Alteration of the Substrate Specificity of the Malonyl-CoA/Acetyl-CoA:Acyl Carrier Protein S-Acyltransferase Domain of the Multifunctional Fatty Acid Synthase by Mutation of a Single Arginine Residue*

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The structural basis for the dual specificity of the malonyl-CoA/acetoyl-CoA acetyl carrier protein S-acyltransferase associated with the multifunctional animal fatty acid synthase has been investigated by mutagenesis. Arginine 606, which is positionally conserved in the transacylase domains of all multifunctional fatty acid and polyketide synthases, was replaced by alanine or lysine in the context of the isolated transacylase domain, and the mutant proteins were expressed in Esherichia coli. Malonyl transacylase activity of the Arg-606 → Ala and Arg-606 → Lys mutant enzymes was reduced by 100- and 10-fold, respectively. In contrast, acetyl transacylase activity was increased 6.6-fold in the Arg-606 → Ala mutant and 1.7-fold in the Arg-606 → Lys mutant. Kinetic studies revealed that selectivity of the enzyme for acetyl-CoA was increased >16,000-fold by the Ala mutation and 16-fold by the Lys mutation. Activity toward medium chain length acyl thioesters was also increased >3 orders of magnitude by mutation of Arg-606, so that the Ala-606 enzyme is an effective medium chain length fatty acyl transacylase. These results indicate that Arg-606 plays an important role in the binding of malonyl moieties to the transacylase domain but is not required for binding of acetyl moieties; these results are also consistent with a mechanism whereby interaction between the positively charged guanidinium group of Arg-606 and the free carboxylate anion of the malonyl moiety serves to position this substrate in the active site of the enzyme.

The animal FAS† consists of two identical polypeptides, each carrying six enzymes and an acyl carrier protein, that are juxtaposed to form two centers for the synthesis of palmitic acid from acetyl- and malonyl-CoA (1–3). The iterative condensation of an acetyl moiety with successive malonyl moieties and acetyl- and malonyl-CoA (1–3). The iterative condensation of an acetyl moiety with successive malonyl moieties and

alteration of the substrate specificity of the enzyme. We have adopted an alternative strategy to identify residues that play a role in promoting catalysis and in determining the substrate specificity of the malonyl/acyl transacylase associated with the multifunctional FAS, for which no three-dimensional structure is available. Our rationale is first to identify from multiple sequence alignments conserved residues that might be candidates for specific roles in catalysis and then to examine the effect of mutating these residues. Using this approach, we have previously identified His-683‡ of the rat FAS as playing an essential role in activation of the catalytic residue Ser-581 (10). Only one other basic residue, Arg-606 in the rat FAS, is universally conserved in the transacylases associated with multifunctional and monofunctional forms of FAS and polyketide synthase (10). Since all of these transacylases can utilize either malonyl-CoA or methylmalonyl-CoA as a substrate, we hypothesized that this residue might facilitate substrate binding by interaction with the free carboxyl group of the malonyl or methylmalonyl moiety and that its replacement might compromise binding of these substrates. A corollary to this hypothesis predicts that, in

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the context of the malonyl-CoA/acetyl-CoA acyl carrier protein transacylase, such a mutation would likely have no negative effect on acetyl binding. We report here the results of experiments designed to test this hypothesis.

EXPERIMENTAL PROCEDURES

Materials—Acetyl-S-pantetheine was prepared by acetylation of pantetheine with acetic anhydride and removal of the acetic acid formed by extraction with ether. The sources of other materials used were reported previously (10).

Construction of Plasmids and Expression of Transacylase Protein—Construction of the transacylase expression plasmid pET23a/388-MAT has been described previously (10). Oligonucleotide-directed in vitro mutagenesis based on the method described by Kunkel et al. (11) was adopted to generate Arg-606 → Ala and Arg-606 → Lys mutants. Correct introduction of mutations and authenticity of the DNA was confirmed by DNA sequencing. Plasmids carrying the mutations were used to transform E. coli BL21(DE3) cells, and expression of the recombinant proteins was induced by 1 mM isopropyl-β-D-thiogalactoside. The recombinant transacylases were recovered from inclusion bodies and refolded in vitro (10, 12).

Determination of Hydrolase Activity Associated with Mutant Transacylases—The Arg-606 → Ala and Arg-606 → Lys mutant transacylases were incubated with malonyl-CoA (100 μM) at 0 °C for 1 h, and the amount of free CoA formed was determined as described earlier (10).

Assay of Malonyl-CoA/Acetyl-CoA Carrier Protein S-Acyltransferase Activity—Transacylase activity was routinely determined at 0 °C using either [1-14C]acetyl-CoA or [2-14C]malonyl-CoA as the substrate and pantetheine as the model acceptor (10). In some experiments acetyl-S-pantetheine was used as the acetyl donor and CoASH as the acceptor; the acetyl-CoA product was isolated and quantitated by high performance liquid chromatography on a C4 reversed phase column (Vydac) as described previously (10). One unit of activity is the amount of product formed in 1 min with 1 μM of substrate/min.

Assay of Medium Chain Length Transacylase Activity—Transfer of medium chain acyl moieties from a pantetheine donor to a CoA acceptor was determined by separating the substrates and products using high performance liquid chromatography. Wild type and mutant transacylases were incubated with 400 μM acetyl-S-pantetheine thioesters and 1 μM CoASH in 100 mM potassium phosphate buffer, pH 6.8 (50-μl volume), at room temperature for 1-15 min, depending on the activity of the preparation. Reactions were stopped by the addition of 1.05 ml of buffer A (25 mM potassium phosphate, pH 5.4, 5% acetonitrile), and the mixture was immediately injected onto a reversed phase high performance liquid chromatography column (Intersil Phenyl, 5 μm, 4.6 × 150 mm, MetaChem, Torrance, CA) maintained at 45 °C. The column was washed with buffer A at 1.5 ml/min for 5 min followed by a three-step linear gradient: to 25% acetonitrile over 3 min, to 55% acetonitrile over 10 min, and to 80% acetonitrile over 2 min. The amount of acetyl-CoA eluted was determined from the observed absorbance at 258 nm using decanoyl-CoA as the standard. Saturating concentrations of donor and acceptor substrates were employed, and the reaction rates were directly proportional to time and enzyme concentration.

Assay of Protein Concentration—Protein concentration was calculated from the absorbance at 280 nm; 1 mg/ml transacylase gives an absorbance of 1.07.

RESULTS

Expression and Purification of Arg-606 → Ala and Arg-606 → Lys Transacylases—Arg-606 was mutated to Ala and Lys in the context of the independently expressed transacylase domain representing residues 429–815 of the rat FAS. The mutant proteins were expressed in E. coli, solubilized in urea, renatured, and purified. Purity of the preparations was estimated to be >95% by SDS-polyacrylamide gel electrophoresis (Fig. 1A). The molecular masses of the proteins were approximately 42 kDa, as predicted, and both mutant proteins were recognized by rabbit anti-rat liver FAS antibodies (Fig. 1B).

Effect of Mutation of Arg-606 on Transacylase Activity—Replacement of Arg-606 with Ala or Lys decreased malonyl transacylase activity by 99 and 91%, respectively, but increased acetyl transacylase activity by 6.6- and 1.7-fold, respectively (Table I). These results demonstrated clearly that Arg-606 is required for optimal transmalonylation but not for transacytalylation.

Kinetic Parameters—Kinetic analysis of the mutant transacylases revealed that the Km for malonyl-CoA was increased 8.5-fold by the Arg-606 → Ala mutation but was relatively unaffected by the Arg-606 → Lys mutation. The value for kcat was lowered about 100-fold by the Arg-606 → Ala mutation and about 9-fold by the Arg-606 → Lys mutation (Table I). With acetyl-CoA as the substrate, kcat increased significantly as a result of both mutations, and the Km decreased slightly. As a result of the Arg-606 → Ala mutation, the calculated specificity constant (kcat/Km) of the transacylase was decreased 3 orders of magnitude with malonyl-CoA as the substrate but was increased almost 2 orders of magnitude with acetyl-CoA as the substrate. On the other hand, replacement of Arg-606 with Lys had a much less drastic effect on the properties of the enzyme: the specificity constant for malonyl-CoA was reduced <1 order of magnitude, whereas that for acetyl-CoA was increased about 2-fold. Thus, based on the calculated values for relative specificity constants, the selectivity of the enzyme for acetyl-CoA was increased >16,000-fold by the Ala mutation and 16-fold by the Lys mutation.

To assess the possibility that the observed lower activity toward malonyl-CoA exhibited by the Arg-606 → Ala and Arg-606 → Lys mutant enzymes might be due to increased hydrolyase activity, we measured the ability of these mutant enzymes to hydrolyze malonyl-CoA. However, the activity of all enzyme preparations was extremely low: wild type, 5.1 milliunits/mg; Arg-606 → Ala, 1.8 milliunits/mg; Arg-606 → Lys, 2.4 milliunits/mg.

Binding of Substrates to the Enzyme—In the absence of a thiol acceptor, almost 80% of the transacylase can be acetylated in the case of both the wild type (10) and Arg-606 mutant enzymes (see Table III). When the transacylase was incubated with equimolar amounts of acetyl-CoA and malonyl-CoA, again almost 80% of both wild type and mutant enzymes was acetylated. Malonyl moieties accounted for 39% of the occupied sites in the wild type transacylase, but only 4 and 11% in the Arg-606 → Ala and Arg-606 → Lys mutant enzymes, respectively (see Table III).

Medium Acyl Chain Length Transacylase Activity—The surprising observation that the catalytic efficiency of the transacylase toward acetyl-CoA was actually increased by the replacement of Arg-606 with either Ala or Lys prompted us to examine
concentrations of acetyl-S,S-pantetheine, pantetheine-SH, and CoASH used were 100 μM, acetyl-CoA, and malonyl-CoA the acceptor (Table II). Under saturating substrate conditions, the wild type enzyme exhibited low medium chain transacylase activity, and the activity decreased as the chain length of the acyl moiety was increased from 8 to 10 carbon atoms (Table II). The Arg-606 → Ala mutant exhibited transacylase activity >3 orders of magnitude higher than that of the wild type enzyme. Replacement of Arg-606 with Lys had a less dramatic effect, increasing octanoyl and decanoyl transacylase activities approximately 2 orders of magnitude.

**DISCUSSION**

Arg-606 is positionedally conserved in all transacylases that function in the substrate-loading reaction of fatty acid and polyketide synthesis, regardless of whether the enzymes are specific for malonyl-CoA or methylmalonyl-CoA or possess dual specificity for acetyl- and malonyl-CoA. Conceivably, a conserved arginine residue might function to enhance catalytic specificity for acetyl- and malonyl-CoA. Since mutation of Arg-606 negatively impacts only the malonyl transacylase activity, this scenario can be entirely discounted. The selective effect of Arg-606 mutation on malonyl transacylase activity is clearly most compatible with the second possibility, namely that this residue specifically forms a salt bridge with the free carboxylate group of the malonyl moiety. Since the arginine is also positionally conserved in polyketide synthases regardless of whether they preferentially utilize malonyl-CoA or methylmalonyl-CoA, it is likely that this residue plays the same role in facilitating the binding of both malonyl and methylmalonyl moieties. Recruitment of a methylmalonyl or malonyl extender unit by the polyketide synthases is clearly regulated at the level of the transacylase as illustrated recently by transacylase domain-swapping experiments (13). Nevertheless, malonyl- and methylmalonyl-specific transacylases share significant sequence similarity, and the structural basis for their different specificities has yet to be elucidated.

The rate of acylation of the active-site serine residue by acetyl and malonyl moieties is too fast to be measured by conventional methods. Earlier quenched flow studies on the whole fatty acid synthase have yielded first order rate constants of ~150 s⁻¹ for acetylation of the active-site serine; however, even at equilibrium only about 50% of the serine sites were occupied (14). Somewhat higher stoichiometries for acylation of the isolated transferase domain were obtained in this study, approximately 0.8 mol of substrate/mol of enzyme. When both acetyl and malonyl moieties are offered simultaneously to the wild type transacylase, malonyl moieties compete effectively for binding to the active-site serine. However, in the Arg-606 → Ala enzyme, and to a lesser extent in the Arg-606 → Lys enzyme, the ability of malonyl moieties to compete with acetyl moieties is severely compromised (Table III). This finding is consistent with the proposed role for Arg-606 in facilitating the initial binding of malonyl moieties to the enzyme.

Removal of the positive charge at residue 606 by replacement with a neutral alanine also had the effect of increasing the acetyl transacylase activity of the enzyme. Indeed the activity toward other uncharged and more hydrophobic acyl moieties was increased even more dramatically so that the acyl chain specificity of the Ala-606 mutant is extended to medium chain lengths. Replacement of Arg-606 with the less basic, slightly smaller Lys residue produced an enzyme with substrate specificity intermediate between wild type and Ala-606 enzymes. Thus, the Lys-606 enzyme exhibited increased activity toward acetyl and medium chain acyl substrates without completely compromising malonyl transacylase activity.

The experiments described provide a rationalization for the
ability of the FAS-related transacylases to catalyze the malonyl transfer reaction. Clearly, in the dual specificity malonyl-CoA/ acyl-CoA:acyl carrier protein S-acyltransferase associated with the multifunctional animal FASs, an additional residue, or residues, must serve to stabilize the acetyl moiety at the substrate binding site. We are presently attempting to identify these amino acids using the same strategy of combining sequence comparisons with mutagenesis experiments.

The finding that a single amino acid replacement, for example Arg-606 → Lys, can effectively extend the acyl chain length specificity of the transacylase invites speculation as to whether such mutations may have occurred in nature. It has not escaped our attention that the ruminant FASs are able to release medium chain length acyl moieties as products by direct transfer to a CoA acceptor (19). This transacylation reaction is catalyzed by the same transacylase domain of the FAS responsible for substrate loading (19). This unique property of the ruminant FASs has been attributed to the unusually permissive substrate binding site. We are presently attempting to identify or residues, must serve to stabilize the acetyl moiety at the substrate binding site. We are presently attempting to identify these amino acids using the same strategy of combining sequence comparisons with mutagenesis experiments.

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<td>Wild type</td>
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<td>0.62 ± 0.04 (80%)</td>
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REFERENCES

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