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Human Acylpeptide Hydrolase

STUDIES ON ITS THIOL GROUPS AND MECHANISM OF ACTION*

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The presence of a cysteine residue(s) near the active site of acylpeptide hydrolase was suggested by inactivation of the enzyme with sulfhydryl-modifying agents and by the substantial protection against inactivation afforded by the competitive inhibitor acetylmethionine. 5,5′-dithiobis-(2-nitrobenzoate) titrations of the native and the denatured enzyme together with analysis for cysteic acid after performic acid oxidation showed that the enzyme contained 12 free SH groups and three disulfide bonds/monomer. Chemical modification with radiolabeled iodoacetamide led to the labeling of Cys-30 and Cys-64 suggesting that one or both of these Cys residues are close to the active site. Modification of one or both of them probably inhibits the enzyme either because of a distortion of the active site or because the adducts present a barrier to the efficient diffusion of substrates into and products out of the active site.

Studies on the mechanism of action of acylpeptide hydrolase have employed *p*-nitrophenyl-*N*-propyl carbamate as a potent active site-directed inhibitor. Enzyme inactivation, which follows pseudo first-order kinetics, is diminished by the competitive inhibitor acetylmethionine. The inhibited enzyme slowly regains activity at a rate that is increased in the presence of the nucleophile hydroxylamine. A general mechanism involving an acyl-enzyme intermediate is supported by evidence for the formation of acetyl-alanyl hydroxamate during hydrolysis of acetyl-alanine *p*-nitroanilide in the presence of hydroxylamine. The effects on V_{max} and K_m during this reaction indicate that hydrolysis of the acyl-enzyme intermediate is rate-limiting.

N-Acylpeptide hydrolase (EC 3.4.19.1) catalyzes the hydrolysis of N^{α}-acylated peptide substrates of various sizes and with different types of acyl groups (acetyl, chloroacetyl, formyl, and carbamyl) to generate an acylamino acid and a peptide with a free NH_2 terminus that is shortened by 1 amino acid (1–4), as described by the equation.

$$\text{acyl-aa}_1\text{-aa}_2\text{- .... aa}_n \rightarrow \text{acyl-aa}_1 + \text{aa}_2 \text{ .... aa}_n \qquad \text{(Eq. 1)}$$

The rates of hydrolysis of different blocked peptides vary considerably, depending on the nature of the first and second amino acids (5, 6). This specificity reflects the nature of the sequences of those isolated proteins acetylated at their NH_2 terminus, suggesting a possible role of this enzyme in co-translational or post-translational modification of the nascent polypeptide chain. The enzyme is also active on small acetylated bioactive peptides (5) such as α-melanocyte-stimulating hormone and ACTH, suggesting a possible biological role in controlling the concentration of these factors.

The complete primary structures of the rat and porcine enzymes, deduced from cDNA sequences, have been reported (7, 8). We (6) and Erlandsson *et al.* (9) have recently ascribed the gene coding for acylpeptide hydrolase to the DNF15S2 locus of human chromosome 3 at region p21, based on the DNA sequence studies of Naylor *et al.* (10). No overall resemblance to that of known proteases was noted. Acylpeptide hydrolase has been classified as a serine protease based on its modification at Ser-587 by diisopropyl fluorophosphate leading to complete inactivation (11). A radiolabeled chloromethyl ketone was employed to identify His-707 as a second active-site residue (11). A comparison with the active-site residues of several other peptidases suggested that acylpeptide hydrolase may be considered as a member of a new family of serine-type proteases (11–15).

Earlier studies in which His-707 of the catalytic triad was identified by labeling with a chloromethyl ketone derivative indicated that Cys-30 was weakly labeled (11). This observation was reminescent of the findings with several other serine proteases including prolyl endopeptidase (16) where there was also weak labeling of a few cysteine residues by peptide chloromethyl ketone inhibitors. These two enzymes have other similarities including the restricted specificity of both enzymes to act on relatively small peptides but not on proteins and the linear arrangement of the residues in their catalytic triads. Indeed, these enzymes along with several lipases and wheat serine carboxypeptidase could belong to a previously unrecognized family of serine proteases with a characteristic αβ-hydrolase fold (13, 17, 18). Except for the G*X*SYG motif around their active-site Ser residues, the overall primary structures of these enzymes bear little resemblance to one another. Hence, prolyl endopeptidase is a monomer, whereas acylpeptide hydrolase is a tetramer with four like subunits of 732 amino acids each. Whether there is any overall similarity in their three-dimensional structures is not yet known.

In this communication, we present the results of studies regarding the mechanism of action of the enzyme from the perspective of its sensitivity to carbamate derivatives, as previously reported for some other serine hydrolases (19). The enzyme has been previously reported to be sensitive to SH-modifying reagents. Further studies on this question as it relates to the mechanism of the enzyme are described herein.

MATERIALS AND METHODS

Acylpeptide hydrolase, isolated from human erythrocytes by the procedure previously described (2), is homogeneous as judged by a number of criteria including the presence of a single band in both denaturing

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and nondenaturing gel electrophoresis. Acetyl-alanine *p*-nitroanilide, *p*-nitrophenyl chloroformate, *N*-propylamine, *N*-methyl-propylamine, C_2-C_6 *p*-nitrophenyl esters, Ac-Met,[1] Ebelactone A, butaneboronic acid, phenylboronic acid, tributyrin, and L-α-dibutyl phosphatidylcholine were purchased from Sigma. All other materials were commercially available reagent-grade products. [^{14}C]iodoacetamide and [^{14}C]pHMB were from New England Nuclear.

Synthesis of PNPPC and PNPMPC—The *p*-nitrophenyl-*N*-propyl carbamate (PNPPC) and *p*-nitrophenyl-*N*-methyl-*N*-propyl carbamate (PNPMPC) were synthesized by a modification of the procedure described by Hosie *et al.* (20). Ten millimoles (2.01 g) of *p*-nitrophenyl chloroformate were dissolved in dry CH_2Cl_2 (15 ml) to which was added, over a period of 1 h, a solution of the appropriate amine (18 mM) in CH_2Cl_2 (5 ml). The reaction mixture was stirred for another 2 h for PNPPC and another 6 h for PNPMPC. The solutions were extracted with water (50 ml), 0.1 N HCl (50 ml), water (50 ml) and then dried over $MgSO_4$ overnight. The dichloromethane solutions were concentrated to dryness and the resulting material recrystallized. Recrystallization of PNPPC from CH_2Cl_2/petroleum ether afforded white needles that melted at 101–102 °C. PNPMPC gave a white oil. PNPMPC was purified by chromatography on Silica gel 60 (230–400 mesh, 30 × 2 cm) using CH_2Cl_2/MeOH (90:10 v/v) as solvent. Purity was established by thin layer chromatography on Silica in the following solvent systems: CH_2Cl_2/methanol (95:5 v/v) for PNPPC and (90:10 v/v) for PNPMPC. Elemental analysis: PNPPC, $C_{10}H_{12}O_4N_2$ (theoretical C, 53.56%; H, 5.39%; N, 12.49%; found C, 53.49%; H, 5.30%; N, 12.71%) and PNPMPC, $C_{11}H_{14}O_4N_2$ (theoretical C, 55.93%; H, 5.93%; N, 11.86%; found C, 55.21%; H, 5.68%; N, 11.68%).

Enzyme Assays—Acylpeptide hydrolase activity was assayed with 4 mM acetyl-alanine *p*-nitroanilide (AANA) in 0.1 M bis-Tris, pH 7.4, as substrate; the rate of appearance of *p*-nitroaniline was measured at 405 nm at 37 °C; ϵ = 7,530 M^{-1} cm^{-1} for *p*-nitroaniline was used for the calculations.

Chemical Modifications by Site-specific Reagents—Inactivations were performed by addition of the inhibitor to an enzyme solution in 0.1 M bis-Tris, pH 7.4, at 37 °C. At different times, aliquots were tested for enzymatic activity with 4 mM AANA as described above. The protective effect of Ac-Met against inactivation by PNPPC was determined by previous incubation of the enzyme with different concentrations of Ac-Met before the addition of the inhibitor.

Restoration of Activity to the Inhibited Enzyme—Acylpeptide hydrolase was incubated with PNPPC (200 μM) or Ebelactone A (300 μM) (21) for 120 and 30 min, respectively. The reaction mixture was diluted 5-fold into buffer containing different concentrations of hydroxylamine in 0.1 M bis-Tris, pH 7.4. Regeneration of activity was assessed by taking aliquots of this solution and diluting it into buffer containing 4 mM AANA; the effect of hydroxylamine on the nonenzymic hydrolysis of PNPPC was taken into account.

Hydroxamate Transfer Experiments during Catalysis—The effect of hydroxylamine on the kinetics of acylpeptide hydrolase-catalyzed hydrolysis of AANA was determined by measuring V_{max} and K_m values as a function of various concentrations of the nucleophile. After the reaction acetyl-alanyl hydroxamate was extracted from the reaction mixture with ethyl acetate and its properties compared with the authentic compound prepared by treating acetyl-alanine symmetric anhydride with hydroxylamine. The presence of acetyl-alanyl hydroxamate was determined by measuring the absorbance at 514 nm of the ferric complex formed in a weak acid ethanolic solution of 2 mM $FeCl_3$. The hydroxamate nature of the derivative obtained after the reactivation of the PNPPC-inhibited acylpeptide hydrolase with hydroxylamine was determined by thin layer chromatography (Silica plates with CH_2Cl_2/methanol (90:10) as solvent). The formation of the ferric complex of the hydroxymate was followed by measuring the absorbance at 504 nm of the ferric complex (22).

K_m, V_{max}, and K_i Determinations—The hydrolysis of esters as substrates was initiated by the addition of the appropriate amount of enzyme to different concentrations of substrates in 0.1 M bis-Tris, pH 7.4, at 37 °C. The rate of hydrolysis for the *p*-nitrophenyl esters was monitored by the increase in absorbance at 405 nm using ϵ = 19,300 M^{-1} cm^{-1} for the calculations. For α-naphthyl butyrate the rate of hydrolysis was monitored by the difference in absorbance at 235 nm using an ϵ = 24,200 M^{-1} cm^{-1}. The hydrolysis of butyryl thiocholine was determined by measuring the appearance of thiocholine with 5,5′-dithiobis-(2-nitrobenzoate) (DTNB) at 405 nm; ϵ = 13,600 M^{-1} cm^{-1} was used. Hydrolysis of L-α-dibutyl phosphatidylcholine and tributyrin was determined by RP-HPLC by determining the decrease of the amount of the compounds at different times after the addition of the enzyme; nonenzymatic hydrolysis was not significant. Analysis was performed using a C_{18} Vydac column (0.46 × 15 cm; particle size, 5 μm) and eluted with a gradient of 0.1% TFA containing acetonitrile in 0.1% TFA. K_i values were determined by adding the enzyme to 0.1 M bis-Tris, pH 7.4, containing different concentrations of inhibitors and AANA as substrate at 37 °C. All of the kinetic parameters were determined according to Cleland (23).

Chemical Analyses—Protein samples for amino acid analysis were dialyzed extensively against water (purified by reverse osmosis) before hydrolysis in 6 M HCl at 110 °C under reduced pressure for 22 or 72 h. For analysis of cysteine as cysteic acid, the samples were treated with performic acid before acid hydrolysis. For analysis of tryptophan, the samples were hydrolyzed in methanesulfonic acid. Amino acid analysis was performed on an amino acid analyzer (Beckman 6300) with System Gold data handling system. For determination of COOH-terminal amino acids, the protein samples were treated with carboxypeptidases Y, A, or B for 2 or 20 h at 25 °C. The proteins were precipitated in 5% sulfosalicylic acid and then centrifuged. The supernatants were subjected to amino acid analysis after neutralization to about pH 2 and the addition of pH 2.2 sodium citrate. The appropriate control values with either carboxypeptidases alone or protein alone were subtracted from the amino acid values found for the complete digest. Total hexoses were determined by the anthrone test using a standard curve constructed with α-mannose. Bovine serum albumin, trypsin (negative control), and acylase I (positive control) were also analyzed for carbohydrate. The presence of glucosamine and galactosamine was determined by amino acid analysis.

pI Determination—Isoelectric focusing was performed with IsoGel-agarose IEF plates (pH 3–7) (FMC BioProducts) in a Pharmacia Multiphor II electrophoresis apparatus at 15 °C for 60 min at constant power. Precast gels and pI markers were used according to the manufacturer's instructions.

Total Protein Digestions, Peptide Purifications, and Sequencing—Native acylpeptide hydrolase was labeled with [^{14}C]iodoacetamide or [^{14}C]pHMB, and the reaction mixtures were dialyzed exhaustively to remove excess radioactive reagents. After concentration in a Centricon 10 concentrator (Amicon) and denaturation in 8 M urea, the protein was reduced and digested with trypsin in 0.8 M bis-Tris, pH 8, and subjected to RP-HPLC as described previously (11). The labeled peptides were repurified, if necessary, and subjected to sequencing as described previously (11).

SH Determination—The free SH content of the native and the reduced enzyme was determined after mixing the protein samples with DTNB and measuring the absorbance at 405 nm; ϵ = 13,600 M^{-1} cm^{-1} was used for the calculations. The SH content was also measured for the enzyme denatured in 6.4 M guanidine-HCl, 0.2 M bis-Tris, pH 7.4, reduced with 25 mM dithiothreitol, and then dialyzed extensively to remove any residual reducing agent. The SH content was also determined as cysteic acid after oxidation of the protein with performic acid.

RESULTS

Amino Acid Composition of Human Red Cell Acylpeptide Hydrolase—The complete protein sequences translated from the cDNA sequences corresponding to the rat and porcine acylpeptide hydrolase enzymes have been reported (7, 8). The pig and rat enzymes are homotetramers of 732 amino acid residues each. A cDNA sequence of a region on the short arm of the human chromosome 3 bears an 87% homology to the rat and porcine cDNA sequences of human acylpeptide hydrolase (6, 9). However, the reported translated protein sequence is different in some parts, because the reported cDNA sequence (10) has deletions of some bases in several places when aligned with the cDNA sequence of acylpeptide hydrolase.

Experimental support for the proposal that the translated cDNA corresponding to the DNF15S2 locus of human chromosome 3-encoded acylpeptide hydrolase was provided during studies in which the sequence of about 30 purified tryptic peptides isolated from the human enzyme comprising nearly 300 amino acids was determined (11). In each case sequence homol-

[1] The abbreviations used are: Ac-Met, acetylmethionine; AANA, acetyl-alanine *p*-nitroanilide; Ac, acetyl; BBA, butaneboronic acid; DTNB, 5,5′-dithiobis-(2-nitrobenzoate); pHMB, *p*-hydroxymercuribenzoate; PNPPC, *p*-nitrophenyl-*N*-propyl carbamate; PNPMPC, *p*-nitrophenyl-*N*-methyl-*N*-propyl carbamate; RP-HPLC, reversed-phase high performance liquid chromatography; TFA, trifluoroacetic acid.

TABLE I
Comparative amino acid composition of human acylpeptide hydrolase with the pig and rat enzymes

	Human (this study)	Pig[a]	Rat[b]
Asp	56.8	52	57
Thr	28.6[c]	33	29
Ser	61.5[c]	67	67
Glu	89.7	83	80
Pro	49.1	47	50
Gly	51.1	57	54
Ala	47.4	44	45
Cys	16.9[d]	18	19
Val	57.9	68	61
Met	14.3[c]	20	19
Ile	21.0	18	24
Leu	75.8	74	75
Tyr	21.1	22	24
Phe	30.2	32	29
His	17.9	16	19
Lys	30.7	28	30
Trp	17.1[e]	16	16
Arg	32.3	37	34

[a] Reported in Ref. 8.
[b] Reported in Ref. 7.
[c] Uncorrected by loss during acid hydrolysis.
[d] Determined as cysteic acid after performic acid oxidation.
[e] Determined after hydrolysis in methane sulfonic acid.

TABLE II
COOH-terminal analysis of human acylpeptide hydrolase by digestion with carboxypeptidases

The digestions were performed with carboxypeptidases A, B, and Y as described under "Materials and Methods." Blank values for the acylpeptide hydrolase alone or carboxypeptidases alone were subtracted.

Amino acid	Amino acid released		
	by carboxypeptidase A	by carboxypeptidase B	by carboxypeptidase Y
	nmol/nmol of subunit		
Ser	0.83	0.81	0.67
Gly	0.57	0.52	0.47
Leu	0.11	0.16	0.13
His	0.06	0.06	0.05

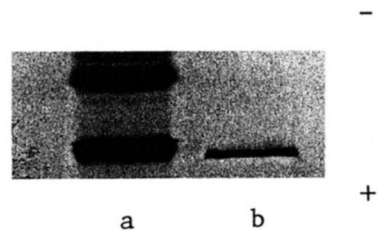


FIG. 1. **Flat-bed agarose isoelectric focusing gel of purified acylpeptide hydrolase.** *Lane a*: glucose oxidase, pI = 4.2 (*lower band*); ovalbumin, pI = 4.8 (*upper band*). *Lane b*: purified acylpeptide hydrolase.

ogy with the rat and porcine enzymes was nearly 90% except for some conservative amino acid replacements. Hence, the deletions in the cDNA sequence (10) are incorrect, because segments of the tryptic peptide sequence do not correspond to the protein sequence translated from the cDNA.

Attempts to determine the NH_2-terminal sequence of the protein by Edman degradation indicated the presence of a blocked NH_2 terminus as determined for the rat and porcine enzymes (24). In the present study, we have performed additional structural studies. The number of amino acids/subunit of the purified human enzyme is given in Table I; the findings are in full agreement with its molecular weight determined by independent means. The results are also consistent with the amino acid composition of the rat and the porcine enzymes within the experimental error for such a large protein (Table I).

Analyses of the COOH terminus of human acylpeptide hydrolase by separate digestions with three types of carboxypeptidases gave the results shown in Table II. Four amino acids were consistently released by each of the three enzymes in good yield, serine, glycine, leucine, and histidine, in decreasing amounts. These results are in accord with the sequence of -His-Leu-Gly-Ser-COOH reported for the porcine and rat enzyme, and they are in agreement with the translated cDNA sequence of the locus DNF15S2 of the human chromosome 3 (10). Analysis for carbohydrate by the anthrone test and amino acid analysis for glucosamine and galactosamine were both negative.

Isoelectric pH of Acylpeptide Hydrolase—Homogeneous human red cell acylpeptide hydrolase was analyzed by isoelectric focusing with ampholines ranging in pH from 3.0–7.0. When the positions of standard proteins relative to their pI were plotted, a polynomial curve fit was used to assign a pI value of 4.1 for acylpeptide hydrolase (Fig. 1). This result indicates the acidic nature of the enzyme and is consistent with its chromatographic behavior observed during the purification of the enzyme (2).

Thiol Groups of Acylpeptide Hydrolase—The amino acid analysis shown in Table I indicates that there are 17 cysteine residues as determined by cysteic acid analysis after performic acid oxidation of the enzyme. This value is in good agreement with the 18 cysteine residues deduced from the translated DNF15S2 locus of the human chromosome 3 (10). To compare this value with that found with SH titrating reagents under different conditions, human acylpeptide hydrolase was titrated with DTNB both in its native form (reduced and unreduced) as well as in the denatured state. For the native enzyme, two SH groups/protein subunit were titrated with DTNB within 1 h, and this value remained constant for up to 4 h. When the enzyme was denatured in 6.4 M guanidine hydrochloride, about 12 sulfhydryl groups/monomer reacted with DTNB. The denatured enzyme was reduced with dithiothreitol and then dialyzed exhaustively to remove the excess reducing agent; 18 SH groups were titrated for the reduced enzyme, an increase of nearly 6 SH groups/molecule of subunit. These results are in excellent agreement with those found by amino acid analysis (Table I) and the value calculated from the cDNA sequence of the DNF15S2 locus. Thus, of the 18 SH groups/subunit, 12 are free and 6 exist as disulfide bonds.

Effect of Thiol-specific Reagents on Enzyme Activity—Acylpeptide hydrolase has been reported to be inactivated with thiol-specific reagents (1, 11). Recently, we reported that it should be classified as a serine protease based on the labeling of its active-site Ser-587 with diisopropyl fluorophosphate resulting in inactivation (11). It was also noted that during labeling of the catalytic His-707 by acetyl leucine chloromethyl ketone one particular Cys residue was also weakly labeled (11). Other serine proteases also suffer inactivation by thiol-specific reagents, but the mechanism of this inactivation is not fully appreciated.

It is conceivable that bulky reagents that react with a Cys at or near the active site could interfere with catalytic activity of this enzyme even if the susceptible group of the enzyme is not part of the catalytic mechanism. To test this hypothesis, various thiol reagents of different sizes were tested to probe the catalytic activity of acylpeptide hydrolase; the results are illustrated in Fig. 2*A*. pHMB inhibited catalytic activity rapidly and completely. With *N*-ethylmaleimide, a higher concentration (5 mM) was required, and a longer period was needed to achieve the total inhibition achieved with HMB. With DTNB (2 mM) and iodoacetamide (10 mM), there was much less inhibition (50 and 15%, respectively. Ac-Met, a competitive inhibitor of acylpeptide hydrolase, efficiently protected against the inacti-

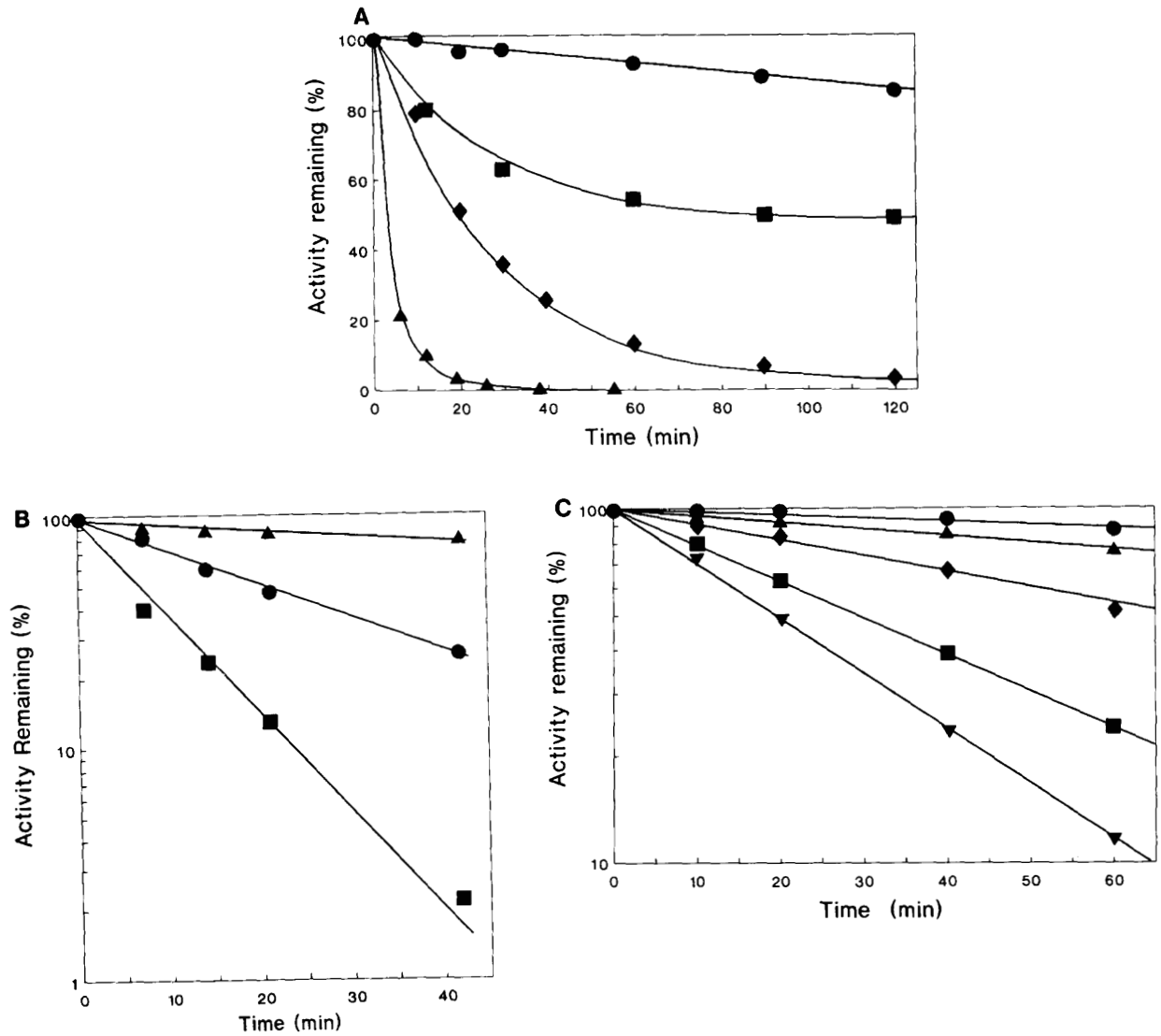


FIG. 2. *A*, inactivation of acylpeptide hydrolase by thiol reagents. The enzyme was incubated with 0.1 mM pHMB (▲), 5 mM *N*-ethyl-maleimide (◆), 2 mM DTNB (■), or 10 mM iodoacetamide (●) in 0.1 M bis-Tris, pH 6.5, at 37 °C. The enzyme activity was determined with AANA as substrate as previously described. *B* and *C*, protection by Ac-Met from inactivation by thiol reagents. The enzyme was preincubated with different concentrations of Ac-Met and then reacted with pHMB (Fig. 3*B*) or *N*-ethyl-maleimide (Fig. 3*C*) in 0.1 M bis-Tris, pH 6.5, at 37 °C (Fig. 3*B*). Ac-Met: none (■); 20 μM (●); 200 μM (▲) (Fig. 3*C*). Ac-Met: none (▼); 10 μM (■); 50 μM (◆), 200 μM (▲); 500 μM (●). The enzyme activity was determined as described in the text.

vation by pHMB and *N*-ethylmaleimide (Figs. 2, *B* and *C*) with effective concentrations in the range of 0.2–0.5 mM, similar to its K_i value (0.2 mM) (25). These results suggested that the inactivation by these three thiol reagents occurred near the active site of the enzyme.

[^{14}C]Iodoacetamide Labeling of Acylpeptide Hydrolase—To determine the identity and the extent of labeling of Cys residues by SH titrants and to identify these sites, studies with radiolabeled iodoacetamide were undertaken. The enzyme (1 mg, 13.3 nmol subunit) in 0.1 M bis-Tris, pH 7.4, was treated with 1 μmol of [^{14}C]iodoacetamide (50 μCi) at 37 °C overnight to achieve a high degree of inactivation. The results in Fig. 2*A* indicate a slow and incomplete inactivation by iodoacetamide. The inactive enzyme had incorporated 1.91 mol of radioactive reagent/mol of protein subunit.

To identify the modified Cys residue, the enzyme was denatured with urea, digested with trypsin, and subjected to RP-HPLC as described under "Materials and Methods." Three radioactive peaks were obtained, as shown in Fig. 3. After further purification, the first peak gave the sequence corresponding to the amino acids 63–65 in the primary structure of the enzyme

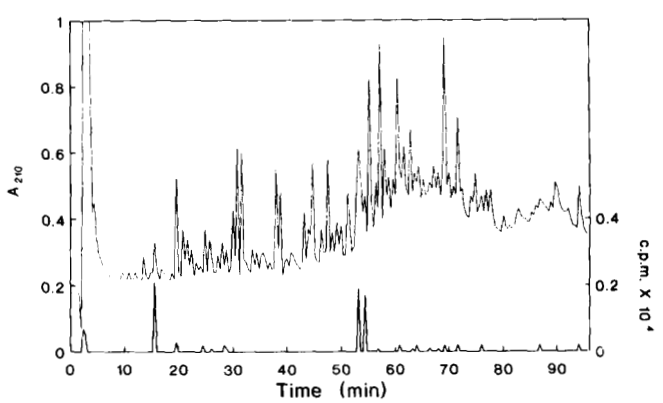


FIG. 3. **RP-HPLC peptide mapping of a tryptic digest of acylpeptide hydrolate after inactivation with [^{14}C]iodoacetamide.** The tryptic peptide map was obtained on a Vydac RP-HPLC column equilibrated in 0.1% TFA and eluted with a gradient of 0–55%, 0.1% TFA, and 80% acetonitrile in 0.1% TFA over a period of 95 min (*upper line*, A_{210}). Fractions were collected, and the radioactivity found in each fraction was plotted (*lower line*).

TABLE III

Amino acid residues of human acylpeptide hydrolase modified by iodoacetamide

Amino acid sequences of the purified radioactive peptides were obtained from [^{14}C]iodoacetamide-labeled acylpeptide hydrolase after tryptic digestion. *Cys* denote positions where carboxyamido methyl cysteine was identified. The numbers given below the sequence are the counts/min found for each cycle of the Edman degradation.

Peak 1	Phe-*Cys*-Arg	(Residues 63–65)
	0 534 257	
Peak 2	Gln-Pro-Ala-Leu-Ser-Ala-Ala-*Cys*-Leu-Gly.....	(Residues 23–32)
	0 26 19 26 3 13 36 1200 831 6	
Peak 3	Gly-Leu-Ser-Arg-Gln-Pro-Ala-Leu-Ser-Ala-Ala-*Cys*-Leu-Gly-Pro...	(Residues 19–33)
	0 5 31 5 18 23 16 97 38 39 39 479 282 144 74	

(Table III). Thus, Cys-64 was a site of modification by iodoacetamide. The second peak gave the sequence corresponding to the first 10 residues in the peptide comprising residues 23–42. The amounts of radioactivity released at each cycle of Edman degradation were determined and, including the amount corresponding to one cycle of carry-over, the majority (93.6%) occurred in the position corresponding to Cys-30 identified as carboxyamide methyl phenylthiohydantoin-cysteine derivative. The third peak gave a similar sequence corresponding to the first 15 residues in the peptide comprising amino acids 19–42 in the sequence of the enzyme. For this peptide, Cys-30 was the site of modification with 59.1% of incorporated radioactivity. It is likely that Cys-30 and Cys-64 correspond to the 2 SH groups that are titrated by DTNB in the native enzyme.

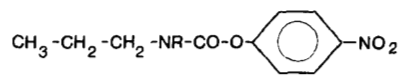
In a similar experiment in which the enzyme was labeled with [^{14}C]pHMB, a radioactive peak was isolated after tryptic digestion and RP-HPLC analysis (data not shown). It gave the sequence corresponding to the first 8 residues in the peptide comprising residues 23–30. However, the reducing conditions employed during Edman degradation precluded determination of the radioactivity released with each cycle of degradation. In an earlier report (11), Cys-30 was also labeled with [^{14}C]acetyl leucine chloromethyl ketone. When prolyl endopeptidase was inactivated at its catalytic His-680 residue by a peptidylchloromethane ketone, it was reported that several Cys residues were also labeled by this reagent; the most predominant of these was Cys-25 (16).

Inhibition by Carbamates—Studies were undertaken to elucidate the roles of Ser-587 and His-707 in the catalytic mechanism of the enzyme and to determine how it was related to the mechanism of related enzymes. Because several lipases and esterases have similarities to acylpeptide hydrolase (13, 17, 18), we decided to study the effects of the carbamate-type inhibitors, because these are especially potent on the activity of these enzymes. Carbamates have also been described to have inhibitory effects on serine proteases (19) and esterases (20, 22). In an attempt to establish the mechanism of inhibition of acylpeptide hydrolase, studies with PNPPC and related compounds were employed (Scheme I).

In this carbamate inhibitor, PNPPC incorporates a nitrogen adjacent to the scissile ester bond. The structure of this compound is unrelated to *N*-acetylated peptides but strongly resembles that of acyl esters shown below to be good substrates for acylpeptide hydrolase.

Acylpeptide hydrolase shows a time-dependent loss of activity in the presence of PNPPC. The decrease in enzyme activity is dependent upon different concentrations of PNPPC (Fig. 4*A*). When the slopes of the rates of inactivation at different PNPPC concentrations were plotted in a secondary plot, the inactivation rate constant was calculated to be about 0.083 min^{-1}. In the presence of Ac-Met (0.2 mM), a competitive inhibitor of acylpeptide hydrolase, there was 82% protection in the presence of 100 μM PNPPC over a period of 1 h (Fig. 4*B*).

The inhibition of acylpeptide hydrolase by PNPPC is consistent with the stabilization of the inhibited enzyme in a carbamyl-



SCHEME I. **Carbamates used with acylpeptide hydrolase.** *R* = *H*, PNPPC; *R* = CH_3, PNPMPC.

serine structure (22) (Scheme II). The essential feature of this proposed mechanism is the presence of a hydrogen bond between the carbamate NH adjacent to the acyl serine intermediate and His-707 of the active site that prevents turnover of the acyl-enzyme intermediate by stabilizing the inhibited enzyme. This scheme is similar to that suggested by Quinn and his colleagues for lipases (20, 22). This interaction should inhibit general base catalysis of decarbamylation by His-707. During normal catalysis a similar hydrogen bond does not exist in the acyl-enzyme intermediate. As a test of the validity of this mechanism, the *N*-methyl-substituted analog of PNPMPC (Scheme II), was synthesized and tested with acylpeptide hydrolase. This compound, which cannot form a hydrogen bond between the acyl-enzyme and His-707 because of the presence of the *N*-methyl group, did not inhibit the enzyme up to a concentration of 1.5 mM. These results are also consistent with Scheme II.

Reversal of the Inhibition—When the inhibited enzyme was diluted, there was a slow return of activity with time (Fig. 5*A*). This behavior is similar to that found for this class of inhibitor with esterases and lipases. The intermediate in the inhibited enzyme can be cleaved by hydroxylamine, which can act as an alternative base in catalyzing the deacylation step resulting in formation of the hydroxamate. The addition of 0.1 M NH_2OH gave a 4-fold increase in the rate at which enzyme activity was restored, and it occurred in a concentration-dependent fashion, *i.e.* there was complete restoration of activity with 0.5 M NH_2OH. Hydroxylamine had no effect on the spontaneous hydrolysis of the carbamate inhibitor, because the initial rates of the nonenzymic hydrolysis in the presence and absence of NH_2OH were the same within experimental error. The hydroxamate nature of the derivative obtained after the reactivation of the PNPPC-inhibited enzyme was determined as the Fe^{3+} complex of the hydroxamate formed in an ethanolic solution of 2 mM $FeCl_3$.

Inhibition of Acylpeptide Hydrolase by Ebelactone A and Restoration of Activity—We previously described the inhibition of acylpeptide hydrolase by Ebelactone A (11, 21). However, the mechanism of inhibition by the long-chain lactone, whose structure is completely unrelated to any of the peptide substrates for this enzyme, was unknown. This compound has been reported to inhibit hog liver esterases, hog pancreatic lipase, and rat liver formyl methionine aminopeptidase (21). For acylpeptide hydrolase, we proposed that the lactone structure opens during the process of inhibition; the acyl serine intermediate that is formed is stabilized by a hydrogen bond to His-707, similar to the carbamate-type inhibitor. Support for this type of structure comes from the profile for the restoration of enzyme activity upon dilution and by hydroxylamines. Thus, as described above for the PNPPC-inhibited enzyme, upon dilution there was also

TABLE IV

Efficiency of cleavage of acyl esters by acylpeptide hydrolase

The V_{max} and K_m values were obtained in 0.1 M bis-Tris buffer, pH 7.4, at 37 °C.

Substrate	V_{max}	K_m	V_{max}/K_m
	mM min^{-1}	*mM*	*min*$^{-1}$
p-Nitrophenylacetate	0.28 ± 0.01	0.63 ± 0.07	0.44 ± 0.03
p-Nitrophenylpropionate	0.76 ± 0.01	0.16 ± 0.01	4.75 ± 0.19
p-Nitrophenylbutyrate	1.44 ± 0.03	0.02 ± 0.01	72.00 ± 6.16
p-Nitrophenylvalerate	0.69 ± 0.04	0.07 ± 0.01	9.85 ± 2.02
p-Nitrophenylexanote	0.48 ± 0.01	0.06 ± 0.01	8.00 ± 1.25
Naphthyl butyrate	1.50 ± 0.02	0.01 ± 0.005	136.36 ± 38.09
Butyryl thiocholine	0.71 ± 0.01	1.00 ± 0.19	0.71 ± 0.02

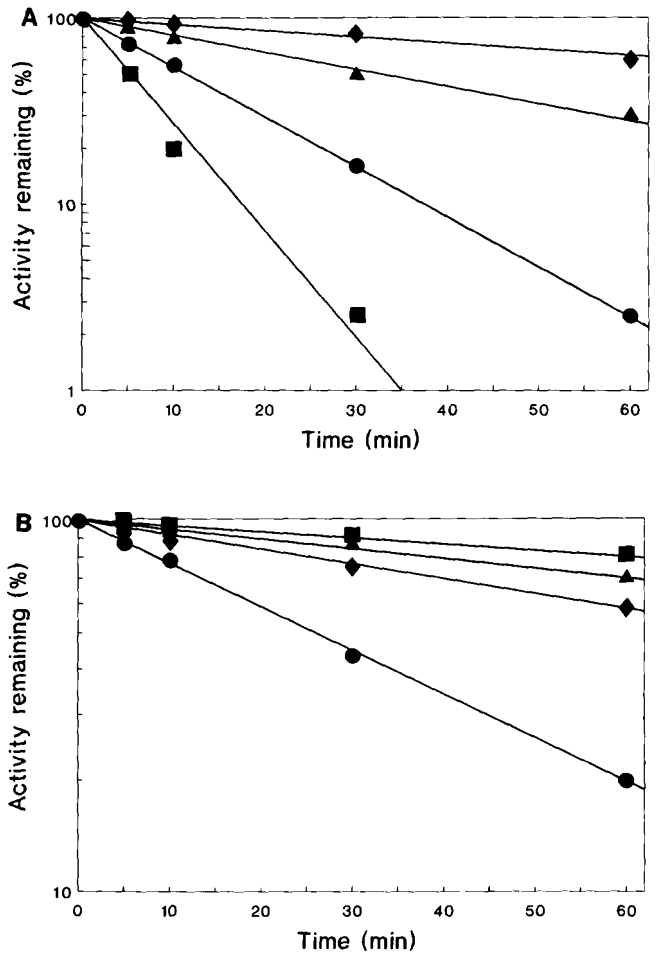


FIG. 4. *A*, inactivation of acylpeptide hydrolase by PNPPC. Semilogarithmic plot of the remaining enzyme activity *versus* time after incubation with various concentration of PNPPC (50 μM (♦), 100 μM (▲), 200 μM (●), and 500 μM (■)) in 0.1 M bis-Tris, pH 7.4, at 37 °C. Enzyme activity was determined with acetyl-alanine *p*-nitroanilide as substrate measuring the rate of appearance of *p*-nitroaniline at 405 nm at 37 °C. *B*, protection by Ac-Met from inactivation of acylpeptide hydrolase by PNPPC. The enzyme was treated with 100 μM PNPPC in 0.1 M bis-Tris, pH 7.4, at 37 °C in the absence or presence of different concentrations of AcMet (0 μM (●), 50 μM (♦), 100 μM (▲), and 200 μM (■)). The enzyme activity was determined with AANA as the substrate as previously described.

restoration of activity to the Ebelactone A-inhibited enzyme (Fig. 5*B*). Hydroxylamine also strongly influenced the rate of reactivation in a pattern remarkably similar to that found for the PNPPC-inhibited enzyme (Fig. 5*A*). It was effective at lower concentrations than with the PNPPC-inhibited enzyme. Thus, in the presence of 0.1 M NH_2OH the rate for the return of activity was 5.39 × 10^{-4} min^{-1} with PNPPC and 5.27 × 10^{-3} min^{-1} with Ebelactone A, respectively, a 10-fold difference.

(±)β-Butyrolactone and 3-hydroxy-2,2,4-trimethyl-3-pentenoic β-lactone, compounds with a β-lactone structure, like Ebelactone A, were ineffective inhibitors of acylpeptide hydrolase. Thus the long fatty chain of Ebelactone A is important for the interaction with the enzyme binding pocket to maximize the inhibition.

pH Dependence Studies—Serine proteases have a catalytically competent histidine residue, which facilitates both the formation and the decomposition of the acyl-enzyme intermediate (26). The ionization of this residue governs the pH dependence of the catalysis and conforms to a simple dissociation curve with a pK_a value of 6.95 ± 0.06 for the inflection point. The same experiment performed using Ebelactone A as inhibitor gave a pK_a of 6.75 ± 0.04. The corresponding value for acetyl-alanine *p*-nitroanilide as substrate was 6.91 ± 0.05. Recently, apparent pK_a values for Ac-Ala-His-Ala and Ac-Ala-Ala-His-Ala substrates were reported as 6.72 and 6.79, respectively (27). Thus, the pH dependence with substrates and inhibitors are the same within experimental error. These results suggest that the inhibitors are indeed active site-directed.

Effect of Hydroxylamine on the Hydrolysis of Substrates—The studies described above on the inhibition of enzyme activity by a stable acyl intermediate are consistent with the presence of an analogous intermediate during normal catalysis. To gain further support for this mechanism, the hydrolysis of acetyl-alanine *p*-nitroanilide was determined in the presence of hydroxylamine. The formation of the acetyl-alanyl hydroxamate obtained was determined as described under "Materials and Methods" by measuring the absorbance of the ferric complex at 514 nm. The effect of different concentrations of hydroxylamine on the Lineweaver-Burk plot is shown in Fig. 6*A*. The parallel pattern of lines, which arises from equal increase in V_{max} and K_m, is consistent with nucleophilic trapping of an acyl-enzyme intermediate whose hydrolysis is the rate-determining step in the absence of nucleophiles. The dependence of V_{max} and K_m on hydroxylamine concentration is illustrated in Fig. 6*B*. These results are completely consistent with the interpretation given above and also with Scheme III. Nucleophilic attack by the active-site serine and consequent loss of *p*-nitroaniline produces the acyl-enzyme intermediate. Hydroxylamine activates by providing an alternate route for decomposition of this intermediate. For such a mechanism, the steady-state activation of the kinetic parameter gives:

$$V_{max} = k_{cat}[E]_T = (k_3 + k_4[NH_2OH])[E]_T \quad \text{where } [E]_T = [E] + [ES] \qquad \text{(Eq. 2)}$$

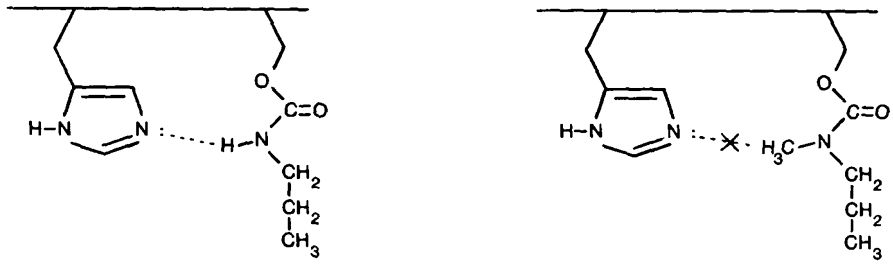
$$K_m = K_s \frac{k_3 + k_4[NH_2OH]}{k_2 + k_3 + k_4[NH_2OH]} \simeq K_s \frac{k_3 + k_4[NH_2OH]}{k_2} \quad \text{if } k_2 \gg k_3 + k_4[NH_2OH] \qquad \text{(Eq. 3)}$$

where K_s is the Michaelis constant for the enzyme substrate complex.

Equations 2 and 3 are based on the supposition that hydrolysis of the acyl-enzyme intermediate is the rate-determining step. These equations predict linear and matching increases in V_{max} and K_m as the hydroxylamine concentration increases. This mechanism is analogous to that of nucleophilic activation described for other serine proteases (28, 29).

Common Inhibitors for Acylpeptide Hydrolase, Lipases, and Esterases—To study the functional relationship between acylpeptide hydrolase and the lipase/esterase family of enzymes, potent inhibitors of these latter enzymes were studied with acylpeptide hydrolase. Butane boronic acid (BBA) and phenyl boronic acid have been reported to be good competitive inhibitors of lipoprotein lipase and carboxyl esterase (30). These compounds were also found to be good competitive inhibitors of acylpeptide hydrolase. The Dixon plots for BBA and phenylboronic acid indicate K_i values of 0.24 ± 0.01 mM and

SCHEME II. **Carbamyl-acylpeptide hydrolase intermediates.**



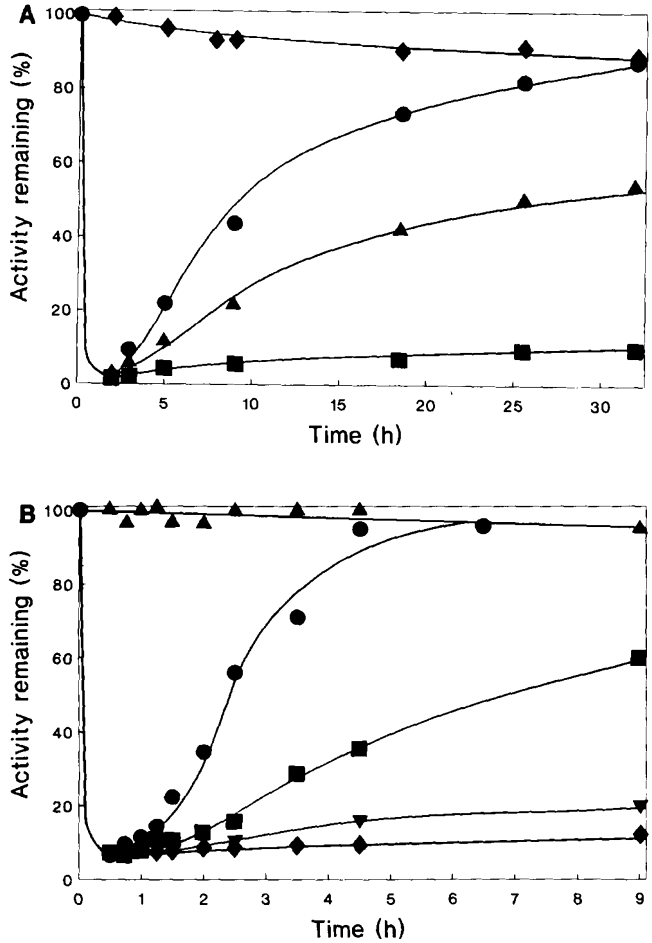


FIG. 5. *A*, long term time courses for inactivation by PNPPC show sequential rapid inhibition, steady-state, and activity return phases. Acylpeptide hydrolase was incubated with 200 μM PNPPC in 0.1 M bis-Tris, pH 7.4, at 37 °C. The inhibited enzyme was diluted into buffer (■), buffer containing 0.1 M NH_2OH (▲), or 0.5 M NH_2OH (●). The spontaneous inactivation of acylpeptide hydrolase (♦) is also represented in the figure. The enzymatic activity was obtained using AANA as substrate. *B*, long term course for the inactivation of the enzyme by Ebelactone A shows sequential rapid inhibition, steady-state, and activity return phase. Acylpeptide hydrolase was incubated with 300 μM Ebelactone A in 0.1 M bis-Tris, pH 7.4, at 37 °C. The inhibited enzyme was diluted into buffer (♦), buffer containing 0.02 M NH_2OH (▼), 0.05 M NH_2OH (■), and 0.1 M NH_2OH (●). The spontaneous inactivation is also represented (▲). The enzymatic activity was obtained using acetyl-alanine *p*-nitroanilide as substrate.

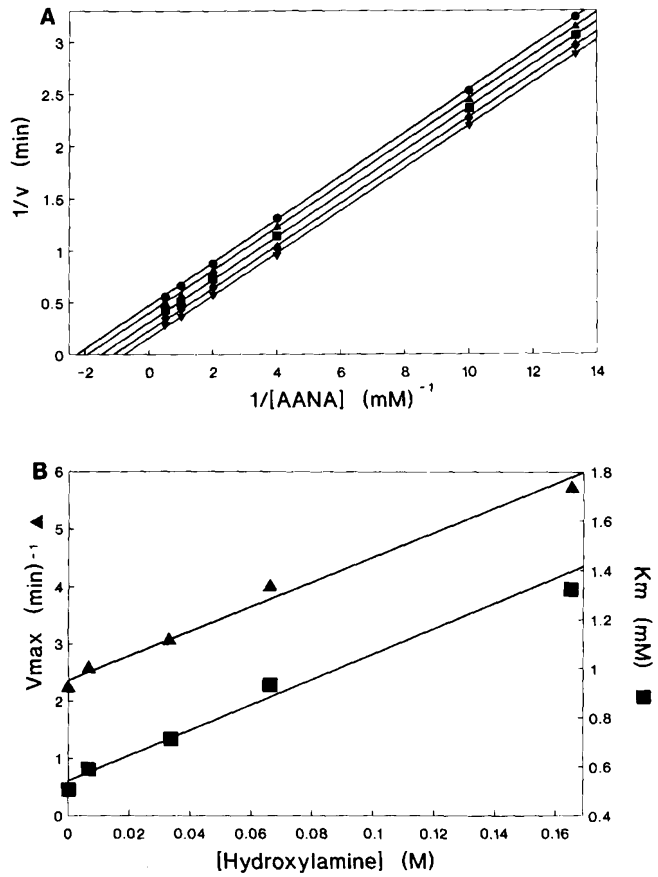
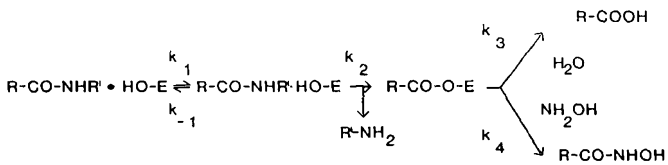


FIG. 6. *A*, hydroxylamine activation of acylpeptide hydrolase-catalyzed hydrolysis of AANA. Varying concentrations of the substrate were incubated in 0.1 M bis-Tris, pH 7.4, at 37 °C in the presence of NH_2OH = 0 M (●), 0.006 M (▲), 0.038 M (■), 0.066 M (♦), and 0.165 (▼). The reaction was started by the addition of an appropriate amount of enzyme. *B*, hydroxylamine concentration dependence of V_{max} and K_m for the acylpeptide hydrolase-catalyzed hydrolysis of AANA.



SCHEME III. **Mechanism of acylpeptide hydrolase-catalyzed hydrolysis of AANA in presence of hydroxylamine.**

0.84 ± 0.07 mM, respectively (Fig. 7, *A* and *B*). These values are similar to the K_m value of acetyl-alanine *p*-nitroanilide with acylpeptide hydrolase. Other boronic acids have also been used as transition-state analog inhibitors of serine proteases (31). Phenyl-*n*-butylborinic acid, which contains the hydrocarbon functionalities of both above-mentioned boronic acid and retains the electrophilic boron, was also tested. A Dixon plot for phenyl-*n*-butylborinic acid gave a K_i value of 22 ± 16 μM (Fig. 7*C*). This compound is the most potent inhibitor thus far found for acylpeptide hydrolase.

Esterase Activity of Acylpeptide Hydrolase—The esterase activity of serine proteases has been previously described (32). Table IV shows the V_{max} and K_m data for substrates different from the usual acyl-di- or -tripeptide substrates reported for the enzyme. For compounds 2–6, which are *p*-nitrophenyl ester derivatives, there is a large increase in activity due to a simultaneous change in K_m and V_{max}. Changing the leaving group

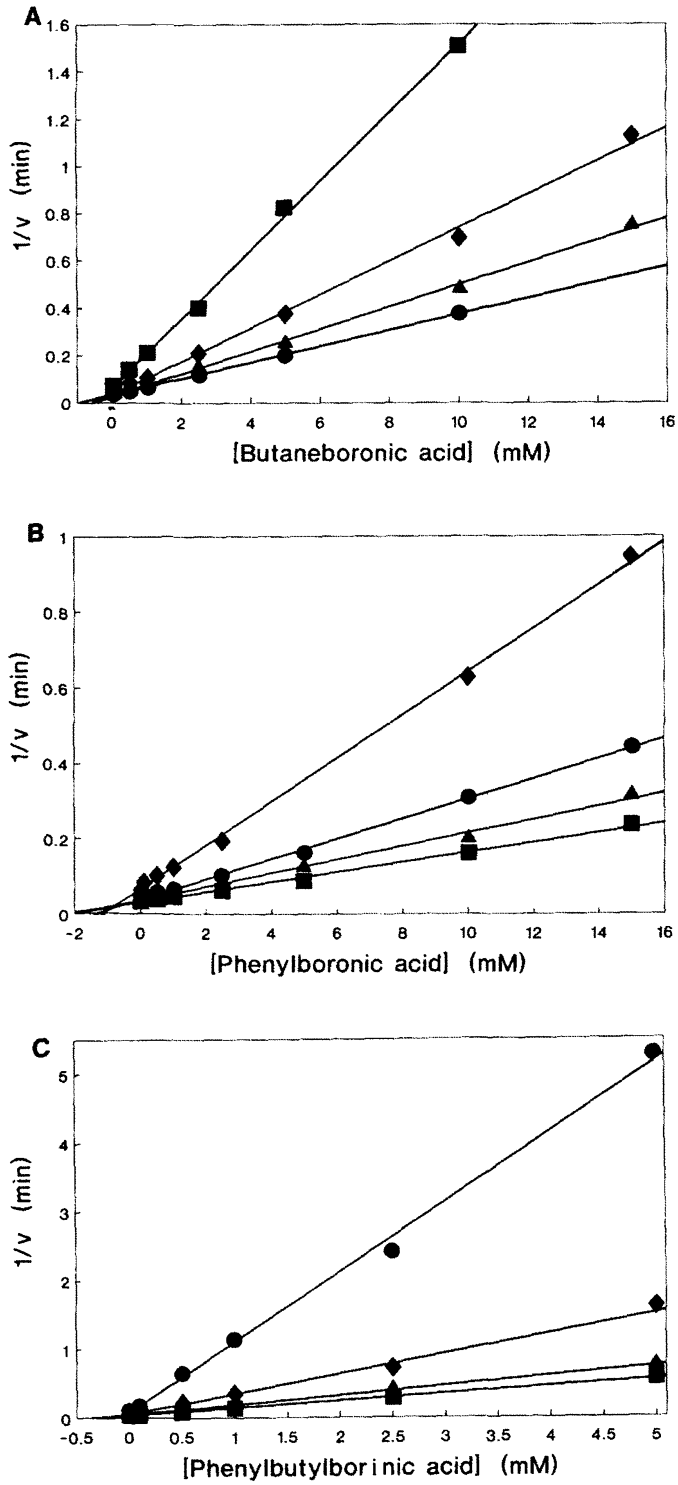


FIG. 7. **Competitive inhibition of BBA (*A*) phenylboronic acid (*B*), and phenyl-*n*-butylborinic acid (*C*) with acylpeptide hydrolase.** The indicated concentrations of the inhibitors were preincubated with enzyme for 10 min in 0.1 M bis-Tris, pH 7.4, at 37 °C. The activity of the enzyme was then measured with its substrate acetyl-alanine *p*-nitroanilide at 0.5, 1.0, 1.5, and 2.0 mM concentrations (from the longest to the smallest slope for each figure) as previously described, at each inhibitor concentration.

from *p*-nitrophenoxide to α-naphthyl oxide doesn't result in a large decrease of enzymatic activity. When the length of the acyl chain is increased from butyrate to hexanoate, there is a decrease of V_{max}, but K_m remains relatively constant. These data confirm the strong affinity of this enzyme for hydrophobic chains and the molecular dimensions required for an effective catalysis. We also investigated other classes of ester derivatives. Butyryl thiocholine was effectively hydrolyzed in the presence of acylpeptide hydrolase (Table I). This compound is commonly used as a good substrate for serum cholinesterase. Acylpeptide hydrolase was also effective with di- and tri-acylglycerol derivatives as substrates. L-α-Dibutyl phosphatidylcholine and tributyrin were found to be substrates for the enzyme. These compounds are also substrates for lipoprotein lipase (33). Glycerol esters having longer fatty acyl chains, β-estradiol 17-valerate or 17-propionate, were not substrates for acylpeptide hydrolase.

DISCUSSION

The results for the inhibition of acylpeptide hydrolase by the carbamate-type inhibitor PNPPC are characteristic of active site-directed irreversible inhibition. First, the inhibition is time-dependent and follows first-order kinetics. Second, with an increasing concentration of inhibitor the enzyme displays saturation kinetics. Third, the enzyme can be protected by the presence of a competitive inhibitor. For lipoprotein lipase, which also has its catalytic His residue nearest the COOH terminus of the protein in its catalytic triad, it has been proposed that after reaction with a carbamate inhibitor, the protein is stabilized in the carbamyl enzyme form, probably by a hydrogen bond with the imidazole ring of the histidine residue (22). Our results also show evidence of a slow hydrolysis of the covalently modified enzyme. As described for lipoprotein lipase, hydroxylamine can increase the rate of hydrolysis of the carbamyl enzyme intermediate as an alternative base involved in the deacylation step, restoring enzymatic activity; a hydroxamate derivative is formed. The lack of inhibition for *N*-disubstituted phenyl carbamates, compared with the *N*-monosubstituted analogs, is analogous to that described for other esterases, such as acetylcholinesterase (34), but a mechanism was not suggested. In this communication, we propose a mechanism for the inhibition, and we test it in studies with the *N*-substituted carbamate. Thus, the *N*-methyl analog PNPMPC cannot form a hydrogen bond with the catalytic His and is thus ineffective as an inhibitor.

A general mechanism involving an acyl-enzyme intermediate was confirmed by the analysis of the acetyl-alanyl hydroxamate obtained during the acylpeptide hydrolase-catalyzed hydrolysis of the blocked peptide and substrate AANA in the presence of hydroxylamine. This nucleophile activates the reaction by providing an alternate route for decomposition of the acyl-enzyme species. Its effect on V_{max} and K_m is consistent with a mechanism that is rate-limited by hydrolysis of this intermediate. The absence of a rate-limiting acylation reaction was recently shown also for prolyl endopeptidase (35).

We and others previously suggested some relationship between acylpeptide hydrolase, acetylcholinesterase, some lipases, as well as prolyl endopeptidase, and dipeptidyl-peptidase IV (13, 18). The serine and the histidine residues of the catalytic triads were identified by chemical modification or site-directed mutagenesis, but the identity of the acid residue of acylpeptide hydrolase is still unknown. Sequence homologies suggested either Asp-542 or Asp-675 as possible candidates for the third member of the catalytic triad of acylpeptide hydrolase. Recent site-directed mutagenesis studies on dipeptidyl-peptidase IV (14) and sequences comparison with acylpeptide hydrolase suggest a possible role of Asp-675. Therefore, all of these hydrolases could share a conserved stretch of almost 200 amino acids with the putative catalytic triad residues organized in a novel sequential order (nucleophile, acid, and histidine) compared with that of chymotrypsin and subtilisin. This topological relationship is strengthened by a similar sensitivity

of some of these enzymes to thiol-modifying reagents and an esterase activity of acylpeptide hydrolase on linear acyl esters, triacylglycerol derivatives, and butyryl thiocholine, usual substrates of the lipases and cholinesterases mentioned above.

The sensitivity of the activity of acylpeptide hydrolase to various thiol reagents can be explained by the size of the reagent, *i.e.* *p*-hydroxymercuribenzoate or *N*-ethylmaleimide could inhibit the enzyme virtually completely but the small iodoacetamide only partially under the same conditions. The identity of the cysteine involved in this reaction was determined as Cys-30 by chemical modification with pHMB and Cys-30 and Cys-64 by iodoacetamide. All of these results suggest that these residues may be located close to the active site but may not be involved directly in the catalytic mechanism. Other studies with SH-directed reagents had shown the same behavior for prolyl endopeptidase (36).

The relationship between Cys-30 and His-707 in acylpeptide hydrolase and Cys-25 and His-680 in prolyl endopeptidase, both near the extremities of each enzyme, is striking but not understood at the present time. Recent limited proteolysis studies showed the existence of two active forms for acylpeptide hydrolase and prolyl endopeptidase (38, 39).[2] Trypsin can selectively cleave an NH_2-terminal 200-residues segment without any loss of activity for both enzymes. The larger subunit in each case contains the catalytic triads. The two fragments of the proteolyzed enzymes did not separate during site-exclusion chromatography under nondenaturing conditions but eluted in place of the native enzymes, indicating that they were strongly associated. For prolyl endopeptidase, this cleavage was associated with an increase in k_{cat} for synthetic substrates (39) suggesting a regulative role of the NH_2-terminal portion on the enzymatic activity (37). The possible presence of cysteine(s) at the NH_2 terminus of these enzymes, as described in this paper, close enough to the active site to influence the substrate's diffusion into the binding pocket, supports the idea of a regulative role of this region on the protein activity.

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[2] A. Scaloni, W. M. Jones, and J. M. Manning, unpublished results.

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