Construction, Expression, and Characterization of a Mutated Animal Fatty Acid Synthase Deficient in the Dehydrase Function*

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The location of the dehydrase domain in the multifunctional animal fatty acid synthase has been determined by engineering a fatty acid synthase mutant deficient in dehydrase activity. A full-length fatty acid synthase cDNA encoding a mutated histidine residue (His878→Ala) was constructed and expressed in insect SF9 cells using a baculoviral vector. The mutated recombinant fatty acid synthase retained all partial activities of the multifunctional complex except the dehydrase and was unable to synthesize fatty acids. β-Hydroxybutyryl moieties were formed by the mutant fatty acid synthase from acetyl-CoA, malonyl-CoA, and NADPH and slowly released as the CoA thioester, confirming that this protein cannot perform the dehydra reaction. This finding points to an important catalytic role for His878 in the dehydrase reaction and establishes that the dehydrase domain is located immediately adjacent to the carboxyl terminus of the transferase domain. Examination of the completed domain map for the animal fatty acid synthase indicates that the catalytic domains are clustered in two groups separated by a central structural core: the ketoacyl synthase, malonyl/acyetyltransferase, and dehydrase in the amino-terminal half and the enoyl reductase, ketoreductase, acyl carrier protein, and thioesterase in the carboxyl-terminal half. A model is proposed in which the two centers for acyl chain initiation, elongation and termination, are formed by the cooperation of the three amino-terminal domains of one subunit with the four carboxyl-terminal domains of the other subunit.

In animals the biosynthesis of fatty acids de novo from malonyl-CoA is catalyzed by a dimer of identical 272-kDa polypeptides, both of which contain seven catalytic domains. The location of six of the catalytic domains, ketoacyl synthase, malonyl/acyetyltransferase, enoyl reductase, ketoreductase, acyl carrier protein, and thioesterase, has been established by identification of the various active site residues within the overall amino acid sequence (1–3), by isolation of catalytically active fragments from limited proteolytic digests of the whole fatty acid synthase (4–7), by identification of regions within the fatty acid synthase that exhibit sequence similarity with various multifunctional proteins (8), by expression of individual catalytic domains as independent recombinant proteins (9), and by identification of the linker regions that separate the individual catalytic domains (8). Nevertheless one of the component domains, the dehydrase, has proven very difficult to map. Several years ago Tsukamoto and Wakil (10) reported that a fragment containing dehydrase activity could be isolated from limited kalikrein digests of the chicken fatty acid synthase. Although it was concluded that the dehydrase domain was localized in the carboxyl-terminal half of the polypeptide, immediately adjacent to the enoyl reductase domain, this claim has not been substantiated by amino-terminal sequencing of the polypeptide fragment. More recently a clue as to the possible location of the dehydrase domain emerged from an analysis of the amino acid sequences encoded in the three open reading frames of the polyketide synthase gene cluster of *Saccharopolyspora erythraea*. The three polypeptides that comprise this polyketide synthase appear to be constructed from "modules" which resemble the animal fatty acid synthase, both in terms of their amino acid sequence and in the ordering of the constituent domains (11, 12). However according to the mechanism proposed for the biosynthesis of the polyketide product, only one of the six modules contains a dehydrase domain. Thus the dehydrase domain was thought likely to lie within a 152-amino acid region unique to module 4, encoded within orf-2 (11, 12). This region exhibits weak sequence similarity with the monofunctional β-hydroxydecanoyl dehydrase of *Escherichia coli* (13). In recent years amino acid sequences have been derived for several other multifunctional proteins that should contain dehydrase domains: the yeast fatty acid synthase (14), the 6-methylsalicylic acid synthase of *Penicillium patulum* (15), the mycocerosic acid synthase of *Mycobacterium tuberculosis* (16), and the animal fatty acid synthase (1–3). A detailed study of the monofunctional *E. coli* dehydrase identified a catalytically important residue, His70, by labeling with a suicide inhibitor (13). Although alignment of the the *E. coli* dehydrase sequence with various candidate regions of the multifunctional proteins did not yield any statistically significant sequence matches (using the ALIGN program, details not shown), it is possible to align a segment of the *E. coli* dehydrase with segments derived from the putative dehydrase domain of the polyketide synthase and unassigned regions of the other multifunctional proteins in such a way as to match up the catalytically important His70 with histidine residues in the other sequences (Table I). From this alignment a conserved sequence element containing a histidine, glycine, and proline can be identified. However the "mo"t" is not universally conserved (Gly is missing in the mycocerosic acid synthase) and a gap must be introduced between the His and Gly components of the motif in the *E. coli* enzyme to preserve the alignment. The basis for this analysis is further complicated by the fact that the *E. coli* dehydrase actually fulfills a slightly different catalytic role than its counterparts in the multifunctional proteins. Whereas the latter dehydrases catalyze the conversion of all 3-hydroxyacyl-S-acyl carrier protein intermediates to their corresponding 2-enoyl thioesters, this *E. coli* enzyme is involved uniquely in the biosynthesis of monounsaturated fatty acids and catalyzes the dehydration of 3-hydroxydecanoyl-S-acyl carrier protein followed by the isom-

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eralization of the 2-enoyl- to the 3-enoyl-thioester. Thus the suicide inhibitor used to identify the active site histidine, 3-decenoyl-N-acetylcysteamine, is actually an analog for the substrate in the isomerase reaction rather than the dehydration reaction. Nevertheless it has been suggested that the same histidine residue is involved in the isomerization and dehydration reactions catalyzed by the E. coli enzyme (17). The uncertainty concerning the appropriateness of comparing dehydrases involved in saturated fatty acid synthesis and unsaturated fatty acid synthesis and the weakness of the sequence match that is obtained does not provide a convincing argument for localization of the dehydrase domain in the animal fatty acid synthase. Furthermore the sequence alignment derived from such a comparison predicts that the dehydrase domain lies in the amino-terminal half of the fatty acid synthase, immediately adjacent to the malonyl/acetyltransferase with the active site histidine at residue 878. Clearly this conflicts with the earlier conclusion of Tsukamoto and Wakil (101, 102) of comparing dehydrases involved in saturated fatty acid synthesis and the weakness of the sequence match that is obtained does not provide a convincing argument for localization of the dehydrase domain in the animal fatty acid synthase. Furthermore the sequence alignment derived from such a comparison predicts that the dehydrase domain lies in the amino-terminal half of the fatty acid synthase, immediately adjacent to the malonyl/acetyltransferase with the active site histidine at residue 878. Clearly this conflicts with the earlier conclusion of Tsukamoto and Wakil (101, 102)

### EXPERIMENTAL PROCEDURES

**Materials**—The sources of materials have been described in detail elsewhere (18). Plasmids pFAS13.20 and pFAS305.20 were constructed by inserting the PCR amplified EcoRI-SalI and EcoRI-NcoI fragments, respectively, of rat fatty acid synthase cDNA into the pUCBM20 vector. Plasmid pFAS203 consists of the full-length fatty acid synthase cDNA inserted into the baculoviral transfer vector pVL1393.

**Construction of a cDNA Encoding the His→Ala Fatty Acid Synthase Mutant**—Construction of a full-length cDNA encoding the rat fatty acid synthase and expression of the catalytically active multifunctional protein in insect S9 cells using baculoviral vectors has been described elsewhere (18). The H878A mutation was introduced into the fatty acid synthase using the polymerase chain reaction, as follows. A portion of the template DNA, pFAS305.20, was amplified using a primer encoding the H878A mutation (FAS878T) in combination with the primer FAS13.20 (Fig. 1). The reaction mixtures, in a final volume of 100 µl, containing 20 mM Tris-HCl (pH 8.5 at 25 °C), 10 mM KCl, 10 mM (NH₄)₂SO₄, 100 µg/ml bovine serum albumin, 0.1% Triton X-100, 2.5–3.5 mM MgSO₄, 0.5 mM of each dNTP, 0.25 µM of each primer, 25–50 ng of template DNA, and 2 units of Vent DNA polymerase. The reaction temperature was cycled 20 times, 1 min at 94 °C, 1 min at 52 °C, and 2 min at 72 °C. The amplified DNA (2178 bp) was restricted with Sall and NsII, and the purified fragment (203 bp) was cloned into appropriately digested pFAS13.20 (Fig. 1) to generate pFAS305.20. The sequence of the mutated SalI-NsII region was verified by dyeoxy sequencing. Finally, an EcoRI-AfII fragment (1637 bp) carrying the mutation was isolated from pFAS878.20 and substituted for the corresponding fragment in the construct carrying the full-length wild-type fatty acid synthase cDNA (transfer plasmid pFAS203) to generate the construct pFAS206 encoding the H878A mutation. Purified DNA from pFAS206 was analyzed by restriction digestion, and the presence of the mutation was reconfirmed in the final construct by dyeoxy sequencing.

**Expression of Recombinant Fatty Acid Synthase**—The recombinant transfer vectors encoding the full-length wild-type fatty acid synthase from the His→Ala mutant, together with linearized baculoviral DNA, were used for cotransfection of S9 cells (19). Recombinant viral clones were purified and screened for recombinant fatty acid synthase expression by SDS-PAGE, using 7.5% polyacrylamide gels (20), and by Western analysis.

**Purification of Recombinant Fatty Acid Synthase**—Purified recombinant viral stocks, at the third passage, were used at a multiplicity of infection of 3–5, to infect 100–200 ml of S9 cell cultures (approximately 1 × 10⁶ cells/ml). The suspension cultures were maintained for 48 h at 27 °C. Cells were harvested by centrifugation and disrupted by 50 mM potassium phosphate buffer (pH 7), 1 mM dithiothreitol, 1 mM EDTA, 0.25% (w/v) sucrose (5 mg of cells per milliliter). The lysate was centrifuged for 10 min at 10,000 × g, and the supernatant was either stored at -70 °C or applied directly to a preparative high performance anion exchange column (TSK-DEAR-5PW, 2.15 × 15 cm, 10 µm) at 20 °C. Proteins were eluted with a phosphate gradient and fractions containing the recombinant fatty acid synthase were located using a convenient and highly sensitive spectrophotometric assay that employs trans-1-decalone (21) as substrate for the ketoreductase component of the multifunctional protein. Fractions containing the recombinant fatty acid synthase were pooled and rechromatographed on the same column. For long term storage of the fatty acid synthase, glyceral was added to 10% (v/v) and the samples frozen at -70 °C. Details of the entire procedure for expression and purification of the recombinant fatty acid synthase are to be published elsewhere (18).

**Enzyme Assays**—Ketoacyl synthase activity was assayed radiochemically by the condensation reaction. ¹⁴CO₂ exchange reaction (22). Transferase activity was assayed, using malonyl-CoA as donor and pantetheine as acceptor, by determining spectrophotometrically the free CoA released in a coupled ATP-dependent lysomal dehydrogenase reaction (7). Ketoreductase was assayed spectrophotometrically at 340 nm; assay systems contained 0.1 mM potassium phosphate buffer (pH 7), 0.15 mM NADPH, enzyme, and 10 mM trans-1-decanol as substrate. Dehydrase activity was measured spectrophotometrically at 270 nm using S-crotonyl-N-acetylcysteamine as substrate. Thioreductase activity was assayed radiochemically by extracting and assaying the [¹⁴C]malonic acid formed from [1-¹⁴C]malonil-CoA during an incubation of 3-min duration (24); assay systems contained in a final volume of 0.1 ml, 25 mM potassium phosphate buffer (pH 9), 20 µg/ml bovine serum albumin, 10 µM [¹⁴C]malonil-CoA (20 nCi), and enzyme. Assay of overall fatty acid synthase activity was performed either spectrophotometrically or radiochemically using [2-¹⁴C]malonyl-CoA as described previously (25). All enzyme assays were conducted at 37 °C, except the transferase, which was assayed at 20 °C. Activity units indicate moles substrate utilized per min. All assays were made at least at two different protein concentrations with the appropriate enzyme and substrate blanks included.

**Fractionation of CoA Thioesters by HPLC**—A Beckman C-18 Ultrasphere column (5 µm, 15 × 0.46 cm) was operated at room temperature with a flow rate of 1 ml/min. Solvent A was 50 mM sodium phosphate buffer (pH 5.5) and solvent B was acetonitrile. The column was eluted with 100% solvent A for 5 min then the proportion of solvent B was increased linearly to 3.2% over 7.5 min, from 3.2 to 4.5% over 15 min and from 4.5 to 9.0% over 10 min.

### RESULTS

**Construction, Authentication, and Expression of the His→Ala Fatty Acid Synthase Mutant**—Due to the large size (approximately 9.5-kilobase pair vector plus 7.5-kilobase pair insert) and dearth of unique restriction sites in the recombinant...
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Transfer vector encoding the rat fatty acid synthase cDNA, it was necessary to use a three-step procedure in order to insert the H878A mutation into the full-length cDNA (Fig. 1). First, for the initial introduction of the mutation by the PCR, we utilized a primer (FAS878T) that encoded the His^878→Ala change and a convenient second primer (FAS13B) that we had synthesized previously for construction of the full-length cDNA (18). In order to minimize the possibility of inadvertently introduction of additional mutations, we transferred only a small fragment (203 bp) of the PCR-amplified region into the final construct. Because of the problem of limited availability of unique restriction sites this process was carried out in two steps involving the initial transfer of a SalI/NsiI fragment into a partial cDNA construct and the subsequent transfer of a EcoRI/AflII fragment into the full-length cDNA (Fig. 1).

Restriction mapping of purified DNA derived from the final transfer vector carrying the desired mutation (pFAS206) generated fragments of the expected size (details not shown). In addition, the presence of the H878A mutation and absence of any additional mutations in the PCR amplified region was confirmed by DNA sequencing (Fig. 2). The transfer vector pFAS206 was then used to generate a recombinant baculovirus encoding the mutated fatty acid synthase and this in turn was used to infect insect Sf9 cells. The recombinant fatty acid synthase was purified from Sf9 cytosol and together with wild-type recombinant and rat liver fatty acid synthase proteins was analyzed by SDS-PAGE and Western blotting (Fig. 3). All three proteins exhibited identical mobility on SDS-PAGE and gave positive reactions when probed with anti-(rat)-fatty acid synthase antibodies in Western analysis.

Characterization of the Catalytic Properties of His^878→Ala Fatty Acid Synthase—The ability of the mutant protein to catalyze each of the partial reactions of the fatty acid synthase was assayed and compared with that of the wild-type recombinant protein (Table II). None of the activities were significantly different between mutant and wild-type except for the dehydrase, which was undetectable in the mutant. When the overall fatty acid synthase activity was assessed spectrophotometrically, by measuring the NADPH oxidation that normally accompanies catalysis of the two reductase steps, the activity of the mutant was found to be only about 1% of that of the wild type. However when the overall fatty acid synthase activity was assessed radiochemically, by measuring the incorporation of [2-14C]malonyl-CoA into fatty acid, no radioactivity was extracted from the

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**Figure 1. Strategy for engineering the His^878→Ala mutation into a full-length fatty acid synthase cDNA.**

1. Introduce mutation by PCR
   -野生H878A
   -转导
   -PCR
   -SaI/SalI
   -LsaI
   -XbOl

2. Clone SaI/NsiI fragment into pFAS13.20
   -野生H878A
   -转导
   -PCR
   -SaI/SalI
   -LsaI
   -XbOl

3. Clone EcoRI/AflII fragment into pFAS203
   -野生H878A
   -转导
   -PCR
   -SaI/SalI
   -LsaI
   -XbOl

**Figure 2. Verification of the His^878→Ala mutation by DNA sequencing.** Plasmid sequencing was carried out by the dideoxy method using a Sequenase® kit. The wild-type CAC (histidine) to GCC (alanine) change in the mutant is indicated.
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Acidification of the reaction mixture into the hexane phase. Clearly muta-
tion of histidine 878 to alanine had completely blocked the ability of the fatty acid synthase to catalyze the dehydrase reaction and the overall biosynthesis of fatty acid. We sus-
ppected that the very low residual rate of substrate-dependent NADPH oxidation associated with the mutant fatty acid synthase might be attributable to the formation of β-hydroxybutyryl moieties on the enzyme and their slow release by transfer to a CoA acceptor. This possibility was investigated by analysis of the reaction products using HPLC (Fig. 4). The reaction was stopped after 7 min by the addition of trichloroacetic acid to 10% final concentration, the mixture was held at 0 °C for 30 min and then protein was removed by centrifugation. The supernatant was neutralized to pH 5.5 with NaOH, and sodium phosphate buffer (pH 5.5) was added to 0.2 M, final concentration. The sample was then fractionated by HPLC (see “Experimental Procedures” for details) and the eluate monitored for radioactivity (shaded columns). B, chromatogram obtained with authentic CoA and CoA thioester standards: I, malonyl-CoA; II, CoA; III, acetyl-CoA, IV, acetoacetyl-CoA, V, β-hydroxybutyryl-CoA, VI, crotonyl-CoA, VII, butyryl-CoA.

### DISCUSSION

Identification of His^878 as a catalytically important residue for the dehydrase function supports our earlier tentative suggestion that the dehydrase might be located immediately adjacent to the malonyl/acyltransferase (26) and is not in accord with the claim that the dehydrase is located immediately adjacent to the enoyl reductase domain (10). Furthermore, this finding indicates that the tentative location of the catalytically important histidine within the dehydrase domains of other multifunctional proteins shown in Table I is most likely correct. Estimates as to the likely locations of the amino- and carboxyl-terminal boundaries for the dehydrase in the rat fatty acid synthase can now be made, based on our previous observation that the boundaries separating the catalytic domains tend to be located at the surface of the protein, are often susceptible to attack by proteases and are less well conserved in amino acid sequence (8). Most recently, our ability to predict the location of these interdomain boundaries enabled us to identify correctly the boundaries of the malonyl/acyltransferase domain and express the transferase activity as a catalytically active recombinant protein consisting of residues 428–815 of the parent fatty acid synthase. Since residue 878 appears to be essential for catalytic activity of the dehydrase, it is clear that the dehydrase domain must be located immediately adjacent to the transferase.

V. S. Rangan and S. Smith, unpublished results.

**TABLE II**

| Enzyme                  | Specific activity | H878 → Ala mutant |
|-------------------------|------------------|-------------------|
| Transferase             | 2.9 × 10^{-3}    | 0.5 × 10^{-3}     |
| Ketoacyl synthase       | 11 × 10^{-3}     | 0.5 × 10^{-3}     |
| Ketoreductase           | 0.72             | 0.67              |

* Determined spectrophotometrically. Radiochemical assay indicated no fatty acids are formed (see text for explanation).
the transferase, perhaps immediately following a proline-rich sequence that extends from Pro^{117} to Pro^{59}2, the location of the dehydrase carboxyl terminus is likely to be prior to the region stretching from 970 to 985, which does not contain a single conserved residue in the rat and chicken fatty acid synthase sequences (8). This estimate would place the dehydrase domain within the limits of residues 829-969. That is close in size both to the 152-residue insertion in the S. erythraea polyketide synthase that is thought to contain the dehydrase function (11, 12) and to the 171-residue monofunctional dehydrase of E. coli (13). A revised version of the domain map is presented in Fig. 5.

It is well established that although the fatty acid synthase monomers contain all of the individual functional domains required for fatty acid synthesis, only the dimer can catalyze the overall reaction. The inability of the individual subunits to synthesize fatty acid was for a long time attributed solely to the fact that catalysis of the condensation reaction could be shown to require participation of both subunits; thus in a head-to-tail oriented pair of subunits, an acetyl moiety attached to the ketoacyl synthase active site cysteine of one subunit condenses with a malonyl moiety attached to the 4'-phosphopantetheine of the other subunit (27). More recently however, clear evidence has been obtained showing that the transfer of substrate from the active site serine of the transferase domain to the 4’ phosphopantetheine has been obtained showing that the transfer of substrate from the active site serine of the transferase domain to the 4’ phosphopantetheine is also an inter-subunit event (28). The revised domain map of the fatty acid synthase reveals that the catalytic domains are clustered in two groups separated by the central structural core: the ketoacyl synthase, malonylacyltransferase, and dehydrase in the amino-terminal half and the enoyl reductase, ketoreductase, acyl carrier protein, and thioesterase in the carboxyl-terminal half (Fig. 5). This arrangement of catalytic domains suggests that the two centers for acyl chain initiation, elongation and termination, may be formed by the cooperation of the ketoacyl synthase, malonylacyltransferase, and dehydrase domains of one subunit with the enoyl reductase, ketoreductase, acyl carrier protein, and thioesterase of the other subunit, as illustrated in the accompanying model (Fig. 6). In this model the dimer is stabilized primarily by interactions between the central structural core regions of the two subunits (7, 8, 29), and the individual catalytic domains are connected by poorly conserved linkers which are exposed at the protein surface and are therefore susceptible to proteolysis (7, 8).

Although the 4'-phosphopantetheine “swinging arm” of the multifunctional fatty acid synthase has long been accepted as a key component in coupling the various partial reactions required for fatty acid synthesis, recent evidence indicates that the simple swinging arm hypothesis alone may not adequately account for the functioning of the complex. Thus, whereas the length of the phosphopantetheine arm is only 20 Å, distances between the active sites have been estimated by fluorescence energy transfer experiments to be considerably greater: for example, 48 Å between the phosphopantetheine and the thioesterase active site and 40 Å between the phosphopantetheine and the nucleotide-binding domains (30, 31). These observations indicate a degree of domain mobility is probably required to accomplish coupling of the partial reactions. We propose in the revised model that such “dynamic interactions” between catalytic domains may be facilitated by flexibility of the inter-domain linkers. The most important feature of the model is the degree of intersubunit communication required to couple the individual reactions such that two centers for acyl chain assembly and release are formed by cooperation of three catalytic domains of one subunit with four catalytic domains of the adjacent subunit. At present it is not known whether these two centers function independently of each other. With the development of a system that allows the engineering of specific fatty acid synthase mutants with altered catalytic residues or modified interdomain linkers, we are now able to test the validity of the proposed model.

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