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Acylpeptide Hydrolase: Inhibitors and Some Active Site Residues of the Human Enzyme*

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Acylpeptide hydrolase may be involved in N-terminal deacetylation of nascent polypeptide chains and of bioactive peptides. The activity of this enzyme from human erythrocytes is sensitive to anions such as chloride, nitrate, and fluoride. Furthermore, blocked amino acids act as competitive inhibitors of the enzyme. Acetyl leucine chloromethyl ketone has been employed to identify one active site residue as His-707. Diisopropylfluorophosphate has been used to identify a second active site residue as Ser-587. Chemical modification studies with a water-soluble carbodiimide implicate a carboxyl group in catalytic activity. These results and the sequence around these active site residues, especially near Ser-587, suggest that acylpeptide hydrolase contains a catalytic triad. The presence of a cysteine residue in the vicinity of the active site is suggested by the inactivation of the enzyme by sulfhydryl-modifying agents and also by a low amount of modification by the peptide chloromethyl ketone inhibitor. Ebelactone A, an inhibitor of the formyl aminopeptidase, the bacterial counterpart of eukaryotic acylpeptide hydrolase, was found to be an effective inhibitor of this enzyme. These findings suggest that acylpeptidase hydrolase is a member of a family of enzymes with extremely diverse functions.

During their biosynthesis, some polypeptide chains are acylated at their N termini (1–3). In eukaryotes, the acyl group is an acetyl moiety but in prokaryotes it is a formyl group. In some but not all mature proteins, this blocking group is removed. The enzyme acylpeptide hydrolase (E.C. 3.4.19.1), which acts very efficiently on peptide substrates (4–8), has properties consistent with its role in cotranslational processing of nascent peptide chains. Thus, acylpeptide hydrolase cleaves primarily Ac-Ala-, Ac-Met-, Ac-Ser-, or Ac-Gly-[1] in peptides with neutral side chains at the second position (6, 9, 10); other blocked amino acids are cleaved at slower rates. If the side chain of the second or third amino acid is charged, the rate of cleavage of the blocked N terminus is considerably reduced (11). This specificity reflects the characteristics of those isolated proteins that have their N-terminal residues acetylated, *i.e.* most of them have Ac-Ala-, Ac-Met-, Ac-Ser-, or Ac-Gly- at their N terminus and many of them also have a charged residue at the second amino acid residue (12), thus reducing the catalytic efficiency of acylpeptide hydrolase. Even though this correlation (11, 12) is consistent with a role for acylpeptide hydrolase in N-terminal processing of nascent polypeptide chains, there are other possible mechanisms (14). In earlier studies on the substrate specificity of acylpeptide hydrolase, we and others (4, 6, 13, 15, 16) reported that peptide substrates of various sizes and with different types of acyl groups at the N terminus (acetyl, chloroacetyl, formyl, and carbamyl) were hydrolyzed to generate an acyl amino acid and a peptide with a free N terminus that was shortened by one amino acid, as described by the equation, acetyl-aa_1-aa_2. . . .aa_n → acetyl-aa_1 + aa_2. . . .aa_n.

The acetyl amino acid product is further cleaved by another enzyme, acylase as follows: acetyl-aa_1 → acetate + aa_1.

For some proteins the newly exposed N terminus is acetylated by acetyl CoA in a reaction catalyzed by an *N*-acetyltransferase; the new blocked N-terminal residue either remains as such or undergoes a second catalytic cleavage to reveal the original third amino acid as the new N terminus. Alternatively, methionine aminopeptidase may remove the initiator methionine residue (14) and then the process of acetylation and deacetylation proceeds.

Neither the order of the events catalyzed by these enzymes nor the coordination of their interaction with the nascent protein chain is completely understood. The process, which can either be cotranslational or posttranslational, is complex since mature proteins can contain Ac-Met, free Met, as well as other acetylated or unblocked amino acids. In addition, a mature protein can be a mixture of acetylated and nonacetylated forms, such as the γ-chain of fetal hemoglobin in which both the free and the *N*-α-acetylated chains are produced. Nevertheless, it seems likely that such processing takes place after the N terminus of the nascent polypeptide chain has emerged from the domain of the ribosome, *i.e.* when it is 30–50 amino acid residues in length (17–19) in order to be exposed to these enzymes. The limiting factors in the efficiency of cleavage by acylpeptide hydrolase are probably the nature of the amino acid side chain adjacent to the N terminus, as described above, or the extent to which the N-terminal region of the nascent polypeptide chain has undergone folding.

The role of acylase in this process is probably an indirect one involved in maintaining acylpeptide hydrolase at maximal efficiency by catalyzing the removal of the acetyl amino acid

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[1] The abbreviations used are: Ac, acetyl; ALCK, acetyl leucine chloromethyl ketone; aa, any amino acid; DFP, diisopropylfluorophosphate; bis-Tris, bis(2-hydroxyethyl)iminotris(hydroxymethyl) methane; PTH, phenylthiohydantoin; Ala_2, dialanine; Ala_3, trialanine; DTT, dithiothreitol; *p*HMB, *p*-hydroxymercuribenzoate; DEP, diethylpyrocarbonate; AANA, acetylalanine *p*-nitroanilide; DTNB, 5,5′-dithiobis(2-nitrobenzoate); EDC, 1-ethyl-3,3′-dimethylaminopropyl carbodiimide; Gly-OEt, glycine ethyl ester; Ac-Ala-OMe, acetyl alanine methyl ester; HPLC, high-performance liquid chromatography; RP, reversed-phase.

product of the acylpeptide hydrolase reaction; these products are potent inhibitors of this enzyme.[2]

There is little information available on the mechanism of action of acylpeptide hydrolase. It has been reported to be inhibited by several types of reagents including diisopropylfluorophosphate, *p*-hydroxymercuribenzoate, diethylpyrocarbonate, acetyl alanine chloromethyl ketone, and some heavy metals such as Hg^{2+}, Zn^{2+}, and Cd^{2+} (6, 8, 16). However, even though the sequences of the enzymes from rat and pig are known (21, 22), there is no information on the nature of the active site or whether it bears any relationship to other known proteolytic enzymes. In the present communication, we describe some reversible and irreversible inhibitors of acylpeptide hydrolase and provide some information on the active site of this enzyme.

We have recently ascribed the gene coding for acylpeptide hydrolase to the DNF15S2 (D3F15S2) locus of human chromosome 3 at region 3p21 (9), based on the sequence studies of Naylor *et al.* (24); deletions within this genetic region occur frequently in small cell lung carcinoma (24). However, alignment of the cDNA sequences of rat acylpeptide hydrolase and the human DNF15S2 locus showed some minor differences. Therefore, another objective of this study was to gain more information on the primary structure of human acylpeptide hydrolase.

MATERIALS AND METHODS

[^{14}C]Acetic anhydride and [^{3}H]DFP were from Du Pont-New England Nuclear. L-1-Tosylamido-2-phenylethyl chloromethyl ketone-trypsin, leucine chloromethyl ketone, DEP, EDC, Gly-OEt, pHMB, DTT, DTNB, Ac-Met, acetylalanine *p*-nitroanilide (AANA), Ac-Ala_2, Ac-Ala_3, trifluoroacetic acid, and ebelactone A were from Sigma. Acetonitrile (HPLC grade) was from Pierce (Rockford, IL).

Synthesis of Acetyl Leucine Chloromethyl Ketone—A solution of 2.5 mmol of acetic anhydride in freshly distilled tetrahydrofuran was cooled to 0 °C. L-Leucine chloromethyl ketone (2.5 mmol) was added, and then 2.5 mmol of triethylamine were added in dropwise fashion. The reaction mixture was stirred for 1 h at room temperature. Ethyl acetate was then added, and the mixture was filtered to remove precipitated triethylamine hydrochloride. Evaporation of the solvent and trituration of the residue with ether gave a powder; the yield was 105 mg of ALCK (20.5% as colorless scales after recrystallization from ethyl acetate, m.p. 70–71 °C). The purity of the sample was established by RP-HPLC where a single peak was observed. Anal. Calc. for $C_9H_{16}ClNO_2$: C, 52.59; H, 7.78; N, 6.81. Found: C, 52.04; H, 7.93; N, 6.55.

For the synthesis of radiolabeled [^{14}C]ALCK, acetic anhydride (500 μCi, 4.35 μmol) in freshly distilled tetrahydrofuran was cooled to 0 °C. A 10-fold molar excess of L-leucine chloromethyl ketone was added and then 4.35 μmol of triethylamine. The reaction mixture was stirred for 1 h at room temperature. Two additions of H_2O were made (1.0 ml), and the sample was dried after each addition. This sample, which was further purified by RP-HPLC, had the same retention time as that of the unlabeled inhibitor. The product contained 106 μCi and 0.93 μmol in a yield of 21% of the theoretical value.

Enzyme Assays—Acylpeptide hydrolase purified to homogeneity from human erythrocytes (5) was assayed with fluorescamine (Fluram) by the determination of new amino groups formed by hydrolysis of Ac-Ala_2 or Ac-Ala_3. Alternatively, AANA was employed as substrate and the rate of appearance of *p*-nitroaniline was measured at 405 nm.

Irreversible Inactivation of Acylpeptide Hydrolase by ALCK—These studies were performed by addition of different concentrations of ALCK to the enzyme in 0.2 M bis-Tris, pH 7.4, at 37 °C. At various times, aliquots were added to a 4 mM solution of Ac-Ala_3 in the same buffer. The kinetics of inactivation were calculated and compared to a control without inhibitors. The protective effect of Ac-Met against inactivation by ALCK was determined by preincubation of the enzyme with different concentrations of Ac-Met prior to the addition of the chloromethyl ketone. The enzyme activity at various times was assayed using a 4 mM AANA solution in 0.2 M bis-Tris, pH 7.4, 37 °C.

Protein Digestions, Peptide Purifications, and Sequencing—Acylpeptide hydrolase preparations labeled with [^{14}C]ALCK or [^{3}H]DFP were denatured by 8 M urea in 0.2 M bis-Tris, pH 7.4, and their disulfide bonds were reduced with 10 mM DTT under N_2 at 37 °C for 1 h. After dialysis the proteins were digested with trypsin (1/30 w/w) in 0.2 M bis-Tris, pH 7.4, containing 2 M urea at 37 °C overnight. The tryptic digests were lyophilized, dissolved in 0.1% trifluoroacetic acid, and chromatographed on a RP-HPLC C_{18} Vydac column (0.46 × 15 cm, 5-μm particle size) equilibrated in the same buffer. The column was eluted for 2 min with 0.1% trifluoroacetic acid and then with a linear gradient of 0–75% (v/v of 80% acetonitrile, 0.1% trifluoroacetic acid in the same buffer) for 115 min at a flow rate of 1 ml/min. The eluate was monitored at 210 nm. Each peak was collected manually, and the peptides were recovered by lyophilization. Each labeled component was further purified on the same column with an optimized gradient of the same solvents. Peptide sequence analyses were carried out using an Applied Biosystems 470 A protein sequencer equipped with an Applied Biosystems 120 A PTH analyzer.

Irreversible Inactivation of Acylpeptide Hydrolase by Ebelactone A—These studies were performed by addition of enzyme in 0.2 M bis-Tris, pH 7.4, at 37 °C containing different concentrations of ebelactone A; this inhibitor was dissolved in 0.1 M sodium phosphate, pH 7, containing 0.5% Triton X-100 and 10% methanol. At various times, aliquots were added to a 4 mM solution of $AcAla_3$ solution containing 0.2 M bis-Tris, pH 7.4, at 37 °C. The kinetics of inactivation were calculated, and these results were compared to a control without inhibitor.

Chemical Modification of Acylpeptide Hydrolase by Site-specific Reagents—In order to investigate the possible involvement of cysteine or histidine residues, *p*-hydroxymercuribenzoate (*p*HMB), dithionitrobenzoic acid (DTNB) and diethylpyrocarbonate (DEP) were tested for their ability to inactivate acylpeptide hydrolase. The inactivation was performed by addition of the inhibitor (0.1–1.0 mM) to an enzyme solution in 0.2 M bis-Tris, pH 7.4 at 37 °C. At different times, aliquots were added to a 4 mM AANA solution in the same buffer. The kinetics of inactivation were calculated comparing the rate of hydrolysis to a control without inhibitor. The protective effect of Ac-Met from inactivation by *p*HMB was determined by previous incubation of the enzyme with different concentrations of Ac-Met prior to the addition of the inhibitor.

RESULTS

Effect of Salts on Acylpeptide Hydrolase—In earlier studies (13, 15), we reported that sodium chloride led to activation of acylpeptide hydrolase when assayed with *p*-nitroanilide substrates. In an extension of these findings, we have investigated the effect of other salts with both acetyl peptide substrates and acetyl *p*-nitroanilide substrates. Sodium fluoride had a strong inhibitory effect on acylpeptide hydrolase with acetyl alanine *p*-nitroanilide as substrate (Fig. 1). We also found that potassium nitrate and sodium bromide are good activators of the enzyme with acetyl alanine *p*-nitroanilide as substrate (Fig. 1). These anions are about as effective as sodium chloride, whereas potassium phosphate has no effect on enzyme activity. Sodium bicarbonate is a weak activator of the enzyme, and sodium sulfate is a weak inhibitor (data not shown). The possibility that these effects are due to changes in ionic strength was excluded. Radhakrisna and Wold (6) found that with acetylated peptides as substrates, chloride was an inactivator rather than an activator. We have also found that chloride inhibits the hydrolysis of acetylated peptide substrates.

Effect of Blocked Amino Acids on Acylpeptide Hydrolase Activity—Acylpeptide hydrolase possesses broad substrate specificity for acyl groups. Peptides with blocking groups such as chloroacetyl and carbamyl are also cleaved by the enzyme together with the first amino acid (5, 13). The capacity of the enzyme to bind other large acyl groups is evident from the data in Fig. 2. For example, benzoyl amino acids and carbobenzyloxy peptides are competitive inhibitors of the enzyme. One of the most efficient competitive inhibitors that we have

[2] A. Scaloni, W. Jones, M. Pospischil, S. Sassa, O. Schneewind, A. M. Popowicz, F. Bossa, and J. M. Manning, unpublished results.

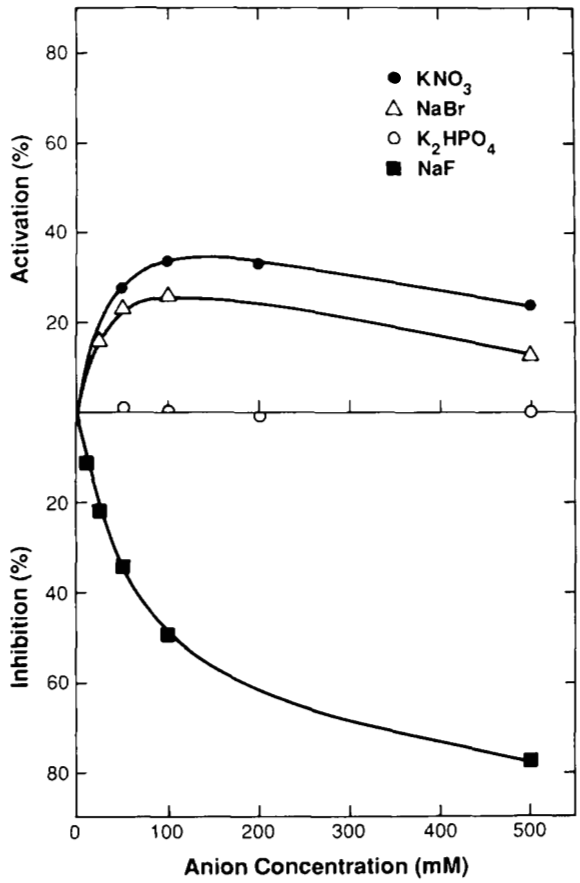

FIG. 1. **Effect of inorganic salts on acylpeptide hydrolase activity.** Enzyme activity toward Ac-Ala_2 and Ac-Ala_3 was measured in the presence of the designated concentrations of KNO_3, NaBr, K_2HPO_4, or NaF. The assays were performed as described under "Materials and Methods."

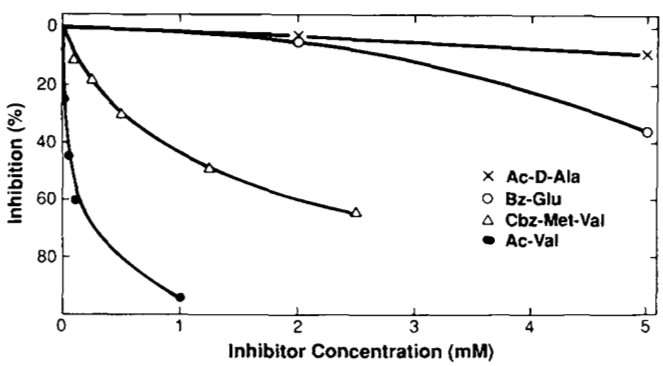

FIG. 2. **Inhibitory effect of blocked amino acids on acylpeptide hydrolase activity.** Enzyme activity toward acetyl alanine *p*-nitroanilide was measured in the presence of different concentrations of Ac-D-Ala, Bz-L-Glu, Cbz-Met-Val, or Ac-L-Val. The assays were performed as described under "Materials and Methods."

found for acylpeptide hydrolase is acetyl-L-valine; 50% inhibition is achieved at a concentration of 0.1 mM (Fig. 2).

The configuration of the blocked amino acid also influences the degree of cleavage by the enzyme. Thus, acylpeptide hydrolase effectively hydrolyzes peptides containing D-amino acids as long as these do not make up the scissile peptide bond. Thus, if a D-amino acid was either the first or second amino acid residue, no cleavage was found. However, the presence of a D-amino acid at the third or fourth position did not influence the rate of hydrolysis; V_{max}/K_m values in the range of 15 for such substrates are similar to those for the best substrates (9). It was of some interest to determine whether a compound with a D-amino acid as the first residue would even be bound by the enzyme. Indeed, Ac-D-Ala was found to bind weakly as described in Fig. 2. A strong competitive inhibitory effect of Ac-Met, a substrate for acylase has been found.[2] As described below, we have used this Ac-Met to protect acylpeptide hydrolase from active site-directed irreversible inhibitors.

Kinetics of Inhibition of Acylpeptide Hydrolase by ALCK—ALCK is a very effective irreversible inhibitor of acylpeptide hydrolase (Fig. 3). A related inhibitor has been reported previously, but the active site residue attacked by this chloromethyl ketone was not identified (16). When the slopes of the rates of inactivation of acylpeptide hydrolase at different ALCK concentrations were plotted in a secondary plot, the inactivation rate constant is calculated to be about 0.03. Ac-Met, a competitive inhibitor of the enzyme,[2] protects acylpeptide hydrolase from inactivation by ALCK (Fig. 4). Complete protection against inhibition is achieved at a concentration of 0.2 mM Ac-Met, a value similar to the K_i for Ac-Met with acylpeptide hydrolase.[2]

Active Site Labeling of Acylpeptide Hydrolase by ALCK—The enzyme (2 mg, 26.6 nmol of subunit) in 0.2 M bis-Tris, pH 7.4, containing 0.1 mM DTT was treated with 400 nmol of [^{14}C]ALCK (45 μCi) for 2 h at 37 °C. In a parallel experiment with unlabeled ALCK, 98% of the enzyme activity was lost in 1 h. After the reaction, the mixture was dialyzed for 24 h against three changes of 0.1 M Tris-HCl, pH 8.5, and then concentrated with a Centricon 10 (Amicon). The inactive enzyme had incorporated 0.7 mol of [^{14}C]ALCK per mol of protein subunit. In order to identify the modified amino acid residues, inactive enzyme was denatured with urea, reduced with DTT, digested with trypsin, and subjected to RP-HPLC as described under "Materials and Methods." Three major radioactive peaks were obtained, as shown in Fig. 5. Each of these peaks was further purified and then sequenced. Peptide 3, which contained the major portion of radioactivity (yield: 10.1%), had a histidine residue at the site of modification; the radioactivity at each cycle of the Edman degradation indicated

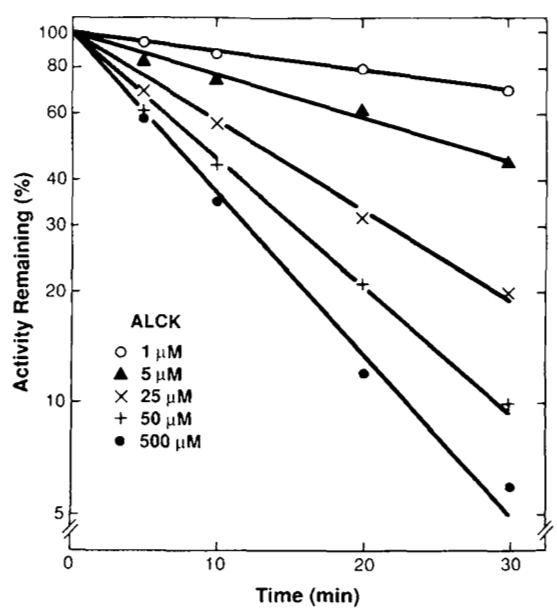

FIG. 3. **Inactivation of acylpeptide hydrolase by ALCK.** Semilogarithmic plot of the remaining enzyme activity *versus* time after incubation with various concentrations of ALCK. The enzyme activity was determined with Ac-Ala_3 as substrate as described under "Materials and Methods."

that the majority was in the position corresponding to His-707 (82.6% recovery of ^{14}C). In confirmation of this result, no histidine PTH-derivative amino acid could be identified probably because the modified PTH amino acid eluted in a different position. It has been previously reported that acylpeptide hydrolase is inhibited by histidine-modifying reagents (6, 16). We verified that diethylpyrocarbonate has a strong inhibitory effect on acylpeptide hydrolase (2% activity remaining after 30 min of incubation with 1 mM DEP).

In peptide 2, the modified amino acid residue was a cysteine; no PTH derivative was found in the expected position in the primary structure of the enzyme (Table I) presumably because the modified cysteine residue either could not form a PTH derivative or formed a derivative that eluted in a different position. However, the extent of labeling of the Cys-12 by ALCK is only 10% that of His-707. Further evidence in support of a cysteine residue near the active site is the finding that *p*HMB inhibits acylpeptide hydrolase; DTNB also inhibits the enzyme although less effectively than *p*HMB. Ac-Met protects acylpeptide hydrolase from inactivation by *p*HMB (data not shown), as described above for protection against inhibition by ALCK. Similar results were obtained using Ac-Val as a protector instead of Ac-Met. It has been reported that acylpeptide hydrolase is inhibited by several types of thiol-modifying reagents (8, 16). Therefore, it seems possible that modification of a cysteine residue by ALCK is at least partially responsible for the observed inactivation. The results suggest that the modified cysteine residue may be located close to the active site even if it is not essential for activity. In this respect, acylpeptide hydrolase is similar to certain members of the subtilisin serine proteases family that are inactivated by modification of a nonessential cysteine residue within the active site (23). The component comprising peak 1 eluted in the same position as the product of reaction between [^{14}-C]ALCK and DTT as determined with a blank reaction. This derivative did not yield a peptide sequence.

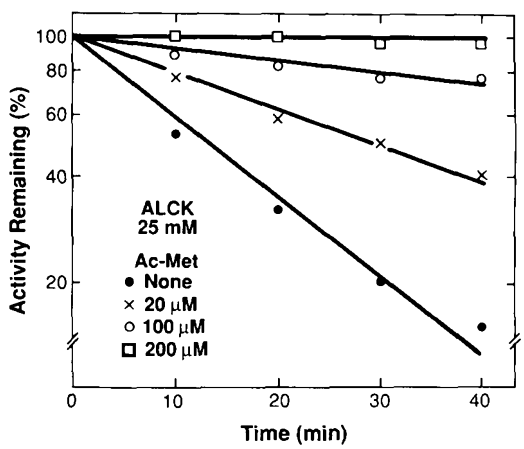

FIG. 4. **Protection by Ac-Met from inactivation of acylpeptide hydrolase by ALCK.** The enzyme was treated with 25 μM ALCK in the absence or presence of different concentrations of Ac-Met. The enzyme activity was determined using acetyl alanine *p*-nitroanilide as substrate.

Active Site Labeling by DFP—It has been previously reported that acylpeptide hydrolase is inhibited by DFP but the residue to which it attaches was not determined (16). In order to identify this residue, the enzyme (2 mg, 26.6 nmol of subunits) in 0.2 M bis-Tris, pH 7.4, was treated with 29 nmol of [^{3}H]DFP (250 μCi) in propylene glycol (final concentrations, 10% v/v) for 2 h at 37 °C. A 10-fold molar excess of cold DFP was then added, and the reaction was allowed to proceed for another 90 min. Ninety-nine % of the enzyme activity was lost. The mixture was dialyzed for 24 h against three changes of 0.2 M bis-Tris, pH 7.4, and then concentrated with a Centricon 10 (Amicon). The inactive enzyme, which had incorporated 0.7 mol of [^{3}H]DFP per mol of protein subunit, was denatured, reduced, digested with trypsin, and subjected to RP-HPLC. Three major radioactive peaks obtained, as described in Fig. 6, were further purified and sequenced. The results shown in Table II were obtained from radiolabeled peptide 2 in Fig. 6 and gave the sequence corresponding to the first 31 residues in the peptide comprising residues 562–592 in the sequence of acylpeptide hydrolase (21, 22). No amino acid PTH derivative was found in the position of the derivatized serine. The amounts of radioactivity released at each cycle of Edman degradation were determined and the majority (79.4%) occurred in the position corresponding to serine 587. The labeled tryptic peptide corresponding to peak 3 gave the same sequence as the one described in Table II and is likely a more hydrophobic peptide, due to a tryptic cleavage to produce a peptide that elutes later in the HPLC column. The radioactivity in peak 1 (Fig. 6) eluted at

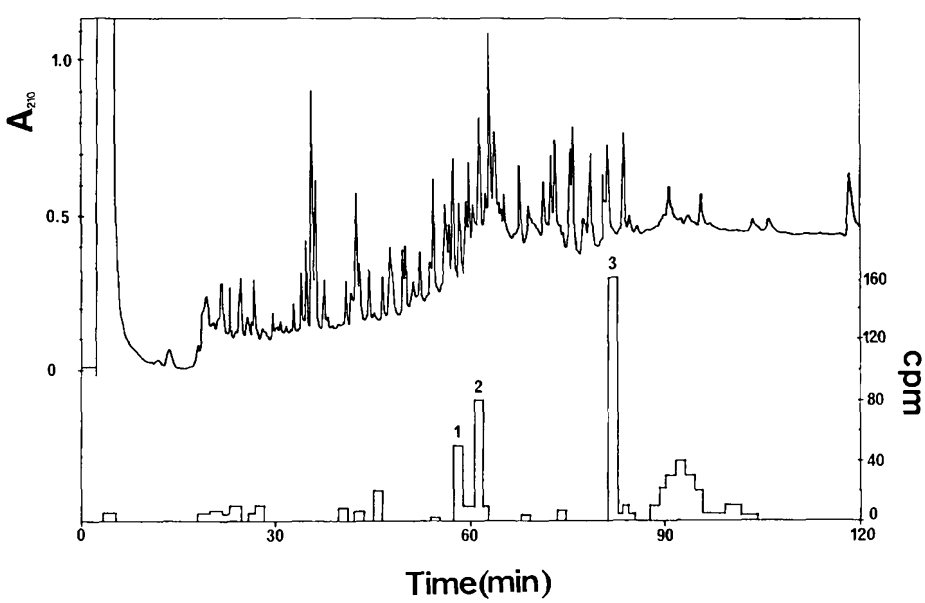

FIG. 5. **HPLC peptide mapping of a tryptic digest of acylpeptide hydrolase after inactivation with [^{14}C] ALCK.** The tryptic peptide map was obtained on a Vydac-RP-HPLC column equilibrated in 0.1% trifluoroacetic acid for 2 min and eluted with a gradient of 0–75%, 0.1% trifluoroacetic acid and 80% acetonitrile in 0.1% trifluoroacetic acid over a period of 115 min. Fractions were collected and the radioactivity found in each fraction was plotted. The radioactive peaks were purified as described under "Materials and Methods."

TABLE I

Amino acid residues of human acylpeptide hydrolase alkylated by ALCK

Amino acid sequences of the purified radioactive peptides were obtained from [^{14}C]ALCK labelled acylpeptide hydrolase after tryptic digestion. H and C denote positions where no PTH derivative could be identified and where the deduced amino acid sequence indicated histidine and cysteine residues. The numbers given in subscripts are the cpm found for each cycle of the Edman degradation.

	Residues
1. S T H A L S E V E V E S D.... 0 0 1289 157 42 13 0 0 0 20 18 14 0	705–717
2. Q P A L S A A C L G.... 17 0 13 15 9 9 19 36 29 3	23–32

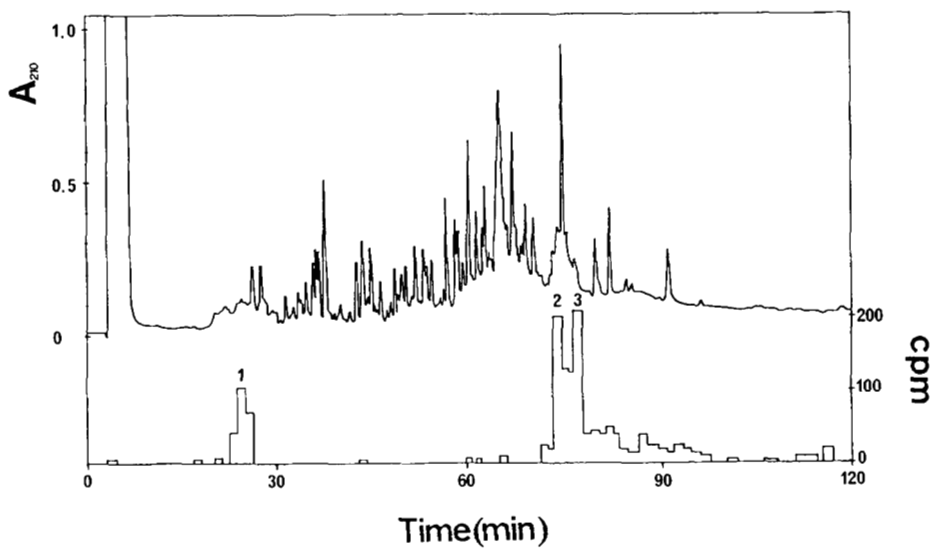

FIG. 6. **HPLC peptide mapping of a tryptic digest of acylpeptide hydrolase after inactivation with [^{3}H] DFP.** The peptide separation was achieved on a Vydac RP-HPLC column equilibrated in 0.1% trifluoroacetic acid for 2 min and eluted with a gradient of 0–75%, 0.1% trifluoroacetic acid and 80% acetonitrile, in 0.1% trifluoroacetic acid over a period of 115 min. Fractions were collected and the radioactivity found in each fraction was plotted.

TABLE II

Active-site serine residue of human acylpeptide hydrolase

The amino acid sequence of the purified radioactive peptide was obtained from [^{3}H]DFP labeled acylpeptide hydrolase after tryptic digestion; S denotes position of the labeled active-site residue from the known amino acid sequence (21, 22). The numbers given as subscripts are the cpm observed for each cycle of the Edman degradation.

	Residues
D V Q F A V E Q V L Q E E M F D A S H V A - 130 0 0 0 0 0 0 0 0 1 0 5 0 0 0 0 0 8 27 21 0	562–592
- L M G G S H G G F L... 2 7 0 11 884 277 31 21 12 9	

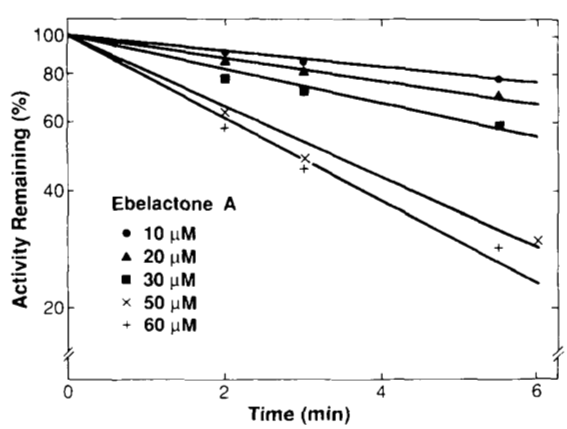

FIG. 7. **Inactivation of acylpeptide hydrolase by ebelactone A.** Semilogarithmic plot of the remaining enzyme activity *versus* time after incubation with different concentrations of ebelactone A. Enzyme activity was determined using Ac-Ala$_3$ as substrate.

the same position of [^{3}H]DFP as determined with a blank chromatogram; it did not yield a sequence.

Involvement of Carboxyl Groups in Enzyme Activity—It has been previously reported that acylpeptide hydrolase is inhibited by 1-ethyl-3,3′-dimethyl aminopropyl carbodiimide in the presence of Gly-OEt (16). We verified this observation and further showed that preincubation of the enzyme with 100 mM Ac-Ala-OMe at 37 °C afforded significant protection from the 64% inactivation from 40 mM EDC/Gly-OEt mixture. These results are consistent with the role of a carboxyl group as the third part of the catalytic triad, although its location is not yet known.

Effect of Chelating Agents and Divalent Cations on Activity—Chelating agents do not measurably affect acylpeptide hydrolase activity, *e.g.* EDTA and 1,10-phenanthroline at concentrations varying from 0.1 to 100 mM did not affect activity. The addition of different concentrations of Ca^{2+} and Mg^{2+} to an enzyme solution exhaustively dialyzed *versus* chelating agents also did not affect the activity.

Sequence of Unlabeled Tryptic Peptides from Acylpeptide Hydrolase—During the isolation of the active site peptide

labeled by [^{14}C]ALCK, several unlabeled peptides were isolated and sequenced (Table III) to obtain more information on the sequence of human acylpeptide hydrolase for comparison with the known sequences for the pig (21) and rat (22) enzymes. The reported sequence of the cDNA from the DNF15S12 locus of human chromosome 3 (24), which we have ascribed to acylpeptide hydrolase (9), contains several deletions and substitutions that would lead to a translated protein significantly different from acylpeptide hydrolase in several regions of the sequence. Our results indicate that the sequence of human acylpeptide hydrolase is very similar to that of the pig and the rat enzymes. However, there are several substitutions, as shown in Table III, for the sequence of some tryptic peptides isolated from the human red cell enzyme. Also shown in Table III are the locations of the active site serine and histidine residues, both of which are located near the C-terminal end of the molecule. The significance of these findings must await the solution of the structure of this enzyme.

Inhibition by Ebelactone—Ebelactone A has been found to be an inhibitor of formyl methionine aminopeptidase (25). As shown in Fig. 7, fairly low concentrations of ebelactone A inhibit acylpeptide hydrolase in a time-dependent manner. This latter result suggests that ebelactone A is an irreversible, covalent inhibitor of the enzyme; its effectiveness is of the same order of magnitude as ALCK. The slopes of the rates of irreversible inactivation for acylpeptide hydrolase at different ebelactone A concentrations are shown in Fig. 7. The inhibition rate constant from the secondary plot is calculated to be about 0.17 min. Several lines of evidence support this suggestion that acylpeptide hydrolase may be the eukaryotic counterpart of prokaryotic *N*-formyl aminopeptidase, *i.e.* each is sensitive to ebelactone A, has a similar molecular weight, and both share similar substrate specificities.

Relationship between Acylpeptide Hydrolase and Acylase—The DNF15S2 locus at region 3p21 of human chromosome 3 reported by Naylor *et al.* (24) encodes for acylpeptide hydrolase, as we have shown earlier (9). This region frequently suffers deletions in small cell lung carcinoma associated with a loss or reduction in acylase activity (44) and acylase messenger RNA (24); such deletions also occur in renal cell carcinoma (45–48). The gene encoding for acylase has been mapped to region 3p21 (20) near but not within the region that encodes acylpeptide hydrolase. Thus, the possibility that both enzymes are encoded by the same gene sequence (9) is not supported by this latter report nor by sequence analysis of some tryptic peptides that we have isolated from porcine acylase. Nevertheless, a functional relationship between these two enzymes is supported by the results of studies on the expression of these two enzymes in various cultured cell lines.[2]

DISCUSSION

Acylpeptide hydrolase can be classified as a serine protease on the basis of the modification of Ser-587 by DFP that leads to inactivation of the enzyme. The known serine proteases can be classified into three different families corresponding

TABLE III

Sequence of some tryptic peptides of human acylpeptide hydrolase

The underlined amino acid residues are those for which there is substitution in the sequence of the human red cell enzyme compared with the sequence of the pig liver enzyme (21), which was used for assignment of the residue number. *X* represents positions where a PTH derivative was not identified. The active-site serine and histidine residues, which are part of the catalytic triad, as indicated by Δ. The identification of the amino acids in parenthesis is tentative.

	Residues
QVLLSEP(E)(E)AAALY(R)	4–18
TVHTEWTQ(R)	45–53
GELLS(R)	90–95
QFLEVWEK	119–126
LK	130–131
SFNLSALEK	132–140
HGPVYEDDC....	141–149
AESFFQTK	173–180
ALDVSASDDEIAR	181–193
TKKPDQA(I)(K)	194–202
GDQFVFYEDWGENMVSK	203–219
LGIR	270–273
SALYYVDLIGGK	280–291
CELLSDDSLAVSSPR	292–306
IVYLQYPSLIPHHQCSQLCLYDWY.....	314–337
VTSVVVDVVPR	340–350
QLGENFSGIYXXLLPL.....	351–366
VVFDSAQ(R)	376–383
SR	384–385
LLTIDXDLMNAQFSTP.....	412–427
VGFLPSAGK	435–443
EQSVLWVSLEEAEPPDI(D)WGIR	444–466
VLQPPQEQEVVQYAGLDFEAILLQPGSPPDK	467–497
MLFPAMLCK	519–527
GSTGFGQDSILSLPGNVGHQDVK	539–561
Δ	
DVQFAVEQVLQEEMFDASHVALMGGSHGGFL	562–592
SPIK	655–658
YIPQVK	659–664
RVPFK	677–681
LLLYPK	699–704
Δ	
STHALSEVEVESD	705–717

to trypsin, subtilisin, or carboxypeptidase Y families (26, 27). Within each family of serine proteases, the primary structures of the enzymes are homologous and the tertiary structures that have been determined to date are similar. The amino acids that surround the active site seryl residue are conserved within each family, *i.e.* Gly-Asp-Ser-Gly-Gly for the trypsin family, Gly-Thr-Ser-Met-Ala for the subtilisin family, and Gly-Glu-Ser-Tyr-Ala for the carboxypeptidase Y family. These sequences have been used to classify newly sequenced proteases to one of these families. Recently, the amino acid sequence around the active site serine of a porcine prolyl endopeptidase (28, 29) has been reported to be Gly-Gly-Ser-Asn-Gly-Gly, which is different from those of the other known families. We have found that for acylpeptide hydrolase the amino acid sequence around the active site serine is Gly-Gly-Ser-His-Gly-Gly, very similar to that of porcine prolyl endopeptidase. The sequence of the active site of these two enzymes, although different from those of the known families, conforms to the consensus sequence Gly-*X*-Ser-*X*-Gly that was proposed for the active site of mammalian serine proteases and esterases (30). After this study was completed, Rawlings *et al.* (31) correctly predicted Ser-587 and His-707 as part of the active site of acylpeptide hydrolase by alignment with the active site of prolyl endopeptidase (prolyl oligopeptidase).

Like acylpeptide hydrolase, porcine prolyl endopeptidase was also labeled on some of its cysteine residues by a peptide chloromethyl ketone. In this respect, these two enzymes would be similar to certain members of the subtilisin protease family whose activities are reduced by modification of a nonessential cysteine residue in the active site (23). The similarities of these proteases involve not only the amino acid sequence surrounding the seryl active site residue, but they all have a similar number of amino acids per monomer; each is inhibited by thiol-modifying reagents (23) and they all have the active site histidyl residue at the C-terminal side of the primary structure with respect to the active site serine. Comparison of the sequence around His-707 with those around the active site histidine residue of other proteases does not reveal significant sequence similarities.

Recently, the three-dimensional structures of two different triacyl glycerol acyl hydrolases (35, 36), wheat serine carboxypeptidase II (36) and acetylcholinesterase from *Torpedo californica* (33) have been described. These enzymes are α/β proteins with common features. They contain a catalytic histidine located at the C-terminal end similar to acylpeptide hydrolase and prolyl endopeptidase. The catalytic serine has the sequence Gly-*X*-Ser-*X*-Ala in wheat carboxypeptidase II (characteristic of the subtilisin family of enzymes), whereas the triacylglycerol acyl hydrolase and acetylcholinesterase contain the sequence Gly-*X*-Ser-*X*-Gly, which is characteristic of the chymotrypsin family of enzymes as acylpeptide hydrolase and prolyl endopeptidase. Like acylpeptide hydrolase, triacyl glycerol acyl hydrolase (38) and acetyl cholinesterase (33, 39) are sensitive to several thiol-modifying reagents; trypsin, chymotrypsin, and subtilisin are not sensitive to organomercurials (40). In addition, some of these enzymes are sensitive to fluoride (Fig. 1).

In order to evaluate any similarity in conformation, we have examined the hydrophobicity profiles for these enzymes in their active site segments. Hydrophobicity analyses have been used extensively in the analysis of protein structure and function (40–41). There is some correlation in the hydrophobicity profile around the active site serine residue for acylpeptide hydrolase, prolyl endopeptidase, and acetylcholine esterase over a range of 150 amino acids. A similar analysis in the region of the active site histidines at the C-terminal region did not show as strong a similarity in the hydropathic profiles. However, the positions of the active site serine and histidine in all three enzymes are located on or near hydrophobic maxima and minima in the profiles, respectively. This similarity would suggest that the overall conformation of the active site in these enzymes may be conserved.

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