Substrate- and pH-dependent contribution of oxyanion binding site to the catalysis of prolyl oligopeptidase, a paradigm of the serine oligopeptidase family

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Abstract

Prolyl oligopeptidase, an enzyme implicated in memory disorders, is a member of a new serine peptidase family. Crystallographic studies (Fülöp et al., 1998) revealed a novel oxyanion binding site containing a tyrosine residue, Tyr473. To study the importance of Tyr473 OH, we have produced prolyl oligopeptidase and its Tyr473Phe variant in *Escherichia coli*. The specificity rate constant, k_{cat}/K_m , for the modified enzyme decreased by a factor of 8–40 with highly specific substrates, Z-Gly-Pro-Nap, and a fluorogenic octapeptide. With these compounds, the decline in k_{cat} was partly compensated for by reduction in K_m , a difference from the extensively studied subtilisin. With the less specific suc-Gly-Pro-Nap, the K_m value, which approximates K_s , was not significantly changed, resulting in greater diminution (~500-fold) in k_{cat}/K_m . The second-order rate constant for the reaction with Z-Pro-prolinal, a slow tight-binding transition-state analogue inhibitor, and the K_i values for a slow substrate and two product-like inhibitors were not significantly affected by the Tyr473 OH group. The mechanism of transition-state stabilization was markedly dependent upon the nature of substrate and varied with pH as the enzyme interconverted between its two catalytically competent forms.

Keywords: amnesia; inhibitor design; oxyanion binding site; site-directed mutagenesis; steady-state kinetic studies; transition state stabilization

Prolyl oligopeptidase (EC 3.4.21.26), previously called prolyl endopeptidase or post-proline cleaving enzyme, is a large intracellular enzyme (molecular mass 80 kD) that preferentially hydrolyzes proline-containing peptides at the carboxy end of proline residues (Wilk, 1983; Polgár, 1994; Cunningham & O'Connor, 1997). It is presumably involved in the maturation and degradation of peptide hormones and neuropeptides (Wilk, 1983; Cunningham & O'Connor, 1997). Prolyl oligopeptidase has recently gained pharmaceutical interest, since specific inhibitors reverse scopolamine-induced amnesia in rats (Yoshimoto et al., 1987; Attack et al., 1991; Portevin et al., 1996). Its activity in plasma correlates with different stages of depression (Maes et al., 1994). A recent study has shown that loss of prolyl oligopeptidase activity counteracts the reduction in inositol(1,4,5)trisphosphate con-

centration caused by Li^+ treatment in depression (Williams et al., 1999). The enzyme 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 (Welches et al., 1993).

Prolyl oligopeptidase is unrelated to the well-known trypsin and subtilisin families and belongs to a new class of serine peptidases (clan SC, family S9), which also includes dipeptidyl peptidase IV, acylaminoacyl peptidase, and oligopeptidase B (Rawlings et al., 1991; Rawlings & Barrett, 1994). These enzymes display distinct specificities and each contains a peptidase domain at the C-terminal region of the single polypeptide chain. In the case of prolyl oligopeptidase, the active site serine and histidine have been identified as Ser554 and His680, respectively (Rennex et al., 1991; Stone et al., 1991). A structural relationship between lipases and the prolyl oligopeptidase family has been supported by similar topology of the catalytic groups and by the similar amino acid sequences around these residues (Polgár, 1992a). The 1.4 Å resolution crystal structures of the enzyme and its complex with Z-Pro-prolinal (Protein Data Bank entries: 1qfm, 1qfs) have most recently been determined (Fülöp et al., 1998). The results have shown that the enzyme contains a peptidase domain with an α/β hydrolase fold, and its catalytic triad (Ser554, Asp641, His680) is covered by

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Abbreviations: PCR, polymerase chain reaction; DTE, dithioerythritol; BSA, bovine serum albumin; EDTA, ethylenediaminetetraacetic acid; Mes, 2-(morpholino)ethanesulfonic acid; Z, benzyloxycarbonyl; suc, succinyl; Nap, 2-naphthylamide; Nan, 4-nitroanilide; Mec, 7-(4-methyl)-coumarinylamide; Pro-H, prolinal.

the central tunnel of an unusual β -propeller, which operates as a gating filter for the active site by excluding large structured peptides.

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 (Polgár, 1987, 1989). In the chymotrypsin-type enzymes, the hydrogen bonds are contributed by the main-chain NH groups of the catalytic Ser195 and another residue, Gly193. In the subtilisin-type enzymes, the side-chain amide of an asparagine replaces the main-chain NH of Gly193. In prolyl oligopeptidase one hydrogen bond is formed between the oxyanion and the main-chain NH group of Asn555, adjacent to the catalytic serine, Ser554. The second hydrogen bond stabilizing the intermediate is unique among serine peptidases as it comes from the OH group of a tyrosine residue, Tyr473 in prolyl oligopeptidase (Fülöp et al., 1998). The tyrosine OH is much more acidic than the peptide NH or amide NH₂ group, and thus it may be a more efficient catalyst in the stabilization of the tetrahedral intermediate.

Here, we describe the construction of prolyl oligopeptidase with modified oxyanion binding site, through substitution of Tyr473 by phenylalanine. Kinetic analysis has indicated that the tyrosine OH group facilitates the catalysis in the transition-state, but this is considerably dependent on the substrate and reaction conditions. This contribution is different from that observed with the thoroughly investigated serine peptidase, subtilisin.

Results and discussion

Recombinant prolyl oligopeptidase

In our earlier studies, the enzyme was prepared from porcine muscle (Polgár, 1994). To study the catalytic importance of oxyanion binding site, site specific mutagenesis was the method of choice. This required the cloning and expression of prolyl oligopeptidase. The gene of the enzyme from porcine brain was a gift of Dr. Hofsteenge (Rennex et al., 1991), and we succeeded in expressing the gene product in *Escherichia coli* (Table 1). The amount of the purified enzyme obtained from the culture medium (~30 mg/1,000 mL, 50% yield) was significantly higher than that produced in a different expression system (Sommer, 1993). It was more convenient to isolate the enzyme from the bacterial cells than from porcine muscle. Thus, the critical precipitation with acetone could be eliminated, and the preparation was accomplished in shorter time. Thanks to the more favorable conditions of preparation, the

recombinant enzyme displayed an activity of approximately two-fold. It cannot be excluded that the enhancement of activity is due to the difference between the muscle and brain enzymes. However, the amino acid sequence deduced from the structure of the muscle enzyme determined at 1.4 Å resolution proved to be identical with that of the porcine brain enzyme (Fülöp et al., 1998).

Hydrolytic activity of prolyl oligopeptidase Tyr473Phe

The importance of oxyanion binding site in the catalysis of serine peptidases has long been known (Henderson, 1970; Kraut, 1977). For estimating the magnitude of contribution, the Asn155 of subtilisin BPN' was replaced with the isosteric Leu (Bryan et al., 1986) or Gly (Carter & Wells, 1990). This lowered the turnover number (k_{cat}) for a specific substrate by 150–300-fold with virtually no change in the Michaelis constant (K_m). It was concluded from these data that the oxyanion binding site only contributed to the transition-state and left the binding unaffected (Bryan et al., 1986; Carter & Wells, 1990). In contrast, for the reaction of the prolyl oligopeptidase Tyr473Phe variant with Z-Gly-Pro-Nap, both k_{cat} and K_m decreased significantly, independent of the ionic strength of the medium (Table 2). Table 2 also shows that with the classic substrate of prolyl oligopeptidase, Z-Gly-Pro-Nap, k_{cat} and K_m for the Tyr473Phe variant decreased about 110–150-fold and 6–16-fold, respectively. As a consequence, the specificity rate constant (k_{cat}/K_m) reduced to a much lesser extent (9–20-fold) than in the case of subtilisin.

We believe that in the Tyr473Phe variant a water molecule occupies the position of the OH of Tyr473, thereby partly compensating for loss of catalytic efficacy. Slight movement of the Phe ring being possible, a molecule of the bulk water very likely binds to the oxyanion, but because of the unfavorable entropy it cannot be as good a catalyst as the Tyr473 OH, which is fixed with the enzyme structure. This is essentially the same as the case of the subtilisin variant having a glycine in place of the asparagine residue. It is interesting to note that in glutathione transferase the sulfur of glutathione forms a hydrogen bond with Tyr6. In the Tyr6Phe variant, a water molecule replaces the missing OH group, thereby filling the void (Xiao et al., 1996). In the prolyl oligopeptidase catalysis, the oxyanion of the tetrahedral intermediate is more basic than a thiol group, so that its hydration is even more probable.

The tyrosine OH group implicated in transition state stabilization is much more acidic ($pK_a = 10.1$) than the corresponding amide group of asparagine residue ($pK_a \approx 15$) or the main-chain NH groups implicated in the reactions of the peptidases of the subtilisin and trypsin families. Hence, protonation of the oxyanion

Table 1. Purification of prolyl oligopeptidase^a

Purification step	Volume (mL)	Total protein (mg)	Activity yield (%)	Rate constant ($\mu\text{M}^{-1} \text{s}^{-1}$)
To DEAE column	380	9,060	100	0.37
From DEAE column	280	720	105	3.99
From Blue-Sepharose	60	331	83	8.00
From Q-Sepharose ^b	8 (176)	11.5 (253)	50	8.26

^aFrom 8,000 mL culture medium.

^bOne run, total in bracket.

Table 2. Kinetic parameters of the reactions of prolyl oligopeptidase^a

	Wild-type		Y473F	
	—	0.5 M NaCl	—	0.5 M NaCl
Z-GP-Nap, pH 8.0				
k_{cat} (s ⁻¹)	71.5 ± 8.5	95.6 ± 5.7	0.48 ± 0.03	0.89 ± 0.07
K_m (μM)	28.9 ± 6.4	14.7 ± 1.5	1.59 ± 0.18	2.67 ± 0.35
k_{cat}/K_m (μM ⁻¹ s ⁻¹)	2.47	6.50	0.301	0.333
Z-GP-Nap, pH 6.0				
k_{cat} (s ⁻¹)	5.86 ± 0.14		0.192 ± 0.006	
K_m (μM)	4.92 ± 0.37		3.11 ± 0.3	
k_{cat}/K_m (μM ⁻¹ s ⁻¹)	1.14		0.0617	
Octapeptide, pH 8.0				
k_{cat} (s ⁻¹)	5.38 ± 0.24	5.55 ± 0.20	0.049 ± 0.003	0.032 ± 0.002
K_m (μM)	2.05 ± 0.34	1.50 ± 0.13	0.77 ± 0.09	0.34 ± 0.06
k_{cat}/K_m (μM ⁻¹ s ⁻¹)	2.62	3.70	0.0636	0.094
suc-GP-Nan				
pH	5.58	7.00	5.50	7.34
k_{cat} (s ⁻¹)	20.8 ± 0.53	21.9 ± 0.841	0.067 ± 0.003	0.064 ± 0.003
K_m (μM)	351 ± 30	407 ± 48	545 ± 37	586 ± 42
k_{cat}/K_m (M ⁻¹ s ⁻¹)	59,300	53,800	123	109

^aThe reactions were measured in the absence and presence of 0.5 M NaCl at 25 °C. With substrate Z-GP-Nap the reaction mixture contained 0.3% acetonitrile, which caused about 20% inhibition of the enzyme activity. Substrate concentrations generally were in the range of 0.2–5 K_m . The enzyme concentrations were 0.54–1.66 and 6.9–38.2 nM for the wild-type and the Y473F variant, respectively. At the low nanomolar level of enzyme concentration, 1 μM bovine serum albumin was added to the reaction mixture. The enzyme concentrations with the octapeptide reaction were similar as in the case of the Z-GP-Nap but considerably higher with suc-GP-Nan, namely, 20–40 and 320 nM for the wild-type and the Tyr473Phe variant, respectively.

could be more effective with the enzymes of the oligopeptidase family, all of them possessing a tyrosine at the oxyanion binding site. In contrast, the specificity rate constants, k_{cat}/K_m , are less affected by elimination of the Tyr473 OH than the subtilisin reaction carried out with the modified oxyanion binding site. The potential advantage of proton transfer from the acidic tyrosine to the basic tetrahedral transition state is not supported by these data.

Kinetic investigations has previously demonstrated that prolyl oligopeptidase exhibits two active forms, one being predominant at pH 6, the other at pH 8 (Polgár, 1991). Structural differences between the two forms have also been detected by intrinsic fluorescence measurements, which clearly indicated that the low-pH form was more unfolded (Polgár, 1995). The effects of oxyanion binding site provide further distinction between the two forms. Notably, at pH 6 the K_m values for the wild-type enzyme and its variant are not too much different, whereas one order of magnitude difference is found between them at pH 8 (Table 2). The difference between the K_m values of the two pH dependent enzyme forms very likely refers to stronger binding associated with the low-pH form. This is supported by the similar change with pH in the true binding constant for Z-Gly-Pro-OH as discussed in a later section. It may be noted that at the physiological pH 7.2 the data are intermediate values of those found at pH 6.0 and pH 8.0.

Since the scissile bond of the naphthylamide derivative is not a genuine peptide bond, we have designed an internally quenched fluorogenic octapeptide (Abz-Gly-Phe-Gly-Pro-Phe-Gly-Phe(NO₂)-Ala-NH₂) that was cleaved by the enzyme at the Pro-Phe bond. For this substrate k_{cat}/K_m was similar to that found with Z-Gly-Pro-Nap, but both k_{cat} and K_m displayed lower values by an order of magnitude (Table 2). The lower k_{cat} can arise from the greater

strength of the peptide bond relative to that of the naphthylamide substrate, while the lower K_m may be due to a stronger binding with the larger octapeptide.

A further important substrate of prolyl oligopeptidase is suc-Gly-Pro-Nan, which is used widely for activity measurements, thanks to its high solubility compared to that of the neutral Z-Gly-Pro-Nan or Z-Gly-Pro-Nap. However, the negatively charged compound is a relatively poor substrate, which can be rationalized in terms of inept binding at subsite S3 that is rather hydrophobic in nature (Fülöp et al., 1998). Apparently, the succinyl group keeps away from the hydrophobic environment and assumes a different, less productive binding mode. It was therefore important to examine whether or not the oxyanion binding site exerts similar effects on the hydrolysis of the charged and neutral substrates.

The rate constants were measured near the maximum activities (Table 2), being at lower pH than that observed with the neutral substrates (L. Polgár, unpubl. result). As compared with the neutral substrates, the K_m values were much higher (350–400 μM) for the suc-Gly-Pro-Nan reactions, while the k_{cat} values remained in the same order of magnitude. The decrease in the specificity rate constant can be very likely ascribed to weaker binding, in agreement with the above discussion about the alternative S3 binding site. That K_m indeed approaches K_s is discussed in the next paragraph. The much higher K_m implies that in an altered binding mode of the substrate the hydroxyl group of Tyr473 facilitates the chemical reaction without interfering with binding. It is noteworthy that the reduction in the specificity rate constant for the Tyr473Phe variant is considerably greater in the unfavorable binding mode of the succinyl derivative (~500-fold) than in the proper position occupied by the neutral substrates (8–40-fold).

Table 3. Inhibition constants for prolyl oligopeptidase^a

	Wild-type		Y473F	
	—	0.5 M NaCl	—	0.5 M NaCl
suc-Gly-Pro-Nan	278 ± 35 ^b	537 ± 47 ^c	360 ± 14 ^b	>600 ^c
Z-Pro-OH		1,280 ± 50		1,570 ± 70
Z-Gly-Pro-OH	253 ± 18	201 ± 8	595 ± 20	513 ± 22
Z-Gly-Pro-OH	21.2 ± 0.5 ^d		16.2 ± 0.8 ^d	

^a K_i values for Z-Pro-OH and Z-Gly-Pro-OH were measured at pH 8.0 with 0.2 μ M Z-Gly-Pro-Nap at 0.3–4 K_i concentrations and are given in μ M. Because of quenching problems with suc-Gly-Pro-Nan, no data were collected above K_i .

^b pH = 5.60, pH optimum.

^c pH = 7.35, pH optimum.

^d pH = 6.35.

To compare the importance of the hydrogen bonding donor on the transition state stabilization of the reactions of various substrates, k_{cat}/K_m is used. This second-order rate constant involves binding and is characteristic of the specificity of competing substrates (Fersht, 1985; Polgár, 1999). An appropriate comparison between these macroscopic rate constants can be made, even if the rate-determining step of the reaction is a physical step. Indeed, a conformational change rather than the chemical step is rate-limiting with both slow and fast substrates, indicating that the conformational change is induced by the substrate (Polgár, 1992b; Polgár et al., 1993). The k_{cat}/K_m values are not controlled by diffusion with either substrate as the highest rate constant is lower by 1–2 orders of magnitude than the limit set by the diffusion.

Relationship between K_m and K_s

Utilizing the inhibitory effect of poor substrates on the hydrolysis of highly specific substrates (Asbóth & Polgár, 1983), we have determined the K_s value of suc-Gly-Pro-Nan. This substrate proved to be an effective inhibitor of Z-Gly-Pro-Nap, and the $K_i = K_s$ values calculated from Equation 1 are shown in Table 3. It is seen that the K_i data agree well with the K_m values obtained from steady-state kinetics (Table 2). This indicates that the K_m actually represents the binding constant for suc-Gly-Pro-Nan.

The minimal mechanism for serine proteases is

where ES' stands for the acyl-enzyme, P_1 is the leaving group (the amine moiety) of the substrate, P_2 is the acid part, and k_2 and k_3 represent the first-order acylation and deacylation rate constants, respectively. Hence, $k_{cat} = k_2 k_3 / (k_2 + k_3)$, and $K_m = K_s k_3 / (k_2 + k_3)$. We have demonstrated by the above inhibition studies that with suc-Gly-Pro-Nan $K_m = K_s$, which requires $k_3 \gg k_2$ and $k_{cat} = k_2$. On the other hand, with Z-Gly-Pro-Nap interpretation of K_m is not straightforward. The introduction of the OH group into the phenylalanine of the Tyr473Phe variant significantly raises the K_m value. Should this mean a reduction in the binding energy for the substrate, the wild-type enzyme appears to use this increment to decrease the transition state energy. Interestingly, this phenomenon is confined to the high-pH form of the enzyme. Such a role for the oxyanion binding site of serine peptidases has not yet been observed.

Interaction of Tyr473 OH with Z-Pro-prolinal

The importance of oxyanion binding site can also be investigated by transition-state analogues. Z-Pro-prolinal is such a compound forming a hemiacetal with the catalytic serine residue of prolyl oligopeptidase, as demonstrated by NMR (Kahyaoglu et al., 1997) and crystallographic studies (Fülöp et al., 1998). It has also been shown that Z-Pro-prolinal is a slow tight-binding inhibitor of prolyl oligopeptidase with $K_i = 0.35$ nM (Bakker et al., 1990). The inhibition follows a mechanism that involves a simple equilibrium between the free and bound enzymes ($E + I \leftrightarrow EI$) with rate constant of association of $160 \text{ mM}^{-1} \text{ s}^{-1}$ (Bakker et al., 1990). We have determined the progress curves with Z-Gly-Pro-Mec under slightly modified conditions (Fig. 1). (1) We used 0.1 mM DTE in the reaction mixture, instead of 1 mM. Notably, DTE reacted with the aldehyde inhibitor and decreased its concentration during the extended incubation as observed in this work and earlier (Kahyaoglu et al., 1997). No interference was found at 0.1 mM DTE. (2) We also used 0.01 μ M BSA to stabilize the enzyme at the low concentrations employed. No reaction of BSA with the aldehyde was observed either. (3) The substrate concentration was reduced by one order of magnitude in order that the condition of $S \ll K_m$ be satisfied. This condition was, in particular, important

Fig. 1. Progress curves for inhibition of prolyl oligopeptidase Tyr473Phe variant with Z-Pro-prolinal. Inhibitor concentrations: +, 15.5 nM; O, 31 nM; Δ, 38.7 nM.

Fig. 2. Inhibition rate constant from Equation 1 as a function of Z-Pro-prolinal concentration. O, Wild-type enzyme; +, Tyr473Phe variant.

for the Tyr473Phe variant that had a K_m much lower ($0.65 \mu\text{M}$) than the wild-type enzyme ($7 \mu\text{M}$). The latter value was lower compared with $16 \mu\text{M}$ found in the previous study (Bakker et al., 1990). It should be noted that it was experimentally impossible to use a substrate concentration lower than $0.2 K_m$ for the reaction with the Tyr473Phe variant. Under the conditions employed, only 92% of the enzyme was present in free state.

The second-order rate constant for inhibition was obtained from the slope of the plot of k of Equation 2 against the Z-Pro-prolinal concentration (Fig. 2). With the wild-type enzyme, the association rate constant proved to be higher ($470 \text{ mM}^{-1} \text{ s}^{-1}$) compared with the earlier data ($160 \text{ mM}^{-1} \text{ s}^{-1}$) (Bakker et al., 1990). For the Tyr473Phe variant, the rate constant was somewhat lower ($248 \text{ mM}^{-1} \text{ s}^{-1}$). Considering that only 92% free enzyme reacted under the conditions employed (see the preceding paragraph), the corrected rate constant is $270 \text{ mM}^{-1} \text{ s}^{-1}$, not far from $470 \text{ mM}^{-1} \text{ s}^{-1}$ obtained with the wild-type enzyme. The reduction caused by the removal of the OH group is negligible in the second-order rate constant for inhibition with respect to that found in the k_{cat}/K_m for the reaction with substrates. This is consistent with the fact that the hemiacetal is a neutral species that cannot accept a hydrogen bond as strong as a charged tetrahedral intermediate can. Accordingly, these data support the view that hemiacetals do not serve as the best model of the catalytic transition-state (Polgár, 1989).

Interaction of Tyr473 OH with product-like inhibitors

Substrate binding separated from the chemical reaction can be investigated by using simple competitive inhibitors. As shown in Table 3, the K_i values for the binding of Z-Pro-OH are similar for the wild-type enzyme and its Tyr473Phe variant. This indicates that the tyrosine OH group does not significantly contribute to the binding of this inhibitor, although the charge on the inhibitor would promote such an interaction. In this respect, the behavior of Z-Pro-OH resembles that of suc-Gly-Pro-Nan. The inhibition follows Equation 1 in the presence of 0.5 M NaCl at pH 8.0 (Fig. 3). It is competitive in nature since an increase in substrate concentration decreases the degree of inhibition at constant inhibitor concentration. Were the inhibition noncompetitive, an increase in

Fig. 3. Inhibition of prolyl oligopeptidase with Z-Pro-OH. The reactions were measured at pH 8.0 in the presence of 0.5 M NaCl .

substrate concentration would cause both v_0 (rate in the absence of inhibitor) and v_i (rate in the presence of inhibitor) to increase by the same factor. In the absence of salt, the data do not properly fit to the theoretical curve derived from Equation 1 and do not give a linear fit when plotting according to Dixon ($1/v_i$ vs. I). Nonlinear (hyperbolic) fit can be obtained with the Dixon plot in the case of partially competitive inhibition. However, in our case the points conformed to a concave rather than a convex curve (not shown). At pH 6.0, even in the presence of 0.5 M NaCl , the direction of deviation from the straight line was similar, but more impressive, which may arise from multiple binding.

With the more specific inhibitor, Z-Gly-Pro-OH, the mechanism follows Equation 1, independent of the reaction conditions. Apparently, the binding mode is precisely defined by a more extensive interaction between the enzyme and the larger inhibitor. It is seen from Table 3 that the inhibitor binds much stronger at pH 6 than at pH 8, both in the wild-type enzyme and its Tyr473 variant. The change in the binding constant is one order of magnitude, similar to that found with the K_m for Z-Gly-Pro-Nap. This raises the possibility that the change in K_m with pH is primarily due to the stronger binding and not to the alteration in the k_3/k_2 . Of course, the argument for variation of K_m with pH cannot be extended to the change in the K_m values for the wild-type and the variant enzymes. On the contrary, the similarity of K_i (Table 3) and the dissimilarity of K_m (Table 2) values for the two enzymes suggests the possibility that $K_m \neq K_s$.

Conclusion

It is an important issue whether the Tyr473 OH interacts with the substrate carbonyl oxygen in the Michaelis complex or this is achieved in the transition state only. As for suc-Gly-Pro-Nan, the OH group of Tyr473 does not appreciably influence the Michaelis complex formation. The K_m value, which in this case corresponds to substrate binding, does not significantly change if the oxyanion binding site has been damaged. This is common with the classic serine peptidase, subtilisin, and suggests that the hydrogen bond with the oxyanion of the substrate develops simultaneously with the formation of the transition state. With the more specific Z-Gly-Pro-Nap and the octapeptide, the K_m decreases when reacting with

the truncated oxyanion binding site. This may be due to the utilization of a part of the binding energy in the stabilization of the transition state. It follows from the present study that the oxyanion binding site exerts different effects on different substrates, and on the same substrate under different reaction conditions. Even if the effect is variable, the oxyanion binding site significantly contributes to the transition state stabilization and suggests that transition state analogue inhibitors can be more effective drugs when bearing a negative oxyanion.

Materials and methods

Cloning and expression of the prolyl oligopeptidase gene

The 2.5 kb insert of porcine brain cDNA encoding from -199 to 2,300 nucleotides of prolyl oligopeptidase was kindly supplied by Dr. J. Hofsteenge (Rennex et al., 1991). The gene cloned into the *EcoRI* site of a pBluescript II KS(+) plasmid (Stratagene, La Jolla, California) was amplified and modified by PCR, using the following primers.

5' primer: 5'-CG GAATTC TAAGGAGG AATTTAAA ATG CTG
 EcoRI SD *DraI* M L

TCC TTC CAG TAC CCC G-3'
 S F Q Y P

3'-primer: 5'-CGG GGTACC G TTA CGG AAT CCA GTC
 KpnI Stop P I W D

GAT GTT CAG-3'
 I N L

The *EcoRI* and *KpnI* restriction sites were used to ligate the gene into the pTrc 99A vector (Pharmacia, Uppsala, Sweden). A Shine-Dalgarno (SD) sequence was also included in the 5'-primer. The PCR amplification was carried out with 1 µg DNA template, 1.5 units of Vent polymerase (New England Biolabs, Beverly, Massachusetts), 400 nM of each primers, 0.3 mM dNTP and 1 µg BSA in 100 µL 20 mM Tris·HCl, pH 8.8, containing 2 mM MgSO₄, 10 mM KCl, 10 mM (NH₄)₂SO₄, and 0.1% Triton X-100. The reaction was initiated with hot start by adding the polymerase at 95 °C and continued in 30 cycles at 95 °C for 1 min denaturation, at 57 °C for 1 min annealing, and at 72 °C for 3 min extension. After gel electrophoresis the PCR product was isolated with a QIAquick Gel Extraction Kit (Qiagen, Hilden, Germany). Then it was digested with *EcoRI* and *KpnI* restriction enzymes, and ligated overnight at 16 °C into the *EcoRI/KpnI* sites of pTrc 99A vector, producing the pTrc/PPO plasmid. The reaction mixture was transformed into *E. coli* DH5α, and the clones displaying prolyl oligopeptidase activity were used to produce sufficient amount of plasmids. For enzyme production *E. coli* JM105 was transformed with the pTrc/PPO plasmid.

Preparation of porcine prolyl oligopeptidase

Escherichia coli JM105 cells harboring the pTrc/PPO expression vector was spread on LB-agar containing 100 µg/mL ampicillin and incubated overnight at 37 °C. A single colony was then transferred into each of two 25 mL LB-medium (100 µg/mL ampicillin) and grown at 37 °C (OD₅₅₀ ~ 0.2; ~3 h). The culture medium

was distributed by 5 mL aliquots into 10 flask containing 800 mL LB-medium containing 100 µg/mL ampicillin and incubated at 37 °C (OD₅₅₀ ~ 0.6; ~8 h; 250 rpm). Then 0.5 mM isopropylthiogalactoside (IPTG) was added and the incubation was continued at 32 °C for 16 h. The cells were collected with centrifugation (15 min, 4,000 g, 4 °C), and the pellet (43.5 g wet weight) was suspended in 100 mL 10 mM phosphate buffer, pH 6.5, containing 10 mM EDTA and 5 mM DTE. The cell suspension was sonicated in four portions with an MSE sonicator over a salted ice bath and centrifuged at 20,000 g.

The slightly opalescent solution was applied to a Whatman 32 DEAE column (5 × 25 cm) equilibrated with 20 mM phosphate buffer, pH 6.6, containing 1 mM EDTA and 5 mM DTE and washed until the effluent was no more opalescent (~800 mL). Fractions of 18 mL were collected at a flow rate of 90 mL/h. Elution of the enzyme was effected with a linear gradient using 800 mL equilibration buffer and 800 mL equilibration buffer containing 0.25 M NaCl. The early fractions having prolyl oligopeptidase activity were combined and concentrated by ultrafiltration on a PM-30 membrane (Amicon, Beverly, Massachusetts) to ~4 mL.

The solution was further purified on a Blue-Sepharose column (2.2 × 62 cm) equilibrated with 20 mM phosphate buffer, pH 7.3, containing 1 mM EDTA and 5 mM DTE. The enzyme eluted with the same buffer, while impurities remained bonded to the column. Fractions of 8 mL were collected, with a flow rate of 45 mL/h. The active fractions contained practically pure enzyme and were concentrated by ultrafiltration to ~4 mL.

An aliquot (15 mg) of the above solution was applied to a Q-Sepharose High Performance column, using a HR 10/30 column with the Pharmacia FPLC system. The column was equilibrated with buffer A (20 mM phosphate buffer, pH 6.5, containing 1 mM EDTA and 5 mM DTE). Prolyl oligopeptidase was eluted at 6 °C with a linear salt gradient developed between 10 and 35% buffer B during 60 min. Buffer B contained 0.8 M NaCl in buffer A. The enzyme eluted at 27% buffer B following two very small peaks. After concentration the enzyme was frozen with liquid nitrogen and stored at -70 °C.

Mutagenesis of prolyl oligopeptidase

The Tyr473Phe mutation was achieved in two steps using the PCR technique. For introduction of the Tyr473Phe mutation, the following primer pair was synthesized.

Sense primer: 5'-CTCTACGGCTtCGGCGGCT-3'

Antisense: 3'-GAGATGCCGAaGCCGCCGA-5'

To the 3'-terminus of the gene the same 3'-primer was used as in cloning the native enzyme (see above), while to the 5'-terminus the following primer was designed.

New 5'-primer: 5'-GCAG GAATTC TAAGGAGG AATT-3'
 EcoRI SD

In the first step of PCR two independent reactions were carried out, using the Vent polymerase, the pTrc/PPO plasmid as template digested with *KpnI* enzyme and (1) the new 5'-primer and the antisense primer, and (2) the 3'-primer and sense primer, each run in 22 cycles. The conditions were similar as in cloning the wild-type enzyme, except that the syntheses in 72 °C were performed

for 1.5 min with the 5'-primer and 1 min with the 3'-primer. The mutated gene was then obtained in two pieces, which were isolated by agarose gel electrophoresis followed by purification with the QIAquick Gel Extraction Kit. In the second step the mixture of the two portions of the gene were used as templates in the reaction mixture, heated to 95 °C, prior to the addition of the Vent polymerase. The temperature was then lowered to 50 °C when the complementary ends of the two DNA portions formed double-stranded DNA. Heating at 72 °C for 5 min allowed the synthesis of the strands. The complete strands were able to bind the terminal primers (new 5'-primer and 3'-primer), which were added to the mixture at 95 °C. The amplification was then carried out in 28 cycles at 95, 57, and 72 °C for 1, 1, and 3 min, respectively. The PCR product was identified on an agarose gel (1%) and purified with a QIAquick kit. The pure product was digested with *EcoRI* and *KpnI* restriction enzymes and isolated with the Sephaglas Band-Prep kit (Pharmacia), and ligated into the pTrc 99A vector as described above for the wild-type enzyme. The production of the Tyr473Phe variant of prolyl oligopeptidase was achieved in *E. coli* JM105 as described for the wild-type enzyme.

Kinetics

The reaction of prolyl oligopeptidase with Z-Gly-Pro-Nap (Bachem Ltd., Bubendorf, Switzerland) was measured fluorometrically, using a Jasco FP 777 spectrofluorometer equipped with a thermostated cell holder. The excitation and emission wavelengths were 340 nm (1.5 nm bandwidth) and 410 nm (5 nm bandwidth), respectively. The gain of photomultiplier was set to "medium." Cells with excitation and emission pathlengths of 1.0 and 0.4 cm, respectively, were used. The substrate with internally quenched fluorescence, Abz-Gly-Phe-Gly-Pro-Phe-Gly-Phe(NO₂)-Ala-NH₂, was prepared with solid phase synthesis, and its hydrolysis was followed similarly as in the case of Z-Gly-Pro-Nap, except that the excitation and emission wavelengths were 337 and 420 nm, respectively.

The reactions with 4-nitroanilide substrates were monitored at 410 nm, using a Cary 1E spectrophotometer. For calculation of the liberation of 4-nitroanilide, a molar extinction coefficient of 8,800 was used (Erlanger et al., 1961).

The Michaelis-Menten parameters, k_{cat} and K_m , were determined with initial rate measurements, using substrate concentrations in the range of 0.2–5 K_m value. Because of the poor solubility of Z-Gly-Pro-Nap, in particular in the presence of 0.5 M NaCl, 0.3% acetonitrile was added to the reaction mixture, which caused about 20% inhibition of prolyl oligopeptidase. The kinetic parameters were calculated with nonlinear regression analysis, using the GraFit software (Leatherbarrow, 1990).

The K_i values, the dissociation constants of the enzyme-inhibitor complex, were calculated from Equation 1 using at least ten inhibitor concentrations.

$$k_i/k_0 = 1/(1 + I/K_i) \quad (1)$$

where k_i and k_0 are pseudo-first-order rate constants determined at substrate concentrations at least 10-fold less than K_m in the presence and absence of inhibitor (I), respectively.

Slow tight-binding inhibition (Morrison & Walsh, 1988) with Z-Pro-prolinal was measured with the progress curve method using Equation 2 (Bakker et al., 1990), and the inactivation rate (k) measured at each inhibitor concentration was calculated by non-

linear least-squares regression. The initial rate equals $-Ak + C$, the final rate is C , and B is the intercept of the final (steady-state) reaction on the ordinate. For these reactions Z-Gly-Pro-Mec substrate was used at concentration of 0.13 μ M, and less than 10% of the substrate was consumed during the reaction. Inhibition of the enzyme was measured spectrofluorometrically (370 nm excitation, 440 nm emission) in 0.1 M Tris/acetate buffer, pH 7.3, containing 0.1 mM DTE and 10 nM BSA. The enzyme concentrations were 0.13 nM and 0.43 nM for the wild-type and the Tyr473Phe variant, respectively.

$$y = Ae^{-kt} + B + Ct. \quad (2)$$

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