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Identification of Carboxyl Residues in Pepstatin-Insensitive Carboxyl Proteinase from *Pseudomonas* sp. 101 that Participate in Catalysis and Substrate Binding[1]

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***Pseudomonas* carboxyl proteinase (PCP), isolated from *Pseudomonas* sp. 101, is the first example from a prokaryote of unique carboxyl proteinases [EC 3.4.23.33] which are insensitive to aspartic proteinase inhibitors, such as pepstatin, diazoacetyl-DL-norleucine methylester, and 1,2-epoxy-3(*p*-nitrophenoxy)propane. To identify the catalytic residue(s) of PCP, chemical modification was carried out using carboxyl residue-specific reagents, carbodiimides. PCP was inactivated effectively by *N,N'*-dicyclohexylcarbodiimide (DCCD) with pseudo-first-order kinetics. For the inactivation, 0.7 mol DCCD was involved per 1 mol PCP. The effects of pH and methanol on the inactivation showed that two carboxyl residues (Asp and/or Glu) were involved in the reaction. The inactivation by DCCD was prevented by a competitive inhibitor, tyrostatin, or a synthetic substrate in a concentration-dependent manner. Based on these data, differential labeling of PCP with DCCD was carried out: Firstly, PCP was treated with cold DCCD in the presence of tyrostatin. After removal of the tyrostatin, which covered the substrate binding site, by dialysis, the PCP was treated with [^{14}C]DCCD to label carboxyl residue(s) essential for its function. Two labeled peptides were isolated by HPLC from a trypsin digest of cold- and [^{14}C]DCCD modified PCP. On analysis of their amino acid sequences, it was revealed that the [^{14}C]-DCCD was bound to Asp^{140} and Glu^{222} of PCP, respectively. Based on these data, it was strongly suggested that Asp^{140} and Glu^{222} of PCP were involved in its catalytic function or substrate binding.**

Key words: carbodiimide, carboxyl proteinase, chemical modification, *Pseudomonas*, pepstatin.

Carboxyl proteinases, formerly called acid proteinases, are distributed in a wide range of organisms, including animals, plants and microbes (*1*). These enzymes are classified into two groups on the basis of their sensitivity to inhibitors: pepstatin (isovaleryl-pepstatin)-sensitive and -insensitive carboxyl proteinases (*2-5*).

Pepstatin-sensitive carboxyl proteinases, represented by porcine pepsin, are blocked by such inhibitors as pepstatin (*6*), S-PI (acetyl pepstatin) (*7*), diazoacetyl-DL-norleucine methylester (DAN) (*8*), and 1,2-epoxy-3-(*p*-nitrophenoxy) propane (EPNP) (*9*). In porcine pepsin, a pair of aspartyl residues, Asp^{32} and Asp^{215}, have been revealed to be essential for its catalytic function (*10*). Such pepstatin-sensitive carboxyl proteinases as renin (*11*), cathepsin D (*12*), chymosin (*13*), penicillopepsin (from *Penicillum janthinellum*) (*14*), and rhizopuspepsin (from *Rhizopus chinensis*) (*15*) also have a pair of active-site aspartyl residues. Therefore, pepstatin-sensitive carboxyl proteinases are called aspartic proteinases. Extensive sequence similarity has been observed among the enzymes belonging to this group (*16*). In particular, the amino acid sequences around the two catalytic aspartyl residues are well conserved as -Asp*-Thr-Gly- (Asp*: catalytic residues).

In 1972, Murao, Oda *et al.* reported that several pepstatin-insensitive carboxyl proteinases are produced by *Scytalidium lignicolum* (*17-20*). None of them, except carboxyl proteinase B, was inhibited by pepstatin, S-PI, DAN or EPNP, carboxyl proteinase B being inhibited by EPNP. Furthermore, these enzymes have unique substrate specificities (*21-26*). The amino acid sequence of carboxyl proteinase B is quite different from those of pepstatin-sensitive carboxyl proteinases reported so far (*27*). The

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Abbreviations: CMC, 1-cyclohexyl-3-(2-morpholinoethyl)carbodiimide metho-*p*-toluenesulfonate; DAN, diazoacetyl-DL-norleucine methylester; DCCD, *N,N'*-dicyclohexylcarbodiimide; EDC, 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride; EPNP, 1,2-epoxy-3-(*p*-nitrophenoxy)propane; GEE, glycine ethyl ester hydrochloride; MES, 2-(*N*-morpholino)ethanesulfonic acid; PCP, *Pseudomonas* carboxyl proteinase; XCP, *Xanthomonas* carboxyl proteinase.

existence of enzymes having characteristics similar to those of *S. lignicolum* has been reported in fungi (*28-32*), bacteria (*33, 34*), and thermophilic bacteria (*35-38*). Recently, it was reported that a pepstatin-insensitive lysosomal carboxyl proteinase related to a fatal neurodegenerative disease, classical late-infantile neuronal ceroid lipofuscinosis, was found in normal human brain (*39*).

Pseudomonas carboxyl proteinase (PCP), a pepstatin-insensitive carboxyl proteinase from *Pseudomonas* sp. 101, is the first carboxyl proteinase to be isolated from a prokaryote and characterized, regardless of its pepstatin sensitivity (*33*). PCP is not inhibited by pepstatin, S-PI, DAN, or EPNP. PCP is inhibited by a competitive inhibitor, tyrostatin (*N*-isovaleryl-tyrosyl-leucyl-tyrosinal; $K_i = 2.6$ nM) (*40*). PCP is produced as a precursor composed of an amino-terminal prepro region (215 residues) and the mature PCP (372 residues) (*41*), which comprises a single chain with one disulfide bond (*42*). The amino acid sequence of PCP shows no similarity to those of any other carboxyl proteinases reported so far. Exceptionally, PCP shows high sequence similarity to *Xanthomonas* carboxyl proteinase (XCP) (51.5% identity, *43*), which was the second pepstatin-insensitive carboxyl proteinase to be isolated from a prokaryote (*34*). In spite of the high sequence similarity, PCP and XCP show quite different substrate specificities (*44*). Moreover, the sequence including the consensus catalytic aspartyl residue, -Asp*-Thr-Gly-, of pepstatin-sensitive carboxyl proteinases does not exist in either PCP or XCP. On the other hand, it was confirmed that PCP has a pair of catalytic carboxyl residues by means of the zinc(II)-PAD (pyridine-2-azo-*p*-dimethylaniline) method (*45*) and kinetic analyses (*46*).

In the present study, chemical modification of PCP using the carboxyl residue-specific reagent, *N,N'*-dicyclohexylcarbodiimide (DCCD) (*47*), and the competitive inhibitor, tyrostatin, was carried out to identify the catalytic residue(s) essential for its function. It was strongly suggested that Asp^{140} and Glu^{222} of PCP are involved in its catalytic function or substrate binding.

EXPERIMENTAL PROCEDURES

Materials—DCCD, 1-cyclohexyl-3-(2-morpholinoethyl)-carbodiimide metho-*p*-toluenesulfonate (CMC), 1-ethyl-3(3-dimethylaminopropyl)carbodiimide hydrochloride (EDC), glycine ethyl ester hydrochloride (GEE), and 2-(*N*-morpholino)ethanesulfonic acid (MES) were purchased from Nacalai Tesque. [^{14}C]DCCD (54 mCi/mmol) and Sephadex G-25 were obtained from Amersham Pharmacia Biotech. The octadecyl silica HPLC column (YMC-pack ODS-AM, 4.6×150 mm) was from Yamamura Chemical Lab. Trypsin was purchased from Wako Pure Chemical. PCP was prepared as described previously (*33*). It gave a single protein band on SDS-polyacrylamide gel electrophoresis. The protein concentration was determined from the absorbance at 280 nm ($E^{1\%}_{1cm} = 16.6$). Tyrostatin was prepared as described previously (*40*).

Proteinase Activity—Proteinase activity was determined by Anson's method with a modification using casein as the substrate (*19*). One unit of enzyme activity was defined as the liberation of 1 μg of tyrosine per ml of reaction mixture per min.

Chemical Modification with Carbodiimides—Chemical modification was carried out at 25°C. A 990 μl sample of PCP (1.3 μM) in 5 mM $CaCl_2$ and 50 mM MES buffer, pH 5.1, was preincubated, and then 10 μl aliquots of solutions of various carbodiimides (final concentration, 1 mM) in DMSO were added at zero time. At the indicated times, 80 μl samples of the reaction mixtures were withdrawn, and then 320 μl of 5 mM $CaCl_2$ and 50 mM sodium acetate buffer, pH 4.8, was added to quench the residual reagents. Kinetic analysis of the inactivation of PCP by DCCD was carried out at pH 5.1 over a range of reagent concentrations (62.5-500 μM). Pseudo-first-order rate-constants were determined from semi-logarithmic plots of residual activity *versus* time. The effects of pH and methanol on the modification by DCCD were investigated by incubation of the enzyme (1.3 μM) with 1 mM DCCD in 5 mM $CaCl_2$ and 50 mM MES (pH 2.8-6.0) in the absence and presence of 30% methanol. The effects of inhibitors or substrates on the modification by DCCD were also studied. PCP (1.3 μM) was treated with DCCD in the presence of tyrostatin (0.2-0.8 μM) or a synthetic peptide substrate, Lys-Pro-Ala-Leu-Phe-Nph-Arg-Leu (43.3-86.6 μM, Nph; *p*-nitrophenylalanine). In the case of the inactivation studies in the presence of tyrostatin, the remaining activity was measured after dialysis to remove the inhibitor.

Differential Labeling of PCP—1st step: A 970 μl sample of PCP (13 μM) in 5 mM $CaCl_2$ and 50 mM MES buffer, pH 5.1, was mixed with 20 μl of a 2 mg/ml methanol solution of tyrostatin, followed by preincubation at 25°C for 10 min. To this mixture was added 10 μl of a 200 mM DMSO solution of cold DCCD (final concentration, 2 mM), and then the modification was conducted at 25°C for 4 h. The reaction was stopped by the addition of 1 ml of 5 mM $CaCl_2$ and 100 mM sodium acetate buffer, pH 4.8. The mixture was dialyzed against 5 mM $CaCl_2$ and 10 mM MES buffer, pH 4.8, at 4°C for 24 h, and then concentrated to 1 mg/ml with an Amicon Centriflo FC25. The residual activity of this preparation was about 82% that of the original sample. 2nd step: A 2.97 ml sample of cold-DCCD modified PCP (13 μM) in 5 mM $CaCl_2$ and 50 mM MES buffer, pH 5.1, was mixed with 30 μl of a 200 mM DMSO solution of [^{14}C]-DCCD (final concentration, 2 mM), followed by incubation at 25°C for 22 h. The reaction was stopped as described above.

Isolation and Purification of the Modified Peptides—The [^{14}C]DCCD-labeled PCP was put on a Sephadex G-25 column (2.5×25 cm) equilibrated with 5 mM $CaCl_2$ and 50 mM sodium acetate buffer, pH 4.8, to remove excess reagents. The void volume fractions were dialyzed and concentrated, and then radioactivity was determined with a BECKMAN LS3801 liquid scintillation counter. The lyophilized enzyme (2.1 mg) was pyridylethylated and digested with 21 μg of trypsin in 2.1 ml of 50 mM Tris-HCl buffer, pH 7.8, containing 50 mM $CaCl_2$ and 2 M urea at 37°C for 24 h. The reaction was terminated by the addition of 42 μl of formic acid. Peptides were separated by HPLC on an ODS-AM reversed phase column, and detected as to the absorbance at 215 nm. Fractions were pooled and lyophilized, and then analyzed by measuring radioactivity. The amino acid sequences of the [^{14}C]DCCD-labeled peptides were determined with an ABI model 476A protein sequencer.

RESULTS

Chemical Modification of PCP with Carbodiimides—Chemical modification of PCP with DCCD, CMC, or EDC led to a loss of proteinase activity, as shown in Fig. 1. PCP was completely inactivated by DCCD within 3 h incubation, while with CMC or EDC, only about 60% of the original activity was lost even after 5 h incubation. The rate of inactivation by DCCD decreased with the addition of GEE as a nucleophile. Accordingly, DCCD was selected as a chemical modification reagent for further experiments.

Effect of the Concentration of DCCD on Inactivation—As shown in Fig. 2, in semi-logarithmic plots of residual activity *versus* time, the inactivation of PCP by DCCD was linear, indicating that the inactivation reaction follows pseudo-first-order kinetics. According to the method of Levy *et al.* (*48*), it turned out that the inactivation of PCP by DCCD was caused by 0.7 mol DCCD per mole PCP (Fig. 2, inset).

Effects of pH and Methanol on Inactivation—The effect of pH on the pseudo-first-order rate constant of the inactivation of PCP by DCCD is shown in Fig. 3. The optimum pH for PCP inactivation was around 3.7, which is very similar to that for proteinase activity with a synthetic peptide substrate (*46*). The plot of relative k_{app}. *versus* pH was bell shaped with pK_a values of 2.8 and 4.6. On the addition of 30% methanol to the reaction mixture, a shift of the pK_a values to neutral pH was observed (pK_a; 4.3 and 5.6, respectively, Fig. 3).

Effects of Tyrostatin and Peptide Substrate on Inactivation—The effects of a competitive inhibitor and a synthetic peptide substrate on the PCP inactivation by DCCD were examined to confirm whether the inactivation was caused by modification of carboxyl residue(s) essential for its function. PCP was inactivated by 1 mM-DCCD at pH 5.1 to the extent of 50% within 20 min. The inactivation of PCP by DCCD was suppressed by the presence of a competitive inhibitor, tyrostatin (0.2-0.8 μM), or a peptide substrate, Lys-Pro-Ala-Leu-Phe-Nph-Arg-Leu (43.3-86.6 μM), in a concentration-dependent manner (Figs. 4 and 5). Plots of protection *versus* tyrostatin or peptide substrate concentration according to the method of Scrutton and Utter (*49*) (Figs. 4 and 5, insets) showed that the inactivation of PCP by DCCD competed with tyrostatin or the peptide substrate.

Differential Labeling of PCP—To identify the carboxyl residue(s) essential for the catalytic function of PCP, differential labeling of the enzyme was carried out. Firstly, the carboxyl group(s) essential for the catalytic function of PCP was covered with tyrostatin, and then the PCP was modified with cold DCCD. As shown in Fig. 6, PCP was inactivated rapidly by DCCD, whereas in the case of PCP treated with tyrostatin in advance, 82% of the original

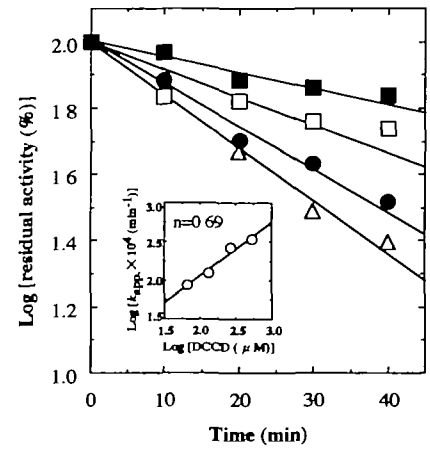

Fig. 2. **Effect of the concentration of DCCD on inactivation.** PCP (1.3 μM) in 1% DMSO, 5 mM $CaCl_2$, and 50 mM MES, pH 5.1, was treated with DCCD at 25°C. The final concentrations were 500 μM (open triangles), 250 μM (filled circles), 125 μM (open squares), and 62.5 μM (filled squares). Inset: the apparent pseudo-first-order rate constants were plotted according to the method of Levy *et al.* (*48*).

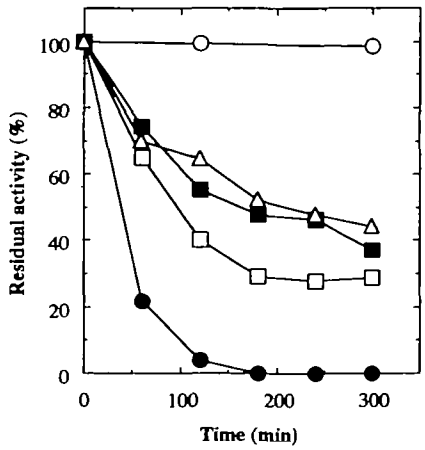

Fig. 1. **Chemical modification of PCP with various carbodiimides.** PCP (1.3 μM) in 1% DMSO, 5 mM $CaCl_2$, and 50 mM MES, pH 5.1, was incubated at 25°C in the absence of a carbodiimide (open circles), and in the presence of 1-mM DCCD (filled circles), 1-mM DCCD, and 50-mM GEE (open squares), 1-mM CMC (filled squares), or 1-mM EDC (open triangles).

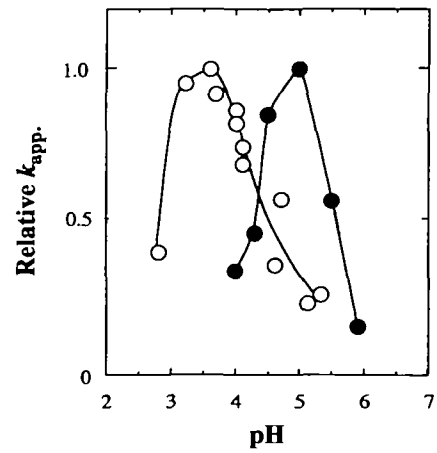

Fig. 3. **Effects of pH and methanol on inactivation by DCCD.** PCP (1.3 μM) in 1% DMSO, 5 mM $CaCl_2$, and 50 mM MES, pH 2.8-6.0, was incubated at 25°C in the absence (open circles) or presence of 30% methanol (filled circles). The k_{app} values were determined from the linear plots of log (residual activity) *versus* time.

activity was retained even after 4 h incubation. After the removal of tyrostatin and other excess reagents by dialysis, the PCP was modified with [^{14}C]DCCD. After 22 h incubation at 25°C, 74% of the enzyme activity was lost (Fig. 6) and about 1.21 mol of [^{14}C]DCCD was incorporated per mol PCP (Table I).

Isolation and Identification of the Modified Peptides—[^{14}C]DCCD-labeled PCP was applied on a Sephadex G-25 column to remove excess reagents, and then dialysed. The modified PCP was then pyridylethylated and subjected to trypsin digestion. The tryptic fragments were applied to an ODS-AM HPLC column. The elution profile of the peptides is shown in Fig. 7. High radioactivity was found to be associated with peptide-A (20%) and peptide-B (25%). These two peptides were subjected to amino acid sequence analysis (Fig. 8). The amino acid sequence of peptide-A was

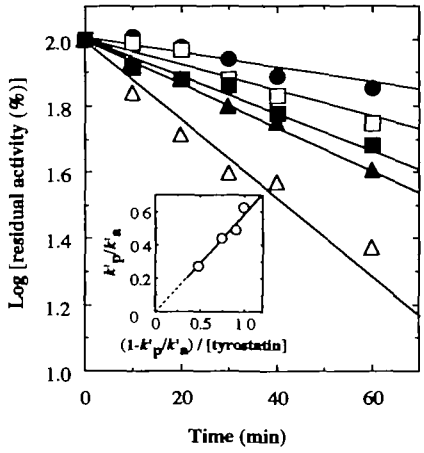

Fig. 4. **Effect of tyrostatin on PCP inactivation by DCCD.** PCP (1.3 μM) in 1% DMSO, 1% methanol, 5 mM $CaCl_2$, and 50 mM MES, pH 5.1, was treated with 1 mM DCCD at 25°C in the absence (open triangles), or presence of 0.8 μM (filled circles), 0.4 μM (open squares), 0.3 μM (filled squares), or 0.2 μM (filled triangles) tyrostatin. After dialysis against 5 mM $CaCl_2$ and 50 mM sodium acetate buffer, pH 4.8, the residual activity of quenched samples was determined. Inset: the apparent pseudo-first-order rate constants obtained in the presence (k'_p) and absence (k'_a) of tyrostatin were plotted according to the method of Scrutton and Utter (*49*).

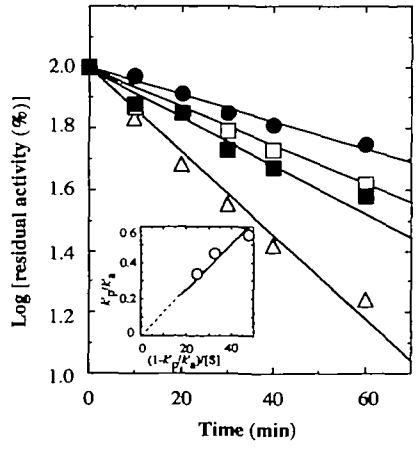

Fig. 5. **Effect of a synthetic peptide substrate on PCP inactivation by DCCD.** PCP (1.3 μM) in 1% DMSO, 5 mM $CaCl_2$, and 50 mM MES, pH 5.1, was treated with 1 mM DCCD at 25°C in the absence (open triangles), or presence of 86.6 μM (filled circles), 65.0 μM (open squares), or 43.3 μM (filled squares) synthetic peptide substrate, Lys-Pro-Ala-Leu-Phe-Nph-Arg-Leu (Nph, *p*-nitrophenylalanine). Inset: the apparent pseudo-first-order rate constants obtained in the presence (k'_p) and absence (k'_a) of the peptide substrate were plotted according to the method of Scrutton and Utter (*49*).

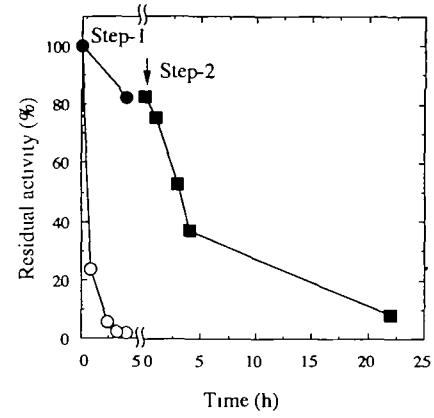

Fig. 6. **Differential labeling of PCP with DCCD.** PCP (13 μM) was treated with cold DCCD in the absence (open circles) or presence (filled circles) of tyrostatin (1st step of differential labeling; see "EXPERIMENTAL PROCEDURES"). Then the enzyme (82% residual activity) was treated with [^{14}C]DCCD (filled squares) (2nd step of differential labeling). The arrow indicates the starting point of step-2.

TABLE I. **Residual activity and [^{14}C]DCCD incorporated into the PCP molecule on differential labeling.**

	Residual activity (%)	Incorporated [^{14}C]DCCD (mol/mol PCP)
Step-1 (after 4 h)	82.4	—
Step-2 (after 22 h)	8.2	1.21

Residual activity is shown as a percentage to the original activity. The detailed experimental conditions are given under "EXPERIMENTAL PROCEDURES."

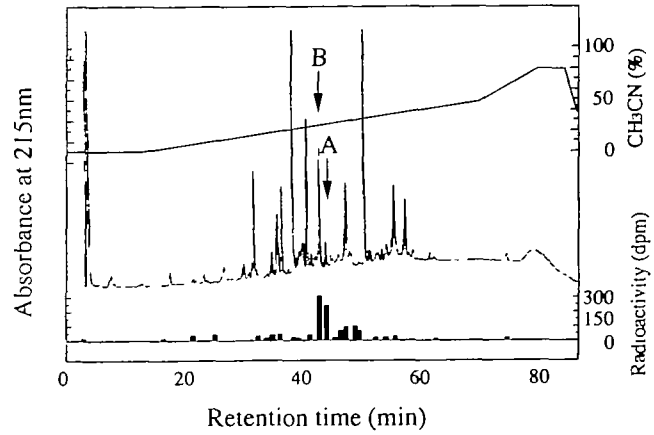

Fig. 7. **HPLC profile of tryptic-peptides derived from differentially labeled PCP.** A portion of the tryptic digest was applied to an ODS-AM column equilibrated with 0.05% trifluoroacetic acid and eluted with a 5-65 min linear gradient of acetonitrile, from 0-48%, at 40°C. The flow rate was 0.8 ml/min. The arrows indicate the elution positions of the ^{14}C-labeled peptides, peptide-A and peptide-B, respectively.

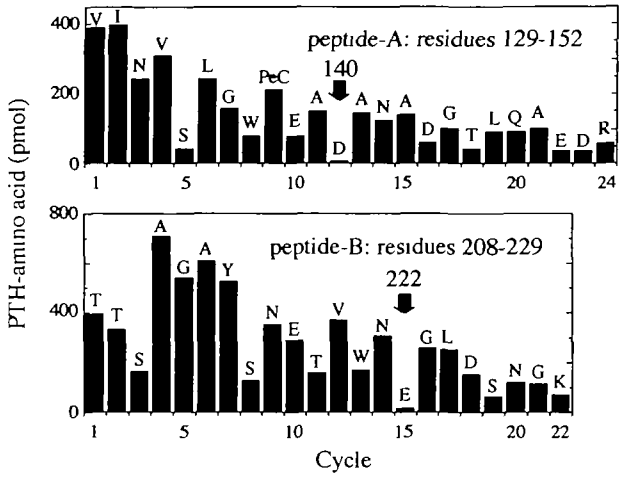

Fig. 8. **Yields of PTH-amino acid on Edman-degradation of ^{14}C-labeled peptides.** The ^{14}C-labeled peptides (peptide-A and peptide-B) were subjected to amino acid sequence analysis. The arrows indicate the undetectable PTH-amino acids.

consistent with Val129-Arg152 of the PCP molecule (*41*). Peptide-B was identified as Thr208-Lys229. On Edman-degradation, a PTH-amino acid was detected, but the amount was very low, at the 12th cycle for peptide-A and the 15th cycle for peptide-B, corresponding to Asp^{140} and Glu^{222}, respectively (Fig. 8).

DISCUSSION

Recently, the existence of pepstatin-insensitive lysosomal carboxyl proteinase in the human brain, related to the fatal neurodegenerative disease, classical late-infantile neuronal ceroid lipofuscinosis, was reported (*39*). This enzyme shows high sequence similarity with PCP and also XCP. Studies on the structure and function relationships of PCP and XCP may contribute to an understanding of the lysosomal enzymes related to lysosomal storage diseases. Moreover, it will be possible to discuss the evolutional origin of pepstatin-insensitive carboxyl proteinases. In the present study, we identified the carboxyl residues essential for the catalytic function of PCP using a combination of carboxyl residue-specific reagents, carbodiimides and a competitive inhibitor, tyrostatin (*40*).

PCP was inactivated most rapidly by DCCD, which exhibits the highest hydrophobicity among the carbodiimides tested. PCP preferably cleaves between two hydrophobic amino acid residues, suggesting that the S1 and S1′ subsites of the enzyme are highly hydrophobic (*46*). This may be the reason why PCP was inactivated by DCCD more rapidly than by other reagents. Pedemonte and Kaplan have described the reaction mechanism underlying chemical modification by carbodiimides (*50*). A protonated carboxyl group is modified by carbodiimides to give an unstable *O*-acylurea, which leads to either rearrangement into a stable *N*-acylurea group or a reaction with a nucleophile (amino group) resulting in the formation of an amide linkage. In the latter case, if the amino group belongs to the enzyme, the amide formation results in cross-linking within the active site. In this experiment, on SDS-polyacrylamide gel electrophoresis, PCP treated with DCCD showed an identical band to that of PCP not treated with DCCD (data not shown). This suggests us that the inactivation of PCP by DCCD was not due to cross-linking or aggregation of enzyme molecules. In the presence of GEE as a nucleophile, no increase in the inactivation rate was observed. These results suggest that DCCD alone is sufficient for inactivation, and that the carboxyl group is likely to be converted to a stable *N*-acylurea. Similar results have been obtained for bovine thrombin (*51*) and adenosine triphosphatase (*52*).

PCP was inactivated by DCCD with pseudo-first-order kinetics in a concentration-dependent manner. With the method of Levy *et al.* (*48*), it was indicated that the inactivation of PCP was caused by 0.7 mol of DCCD per mole PCP. The effect of pH on the inactivation was examined. The plot of relative k_{app}. *versus* pH was bell shaped with pK_a values of 2.8 and 4.6. These pK_a values are similar to those of k_{cat} *versus* pH (pK_a; 2.97 and 4.92, *46*). In addition, the effect of methanol was also examined. It is well known that the reduction of permittivity on the addition of methanol causes a shift of the pK_a value of a carboxyl residue to neutral pH, but not that of an imidazole residue (*53*). In this study, a shift of the pK_a value to neutral on the addition of methanol was observed (Fig. 3). Based on these results, we can say that two carboxyl residues (Asp and/or Glu) are involved in the inactivation of PCP by DCCD. Similar results have been obtained for xylanase A from *Schizophyllum commune* (*54*), end-β-1,4-glucanase (*55*), and glucoamylase from *Aspergillus niger* (*56*).

The inactivation of PCP by DCCD was depressed by the addition of tyrostatin or a peptide substrate in a concentration-dependent manner. According to Scrutton and Utter plots (*49*), it was indicated that the inactivation of PCP by DCCD competed with tyrostatin or a peptide substrate (Lys-Pro-Ala-Leu-Phe-Nph-Arg-Leu). PCP cleaves this peptide between Phe and Nph with a high k_{cat}/K_m value (8.16 $\mu M^{-1} \cdot s^{-1}$, *44*). Accordingly, its hydrolysis products, Lys-Pro-Ala-Leu-Phe or Nph-Arg-Leu by PCP, may compete with the incorporation of DCCD. From these results, it was strongly suggested that DCCD is useful for identifying catalytic residues. Thus, we carried out differential labeling of PCP using DCCD.

The differential labeling of PCP with the combination of DCCD and tyrostatin, and the following HPLC analysis of the labeled peptides revealed that [^{14}C]DCCD was incorporated into peptides-A and -B. These peptides were identified as Val129-Arg152 and Thr208-Lys229 of the PCP molecule, respectively. On amino acid sequence analysis of peptide-A, a PTH-amino acid corresponding to Asp^{140} was detected, but the amount was very low, indicating that Asp^{140} of PCP was modified by [^{14}C]DCCD. On amino acid sequence analysis of peptide-B, a PTH-amino acid corresponding to Glu^{222} was detected, but the amount was very low, indicating that Glu^{222} was also modified by [^{14}C]-DCCD. Other glutamic acid or aspartic acid residues were not affected at all. Thus, chemical modification of Asp^{140} and Glu^{222} of PCP by DCCD was blocked by a competitive inhibitor, tyrostatin. A comparison of the primary sequences of PCP and XCP around Asp^{140} and Glu^{222} is shown in Fig. 9. Asp^{140} of PCP is converted to Ala in the XCP molecule. In general, catalytic residue(s) and their vicinities are well conserved among enzymes exhibiting high

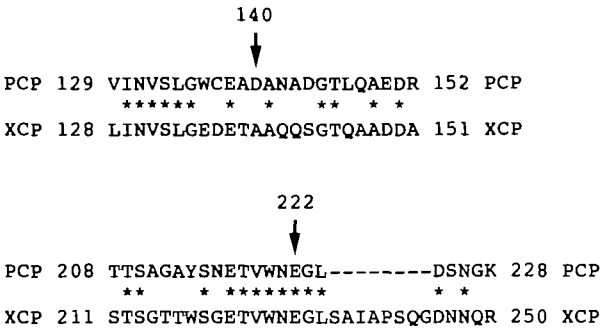

Fig. 9. **Comparison of the amino acid sequences of PCP and XCP around labeled residues.** The amino acid sequences are numbered from the NH_2-terminal residues of the mature enzymes. Identical amino acids indicated by asterisks. The arrows indicate the [^{14}C]DCCD-labeled residues of PCP.

sequence similarity. Therefore, Asp^{140} of PCP was thought to be a residue related to substrate binding rather than a catalytic residue. On the other hand, Glu^{222} of PCP is conserved in the XCP molecule. The upstream region of this residue was also well conserved in both enzymes. This suggests that Glu^{222} may be one of the catalytic residues.

Thus, it was strongly suggested that Asp^{140} and Glu^{222} of the PCP molecule play important roles in the catalytic reaction or substrate binding. We are now trying to identify the catalytic residue(s) by site-directed mutagenesis based on the high sequence similarity between PCP and XCP (51.5% identity, *43*). Furthermore, analysis of the three-dimensional structure is under way. We hope to identify the catalytic residues of PCP and XCP by means of these three different methods (chemical modification, site-directed mutagenesis and three-dimensional structure analysis).

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