Human fatty acid synthase: Role of interdomain in the formation of catalytically active synthase dimer

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The human and animal fatty acid synthases are dimers of two identical multifunctional proteins (M_r 272,000) arranged in an antiparallel configuration. This arrangement generates two active centers for fatty acid synthesis separated by interdomain (ID) regions and predicts that two appropriate halves of the monomer should be able to reconstitute an active fatty acid synthesizing center. This prediction was confirmed by the reconstitution of the synthase active center by using two heterologously expressed halves of the monomer protein. Each of these recombinant halves of synthase monomer contains half of the ID regions. We show here that the fatty acid synthase activity could not be reconstituted when the ID sequences present in the two recombinant halves are deleted, suggesting that these ID sequences are essential for fatty acid synthase dimer formation. Further, we confirm that the ID sequences are the only regions of fatty acid synthase monomers that showed significant dimer formation, by using the yeast two-hybrid system. These results are consistent with the proposal that the ID region, which has no known catalytic activity, associates readily and holds together the two dynamic active centers of the fatty acid synthase dimer, therefore playing an important role in the architecture of catalytically active fatty acid synthase.

The fatty acid synthase (FAS; EC 2.3.1.85) catalyzes the synthesis of long-chain saturated fatty acids—palmitate, in the presence of acetyl-CoA as primer, malonyl-CoA as the two-carbon donor, and NADPH as the source of reduction of the various intermediates produced (1–3). Palmitate synthesis is a complex process involving the participation of seven catalytic enzymes and an acyl carrier protein (ACP) with its 4′-phosphopantetheine group, to which the various acyl intermediates are attached covalently. In prokaryotes, the enzymes are associated loosely with each other and can be separated readily by conventional procedures (1–3). In animals, including humans, the seven partial activities of FAS and ACP are linked together covalently in a single polypeptide chain encoded by a single gene (1–4). The native enzyme has a molecular weight of 544,000 and is composed of two identical subunit proteins, each containing all of the component activities plus ACP. The dimer formation is essential for FAS function (1, 2, 5–8). The organization of these activities along the multifunctional polypeptide from the N terminus to the C terminus is as follows (Fig. 1): β-ketoacyl synthase (KS), acetyl/malonyl transacylase (AT/MT), β-hydroxyacyl dehydratase (DH), enoyl reductase (ER), β-ketoacyl reductase (KR), ACP, and thioesterase (TE). In addition, these activities were grouped in three separate domains, domain I (DI), domain II (DII), and domain III (DIII; Fig. 1 and refs. 1, 2, 9–13). This order and the arrangement of these activities was based initially on proteolytic mapping studies of FAS (1, 2, 9–13) and was verified by the amino acid sequences predicted from the FAS cDNA sequences of human (4), chicken (14–16), rat (17–18), goose (19), and mouse (20). The sequence comparisons and the expression of rat, chicken, and human FAS cDNAs in heterologous systems helped in refining the arrangement of component activities and the domain nature of the multifunctional subunits of FAS (21–24). These studies led to the conclusion that in each monomer, KS, AT/MT, and DH are located in DI (amino acids 1–980); ER, KR, and ACP are located in DII (amino acids 1,631-2,208); and TE is located in DIII (amino acids 2,209–2,504; refs. 21–24). DI and DII are separated by an interdomain (ID) polypeptide of 649 amino acids (residues 981–1,630) of unknown function (22). Generally, it is assumed that ID regions of the FAS monomers play a role in the dimer formation (1–3). The antiparallel arrangement of the FAS monomers in the dimer brings together DI of one subunit and DII and DIII of the second subunit, generating an active center for palmitate synthesis (Fig. 1 and refs. 1, 2, 5–8). Thus the FAS dimer contains two active centers for fatty acid synthesis, and both these centers have been shown to be simultaneously active (1, 2, 5–8). In addition, the dimer structure also predicted that two appropriate halves of the monomer should reconstitute an active fatty acid-synthesizing center.

We have reported recently the expression and purification of the two halves of human FAS expressed in *Escherichia coli* as thioredoxin (TRX) fusion proteins containing six-His tags (23, 24). TRX-hFAS-dI (residues 1–1,297) contains KS, AT/MT, DH, and the N-terminal half of ID (ID-N). TRX-hFAS-dII-III (residues 1,296–2,504) contains the C-terminal half of ID (ID-C) followed by ER, KR, ACP, and TE (24). When the purified recombinant proteins were mixed in a reaction mixture containing acetyl-CoA, malonyl-CoA, and NADPH, they reconstituted an active center capable of long-chain fatty acid synthesis (24). The rate of fatty acid synthesis was linear without a lag, depended on the presence of both recombinant proteins, and reached a constant level when they were present in equivalent amounts, indicating that the reconstitution of an active fatty acid-synthesizing site required the presence of every partial activity associated with the subunit protein (24). These studies confirmed the head-to-tail dimer structure of FAS with two active centers proposed earlier (1, 2, 5–8).

In this report, we show that the ID regions present in these recombinant proteins are essential for the reconstitution of FAS activity. Furthermore, by using the yeast two-hybrid system, we demonstrate that the ID sequences interact strongly with each other compared with the other regions of the FAS dimer that may come together to form the active centers of FAS. The importance of the interaction between the ID regions of the FAS monomers in dimer formation and in the generation of two dynamic active centers of fatty acid synthesis is discussed.

Materials and Methods

Materials. The TAYLON resin was obtained from CLONTECH, and pET32 vectors were obtained from Novagen. Stephen J.

Abbreviations: FAS, fatty acid synthase; DI, DII, and DIII, FAS domains I, II, and III; ID, interdomain; ID-N, N-terminal half of ID; ID-C, C-terminal half of ID; DB, DNA-binding domain of GAL4; ACT, activation domain of GAL4; KS, ketoacyl synthase; TE, thioesterase; TRX, thioredoxin; ACP, acyl carrier protein.

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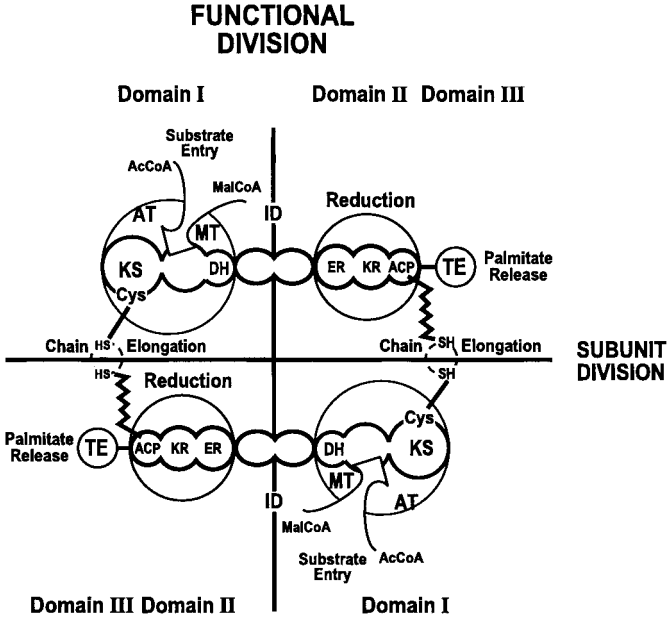

Fig. 1. The structural and functional organization of animal and human FAS dimer. The linear arrangement of the component activities and their domains are indicated in the subunits of FAS. The dimer formation results in two active centers. The functional division indicates the participation of DI of one subunit and DII and DIII of the second subunit in generating the active center. The ID regions (ID) have no known catalytic activities but play an essential role in the structural organization of catalytically active FAS. AT, acetyl transacylase; MT, malonyl transacylase; ER, enoyl reductase; KR, β-ketoacyl reductase; DH, β-hydroxyacyl dehydratase.

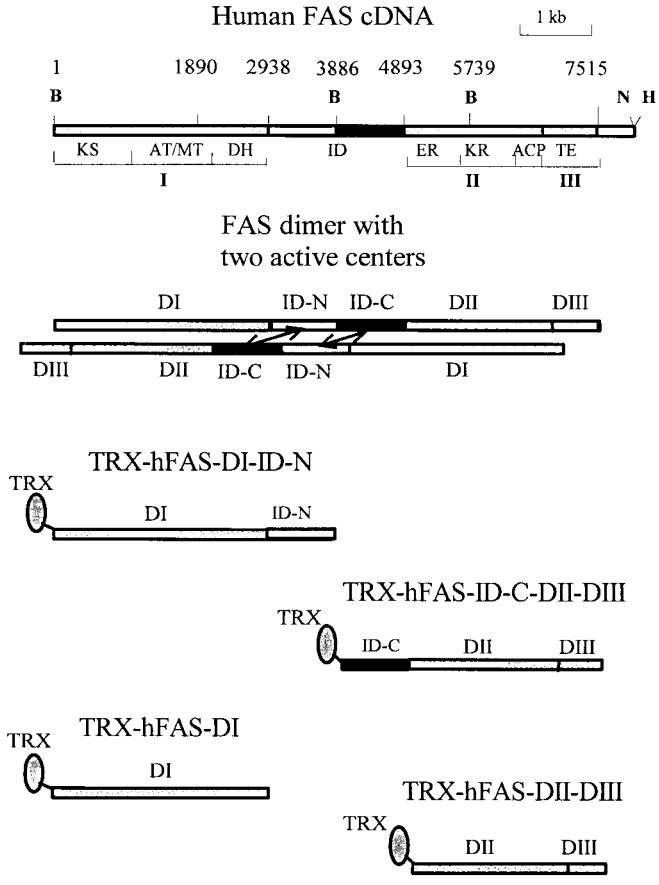

Fig. 2. A representation of the cDNA of human FAS DI and DII-DIII plasmids used in identifying the importance of the ID region in the FAS dimer formation. The top bars represent, respectively, human FAS monomer and dimer. The cDNA nucleotide sequences coding for various constituent activities (speckled bars) and the ID (filled bars) are indicated. The N- (ID-N) and C-terminal (ID-C) halves of the ID are represented as gray and black bars, respectively. The human FAS cDNA nucleotide numbers on the top bar indicate the location of the restriction sites for *Bam*HI (B), *Hin*dIII (H), and *Not*I (N), and the boundaries of the coding sequences present in the recombinant expression plasmids. The cartoon describing the FAS dimer with two active centers is drawn by using the descriptors shown for human FAS cDNA shown in the top line. The construction of the plasmids used for the expression of human FAS recombinant protein halves containing ID halves is described as before (23, 24), except that their names have been changed to include the ID sequences present in them. The constructs lacking ID regions, TRX-hFAS-DI and TRX-hFAS-DII-III, are described in *Materials and Methods*. TRX, thioredoxin (as N-terminal fusion present in the recombinant protein).

Elledge, Department of Biochemistry and Molecular Biology, Baylor College of Medicine (Houston), provided the yeast two-hybrid system vectors and strains. The sources of all other reagents were described (23, 24).

Media and Growth Conditions. The *E. coli* cells harboring various human FAS expression plasmids were grown and induced as described (23, 24). The yeast strains, Y10 [*MATa*, *gal4*, *gal80*, *his3*, *trp1–901*, *ade2–101*, *ura3–52*, *leu2–3*, *112*, *+URA3*::*GAL* → *lacZ*, *LYS2*::*GAL(UAS)* → *HIS3*, *chy*^{r}], and Y187 (*MAT*α, gal4, gal80, his3, trp1–901, ade2–101, ura3–52, leu2–3, 112, met, *+URA3*::*GAL* → lacZ), were maintained and grown in a medium containing 1% (wt/vol) yeast extract, 2% (wt/vol) peptone, and 2% (wt/vol) glucose (yeast extract/peptone/dextrose). Synthetic complete medium lacking either tryptophan or leucine or both (25) was used to maintain plasmids derived from pAS2 and pACT2 vectors. All experiments were performed with mid-logarithmic phase cells grown to an optical density of 0.4–0.8 at 600 nm.

Construction of Plasmids for the Expression of Human FAS DI and DII-DIII Lacking the ID Sequences in *E. coli*. Earlier, we described the construction of the pET32-based *E. coli* expression plasmids TRX-hFAS-dI and TRX-hFAS-dII-III, which are now called TRX-hFAS-DI-ID-N and TRX-hFAS-ID-C-DII-DIII, respectively, to describe more accurately the sequences present in these two halves of human FAS (refs. 23 and 24 and Figs. 1 and 2). To construct the DI expression vector lacking the sequences coding for the ID-N region, the 3.9-kb *Bam*HI fragment (Fig. 2) was isolated and recloned in pET32b vector that was cut with *Bgl*II and *Bam*HI to generate a new TRX-hFAS-DI-ID-N. Next we amplified a cDNA fragment containing 899 bp by PCR with pET32b-DI-ID-N as a template and a forward primer (nucleotides 2,056–2,076) and a reverse primer (nucleotides 2,940–2,955) containing a *Hin*dIII site at the 3′ end. The PCR product and the new TRX-hFAS-DI-ID-N plasmid described above were digested with *Sst*I (nucleotide 2,088) and *Hin*dIII and ligated to generate TRX-hFAS-DI, which lacks the ID-N region (Fig. 2). To generate the DII–DIII expression plasmid lacking the ID-C region, we first amplified a 1.5-kb PCR fragment by using TRX-hFAS-ID-C-DII-III as a template and a forward primer (nucleotides 4,837–4,857) containing an *Eco*RI site at the 5′ end and a reverse primer (nucleotides 6,311–6,330). The PCR product was cut with *Eco*RI and blunt ended by filling in with Klenow fragment of DNA polymerase (26). Then the product was cut with *Bam*HI, and the 908-bp fragment was isolated. The fragment was cloned into TRX-hFAS-ID-C-DII-III that was cut with *Bgl*II, located in the pET32a vector and blunt ended, and then cut with *Bam*HI to generate TRX-hFAS-DII-III, which lacks the ID-C region.

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Cloning of the Human FAS cDNA Coding for the ID, KS, and ACP Regions into the Yeast Two-Hybrid System Vectors. A fragment (1,955 bp) of human FAS cDNA coding for the ID region of human FAS (4) was amplified by PCR by using a forward primer (nucleotides 2,938–2,956) and a reverse primer (nucleotides 4,876–4,893). Both primers contain *Nco*I recognition sequences. In addition, the reverse primer contains in-frame a translation termination codon, TAG. The PCR product was purified and digested with *Nco*I and cloned at the *Nco*I site of pAS2 and pACT2, the yeast two-hybrid system vectors (27, 28). The orientation of the *Nco*I fragment in these vectors was confirmed by using various restriction enzymes. In the correct orientation, the pAS2-ID and pACT2-ID (Fig. 3*B*) will express the entire ID amino acid sequences of human FAS from residues 979–1,631 fused in-frame with the C-terminal end of either the DNA-binding domain (DB) or the activation domain (ACT) of the GAL4 protein, respectively (27, 28). The plasmids pAS2-ID-N and pACT2-ID-N that contain the ID-N region (residues 979–1,295) fused in-frame with DB and ACT of GAL4, respectively, were obtained by digesting pAS2-ID and pACT2-ID with *Bam*HI to delete the ID-C regions, followed by religation (Fig. 3*B*). The constructs pAS2-ID-C and pACT2-ID-C (Fig. 3*B*), which contain the ID-C, were obtained by first cloning the 1.9-kb *Bam*HI fragment (nucleotides 3,885–5,739) in pET32c vector, isolating a 1.6-kb *Nco*I fragment (nucleotides 3,885–5,486), and cloning it at the *Nco*I site of both the pAS2 and pACT-2 vectors. The resulting clones, pAS2-ID-C and pACT2-ID-C, contain the cDNA coding for the C-terminal half of the ID (residues 1,296–1,631) and an additional 196-aa sequence from the enoyl reductase component of human FAS (4, 23).

In addition, the 1–1,890-bp fragment of human FAS cDNA containing the N-terminal coding sequences was cloned into pAS2 and pACT2 vectors as an *Nco*I fragment obtained from the pET32a vector that contained full-length human FAS cDNA (23). The resulting constructs, pAS2-KS and pACT2-KS, which express DI amino acid sequences from residues 1–630 (KS and part of AT/MT), were fused to the DB and ACT of GAL4 protein, respectively (Fig. 3*B*).

The cloning of the human FAS cDNA coding for ACP and TE into pACT2 was achieved as follows. The 2,400-bp fragment of human FAS cDNA coding for the C-terminal amino acid sequences from residues 1,897–2,505 was isolated from a pET32c human FAS DII-DIII construct as a *Bam*HI-*Xho*I (23) and cloned into a pACT2 vector that was digested with the same restriction enzymes. The resulting plasmid, pACT2-ACP-TE, expresses human FAS ACP and TE fused with the GAL4 activation domain (Fig. 3*B*).

All of the human FAS constructs described above (see also Fig. 3*B*) were sequenced with appropriate human FAS primers to confirm the in-frame fusion of FAS sequences with the DNA-binding or activation domains of the GAL4 protein.

Miscellaneous Procedures. Measurement of the protein concentration, SDS/PAGE analysis, DNA sequencing, Western blotting (4, 22–24), yeast transformation, and measurement of β-galactosidase were performed as described (29, 30).

Results

The Role of the ID Region in the FAS Dimer Formation. Catalytically active FAS is a homodimer arranged in an antiparallel configuration forming two active centers of fatty acid synthesis (Figs. 1 and 2). The reconstitution of FAS activity by TRX-hFAS-DI-ID-N and TRX-hFAS-ID-C-DII-III (Table 1 and ref. 24) could have been achieved by bringing these recombinant proteins together through the interactions between the polypeptide components of the constituent enzymatic activities or the ID regions present in them (Fig. 2). To understand the role of the ID sequences in the FAS dimer formation, we have constructed

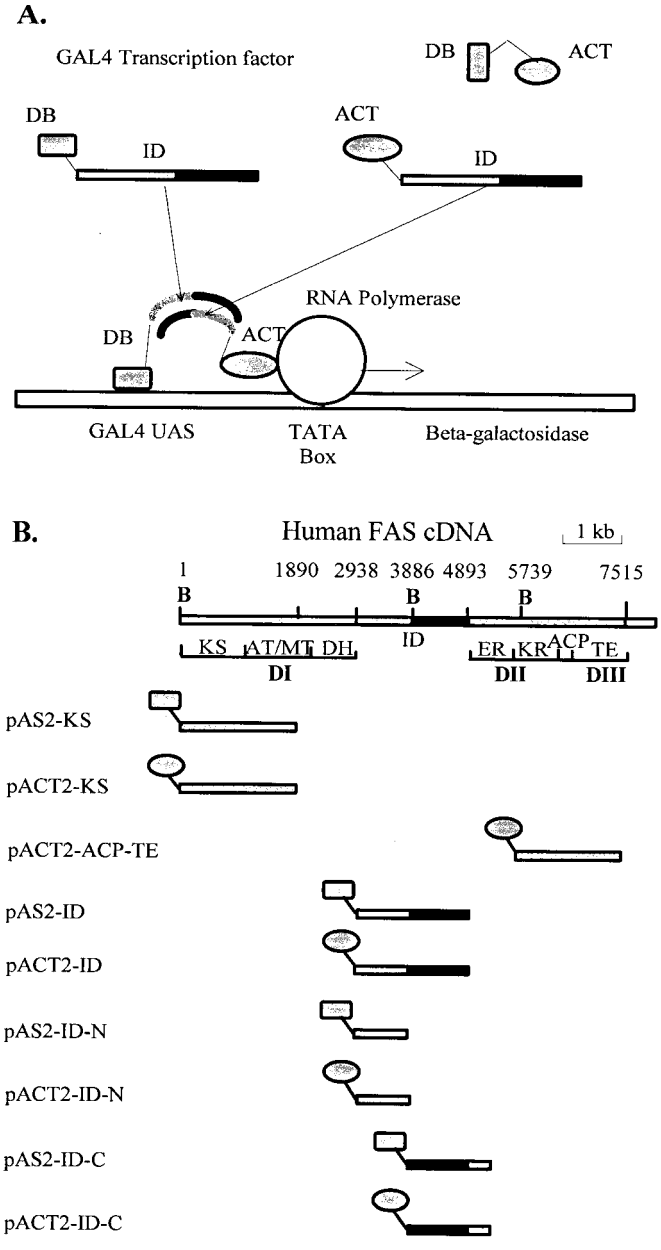

Fig. 3. (*A*) A schematic representation of how human FAS ID fusions of GAL4-DB (filled rectangle) and GAL4-ACT (filled oval) might interact to reconstitute the GAL4 transcription factor to activate the β-galactosidase gene. The head–tail interaction of ID regions will bring together DB and ACT to function as a ''native'' GAL4 transcription factor that binds to the upstream activation sequence (UAS) and activates transcription of the β-galactosidase gene. (*B*) Description of plasmids used in the yeast two-hybrid system for determining the regions in the FAS monomer that are involved in the dimer formation. The yeast two-hybrid system vectors pAS2 and pACT2 contain the N-terminal DB (GAL4-DB) and the C-terminal ACT (GAL4-ACT), respectively. The construction of the yeast two-hybrid system plasmids containing the human FAS sequences fused in-frame with the C terminus of GAL4-DB and GAL4-ACT coding sequences is described in *Materials and Methods*. The depiction of various bars and the significance of nucleotide numbers, etc., are as described in Fig. 2.

TRX-hFAS-DI and TRX-hFAS-DII-III, which lack these ID regions, as described in *Materials and Methods* and in Fig. 2. The FAS activity was reconstituted with TRX-hFAS-DI-ID-N and TRX-hFAS-ID-C-DII-dIII (Table 1), as reported earlier (24).

Table 1. ID requirement for fatty acid synthesizing center generated by reconstitution of various recombinant domains

TRX-hFAS fusions	Partial activities, nmol/min per mg						Fatty acids, cpm
	AT	MT	DH	KR	ER	TE	
DI-ID-N	752	594	119				685
ID-C-DII-III				1,158	43	98	90
DI	475	257	ND				32
DII-III				630	68	80	72
DI-ID-N + ID-C-DII-III							24,750*
DI-ID-N + DII-III							240
DI + ID-C-DII-dIII							46
DI + DII-III							31

The construction of TRX-hFAS fusion plasmids is described in *Materials and Methods* and in Fig. 2. The expression of recombinant proteins in *E. coli*, purified by using TALON resin, and the determination of partial activities, were performed as described (23, 24). The reconstitution of FAS activities from the various recombinant halves of the human FAS subunits and the determination of fatty acid synthesis were carried out by using [2-^{14}C] malonyl-CoA, acetyl-CoA, and NADPH as substrates (24). AT, acetyl transacylase; MT, malonyl transacylase; DH, β-hydroxyacyl dehydratase; KR, β-ketoacyl reductase; ER, enoyl reductase; ND, not determined.
*These counts represent 9.8 nmol of fatty acids synthesized.

However, FAS activity could not be reconstituted if either or both of these recombinant proteins lack their respective ID regions (Table 1 and Fig. 2). These results suggest that these ID regions are essential for the association of the two halves of FAS-recombinant proteins and the generation of the FAS active center. Moreover, the N- and C-terminal halves of the ID sequences interact in an antiparallel fashion.

Utilization of the Yeast Two-Hybrid System to Test the Interaction of the ID Regions of FAS. We used the yeast two-hybrid system that was designed to detect interaction/dimer formation between two proteins (27, 28) to test whether the FAS ID peptides can form dimers. On the basis of the principle of this system, the expected head-to-tail dimer formation between the ID peptides of hFAS fused with GAL4 DB and ACT could lead to the reconstitution of the GAL4 transcription factor that then binds to GAL4 upstream activation sequence and activates the transcription of the β-galactosidase gene, as illustrated in Fig. 3*A*. The levels of expression of β-galactosidase activity reflect the extent of the interaction between the sequences fused to the DNA-binding and activation domains. We tested the full-length ID region as it exists in native FAS; the ID-N and ID-C segments as they exist in TRX-hFAS-DI-ID-N and TRX-hFAS-ID-C-DII-III; and the N-terminal KS and C-terminal ACP regions of the FAS subunit

Table 2. Determination of the sequences that interact in human FAS dimer formation by using the yeast two-hybrid system

GAL4-DB plasmids	GAL4-ACT plasmids	β-Gal Activity
pAS2	pACT2	2
pAS2-ID	pACT2	10
pAS2	pACT2-ID	10
pAS2-ID	pACT2-ID	487
pAS2-ID ←	pACT2-ID ←	13
pAS2-ID-N	pACT-ID	92
pAS2-ID-C	pACT2-ID	182
pAS2-ID-N	pACT2-ID-C	271
pAS2-ID-N ←	pACT2-ID ←	13
pAS2-ID-C ←	pAS2-ID-C ←	13
pAS2-ID-C	pACT2-ACP-TE	16
pAS2-ID-C	pACT2-KS	56
pAS2-KS	pACT2-ACP-TE	18

Plasmids pAS2 and pACT2 are the vectors that contain sequences coding for the DNA-binding and activation domains (GAL4-DB and GAL4-ACT) to which the FAS sequences are fused in-frame at their C-terminal ends. The plasmid constructs used in these experiments are described in Fig. 2 and in *Materials and Methods*. The left arrow (←) indicates that the cDNA sequences coding for ID are fused in the opposite orientation to either the GAL4-DB or the GAL4-ACT domain and hence do not code for the ID sequences. These constructs are not described in Fig. 2. β-Galactosidase (β-Gal) activity is measured in permeabilized yeast cell suspensions by using *O*-nitrophenyl-β-D-galactoside as substrate. The formed *O*-nitrophenol was measured at 420 nm, and the optical density of cell suspension was measured at 600 nm. One unit of β-Gal activity equals 0.001 OD_{420}/OD_{600}/min. The average values of three independent transformants are shown. The variation among replicates is <5%.

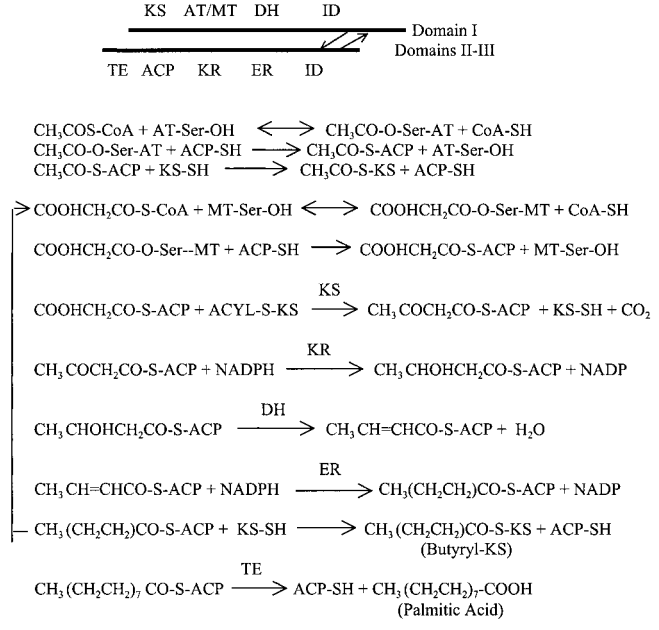

Fig. 4. FAS active center constituted by DI and DII–DIII and the individual catalytic reactions involved in the synthesis butyryl-intermediate and its subsequent elongation to palmitate. As described above, 10 reactions are involved in the formation of butyryl–*S*-KS, which undergoes bracketed reactions six more times to generate palmitoyl-*S*-ACP, which is released by TE. The complexity and the repetitive nature of these reactions are the basis of our proposal that the ACP carrying the acyl-intermediates and the catalytic activity are in dynamic state and that the ID sequences that do not participate in these catalytic reactions play an important role in holding together the two subunits of FAS.

that come together in the FAS dimer (1, 2) to determine whether they are involved in the FAS dimer formation, as measured by the levels of the β-galactosidase expression in yeast cells. The construction of the yeast plasmids containing various FAS-coding regions in-frame with the C-termini of DB and ACT is described in *Materials and Methods* and in Fig. 3*B*. In these experiments, we used several constructs as controls that included the yeast vectors, which are devoid of the coding sequences for FAS, and those that contained FAS-coding sequences cloned in the wrong orientation. The latter constructs express nonFAS-related sequences of unknown length fused to DNA-binding and activation domains. On the basis of the levels of β-galactosidase expression (Table 2), significant interaction that resulted in the dimer formation occurred between the hFAS sequences containing the ID sequences only. The β-galactosidase expression observed when the DB and ACT domains are fused to the N- and C-terminal halves of the ID is consistent with the antiparallel interactions mediated by the N- and C-terminal portions of the ID. The relatively high levels of β-galactosidase activities observed when the full-length ID sequences were fused to the DB (DB-ID) and the ACT (ACT-ID) domains (Table 2) are consistent with the expectation of a more extensive interaction with the entire interdomain sequences present in FAS monomers.

Discussion

The amino acid sequences of interdomain regions of hFAS, rat FAS, and chicken FAS show very low similarity (<20% identity), indicating lack of conservation and possibly lack of catalytic activity in this region of FAS. The results shown above demonstrate that the FAS dimer formation is facilitated by the ID dimer regions, which also act as a spacer region separating the two active centers (Fig. 1). On the basis of the antiparallel nature of the FAS dimer formation, the ID sequences are expected to interact noncovalently in an antiparallel fashion. The presence of the ID regions (ID-N and ID-C) in the TRX-hFAS-DI-ID-N and TRX-hFAS-ID-C-DII-III recombinant proteins was essential for the formation of the FAS active center (Table 1, Fig. 1). In addition, the activation of β-galactosidase expression by the N- and C-terminal halves of ID fused to DB and ACT was consistent with the antiparallel association of the ID sequences (Fig. 2, Table 2). This association leads to the generation of two dynamic active centers of FAS, each performing 52 reactions starting with acetyl-CoA and malonyl-CoA, yielding palmitate (1–3). The dynamic nature of FAS active centers is essential for carrying the reactions catalyzed by the various component enzymes that are distributed between the two polypeptides (Figs. 1 and 4). Although we know that the active thiols of ACP and KS are juxtaposed within 2 Å for the condensation reaction (1, 2), we do not yet know the proximities of each of the remaining component activities to the acyl-ACP substrates involved in FAS catalysis. However, the individual reactions of FAS that participate in the synthesis of the first saturated C_4 intermediate and the reactions involved in its further elongation to palmitate (Fig. 4) suggest that the two polypeptides forming the active center might be capable of swinging in and out to help bring the appropriate substrates to the appropriate catalytic sites. One might expect also that the swinging arm of ACP, the 4-phosphopantetheine to which acyl-intermediates are attached, plays an important role in facilitating the dynamic states of substrate–enzyme interactions (1–2). On the basis of the routinely purified human FAS preparation with a specific activity of 1,200–1,600 nmol/min/mg, each FAS molecule performs 500–700 reactions/sec, indicating the complexity of the overall reactions. These dynamic interactions of FAS component enzymes require a structural organization in which the polypeptides containing the individual component enzymes are brought and held together in a way that facilitates their actions. We propose, therefore, that the ID peptide, which is catalytically inactive, is the region of the FAS subunits that performs this function. Its role in FAS architecture, therefore, is essential for the function of this multifunctional enzyme.

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