Reengineering the Specificity of a Serine Active-site Enzyme

TWO ACTIVE-SITE MUTATIONS CONVERT A HYDROLASE TO A TRANSFERASE*

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Two residues are known to play important catalytic roles in fatty acyl-thioester hydrolase, thioesterase II: Ser-101, the site of a covalent acyl-enzyme intermediate, and His-237 which is within hydrogen bonding distance of Ser-101 and facilitates catalysis by increasing the nucleophilicity of this residue. In this study we have examined the effect of mutations at these two residues on the ability of the enzyme to function as a hydrolase and, in the presence of a thiol acceptor, as an acyltransferase. In the hydrolase reaction \(k_{cat}\) values for the wild-type, H237R, S101C, and S101C/H237R thioesterases enzymes were 0.11, <0.002, 0.10, and <0.002 s\(^{-1}\), respectively, and at steady state, the proportion of each enzyme present as the covalent acyl-enzyme intermediate was 11, 91, 71, and 100%, respectively. In the acyltransferase reaction no activity could be detected for the wild-type or H237R enzymes but the specific activities of the S101C and S101C/H237R thioesterases were 170 and 1300 nmol/min/mg of protein, respectively. From this data we conclude the following: the wild-type enzyme functions exclusively as a hydrolase. The H237R mutant acts ineffectively as a hydrolase primarily because the deacylation reaction is drastically retarded. The S101C enzyme functions well as a hydrolase, even though the rate of deacylation is adversely affected, and this enzyme can also perform as an acyltransferase. Mutation of both catalytic residues leads to a complete loss of hydrolase activity and the S101C/H237R mutant functions as an effective acyltransferase exhibiting \(k_{cat}\) values higher than those of the wild-type enzyme acting as a hydrolase. This study reveals that, with only two amino acid replacements, an enzyme capable of functioning exclusively as a hydrolase can be converted into an equally active enzyme performing solely as an acyltransferase.

Thioesterases have been implicated as important enzymes in a variety of metabolic pathways including the biosynthesis of fatty acids (1–5), polyketides (6, 7), and peptide antibiotics (8, 9). Although only the thioesterases involved in fatty acid synthesis have been studied extensively, it appears likely that the role of thioesterases associated with all of these pathways is to release the fatty acid, polyketide or peptide, from the phosphopantetheine which provides the thiol template for assembly of the product.

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Currently 2 residues have been identified as playing a catalytic role in the thioesterases: Ser-101 (numbering for rat mammary gland thioesterase II located in the serine esterase motif Gly-Xaa-Ser-Xaa-Gly (3, 10–12) and His-237 found within Gly-Xaa-His motif (13–17). Replacement of the active-site histidine of rat mammary gland thioesterase II with arginine, leutine (15), or alanine (13) reduced the catalytic activity by 2–3 orders of magnitude. Substitution of the active-site serine with cysteine in rat thioesterase I (16) and rat mammary gland thioesterase II (13, 17) reduced activity only slightly. In this paper we analyze in detail the effects of mutations at the active-site seryl and histidyl residues of thioesterase II and show that a double mutation, S101C/H237R converts this hydrolase into an excellent acyltransferase.

EXPERIMENTAL PROCEDURES

Materials—Lysyl endopeptidase from Achromobacter lyticus was bought from Wako BioProducts (Dalas, TX) and (1-14C)palmitoyl-CoA (64 Ci/mol) was obtained from Amersham Corp. Other chemicals were purchased from Sigma and Aldrich.

Mutant Construction and Expression—The cDNAs coding for the single mutants of thioesterase II, S101C, and H237R, cloned into a modified plLA502 vector (15, 17) were used for construction of a cDNA encoding the double mutant. Standard recombinant DNA techniques (15) were utilized if not otherwise indicated. The H237R and S101C single mutant cDNAs were restricted with BamHI and EcoRI enzymes and the 5.2-kilobase pair fragment from the S101C mutant cDNA, containing the vector sequence and the mutated S101C codon, was ligated to the 0.9-kilobase pair fragment from the H237R mutant cDNA, which contained the mutated H237R codon. The resulting plasmid containing the double mutation, S101C and H237R, was cloned into Escherichia coli DH5 cells and expression of the encoded protein was induced at 42 °C in a 0.1% culture of TB medium (24 g/l yeast extract, 12 g/l tryptone, 16.43 g/l K2HP04, 2.3 g/l KH2PO4, 1 m/l glycerol, 50 mg/l carbenicillin). The enzyme was purified as described previously (15, 19). A final purification step was added that involved chromatography on a high performance anion-exchange column (TSK-DEAE-5PW, 2.15 × 15 cm, 10 mm, Bio-Rad) using a NaCl gradient (0–0.15 m over 27 min) in 50 mm Tris-HCl, pH 7.8/1 m EDTA at a flow rate of 7 ml/min. All chromatographic and storage buffers contained dithiothreitol (1 or 2 m) unless otherwise stated.

Assay of Mutant Activity—Thioesterase activity of the mutant was determined spectrophotometrically with acyl-CoA (17) and p-NP-Dec (3) as the substrates. The use of p-NP-Dec as substrate was particularly advantageous for an assay of activities of thioesterase II mutants that contained a cysteinyl residue at the active site, position 101; these mutants are rapidly inactivated by 5,5'-dithiobis(2-nitrobenzoate) and cannot be assayed by the usual procedure that allows direct reaction, with 5,5'-dithiobis(2-nitrobenzoate), of the CoA thiol released following formation of the covalent acyl-enzyme intermediate. Prior to assay dithiothreitol was removed from all enzyme preparations by gel filtration. Specific activities were calculated using the amount of oxidized form of the enzyme (i.e. Cys-101 present exclusively in the free thiol form) that was estimated from the absorbance profile on HPLC (see below). All data were corrected for non-enzymatic release of p-nitrophenol. The kinetic

1 The abbreviations used are: 1, liter(s); p-NP-Dec, p-nitrophenyl decanolate; 2-ME, 2-mercaptoethanol; HPLC, high performance liquid chromatography; ESIMS, electrospray ionization mass spectrometry.
parameters are presented as an average from five different methods of calculation: Lineweaver-Burk, Eadie-Hofstee, Hanes-Woolf, direct linear plot, and non-linear regression.

**Reverse-phase HPLC—Proteins, substrates, and products were separated on a Vydac C4 reverse-phase HPLC column (5 µm, 300-Å pore size, 4.6 × 25 cm) using acetonitrile gradients generated from 0.1% trifluoroacetic acid in water (solvent A) and 0.1% trifluoroacetic acid in acetonitrile (solvent B) according to the following programs: system I: 30% solvent B for 5 min, followed by a three-step linear gradient (30–42% B over 5 min, 42–56% B over 28 min, and 56–75% B over 2 min; system II: five-step linear gradient (19–14% B over 6 min, 14–27% B over 15 min, 27–35% B over 16 min, 35–65% B over 30 min, and 65–90% B over 10 min); system III: as the system I except that the first step lasted for 10 min. The flow rate was 0.7 ml/min for all separation systems.

**Online reverse-phase HPLC/ESIMS—Separation of proteins, substrates, and products of the acyltransferase reaction was performed on a microbore HPLC system (Michrom Biosources, Pleasanton, CA) equipped with a reverse-phase C4 column (5 µm, 300-Å pore size, 0.1 × 15 cm) using a flow rate of 40 µl/min at 40 °C. The gradient systems were similar to those described above. The flow was split directly in the UV cell allowing 4 µl/min to be delivered to the mass spectrometer through a modified VG BioQ probe. A sheath liquid (0.2% trifluoroacetic acid in methanol) was added at a flow rate of 2 µl/min prior to nebulization. ESIMS was performed on a VG Biogas quadrupole mass spectrometer (VG Biotech/Visions, Altrincham, United Kingdom). The ionization was controlled and data analyzed using a Labspec® software (VG Biotech/Visions, Altrincham, U.K.). A mixture of heart horse myoglobin and PEG 550 was used to calibrate the mass scale within the 300–1300-Da range.

**RESULTS AND DISCUSSION**

**General Properties of the S101C,H237R Thioesterase II—** Approximately 30 mg of the double mutant was isolated from the 2-1 culture, and its authenticity was confirmed by ESIMS. The experimentally determined value for the average molecular mass (29,508.8 ± 3.2 Da) was in good agreement with that predicted from the primary sequence with a NH2 terminally unblocked enzyme (29,506.2 Da) that is consistent with our earlier finding that the recombinant wild-type thioesterase II (19) and H237R, H237L (15, 17) mutants (15, 17) accumulated in E. coli cytosol as deformedylated proteins. The thioesterase mutant, unlike the recombinant wild-type thioesterase II, when stored without dithiothreitol, was slowly converted to a +32.0 Da form (Fig. 1A). A similar +32.0 Da form was produced on aging of the single mutant S101C thioesterase II in the absence of dithiothreitol. We conclude that these +32.0 Da-forms resulted from the oxidation of the Cys-101 thiol to the absence of dithiothreitol. We conclude that these +32.0 Da forms did not result from the wild-type or the H237R enzymes upon aging; the presence of 2 mM dithiothreitol on storage almost entirely prevented formation of the +32.0 Da-forms; the +32.0 Da species did not form covalent acyl-enzyme intermediates (see below) and the increase in the amount of the +32.0 Da form was accompanied by a lowering of the specific activity of the enzyme (details not shown).

On incubation of the double mutant with p-NP-Dec, a brief presteady state burst of p-nitrophenol release was observed and was followed by a very slow liberation of p-nitrophenol. The slow release of p-nitrophenol from 20 µM p-NP-Dec measured at pH 7, 8.2, and 9.2 was 0.5, 1.8, and 3.4 nmol/min mg, respectively, that compares to 160 and 150 nmol/min mg determined for the wild-type thioesterase II and S101C mutant, respectively, at pH 8 (optimal pH). These data are in agreement with a small hydrolyase activity (<0.3%) that was reported for the S101C,H237A thioesterase II mutant (13). Evidently the replacement of His-237 in the S101C thioesterase II, as well as in the wild-type enzyme (13, 15), significantly reduces hydrolyase activity confirming an important catalytic role for this histidyl residue.

**Acylation of the Double Mutant—** Examination of the products of the reaction of p-NP-Dec or decanoyl-CoA with S101C,H237R thioesterase II (Fig. 1B) revealed that the double mutant was entirely converted to the +154.3 Da form. The increase in the molecular mass was in an excellent agreement with the value expected for the covalent binding of a single decanoyl residue (+154.4 Da). Similar results were observed when palmitoyl-CoA (+242.0 Da versus expected +239.4 Da) was used as substrate. The extent of acylation varied less than 5% over the pH range 6.6–9.2 and reached a maximum in the presence of only a slight excess of substrate. For example, 100% of the double mutant was converted to the +154.3 Da form in the presence of only a 1.5-fold molar excess of decanoyl-CoA. These data indicate that whereas formation of the acyl-enzyme intermediate proceeds freely with the double mutant, subsequent hydrolysis is seriously impaired leading to accumulation of the acyl-enzyme species. In contrast, the oxidized form of the double mutant (putative cysteine sulfinic acid derivative) did not generate a +154.3 Da species on incubation with p-NP-Dec indicating that this form cannot generate the covalent acyl-enzyme intermediate.

The acyl moieties were removed from the double mutant acyl-enzyme by treatment with hydroxylamine in 8 M urea at pH 7 indicating that they were attached to the enzyme via thioester linkage (data not shown). To verify that Cys-101 is the acylated residue, the double mutant was labeled with [14C]palmitoyl-
mitoyl-CoA, digested with lysyl endopeptidase for 16 h, the radioactive peptides were separated by HPLC, and identified by on-line HPLC/ESIMS (Table I). Four radioactive peptides were found, and three of them were products of partial digestion. All radioactive peptides contained Cys-101, the cysteine that replaced the active-site serine, while none contained any of the other 3 cysteinyl residues present on thioesterase II (positions 18, 31, and 256) confirming Cys-101 as the site of acylation. Similar results were obtained when the double mutant was acylated with decanoyl-CoA (data not shown). The incomplete digestion of the acylated double mutant enzyme was surprising in view of the fact that cleavage of all lysyl peptide bonds was observed in 4 M urea after only 8 h digestion of the carboxymethylated enzyme (no fatty acyl present). We hypothesize that the presence of an acyl-chain covalently bound to Cys-101 makes Lys-93, Lys-115, and, to some extent, Lys-117 unavailable for cleavage by the endopeptidase. This protection from protease attack could result from interaction of the acyl-chain with a hydrophobic region of the thioesterase that extends in this peptide from residues 84–99, 97–98, and 105–112 (hydrophilicity index ≤ −1 in the Kyte-Doolittle scale, window 7).

Additional support for localization of the fatty acyl hydrophobic binding site comes from comparison of the rat thioesterase II sequence with two structurally related thioesterases, one having a similar substrate specificity and the other having a different substrate specificity, viz a thioester hydrolase in the presence of the fatty acyl chain. We suggest, therefore, that this is the region of thioesterase II that encodes the fatty acyl-binding site.

Decaylation by 2-ME and Other Thiol dissociations of the acyl enzyme intermediate formed by the double mutant thioesterase II revealed that in the presence of certain thiol reagents the acyl chain was rapidly removed from Cys-101. Incubation of the double mutant with p-NP-Dec and 2-ME resulted in a rapid and steady release of p-nitrophenol. The reaction was pH dependent (Fig. 3) and involvement of a moiety with a pKα above 8, perhaps a thiol, was indicated by the pH/activity profile. This thiol could be the one associated with the enzyme active-site cysteine and/or that associated with the 2-ME substrate.

Nucleophilic substitution at decanoyl-Cys-101 thioester by 2-ME would form decanoic acid (2-hydroxymercaptoethyl) ester releasing free enzyme thiol available for reacylation. To determine whether the double mutant was functioning as a transferase, we identified the products of the reaction. When p-NP-Dec was used as acyl donor and CoA as acceptor, decanoyl-CoA was identified, from its characteristic retention time by HPLC (10 min using solvent system III) and from its molecular mass, 922.3 Da, determined by HPLC/ESIMS. Similarly, the product

### Table I

| Residue number | Mutation index | Difference in mutation indices |
|----------------|----------------|------------------------------|
| 8              | L              | 16                           |
| 20             | K              | 8                            |
| 22             | K              | 6                            |
| 105            | K              | 4                            |
| 112            | L              | 2                            |
| 115            | K              | 1                            |

**FIG. 5.** Comparison of the frequency of non-conservative mutations between thioesterases with different specificity. Rat thioesterase II was aligned with duck thioesterase II and the grsT protein using the ALIGN program (Protein Identification Resources, Washington, D.C.), and the mutation index was calculated for each alignment using a 20-amino-acid window (23), smoothed, and shown in panel A. The mutation index is a measure of the frequency of accepted non-conservative mutations. Panel B presents the smoothed arithmetical difference between mutation indices for the grsT protein versus rat thioesterase II and rat thioesterase II versus duck thioesterase II. The residue numbers given are those of the rat enzyme.
of the catalyzed reaction between 2-ME and p-NP-Dec was established as decanoic acid (2-hydroxy)mercaptoethyl ester by comparison of HPLC retention time with the value determined for the synthesized standard (both 24 min using solvent system III). Identification of the reaction products confirmed our conclusion that the double mutation of thioesterase I1 converted the enzyme into an acyl-thioester hydrolase and an acyltransferase.

Considering the available data, the reaction catalyzed by the double mutant can be described as a sequence of two reactions with no formation of a ternary complex:

\[ E + p\text{-NPDec} \rightarrow E\text{-p-NPDec} \rightarrow E\text{-Dec} + p\text{-NP} \quad (\text{Eq. 1}) 
\]

\[ E\text{-Dec} + 2\text{-ME} \rightarrow E\text{-Dec}\text{-2-ME} \rightarrow E\text{-Dec} + 2\text{-ME} \]

The observation that the enzyme can be fully acylated with a minimal excess of p-NP-Dec indicates that the last reaction of the first equation is functionally irreversible. In this case the apparent dissociation constant for the binding of p-NP-Dec to the enzyme is almost zero and the steady-state equation for the reaction involving two substrates reduces to (26):

\[
\frac{1}{v} = \left( K_m (p\text{-NPDec}) \right) \left( \frac{1}{V_{\text{max}}} \right) + \left( K_m (2\text{-ME}) \right) \left( \frac{1}{V_{\text{max}}} \right)
\]

At high concentrations of either substrate, this equation simplifies to the Michaelis-Menten equation for a single-substrate reaction. To investigate kinetics of the two reactions, the double mutant was incubated with p-NP-Dec as substrate and 2-ME as acceptor. Calculated \( K_m \) and \( k_{\text{cat}} \) values are presented in Table II. The \( k_{\text{cat}} \) values calculated for the two half-reactions are not exactly identical. However, since under experimental conditions full saturation is not attained, the difference between these values seems to be acceptable. The turnover number for the acyltransferase reaction catalyzed by the double mutant is more than 10-fold higher than \( k_{\text{cat}} \) values calculated for hydrolysis of p-NP-Dec and decanoyl-CoA, respectively, by wild-type thioesterase II (17). These results demonstrate that the mutations of S101C and H237R in thioesterase II converted the acyl-thioester hydrolase into a highly effective acyltransferase.

The \( K_m \) values calculated for 2-mercaptoethanol, dithiothreitol, cysteamine, and CoA are very similar (Table II), indicating that there is no preferential binding of any of these acceptors. However, all effective acceptors contain the HS-CH2-CH-moiety that may be recognized by the enzyme.

### Table II

| Substrate | \( K_m \) | \( k_{\text{cat}} \) |
|-----------|----------|----------------|
| p-NP-Dec  | (1.5 ± 0.1) \times 10^{-3} | 1.21 ± 0.04 |

### Table III

| Thioesterase II | \( k_{\text{cat}} \) (hydrolysis) | Acylated form* | Transfer to 2-ME* |
|-----------------|-------------------------------|----------------|-----------------|
| Wild-type       | 0.11                          | 11.2 ± 4.4     | 0               |
| H237R           | <0.002                        | 90.5 ± 4.0     | 0.0             |
| S101C           | 0.10                          | 71.2 ± 2.6     | 170             |
| S101C, H237R    | <0.002                        | 88.5 ± 0.6     | 1300 ± 98       |

* Calculated from HPLC absorbance profile. Substrate (70–110 \( \mu \)M) concentration was at least 10-fold over the enzyme concentration. Up to 25% of substrate was hydrolyzed at the moment of injection.

### Comparison of the Wild-type and Mutant Thioesterases II

The properties of the wild-type thioesterase and its double and single mutants were compared according to several criteria: their ability to catalyze the hydrolysis of p-NP-Dec and acyl-CoA esters, the levels of acyl-enzyme formed on incubation with these substrates, and their ability to catalyze acyltransfer between p-NP-Dec and 2-ME (Table III). A low fraction of acyl-enzyme intermediate, 10%, and no transfer product were found in the case of the wild-type thioesterase II indicating that acylation is the rate-limiting step and hydrolysis of the acyl-enzyme occurs rapidly. The role of the His-237 in activating the active-site residue Ser-101 has been substantiated by our demonstration that the 2 residues are within hydrogen-bonding distance of each other (17) and by the finding that mutations at His-237 result in dramatically reduced hydrolysis activity (13, 15). It seems likely that His-237 may also assist the deacylation reaction by acting as a general base, facilitating the nucleophilic substitution by water on the acyl-enzyme intermediate as it is generally believed to occur for the serine proteases. In fact, for the H237R mutant, 90% of the enzyme was found to be acylated in the presence of p-NP-Dec suggesting that the decreased hydrolysis activity of the mutant results mainly from impaired hydrolysis. The S101C mutant can catalyze both the hydrolysis and acyltransferase reactions and in this case too, the acyl-enzyme intermediate accumulated to relatively high levels (Table III). The acyltransferase activity is a novel property of the enzyme acquired by the mutation S101C and presumably results from
creased nucleophilicity of the acylated amino acid, Cys versus Ser (17), and from reduced enzyme hydrolytic activity. Taking into account that hydrolysis of thioesters (acyl-Cys) requires lower activation energy than hydrolysis of esters (acyl-Ser) and that the water concentration was 55.5 m and the 2-ME only 20 m, it seems likely that water positioning must be partially obstructed in the S101C mutant. Finally, in the case of the double mutant (S101C,H237R) replacement of His-237 with arginine leads to the almost complete elimination of hydrolase activity, leaving an enzyme that functions exclusively as an acyltransferase.

Speculation on the Evolutionary Significance of the Simple Hydrolase to Aeryltransferase Conversions—We have previously pointed out (17) that structurally and functionally related serine active-site enzymes in the thioesterase family utilize both types of serine codons, TCN and AGY, that could have been interchanged via codons for cysteine (TGY), since substitution of cysteine for the active-site seryl residue generates functionally active enzymes from thioesterases using both types of codon (16, 17). The present study reveals that a two-step transformation of hydrolase to acyltransferase can be made via a catalytically active intermediate. These findings raise the possibility that a primordial cysteine active-site enzyme (exemplified by the thioesterase II S101C mutant described herein) possessing both hydrolase and acyltransferase activity could have given rise to two families of enzymes, one having exclusively hydrolase activity (exemplified by the thioesterase having exclusively hydrolase activity (exemplified by the thioesterase family), or the other exclusively acyltransferase activity (exemplified by the acyltransferase II S101C,H237R mutant). This evolutionary transition could have occurred via single nucleotide changes, one resulting in replacement of the active-site cysteinyl with a seryl residue forming the hydrolase family, the other resulting in replacement of the active-site histidine forming the acyltransferase family. Although we are unaware of the existence of a naturally occurring acyltransferase that is structurally related to thioesterase II, one of the genes of the bialophos peptide antibiotic gene cluster encodes a thioesterase II-like enzyme with putative active-site cysteinyl and histidyl residues (9), analogous to the S101C mutant described in our study. The exact catalytic role of this enzyme, be it hydrolase or transferase, has yet to be ascertained.

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