Characterization of the Interthiol Acyltransferase Reaction Catalyzed by the β-Ketoacyl Synthase Domain of the Animal Fatty Acid Synthase†

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ABSTRACT: The enzyme activity responsible for translocation of saturated acyl chains from the 4′-phosphopantetheine of the acyl carrier protein to the active site cysteine of the β-ketoacyl synthase in the animal fatty acid synthase has been identified. An enzyme assay was devised that allows uncoupling of the interthiol transfer step from the condensation reaction. Experiments with various fatty acid synthase mutants indicate clearly that catalysis of the transfer of saturated acyl moieties from the 4′-phosphopantetheine thiol to the active site cysteine thiol, Cys-161, is an inherent property of the β-ketoacyl synthase domain. Catalytic efficiency of the interthiol transferase increases from C2 to C12 and decreases with increasing chain-lengths beyond C12. Malonyl, β-hydroxybutyryl, and crotonyl thioesters are not substrates for the transferase, whereas the β-ketobutyryl moiety is a poor substrate. These features of the substrate specificity are exactly as predicted for a transferase that fulfills the proposed role in the fatty acid synthase reaction sequence and indicate that this activity plays an important role in determining the overall specificity of the β-ketoacyl synthase reaction.

In animals, the *de novo* biosynthesis of fatty acids is catalyzed by a homodimer of 272-kDa polypeptides containing seven catalytic domains: β-ketoacyl synthase (EC 2.3.1.41), malonyl-CoA/acetyl-CoA:ACP[1] *S*-acyltransferase (EC 2.3.1.38/39), β-hydroxyacyl-ACP dehydrase (EC 4.2.1.61), enoyl-ACP reductase (EC 1.3.1.10), β-ketoacyl-ACP reductase (EC 1.1.1.100), and ACP and thioesterase (EC 3.1.2.14); for reviews see refs *1*−*3*. This multifunctional protein is known as the fatty acid synthase, FAS (EC 2.3.1.85). The key feature of the *de novo* pathway is the sequential extension of an alkanoyl chain, two carbon atoms at a time, by a series of Claisen condensation reactions. The "primer" substrate, usually an acetyl moiety, is first translocated from CoA to the FAS in a reaction catalyzed by the malonyl-CoA/acetyl-CoA:ACP S-acyltransferase[2] (Figure 1, step 1). The first covalent intermediate in the translocation pathway is located at Ser-581 (numbering is for rat FAS), the active site residue of the malonyl/acetyl transferase. Subsequently, the malonyl/acetyl transferase catalyzes translocation of the acetyl moiety to the thiol group of 4′-phosphopantetheine, which is attached to Ser-2151 in the ACP domain (step 2). In the final translocation step, the acetyl moiety is transferred from the 4′-phosphopantetheine to Cys-161, the active site residue of the β-ketoacyl synthase (step 3). The loading of malonyl moieties follows the same path (steps 1 and 2) except that no transfer occurs between the 4′-phosphopantetheine and Cys-161 thiols. Condensation of the acetyl and malonyl moieties ensues, catalyzed by the β-ketoacyl synthase, resulting in the formation of β-ketobutyryl-*S*-4′-phosphopantetheine and the release of CO_2 (step 4). Following complete reduction of the β-carbon atom in a series of three reactions catalyzed successively by the β-ketoacyl-ACP reductase, β-hydroxyacyl-ACP dehydrase, and enoyl-ACP reductase, the butyryl moiety formed is translocated back to Cys-161 in preparation for condensation with a second malonyl moiety (step 3).

In most tissues, seven cycles of elongation and reduction take place resulting in the formation of a C_{16} acyl moiety that is released by the thioesterase as free palmitic acid. The transferase activity responsible for translocation of successive saturated acyl moieties, C_2 to C_{14}, from ACP to the β-ketoacyl synthase active site, has not been characterized (identified by a question mark at step 3 in Figure 1). Since the activity of the malonyl/acetyl transferase decreases markedly with saturated acyl moieties of increasing chain length (*4*), it seems unlikely that this enzyme could be responsible for the transesterification of acyl moieties having 2−14 carbon atoms between the 4′-phosphopantetheine and Cys-161 thiols. The most likely possibility then is that the β-ketoacyl synthase possesses the necessary interthiol acyltransferase activity. The objective of this study was to devise an assay that would permit uncoupling of the interthiol acyltransferase and condensation reactions, allowing us to test the hypothesis that the interthiol acyltransferase reaction is catalyzed by the β-ketoacyl synthase domain, to identify residues essential for catalysis of the interthiol acyltransferase reaction, and to determine whether the substrate specificity of the enzyme is consistent with the proposed role in the FAS reaction sequence.

MATERIALS AND METHODS

Materials. The pantetheinyl thioester of decanoic acid was prepared from decanoyl chloride and pantetheine as described previously (*5*). Radiolabeled malonyl-CoA, acetyl-CoA, and palmitoyl-CoA were obtained from Moravek Biochemicals (Brea, CA).

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[1] Abbreviations: ACP, acyl carrier protein; FAS, fatty acid synthase; CoASH, reduced form of CoA; HPLC, high performance liquid chromatography; DTT, dithiothreitol.

[2] Subsequently referred to as the malonyl/acetyl transferase.

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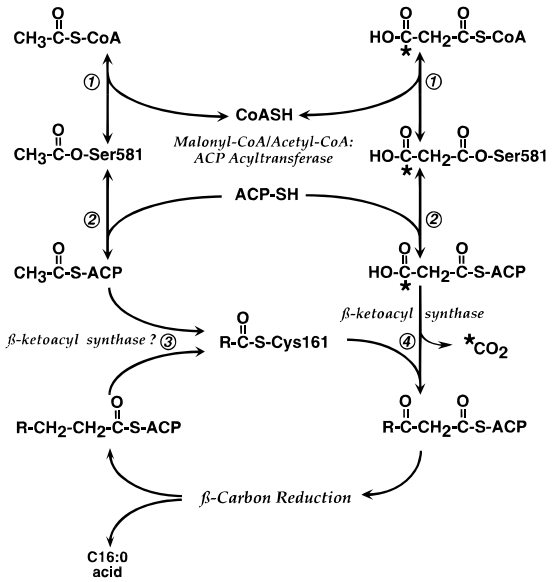


FIGURE 1: Substrate loading, acyl transfer, and condensation reactions catalyzed by the animal FAS. Acetyl and malonyl moieties are first loaded onto Ser-581, the nucleophilic residue of the dual specificity enzyme malonyl/acetyl transferase (step 1). Subsequently, acetyl and malonyl moieties are transferred to the 4′-phosphopantetheine of the ACP domain (step 2). The acetyl moiety, but not the malonyl moiety, is transferred from the 4′-phosphopantetheine to the active site cysteine of the β-ketoacyl synthase domain (step 3). Condensation of the acetyl moiety located at Cys-161 with the malonyl moiety attached to the 4′-phosphopantetheine is catalyzed by the β-ketoacyl synthase (step 4). Decarboxylation of a malonyl moiety attached to the 4′-phosphopantetheine thiol can also occur at a relatively slow rate when no acetyl moiety is present on the Cys-161 thiol; in this case, the decarboxylation is not accompanied by a condensation reaction. Following complete β-carbon reduction, the saturated butyryl moiety is transferred back to Cys-161, in preparation for the second condensation. Thus, each subsequent condensation reaction utilizes a saturated acyl moiety with two more carbon atoms than in the previous reaction so that R can be CH_3, $CH_3(CH_2)_2$, $CH_3(CH_2)_4$, etc. After seven cycles of condensation and β-carbon reduction, palmitic acid is released as the product. The mechanism of transfer of the saturated acyl moieties from ACP to Cys-161, step 3, has not been previously characterized and is the subject of this study.

[1-^{14}C]Decanoyl-CoA was synthesized from the sodium salt of [1-^{14}C]decanoic acid (Sigma Chemical Co., St. Louis, MO) using the mixed anhydride method, essentially as described previously (*6*). Triethylamine was replaced with the 15-crown-5 ether (Sigma) to activate sodium decanoate. When the reaction was complete, the mixture was acidified and extracted with hexane. [1-^{14}C]Decanoyl-CoA was purified from the aqueous phase using a C18 SepPak cartridge (Millipore,Waters Chromatography Division, Milford, CT). The cartridge was washed successively with water and 20% methanol, and then the product was eluted with 100% methanol. Radiochemical purity, assessed by reversed-phase HPLC (system 6) as described below, was 97.3% and the product contained no free CoASH.

Butyryl and crotonyl pantetheine thioesters were synthesized from the appropriate acid chloride or anhydride and pantethine, which had been reduced to pantetheine with a 1.2-fold excess of tributyl phosphine (Aldrich Chemical Co., Inc., Milwaukee, WI). Diketene (Aldrich Chemical Co.) was employed to synthesize β-ketobutyryl pantetheine thioester (*7*), and the *p*-nitrophenyl monoester of malonic acid (synthesized by Dr. Brian Sedgwick) was utilized for synthesis of malonyl pantetheine mono thioester (*8*). β-Hydroxybutyryl pantetheine thioester was synthesized by the mixed anhydride method from sodium (*R*)-β-hydroxybutyrate (Aldrich) after activation with 15-crown-5 ether as described above. Generally, when the reaction was complete, the pH was adjusted to 2−3, the mixture was extracted three times with petroleum ether, and the product purified from the aqueous phase using a C18 SepPak cartridge. Purity of the products, determined by HPLC was >92%, except for malonyl-pantetheine (72%) and β-hydroxybutyryl pantetheine (82%). None of the preparation contained free pantetheine.

Pantetheine used as an acceptor in acyltransfer reactions was derived from pantethine (Sigma) by reduction with a 2-fold excess of tris(2-carboxyethyl)phosphine (Calbiochem, San Diego, CA). Freshly prepared solutions of CoASH (Sigma) and *N*-acetylcysteamine (Aldrich) were stabilized by the inclusion of equimolar concentration of tris(2-carboxyethyl)phosphine. The presence of tris(2-carboxyethyl)phosphine had no effect on acyltransferase reaction rates (data not shown).

The sources of other materials used in this study were reported previously (*9*−*11*).

In Vitro Mutagenesis. The wild-type recombinant baculoviral transfer vector encoding the 2505-residue rat FAS was constructed as described earlier (*9*). *In vitro* site-directed mutagenesis was carried out by the overlap polymerase chain-reaction method (*12*), using Vent DNA polymerase. Details of the strategy employed to generate most of the mutants used in the present study have been described previously (*11*). Briefly, the mutated DNA fragment from overlap polymerase chain reaction was used to replace the corresponding region of the parent partial cDNA construct in pUCBM 20 vector and introduced into *Escherichia coli* DH5α competent cells (*13*). The sequence of the amplified region was confirmed, and the fragment was moved stepwise into the full-length FAS cDNA cloned in either transfer vector pBacPak9 or modified pFastBac1. The malonyl/acetyl transferase domain mutant cDNA construct, pET S581A, was obtained from Dr. V. S. Rangan (*14*) and used to generate the Ser-581 → Ala FAS mutant. An appropriate cDNA fragment from this construct was used to replace the corresponding region of pFAS74.20. The sequence was confirmed, and the cDNA fragment containing the mutation moved into the full-length FAS cDNA construct in a modified pFastBac1 transfer vector.

The final cDNA constructs were used to generate recombinant baculoviral stocks in *Sf*9 insect cells using standard insect cell culture techniques (*15*).

*Expression and Purification of Mutant FASs. Sf*9 cells were infected with purified recombinant viruses, cultured for 48 h at 27 °C, and the mutant FAS proteins isolated as described previously (*9*, *10*). The protein purification procedure was modified by the addition of 10% v/v glycerol to all buffers used during chromatography.

Enzyme Assays. Assays for the partial reactions of the FAS and the overall FAS reaction have been described previously (*16*).

Interthiol Acyltransferase Assay. A model reaction involving transesterification of decanoyl moieties between pantetheine and CoA thiols was routinely employed to assay this activity. Enzyme was incubated with the acyl thioester and free thiol acceptor in 225 mM potassium phosphate buffer, final volume 0.1 mL, at 37 °C for 1 min. Reactions were stopped by dilution with 10 vol of a mixture of the

starting buffer for HPLC and 1 M potassium phosphate, pH 5.4 (3:1, v/v). The material was analyzed immediately, as described below.

HPLC Systems for Separation of Substrates and Products of the Interthiol Acyltransferase Reactions. The chromatographic systems used to separate the CoA and pantetheine thioesters of the various acyl moieties tested as potential substrates in the model interthiol acyltransferase reaction are outlined below. Compounds were identified and quantitated by comparison of the elution position and UV absorbance with those of authentic standards. Pantetheine thioesters were detected at 232 nm and CoA thioesters at 258 nm. In some instances, compounds were also quantitated by measuring the associated radioactivity (acetyl, malonyl, decanoyl, and hexadecanoyl transfer from radiolabeled acyl-CoAs to pantetheine). In all cases, the CoA ester eluted ahead of the corresponding pantetheine ester, and both thioesters separated from the free thiols, CoASH and pantetheine.

System 1 (Acetyl and Malonyl Transfer). A C18 SpheriSorb, 300 Å, 5 μm, 4.6 × 250 mm column (PhaseSeparation Ltd., Deeside, Clwyd, U.K.) was developed at 35 °C, 1 mL/min, in 50 mM sodium phosphate, pH 5.5, for 5 min followed by a three-step gradient, to 27% 50 mM sodium phosphate, pH 5.5/10% acetonitrile over 5 min, to 37% 50 mM sodium phosphate, pH 5.5/10% acetonitrile over 10 min, and to 100% 50 mM sodium phosphate, pH 5.5/10% acetonitrile over 10 min.

System 2 (β-Ketobutyryl and β-Hydroxybutyryl Transfer). A Phenyl Intersil, 5 μm, 4.6 × 150 mm column (MetaChem Technologies, Torrance, CA) was developed at 45 °C, 1.5 mL/min, in 25 mM potassium phosphate, pH 5.4/3% acetonitrile for 5 min followed by a four-step gradient, to 9.8% acetonitrile over 2 min, to 11.65% acetonitrile over 1 min, to 25.9% acetonitrile over 7 min, and to 40.2% acetonitrile over 1 min.

System 3 (Butyryl and Crotonyl Transfer). The Phenyl Intersil column was developed at 45 °C, 1.5 mL/min, in 25 mM potassium phosphate, pH 5.4/5% acetonitrile for 5 min followed by a four-step gradient, to 24% acetonitrile over 2 min, 24% acetonitrile for 1 min, to 34.5% acetonitrile over 7 min, and to 40.2% acetonitrile over 1 min.

System 4 (Hexanoyl Transfer). The Phenyl Intersil column was developed at 45 °C, 1.5 mL/min, in 25 mM potassium phosphate, pH 5.4/5% acetonitrile for 5 min followed by a four-step gradient, to 28.8% acetonitrile over 2 min, 28.8% acetonitrile for 1 min, to 45.9% acetonitrile over 6 min, and to 81% acetonitrile over 2 min.

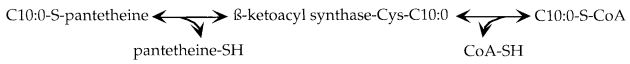
System 5 (Octanoyl, Decanoyl and Dodecanoyl Transfer). The Phenyl Intersil column was developed at 45 °C, 1.5 mL/min, in 25 mM potassium phosphate, pH 5.4/5% acetonitrile for 5 min followed by a four-step gradient, to 28.8% acetonitrile over 2 min, 28.8% acetonitrile for 1 min, to 57.3% acetonitrile over 10 min, and to 81% acetonitrile over 2 min.

System 6 (Tetradecanoyl and Hexadecanoyl Transfer). The Phenyl Intersil column was developed at 52 °C, 1.5 mL/min, in 25 mM potassium phosphate, pH 5.4/9.8% acetonitrile for 5 min followed by a four-step gradient, to 33.5% acetonitrile over 2 min, 33.5% acetonitrile for 1 min, to 70.6% acetonitrile over 13 min, and to 81% acetonitrile over 2 min.

Formation of a Covalent Acyl-FAS Thioester Intermediate. The FAS triple mutant, Ser581Ala/Ser2151Ala/Ser2302Ala (8 μM), was incubated with 0.1−1 mM [1-^{14}C]decanoyl-CoA at 37 °C for 2 min. The reaction was stopped by addition of a trichloracetic acid/ethanol mixture (10% and 50%, final concentrations, respectively). Bovine serum albumin (0.25 mg) was added as carrier and the reaction mixture was held at 0 °C for 15 min. The precipitate was collected by centrifugation, washed four times with 10% trichloracetic acid/50% ethanol, dissolved in 6 M guanidine hydrochloride, and radioactivity determined using EcoLite scintillation fluid (ICN Pharmaceutical Inc.). Blank values obtained by omitting FAS from the assay were always below 100 cpm. Lability of the [1-^{14}C]decanoyl-FAS to performic acid treatment was assessed as described previously (*17*).

RESULTS AND DISCUSSION

Uncoupling of the Interthiol-Acyltransferase and Condensation Reactions. The acyltransferase responsible for moving saturated acyl moieties, C_2 to C_{14}, from the ACP to the β-ketoacyl synthase domain must be capable of translocating these acyl moieties between the thiol of a pantetheine moiety and the thiol of Cys-161. Assuming that this transesterification reaction likely is readily reversible, we designed an assay using two pantetheine-containing compounds, pantetheine and CoA to mimic the role of ACP. Thus, decanoyl-*S*-pantetheine was used as a model acyl donor and CoASH as a model acceptor. The use of different thiol compounds as acyl donor and acceptor facilitated chromatographic distinction between the substrate and product in the acyltransferase reaction.



Initially, a decanoyl moiety was chosen as the acyl group, because medium chain-length acyl thioesters are poor substrates for both the malonyl/acetyl transferase (*18*) and thioesterase (*19*) domains of FAS. Dependence of reaction velocity on the concentration of both substrate and acceptor was readily demonstrable (Figure 2). Translocation of malonyl and acetyl moieties between FAS and CoA thioester involves the obligatory formation of a covalent acyl-FAS intermediate at residue Ser-581 within the malonyl/acetyl transferase domain (*20*, *21*). Mutation of this active site serine residue to alanine, in the context of the isolated malonyl/acetyl transferase domain completely eliminates malonyl/acetyl transferase activity (*14*). However, introduction of this mutation into the FAS had little effect on the decanoyl-*S*-pantetheine:CoA *S*-acyltransferase activity, indicating that the malonyl/acetyl transferase domain is not responsible for catalysis of the interthiol medium chain-length acyltransferase reaction (Figure 2A). These results revealed that the FAS possesses an intrinsic interthiol acyltransferase activity, distinct from the malonyl/acetyl transferase, that can be assayed in the absence of an accompanying condensation reaction.

Localization of the Domain Responsible for Catalysis of the Interthiol Acyltransferase Reaction. In order to assign the interthiol acyltransferase activity to a specific region of the multifunctional protein we utilized several recombinant FAS mutants: Cys-161 → Ser, Cys-161 → Thr, Lys-326 → Ala, and Lys-326 → Leu. The properties of the first three mutants have been described previously (*16*). All four mutants lacked the ability to synthesize fatty acids and the only partial activity affected by the mutation was the condensation reaction catalyzed by the β-ketoacyl synthase domain (details not shown). An exception was the Cys-161

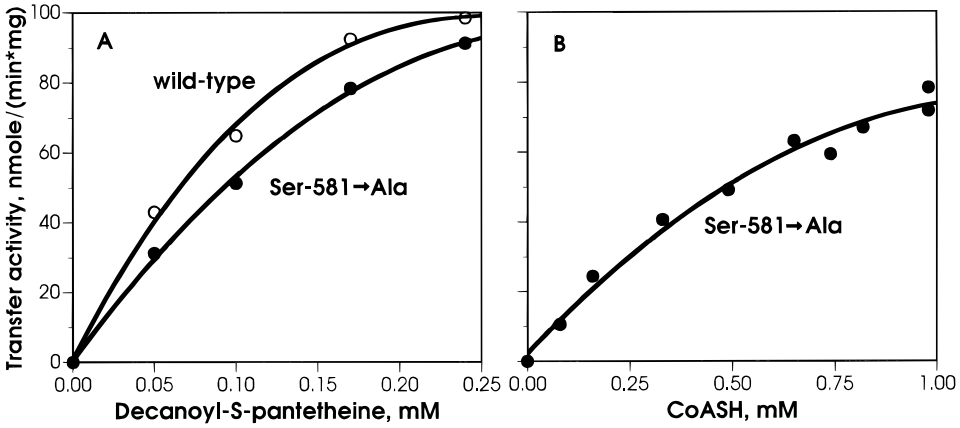


FIGURE 2: Transfer of decanoyl from pantetheinyl thiol to CoASH by FAS and malonyl/acetyl transferase FAS mutant. Enzymes were incubated with substrate and acceptor in 0.225 M potassium phosphate, pH 7, at 37 °C for 1 min. The reaction products were analyzed by HPLC. (A) CoASH acceptor at 1 mM. (B) Decanoyl-pantetheine donor at 0.17 mM.

Table 1: Interthiol Decanoyl Transfer by FAS Mutants[a]

FAS mutation	targeted domain	transferase activity (% of wild-type)[b]
Ser-581 → Ala	malonyl/acetyl transferase	72.7 ± 0.1
Ser-2151 → Ala	ACP	79.8 ± 1.3
Ser-2302 → Ala	thioesterase	86.9 ± 0.8
triple mutant[c]		90.5 ± 0.9
Lys-326 → Ala	β-ketoacyl synthase	0.9 ± 0.0
Lys-326 → Leu	β-ketoacyl synthase	0.2 ± 1.1
Cys-161 → Ser	β-ketoacyl synthase	0.2 ± 0.1
Cys-161 → Thr	β-ketoacyl synthase	0.9 ± 0.6

[a] FAS enzymes were incubated with 0.2 mM decanoyl-pantetheine and 0.8 mM CoASH in 225 mM potassium phosphate buffer, pH 7, at 37 °C for 1 min. The reaction products were analyzed by HPLC. [b] Specific activity determined for the wild-type enzyme was 105.5 ± 6.6 nmol/(min mg) of decanoyl moieties transferred to CoASH. [c] Ser-581 → Ala, Ser-2151 → Ala, Ser-2302 → Ala.

→ Ser mutant which retained about 5% β-ketoacyl synthase activity (*16*).

As shown above, mutation of Ser-581 to Ala had little affect on interthiol acyltransferase activity. Similarly, mutation of Ser-2151 to Ala, which prevents attachment of the 4′-phosphopantetheine prosthetic group to the ACP domain (*11*), had little effect on decanoyl-S-pantetheine:CoA acyltransferase activity, indicating that this domain does not participate in the reaction with the model substrates (Table 1). Replacement of the active site serine residue of the thioesterase domain with alanine also did not appreciably reduce activity, indicating that the interthiol acyltransferase activity was not associated with this domain (Table 1). Replacement of all three residues, Ser-581, Ser-2151, and Ser-2302, with alanine in a triple mutant had no significant affect on interthiol acyltransferase activity. However, replacement of the β-ketoacyl synthase active site Cys-161 with Ser or Thr lowered interthiol acyltransferase activity by more than 2 orders of magnitude. This result was consistent with the involvement of the β-ketoacyl synthase active site cysteine as the recipient residue in the interthiol acyltransferase reaction, as originally suggested for the *E. coli* FAS system (*22*). Lys-326, which is positionally conserved in β-ketoacyl synthases (*23*) and is essential for catalysis of the overall condensation reaction (*11*), when mutated to Ala or Leu, also resulted in dramatically lowered interthiol acyltransferase activity. This effect of these mutations does not appear to result from a global conformational change in the FAS since the Lys326Ala and Lys326Leu mutants exhibit the same resistance to limited proteolysis by trypsin as does the wild-type FAS and they are able to complement mutant FASs that lack functional dehydrase, ACP, or thioesterase domains (*16*). Neither is the effect likely to result from perturbation of the conformation of the β-ketoacyl synthase domain since the rate of decarboxylation of malonyl moieties bound to the ACP phosphopantetheine thiol, catalyzed by the β-ketoacyl synthase domain of the mutants (see Figure 1), is similar to that of the wild-type FAS (details not shown). Implication of both Cys-161 and Lys-326 of the β-ketoacyl synthase domain provided support for the hypothesis that the interthiol acyltransferase reaction is indeed catalyzed by this domain.

Kinetics of the Interthiol Acyltransferase Reaction. Again, for the reasons outlined above, the FAS triple mutant, Ser581Ala/Ser2151Ala/Ser2302Ala, was used to investigate the kinetics of decanoyl transfer by β-ketoacyl synthase. The reaction was assayed in both directions, from CoA thioester to pantetheine and from pantetheine to CoASH, at different concentrations of donor and acceptor. Double reciprocal plots of the data yielded a series of parallel straight lines and secondary plots of slopes and intercepts also gave straight lines (Figure 3). This type of kinetic data is indicative of formation of binary complexes in a two-step transfer reaction, referred to as a ping-pong mechanism (*24*). The kinetic constants calculated from the secondary plots for the decanoyl transfers, as well as those for several other acyltransfer reactions performed at only a single concentration of acceptor or donor, are presented in Table 2. Pantetheine and CoA both function well in the role of acyl donor or acceptor, yielding values for k_{cat} in the range ~4 s^{-1}. However the K_m for decanoyl pantetheine was significantly lower than for decanoyl-CoA. *N*-Acetyl cysteamine, which does not contain a complete pantetheine moiety, functions less efficiently in either role, and values for k_{cat}/K_m are 1−2 orders of magnitude lower than values obtained with the pantetheine-containing compounds. Dithiothreitol is a very poor acceptor in the interthiol acyltransferase reaction. These results supported our initial premise that pantetheine-containing compounds should be valid model substrates, replacing ACP in the interthiol acyltransferase reaction.

Dependence of the Interthiol Acyltransferase Reaction on pH. The effect of pH on interthiol acyltransferase activity was assessed using the triple mutant FAS, Ser581Ala/Ser2151Ala/Ser2302Ala, and compared with that of the

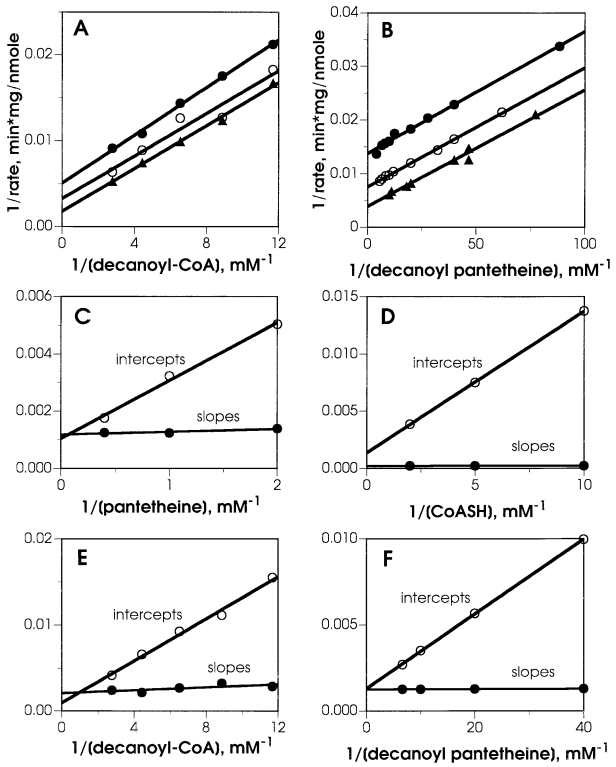


FIGURE 3: Kinetic analysis of the interthiol acyltransferase reaction. The triple mutant Ser581Ala/Ser2151Ala/Ser2302Ala (10 μg/0.1 mL) was incubated with various concentrations of donor and acceptor in 0.225 M phosphate buffer, pH 7.6, at 37 °C for 1 min. The reactions were analyzed by HPLC as described in Materials and Methods. Panels A, C, and E show data for decanoyl transfer from CoA to pantetheine (●, 0.5, ○, 1.0; and ▲, 2.0 mM in panel A) whereas panels B, D, and F show data for decanoyl transfer from pantetheine to CoASH (●, 0.1, ○, 0.2; and ▲, 0.5 mM in panel B). Panels C−F display secondary plots in which the both the intercepts on the 1/v axes (○), and the slopes (●) of the primary plots are plotted against 1/s. In the secondary plot, the intercept on the 1/v axis yield the value for 1/V, and the intercept on the 1/s axis yields the value for $-1/K_m$.

nonenzymic rate of transfer (Figure 4). The pH optimum for the enzyme-catalyzed reaction was between pH 7.4 and 8.0, whereas the rate of nonenzymatic acyltransfer increased steadily over the entire pH range studied. At pH 7.6, the catalytic enhancement factor, k_{cat}/k_{chem} is 2×10^5 mM^{-1} or, at 2.5 mM pantetheine, $k_{cat}/k(app)_{chem}$ is 4.2×10^4. The pH/activity profile for the enzyme-catalyzed acyltransfer was identical regardless of whether CoA or pantetheine was used as the decanoyl donor or acceptor and resembles a simple ionization curve of a single group, the enzyme being in the active form when that group is in its basic form. The apparent pK_a of this residue, approximately 6.9, is consistent with the possible involvement of a histidine residue in the reaction. On the basis of multiple sequence alignments and theoretical considerations, Siggaard−Anderson has proposed key roles for both lysine and histidine residues in the overall β-ketoacyl synthase reaction (*23*).

Formation of a Covalent Acyl-S-β-Ketoacyl Synthase Intermediate. The ping-pong kinetics observed for the interthiol decanoyltransferase reaction catalyzed by the β-ketoacyl synthase domain indicated that a covalent acyl-enzyme intermediate is formed prior to transfer of the acyl moiety to the acceptor molecule. This possibility was further investigated using the triple mutant Ser581Ala/Ser2151Ala/Ser2302Ala in which all potential sites for covalent attachment of acyl moieties have been eliminated except for Cys-161. This FAS cannot load substrates *via* Ser-581, the active site residue of the malonyl/acetyltransferase, and since it lacks the 4′-phosphopantetheine moiety that is normally attached to Ser-2151, it cannot form covalent acyl-enzyme intermediates at this site. In addition, this FAS lacks thioesterase activity and therefore cannot destroy acyl-CoA substrates by thioester hydrolysis and nor can it form covalent acyl-enzyme intermediates *via* Ser-2302. On incubation of the triple mutant FAS with [1-^{14}C]decanoyl-CoA, a covalent [1-^{14}C]decanoyl-FAS complex was formed (Figure 5). Saturation of the available binding sites was reached with ∼0.5 mM substrate, but the stoichiometry was only ∼0.5 mol decanoyl moieties bound per mol FAS subunit. The same stoichiometry was obtained using [1-^{14}C]butyryl-CoA at a single concentration, 0.7 mM (Figure 5). All of the decanoate was released from the FAS on exposure to performic acid indicating that the [1-^{14}C]decanoyl moiety was linked to the FAS by a thioester bond. The reason for the low stoichiometry is not readily apparent. One possibility is that in some FAS molecules the Cys-161 thiol is blocked, perhaps by some metabolite that bound to the FAS *in vivo*, prior to purification of the protein. Indeed this does appear to be the case since we found that prior exposure of the triple mutant FAS to either CoASH or neutral hydroxylamine increased subsequent binding of decanoyl moieties by 10−15%. Both procedures might result in removal of the bound moiety, by a catalyzed transfer to the CoASH acceptor or by the chemical formation of a hydroxamate. However, this explanation cannot entirely account for the low stoichiometry and, at this time, we cannot entirely rule out the possibility that there are restraints on the FAS that prohibit the simultaneous acylation of the two Cys-161 thiols present in the homodimer. Despite this uncertainty over the stoichiometry, these experiments indicate clearly that, in the triple mutant, the site of acyl-FAS formation is a thioester, and since the only remaining site for formation of a covalent acyl-enzyme species in this mutant is Cys-161, this residue is the nucleophile in the interthiol acyltransferase reaction.

Substrate Specificity of the Interthiol Acyltransferase Reaction Catalyzed by the β-Ketoacyl Synthase. On the basis of prior knowledge of the properties of the animal FASs, it is possible to predict certain features of the interthiol acyltransferase responsible for translocation of acyl chains between the 4′-phosphopantetheine and the β-ketoacyl synthase active site cysteine residue. An earlier detailed analysis of the distribution of covalent intermediates in the FAS reaction revealed that the concentration of saturated acyl moieties associated with the FAS decreased with increasing chain length up to C14, indicating that the initial condensation steps are slower than analogous reactions with longer chain-length intermediates (*25*). In addition, from the ratio of intermediates associated with the β-ketoacyl synthase active site cysteine and 4′-phosphopantetheine thiols (cysteine/pantetheine), these authors calculated the equilibrium constants for transesterification of acyl moieties between cysteine and 4′-phosphopantetheine; for chain-lengths shorter than C12 the ratios were >1, indicating that these intermediates are readily transferred from the 4′-phosphopantetheine (the site of formation of the saturated acyl moieties) to the cysteine in preparation for further elongation. The ratio became progressively smaller for C14, C16, and C18, where the value was 0.05, indicating a decreasing tendency for these

Table 2: Kinetic Parameters of the Decanoyl Transfer Reaction Catalyzed by the FAS Triple Mutant[a]

decanoyl transfer		k_{cat} (s^{-1})	K_m (mM)	k_{cat}/K_m (s^{-1} mM)
from CoA to pantetheine	donor	4.8	1.3	3.7
	acceptor	4.4	2.0	2.2
from pantetheine to CoA	donor	3.5	0.17	20.6
	acceptor	3.4	0.92	3.7
from pantetheine to CoA (0.5 mM)	donor	1.2	0.06	20.0
from *N*-acetylcysteamine to CoA (0.5 mM)	donor	0.023	0.059	0.39
from CoA (0.23 mM) to pantetheine	acceptor	0.69	0.33	2.1
from CoA (0.23 mM) to *N*-acetylcysteamine	acceptor	0.34	2.9	0.12
from CoA (0.23 mM) to dithiothreitol	acceptor	1.7	850	0.002

[a] The triple mutant was incubated with a decanoyl-thioester donor and an acceptor in 200 mM potassium phosphate buffer, pH 7.6, at 37 °C for 1 min. The reaction products were analyzed by HPLC as described in the Materials and Methods. Kinetic data in the upper panel were calculated from the secondary plots shown in Figure 3, those in the lower panel were derived from primary double reciprocal plots derived using a single concentration of either the donor or acceptor.

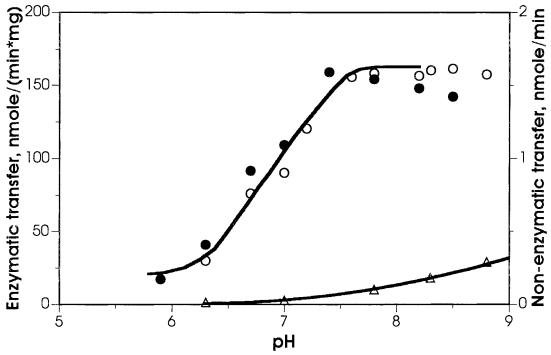


FIGURE 4: Dependency of the interthiol acyltransferase activity on pH. The triple mutant, Ser581Ala/Ser2151Ala/Ser2302Ala (10 μg/0.1 mL), buffered with 0.225 M potassium phosphate at the final pHs indicated, was incubated with either 0.23 mM radioactive decanoyl-CoA and 2.5 mM pantetheine (○) or 0.24 mM decanoyl-pantetheine and 0.85 mM CoA (●) at 37 °C for 1 min and the reaction products analyzed by HPLC. Rates of nonenzymatic reaction between decanoyl-CoA and pantetheine are shown as △; rates of nonenzymatic reaction between decanoyl-pantetheine and CoA were slightly lower (data not shown).

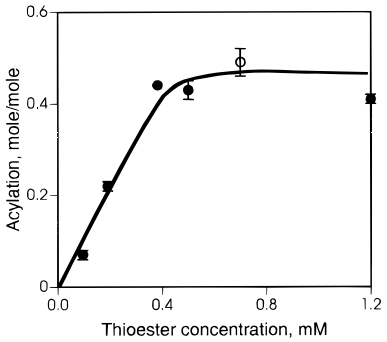


FIGURE 5: Acylation of the FAS triple mutant, Ser581Ala/Ser2151Ala/Ser2302Ala, by decanoyl- and butyryl-CoA. The triple mutant (30 μg) in 0.25 M potassium phosphate buffer, pH 7, 1 mM EDTA, 1 mM DTT, and 10% glycerol was incubated with [1-^{14}C]decanoyl-CoA (●) or butyryl-CoA (○) at 37 °C for 2 min. Radioactive substrate covalently bound to the protein was determined as described in the Materials and Methods. All of the radiolabeled fatty acid bound to the FAS could be released by treatment with performic acid and extracted into petroleum ether; no radioactivity was released in control samples treated with formic acid.

intermediates to be moved to the site favoring further elongation. Thus, one might predict that the acyl transferase responsible for moving acyl chains between the 4′-phosphopantetheine and cysteine thiols would exhibit a preference for medium chain-lengths and have relatively low activity with C2 and C4 and decreasing activity for chain-lengths

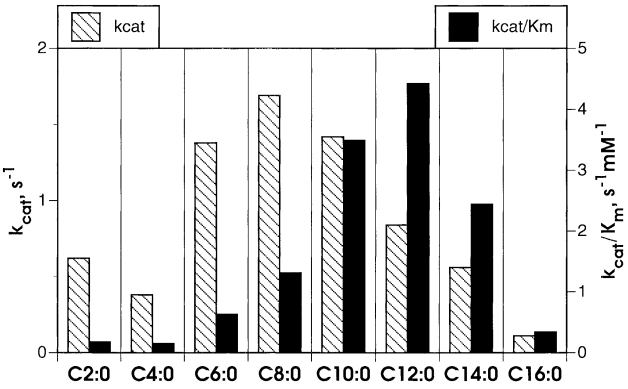


FIGURE 6: Chain-length specificity of the interthiol acyltransferase associated with the β-ketoacyl synthase domain of the FAS. The triple mutant, Ser581Ala/Ser2151Ala/Ser2302Ala (10 μg/0.1 mL for C4:0 to C14:0 and 30 μg/mL for C2:0 and C16:0) was incubated with acyl-CoAs and 2.5 mM pantetheine in 0.225 M potassium phosphate buffer, pH 7, at 37 °C for 1 min. The reaction products were analyzed by HPLC as described in Materials and Methods.

>C12. The interthiol acyltransferase activity exhibited by the FAS triple mutant Ser581Ala/Ser2151Ala/Ser2302Ala was compared using various acyl-CoA compounds as acyl donors and pantetheine as acceptor to determine whether the specificity was consistent with the proposed role in the FAS reaction sequence (Figure 6). The values for catalytic efficiency followed exactly the predicted pattern, increasing with increasing acyl chain-length from C2 to C12, then decreasing with increasing chain-length above C12. The values for k_{cat} alone followed a similar trend but peaked at shorter acyl chain-length. The turnover number for the interthiol acyltransferase, ~4 s^{-1} for C10, is approximately the same order of magnitude as that of the overall FAS reaction, ~5 complete condensation and β-carbon reduction cycles per second.

The FASs are distinguished from the structurally related modular polyketide synthases in that they cannot effectively elongate acyl chains in which the β-carbon atom has not been fully reduced to a saturated carbon, For example, β-hydroxybutyryl moieties formed as a result of the first condensation and β-ketoreduction by a mutant FAS lacking dehydrase activity cannot be elongated further (*10*). On the other hand, β-ketobutyryl moieties formed by the wild-type FAS in the absence of NADPH can undergo a second round of elongation, resulting in the formation of triacetic acid lactone. This condensation reaction proceeds relatively slowly however, compared to the rate of elongation in the presence of NADPH, when full β-carbon reduction is

Table 3: Transfer of Malonyl and Butyryl Intermediates by the Triple Mutant[a]

thioester	transferase activity [nmol/(mg min)]	%
butyryl-*S*-pantetheine	67.1 ± 2.1	100
β-ketobutyryl-*S*-pantetheine	4.4 ± 0.9	6.5
β-hydroxybutyryl-*S*-pantetheine	0.0 ± 0.5	0
crotonyl-*S*-pantetheine	0.0 ± 0.2	0
malonyl-*S*-pantetheine	0.0 ± 0.2	0

[a] The triple mutant was incubated with 4 mM acyl-*S*-pantetheine thioester and 0.5 mM CoASH in 200 mM potassium phosphate buffer, pH 7.6, at 37 °C for 1 min. The reaction products were analyzed by HPLC as described in the Materials and Methods.

possible (*26*). One possible explanation of these findings is that the β-ketobutyryl moiety is a much poorer substrate than is the butyryl moiety for transfer from the 4′-phosphopantetheine to the β-ketoacyl synthase active site cysteine, whereas β-hydroxybutyryl, and perhaps crotonyl moieties too, cannot be transferred at all. This possibility was investigated by comparing the interthiol acyltransferase activity associated with the FAS triple mutant, Ser581Ala/Ser2151Ala/Ser2302Ala, with the various 4-carbon substrates. The results were entirely consistent with this hypothesis and again emphasized the important role played by the interthiol acyltransferase in determining which intermediates can be subjected to further elongation (Table 3). Finally, the active site cysteine of the β-ketoacyl synthase cannot be occupied by malonyl moieties so that one would predict that the interthiol acyltransferase cannot recognize malonyl thioesters as potential substrates. Again, this anticipated feature of the interthiol acyltransferase could be confirmed experimentally using the FAS triple mutant (Table 3).

In conclusion, characterization of the various FAS mutants described in this study demonstrates that translocation of saturated acyl moieties between 4′-phosphopantetheine and Cys-161 is catalyzed by the β-ketoacyl synthase domain of the FAS. Two residues have been identified that play critical roles in the reaction, Cys-161 and Lys-326. Since strong evidence is presented indicating that a covalent acyl-enzyme thioester is formed as an intermediate in the interthiol acyltransferase reaction catalyzed by the triple mutant Ser581Ala/Ser2151Ala/Ser2302Ala and since Cys-161 is the thiol that carries the saturated acyl chain that participates in the condensation with malonyl moieties in the chain elongation reaction, it is reasonable to conclude that Cys-161 is the nucleophile for the interthiol acyltransferase reaction. A precise role for Lys-326 has yet to be established, although other investigators have suggested possible roles for a lysine residue in catalysis of the condensation reaction, for example, by interacting with the substrate carbonyl (*23*) or by activating the cysteine nucleophile (*27*). Experiments are presently under way to evaluate these possibilities. Finally, the experimentally observed substrate specificity of this interthiol acyltransferase is entirely consistent with a role for this activity in determining the overall substrate specificity of the β-ketoacyl synthase reaction. In particular, the absence of significant activity toward β-keto, β-hydroxy, and $\alpha\beta$-enoyl intermediates ensures that only saturated acyl chains are subjected to further elongation so that the final product is a fully saturated long-chain fatty acid. This feature of the β-ketoacyl synthase enzyme may well distinguish the FASs from the modular polyketide synthases where the β-ketoacyl synthases are able to elongate intermediates with incompletely reduced β-carbon atoms (*28*).

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