A Catalytic Role for Histidine 237 in Rat Mammary Gland Thioesterase II*

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The involvement of a histidyl residue in the catalytic mechanism of thioesterase II, a serine active-site enzyme that catalyzes the chain terminating reaction in de novo fatty acid synthesis, has been inferred from studies with the inhibitor diethyl pyrocarbonate. Its likely location has been predicted by identification of conserved residues in related thioesterases and ultimately confirmed by site-directed mutagenesis. Diethyl pyrocarbonate inactivated the enzyme with a second-order rate constant of 49 M⁻¹ s⁻¹ at pH 6, 10 °C. Data analysis indicated that although several residues reacted with the reagent, modification of a single residue was responsible for the inactivation. Removal of a single ethoxycarbonyl moiety by treatment with neutral hydroxylamine completely restored enzyme activity. Prior ethoxycarbonylation of the histidyl residue blocked the ability of the active-site serine to react with phenylmethanesulfonyl fluoride. Comparison of the amino acid sequences of five structurally related proteins indicated that only 1 histidine has been completely conserved. Replacement of this residue in rat thioesterase II (His-237) with arginine and leucine by mutagenesis reduced the catalytic activity by 2–3 orders of magnitude. The activity of the mutant thioesterases, unlike that of the wild-type enzyme, was relatively insensitive to inhibition by diethyl pyrocarbonate and phenylmethylsulfonyl fluoride. These studies provide strong evidence that His-237 is involved directly in catalysis and suggest that its role is to increase the nucleophilic character of the active-site Ser-101 by acting as a proton acceptor thus facilitating acylation of the seryl residue. The mechanism appears to share certain common features with the charge-relay system characteristic of other esterases.

In animal tissues medium and long chain fatty acids are assembled de novo on the pantetheinyl thiol of the multifunctional fatty acid synthase. Termination of growth of the acyl chain can be effected through the intervention of a thioesterase which removes the acyl moiety from the pantetheinyl residue, releasing it as a free fatty acid. In most tissues it is the resident thioesterase I domain of the fatty acid synthase which frees the acyl chain once it has reached 16 carbons in length. However, in some specialized tissues, such as non-ruminant mammary glands (2–4) and avian uropygial glands (5), a discrete monofunctional, monomeric protein, thioesterase II, catalyzes the hydrolysis of medium chain acyl moieties from the fatty acid synthase.

The amino acid sequences of rat (6–8) and duck (9) thioesterase II enzymes have been derived. Both enzymes are inactivated by serine esterase inhibitors, phenylmethylsulfonyl fluoride and diisopropyl fluorophosphate (4, 10), and the serine active-site peptide, containing a GSXGXG motif, has been identified (11). It has been shown by chemical modification that none of the 3 cysteinyl residues in thioesterase II is involved in the hydrolysis of acylthioester model substrates (12), but preliminary data have implicated the possible involvement of a histidyl residue (10, 13).

In the present paper we report evidence, from amino acid modification experiments and sequence comparisons, for the catalytic role of a histidyl residue in thioesterase II and confirm the identity of this histidine by site-directed mutagenesis.

**EXPERIMENTAL PROCEDURES**

Materials—Thioesterase II was purified from lactating mammary gland as described previously (4). The molar concentration of this enzyme was determined from its molar absorption coefficient, ε₉₀₀₀₀ = 0.85 M⁻¹ cm⁻¹ (14) and molecular mass of 29,500 Da (8).

Reactions with DEPC—DEPC was diluted with acetonitrile, and its concentration was measured with imidazole (15). Acetonitrile, up to 2% and for at least a 4-min incubation at 10 °C, had no effect on thioesterase activity. Control samples received the same amount of acetonitrile. Thioesterase was prepared in 50 mM potassium phosphate buffer (pH 6.0), 80 mM sodium chloride, 1 mM EDTA. Chemical modification was carried out at 10 °C, and periodically portions of the reaction mixture were withdrawn and added to an equal volume of 20 mM imidazole in 10 mM potassium phosphate (pH 7.5), 1 mM EDTA. Enzyme activity was assayed spectrophotometrically with decanoylpantetheine (80 μM) as a model substrate (16) or, where indicated, with a "natural substrate," S-acyl-(fatty acid synthase), where the resident thioesterase domain has been removed from the fatty acid synthase by trypsinization so that release of the fatty acid product is dependent on the presence of an exogenous thioesterase (17). The presence of imidazole had no effect on thioesterase II activity. The extent of histidine modification was calculated from the change in absorbance caused by the formation of N-ethoxycarbonyl-histidyl moieties, Δε = 3,200 M⁻¹ cm⁻¹ (18). Spectral and enzyme kinetics data were recorded using a Gilford Response model 237.

Reactivation with Hydroxylamine—Thioesterase II was first modified with DEPC at 15 °C; then the temperature was raised to 25 °C, and neutral hydroxylamine (2 M) was added to a final concentration of 0.5 M. Portions of the reaction mixture were withdrawn periodically for determination of enzyme activity.

Effect of Substrates and Products on Inactivation—Reagents were mixed with thioesterase II at 15 °C, and DEPC was added to a final
concentration of 0.1 mM. Periodically, portions of the reaction mixture were withdrawn, excess DEPC was neutralized with an equal volume of 20 mM imidazole, and a 40-μl portion of the reaction mixture taken for enzyme activity measurement (0.4 ml, final volume).

Plasmids, cDNA, and Bacterial Strains—Thioesterase II cDNAs, cloned into pUC120 and pJLA502 in XL1-Blue cells (Stratagene), were used for recombinant DNA constructions and enzyme expression, respectively (19). Standard recombinant DNA techniques (20) were utilized if not otherwise indicated.

Site-directed Mutagenesis—Mutations were introduced into the thioesterase in the pUC120 vector using the polymerase chain reaction, and the cDNAs were cloned as described previously (19). The strategy for mutant construction is summarized in Fig. 1. Two pairs of oligonucleotides were used in the polymerase chain reaction and are shown below with the altered nucleotides as lowercase letters. Reaction i is 5'-TGGTCACTGAGCATG, a primer that introduces a BstEII site at the same location (noncoding strand of thioesterase 11), and ii is 5'-AACATGGAGGATCCCC, a primer corresponding to the pUC multiple cloning site (noncoding strand of thioesterase II); thus the amplified cDNA from this reaction encodes a mutated codon and the remainder of the carboxyl-terminal region of thioesterase II. Reaction ii is 5'-TGGTCACTGAGCATG, a primer that introduces a BstEII site at the same location (noncoding strand of thioesterase II), and 5'-AACATGGAGGATCCCC, a primer corresponding to the 5' end of the coding strand and containing a Ncol site; thus the amplified cDNA from this reaction encodes the remainder of the thioesterase toward the amino terminus. Ncol-BstEII and BstEII-EcoRI fragments, derived from reactions ii and i, respectively, were first cloned into a convenient pUC120 construct with appropriately positioned Ncol, BstEII, and EcoRI sites. The choice of a plasmid containing the thioesterase I cDNA was based only on the convenient location of the three restriction sites; no thioesterase I cDNA was retained in the final construct. Finally, the entire Ncol-EcoRI fragment was transferred into the expression vector pJLA502 (21). Mutants were identified by dilutely sequencing (T7 Sequencing Kit, Pharmacia LKB Biotechnology Inc.).

Enzyme Expression and Purification—A 500-ml culture of a mutant construct in XL1-Blue cells was grown at 30 °C in TB medium (19) to an OD 600 of 2. The culture was warmed to 42 °C and transferred to 1.5 liter of TB medium which had also been prewarmed to 42 °C. The cells were grown for 6 h with approximately 0.5 liter/min oxygen supply. Every hour the pH was adjusted to 6.8 with 0.5 M KOH. Additional sterile glycerol was supplied in 15-ml portions after 2 and 4 h of incubation. Typically 2.2 liters of culture produced 13-17 g of wet cells. The cells were lysed, and thioesterase II mutants were purified as described earlier (19). During purification, thioesterase II mutant proteins were detected by dot blotting onto nitrocellulose, incubating with goat anti-rat thioesterase II antibodies, and staining by the ABC immunoperoxidase procedure (22).

Assay of Mutant Activity—Thioesterase activity of His-237 mutants was determined colorimetrically with either [1-14C]palmitoyl-CoA or S-[14C]acyl-(trypsinized fatty acid synthase) as a substrate (16, 19); no bovine serum albumin was included in the assay.

Mass Spectrometry—Electrospray ionization mass spectrometry was performed using a VG Bio-Q mass spectrometer as described previously (23).

RESULTS

Reaction with DEPC—Treatment of thioesterase II with a large excess of DEPC at pH 6 and 10 °C resulted in a rapid, time-dependent loss of enzyme activity. The low pH and temperature were chosen to increase the specificity of DEPC reaction with histidines (24) and to decrease the rate of the modification. To interpret the kinetics of inactivation the short half-life of the inhibitor in aqueous solution was taken into account. The first-order rate constant for the hydrolysis for DEPC, k′, was determined as 0.0032 min⁻¹ (data not shown), and it was then used to calculate the kinetics of inactivation according to the equation

\[ \ln(A/A_0) = -(k/k')\ln([\text{DEPC}]) + (1 - e^{-k't}) \]

where (A/A0) is the fraction of activity remaining after time t, k is the second-order rate constant for inhibition of enzymatic activity, and [DEPC]0 is the initial concentration of DEPC (25). The inactivation of thioesterase activity was found to follow pseudo first-order kinetics, and the rate constants were proportional to DEPC concentration (Fig. 2). The order of the reaction with respect to DEPC (26) was 1/2, and the second-order rate constant was calculated as 48.9 ± 3.6
M⁻¹ s⁻¹. Rate constants for inactivation by DEPC have been reported ranging from 0.017 M⁻¹ s⁻¹ at pH 6, 25 °C for fungal chloroperoxidase (27) to 31 M⁻¹ s⁻¹ at pH 6, 20 °C for pig lactate dehydrogenase (28) with average values in the 1–10 M⁻¹ s⁻¹ range. Thus thioesterase II appears to be one of the most sensitive enzymes to inactivation by this reagent.

Characterization of DEPC-modified Residues—In the pH range 6–7, the reaction of DEPC with proteins is relatively specific for histidyl residues, although there have been reports of other residues being modified (for review see Ref. 24). Normally, the difference spectrum between DEPC-treated and native enzyme exhibits a maximum near 240 nm because of the formation of an N-ethoxycarbonyl-histidyl residue (29). However, a similar effect has been observed for the modification of a cysteinyl residue with DEPC (30). The inhibition of thioesterase II by DEPC was accompanied by the appearance of an absorbance maximum at 236 nm in the difference spectrum (data not shown). No decrease in the absorbance at 278 nm was observed, eliminating the possibility that a tyrosyl residue was modified simultaneously (29). The inhibition of thioesterase II activity was completely reversed by neutral hydroxylamine (0.55 mM) within 10 min at 25 °C (Fig. 3B). The recovery of the activity was accompanied by a decrease in the absorbance at 236 nm. From this absorbance change we calculated that reactivation of the enzyme was accompanied by the removal of 1.1 mol of ethoxycarbonyl moiety/mol of enzyme. Neutral hydroxylamine is known to remove the ethoxycarbonyl group from modified histidine, tyrosine, and serine but not from lysine, arginine, and terminal amino groups (15, 24). Ethoxycarbonyl groups are not normally removed from cysteinyl residues by this treatment (24) although there may be exceptions to this rule (30). Since modification of accessible cysteine residues on thioesterase II with either 5,5′-dithiobis(nitrobenzoate) or methyl methanethiosulfonate does not alter the ability of the enzyme to hydrolyze model thioester substrates (12), we considered it most likely that inactivation of the enzyme by DEPC resulted from the modification of 1 or more histidyl residues although involvement of a seryl residue(s) could not be entirely discounted at this point.

Number of Residues Essential for Activity—Under the conditions described, 85% inactivation of thioesterase II was accompanied by modification of 2.3 histidyl residues (Fig. 3A). A classically employed method for the estimation of the number of residues essential for enzyme activity, involving extrapolation to zero of the plot of activity loss against the number of residues modified, gave an intercept on the x axis equal to 0.9 (data not shown). However, this method may not give accurate results in cases in which inactivation is very rapid. Therefore, we have used the statistical method of Tsou (31, 32), which describes the number of residues modified, m, according to Equation 2,

\[
m = n(1 - x) = n - p(A/A_0)^{10} - (n - p)(A/A_0)^{10}
\]

where \(n\) is the total number of modifiable residues, \(x\) is the total fraction of residues remaining, \(p\) is the number of residues, including \(i\) essential residues, which react with a given constant rate, \(k_i\); \((n - p)\) residues react with a different rate constant, \(k_{ii}\), and \(a\) is the coefficient in the equation \(k_i = a k_{ii}\). Equation 2 can be rearranged to Equation 3.

\[
log[nx/(A/A_0)] = log(n - p) + [(a - 1)/a]log(A/A_0)
\]

All possible combinations of values for \(i\), \(n\), and \(p\) from 1 to 6 were examined, and satisfactory results were obtained for \(i = 1\) and \(n = 5\) or \(n = 6\) (Fig. 3C). An exact determination of the value for \(p\) by this method is not possible because of insignificant changes in the linear regression coefficient, from 0.990 to 0.989 for \(p\) values ranging from 1 to 6. The calculated value of \(a\) was 0.35 for \(n = 5\). These results show that only 1 residue out of several modified is essential for thioesterase II activity. This finding is in accord with the observation that removal of only one ethoxycarbonyl moiety by treatment with hydroxylamine is sufficient to reactivate the enzyme completely (Fig. 3B).

Effect of Substrates and Products on Inactivation—None of the reagents examined protected thioesterase II from inactivation by DEPC. On the contrary, in the presence of the substrates decanoyl-CoA and decanoylpantetheine approximately a 3-fold increase of the second-order rate constant of inactivation was observed (Table 1). Decanoyl-N-acetylcysteamine and decanoylbenzenethiol (poor substrates) and decanoic acid, pantetheine, and acetyl-CoA (not substrates) had no effect on the rate of inactivation. Control experiments established that the enhanced rate of inactivation could not be attributed to carryover of substrates into the subsequent thioesterase assay, and a similar enhanced inactivation was evident when thioesterase activity was assessed using S-acyl-fatty acid synthase), the natural substrate (data not shown). In contrast to the stimulative effect of substrates on the rate of inactivation by DEPC, substrates protected the thioesterase from inactivation by PMSF (details not shown). Since PMSF inactivates the enzyme by sulfonylation of the residue Ser-101 (11), this result ruled out the possibility that inactivation by DEPC might involve the active-site serine and lent

### Table I

**Effect of substrates and products on inactivation of thioesterase II by DEPC**

Thioesterase II (4.4 μM) at pH 6 was preincubated with a substrate or product at the indicated concentration and incubated with DEPC (0.1 mM) at 15 °C. The second-order rate constants for inactivation, \(k\), were calculated and compared with the catalytic constant, \(k_{cat}\). The values of \(k_{cat}\) taken from previously published data (33), are presented for those reagents that can act as substrates.

| Additions          | Concentration | \(k\)  | \(k_{cat}\) |
|-------------------|---------------|-------|------------|
| None              |               | 54    |            |
| Substrates        |               |       |            |
| Decanoylpantetheine | 0.16          | 166   | 0.62       |
| Decanoyl-CoA      | 0.54          | 188   | 0.52       |
| Decanoyl-N-acetylcysteamine | 0.54 | 74    | 0.04       |
| Decanoylbenzenethiol | 0.64  | 54    | 0.08       |
| Others            |               |       |            |
| Decanoic acid     | 0.54          | 78    |            |
| Acetyl-CoA        | 0.54          | 64    |            |
| Pantetheine       | 0.54          | 63    |            |

**Fig. 3. Time course of number of histidine residues modified during inactivation and reactivation.** A, thioesterase II (19.3 μM) was inactivated with DEPC (0.25 mM) at 10 °C. Enzyme activity (□) was measured, and the number of modified histidines (○) was calculated from the difference spectra at 236 nm. B, reactivation with hydroxylamine. C, Tsou analysis of the data. The inactivation data in A were plotted using Equation 3 with \(p = 1\), \(n = 5\) (○) and \(n = 6\) (□); the linear regression coefficients, \(r^2\), were 0.989 and 0.990, respectively.
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Fig. 4. Resistance of the DEPC-inactivated thioesterase II to sulfonilation by PMSF. The wild-type thioesterase (19.1 μM) was incubated with DEPC (0.5 mM) for 5 min at pH 6, 10°C. The pH of the reaction mixtures was adjusted to 7.5 with KOH (0.5 M); PMSF (final concentration 0.5 mM) was added, and the samples were kept for 20 min at 20°C. Unreacted PMSF was removed using a Bio-Gel P-30 (Bio-Rad) spin column, neutral hydroxylamine (0.5 M final concentration) was added, and the samples were incubated for 20 min at 20°C. Thioesterase activity was determined spectrophotometrically with decanoyl-CoA (80 μM) as substrate. Thioesterase in the control reaction (no inhibitors added) lost 10% activity during the whole procedure. Bars represent data from duplicate determinations of enzyme activity before (solid bars) and after hydroxylamine treatment (hatched bars).

Further support to the conclusion that inactivation by DEPC is caused by the ethoxycarbonylation of a single histidyl residue.

Reaction of PMSF with DEPC-modified Thioesterase—The possibility that the DEPC-modified thioesterase II was deficient in its ability to form the acylenzyme intermediate was tested using PMSF, a transition state inhibitor for serine active-site esterases. Sulfonylation of the active-site serine by PMSF was assessed indirectly by determining whether enzyme activity could be restored by removing the ethoxycar- bonyl moiety with hydroxylamine (Fig. 4). Indeed, exposure of the DEPC-inactivated thioesterase to PMSF did not prevent the subsequent reactivation by hydroxylamine, indicating that ethoxycarbonylation of the essential histidyl residue protected the active-site serine against sulfonilation by PMSF. This result implies that the presence of a histidyl residue at the active site is required for activation of the Ser.

Selection of a Candidate Active-site Histidyl Residue—On the assumption that a catalytically important histidyl residue would likely be conserved in related thioesterases we attempted to identify candidate residues by comparison of the amino acid sequences of several such enzymes. A comparison of the amino acid sequences of the duck and rat thioesterase II enzymes (6) had revealed that 4 histidines were conserved at positions 100, 124, 134, and 237 (rat numbering). We had also noticed that although thioesterase I, a component of the rat fatty acid synthase, shares very limited overall sequence identity with the thioesterase II enzymes, a region near the carboxyl terminus exhibits some similarity with the thioesterase II region around His-237, both in primary and predicted secondary structure (34). The sequences of the thioesterase I domains associated with the mouse (35) and chicken (36, 37) fatty acid synthases are also highly conserved in the carboxy-terminal region. More recently the amino acid sequences of two putative thioesterases associated with bacterial peptide antibiotic synthases have been deduced (38). These enzymes may be responsible for removal of S-peptidyl moiety from the 4′-phosphopantetheine of the synthase complex. Although these bacterial enzymes exhibit remarkably extensive sequence identity with the rat and duck thioesterase II enzymes (ALIGN scores for the entire sequences range from 10 to 16 S.D. details not shown), only 2 histidyl residues are conserved in all four sequences, those corresponding to His-100 and His-237 in rat thioesterase II. Thus, the only histidine that is conserved in enzymes of the animal thioesterase I, the animal thioesterase II, and the bacterial peptide thioesterases is that corresponding to His-237 in rat thioesterase II. In all cases except the duck thioesterase II, this histidine is contained within a Gly-Asp-His tripeptide, a motif that has been recognized as containing a catalytically important histidine in mammalian carboxylesterases (39). The location of the conserved histidine in these enzymes is shown in Table II.

Construction and Characterization of His-237 Mutants—Two mutated sequences coding for His237Leu and His237Arg were constructed, recombinant DNAs cloned, and the mutant proteins expressed in Escherichia coli cells. The isolated His237Leu and His237Arg recombinant proteins were estimated to be sodium dodecyl sulfate-polyacrylamide gel electrophoresis to be 79 and 87% pure, respectively. As estimated by Western immunoblotting of crude E. coli extracts and the yield of purified enzyme, the amount of His237Leu mutant expressed (70 μg of isolated/g of wet cells) was considerably lower than either the wild-type recombinant or His237Arg mutant enzyme (460 μg of protein/g of wet cells). The reason for the lower expression of this mutant is unknown.

The molecular masses of the His237Leu and His237Arg mutant enzymes were determined by electrospray ionization mass spectrometry to be 29,452.5 ± 3.3 and 29,496.5 ± 4.2 Da, respectively, very close to the values predicted for the 263-residue, amino-terminally unblocked protein in which 1 histidine is replaced by either a leucine (29,447.1 Da) or an arginine (29,490.1 Da). Our earlier study had demonstrated that recombinant wild-type thioesterase II accumulates in E. coli cytosol as a 263-residue amino-terminally unblocked polypeptide (19).

Kinetic Properties of the Mutant Thioesterases—The purified mutants, His237Arg and His237Leu, had no measurable activity when assayed spectrophotometrically with either the model or natural substrates (data not shown). However, some activity was detected using more sensitive radiochemical assays with S-[14C]acyl-(fatty acid synthase) and [1-14C]palmitoyl-CoA as natural and model substrates, respectively (Table III). The kinetic studies with the natural substrate revealed that the His237Arg mutation caused a decrease in the value for Km of approximately 3 orders of magnitude, with only a small change in Km. Thus, the catalytic efficiency of this mutant was decreased approximately 700-fold relative to the wild type. Activity of the His237Leu mutant was too low to be detected, the sensitivity of the assay being limited by the significant endogenous rate of hydrolysis of the S-acyl-(fatty

| Enzyme                  | Sequence       | Ref. |
|------------------------|----------------|-----|
| Rat mammary thioesterase II | 232-MI,FQDDFY  | 6, 7|
| Duck uropygial thioesterase II | 221-SL,RQDDFY  | 9   |
| Bacillus brevis gramicidin synthase | 225-MFY,DDFY  | 38  |
| Streptomyces hyosporicu bialophis thioesterase | 215-EMQDDFY  |     |
| Rat fatty acid synthase thioesterase | 271-IEFDBRT  | 34  |
| Rabbit carboxylesterase | 436-TYVQDDGKD  | 39  |

a Personal communication from Dr. Charles Thompson, Institut Pasteur, Paris, France.

b The mouse and chicken thioesterase I domains have identical sequences in this region (35-37).

Table II

Amino acid sequences in the vicinity of conserved histidine in related serine esterases

[Table contents are not visible in this text format.]

C. Thompson, personal communication.
buffers were used for the pH range 6.1-7.5, 7.5-8.2, 8.1-10.1, and the catalytic process is quantitatively much less than that of pK of about 7.2. The contribution of this ionizable residue to thioesterase activity even above pH 9; however, significant chemical hydrolysis of the substrate at pH > 11 precluded further exploration of this phenomenon. These findings are consistent with the mutations having removed a catalytically important residue, the ionization state of which alters between pH 6 and 7.5.

Effect of DEPC and PMSF on Mutant Thioesterase Activity—If the inhibitory effect of DEPC on the normal thioesterase II is primarily the consequence of modification of His-237, then it follows that mutations at position 237 should reduce or even abolish sensitivity to this reagent. Indeed both the His237Arg and His237Leu mutant enzymes were inactivated less than 25% by DEPC treatment, as compared with 95% inactivation for the wild-type thioesterase (Table IV). The small loss of activity in the mutant enzymes may reflect modification of other residues, not necessarily histidines. Sensitivity to PMSF, an active-site serine-directed reagent was also greatly reduced in the mutants. Thus, under conditions that induce 98% inactivation of the wild-type enzyme, activity of the His237Leu mutant was unaffected, and that of the His237Arg mutant was reduced only by 23%. A 2-fold increase in the concentration of DEPC and PMSF did not produce further inactivation of the mutant thioesterases (data not shown).

**DISCUSSION**

The activity of thioesterase II was more than 95% inhibited by exposure to diethyl pyrocarbonate, a reagent that is known to modify histidyl residues with some degree of selectivity. From consideration of the spectral changes in the enzyme associated with the modification, the ability of hydroxylamine to reverse both the inhibition, and the spectral changes and the insensitivity of the enzyme to thiol-directed reagents we concluded that the inactivation was unlikely to result from the modification of tyrosyl, seryl, lysyl, arginyl, or cysteinyl residues, or terminal amino groups. Although several histidyl residues on the thioesterase appear to be modified by DEPC, one reacts considerably more rapidly, and removal of only one of the ethoxycarbonyl-His moieties with hydroxylamine is sufficient to reactivate the enzyme completely. These results provided a strong indication that a histidyl residue plays an important role in catalysis by thioesterase II. By looking for conserved histidyl residues in several structurally related proteins we were able to identify a single outstanding candidate for this role, His-237.

Mutation of His-237 to either arginine or leucine caused a decrease in the catalytic efficiency of 2-3 orders of magnitude. The residual activity associated with both mutants was relatively insensitive to inhibition by DEPC, supporting our hypothesis that His-237 is the critical residue modified during inactivation of the wild-type thioesterase by DEPC. Collectively, the results of the chemical modification experiments, the search for a conserved histidyl residue, and the mutagenesis experiments provide compelling evidence for an important role for His-237 in catalysis. Furthermore, the greatly diminished ability of ethoxycarbonyl-His-237 wild-type and His-237 mutant thioesterases to react with PMSF, a transition state inhibitor for serine active-site esterases, suggests

**TABLE III**

| Enzyme          | Natural substrate | Model substrate specific activity |
|-----------------|-------------------|----------------------------------|
|                 | $k_{cat}$ | $K_m$  | $k_{cat}/K_m$ | nmol/min/mg |
| Wild type       | 0.66    | 3.9    | 169           | 1.42        |
| His237Arg       | 0.0005  | 1.9    | 0.25          | 0.008       |
| His237Leu       | <0.03   | 0.019  |               |             |

**FIG. 5.** pH dependence of the activity of His-237 mutants. His237Leu (●) and His237Arg (○) were incubated for 2 min with [14C]palmitoyl-CoA (2 μM) at 30 °C. Potassium phosphate (50 mM), Tris-HCl (50 mM), borate (25 mM), and sodium phosphate (25 mM) buffers were used for the pH range 6.1-7.5, 7.5-8.2, 8.1-10.1, and 11.1-11.7, respectively. The points represent average values of data from four experiments. The pH activity profile for the wild-type thioesterase II (no points) is taken from data published previously.

acid synthase). In the model substrate assay, activity of the two mutant enzymes was estimated to be reduced on average by 2 orders of magnitude compared with the wild type. Because of the difficulties in obtaining reliable kinetic parameters with surface-active acyl-CoA substrates only the specific activities of the enzymes are reported.

The pH-velocity profile of thioesterase II indicated that the ascending portion resembled the ionization curve for a single group with a $K_a$ of about 6.7 (4), consistent with the involvement of a histidyl residue in catalysis. The profile was altered by mutation of His-237 (Fig. 5). Thus the pH optimum of the His237Leu mutant was shifted significantly to higher pH, and the profile resembled the ionization curve for a group with a $K_a$ of about 7.2. The contribution of this ionizable residue to the catalytic process is quantitatively much less than that of His-237. The His237Arg mutant continued to show increasing enzyme activity even above pH 9; however, significant chemical hydrolysis of the substrate at pH > 11 precluded further exploration of this phenomenon. These findings are consistent with the mutations having removed a catalytically important residue, the ionization state of which alters between pH 6 and 7.5.

**TABLE IV**

| Inhibitor | Wild-type | His237Leu | His237Arg |
|-----------|-----------|-----------|-----------|
| DEPC      | 4.6 ± 11.8| 73.2 ± 1.3| 81.2 ± 2.4|
| PMSF      | 2.2 ± 5.2 | 99.5 ± 2.2 | 76.6 ± 1.0 |
that His-237 cooperates with the active-site Ser-101 to facilitate catalysis.

The observation that substrates accelerated the inactivation by DEPC is not without precedent since a similar phenomenon was observed for inactivation of phosphoenolpyruvate carboxykinase by DEPC in the presence of the substrate bicarbonate (40). In the case of the thioesterase, it is conceivable that the strong hydrogen bond linking Ser-101 and His-237 in the resting enzyme could be replaced by a weaker interaction, possibly with water, in the transition state (41). Such a change would decrease protonation of the histidyl residue and consequently increase its reactivity toward DEPC.

The classical model for the serine active-site proteases involves a catalytic triad of serine, histidine, and aspartate residues which form a charge relay system that shifts the proton from the active-site serine, increasing the nucleophilicity of this residue and facilitating formation of the tetrahedral intermediate. From the present study, which reveals a cooperative interaction between Ser-101 and His-237, it would appear likely that a similar mechanism is operative in the thioesterases. However, at present there is no experimental evidence available to implicate a third member of the catalytic triad.

The mutagenesis experiments do in fact reveal some differences in the proteases and thioesterases. For example, the decrease in $k_{cat}$ observed in the histidine active-site mutants of thioesterase II, about 2–3 orders of magnitude, is considerably less than that observed with equivalent mutants of subtilisin. From mutagenesis studies it has been estimated that the active-site His-64 of the protease contributes a rate enhancement of the order of $10^6$ times (42). Nevertheless, subtilisin and thioesterase II exhibit similar $k_{cat}$ values with substrates so the lower rate enhancement contributed by the active-site histidine in the thioesterase might reflect the lower free energy of activation required for hydrolysis of thioesters. Second, in the absence of the catalytically important His-64 substrate so the lower rate enhancement contributed by the His-237 Arg mutant at higher pH is that the guanidyl group of Arg-237 in the wild-type thioesterase.

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Second, in the absence of the catalytically important His-64 at higher pH is that the guanidyl group of Arg-237 in the wild-type thioesterase.

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