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# Review

# The prolyl oligopeptidase family

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**Abstract.** A group of serine peptidases, the prolyl oligopeptidase family, cannot hydrolyze peptides containing more than about 30 residues. This group is unrelated to the classical trypsin and subtilisin families, and includes dipeptidyl peptidase IV, acylaminoacyl peptidase and oligopeptidase B, in addition to the prototype prolyl oligopeptidase. The recent crystal structure determination of prolyl oligopeptidase (80 kDa) has shown that the enzyme contains a peptidase domain with an $\alpha/\beta$ hydrolase fold, and its catalytic triad is covered by the central tunnel of an unusual seven-bladed $\beta$-propeller. This domain operates as a gating filter, excluding large, structured peptides from the active site. The binding mode of substrates and the catalytic mechanism differ from that of the classical serine peptidases in several features. The members of the family are important targets of drug design. Prolyl oligopeptidase is involved in amnesia, depression and blood pressure control, dipeptidyl peptidase IV in type 2 diabetes and oligopeptidase B in trypanosomiasis.

**Key words.** Serine peptidases; oligopeptidases; prolyl oligopeptidase; dipeptidyl peptidase; oligopeptidase B; acylaminoacyl peptidase; $\beta$-propeller structure; catalytic mechanism.

## Introduction

Compared with the classic trypsin and subtilisin families, the prolyl oligopeptidase family represents a relatively new class of serine peptidases. It was first described in 1991, based on the amino acid sequence homology of prolyl oligopeptidase, dipeptidyl peptidase IV and acylaminoacyl peptidase [1]. The fourth major member of the group was added when the amino acid sequence of oligopeptidase B was determined [2]. Several complementary DNAs (cDNAs) related to the proteins of the prolyl oligopeptidase family have also been cloned from various sources. Some of them lacking the catalytic serine residue were inactive. The prolyl oligopeptidase group called family S9 has been grouped with other families into the SC clan of serine peptidases [3]. Based on the structural relationship between these enzymes and lipases [4], and on secondary structural studies [5], these enzymes have been considered as members of the $\alpha/\beta$-hydrolase fold enzymes.

The amino acid sequence homology of the four basic peptidases is rather low, but they apparently share a similar three-dimensional (3D) structure. They display distinct specificities and represent different types of peptidases. Thus, prolyl oligopeptidase and oligopeptidase B are endopeptidases and are found in the cytosol. Acylaminoacyl peptidase and dipeptidyl peptidase IV are exopeptidases, acylaminoacyl peptidase is a cytoplasmic omega peptidase, whereas dipeptidyl-peptidase IV is a membrane-bound enzyme that cleaves a dipeptide from the amino terminus of oligopeptides.

The catalytically competent residues, the so-called catalytic triad (Ser, Asp and His), are concentrated in the carboxyl terminal region within about 130 residues. The amino acid sequence homology is more significant in the carboxyl terminal domain than in the amino terminal half

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of the molecules. The enzymes of the family are much larger (about 80 kDa) than are the classic serine proteases, trypsin and subtilisin (25–30 kDa). The membrane-bound dipeptidyl-peptidase is somewhat larger than the other family members, owing to its transmembrane region. The enzymes do not have a zymogen (or proenzyme) form; they are apparently synthesized as active peptidases.

The most notable property of the family members is their selectivity, which is restricted to oligopeptides comprising not more than about 30 amino acid residues. The main feature of this regulatory mechanism has recently been understood in the light of the three-dimensional structure of prolyl oligopeptidase [6, 7]. Since the structural basis of the catalytic function of prolyl oligopeptidase has also been studied in detail, this review will primarily focus on this enzyme.

The members of the prolyl oligopeptidase family are involved in important physiological processes, and attract considerable pharmaceutical interest.

## Prolyl oligopeptidase

Prolyl oligopeptidase (EC 3.4.21.26) was discovered in the human uterus as an oxytocin-degrading enzyme [8, 9]. The peptidase was originally named post-proline cleaving enzyme since it preferentially hydrolyzed the peptide bond on the carboxyl side of proline residues. Several enzymes with similar specificity, like tyrotropin-releasing hormone deaminase, endooligopeptidase B, brain kinase B and others were then described, which later proved to be identical with prolyl oligopeptidase. The selectivity of the enzyme for oligopeptides was discovered in the 1970s [10]. The name prolyl endopeptidase was first recommended by the Enzyme Nomenclature, but later it was changed to prolyl oligopeptidase, emphasizing the special characteristics of the enzyme.

## Biological relevance

Proline is an imino rather than an amino acid, and thus most peptidases are not able to hydrolyze the peptide bond at proline residues. Hence, proline residues can protect biologically active peptides against enzymatic degradation. Indeed, most peptide hormones and neuropeptides comprise one or more proline residues, and the processing and degradation of such peptides often requires the use of proline-specific enzymes. Several reviews have discussed the role of proline in the metabolism of biologically active peptides [11–13].

Prolyl oligopeptidase is present in most organisms and tissues. Its preparation, assay, sensitivity to cis-trans isomerization, ionic strength and organic solvents have been reviewed [14]. Purification of the recombinant enzyme expressed in *Escherichia coli* is more readily achieved compared with the isolation from different tissues [15]. Prolyl oligopeptidase has been cloned from different sources, including porcine brain [16], *Flavobacterium meningosepticum* [17–19], human lymphocytes [20, 21], mouse brain [22], bovine brain [23], *Sarcophaga peregrina* [24], *Aeromonas hydrophila* [25] and *Pyrococcus furiosus* [26, 27]. The structure and localization of the mouse prolyl oligopeptidase gene has also been accomplished [28]. Prolyl oligopeptidase may exhibit the highest concentration among brain peptidases, and thus it is implicated in a variety of disorders of the central nervous system.

A decrease in serum prolyl oligopeptidase activity has been observed in patients suffering from different stages of depression [29]. Peptides involved in the pathophysiology of depression, for example thyroliberin, $\beta$-endorphin, substance P and arginine-vasopressin, are known to be substrates of the enzyme. In contrast, serum prolyl oligopeptidase activity is increased in patients with mania and schizophrenia [30]. Both the antidepressant fluoxetine and the antimanic drug valproate restore prolyl oligopeptidase activity to normal levels [30]. Lithium is the simplest and oldest therapeutic agent for the treatment of depression. Recent studies indicate that a lithium-sensitive signal transduction involves inositol (1,4,5)-trisphosphate, the biosynthetic pathway of which is regulated by prolyl oligopeptidase [31, 32]. Low prolyl oligopeptidase activity may cause neurotrophic effects through elevation of inositol (1,4,5)-triphosphate, and this process is reversed by lithium treatment. Lower serum activity of prolyl oligopeptidase has also been observed in anorexia and bulimia nervosa [33].

Specific inhibitors of prolyl oligopeptidase have revealed a further physiological role of the enzyme. The first effective inhibitor, benzyloxycarbonyl-Pro-prolinal [34], was shown to reverse scopolamine-induced amnesia in rats [35]. This was confirmed by a number of studies using various inhibitors [36–39]. These inhibitors are described as cognitive enhancers, and some (Ono-1603, JTP-4819, and S-17092-1) entered clinical trials [40].

Several lines of evidence indicate that prolyl oligopeptidase could contribute to the symptomatology of Alzheimer disease, in particular to the memory loss. It was reported that inhibitors of prolyl oligopeptidase suppress the formation of amyloid $\beta$-peptide in neuroblastoma cells [41], and prevent amyloid-like deposition in the mouse model of accelerated senescence [42]. In contrast, the involvement of prolyl oligopeptidase in the amyloid-related etiology of Alzheimer disease is not supported by other findings indicating that specific inhibitors do not affect the formation and degradation of $\beta$-amyloid peptides and $\beta$-amyloid precursor protein [43]. This is, indeed, expected, as prolyl oligopeptidase does not hydrolyze large peptides and proteins.

Prolyl oligopeptidase also has a role in the regulation of blood pressure by participating in the renin-angiotensin system through metabolism of bradykinin and angiotensins I and II [44].

**Structural aspects**

Prolyl oligopeptidase is a single-chain protein with a molecular mass about three times that of trypsin or subtilisin. Following the cDNA cloning of porcine brain enzyme [16], a molecular mass of 80,751 Da was deduced from the 710-residue sequence. The peptidase domain of this large molecule is located at the carboxyl terminus. The active site serine and histidine were identified as Ser554 [16] and His680 [45], respectively, in the pig brain enzyme. This order of the catalytic residues is the reverse of that found with the trypsin and subtilisin amino acid sequences, but corresponds to some lipase sequences. A structural relationship between lipases and the peptidase domain of oligopeptidases has been indicated by the similar topology of the catalytic groups and by the homologous amino acid sequences around these residues. [4]. Comparison of the sequences of lipases and oligopeptidases suggested that the third member of the catalytic triad was Asp641 [4].

The most important structural information was obtained from the 1.4-Å-resolution crystal structures of prolyl oligopeptidase and its complex with benzyloxycarbonyl-Pro-prolinal (Protein Data Bank codes 1qfm and 1qfs) [6]. The enzyme has a cylindrical shape of an approximate height of 60 Å and diameter of 50 Å. It consists of two domains, a peptidase and a seven-bladed $\beta$-propeller (fig. 1).

The peptidase or catalytic domain is built up of residues 1–72 and 428–710, and the residues between these two portions constitute the propeller domain. As predicted earlier [4, 5], the peptidase domain exhibits a characteristic $\alpha/\beta$ hydrolase fold [46–50], and contains a central eight-stranded $\beta$ sheet with all strands except the second one aligned in a parallel manner. The $\beta$ sheet is significantly twisted, and it is flanked by two helices on one side and six helices on the other side. It may be noted that there is a proline-specific enzyme, prolyl aminopeptidase, or as originally named, proline iminopeptidase, which displays a similar catalytic structure, but does not has a propeller domain [51]. This enzyme belongs to the S33 family rather than to the S9 family discussed here.

The propeller domain is based on a sevenfold repeat of four-stranded antiparallel $\beta$ sheets. The sheets are twisted and radially arranged around their central tunnel. They pack face to face, and the predominantly hydrophobic interaction provides most of the required structural stability. All the other known propeller proteins have evolved ways to close the circle ('Velcro') between their first and last blades [52]. In the $\beta$ subunit of G proteins, for example, the Velcro is closed by the main-chain hydrogen bonds formed between one $\beta$ strand from the amino terminus and three antiparallel $\beta$ strands from the carboxyl terminus. Although the six-, seven- and eight-bladed propellers close the Velcro in a similar way [52, 53], the smaller four-bladed proteins (hemopexin and collagenase C-terminal domain) form a disulphide bond between the first and last blades [54, 55]. The circular structure is not stabilized in prolyl oligopeptidase via these mechanisms. There are only hydrophobic interactions between the first and last blades. The $\beta$-propeller is held to the catalytic domain of prolyl oligopeptidase by the two connecting polypeptide main chains, with hydrogen bonds and salt bridges, but mainly with hydrophobic forces.

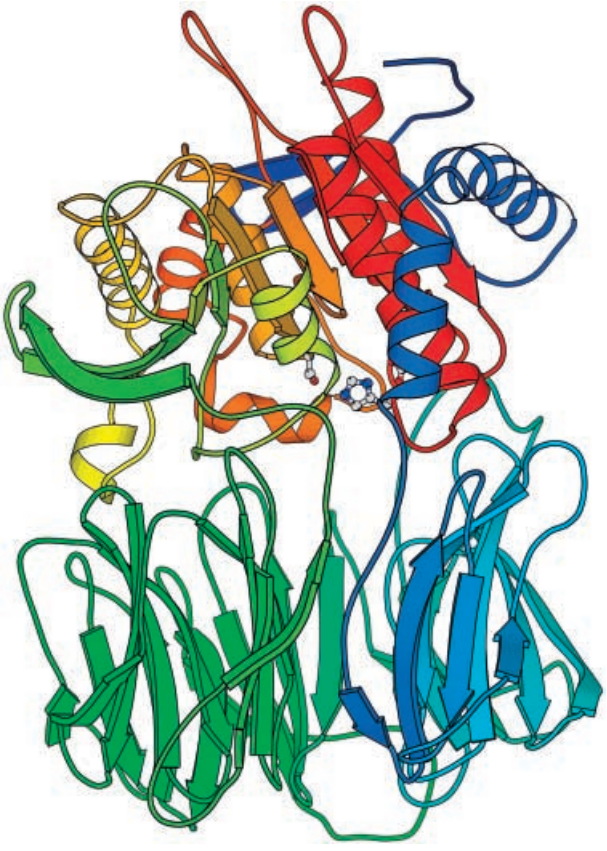

Figure 1. Structure of porcine prolyl oligopeptidase. The ribbon diagram is color-ramped blue to red from the amino to carboxyl terminus. The catalytic residues are shown in ball and stick representation. From [6] with permission.

**The active site**

The catalytic triad (Ser554, Asp641, His680) is located in a large cavity at the interface of the two domains [6]. Ser554 is found at the tip of a very sharp turn, referred to as nucleophile elbow, which is characteristic of hydrolases of the $\alpha/\beta$ type [46]. Consequently, the serine OH group is well exposed and readily accessible to the cat-

alytic imidazole group on one side and to the substrate on the other. His680 is found in the middle of a loop. One of the oxygen atoms of Asp641 is in the plane of the imidazole ring of His680, providing ideal position for hydrogen bond formation, and it is also hydrogen bonded to a well-ordered water molecule. However, $^1$H nuclear magnetic resonance (NMR) studies have indicated that the hydrogen bond between Asp641 and His680 should be weaker than in trypsin or chymotrypsin [56] The other oxygen atom of the carboxylate of Asp641 is coordinated by two main-chain NH groups (Arg643, Val644). The location and geometry of the members of the triad are very similar to those found in other $\alpha/\beta$ hydrolases providing the same 'handedness' [46], which is opposite to that of trypsin and subtilisin.

The first information about substrate binding was provided by the 3D structure of the enzyme complexed with the transition state analogue aldehyde inhibitor, benzyloxycarbonyl-Pro-prolinal [6]. As shown in figure 2, the catalytic serine side chain attacks the aldehyde carbon atom and forms a covalent hemiacetal adduct. The S1 specificity pocket ensures hydrophobic environment and a snug fit for the proline residue. The specificity is enhanced by ring stacking between the indole ring of Trp595 and the substrate/inhibitor proline residue.

The oxyanion binding site is an essential feature of the serine peptidase catalysis [cf. 46, 57, 58]. The negatively charged oxyanion of the tetrahedral intermediate is generated from the carbonyl oxygen of the scissile bond and becomes stabilized by two hydrogen bonds. Distinct from the classic serine peptidases, one of the groups of the oxyanion binding site in prolyl oligopeptidase is the main-chain NH group adjacent to the catalytic serine (Asn555). This is a direct consequence of the opposite handedness [46, 59] of the catalytic triad of $\alpha/\beta$ hydrolase-like enzymes. The second group stabilizing the oxyanion in prolyl oligopeptidase is unique among hydrolases, since the hydrogen bond is provided by the OH group of a tyrosine residue (Tyr473 shown in fig. 2) [6]. This hydrogen-bonding pattern was also found in the complexes with an octapeptide and a productlike inhibitor [60].

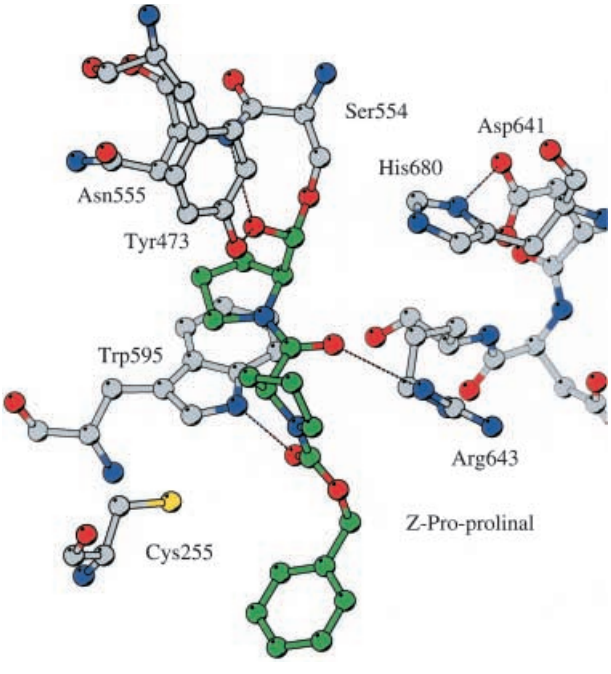


Figure 2. The active site of porcine prolyl oligopeptidase. The carbon atoms of the enzyme and the covalently bound inhibitor, benzyloxycarbonyl-Pro-prolinal, are colored gray and green, respectively. Dashed lines indicate hydrogen bonds. From [6] with permission.

The binding of the peptide main chain to the enzyme is different with prolyl oligopeptidase and the classic serine peptidases. Whereas trypsin and subtilisin form antiparallel $\beta$ sheets with the NH groups of substrate main chains, this is not possible with the proline-containing substrate lacking the main-chain NH group. Thus instead of the S1–P1 hydrogen bond of the classic peptidases, with prolyl oligopeptidase the S2–P2 hydrogen bond is important, which is formed between the carbonyl oxygen of residue P2 and the guanidinium group of Arg643, as is seen from the binding of benzyloxycarbonyl-Pro-prolinal (fig. 2) [6]. More detailed information has been obtained from the binding of an octapeptide to an inactive prolyl oligopeptidase, in which the catalytic serine was replaced by an alanine [60]. The binding mode of P1–P3 residues (Phe-Gly-Pro) was similar to that of benzyloxycarbonyl-Pro-prolinal. The P1 proline ring was stacked against Trp595, whose indole nitrogen was bound to the carbonyl oxygen of P3 Phe. The carbonyl oxygens of glycines at P2 and P4 positions were hydrogen bonded to the guanidinium group of Arg643. The P1′–P2′ (Phe-Gly) portion was bound close to the catalytic His680. The aromatic side chain of Phe residue at the P1′ position projected back to the cavity to stack against the peptide bond of the P1 and P2 residues. The carboxyl terminal P3′ and P4′ residues, as well as the blocking amino terminal 2-aminobenzoyl group, presumably do not bind to the protein because they are poorly defined in the electrondensity map. Therefore, it can be concluded that the enzyme binds no more than six residues (P4–P2′).

**Selection of oligopeptides**

Proteases have a variety of strategies for selecting substrates in order to prevent uncontrolled protein degradation. The narrow entrance of the propeller (~4 Å) opposite to the active site is much smaller than the diameter of an average peptide (6–12 Å), but it could be enlarged by partial separation of the unclosed blades 1 and 7. Such a possibility could provide access to the active site and at the same time would protect proteins and larger, struc-

tured peptides from accidental hydrolysis. This mechanism was verified by engineering a disulphide bond between blades 1 and 7, which inactivated the enzyme [7]. Specifically, a cysteine was substituted for Gln397 since blade 1 already contains a cysteine (Cys78) in an appropriate position for disulphide bond formation with residue 397. The disulphide bond was generated very slowly in the expressed enzyme, but the reaction rate was substantially increased by addition of oxidized glutathione and by increasing the pH. The presence of the disulphide bond in the enzyme variant was demonstrated by X-ray crystallography. Accordingly, the regulatory mechanism of prolyl oligopeptidase involves oscillating propeller blades acting as a gating filter during catalysis. There is a major difference between prolyl oligopeptidase of the hyperthermophilic *Pyrococcus furiosus* and its mesophilic counterparts, as the hyperthermophilic enzyme has the capacity to hydrolyze proteins such as azocasein [27, 61]. Furthermore, it is the only prolyl oligopeptidase for which autoproteolysis has been demonstrated [27, 61]. These results may indicate that the propeller of the hyperthermophilic enzyme opens to a large extent at high temperature, so that it allows azocasein to enter the cavity of the enzyme, where the hydrolysis occurs.

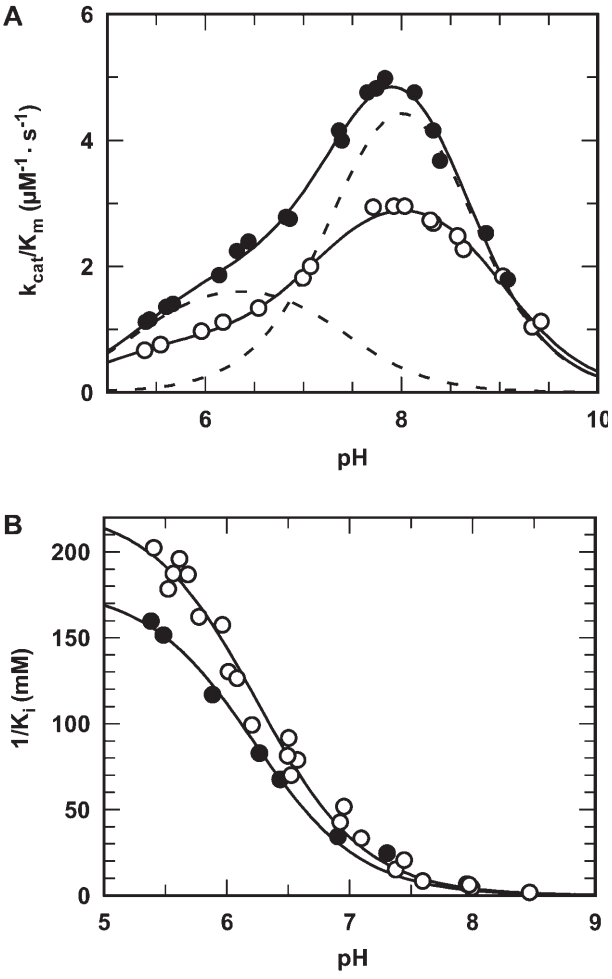


Figure 3. (*A*) pH-rate profiles for the reactions of porcine prolyl oligopeptidaase with 2-aminobenmzoyl-Gly-Phe-Gly-Pro-Phe-Gly-Phe($NO_2$)-Ala-$NH_2$. The reactions were performed in the presence (●) and absence (○) of 0.5 M NaCl. (*B*) formation of enzyme-inhibitor complex as a function of pH. The association constants ($1/K_i$) were determined for benzyloxycarbonyl-Thr-Arg-Arg-OH in the presence (●) and absence (○) of 0.5 M NaCl. From [60] with permission.

## Kinetic properties

The simplest way of estimating the p$K_a$ values of catalytically competent groups utilizes pH-$k_{cat}/K_m$ profiles. For example, the pH dependence curves for the subtilisin and chymotrypsin reactions have revealed a p$K_a$ of ~7 for the catalytic histidine. Unlike the sigmoid or bell-shaped pH-rate profiles observed with the classic serine peptidases, the pH dependence for prolyl oligopeptidase is more complicated. This has been shown with several substrates [60, 62, 63]. As seen in figure 3A, the data conform to a doubly bell shaped curve, which arises from modification of the usual bell-shaped curve by an additional ionization event involving a group with an apparent p$K_a$ of ~7. The resulting pH dependence is composed of two active enzyme species, which are illustrated by broken lines in figure 3A. The two lower p$K_a$ values of ~5 and ~7 are apparent dissociation constants, and cannot be assigned to His680. This is supported by the observation that the relative activities of the two forms change with different substrates, leading to the alteration in p$K_a$ values. Similar effects on the p$K_a$ values are also seen when the kinetic parameters are compared in the absence and presence of 0.5 M NaCl [60]. The possible structural differences between the two pH-dependent forms have also been detected by intrinsic fluorescence measurements, which clearly indicated that the low-pH form is more unfolded [64].

The structure of the productlike inhibitor benzyloxycarbonyl-Gly-Pro-OH complexed with prolyl oligopeptidase has shown that the carboxyl group of the inhibitor forms a hydrogen bond with the NE2 of His680 [60]. Since the carboxyl group makes a stronger interaction with the protonated than with the neutral histidine, the real p$K_a$ of the catalytic histidine can be titrated (figure 3B). This value was 6.25 [60], lower than that of the well-studied serine proteases. In contrast to the complex pH dependence for $k_{cat}/K_m$, the titration method gave a well-defined single p$K_a$ value.

An additional difference of prolyl oligopeptidase from the classic serine peptidases concerns the rate-limiting step of the catalysis. Kinetic deuterium isotope effects clearly indicated in chymotrypsin reactions that the rate-limiting step involves general base/acid catalysis, because the reactions proceed 2–3 times slower in deuterium oxide than in water [cf. 57, 58]. In contrast, no sig-

nificant isotope effects were found in the reactions of prolyl oligopeptidase with different substrates [62, 65], which together with the lack of leaving group effects on the acylation rate constant $k_{cat}/K_m$ [66] indicated that a substrate-induced conformational change may be the rate-limiting step.

The peptide bond at the amino-terminal side of proline residues is a mixture of cis and trans isomers, the trans conformation being more abundant. Prolyl oligopeptidase hydrolyzes the trans isomer only. Therefore, at high prolyl oligopeptidase concentrations, when the enzyme reaction is faster than the cis-trans conversion, a biphasic kinetics may be obtained. The faster reaction depends on the enzyme concentration, whereas the second phase is independent of enzyme concentration, but depends on the rate of the cis-trans isomerization. Consequently, while measuring rate constants, care should be taken that the substrate isomerization be negligible with respect to the enzymatic reaction [14].

**Transition state stabilization**

The mechanism of action of serine peptidases involves an acyl enzyme intermediate. Both the formation and the decomposition of the acyl-enzyme proceed through the formation of a negatively charged tetrahedral intermediate that is stabilized by the oxyanion binding site providing two hydrogen bonds to the oxyanion [cf. 57, 58, 67]. In the chymotrypsin-type enzymes the hydrogen bonds are contributed by the main-chain NH groups of the catalytic Ser195 and the nearby Gly193. In the subtilisin-type enzymes the side-chain amide of an asparagine replaces the main-chain NH of Gly193. In prolyl oligopeptidase one of the hydrogen bonds is formed between the oxyanion and the main-chain NH group of Asn555, adjacent to the catalytic serine, Ser554. The second hydrogen bond is unique among serine peptidases as provided by the OH group of tyrosine Tyr473 (fig. 2) [6]. Experiments with the Y473F variant of prolyl oligopeptidase have shown that the Tyr473 OH is indeed markedly contributed to the transition state stabilization, but the effects are greatly dependent upon the nature of substrate and vary with pH as the enzyme interconverted between its two catalytically competent forms [15].

**Effect of the propeller domain on the catalytic action**

The Cys255 side chain, which is close to the S1 and S3 binding subsites (fig. 2), accounts for the inhibition of prolyl oligopeptidase with bulky thiol-specific reagents. Prolyl oligopeptidase isolated from *F. meningsepticum,* which is not inhibited with thiol-reacting agents, has a threonine residue in place of Cys255. In contrast to the wild-type porcine brain enzyme, the Cys255Thr variant was not inhibited with *N*-ethylmaleimide, indicating that Cys255, of the 16 free cysteine residues, exclusively accounts for the enzyme inhibition [63]. Unlike the wild-type enzyme that showed a double bell-shaped pH-rate profile, the modified enzyme displayed a single bell-shaped pH dependence with the elimination of the high-pH enzyme form. The high p$K_a$ (9.77) of Cys255 determined by titration with *N*-ethylmaleimide excluded the possibility that ionization of the thiol group was responsible for generation of the two active enzyme forms. The impaired activity of the enzyme variants could be rationalized in terms of weaker binding, which manifests itself in high $K_m$ for substrates and high $K_i$ for inhibitors. The results have indicated that in addition to the regulatory effect, the propeller domain, originally independent of the catalytic domain, also offers a residue that significantly modifies the catalytic action of the peptidase domain.

## Oligopeptidase B

Oligopeptidase B (EC 3.4.21.83) was first isolated from *E. coli* cells with trypsin-like specificity, cleaving peptides at lysine and arginine residues [68]. The enzyme is also present in protozoan parasites, such as *Trypanosoma cruzi*, the causative agent of Chagas disease in humans [69], and the African trypanosomes that produce the diseases nagana and sleeping sickness in cattle and humans, respectively. It has been shown that oligopeptidase B hydrolyzes peptides with dibasic sites much faster than monobasic substrates [70–72].

**Biological relevance**

The role of oligopeptidase B in *E. coli* has not yet been revealed. More information is available about the enzyme from trypanosomes. Commonly used trypanocidal drugs inhibit oligopeptidase B from the African trypanosome *Trypanosoma brucei* [73, 74]. Studies on the mechanism of the host cell invasion by *T. cruzi* have shown that the enzyme generates an active $Ca^{2+}$-agonist from a cytosolic precursor molecule [75–78]. Antibodies to the recombinant oligopeptidase B inhibited both peptidase activity and $Ca^{2+}$-signalling [76]. Oligopeptidase B activity correlates with blood parasitemia level. Since live trypanosomes do not release oligopeptidase B in vitro, it was proposed that disrupted cells of *T. brucei* releases it into circulation, where it retains full catalytic activity and may thus play a role in the pathogenesis [79].

**Structural aspects**

Oligopeptidase B has been isolated from several sources [80], including *E. coli* [68, 81], *T. cruz*i [82] and *T. brucei* [71, 83]. It has been demonstrated by osmotic shock that the *E. coli* enzyme is a cytosolic protein [68]. In contrast,

the enzyme was reviewed as a periplasmic peptidase [84], but no reference was given to the original article. The most convenient preparation utilizes the expression of the recombinant enzyme in *E. coli.* Such purifications were reported for the enzymes from *E. coli* [2, 85], *T. cruzi* [76], *T. brucei* [86] and *Moraxella lacunata*. The gene product of the latter species was also crystallized [87], but no further determination has been published until recently. The *T. cruzi* oligopeptidase B formerly was termed alkaline proteinase [70], and its molecular mass was estimated at 120 kDa when active enzyme was applied to the SDS polyacrylamide gel [75]. In contrast, its molecular mass was estimated at 80 kDa by SDS polyacrylamide gel electrophoreses (PAGE), when the enzyme was denatured at 100 °C prior to the gel electrophoresis [76]. Gel filtration profiles for the native and recombinant enzymes suggested that the protein is a dimer [76], in contrast to the monomeric enzyme found in *E. coli* [80]. However, the *E. coli* enzyme tends to aggregate at high concentration and at low temperature. Thus, it was not possible to perform NMR measurements below 10 °C [56]. The catalytic triad of *E. coli* enzyme is composed of Ser532, Asp617 and His 652 [2]. Oligopeptidase B from *E. coli* is about 25% identical to prolyl oligopeptidase of porcine brain [2]. The homology is higher in the peptidase domain, in particular around the catalytic groups, than in the propeller domain.

Based on the structure of prolyl oligopeptidase [6], a three-dimensional model was constructed for oligopeptidase B [88]. This revealed that the S2 binding site contains Asp460 and Asp462, which interact with the substrate arginine at the P2 position. The P1 arginine was placed into a nonionic environment. However, our preliminary results indicate that the P1 arginine very likely binds to Glu576 and Glu578, which constitute a similar pair of carboxylate ions as found on the S2 subsite [L. Polgár, unpublished result]. Indeed, Glu576 occupies the same steric position as Trp595 in prolyl oligopeptidase. This residue is known to interact with the P1 proline ring of the substrate.

**Kinetic properties**

Kinetic studies on oligopeptidase B have revealed several special and unusual properties of the enzyme, not found with the classic serine peptidases [70, 72, 89]. Thus, the reactions are rather sensitive to ionic strength, in particular with substrates containing arginine residues both at the P1 and P2 positions. In fact, the rate decreases by more than one order of magnitude upon addition of 1 M NaCl. The reaction is very sensitive in the region of low ionic strength, and reproducible rate constants can more conveniently be measured at high ionic strength. The $k_{cat}/K_m$ for benzyloxycarbonyl-Arg-Arg-Amc, one of the best substrates, is extremely high, 63 $\mu M^{-1}$ $s^{-1}$ at low ionic strength, which is not far from the diffusion limit [72]. The preference for adjacent arginine residues suggests that oligopeptidase B may be a new type of processing enzyme. The arginine residues probably bind through salt bridges to enzymatic carboxyl groups, and the electrostatic interaction is depressed by the high ionic strength.

As observed with prolyl oligopeptidase, the pH-rate profile for oligopeptidase B is also complicated by ionization of at least one enzymatic group not directly involved in catalysis. The deviation from simple curves is significant at low ionic strength. The effect of this group is practically abolished in the presence of 1 M NaCl.

The importance of the positive charge of arginine was studied by using a neutral, structurally similar citrulline derivative. The sharp pH-$k_{cat}/K_m$ profile and the very low rate constant for benzoyl-citrulline ethylester revealed an additional ionizing group, and indicated that the positive charge of arginine was essential for catalysis [89].

The second-order acylation rate constants, $k_{cat}/K_m$, are similar for the more reactive ester and the corresponding less-reactive amide substrates. This suggested that their chemical reactivity did not prevail in the rate-limiting step. Furthermore, kinetic deuterium isotope effects indicated that the rate-limiting step for $k_{cat}/K_m$ was primarily governed by conformational changes, as in the case of prolyl oligopeptidase.

Oligopeptidase B is inhibited by high concentration of substrate. The mechanism of inhibition markedly varies with the reaction conditions, like pH and ionic strength. Only under special conditions do the data fit the theoretical curve, and this implies that the inhibitory effect is mediated by a second molecule of the same substrate.

An interesting feature of the oligopeptidase B reactions is their unusually low temperature optimum with amide substrates, which are slightly below 25 °C, whereas with an ester substrate a linear Eyring plot is obtained up to 39 °C. This may be explained in terms of a change in the rate-determining step, with increasing temperature in the case of the amide substrates. The positive entropies of activation point to substantial reorganization of water molecules upon substrate binding [89].

Extensive kinetic studies were also conducted on prolyl oligopeptidase of the hyperthermophilic archaeon *Pyrococcus furiosus* at 85 °C [61]. The results have shown that the mesophilic and the hyperthermophilic enzymes share several characteristics. Thus the pH-rate profiles indicated three catalytically influential ionization groups. Temperature dependence of the p$K_a$ values revealed a heat of ionization of 4.7 kJ/mol for one p$K_a$ and 22 kJ/mol for another, suggesting the catalytic involvement of a carboxyl group and an imidazole group, respectively. Also, the rate constant significantly increased at high ionic concentration, owing to a decrease in $K_m$. The kinetic deuterium isotope effects on $k_{cat}$ was 2.2, indicating general

base/acid catalysis in the rate-limiting step related to $k_{cat}$. This result was contrasted with the lack of isotopic effect for the $k_{cat}/K_m$ for the porcine enzyme reaction. However, the two different rate constants may stand for different catalytic processes.

## Dipeptidyl-peptidase IV

Dipeptidyl-peptidase IV (EC 3.4.14.5) is different from both prolyl oligopeptidase and oligopeptidase B in that it is a dimer, an exopeptidase, a glycoprotein and an ectoenzyme bound to the cell membrane. It cleaves dipeptides with penultimate proline or to a lesser extent with alanine from the amino terminus of oligopeptides [13, 90]. Its cellular localization and enzymatic properties are also different from those of other dipeptidyl-peptidases. Specifically, dipeptidyl-peptidases I and II are localized in lysosomes, whereas dipeptidyl-peptidase III is found in the cytoplasm. The specificity of dipeptidyl-peptidase II is similar to that of dipeptidyl-peptidase IV, but neither dipeptidyl-peptidase I nor dipeptidyl-peptidase III can cleave at proline residue. Dipeptidyl-peptidase IV was first identified as glycylproline naphthylamidase [91] and also called dipeptidyl aminopeptidase or postproline dipeptidyl peptidase. The enzyme has been purified from a variety of tissues, like kidney [92–94], small intestine [95] and liver [90, 96, 97]. The intact membrane form can be prepared by solubilizing the enzyme with Triton X-100, whereas the soluble form is obtained by autolysis of microsomes incubated at low pH or by digestion of membranes with papain, which cleaves a peptide bond of the stalk linking the peptidase to the membrane.

### Biological relevance

Dipeptidyl-peptidase IV appears to have several different physiological functions. It is involved in peptide degradation and amino acid scavenging at the intestinal ad renal brush-border membranes. Using gliadin, a proline-rich protein, in feeding rats that were normal and genetically deficient in dipeptidyl-peptidase IV, the control groups maintained their weights, whereas the deficient group experienced a significant weight loss [98]. The amount of the enzyme in rat small intestine can be increased by feeding a high-proline-containing diet [99].

Dipeptidyl-peptidase IV also extinguishes signals by cleaving exterior peptide mediators, functions as receptor and participates in signal transduction or in adhesion. It has been suggested that the enzyme is involved in cell-extracellular matrix interactions, in particular with collagens [100]. The binding site of the enzyme against collagen may be the cysteine-rich domain distinct from the catalytic domain [101].

Dipeptidyl-peptidase IV was also demonstrated to exist on the surface of human lymphocytes as an ectoenzyme [102], which suggested its implication in immunological processes. Dipeptidyl-peptidase IV was then identified as CD26, a T-lymphocyte surface antigen. The enzyme was considered to participate in regulation of proliferation and differentiation of the lymphocytes, and in production of lymphokines [103–105]. The T-cell activation was shown to require dipeptidyl-peptidase activity [106–108]. However, experiments with an inactive variant of dipeptidyl-peptidase suggested that the peptidase activity is considerably, but not absolutely necessary for the enhancement of interleukin 2 production by Jurkat cells [109].

It has been reported that dipeptidyl-peptidase IV binds adenosine deaminase on T cells [110] and that this association is an important immunoregulatory mechanism which does not rely on the dipeptidyl-peptidase activity [111]. Point mutations in the propeller domain abolished the adenosine deaminase binding [112]. The interaction between the two proteins has been utilized for purification of dipeptidyl-peptidase by affinity chromatography [113].

Most important, dipeptidyl-peptidase IV cleaves the amino-terminal His-Ala or Tyr-Ala dipeptides from glucagons-like peptide-1 (GLP-1) and gastric inhibitory polypeptide (GIP), both stimulating insulin release. Administration of specific dipeptidyl-peptidase IV inhibitors reduces the degradation of the peptides and enhances insulin secretion. The use of such inhibitors as drugs appears to be very promising in treating type 2 diabetes [114, 115].

Dipeptidyl-peptidase IV has been intensively investigated. Medline shows up more than a thousand papers, most of them concerned with the biological and pathological aspects of the enzyme. Determination of its activity may be a diagnostic tool to differentiate between healthy and diseased states in various pathological cases [13].

### Structural aspects

The amino acid sequences of dipeptidyl-peptidase IV have been determined from different species by complementary DNA (cDNA) cloning and sequencing. The human [116, 117], murine [118] and rat [119] enzymes have 766, 760 and 767 amino acid residues. The bacterial enzymes display shorter peptide chains: *Flavobacterium meningosepticum* 711 [120], and *Porphyromonas gingivalis* 723, including a 16-residue signal peptide [121]. From the *Pseudomonas* sp. WO24 two forms of dipeptidyl-peptidase were isolated, an 84-kDa and an 82-kDa species, which exhibited similar specific activities [122]. The *P. gingivalis* enzyme displayed a broader specificity, as it was capable of splitting not only the Gly-Pro deriva-

tive but also Ala-Pro, Lys-Pro and Phe-Pro-derivatives. The rat enzyme has a short cytoplasmic tail (6 residues), a transmembrane section (23 residues) and a long extracellular peptide chain (738 residues) [119]. The extracellular part contains several glycosylation sites, mostly in the amino-terminal half. This glycosylation accounts for the difference in the molecular mass between the predicted 88-kDa and the native 109-kDa forms. The essential residues of the catalytic triad of the mouse enzyme are Ser624, Asp702 and His734 [123], which is consistent with the order of the triad residues and their topology of the enzymes of SC clan.

The gene structure of human dipeptidyl-peptidase has been elucidated [124]. It spans about 70 kbp and contains 26 exons. The promoter region has no TATA box, which is characteristic of housekeeping genes. The widely varying transcriptional activity of the gene may explain why the enzyme is expressed ubiquitously, but at different levels in the different tissues [124–126].

**Kinetic properties**

The activity of dipeptidyl-peptidase IV is conveniently measured with substrates that contain a chromophore or fluorophore attached to the carboxyl group of the dipeptide Gly-Pro, such as Gly-Pro-4-nitroanilide or Gly-Pro-2-naphthylamide. Besides the preferred proline residue at the P1 subsite, the enzyme hydrolyzes substrates with alanine, hydroxyproline, oxazolidine, thiazolidine, azetidine and similar derivatives [127, 128]. The proline-containing substrate requires that the peptide bond between the P1 and P2 residues be in the trans conformation [129]. The N-terminal amino group of the substrate must not be acylated, but the peptide with methylated amino group is hydrolyzed by dipeptidyl peptidase IV [127].

The specificity of dipeptidyl-peptidase IV was studied with bovine growth-hormone-releasing factor (GRF) to develop analogues of this peptide, which are not hydrolyzed by the enzyme. A stable derivative of GRF(1–29)$NH_2$ was obtained with a derivative containing D-Ala at the P2 position [130]. The N-terminal Tyr-Ala dipeptide is effectively cleaved from GRF, but substitution of His-Val for Tyr-Ala protects GRF from hydrolysis by dipeptidyl peptidase IV without destroying the peptide potency [131]. Studies with GRF of different peptide lengths have shown that the specificity extends beyond the P1′ residue. Indeed, requirement for L residue in position P4′ indicates that the enzyme binding site extends to the S4′ subsite [132].

The action of inhibitors against dipeptidyl peptidase IV has also been studied. Interestingly, simple tripeptides – diprotin A (Ile-Pro-Ile) and diprotin B (Val-Pro-Leu) – proved to be specific inhibitors of the enzyme [133, 134]. Aminoacylpyrrolidine-2-nitriles formed a covalent imidate adduct with the catalytic serine [135]. Dipeptide diphenyl phosphonate esters were slow irreversible inhibitors [136, 137], whereas the most effective inhibitors contained an amino boronic acid analogue of proline. Thus, Pro-boroPro had a $K_i$ of 16 pM [138].

**Acylaminoacyl peptidase**

Acylaminoacyl peptidase (EC 3.4.19.1) has also been referred to by the names acyl-amino-acid-releasing enzyme, acylpeptide hydrolase and acylaminoacyl peptide hydrolase. It catalyses the removal of an N-acylated amino acid from blocked peptides [139]. The enzyme cleaves a variety of peptides with different N-terminal acyl groups, such as acetyl, chloroacetyl, formyl and carbamyl [140]. The blocked peptide containing two to three amino acids is hydrolyzed faster than the longer peptides [141]. N-terminally blocked proteins, however, are not substrates for the enzyme [142].

**Structural aspects**

In contrast to prolyl oligopeptidase and oligopeptidase B, which are monomers, and dipeptidyl peptidase IV, which is a dimer, acylaminoacyl peptidase is composed of four identical subunits. The enzyme was purified from several sources, including human erythrocytes [139], ovine liver [142], bovine liver [143], rabbit muscle [144], bovine lens [145] and porcine intestinal mucosa [146]. The complete amino acid sequences of human, porcine and rat acylaminoacyl peptidase have been reported [147–149]; they are all 732 amino acids long, and their sequences are over 90% identical to each other. The members of the catalytic triad were identified by sequence homology studies [1, 4] and chemical modifications by inactivating the enzyme by diisopropyl fluorophosphates at Ser587 and by chloromethyl compounds at His707 [150]. The third member of the triad is Asp675, which was confirmed by site-specific mutagenesis [151]. Acylaminoacyl peptidase has been crystallized [152], but the three-dimensional structure of the protein has not yet been reported. Besides eukaryotic cells, acylaminoacyl peptidase has also been purified and cloned from the thermophilic archaeon *Pyrococcus horikoshii* [153]. This enzyme is shorter by 100 residues than the mesophilic acylaminoacyl peptidases, and it appears to be a dimer rather than a tetrameric enzyme. Interestingly, the specific activity of the enzyme increased by one order of magnitude during incubation at 95 °C for a few hours, suggesting a modification in the protein structure [153].

**Kinetic properties**

The activity of acylaminoacyl peptidase can readily be measured with acetylalanine *p*-nitroanilide as substrate

[139]. The enzyme exhibits different pH optima depending on the substrate used [140]. For instance, with acetylglutamate *p*-nitroanilide, the pH optimum is about pH 6, whereas for acetylalanine *p*-nitroanilide it is about 8.4. The $K_m$ values for the acetylated L-alanine peptides vary very little from the dipeptide to the tetrapeptide ($K_m$ values are close to 0.7 mM), whereas $k_{cat}$ decreases with the length of the peptide [146]. Interestingly, Ac-Ala-Ala-OH and Ac-Ala-Ala-Ala-OH are somewhat better substrates than Ac-Ala-*p*-nitroanilide, although the *p*-nitroanilide is a better leaving group than an amino acid. Ac-Ala-OH is a productlike inhibitor ($K_i$ = 0.4 mM), having higher inhibitory activity than Ac-D-Ala-OH ($K_i$ = 17 mM) [146].

## Biological relevance

Human acylaminoacyl peptidase is encoded by the DNF15S2 locus on the short arm of chromosome 3 at region 21 [141], which suffers deletions in small cell lung carcinomas and renal carcinomas, resulting in deficiency in the expression of the enzyme [154–156]. However, the potential role of acylaminoacyl peptidase in the malignant state of these cell lines has not yet been established.

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