. J. Biol. Chem. *271*, 27795–27801.
14. Stein, J.P., Jr. (1976). Studies on the active site of β-hydroxydecanoyl thioester dehydrase from *Escherichia coli*. PhD thesis, Harvard University, Cambridge, Massachusetts.
15. Witkowski, A., Joshi, A.K., and Smith, S. (2004). Characterization of the β-carbon processing reactions of the mammalian cytosolic fatty acid synthase: role of the central core. Biochemistry *43*, 10458–10466.
16. Keatinge-Clay, A.T., and Stroud, R.M. (2006). The structure of a ketoreductase determines the organization of the β-carbon processing enzymes of modular polyketide synthases. Structure *14*, 737–748.
17. Smith, S. (2006). Structural biology. Architectural options for a fatty acid synthase. Science *311*, 1251–1252.
18. Jenni, S., Leibundgut, M., Boehringer, D., Frick, C., Mikolàsek, B., and Ban, N. (2007). Structure of fungal fatty acid synthase and implications for iterative substrate shuttling. Science *316*, 254–261.
19. Lomakin, I.B., Xiong, Y., and Steitz, T.A. (2007). The crystal structure of yeast fatty acid synthase, a cellular machine with eight active sites working together. Cell *129*, 319–332.
20. Witkowski, A., Joshi, A.K., and Smith, S. (1997). Characterization of the interthiol acyltransferase reaction catalyzed by the β-ketoacyl synthase domain of the animal fatty acid synthase. Biochemistry *36*, 16338–16344.
21. Anderson, V.E., and Hammes, G.G. (1985). Distribution of reaction intermediates on chicken liver fatty acid synthase. Biochemistry *24*, 2147–2154.
22. Helmkamp, G.M., Jr., Brock, D.J., and Bloch, K. (1968). β-hydroxydecanoyl thioester dehydrase. Specificity of substrates and acetylenic inhibitors. J. Biol. Chem. *243*, 3229–3231.
23. Alekseyev, V.Y., Liu, C.W., Cane, D.E., Puglisi, J.D., and Khosla, C. (2007). Solution structure and proposed domain recognition interface of an acyl carrier protein domain from a modular polyketide synthase. Protein Sci. *16*, 2093–2107.
24. Jorio, H., Tran, R., Meghrous, J., Bourget, L., and Kamen, A. (2006). Analysis of baculovirus aggregates using flow cytometry. J. Virol. Methods *134*, 8–14.
25. Reed, M.A., Schweizer, M., Szafranska, A.E., Arthur, C., Nicholson, T.P., Cox, R.J., Crosby, J., Crump, M.P., and Simpson, T.J. (2003). The type I rat fatty acid synthase ACP shows structural homology and analogous biochemical properties to type II ACPs. Org. Biomol. Chem. *1*, 463–471.
26. Joshi, A.K., Zhang, L., Rangan, V.S., and Smith, S. (2003). Cloning, expression, and characterization of a human 4′-phosphopantetheinyl transferase with broad substrate specificity. J. Biol. Chem. *278*, 33142–33149.
27. Rasmussen, J.T., Börchers, T., and Knudsen, J. (1990). Comparison of the binding affinities of acyl-CoA-binding protein and fatty-acid-binding protein for long-chain acyl-CoA esters. Biochem. J. *265*, 849–855.
28. Schwede, T., Kopp, J., Guex, N., and Peitsch, M.C. (2003). SWISS-MODEL: an automated protein homology-modeling server. Nucleic Acids Res. *31*, 3381–3385.
29. Guex, N., and Peitsch, M.C. (1997). SWISS-MODEL and the Swiss-PdbViewer: an environment for comparative protein modeling. Electrophoresis *18*, 2714–2723.