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Mutational Analysis of the Active Site of *Pseudomonas fluorescens* Pyrrolidone Carboxyl Peptidase

O. LE SAUX,* T. GONZALES, AND J. ROBERT-BAUDOUY

Laboratoire de Génétique Moléculaire des Microorganismes et des Interactions Cellulaires, Unité Mixte de Recherche, Centre National de la Recherche Scientifique, Institut National des Sciences Appliquées, 69621 Villeurbanne Cedex, France

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On the basis of chemical inhibition studies and a multiple alignment of four pyrrolidone carboxyl peptidase (Pcp) amino acid sequences, seven conserved residues of the *Pseudomonas fluorescens* Pcp, which might be important for enzyme activity, have been modified by site-directed mutagenesis experiments. Wild-type and mutant Pcps were expressed in *Escherichia coli*, purified, and characterized by the ability to cleave the synthetic chromogenic substrate pyroglutamyl-β-naphthylamide and the dipeptide pyroglutamyl-alanine. Substitution of Glu-10 and Glu-22 by Gln led to enzymes which displayed catalytic properties and sensitivities to 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide similar to those of the wild-type Pcp. These residues are not essential for the catalytic activity. Replacement of Asp-89 by Asn and Ala resulted in enzymes which retained nearly 25% of activity and which had no activity, respectively. Substitution of the Cys-144 and His-166 residues by Ala and Ser, respectively, resulted in inactive enzymes. Proteins with changes of Glu-81 to Gln and Asp-94 to Asn were not detectable in crude extract and were probably unstable in bacteria. Our results are consistent with the proposal that Cys-144 and His-166 constitute the nucleophilic and imidazole residues of the Pcp active site, while residue Glu-81, Asp-89, or Asp-94 might constitute the third part of the active site. These results lead us to propose Pcps as a new class of thiol aminopeptidases.

Pyrrolidone carboxyl peptidases (Pcps) (EC 3.4.11.8) are aminopeptidases able to specifically remove the L-pyroglutamyl residue from the amino termini of polypeptides and synthetic chromogenic substrates, such as L-pyroglutamyl-β-naphthylamide (pGlu-βNA) (1). The Pcp activity was first reported three decades ago (12) and has since been demonstrated in bacteria, plants, and animal and human tissues (33). Preliminary studies of Pcps have concentrated on their purification from bacterial strains in order to investigate the biochemical properties of these unusual enzymes (16, 32, 34). The physiological function of the bacterial Pcps has not been elucidated, but these enzymes have been used in protein sequencing to unblock proteins and polypeptides with an N-terminal pyroglutamyl residue prior to Edman sequential degradation (3). Various studies of Pcps have demonstrated the occurrence of at least two different types of Pcps. Enzymes of the second type, found in mammals, are dimeric ectoenzymes and have been characterized as metallopeptidases specific for a hormonal tripeptide, the thyrotropin-releasing hormone (10, 23, 31). Enzymes of the first type are present both in mammalian tissues (7) and in numerous bacterial species (3). They show a general specificity for N-terminal pyroglutamyl residues and have been characterized as thiol aminopeptidases. On the basis of biochemical studies, classes I and II have been shown to catalyze similar reactions by different mechanisms, which suggests that these enzymes are not related in an evolutionary way (3). Up to now, genes coding for Pcps of three gram-positive bacteria, *Streptococcus pyogenes* (4, 8), *Bacillus subtilis* (2), and *Bacillus amyloliquefaciens* (37), and one gram-negative bacterium, *Pseudomonas fluorescens* (14), have been cloned and sequenced. The products of the four *pcp* genes were expressed in *Escherichia coli* and purified, and their biochemical properties were characterized. Recent inhibition studies confirmed that these Pcps belong to the thiol enzyme family since they are completely inhibited by micromolar concentrations of thiol-blocking reagents, such as iodoacetamide (14). Furthermore, these studies also indicated the involvement of acidic residues, in addition to a His residue, in the enzymatic activity on the basis of experiments with 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDAC) at acidic pH and diethylpyrocarbonate. The deduced amino acid sequences of the four *pcp* gene products displayed no significant homology with other thiol peptidases, such as the well-known papain-like family. It can be inferred that amino acids essential for Pcp activity might be conserved within the Pcp polypeptides. A multiple alignment of the four Pcp polypeptide sequences allowed for estimation of 31% amino acid identity among the four Pcps but with two highly conserved segments (amino acids 81 to 100 and 133 to 145; see Fig. 1), in which at least 75% of the residues are identical. It is known that the catalytic site of Cys peptidases contains Asp, Glu, Asn, or Gln in addition to Cys and His (24). Of the three Cys residues that are present in the *P. fluorescens* enzyme, only one (Cys-144) is conserved at the end of the stretch from amino acids 133 to 145 and may be the nucleophilic residue. His is also essential for the catalysis, since the residue forms a thiolate-imidazolium ion pair with the nearby Cys, promoting thiol group nucleophilicity. Although it is not located in one of the two highly conserved stretches, His-166 is perfectly conserved within the Pcp family. This His may correspond to the imidazole-reactive residue. Moreover, the first highly conserved segment (amino acids 81 to 100) contains residues such as Glu-81, Asp-89, and Asp-94, which could likely be implicated in the active site.

In this study, we have introduced single amino acid substitutions at seven positions (E-10, E-22, E-81, D-89, D-94, C-144, and H-166) in the *P. fluorescens* Pcp sequence in order

* Corresponding author. Mailing address: Laboratoire de Génétique Moléculaire des Microorganismes et des Interactions Cellulaires, UMR CNRS 5577, Institut National des Sciences Appliquées, 20 avenue A. Einstein, 69621 Villeurbanne Cedex, France. Phone: 33/72-43-80-88. Fax: 33/72-43-87-14. Electronic mail address: lesaux@insa.insa-lyon.fr.

to determine residues that are required for its catalytic activity. Mutant and wild-type enzymes were expressed in *E. coli*, which does not naturally contain Pcp activity, and their enzymatic properties were established by using the chromogenic substrate pGlu-βNA and the dipeptide pGlu-Ala. The sensitivity of the native and modified Pcps to EDAC was also tested.

MATERIALS AND METHODS

Bacterial strains, growth conditions, and DNA manipulation. *E. coli* NM522 [*supE thi* Δ(*lac-proAB*) Δ*hsd-5* (r_k^-, m_k^-) (F′ *proAB lacI*^{q} Δ*lacZ* M15)] was used for overexpression and plasmid manipulations. Growth media were described by Miller (21). When required, Difco agar (12 g · liter^{-1}) was added. Antibiotics (ampicillin and chloramphenicol) were used at 50 μg · ml^{-1}. Plasmid DNA isolation, digestion, purification, ligation, electrophoresis, and bacterial transformation were carried out as described by Sambrook et al. (29). All enzymes were obtained from Boehringer Mannheim.

Site-directed mutagenesis. Oligonucleotides were designed according to the unique site elimination (USE) procedure (Pharmacia) and purchased from Eurogentec (see Table 1). Experimentation instructions, provided with the USE mutagenesis kit, followed the protocol developed by Deng and Nickoloff (11). The plasmid template was pAM1, a pUC19 plasmid carrying the *P. fluorescens pcp* gene on a 1.3-kb *Ssp*I-*Bam*HI fragment (14). The unique *Nco*I site is located at the end of the insert, outside the *pcp* open reading frame. Cys and His amino acid substitutions were chosen in agreement with the guidelines of Bordo and Argos (5) for changes giving the least local disturbance. Similarly, Asp and Glu residues were changed to Asn and Gln, respectively, to introduce the minimum structural disruption, since the side chain of these amino acids is similar to that of the nonmodified amino acids but lacks the capacity to transfer protons. Mutated DNAs were sequenced to examine the codon substitutions according to the chain termination method (30) on double-stranded DNA templates, using the Pharmacia T7 sequencing kit and α-^{35}S-dATP (Amersham International).

Enzyme purification. Mutant enzymes were overproduced in *E. coli* NM522 harboring a pKSM710-derived plasmid (19) with a 0.8-kb *Hin*dIII-*Stu*I fragment containing the entire wild-type and mutant *pcp* genes. The cultures were grown in 200 ml of Luria-Bertani medium under various conditions of temperatures and time of induction. Gene expression was induced by adding 0.8 mM isopropyl-β-D-thiogalactoside (IPTG) at the mid-log phase of cell growth. All purification steps were conducted at 4°C in the presence of 2 mM 1,4-dithiothreitol and 10 mM EDTA. Cells were centrifuged at 5,000 × *g* for 15 min and resuspended in 10 ml of extraction buffer (50 mM potassium phosphate, pH 7). The bacteria were disrupted in a French pressure cell (Aminco), and the cell debris was eliminated by centrifugation at 27,000 × *g* for 15 min. Protamine sulfate was added to the supernatant at a final concentration of 0.15%, and nucleic acids were removed by centrifugation at 12,000 × *g* for 20 min. The supernatant was subjected to a fractionated precipitation by gradually adding solid ammonium sulfate. Proteins precipitating between 40 and 55% (wt/vol) ammonium sulfate saturation were collected by centrifugation at 38,000 × *g* for 15 min and resuspended in 1 ml of extraction buffer containing 1.2 M ammonium sulfate. The proteins (10 mg) were applied to a Progel-TSK phenyl-5PW column (0.75 by 7.5 cm; Supelco) previously equilibrated with the extraction buffer containing 1.2 M ammonium sulfate. The column was extensively washed with the same buffer, and the bound proteins were eluted at 0.8 ml · min^{-1}, with a decreasing linear gradient of ammonium sulfate, by using a fast protein liquid chromatography (FPLC) system (Waters). Fractions containing Pcp activity were collected, pooled, dialyzed against the extraction buffer supplemented with 200 mM NaCl, and concentrated with a Centricon-10 filtration device (Amicon). A Superose 12 HR10/30 gel filtration column (1 by 30 cm; Pharmacia LKB), previously equilibrated with extraction buffer containing 200 mM NaCl, was used for the ultimate step of the purification. Proteins were eluted for 2 h at 0.2 ml · min^{-1} in the same FPLC system. Pcp-containing fractions were pooled, dialyzed against the extraction buffer, and either used immediately for enzyme assays or stored at −20°C.

Analytical methods and enzyme assays. Protein concentrations were determined as described by Bradford (6). Crude extract and purified proteins were analyzed by denaturing (sodium dodecyl sulfate [SDS]) or native polyacrylamide gel electrophoresis (PAGE) by the method of Laemmli (17). PAGE was performed at 4°C, and qualitative detection of Pcp activity was carried out directly after the electrophoresis as previously described (20), using pGlu-βNA (Sigma) as a substrate. Alternatively, gels were stained with Coomassie brilliant blue or immunostained.

Qualitative detection of Pcp activity on agar plates was based on the method previously described by Mulczyk and Szewczuk (22). Quantitative detection of activity was based on the method described by Lee et al. (18), using pGlu-βNA as the substrate. Enzyme assays were performed with 900 μl of 50 mM phosphate buffer (pH 7). After 2 min at 30°C, 100 μl of 10 mM pGlu-βNA in methanol was added and the initial reaction rates were determined from the increase in absorbance of βNA released, measured with a spectrophotometer at 340 nm (ε_{340} = 2,070 M^{-1} cm^{-1}). One unit of Pcp activity was defined as the amount required to liberate 1 μmol of βNA per min.

Enzyme sensitivity to EDAC was assayed by incubating the enzyme for 10 min at 30°C with 10 mM inhibitor in 100 mM 2-(*N*-morpholino)ethanesulfonic acid (MES) buffer at pH 5.5 before measuring the residual activity. Controls included enzymes incubated in equal volumes of buffer for the same period at the same pH and without EDAC.

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P.fl  MRI.VLLTGFEPFDQDPVNPSWEAVRQLDGVQLGSDVKIVARRLPCAFATAGECLTRLID  59
S.py  M..KILVTGFDPFGGEAINPALEAIKKLPATIHG.AE.IKCIEVPTVFQKSADVLQQHIE
B.su  MRKKVLITGFDPFDKETVNPSWEAAKRLNGFETEEAI.ITAEQIPTVFRSALDTLRQAIQ
B.am  MEKKVLLTGFDPFGGETVNPSWEAVKRLNGAAEGPAS.IVSEQVPTVFYKSLAVLREAIK
Cons. M   *L*TGF*PF* ***NP* EA **L *   * *  I    *P *F ** * L   I*
                  ▲          ▲
                  10         22

P.fl  ELHPAMVIATGLGPGRSDISVERVAININDARIPDNLGEQPIDTAVVADGPAAFFTTLPI 119
S.py  SFQPDAVLCIGQAGGRTGLTPERVAINQDDARIPDNEGNQPIDTPIRADGKAAYFSTLPI
B.su  KHQPDIVICVGQAGGRMQITPERVAINLADARIPDNEGHQPIDEEISPDGPAAYWTRLPV
B.am  KHQPDIIICVGQAGGRMQITPERVAINLNEARIPDNEGNQPVGEDISQGGPAAYWTGLPI
Cons. * *P* ** *G * GR  ** ERVAIN **ARIPDN G*QP*** * **G AA*** LP*
                        ▲     ▲  ▲
                        81    89 94

P.fl  KAMVKAVREAGIAASVSQTAGTFVCNQVFYLLQHALA..GSGVRSGFIHVPFLPEQVAG. 176
S.py  KAMVAAIHQAGLPASVSNTAGTFVCNHLMYQALYLVDKYCPNAKAGFMHIPFMMEQVVDK
B.su  KRMTAKMKEHGIPAAVSYTAGTFVCNYLFYGLMDHISRTSPHIRGGFIHIPYIPQQTIDK
B.am  KRIVEEIKKEGIPAAVSYTAGTFVCNHLFYGLMDEISRHHPHIRGGFIHIPYIPEQTLQK
Cons. K **  *** G**A*VS TAGTFVCN **Y     **   *  **GF*H*P** *Q*
                           ▲              ▲
                           144            166

P.fl  SQRPSMALDAMVAGLQAAVLTA..WHTPVDVKEAGGQVS                     213
S.py  PNTAAMNLDDITRGIEAAIFAIVDFKDRSDLKRVGGATH
B.su  T.APSLSLDTIVRALRIAAVTAAQYDE..DVKSPGGTLH
B.am  S.APSLSLDHITKALKIAAVTAAVHED..DIETGGGELH
Cons. *  ****LD ** **  A  *    **  D**  GG
```

FIG. 1. Alignment of the amino acid sequences of Pcps known to date. The two regions of highest homology are underlined. Abbreviations: *P. fl*, *P. fluorescens*; *S. py*, *S. pyogenes*; *B. su*, *B. subtilis*; *B. am*, *B. amyloliquefaciens*; Cons., consensus. Conservative amino acid substitutions (*) and mutagenesis targets (▲) are indicated.

Western blotting. Western blotting (immunoblotting) was performed by the method of Towbin et al. (36). After electrophoretic transfer of the proteins, nitrocellulose membranes were blocked for 2 h at 37°C by immersion in 3% gelatin in Tris-buffered saline (TBS) (30 mM Tris-HCl, 150 mM NaCl; pH 7.2). They were incubated for 2 h at room temperature with rabbit anti-*S. pyogenes* Pcp antibody (13), which had been purified as previously described (28) and diluted in TBS containing 0.05% Tween and 1.0% gelatin. After subsequent washing, the membranes were incubated in the same buffer containing peroxidase-conjugated goat anti-rabbit immunoglobulin G. Serological reactions were detected with a bioluminescent enhanced chemiluminescence (ECL) kit (Amersham). Semiquantitative comparison of immunodetected proteins was performed with a Bio-Profil BIO-1D (version 6.02) imager system.

Expression of mutated Pcp gene products by using the T7 promoter-polymerase system. The wild-type and mutated Pcps were expressed via the T7 promoter located upstream of the *lac* promoter of the pKSM710 system. The plasmids carrying the mutant genes were introduced into *E. coli* K38, which carries the compatible plasmid pGP1-2. Overexpression of plasmid-encoded proteins was done by using the T7 promoter-polymerase system as described by Tabor and Richardson (35). The products were labelled with [^{35}S]methionine (Amersham International) for 15 min, and the [^{35}S]methionine was chased with an excess of cold methionine and cysteine to monitor possible degradation of the labelled proteins. The expression was performed at various temperatures (25 to 37°C). Samples were taken at different times and diluted with Laemmli buffer.

Thin-layer chromatography. After Pcps were incubated with 1 mM pGlu-Ala for 2 h at 30°C, thin-layer chromatography was carried out on silica gel plates (Kieselgel 60 F_{254}; Merck). Elution was done with butanol-acetic acid-water (4:2:2, vol/vol), and Ala spots were revealed with ninhydrin (1% in methanol).

RESULTS

Site-directed mutagenesis. On the basis of a multiple alignment of the four amino acid sequences of Pcps (Fig. 1) and inhibition studies, site-directed mutagenesis experiments were performed with the *P. fluorescens pcp* gene in order to examine the putative roles of various conserved amino acids in catalysis. Cys-144 was replaced by Ala and His-166 was substituted by Ser, giving C144A and H166S mutants, respectively. Highly

TABLE 1. Amino acid substitutions, codon replacements, and oligonucleotide sequences used for site-directed mutagenesis

Amino acid change[a]	Codon change	Nucleotide primer[b]
E10Q	GAA→CAA	GATCAAAGGGTT**G**GAAACCCGTC
E22Q	GAA→CAA	GGCGCACAGCTT**G**CCAGGAGGGG
E81Q	GAA→CAA	CGCCACCCGTT**G**GACTGAGATAT
D89N	GAT→AAT	GGGATGCGTGCAT**T**ATTGATGTT
D89A	GAT→GCT	GGGATGCGTGCA**G**CATTGATGTT
D94N	GAT→AAT	CACCCAGATTAT**T**GGGGATGCG
C144A	TGT→GCT	AACCTGATTA**GC**CACGAACGTCC
H166S	CAC→AGC	GAAACGGCACG**CT**GATAAACCC
*Nco*I site elimination	CCATGG→CCATCG	GGCAGACGCCAT**C**GATAAGCACTTC

[a] Mutants are abbreviated to the single-letter code for the wild-type amino acid, which is followed by its codon position and the amino acid replacement.
[b] Base changes are underlined.

conserved acidic residues Glu-10, Glu-22, Glu-81, Asp-89, and Asp-94 were also changed, to Gln or to Asn (E10Q, E22Q, E81Q, D89A, D89N, and D94N). All substitutions and codon changes are summarized in Table 1.

Expression of mutants. Mutated *pcp* genes were expressed under the control of the *lac* promoter of plasmid pKSM710. Different culture conditions were tested to define the optimal parameters for overexpression. The C144A and H166S mutants were overexpressed under the standard conditions (37°C) used for wild-type enzyme production. Maximal expression was obtained at 30°C for E10Q and E22Q mutants. The E10Q, E22Q, and H166S proteins were slightly unstable, with patterns of degradation observable on Western blots (Fig. 2A), since lower-molecular-weight proteins were recognized by anti-Pcp antibody. Despite this degradation, significant amounts of proteins were obtained, allowing for purification. The D89A and D89N mutants were not detectable in crude extract when overexpressed at a temperature higher than 30°C. Maximal production was obtained when the induction was carried out at 25°C, although the amount of both mutated proteins in the crude extract represented only 10% of the quantity of native Pcp. No efficient condition was found for overexpression of the two other mutants, E81Q and D94N, and qualitative enzymatic tests, SDS-PAGE, and Western blotting analysis were inefficient at detecting them. Therefore, these two mutated enzymes were not available for the subsequent characterizations.

Purification of modified Pcps. The wild-type and mutated Pcp proteins were purified in order to study their chromatographic behavior and catalytic properties. The C144A, H166S, E10Q, and E22Q mutants displayed the same biochemical properties as the wild-type protein, indicating that these mutations did not result in conformational changes. They showed the same hydrophobic-interaction pattern on the Progel-TSK phenyl-5PW column, the same elution profile on Superose gel filtration corresponding to a native dimeric mass of 41 kDa, and an identical molecular mass of 23 kDa for the subunits immunodetected after SDS-PAGE (Fig. 2A). Mutations of the D-89 residue (D89A and D89N) dramatically affected the enzyme behavior, as the corresponding proteins were inactivated during the first step of the purification procedure, Progel-TSK phenyl-5PW chromatography. Nevertheless, some activity was recovered after gel filtration of the crude extract. The molecular masses of the mutants were not modified, with values of 41 and 23 kDa for the native dimer and the subunit, respectively. No deterioration pattern was seen with the purified wild-type and mutated Pcps (Fig. 2B). No degradation of the E81Q and D94N enzymes could be observed by using the T7 polymerase expression system or the Western blotting technique (data not shown).

Mutated-Pcp apparent conformation. The available mutated Pcps loaded on a nondenaturing polyacrylamide gel displayed various electrophoretic behaviors. The wild type and three of the modified Pcps (E10Q, E22Q, and D89N) showed activity under the conditions described in Materials and Methods (Fig. 3). The PAGE patterns of the native Pcp, mutants E10Q, C144A, and H166S, and the partially purified D89A and D89N proteins were similar (Fig. 4). In contrast, the E22Q Pcp migrated more slowly than the wild-type protein.

Catalytic properties of mutants. The Pcp activities were assessed by using pGlu-βNA and pGlu-Ala as substrates. Only two mutants, E10Q and E22Q, were able to hydrolyze these standard substrates, and kinetic parameters were established.

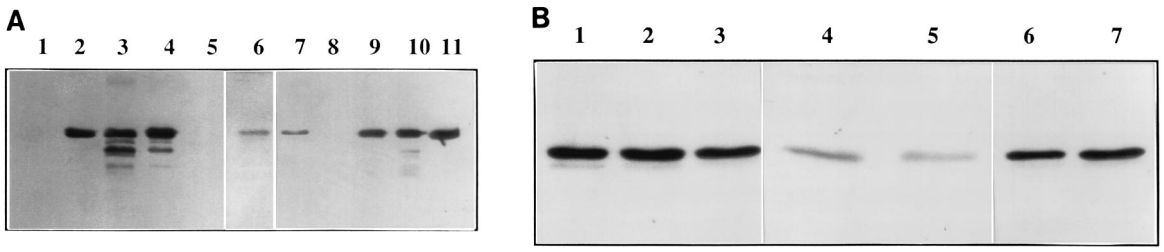

FIG. 2. Western blot analysis of wild-type and mutant Pcps. (A) Crude extracts (30 μg) from cells expressing the wild-type and mutant Pcps were heat treated, and proteins were separated by SDS-PAGE and transferred to a nitrocellulose sheet as described in Materials and Methods. The proteins were immunostained with a polyclonal antibody raised against *S. pyogenes* Pcp. Revelation was performed with a peroxidase-conjugated antibody and the ECL kit chemiluminescent substrate. Lane 1, *E. coli* NM522 harboring pKSM710 plasmid without an insert; lane 2, wild-type enzyme; lanes 3 to 10, mutants E10Q, E22Q, E81Q, D89A, D89N, D94N, C144A, and H166S, respectively; lane 11, pure Pcp. (B) Purified Pcps were heat treated, subjected to SDS-PAGE, transferred to a nitrocellulose sheet, and immunostained as described in Materials and Methods. The Pcps were purified by two chromatography steps: hydrophobic interactions on a Progel-TSK phenyl-5PW column and gel filtration on Superose 12 HR10/30, except for the D89A and D89N proteins, which were obtained without previous hydrophobic-interaction chromatography. Samples of 3 μg of total proteins for D89A and D89N and 5 μg for the wild type and the other modified Pcps were used. Lanes 1 to 7, wild-type enzyme and mutants E10Q, E22Q, D89A, D89N, C144A, and H166S, respectively.

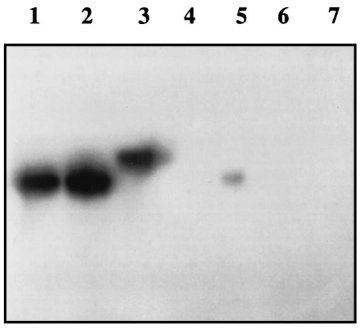

FIG. 3. Pcp activity of the purified mutated enzymes. Purified or partially purified D89A and D89N proteins directly obtained after gel filtration chromatography as described in Materials and Methods (D89A and D89N, of total proteins) were separated by native PAGE at 25 mA and 4°C. Samples of 10 μg were used, except for D89A and D89N (6 μg). The Pcp activity was revealed as described by Miller and Mackinnon (20). Lanes 1 to 7, wild-type enzyme and mutants E10Q, E22Q, D89A, D89N, C144A, and H166S, respectively.

Their catalytic and Michaelis constants were not affected by the mutations (Table 2). In addition, the mutated Pcps displayed comparable sensitivities to EDAC (35, 26, and 47% of residual activity for E10Q, E22Q, and native Pcp, respectively). The other mutants displayed low or no activity. The K_m of D89N was determined on crude extract because of the lack of stability of this enzyme during the purification steps. A value lower than that of the wild-type Pcp (50 ± 10 μM) was found. The specific activity of D89N was estimated as 0.5 ± 0.1 $\mu mol \cdot min^{-1} \cdot mg^{-1}$ (wild type, 2.1 ± 0.6 $\mu mol \cdot min^{-1} \cdot mg^{-1}$) from a crude extract after comparison of its level of synthesis with that of the native Pcp. On the basis of this estimation, the D89N mutant retained about 25% of wild-type activity. No inhibition measurement with EDAC could be performed, since D89N activity was also completely lost at pH 5.5. The D89A protein showed no activity toward any standard substrates. The modification of Cys-144 to Ala resulted in a protein also deficient for enzymatic activity with pGlu-βNA and pGlu-Ala as substrates. The H166S enzyme exhibited a very low catalytic activity with pGlu-Ala as a substrate after prolonged incubation but none with pGlu-βNA.

DISCUSSION

Previous chemical inhibition studies have suggested the nature of the amino acids required for *P. fluorescens* Pcp activity (14). On the basis of a multiple alignment of the four known

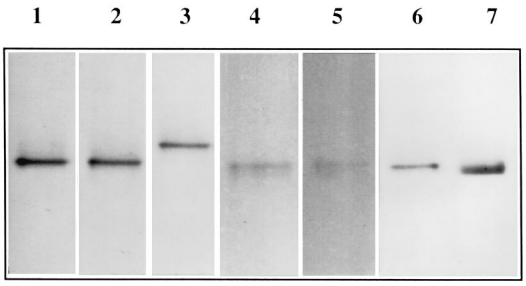

FIG. 4. Native PAGE of purified and partially purified proteins obtained without previous hydrophobic-interaction chromatography. The proteins were separated at 25 mA and 4°C. Samples of 5 μg of proteins were used except for D89A and D89N (3 μg). The gel was stained with Coomassie brilliant blue. Lanes 1 to 7, wild-type enzyme and mutants E10Q, E22Q, D89A, D89N, C144A, and H166S, respectively.

TABLE 2. Hydrolytic activities toward pGlu-βNA and pGlu-Ala substrates[a]

Pcp	Pcp activity		K_m (μM)	k_{cat}[b] (s^{-1})	k_{cat}/K_m ($s^{-1}\ \mu M^{-1}$)
	pGlu-βNA	pGlu-Ala			
Wild type	+	+	210 ± 30	31.4 ± 5	0.149 ± 0.027
E10Q	+	+	240 ± 60	28.0 ± 8	0.117 ± 0.034
E22Q	+	+	220 ± 90	35.1 ± 7	0.159 ± 0.029
E81Q	ND[c]	ND	ND	ND	ND
D89A	−	−	ND	ND	ND
D89N	+	+	50 ± 10[d]	7.4[e]	0.148[e]
D94N	ND	ND	ND	ND	ND
C144A	−	−	ND	ND	ND
H166S	−	+/−[f]	ND	ND	ND

[a] Hydrolysis of pGlu-Ala was visualized by thin-layer chromatography after revelation with ninhydrin. The kinetic constants of the wild-type and modified Pcps were determined with the chromogenic substrate pGlu-βNA. Data are means ± standard errors.
[b] k_{cat}, catalytic constant.
[c] ND, not detected.
[d] Measured on crude extract.
[e] Estimated on crude extract on the basis of optic densitometric measurement on Western blot.
[f] +/−, weak activity detected after 15 h of incubation at room temperature.

Pcp sequences, putative residues that may constitute the catalytic site of this enzyme have been proposed: Glu-10, Glu-22, Glu-81, Asp-89, Asp-94, Cys-144, and His-166. The involvement of these different residues in the catalytic mechanism was analyzed in this study by introducing single amino acid substitutions by site-directed mutagenesis.

It has been previously shown that most amino acid substitutions were tolerated and had no deleterious effect on the protein structure (27), but since the mutations of critical structural residues might modify the protein and consequently the catalytic ability, the apparent conformation and stability of mutated Pcps have been estimated by chromatography (dimeric structure), PAGE (dimeric structure and surface charge variations), and Western blotting analysis. Since the degradation patterns are observable only with crude extracts (Fig. 2A) and not with the purified proteins (Fig. 2B), deterioration likely occurred in vivo or during the first step of the extraction.

Replacement of Cys-144 and His-166 by Ala and Ser, respectively, completely abolished the aminopeptidase activity without detectable modification of the protein conformation. Thus, it appears that these two amino acids are essential for the Pcp activity. We propose Cys-144 and His-166 as, at least, a catalytic dyad for *P. fluorescens* Pcp.

Other substitutions were introduced at various positions in order to determine the other residue involved in the catalytic process. Six mutations of acidic amino acids were performed, and the modified proteins were characterized. Despite their low degree of stability, E10Q and E22Q mutants could be easily characterized. Although E22Q was homodimeric, it migrated slowly in PAGE, indicating a possible variation of protein charge, a slight conformational change, or an external localization of the E-22 residue (Fig. 4). However, it is unlikely that E-10 and E-22 are involved in the catalytic process, since no significant differences from the native Pcp were found, as they retained catalytic properties and sensitivity to EDAC. The modification of Glu-81 and Asp-94 residues, to Gln and Asn, respectively, resulted in enzymes that could not be detected in *E. coli* crude extracts. The absence of these proteins could be the result of the loss of the side chain charges at physiological pH, since replacement induced little steric disturbance. These conserved amino acids likely have a critical role in folding or

general stability. Another possibility is that a protease cleavage site could be introduced with the mutation. Thus, no characterization could be done and the exact function of these residues is still unknown.

In papain, often considered a model of thiol peptidases, the third part of the catalytic site is not directly involved in the catalysis process, but the amide oxygen of the Asn side chain is hydrogen bonded to the histidine imidazole ring, creating a Cys-His-Asn triad. Thus, the proposed role for Asn is to direct the imidazole ring in a favorable orientation for the catalysis mechanism, and this might also assist the stabilization of the thiolate-imidazolium ion pair (26). In contrast to the papain-like enzymes, some thiol peptidases do not contain Asn but, rather, Asp or Glu as the third component (25), and these are likely to have a catalytic mechanism similar to that of Ser proteases, since the charge of the side chain is different at the intracellular pH. Consequently, any amino acid substitution occurring on the third catalytic component leads to an enzyme with dramatically reduced activity (9, 15).

Because of partial purifications and characterizations, the function of the Asp-89 residue is not clear, since D89N retained 25% of wild-type activity and displayed a decreased K_m, which might be because the substrate binds slightly better to this mutated enzyme, while D89A, carrying a less conservative change, showed a complete loss of activity (Table 2). Moreover, it is worth noting that Asp-89 is not fully conserved among the four known Pcp sequences (Fig. 1). Asp is replaced by Glu in the *B. amyloliquefaciens* Pcp sequence, but the differences between side chains reside only in the carbon chain length, the acidic function, and the overall structure being maintained. Given the instability of mutants D89A and D89N and the lack of information about the E81Q and D94N proteins, no obvious catalytic function could be assigned, at this point, to one of these acidic residues.

The mutational analysis of the *P. fluorescens* Pcp active site presented here fits into the now well-accepted picture of the peptidase catalytic machinery. Indeed, Cys-144 and His-166 are essential for catalysis. A third acidic component, which may be one of three residues, Glu-81, Asp-89, or Asp-94, is necessary for the Pcp enzymatic activity, as shown in previous studies (14). Nevertheless, the amino acid environment of these residues does not resemble the active site environment of other thiol or Ser proteases, and their primary structure arrangement is not similar to those previously described, suggesting that Pcps constitute a novel class of thiol peptidases with regard to the nomenclature proposed by Rawlings and Barrett (24).

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ADDENDUM IN PROOF

After submission of this work, the cloning of a gene encoding a Pcp from *Staphylococcus aureus* was reported. Sequence data confirmed our results and hypothesis about residues involved in the catalytic site (J. M. Patti, A. Schneider, N. Garza, and J. O. Boles, Gene **166:**95–99, 1995).

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