His507 of acylaminoacyl peptidase stabilizes the active site conformation, not the catalytic intermediate

András L. Kiss[a], Zoltán Szeltner[a], Vilmos Fülöp[b], László Polgár[a,*]

[a]*Institute of Enzymology, Biological Research Center, Hungarian Academy of Sciences, P.O. Box 7, H-1518 Budapest 112, Hungary*
[b]*Department of Biological Sciences, University of Warwick, Gibbet Hill Road, Coventry CV4 7AL, UK*

Received 11 May 2004; revised 17 June 2004; accepted 24 June 2004

Available online 1 July 2004

Edited by Judit Ovádi

Abstract Acylaminoacyl peptidase is a member of the prolyl oligopeptidase family. Amino acid sequence alignment suggests that the stabilization of the tetrahedral intermediate should be mediated by His507 rather than by a tyrosine residue found in the other family members of this serine peptidase group. The pH dependence of k_{cat}/K_m did not reveal any effect of His507. Substitution of an alanine for His507 gave the same bell-shaped pH rate profile with the same pK_a values (7.0 and 8.7). However, the value of the rate constant was 85 times lower with the modified enzyme, which indicated that His507 is an important residue that is probably involved in the formation of the 3-dimensional structure.

Keywords: Oligopeptidase; Acylaminoacyl peptidase; Oxyanion binding site; Catalytic mechanism

1. Introduction

Acylaminoacyl peptidase (acyl-amino-acid-releasing enzyme, acylpeptide hydrolase) belongs to the recently identified prolyl oligopeptidase family of serine peptidases cf. [1,2], which is different from the classic serine peptidases, like trypsin and subtilisin. Prolyl oligopeptidase is implicated in the metabolism of neuropeptides and is involved in amnesia and depression cf. [3]. Crystal structure determination has revealed that it contains a peptidase and an unusual β-propeller domain that accounts for the oligopeptidase activity by excluding large peptides and proteins from the active site [4]. Sequence alignment of important members of the family, like prolyl oligopeptidase, acylaminoacyl peptidase and dipeptidyl peptidase IV [1], indicated that the relationship is the closest between prolyl oligopeptidase and acylaminoacyl peptidase. The homology in the family is always higher for the peptidase than for the propeller domain. Indeed, prolyl oligopeptidase has a seven-bladed propeller, whereas that of dipeptidyl peptidase is eight-bladed, as shown by the most recently determined crystal structure of dipeptidyl peptidase IV [5–9]. Sequence alignment suggests that the propeller domain of acylaminoacyl peptidase is seven-bladed.

In contrast to prolyl oligopeptidase and oligopeptidase B, which are monomers both in solution and in crystal structure [4], and dipeptidyl peptidase, which is a dimer [5–9], mammalian acylaminoacyl peptidases are composed of four identical subunits. The enzyme catalyzes the removal of an *N*-acylated amino acid from blocked peptides cf. [10]. The blocked peptides containing two or three amino acids are hydrolyzed faster than the longer peptides, but *N*-acylated proteins are not substrates for the enzyme [10]. The members of the catalytic triad were identified by sequence homology studies [1,11] and chemical modifications by inactivating the enzyme by diisopropyl fluorophosphate at Ser587 and by chloromethyl compounds at His707. The third member of the triad is Asp675, which was confirmed by site-specific mutagenesis cf. [10].

A major catalytic difference from the trypsin- and subtilisin-type enzymes concerns the stabilization of the negatively charged tetrahedral intermediate. This is achieved by the oxyanion binding site, which provides two hydrogen bonds to the oxyanion. In the trypsin-type enzymes, the hydrogen bonds are contributed by two main chain NH groups, whereas in the subtilisin and its homologs one of the hydrogen bonds originates from the side chain amide of an asparagine residue cf. [12]. In prolyl oligopeptidase, one hydrogen bond is formed between the oxyanion and the main chain NH group of Asn555, adjacent to the catalytic Ser554. The other bond comes from the OH group of Tyr473 [4]. Sequence alignment suggests that a tyrosine residue in the same position is encountered in all oligopeptidases, except in acylaminoacyl peptidase, where a histidine (His507) occupies the place of the tyrosine (Fig. 1). The His507 is conserved throughout different organisms, from bacteria to mammals. In this paper, we have investigated whether His507 is involved in the catalysis.

2. Materials and methods

2.1. Preparation of porcine liver acylaminoacyl peptidase

All preparations were carried out at 4 °C, unless otherwise stated. 2.5 kg porcine liver was homogenized in 3 L of buffer 1 (50 mM phosphate buffer, pH 7.2, containing 1 mM ethylenediaminetetraacetic acid (EDTA), 5 mM, 2-mercaptoethanol, and 5 mM Na_2SO_3). The mixture was heated to 64 °C for 7 min as described earlier [13], then cooled on ice and centrifuged. The supernatant was further purified on a DE52 column (56 × 230 mm, Whatman International Ltd. Maidstone,

* Corresponding author. Fax: +36-1-466-5465.
E-mail address: polgar@enzim.hu (L. Polgár).

Abbreviations: PCR, polymerase chain reaction; EDTA, ethylenediaminetetraacetic acid

0014-5793/$22.00
doi:10.1016/j.febslet.2004.06.054

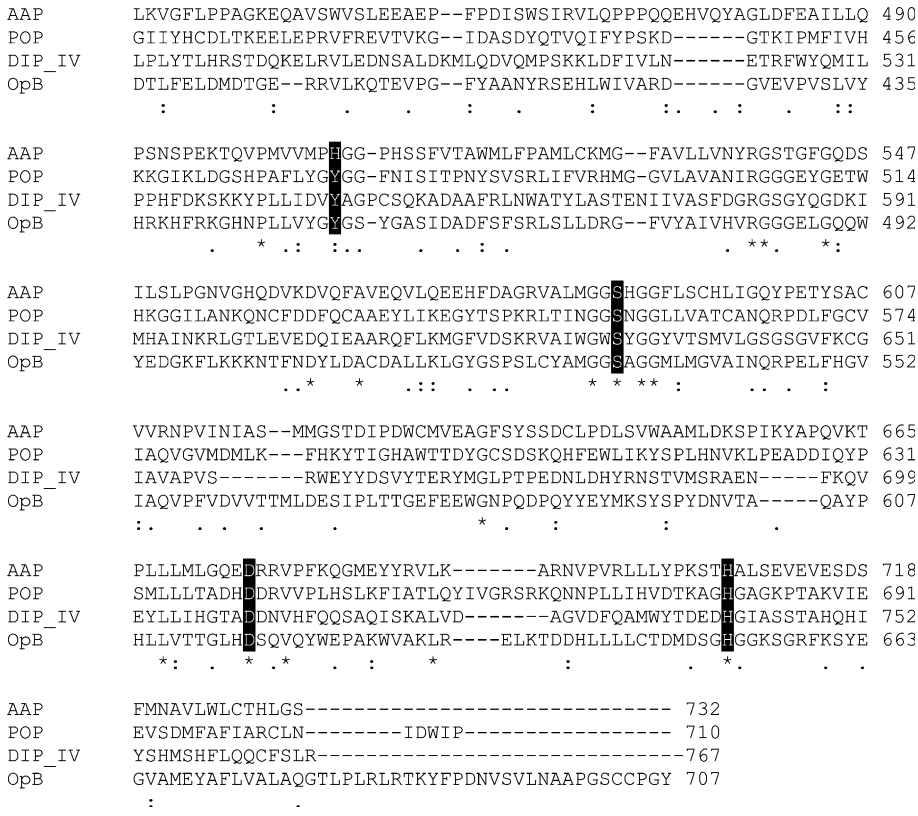

Fig. 1. Multiple sequence alignment of the peptidase domains of oligopeptidases. The amino acid sequences are AAP, porcine liver acylaminoacyl peptidase [17]; POP, pig brain prolyl oligopeptidase [18]; DIP IV, rat liver dipeptidyl peptidase IV [19]; and OpB, *E. coli* oligopeptidase B [20]. The oxyanion binding site Tyr and the Ser, His, and Asp of the catalytic triad are highlighted.

England) equilibrated with buffer 2 (buffer 1 without Na_2SO_3) and the enzyme was eluted with a linear salt gradient (0–1 M NaCl, 1000 ml of each component of buffer 2). The active fractions were combined and the enzyme was precipitated by the addition of $(NH_4)_2SO_4$ to 50% saturation. After incubation for 2 h, it was centrifuged and the precipitate was dissolved in buffer 1. The solution was passed through a Cibacrone Blue column (28×170 mm, Sigma–Aldrich). The active fractions were collected and after concentration in Centricon membrane, MWCO 30 kDa (Millipore), the final purification was achieved on a MonoQ HR10 column (Pharmacia LKB). Acylaminoacyl peptidase was eluted with a linear gradient from 0.25 to 0.45 M NaCl in 20 mM phosphate buffer, pH 7.2, containing 1 mM EDTA, using the FPLC system. The yield was 10 mg.

2.2. Cloning of wild type N-terminally His-tagged acylaminoacyl peptidase gene

The porcine acylaminoacyl peptidase gene from a pET23a vector (obtained from S. Tsunasawa [14]) was opened with *Bam*HI restriction endonuclease and was amplified with the polymerase chain reaction (PCR) method, using the primers 3′-gatgcgtgtgtgaacccgtcg-attcctaggtga-5′ (5′-primer) and 5′-atcagtacatatggaacgtcaggtgctgctg-3′ (3′-primer), containing *Bam*HI and *Nde*I recognition sites (underlined), respectively. The acylaminoacyl peptidase gene was digested with *Nde*I and *Bam*HI endonucleases, purified with 1% agarose gel-electrophoresis and ligated into the pET15b plasmid, digested with the same restriction enzymes. The pET15b plasmid adds a His-tag to the N-terminus of the protein, which can be cleaved by thrombin. Finally, the plasmid was amplified in *Escherichia coli* DH5α strain.

2.3. Constructing the H507A mutant acylaminoacyl peptidase gene bearing a His-tag at the N-terminus

The pET23a plasmid opened with *Bam*HI was employed as a template for introducing the mutation with PCR. In the first step, the gene was amplified between nucleotides 1 and 1539, using the 5′-primer (preceding paragraph) and antisense primer coding for the mutation (5′-gtccccctgcaggcatgacca-3′). An extra *Pst*I recognition site (underlined) was also introduced with the primer. In the second step, the gene was amplified between nucleotides 1501 and 2199, using the 3′ primer (preceding paragraph) and the sense primer coding for mutation (5′-tggtcatgcctgcaggggac-3′). The two gene fragments were fused and amplified with another PCR in the presence of the 3′ and 5′ primers. The mutant gene was digested with *Nde*I and *Bam*HI enzymes and ligated into pET15b vector opened with the same restriction enzymes.

2.4. Preparation of recombinant acylaminoacyl peptidase proteins

The same preparation method was used for the wild type enzyme and the H507A variant. The pET15b plasmid harboring the acylaminoacyl peptidase gene was transformed into BL21(DE3)-pLysS *E. coli* cells. 5–6 single cell colonies grown up on agar plate were added to each of 20×600 ml of LB medium containing 100 μg/ml ampicillin and 25 μg/ml chloramphenicol. The induction was performed with 13 μM isopropyl β-D-thiogalactoside at about OD 0.6. The cells were incubated at 32 °C for 70 h. The next preparation steps were carried out at 4 °C. The cell paste (75 g wet weight, obtained from 20×600 ml) was sonicated in 260 ml of 50 mM phosphate buffer, pH 8.0, and 2 mM Na_2SO_3. The supernatant of the sonicate was filtered and applied to a Ni–NTA column (Qiagen Gmbh, Hilden, Germany). The acylaminoacyl peptidase was stepwise eluted with 20, 50, 100 and 200 mM imidazole. The active fractions (50 and 100 mM imidazole) were concentrated by ultrafiltration to 2 ml and washed to remove imidazole. To eliminate the His-tag, 10 U of thrombin/mg acylaminoacyl peptidase was added. The reaction mixture was incubated overnight at room temperature and purified on a MonoQ HR10, and then a Superose 12 column in buffer 2. The protein concentration of the pure enzyme was calculated from the absorbance at 280 nm by using M_r value of 324 957 for the tetramer and A_{280} (0.1%) of 1.48. The overall yield was 1 mg protein from the wild type and 120 μg from H507A enzyme variant. The latter exhibited much greater tendency for denaturation than did the former.

2.5. Kinetics

The reaction of acylaminoacyl peptidase with acetyl-Ala-4-nitroanilide (Bachem Ltd., Bubendorf, Switzerland) was measured spectrophotometrically, using a Cary 100 spectrophotometer equipped with a Peltier cell holder accessory and a temperature controller. The liberation of 4-nitrophenol was monitored at 410 nm. The first-order rate constants were measured at substrate concentrations lower than 0.1 K_m and were calculated by nonlinear regression analysis, using the GraFit software [15]. The specificity rate constants, k_{cat}/K_m, were obtained by dividing the first-order rate constant by the total enzyme concentration in the reaction mixture. Theoretical curves for bell-shaped pH rate profiles were calculated by nonlinear regression analysis [15]. The buffer used in a wide pH range contained 25 mM 2-(morpholino)ethanesulfonic acid, 25 mM acetic acid, 75 mM Tris, 25 mM glycine, 1 mM EDTA and 0.3 M NaCl. The buffers were adjusted to the appropriate pH values with 1 M HCl or 1 M NaOH. The substrate was dissolved in dimethylsulfoxide. The concentration of the organic solvent in the reaction mixture was less than 0.006%. The substrate concentration was 1.38 μM, and the enzyme concentrations were 3 and 60 nM for the wild type and the H507A variant, respectively. The analyses were carried out at 25 °C.

3. Results and discussion

The catalytic triad, which consists of serine, histidine and aspartate residues, is an essential part of the catalytic machinery of serine peptidases. The catalytic histidine functions as a general base catalyst that assists the proton transfer from the serine OH to the leaving group of the substrate through the formation of a tetrahedral intermediate formed on the reaction path. This process generates an acyl enzyme, which is subsequently hydrolyzed by the reverse mechanism of acylation cf. [12].

The pK_a of histidine is about 7, and thus it should be seen in the pH-rate profile of serine protease reactions, because on protonation the histidine becomes catalytically inactive. Therefore, we have determined the pH dependence of the specificity rate constant for acylaminoacyl peptidase (k_{cat}/K_m), which is affected by the ionization state of the imidazole group. Fig. 2 shows that the k_{cat}/K_m follows a bell-shaped pH dependence having two pK_a values, one refers to the catalytic histidine, His707 (p$K_1 = 7.0$), the other probably to conformational changes (p$K_2 = 8.7$). A similar bell-shaped curve was observed with chymotrypsin [12], whereas with subtilisin, in the absence of conformational changes, a simple sigmoid curve is obtained with a single pK_a of about 7.2 [12].

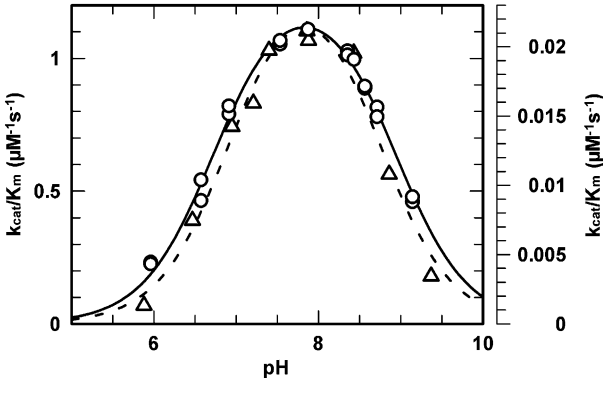

Fig. 2. The pH dependence of k_{cat}/K_m for the acylaminoacyl peptidase reactions. Wild type enzyme (○, solid line, left ordinate), parameters for the curve: p$K_1 = 7.01 \pm 0.03$, p$K_2 = 8.65 \pm 0.03$, and $k_{cat}/K_m = 2.28 \pm 0.05$ μM^{-1} s^{-1}. H507A variant (△, dashed line, right ordinate), parameters for the curve: p$K_1 = 6.94 \pm 0.07$, p$K_2 = 8.69 \pm 0.08$, and $k_{cat}/K_m = 0.0269 \pm 0.002$ μM^{-1} s^{-1}.

While the bell-shaped-curve in Fig. 2 implies the participation of the catalytic histidine base (His707), it does not support the contribution of a second histidine, i.e., His507, from the oxyanion binding site. The imidazole group contains two nitrogen atoms, one nitrogen is always protonated in the pH range studied and the other may be protonated or unprotonated, depending on the pH. Of course, only the protonated nitrogen is able to offer a hydrogen bond to the oxyanion. However, such a contribution of His507, i.e., an increase in rate, is not seen in Fig. 2 below pH 7, where the protonation of His507 would compensate for the decrease in rate when His707 becomes protonated.

Only a very low pK_a for His507 (<5) would be consistent with the bell-shaped curve of Fig. 2, when the neutral imidazole side chain serves as the oxianion binding site. Such a decrease in the pK_a of His507 can be elicited by nearby positive charges of Lys or Arg residues. However, the electrostatic effects of the charges should be shielded by an increase in ionic strength of the medium. Moreover, addition of 0.3 M NaCl did not influence the pH-rate profile shown in Fig. 2. A further explanation of the electrophilic catalysis by His507 may be that a water molecule that binds to His507 serves as the oxianion binding site. The contribution of the bound water molecule, however, would be affected by the dissociation of His507, and this would perturb the bell-shaped curve. In addition, such a mechanism would be entropically unfavorable.

It may be assumed that in a special enzymatic environment the pK_a of His507 may be so high that the imidazole ring is protonated up to pH 8.7 and this would account for the decrease of k_{cat}/K_m in the alkaline pH region. However, such an environment that could raise the pK_a by 1.7 units is unlikely. If the NH group of the imidazole ring, which holds the proton over the entire pH range studied, provided the hydrogen bond, then the effect of ionization of the other nitrogen would be seen around pH 7, in contrast to the present finding. Consequently, the participation of His507 in the stabilization of the oxyanion is not probable.

The above data do not rule out that His507 might exhibit an unusually high pK_a, and the protonated imidazole serves as a catalyst up to pH 8.7, where the protonated imidazole does not even lose the proton. A convincing evidence that excludes His507 as a catalytic entity can be obtained by using the H507A variant of acylaminoacyl peptidase. To this end, we have expressed the porcine liver acylaminoacyl peptidase in *E. coli*. The yield was rather low, but enough for the comparison of the recombinant enzyme with that isolated from porcine liver. The kinetic parameters were identical for the natural and the recombinant enzymes within experimental error. The acylaminoacyl peptidase was then modified by substituting an alanine for the His507. The pH rate profiles were similar for the native enzyme and the H507A variant (Fig. 2), suggesting that the ionization of His507 does not significantly influence the pK_a values and does not participate in the stabilization of the oxyanion of the tetrahedral intermediate.

Although the pK_a values extracted from the pH-rate profiles are not affected by the removal of His507, the k_{cat}/K_m value for the H507A variant was 85 times less than that for the wild type enzyme (Fig. 2). Thus, His507 is an important residue, even though it is not a catalytic group. It is probable that His507 has structural importance; its side chain may interact with

other groups, ensuring the proper enzyme conformation for catalysis. His507 must be close to the active site so that a small conformational change may elicit substantial catalytic effects. Because we do not see the effect of either a histidine or a tyrosine residue, the latter could be seen above pH 9, it is possible that two main chain NH groups are involved in the stabilization as found in the chymotrypsin-type enzymes. The role of His507 in the stabilization of the protein structure is also supported by the lower yield obtained with the H507A variant.

The conformational change caused by the modification of His507 could exert an effect on the oligomerization of the protomers or dimers. However, both the wild type and the modified enzyme eluted at the same position during chromatography on a Superose 12 column, indicating that the tetramer structure was preserved with the H507A variant.

It can be concluded that His507 does not contribute to the stabilization of the tetrahedral intermediate by providing a hydrogen bond to the oxyanion. This is not surprising because the imidazole group is unprotonated in the catalytically effective pH range. Therefore, we assume that the main chain folding of the segment holding the His507 residue is different from the corresponding segment of prolyl oligopeptidase and dipeptidyl peptidase IV. Protein structure prediction based on amino acid sequence alignment is not always straightforward. For example, a seven-bladed propeller was assigned for dipeptidyl peptidase IV on the basis of low homology with prolyl oligopeptidase [16], whereas later crystal structure determination established an eight-bladed propeller [5–9].

Acknowledgements: The authors thank Dr. S. Tsunasawa for providing the acylaminoacyl peptidase clone. Thanks are due to Ms. I. Szamosi for excellent technical assistance. This work was supported by the Wellcome Trust (Grant No. 066099/01/Z) and the Hungarian National Science Fund (Grant No. T 046057). V.F. is a Royal Society University Research Fellow.

References

[1] Rawlings, N.D., Polgár, L. and Barrett, A.J. (1991) Biochem. J. 279, 907–908.
[2] Polgár, L. (2002) Cell. Mol. Life Sci. 59, 349–362.
[3] Polgár, L. (2002) Curr. Med. Chem. Central Nervous System Agents 2, 251–257.
[4] Fülöp, V., Böcskei, Z. and Polgár, L. (1998) Cell 94, 161–170.
[5] Rasmussen, H.B., Branner, S., Wiberg, F.C. and Wagtmann, N. (2003) Nat. Struct. Biol. 10, 19–25.
[6] Thoma, R., Loffler, B., Stihle, M., Huber, W., Ruf, A. and Hennig, M. (2003) Structure 8, 947–959.
[7] Engel, M., Hoffmann, T., Wagner, L., Wermann, M., Heiser, U., Kiefersauer, R., Huber, R., Bode, W., Demuth, H.-U. and Brandstetter, H. (2003) Proc. Natl. Acad. Sci. 100, 5063–5068.
[8] Hiramatsu, H., Kyono, K., Higashiyama, Y., Fukushima, C., Shima, H., Sugiyama, S., Inaka, K., Yamamoto, A. and Shimizu, R. (2003) Biochim. Biophys. Res. Commun. 302, 849–854.
[9] Oefner, C., D'Arcy, A., Mac Sweeney, A., Pierau, S., Gardiner, R. and Dale, G.E. (2003) Acta Crystallogr. Sect. D 59, 1206–1212.
[10] Jones, W.M., Scaloni, A. and Manning, J.M. (1994) Methods Enzymol. 244, 227–231.
[11] Polgár, L. (1992) FEBS Lett. 311, 281–284.
[12] Polgár, L. (1989) Mechanisms of Protease Action. CRC Press, Boca Raton, FL. pp. 87–122.
[13] Radhakrishna, G. and Wold, F. (1989) J. Biol. Chem. 264, 11076–11081.
[14] Mitta, M., Miyagi, M., Kato, I. and Tsunasawa, S. (1998) J. Biochem. 123, 924–931.
[15] Leatherbarrow, R.J. (1989) GraFit, Version 5. Erythacus Software Ltd., Staines, Middlesex.
[16] Abbott, C.A., McCaughan, G.W., Levy, M.T., Church, W.B. and Gorrell, M.D. (1999) Eur. J. Biochem. 266, 798–810.
[17] Mitta, M., Asada, K., Uchimura, Y., Kimizuka, F., Kato, I., Sakiyama, F. and Tsunasawa, S. (1989) J. Biochem. (Tokyo) 106, 548–551.
[18] Rennex, D., Hemmings, B.A., Hofsteenge, J. and Stone, S.R. (1991) Biochemistry 30, 2195–2203.
[19] Ogata, S., Misumi, Y. and Ikehara, Y. (1989) J. Biol. Chem. 264, 3596–3601.
[20] Kanatani, A., Masuda, T., Shimoda, T., Misoka, F., Lin, X.S., Yoshimoto, T. and Tsuru, D. (1991) J. Biochem. (Tokyo) 110, 315–320.