Dibromopropanone Cross-linking of the Phosphopantetheine and Active-site Cysteine Thiols of the Animal Fatty Acid Synthase Can Occur Both Inter- and Intrasubunit

REEVALUATION OF THE SIDE-BY-SIDE, ANTIPARALLEL SUBUNIT MODEL* (Received for publication, December 21, 1998, and in revised form, January 18, 1999)

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The objective of this study was to test a new model for the homodimeric animal FAS which implies that the condensation reaction can be catalyzed by the aminoterminal β-ketoacyl synthase domain in cooperation with the penultimate carboxyl-terminal acyl carrier protein domain of either subunit. Treatment of animal fatty acid synthase dimers with dibromopropanone generates three new molecular species with decreased electrophoretic mobilities; none of these species are formed by fatty acid synthase mutant dimers lacking either the active-site cysteine of the β-ketoacyl synthase domain (C161A) or the phosphopantetheine thiol of the acyl carrier protein domain (S2151A). A double affinity-labeling strategy was used to isolate dimers that carried one or both mutations on one or both subunits; the heterodimers were treated with dibromopropanone and analyzed by a combination of sodium dodecyl sulfate/polyacrylamide gel electrophoresis, Western blotting, gel filtration, and matrix-assisted laser desorption mass spectrometry. Thus the two slowest-moving of these species, which accounted for 45 and 15% of the total, were identified as doubly and singly cross-linked dimers, respectively, whereas the fastest moving species, which accounted for 35% of the total, was identified as originating from internally cross-linked subunits. These results show that the two polypeptides of the fatty acid synthase are oriented such that head-to-tail contacts are formed both between and within subunits, and provide the first structural evidence in support of the new model.

In animals, the de novo synthesis of long-chain fatty acids from acetyl- and malonyl-CoA is catalyzed by a single protein, the FAS, that consists of two identical, multifunctional polypeptides (1–3). Although all of the functional domains are present on a single polypeptide, coupling of the individual activities necessary for catalysis of the overall reaction of fatty acid synthesis is performed only by the dimer (4, 5). In 1981, Stoops and Wakil (6) found that the bifunctional reagent dibromopropanone reacts with the FAS dimer such that the 4′-phosphopantetheine thiol of the ACP domain becomes cross-linked with the active site cysteine thiol of the β-ketoacyl synthase domain. The electrophoretic mobility of FAS on SDS-polyacrylamide gels was dramatically reduced following treatment with dibromopropanone, indicating that the cross-linking had occurred between the two subunits. Approximately two molecules of the reagent were incorporated into the fully cross-linked dimer, and the ability to synthesize palmitate was lost. These observations inspired a model for the FAS in which the two subunits are arranged in head-to-tail orientation, so that the β-ketoacyl synthase of one subunit is juxtaposed with the ACP domain of the opposite subunit and two sites for palmitate synthesis are formed per dimer (7).

Support for the two-site model was provided by experimental evidence showing that the FAS dimer, in which the chain-terminating reaction had been blocked, assembles and retains two long-chain fatty acyl moieties (8, 9). Subsequently, sequencing (10–12) and mutagenesis (13) of the animal fatty acid synthase established unequivocally the arrangement of the functional domains in the FAS polypeptide as, starting from the amino terminus, β-ketoacyl synthase, malonyl/acyetyltransferase, dehydrase, enoyl reductase, β-ketoacyl reductase, ACP, and thioesterase. The dehydrase and enoyl reductase domains are separated by a region of approximately 600 amino acids that has not yet been ascribed a specific function. Initially, only the condensation reaction was believed to be catalyzed across the subunit interface, but later, experimental evidence was obtained which demonstrated that the translocation of acetyl and malonyl moieties from CoA ester to the 4′-phosphopantetheine of the ACP domain also requires the dimeric form of the protein (14). Thus it has become generally accepted that the two subunits lie side-by-side in a fully extended, antiparallel configuration so that each of the two centers for palmitate synthesis requires cooperation between catalytic domains located in the amino-terminal half of one subunit with those of the carboxyl-terminal half of the companion subunit (1, 3, 15).

This side-by-side, head-to-tail model had not been subjected to rigorous testing until recently when we introduced an in vitro mutant complementation strategy to map the functional topology of this multifunctional complex (16–18). The approach requires the engineering of various modified FASs, in which the activity of one of the functional domains is specifically compromised by mutation. Heterodimers formed from subunits containing different, single mutations may be capable of fatty acid synthesis if the two mutations are located on domains that normally cooperate with each other across the subunit inter-

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‡The abbreviations used are: FAS, fatty acid synthase; ACP, acyl carrier protein; MALDI-TOF, matrix-assisted laser desorption ionization/time of flight.
face. To date, the results of this ongoing mutant complementation analysis have confirmed several key features of the original. For example, mutations in either the \( \beta \)-ketoacyl synthase or malonyl/acyltransferase domain complement mutations in either the ACP or thioesterase domains, whereas mutations in the ACP and thioesterase domains fail to complement each other (17, 18). These findings confirmed that the \( \beta \)-ketoacyl synthase and malonyl/acyltransferase domains, located in the amino-terminal half of the FAS polypeptide, can contribute to the same center of palmitate synthesis as do the ACP and thioesterase domains of the companion subunit. On the other hand, the complementation analysis also revealed some unanticipated properties that could not be explained by the prevailing model. First, conversion of the \( \beta \)-hydroxyacyl-ACP to the enoyl-ACP was found to be catalyzed by cooperation of the ACP and dehydrase domains of the same subunit (17). Because the dehydrase and ACP domains are separated by more than 1000 residues, this finding provided the first experimental evidence indicating that the two constituent polypeptides are not positioned side-by-side in a rigid, fully extended, antiparallel orientation but are coiled in such a way as to permit functional contacts between domains distantly located on the same polypeptide. Second, both the substrate translocation and condensation reactions were found to be catalyzed by cooperation of the malonyl/acyl transferase and \( \beta \)-ketoacyl synthase domains, respectively, with the ACP domains of either subunit (18). Because the active centers of \( \beta \)-ketoacyl synthase and malonyl/acyl transferase domains are separated from the 4'-phosphopantetheine moiety by more than 1500 residues, this finding raised further doubts as to the validity of the fully extended, rigid antiparallel polypeptide model.

We have proposed a revised model that retains the concept of head-to-tail orientation of the two subunits but also allows for head-to-tail interactions within each subunit (18, 19). At present, because the evidence in support of the revised model is derived entirely from functional studies performed with mutant FASs, we are now exploring experimental approaches that can provide structural data with which to test the validity of the new model. The results of the mutant complementation analysis imply that the active-site cysteine residue of the \( \beta \)-ketoacyl synthase domain of each subunit must lie close to the 4'-phosphopantetheine thiol of both subunits. This finding would appear to be inconsistent with the conclusion drawn from the original dibromopropanone cross-linking experiments by Stoops and Wakil (6), namely that cross-linking occurs exclusively between subunits. However, the fact that three molecular species with retarded electrophoretic mobilities are formed by treatment with dibromopropanone has never been explained. Because an immediate inference of the revised model is that cross-linking would be expected to occur both within and between subunits, we have reinvestigated the specificity of the dibromopropanone reaction with the FAS to determine whether one of these species might represent an internally cross-linked subunit.

**EXPERIMENTAL PROCEDURES**

**Materials**—1,3-Dibromopropanone was purchased from Alpha \\&bar, Ward Hill, MA, and purified by high performance liquid chromatography as described previously (20).

**Construction of cDNAs Encoding His\(_c\) and FLAG-Tagged FASs and Expression of the Proteins in S9 Cells**—The strategies for construction of cDNAs encoding the wild-type FAS, single domain-specific mutants and for introduction of His\(_c\) or FLAG tags have been described in detail previously (16, 18, 21, 22). The carboxy-terminal FLAG-tagged C161A mutant was constructed by first generating a mutated partial cDNA fragment by polymerase chain reaction amplification, using a primer set M161T3/54B1152 and pFAS 74.20 (partial FAS cDNA in pUCBM20) as template (21). Authenticity of the amplification product was confirmed by DNA sequencing, and the fragment was moved stepwise into the full-length, wild-type carboxyl-terminal FLAG-tagged construct. The quadruple mutant (C161A,S581A,S2151A,S2302A) was assembled stepwise by incorporating partial cDNA fragments carrying individual mutations in to full-length FAS cDNA. At each step, successful introduction of the new substituting residue was confirmed by DNA sequencing. The final FAS 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 according to the manufacturer instructions. S9 cells were then infected with the purified recombinant viruses and cultured for 48 h at 27 °C. The tagged FAS proteins were partially purified from the cytosol as described earlier (21) and then subjected to final purification by affinity chromatography (22); glyceral (10%, v/v) was included in all buffers used for chromatography.

**Formation and Purification of Heterodimeric FASs**—To confirm the identity of each of the mutant FAS proteins, portions of the cell extract were run on a sodium dodecyl sulfate (SDS)-polyacrylamide gel, blotted to nitrocellulose membranes and exposed successively to either murine anti-FLAG M2 antibodies (Eastman Kodak Co.) and nickel-nitrilotriacetic acid/agarose (Qiagen, Inc., Santa Clarita, CA) as described previously (22). The FAS subunits were detected by preincubation with a monoclonal antibody (FAS-10) directed against a eukaryotic protein (19). The latter heterodimer was used in lieu of a C161A,S2151A/FLAG type heterodimer (presently unavailable), because the S581A and S2302A mutations, which compromise specifically the malonyl/acyl transferase and \( \beta \)-ketoacyl synthase activities, respectively (17, 18), were found to have no effect on the reaction of the FAS with dibromopropanone (see "Results"). For clarity, this mutant is identified in the text by the two mutations that are relevant to this study, namely C161A,S2151A.

**Modification of FAS with Dibromopropanone**—The FAS storage buffer was replaced with 0.2 M sodium phosphate, pH 7, containing 1 mM EDTA, either by successive dilution and concentration in Centri-con-50 devices (Amicon, Inc., Beverly, MA), or centrifugation through a gel filtration column (23). Fresh solutions of dibromopropanone were prepared by evaporation of the benzene solvent from the stock solution and redissolution of the reagent in phosphate buffer. The concentration of the reagent was determined before every experiment as described previously (20). The cross-linking reaction was carried out at room temperature according to the manufacturer instructions. After completion of the reaction, the samples were dialyzed against 50 mM Tris/HCl, pH 8, and precipitated with 100% acetone. Mass Spectrometry—The MALDI-TOF experiments were performed under conditions that result in protein denaturation and obliteration of specific noncovalent interactions. Mass spectra were recorded on a Voyager DE STR instrument (PerSeptive Biosystems, Framingham, MA), equipped with a two-stage ion source, delayed extraction (24), and a high current detector. The Voyager/Gramps software supplied with the instrument was used for instrument control and data analysis. All measurements were carried out in the positive ion mode. The linear path length in this instrument is 2.0 m. Desorption ionization was initiated with a nitrogen laser operating at 337 nm with a repetition rate of 3 Hz. The overall acceleration voltage used was 25 kV. A sampling rate of 20 ns (50 MHz) and an input filter of 20 MHz were used for data collection. Each spectrum represents the sum of 150–250 individual laser shots, and mass measurements were done automatically using the instrument.
default calibration. FAS samples (0.5 μl in 5 mM ammonium acetate, pH 7.8) were mixed with an equal volume of matrix solution directly on the stainless steel sample target plate and allowed to dry at room temperature. The matrix solution consisted of 10 g/liter of sinapinic acid dissolved in 0.3% aqueous trifluoroacetic acid:acetone (2:1, v/v).

Figure 1—Modification of Fatty Acid Synthase by Dibromopropane—The storage buffer was replaced with the reaction buffer using two consecutive centrifuged gel filtration columns. The enzyme during reaction was at 0.9 mg/ml, and dibromopropanone was at 1.5-fold molar excess over FAS subunit concentration. A, SDS-polyacrylamide gel electrophoresis analysis of dibromopropane-modified FAS. Lane 1, wild-type FAS; lane 2, wild-type FAS after 60 s of exposure to dibromopropanone. Phosphorylase b cross-linked dimers (195 kDa) and hexamers (584 kDa), which exhibit near ideal electrophoretic behavior under the conditions employed, migrate close to the bottom and top of the gel, respectively. B, inhibition of fatty acid synthesis and β-ketobutyryl CoA reduction activities of FAS by dibromopropanone and quantitation of the residual species i (all expressed as percent of 0 time values).

Enzyme Assays—Overall fatty acid synthesizing activity was measured spectrophotometrically (25); one unit of FAS activity is defined as 1 μmol of NADPH oxidized per min at 37 °C. β-Ketobutyryl-CoA reductase activity of FAS was determined by monitoring the accompanying oxidation of NADPH as described earlier (17). The interthiol decanoyl transferase activity of the β-ketoacyl synthase domain FAS was monitored by high performance liquid chromatographic separation of substrates and products as described previously (26).

Hydrolysis of Propanoyl Bond by High pH—The cross-linked products were first separated on 1.5-mm SDS-polyacrylamide gels. Protein bands, revealed using an imidazole-zinc negative stain (27), were cut out; and metal ions were removed by incubation of the gel pieces successively with 0.5 M EDTA, pH 8, and water, each for 10–15 min. The proteins were then electroeluted in a Centricon (Amicon) using Centricon-50 or -100 units. During electroelution, the Tris-glycine-0.1% Triton X-100 buffer was replaced with the reaction buffer using two consecutive centrifuged gel filtration columns. The enzyme during reaction was at 0.9 mg/ml, and dibromopropanone was at 1.5-fold molar excess over FAS subunit concentration. A, SDS-polyacrylamide gel electrophoresis analysis of dibromopropane-modified FAS. Lane 1, wild-type FAS; lane 2, wild-type FAS after 60 s of exposure to dibromopropanone. Phosphorylase b cross-linked dimers (195 kDa) and hexamers (584 kDa), which exhibit near ideal electrophoretic behavior under the conditions employed, migrate close to the bottom and top of the gel, respectively. B, inhibition of fatty acid synthesis and β-ketobutyryl CoA reduction activities of FAS by dibromopropanone and quantitation of the residual species i (all expressed as percent of 0 time values).

RESULTS

Reaction Conditions for Inactivation and Cross-linking of FAS by Dibromopropanone—In agreement with the earlier findings of Stoops and Wakil (6, 28), as a consequence of treatment of the FAS with dibromopropanone, we observed the formation of three new molecular species with retarded electrophoretic mobilities on SDS-polyacrylamide gels (Fig. 1A, species ii, iii, and iv). Typically, when a centrifuged gel filtration column was used to remove dithiothreitol from the preparation and the FAS was exposed to a 1.5-fold molar excess of reagent for 60 s, the species exhibiting retarded electrophoretic mobilities accounted for more than 80% of the total FAS species present. Formation of the three new molecular species occurred extremely rapidly and was accompanied by a loss in the ability of the protein to synthesize fatty acids; only 4% of the FAS activity remained after 60 s exposure to dibromopropanone (Fig. 1B). The rate of loss in ability to reduce β-ketobutyryl-CoA to butyryl-CoA was slower; about 15% of the activity remained after 60 s (Fig. 1B). For the overall FAS reaction, complete integrity of both the β-ketoacyl synthase active-site cysteine and the phosphopantetheine of the ACP domain are crucial. For the reduction of β-ketobutyryl-CoA by the FAS, the β-ketoacyl synthase active-site cysteine is not required. Translocation of the β-ketobutyryl moiety from CoA to the ACP domain, reduction of the keto group and translocation of the product back to CoASH involves only the malonyl/acyetyltransferase, the phosphopantetheine thiol and the β-carbon processing enzymes. The difference in rate of loss of these two activities suggested that perhaps the active-site cysteine residue is the primary target of the reagent, and indeed the rate of disappearance of the original species i paralleled closely the rate of loss of β-ketobutyryl-CoA reductase activity rather than the rate of loss of overall fatty acid synthesizing activity.

This inference was further supported by the results of an experiment that compared the loss in β-ketobutyryl-CoA reductase and interthiol acyltransferase activities of FAS treated with different molar ratios of dibromopropanone. The interthiol acyltransferase reaction is catalyzed exclusively by the β-ketoacyl synthase domain and uses model substrates to assess the ability of the β-ketoacyl synthase to transfer acyl moieties between substrate and acceptor thioles via the cysteine nucleophile (26). Two different homodimeric FAS mutants were employed for this experiment, C161A, in which the β-ketoacyl synthase nucleophile is replaced, and S2151A, in which the site of posttranslational insertion of the phosphopantetheine is removed. In the presence of a two-fold molar excess of reagent, 90% of the interthiol acyltransferase activity associated with the S2151A mutant was eliminated (Fig. 2). Under identical conditions, less than 20% of the β-ketobutyryl-CoA reductase activity of the C161A mutant was lost. To achieve 90% loss in β-ketobutyryl-CoA reductase activity with this mutant, more than a 25-fold molar excess of reagent was required. In contrast, only a 1.5-fold molar excess of reagent was required to eliminate 85% of the β-ketobutyryl-CoA reductase activity associated with the wild-type FAS (Fig. 1B). Clearly in the absence of Cys-161, the phosphopantetheine thiol attached to Ser-2151 is a relatively poor target for dibromopropanone.

These results are consistent with Cys-161 being the primary target for the reagent in the wild-type FAS. Reaction of the second alkylating group of the bifunctional reagent with the phosphopantetheine thiol likely is facilitated by the proximity of the phosphopantetheine to the cysteine nucleophile, rather than by the inherent reactivity of the phosphopantetheine thiol.
Identification of the Three Molecular Species Formed by Reaction of Dibromopropanone with the FAS—Three separate approaches were used to identify the three electrophoretic species formed uniquely as the result of exposure of the FAS to dibromopropanone. First, the electrophoretic species formed by treatment of a panel of mutant homo- and heterodimers were analyzed by general protein staining. Second, the presence or absence of individual subunits in each electrophoretic species was assessed by detection of the specific tags using Western blotting. This procedure clearly distinguishes between FAS polypeptides containing the His_{sg} and FLAG tags because neither are the His_{sg}-tagged homodimeric FASs recognized by the anti-FLAG antibodies nor are the FLAG-tagged homodimers by the anti-His antibodies (22). Third, the molecular masses of the various species formed as the result of dibromopropanone treatment of the mutant FASs were assessed by gel filtration and mass spectrometry.

The relative proportions of species i–iv present in the dibromopropanone-treated FAS were dependent on the method used to remove dithiothreitol from the preparations. Considerably more of the unmodified FAS species i remained when either the centrifuged gel filtration column or dialysis procedures were employed than when the Centricon-50 device was used. The proportion of species iii formed was also higher when the latter procedures were used. We attribute these quantitative differences to the susceptibility of the Cys-161 thiol to oxidation. The dialysis procedure is inherently slow, and the centrifuged gel filtration column procedure potentially exposes the FAS protein solution to a very large surface area after loading on the column and spanning out the solvent. The Centricon procedure, on the other hand, is relatively speedy and does not expose the FAS solution unduly to air. Typically then, using the Centricon procedure to remove dithiothreitol, more than 95% of the wild-type FAS could be cross-linked by dibromopropanone. Under these conditions, the major products formed are species iv, 45 ± 5%, and species ii, 35 ± 2%; species iii and species i account for only 15 ± 5 and 5 ± 2%, respectively. All subsequent experiments were performed using FASs that were freed of dithiothreitol using the Centricon procedure.

On treatment with dibromopropanone, neither FAS dimers containing the C161A mutation in both subunits nor those containing the S2151A in both subunits formed any of the unique electrophoretic species produced by the wild-type FAS, as revealed by Pro-Blue protein staining (Fig. 3, compare A and B). This finding confirmed the original observation of Stoops and Wakil (28) that only a cysteine and a pantetheine thiol are involved in the reaction of dibromopropanone with the chicken FAS and demonstrated that these thiols are located at positions 161 and 2151 in the rat FAS. Because mutants lacking the thiol at residue 2151 are still able to react with dibromopropanone at the Cys-161 thiol (see Fig. 2), it is clear that derivatization of FAS at a single site, i.e. without cross-linking, does not alter its electrophoretic mobility.

Dibromopropanone-treatment of a heterodimer consisting of one FLAG-tagged wild-type subunit and one His_{sg}-tagged subunit carrying the S2151A mutation also resulted in the formation of only one new species, this time species iii, as revealed by staining with Pro-Blue (Fig. 3D, panel 1). However, in this case, both the FLAG-tagged (C161A) and His_{sg}-tagged (S2151A) subunits were present in species iii (Fig. 3D, panels 2 and 3). Because the heterodimer containing the C161A and S2151A mutations on opposite subunits can form neither intrasubunit cross-links nor double intersubunit cross-links, and because species iii contains both subunits, it follows that species iii must be a singly cross-linked dimer.

A similar result was obtained by dibromopropanone treatment of a heterodimer consisting of one FLAG-tagged wild-type subunit and one His_{sg}-tagged subunit carrying the S2151A mutation. Species ii and iii were formed but not species iv, as evidenced by staining with Pro-Blue (Fig. 3E, panel 1) and again species ii contained only the wild-type subunit, whereas species iii contained both the wild-type and the C161A mutant subunits (Fig. 3E, panels 2 and 3). These results support the earlier inference that species ii is a cross-linked intra-subunit, whereas species iii is a singly cross-linked dimer.

For comparison, the analysis of the products formed by dibromopropanone treatment of a wild-type dimer containing independently tagged subunits was also included in this series of experiments. All three species formed characteristically as a result of exposure to dibromopropanone (species ii, iii, and iv) are in evidence, as revealed by staining with Pro-Blue (Fig. 3A, panel 1), and all three contain both wild-type subunits, as revealed by Western blotting (Fig. 3A, panels 2 and 3). In this case, either of the wild-type subunits is able to form intrasubunit cross-links so that species ii contains both FLAG-tagged and His_{sg}-tagged subunits. The observation that species iv, which contains both subunits, is formed only when the Cys-161 and phosphopantetheine thiols are preserved on both subunits is consistent with this species representing a doubly cross-linked dimer in which the Cys-161 thiols of each subunit are cross-linked to the phosphopantetheine thiols of the opposite subunit.

Estimation of the Molecular Masses of the Cross-linked FAS Species by Gel Filtration and Mass Spectrometry—When the products of dibromopropanone treatment of the wild-type FAS were subjected to gel filtration, under nondenaturing conditions, a single protein zone emerged from the column with an elution time characteristic of the FAS dimer (details not shown), indicating that no oligomers larger than the dimer are formed by cross-linking.

The three molecular species produced by dibromopropanone treatment of the wild-type FAS were isolated from the acrylamide gel by electroelution, and most of the free SDS was
removed using SDS-OUT™ (Pierce). However, all attempts to remove the residual protein-bound SDS, by dialysis against 0.25% octyl β-glucoside or by extraction with organic solvent, produced a largely insoluble residue that yielded no detectable signal in MALDI-TOF mass spectrometry. We therefore resorted to determining the masses of the various cross-linked species by subjecting to mass spectrometry the entire mixtures of products formed by each of the FAS mutants, without prior exposure to SDS.

Given that species in the MALDI ion source can generate ions only by addition of an integral number of charged particles (protons in this case), one can distinguish between monomers and dimers. FAS dimers (546 kDa) can theoretically give rise to peaks at \( m/z \) 546 (1+), \( m/z \) 273 K (2+), \( m/z \) 182 K (3+), \( m/z \) 136 K (4+), \( m/z \) 109 K (5+), \( m/z \) 91 K (6+), etc., whereas FAS monomers can give rise to peaks at \( m/z \) 273 K (1+), \( m/z \) 136 K (2+), \( m/z \) 91 K (3+) etc. Thus peaks representing odd multiply charged states of the dimer provide a unique signature for the presence of the cross-linked dimeric form of the protein. None of these species is produced in any significant amount from the native wild-type FAS, consistent with there being no covalent links between the subunits (Fig. 4A).
The conditions under which MALDI-TOF experiments are carried out almost always result in near complete disruption of any noncovalent associations that may be present. Nevertheless, the trace of m/z 182 K species detected was reproducible and persisted after incubation of the FAS with 3 mM dithiothreitol at 20 °C for 15 min, suggesting that it did not represent an artifact resulting from disulfide bond formation between subunits. This phenomenon, termed “nonspecific protein ion clustering” in MALDI mass spectra has been observed previously and reported by others (29, 30). In contrast, wild-type FAS that had been exposed to dibromopropanone generated strong peaks at both m/z 182 K and m/z 109 K species (Fig. 4B), confirming that cross-linking had occurred between subunits.

The mass spectrum of the dibromopropanone-treated wild-type FAS exhibits a molecular ion distribution skewed toward the doubly charged species and the singly charged molecular ion (m/z 546 K, in this case), which is typically found in MALDI-TOF mass spectra, is missing. The observed molecular ion distribution is a product of both the propensity of a molecule for acquisition of charges under the conditions of ionization and sensitivity of the detector for molecular ions at various m/z values. Given, that there is little data available on MALDI-TOF analysis of proteins larger than 200 kDa, it is not clear at the moment whether the absence of the singly charged species (m/z 546 K) is characteristic of large protein molecules, or is an artifact resulting from detector sensitivity bias.

The dimer signature species, m/z 182 K and m/z 109 K, were also formed from heterodimers consisting of one subunit bearing the C161A mutation and the other bearing the S2151A mutation (Fig. 4D). Because this heterodimer can only form a single intersubunit cross-link (species iii, Fig. 3D), the mass spectrometric data confirm that the electrophoretic species iii (present at the level of ~35%) is indeed a cross-linked dimer. Heterodimers consisting of a wild-type subunit paired with a C161A,S2151A doubly mutated subunit, after dibromopropanone treatment, did not generate either the m/z 182 K or m/z 109 K species (Fig. 4C). Thus the electrophoretic species ii (present at the level of ~30%) derived from this heterodimer (Fig. 3C) cannot be a dimer and must therefore represent an internally cross-linked subunit.

Because the electrophoretic species i and ii as well as species iii and iv have identical molecular masses, 272 and 544 kDa, respectively, it is clear that the various FAS species exhibit anomalous electrophoretic behavior. The reason is not known but likely results from differences in SDS-binding capacities and/or shape of the various species.

Reversal of Cross-linking by Exposure to Elevated pH—Given that only the Cys-161 and phosphopantetheine thiols participate in formation of the cross-linked species iv and that both thiols must be available on both subunits in order that species iv can be formed, species iv must contain two cross-links. However, because cross-linking can occur both inter- and intrasubunit, it is possible that a doubly cross-linked dimer could be formed by two intrasubunit cross-links that interlock the subunits like two links in a chain. Were this the case, then cleavage of one or both of the cross-links would generate only single subunit species, and species ii, the single subunit cross-linked internally, would be expected to be formed as an intermediate. On the other hand, if species iv is in fact a dimer containing two intersubunit cross-links, then cleavage of one of the cross-links ought to generate an intermediate corresponding to the singly cross-linked dimer, identified as species iii. To distinguish between these two possibilities, we made use of the inadvertent discovery that propanone cross-links are susceptible to cleavage by mild alkaline treatment. This treatment has no effect on the integrity of FAS that has not been exposed to dibromopropanone (Fig. 5, lanes 2 and 3). Species iv, formed by treatment of the wild-type FAS with dibromopropanone and isolated by preparative SDS-polyacrylamide electrophoresis is approximately 70–75% pure (Fig. 5, lane 4). Exposure of species iv to high pH for 1 and 2 h resulted in the appearance of increasing amounts of species iii but no species ii (Fig. 5, lanes 5 and 6). This result ruled out the possibility that cross-linked dimers could be formed by linking the two subunits through two intrasubunit cross-links. Thus species iv, which accounts for 45% of the cross-linked products, results from two intersubunit cross-links, and species ii, which accounts for 35%, results from a single intrasubunit cross-link. The singly cross-linked dimer, species iii, which accounts for 15% of the products, may be formed as a consequence of oxidation of a small fraction of the participating thiois at residues 161 and/or 2151, and/or from the presence of a small amount of apo-ACP in the preparation, and/or reaction of the second bromine atom with water.

These findings provide a simple explanation for the previously perplexing observation that, whereas cross-linking of the native FAS yields three unique species, cross-linking of FAS...
that has been nicked by trypsin yields only one new species. FAS that has been treated with trypsin is essentially a “nicked dimer” consisting of pairs of 125-kDa amino-terminal and 95-kDa carboxyl-terminal polypeptides (31); treatment with dibromopropanone gives only a single molecular species of 220 kDa (9). Clearly, the species formed by cross-linking between these two nicked subunits and the species formed by cross-linking of the 125- and 95-kDa species originating from the same subunit will be indistinguishable.

DISCUSSION

The two residues implicated by Stoops and Wakil (6, 28) as participants in the reaction of the chicken FAS with dibromopropanone correspond in the rat FAS to Cys-161, the nucleophile for the β-ketoacyl synthase reaction, and the phosphopantetheine at Ser-2151. When either of these residues is mutated to alanine, functionality of the targeted domain is compromised (16, 19). In the series of heterodimers engineered for this study, one or both subunits contained, at positions 161 and 2151, either the wild-type residues or the C161A or S2151A mutations, or both. Thus, dimers consisting of a wild-type subunit paired with either a C161A or S2151A subunit exhibited approximately half of the fatty acid synthesizing activity characteristic of the wild-type FAS as would be anticipated. On the other hand, dimers consisting of a C161A mutant subunit paired with a S2151A mutant subunit and those consisting of a wild-type subunit paired with a subunit containing both the C161A and S2151A mutations, exhibited only about 5–6% of the activity of the wild-type FAS (see “Experimental Procedures”). We have previously noticed that mutations in the β-ketoacyl synthase domain, particularly at the active-site cysteine appear to influence catalytic activity at the remaining functional center (16–18). The reason for these lower than expected activities is not immediately obvious and is the subject of current investigation. Nevertheless, it should be emphasized that in this study the FAS mutants are used solely as tools to identify each of the three cross-linked species formed by treatment of the wild-type FAS with dibromopropanone. Quantitative assessments of the proportions of the three species formed by the wild-type FAS can be made independently of any consideration of the specific activities of the various mutant FASs.

The classical model for the FAS that has prevailed since the early 1980s visualized the two subunits as lying side-by-side in a rigid, extended, antiparallel configuration. The first indication that this model might be inadequate came from a mutant complementation analysis that was designed to map the functional interactions occurring between domains of the FAS dimer. Several lines of evidence presented in this report support the hypothesis that, in the wild-type FAS dimer, the active-site cysteine residue of the β-ketoacyl synthase domains are positioned close to the phosphopantetheine thioles of both subunits: 1) species ii has a unique electrophoretic mobility that distinguishes it from unmodified subunits, subunits that are derivatized at only the Cys-161 site, and intersubunit cross-linked species; 2) species ii has a molecular mass consistent with that of a single subunit; and 3) species ii is formed only from a wild-type subunit; the absence of either of the thioles at residues 161 or 2151 precludes its formation.

This inference is entirely consistent with the conclusions drawn from mutant complementation analysis that mapped the functional interactions occurring between domains of the FAS dimer. These experiments indicated that functional contacts could be made between the β-ketoacyl synthase and ACP domains associated with the same or the companion subunits. Thus reinterpretation of the specificity of the reaction of the FAS with dibromopropanone provides the first structural evidence in support of conclusions derived from the mutant complementation analysis. Based on the relative proportion of the FAS species that undergo inter- and intrasubunit cross-linking, one can conclude that in the resting wild-type FAS, as much as 35% of the dimers adopt a conformation in which the active site cysteine residue Cys-161 is juxtaposed with the phosphopantetheine moiety attached to residue Ser-2151 of the same subunit. Thus the old model for the FAS (1–3) must be revised to accommodate the finding that head-to-tail structural and functional contacts are possible both between and within subunits. This new model implies a much greater flexibility in the interaction of domains within and between subunits than was implied by the earlier side-by-side, head-to-tail model.

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