Unusual Secondary Specificity of Prolyl Oligopeptidase and the Different Reactivities of Its Two Forms toward Charged Substrates

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ABSTRACT: Prolyl oligopeptidase belongs to a new family of serine proteases which contains both exo- and endopeptidases, and this suggests that the enzyme binds its substrate in a special manner. Its secondary specificity, i.e., its interaction with the other residues linked to the proline that accounts for the primary specificity, has been investigated by using peptide substrates of various length and charge. Elongation of the classic dipeptide substrate Z-Gly-Pro-2-naphthylamide with 1–3 residues (Gln, Ala-Gln, Ala-Ala-Gln, and Ala-Lys-Gln) resulted in decreased specificity rate constants. This indicated a limited binding site for prolyl oligopeptidase, a major difference from the finding with other serine endopeptidases. Insertion of charged residues into the substrates, such as lysine or aspartic acid, considerably affected the rates and the pH–rate profiles. The rate constants were higher with the positively charged peptides and lower with the substrates bearing a negative charge. These electrostatic effects were reduced at high ionic strength. The results can be interpreted in terms of a negatively charged active site, which exists at high pH and exerts electrostatic attraction or repulsion toward charged substrates. The pH dependencies of the rate constants with neutral substrates exhibited roughly bell-shaped curves, whereas with charged substrates the existence of two active enzyme forms was clearly demonstrated. The physiologically competent high pH form preferred positively charged substrates (Z-Lys-Pro-2-(4-methoxy)naphthylamide, Z-Ala-Lys-Gln-Gly-Pro-2-naphthylamide), whereas the low pH form reacted faster with the negatively charged substrate (Z-Asp-Gly-Pro-2-naphthylamide). The pH dependencies obtained with neutral substrates provided the first experimental evidence that two reactive enzyme forms when having similar reactivities may exhibit a single apparent bell-shaped curve. The kinetic results are rationalized in terms of a tunnel-like binding site which includes the catalytic residues. This approach renders it possible for the oligopeptidases to exclude large peptides from their active site.

Prolyl oligopeptidase (Nomenclature Committee, 1992), previously called prolyl endopeptidase, is a large (76-kDa) intracellular serine endopeptidase that preferentially hydrolyzes proline-containing peptides at the carboxy end of proline residues. It also splits peptide bonds having alanine in place of the proline, but this cleavage is slower by 1–2 orders of magnitude [cf. Wilk (1983)]. The enzyme may be involved in the maturation and degradation of peptide hormones and neuropeptides (Wilk, 1983; Mentlein, 1988) and may also be implicated in amnesia (Yoshimoto et al., 1987) and Alzheimer's desease (Ishiura et al., 1990). Prolyl oligopeptidase belongs to a new family of serine proteases, which has no evolutionary relationship to the extensively studied trypsin and subtilisin families (Rawlings et al., 1991). Unusually, the new family contains not only endopeptidases but also exopeptidases, which are known to have restricted binding sites. This prompted us to examine the specificity of prolyl oligopeptidase toward longer peptides, which are usually better substrates for the common serine endopeptidases.

In the case of the small (25–30-kDa) extracellular serine proteases, like chymotrypsin or subtilisin, the ionization of the essential imidazole is not affected by the dissociation of other groups and follows a simple pH–rate profile dependent on a pK_a of about 7. (The deviation found with chymotrypsin in the alkaline region will be discussed later.) In contrast, prolyl oligopeptidase exhibits a doubly sigmoidal curve, which indicates that two forms of different activities operate in the range between pH 5 and 9 (Polgár, 1991). The low pH form displays a significant kinetic deuterium isotope effect, as usually observed in the serine protease catalysis, whereas the high pH form, which is physiologically competent, has practically no kinetic isotope effect. These results have suggested that a general base/acid-catalyzed acylation step is rate-limiting in the lower pH range and that an isotopically silent step, probably a conformational change preceding acylation, dominates the reaction in the physiological pH range (Polgár, 1991).

The activity of prolyl oligopeptidase was found to be remarkably dependent on ionic strength (Polgár, 1991). Therefore, in the present work charged substrates have also been examined. The results have shown that electrostatic interactions markedly affect the reactivity of the catalytically competent, high pH form of the enzyme, which presumably bears considerably negative charges.

EXPERIMENTAL PROCEDURES

Substrates. Z-Gly-Pro-Nap[1] was purchased from Bachem, Inc., and Z-Gln-Gly-Pro-Nap, Z-Ala-Ala-Gln-Gly-Pro-Nap, and Z-Ala-Lys-Gln-Gly-Pro-Nap were prepared as described (Polgár et al., 1991). The syntheses of Z-Asp-Gly-Pro-Nap and Z-Ala-Gln-Gly-Pro-Nap were performed similarly and will be published elsewhere.

Prolyl Oligopeptidase. The enzyme was prepared from pig muscle as described (Polgár, 1991), using the following modifications. (i) The time for extracting prolyl oligopeptidase from muscle was shortened. (ii) The overnight dialysis was replaced by a rapid gel filtration. (iii) The Q-Sepharose chromatography was substituted by the more effective chro-

[1] Abbreviations: Z, benzyloxycarbonyl; Nap, 2-naphthylamide; (4M)-Nap, 2-(4-methoxy)naphthylamide; Nan, *p*-nitroanilide; Suc, succinyl; DTE, dithioerythritol.

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matography on a Blue A column (Yamakawa et al., 1986). (iv) A more sensitive salt gradient was used with the FPLC chromatography. In short, the enzyme extracted from 750 g of pig muscle was incubated at room temperature for 15 min instead of 30 min and was fractionated by acetone and ammonium sulfate. The precipitate was extracted with 100 mL of standard buffer (20 mM phosphate, 1 mM EDTA, 5 mM Na_2SO_3) and centrifuged at 10000*g*, at 4 °C for 20 min. The extraction was repeated with 50 mL of standard buffer. The combined supernatants (170 mL) were loaded onto a Sephadex G-50 column (8 × 50 cm) equilibrated with the standard buffer. It was important that some colored proteins were separated from the prolyl oligopeptidase during the chromatography. The fractions containing the enzyme (320 mL) were combined and chromatographed on a DEAE-cellulose column as described (Polgár, 1991). It should be noted that after DEAE chromatography the enzyme activity increased. The active fractions from the DEAE-cellulose column were concentrated on an Amicon YM 30 membrane, diluted about 10-fold with 20 mM phosphate buffer, pH 7.2, containing 1 mM EDTA and 1 mM DTE and concentrated again. This solution from two batches (1–2 mL from 2 × 750 g of muscle) was applied to a Dyematrex Blue A column (Pierce Chemical Co.; 1 × 13 cm), which was previously washed with 20 mM phosphate buffer, pH 7.2, containing 1 mM EDTA and 1 mM DTE. The enzyme was eluted in the same buffer at a flow rate of 24 mL/h. The prolyl oligopeptidase was found in the break-through protein peak. The activity peak contained 10–15% of the total absorbance applied to the column. A total of 75–80% of the protein was eluted with 1.5 M KCl in the equilibration buffer as an inactive peak. The rest was eluted with 8 M urea. The enzyme (6.0–7.5 mL, 0.8–1.1 absorbance at 280 nm) was concentrated by ultrafiltration and stored in the presence of 40% ethylene glycol at −18 °C until further purification by FPLC chromatography. It is worth noting that the chromatography at pH 6.5 was less effective as some of the enzyme was not eluted with the equilibration buffer.

Prior to the FPLC chromatography, the concentration of ethylene glycol in the enzyme solution was reduced by a 10-fold dilution with 20 mM phosphate buffer, pH 6.5, containing 1 mM EDTA and 1 mM DTE. The prolyl oligopeptidase was subsequently concentrated to about the original volume by using an Amicon YM 30 membrane. The enzyme was then applied to a Mono Q HR 5/5 column (1–2 mg in one run) and eluted with NaCl gradient using 20 mM phosphate buffer, pH 6.5, containing 1 mM EDTA and 1 mM DTE (solvent A) and 0.5 M NaCl in the same buffer (solvent B). The gradient, used between 18 and 30%, was developed with a flow rate of 0.5 mL/min during 22 min. The active peak was eluted at about 24% solvent B, and it contained about 1 mg/mL protein (30–40% of the amount loaded onto the column). The most significant contamination, which traveled slightly before the prolyl oligopeptidase during SDS gel electrophoresis, was eluted from the Mono Q column by washing with 100% solvent B. The yield approximately doubled as a result of the modifications. A total of 2.5–3 mg of prolyl oligopeptidase was obtained from 1.5 kg of muscle. The enzyme was stored in the presence of 40% ethylene glycol at −18 °C.

Kinetics. The activities of prolyl oligopeptidase with naphthylamide substrates were determined as described (Polgár, 1991) by using a Jasco FP 777 spectrofluorometer at 340 and 410 nm as excitation and emission wavelengths, respectively. The 2-(4-methoxy)naphthylamide derivative was measured in the same way. The hydrolysis of *p*-nitroanilide substrate was monitored spectrophotometrically at 410 nm (Erlanger et al., 1961).

Table I: Reaction Conditions for Measuring the Pseudo-First-Order Rate Constants

substrate	[substrate] (μM)	[enzyme] (nM)	solvent (%)
Z-Gly-Pro-Nap	0.5–2	3–6	0.028 AN
Z-Lys-Pro-(4M)Nap	0.4–0.7	3–6	
Z-Gln-Gly-Pro-Nap	1–2	10–40	0.017 DMF
Z-Gln-Ala-Pro-Nap	1–2	5–10	0.017 DMF
Z-Asp-Gly-Pro-Nap	1–2	40–80	0.020 DMF
Z-Ala-Gln-Gly-Pro-Nap	1–2	5–10	
Z-Ala-Ala-Gln-Gly-Pro-Nap	1–2	5–20	0.033 DMF
Z-Ala-Lys-Gln-Gly-Pro-Nap	1–2	5–20	0.011 AN
Suc-Gly-Pro-Nan	3–6	100–200	0.030 DMF

The rate constants were measured under first-order conditions (Table I), i.e., at substrate concentrations lower than K_m. The second-order rate constants were obtained by dividing the first-order rate constant by the enzyme concentration used in the reaction mixture. Where solubility of the substrate permitted, the K_m values were also determined from Michaelis–Menten kinetics. Both the first-order rate constants and the Michaelis–Menten parameters were calculated by nonlinear regression data analysis.

The pH dependence of the rate contants were measured in 50 mM Hepes buffer containing 1 mM EDTA and 1 mM DTE. The ionic strength of the buffer was always adjusted with sodium chloride to $I = 0.05$ M. Alternatively, the ionic strength was maintained constant at 0.05 M by using a three-component buffer mixture as recommended by Ellis and Morrison (1982). Following the reaction, the pH of each sample was determined and found to be identical with the starting value. Theoretical curves for a bell-shaped pH–rate profile were calculated by nonlinear regression analysis according to

$$k = k(\text{limit})[1/(1 + 10^{-pH}/10^{-pK_I})] \times [1/(1 + 10^{-pK_{II}}/10^{-pH})] \quad (1)$$

where k(limit) stands for the pH-independent maximum rate constant and K_I and K_{II} represent the acidic ionization constant of the base and acid, respectively. A more complex pH dependence, which involves two active enzyme forms interconverting with pH (EH_2 and EH in eq 2), was calculated by using eq 3 (Polgár, 1991), where $k(\text{limit})_1$ and $k(\text{limit})_2$ stand for the pH-independent rate constants of the two active enzyme forms and K_1, K_2, and K_3 are the dissociation constants of three enzymic groups whose state of ionization affects the rate constant. The interconversion of the active forms depends on K_2. For curve fitting a nonlinear regression data analysis program was used:

$$\underset{EH_3}{\text{EH(ImH)XH}} \rightleftharpoons \underset{EH_2}{\text{EH(Im)XH}} \rightleftharpoons \underset{EH}{\text{EH(Im)X}} \rightleftharpoons \underset{E}{\text{E(Im)X}} \quad (2)$$

inactive active active inactive

where X is an unknown ionizing group whose dissociation perturbs the pK_a of the imidazole group (Im) of the catalytic histidine.

$$k = k(\text{limit})_1[1/(1 + 10^{-pH}/10^{-pK_1})] \times [1/(1 + 10^{-pK_2}/10^{-pH})] + k(\text{limit})_2 \times [1/(1 + 10^{-pH}/10^{-pK_2})][1/(1 + 10^{-pK_3}/10^{-pH})] \quad (3)$$

Table II: Effects of Solvents on the Reactions of Prolyl Oligopeptidase[a]

substrate	k^b (mM^{-1} s^{-1})	solvent[c] (%)
Z-Gly-Pro-Nap	1580 ± 190	AN (1.03)
	2000 ± 242	AN (0.53)
	2630 ± 286	AN (0.25)
	3050 ± 176	AN (0.028)
	650 ± 110	DMF (1.03)
	1900 ± 220	DMF (0.21)
	1750 ± 230	D (1.03)
Z-Gly-Pro-Nan	740 ± 88	AN (0.20)
	480 ± 66	D (1.0)
	125 ± 22	D (5.0)
	32 ± 7	D (10.0)

[a] Measured in 0.05 M Hepes buffer, pH 8.0, containing 0.5 M NaCl. [b] Values are given as the mean and standard deviation for the rate constants of 3–5 measurements. [c] Solvents: AN, acetonitrile; DMF, dimethylformamide; D, dioxane.

Values of p^2H of deuterium oxide (99.8%) solutions were obtained from pH meter readings according to the relationship p^2H = pH(meter reading) + 0.40 (Glasoe & Long, 1960).

RESULTS

Solvent Effects. Uncharged amino acid or peptide derivatives are often sparingly soluble in water, and therefore, their hydrolysis is usually carried out in the presence of organic solvents. For example, the reactions of prolyl oligopeptidase are sometimes measured at dioxane concentrations as high as 15–20% (Yoshimoto et al., 1978). Prolyl oligopeptidase, however, is remarkably sensitive to organic solvents. This can be seen from Table II which shows that even 1% acetonitrile or dioxane inhibits the enzyme by about 50%, and in the presence of 10% dioxane the activity is less than 5%. The maximum activity was calculated by plotting the rate constants against the solvent concentration and by extrapolating the data to zero acetonitrile concentration (not shown). Such a calculation has shown that the inhibition at the lowest solvent concentration (0.028%) was less than 3%. Although at low solvent concentration the reaction can only be measured at low substrate concentration, the acylation rate constants can readily be determined under first-order conditions, in particular when the sensitive spectrofluorometric method is employed. A comparison of the effects of different solvents (Table II) shows that dimethylformamide is more detrimental than acetonitrile or dioxane. However, some of the substrates dissolved better in dimethylformamide than in the two other solvents.

Elongated Substrates. Longer peptides are usually hydrolyzed by proteases more rapidly than single amino acid derivatives. The rate enhancement may be of several orders of magnitude, as exemplified by the elastase catalysis, but may also be much less, as found with the trypsin catalysis [cf. Polgár (1987, 1989)]. In contrast, prolyl oligopeptidase exhibits diminished activity toward elongated substrates. It can be seen from Table III that the classical dipeptide substrate (compound I) displays a significantly higher rate constant than its extended neutral tripeptide (compound II), tetrapeptide (compound V), and pentapeptide (compound VI) derivatives, independent of the absence or presence of salt. Also, the charged compound VII, which is more active in the absence of salt than the analog compound VI, exhibits still lower activity than compound I. Even if some compounds of a more extended range of substrates would display higher reactivity, as a consequence of some special side chain effects, the present finding seems to be rather unusual for an endopeptidase and suggests that a long cleft, characteristic of the binding modes of endopeptidases [cf. Polgár (1987, 1989)], may be perturbed or may be entirely different in the substrate binding by prolyl oligopeptidase.

Side Chain Effects. The comparison of compounds II and III (Table III) shows that prolyl oligopeptidase prefers Ala over Gly at subsite S_2. Interaction of Lys with the same subsite also improves the substrate reactivity but at low ionic strength. In the presence of 0.5 M NaCl, the rate constants with compounds I and VIII are similar. It should be noted that the effect of the methoxy group (compound VIII) on the leaving ability of naphthylamide appears to be negligible (Polgár, 1992). These data indicate that some negative charge(s) at or near the active site of the enzyme facilitates the binding of compound VIII, and this electrostatic effect is compensated by the increased concentration of NaCl. The importance of charge effects is also apparent from the comparison of the rate constants for compound VIII in the presence and absence of salt. In contrast to the neutral substrates, compound VIII reacts slower in the presence of 0.5 M NaCl, displaying a ratio of $k_{NaCl}/k_0 = 0.8$ (Table III). Similar effects can be observed when the Lys residue is located at position P_4 (compound VII). Thus, charge effects are also important at this position, as seen from a comparison with compound VI.

Whereas a positive charge makes the peptide a better substrate (compound VIII), introduction of the negatively charged succinyl group results in a very poor substrate (compound IX). Compound IX is a nitroanilide rather than a naphthylamide, but the lower reactivity of the nitroanilide substrate (Table II) cannot account for the large difference between the rate constants of compounds I and IX [cf. Polgár (1992)]. Similar charge effects were observed when compound IV holding a negative charge at position P_3 is compared to the neutral compound II. Unexpectedly, in the presence of NaCl the rate constants for compound IV and the neutral substrates increased similarly, which indicates that factors other than the charge effects are also important. More information about this problem can be obtained from the comparison of the rate constants over a wider pH range.

pH Dependence of Rate Constants. The formation of hydrolysis of the intermediate acylenzyme in serine protease catalysis are promoted by a histidine residue, which operates as a general base/acid catalyst and exhibits a pK_a of about 7 [cf. Polgár (1987, 1989)]. The ionization of this residue governs the pH dependence of the catalysis, which conforms to a simple dissociation curve. However, the acylation of prolyl oligopeptidase with Z-GP-Nap is more complicated. It exhibits a doubly sigmoidal curve, which tends to decrease above pH 9 (eq 3). This implies a mechanism (eq 2) involving two enzyme forms of different activities, which interconvert with changing pH (Polgár, 1991). With the neutral extended substrates Z-QGP-Nap (compound II), Z-AQGP-Nap (compound V), and Z-AAQGP-Nap (compound VI), the two active enzyme forms are not as apparent as with Z-GP-Nap. Several repeated experiments with Z-AAQGP-Nap, however, have shown that the experimental points were always below a simple bell-shaped curve at pH $\approx$ 6 and above the curve at pH $\approx$ 7.2 (Figure 1). In some other cases, significant deviation from the bell-shaped pH–rate profile was not observed (Z-AQGP-Nap and Z-QGP-Nap in the presence of salt, Table IV). This is obviously due to the similar activities of the two enzyme forms toward these neutral substrates, as seen from the comparison $k(\text{limit})_1$ and $k(\text{limit})_2$ values in Table IV. The charged substrates, on the other hand, displayed markedly

Table III: Secondary Interactions in Prolyl Oligopeptidase Catalysis[a]

substrate no.	P_5 P_4 P_3 P_2 P_1	k (mM^{-1} s^{-1}) no salt	k (mM^{-1} s^{-1}) +0.5 M NaCl	k_{NaCl}/k_0
I	Z-Gly-Pro-Nap	1240 ± 80	3050 ± 280	2.5
II	Z-Gln-Gly-Pro-Nap	195 ± 14	510 ± 30	2.6
III	Z-Gln-Ala-Pro-Nap	760 ± 50	2020 ± 120	2.6
IV	Z-Asp-Gly-Pro-Nap	33 ± 5	82 ± 4	2.5
V	Z-Ala-Gln-Gly-Pro-Nap	228 ± 11	564 ± 55	2.5
VI	Z-Ala-Ala-Gln-Gly-Pro-Nap	300 ± 30	787 ± 30	2.6
VII	Z-Ala-Lys-Gln-Gly-Pro-Nap	687 ± 70	575 ± 60	0.8
VIII	Z-Lys-Pro-(4M)Nap	4340 ± 210	2950 ± 250	0.7
IX	Suc-Gly-Pro-Nan	7 ± 1	14 ± 2	2.0

[a] 0.05 M Hepes, pH 8.0.

Table IV: Kinetic Parameters for the Reactions of Prolyl Oligopeptidase with Different Substrates

condition	$k(\text{limit})_1$ (mM^{-1} s^{-1})	$k(\text{limit})_2$ (mM^{-1} s^{-1})	pK_I	pK_2 or pK_I	pK_3 or pK_{II}
Z-QGP-Nap					
H_2O	197 ± 13	238 ± 9	5.35 ± 0.08	6.84 ± 0.37	8.69 ± 0.07
H_2O/NaCl		563 ± 21		5.96 ± 0.07	9.02 ± 0.10
Z-AQGP-Nap					
H_2O		278 ± 11		5.69 ± 0.07	8.67 ± 0.10
H_2O/NaCl		667 ± 23		6.43 ± 0.06	8.80 ± 0.07
Z-AAQGP-Nap					
H_2O	260 ± 48	332 ± 9	4.92 ± 0.22	6.46 ± 0.48	8.98 ± 0.06
H_2O/NaCl	414 ± 153	849 ± 23	5.24 ± 0.34	6.46 ± 0.28	9.20 ± 0.08
Z-AKQGP-Nap					
H_2O	243 ± 31	903 ± 50	5.25 ± 0.18	7.24 ± 0.11	8.75 ± 0.07
H_2O/NaCl	98 ± 11	681 ± 8	3.64 ± 1.37	6.27 ± 0.03	8.78 ± 0.03
Z-KP-(4M)Nap					
H_2O	810 ● 240	6180 ± 450	5.58 ± 0.40	7.64 ± 0.12	9.25 ± 0.10
Z-DGP-Nap					
H_2O	305 ± 187	45 ± 14	5.55 ± 0.39	6.07 ± 0.49	8.28 ± 0.34

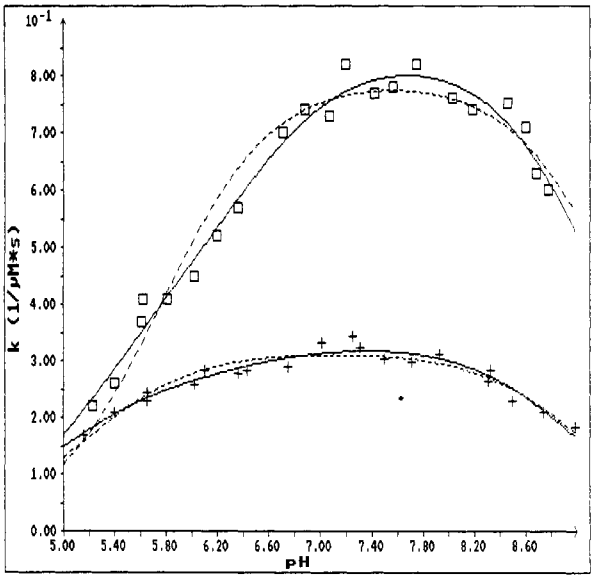

FIGURE 1: pH dependence of acylation of prolyl oligopeptidase with Z-AAQGP-Nap. The rate constants were measured at 25 °C in 0.05 M Hepes buffer (+) and in 0.05 M Hepes buffer containing 0.5 M NaCl (□). The continuous lines represent theoretical curves calculated by using eq 3 with the parameters shown in Table IV. The dashed lines show bell-shaped curves according to eq 1 with parameters $k(\text{limit})$ = 317 mM^{-1} s^{-1}, pK_I = 5.16, pK_{II} = 9.06 (+), and $k(\text{limit})$ = 798 mM^{-1} s^{-1}, pK_I = 5.76, pK_{II} = 9.35 (□). Curve fittings were performed by nonlinear regression analysis.

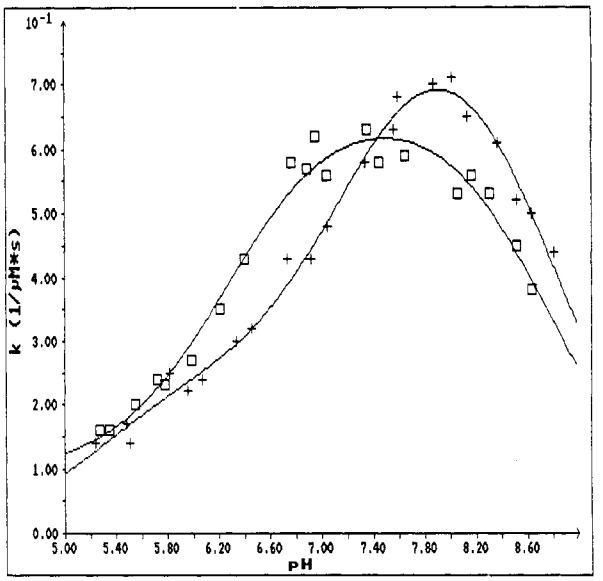

FIGURE 2: pH dependence of acylation of prolyl oligopeptidase with Z-AKQGP-Nap. The rate constants were measured at 25 °C in 0.05 M Hepes buffer (+) and in 0.05 M Hepes buffer containing 0.5 M NaCl (□). The continuous lines are theoretical curves calculated by using eq 3 with the parameters shown in Table IV. Curve fittings were performed by nonlinear regression analysis.

different profiles as will be demonstrated in the following paragraph.

Electrostatic Effects. The importance of electrostatic effects in substrate binding is apparent from Table III which shows that substrates containing a postively charged lysine residue exhibit inverse salt effects at least at pH 8.0. Figure 2 illustrates the pH–rate profiles for Z-AKQGP-Nap in the absence and in the presence of 0.5 M NaCl, and Table IV shows the related kinetic parameters. It is seen that the inverse salt effect only prevails at the alkaline end of the pH range shown in Figure 2. Interestingly, the sharpened maximum curve obtained in the absence of salt widens in the presence of NaCl, resulting in a pH–rate profile more similar to those of the neutral substrates.

With the lysine-containing substrate, the decline of the curves characterized with pK_3 seems to be unrelated to the

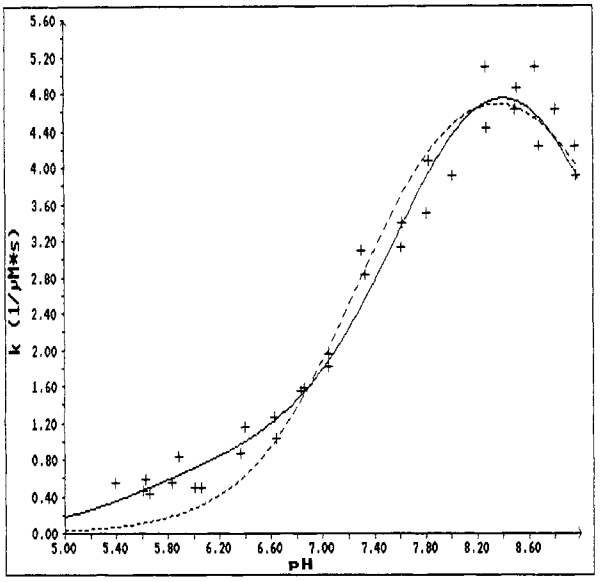

FIGURE 3: pH dependence of acylation of prolyl oligopeptidase with Z-KP-(4M)Nap. The rate constants were measured at 25 °C in 0.05 M Hepes buffer. The continuous line is a theoretical curve calculated by using eq 3 with the parameters shown in Table IV. The dashed line represents a bell-shaped curve according to eq 1. The parameters for eq 1 are k(limit) = 5490 mM^{-1} s^{-1}, pK_I = 7.28, and pK_{II} = 9.44. Curve fittings were performed by nonlinear regression analysis.

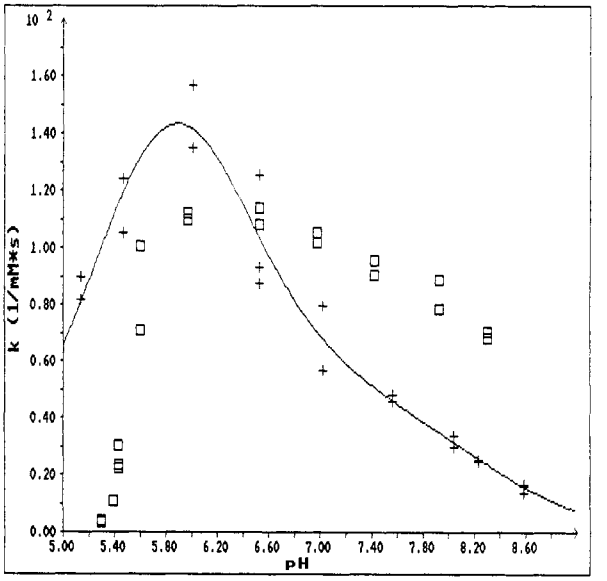

FIGURE 4: pH dependence of acylation of prolyl oligopeptidase with Z-DGP-Nap. The rate constants were measured at 25 °C in 0.05 M Hepes buffer (+) and in 0.05 M Hepes buffer containing 0.5 M NaCl (□). The continuous line is a theoretical curve calculated by using eq 3. The parameters of the curve are shown in Table IV. Curve fittings were performed by nonlinear regression analysis.

dissociation of the side chain amino group because this group is protonated practically over the entire pH range studied ($pK_a \approx 10.5$) and because a similar value of pK_3 is also associated with the pH dependence of Z-AAQGP-Nap. Additional information about the decline of the pH-dependence curve at alkaline pH was obtained from the Michaelis parameters which could be determined in the case of the fairly soluble Z-AKQGP-Nap. It was found that the K_m increased considerably with increasing pH (15 μM at pH 7 and 60 μM at pH 9) while k_{cat} leveled off in the alkaline pH range. As $K_m = K_s k_3/(k_2 + k_3)$ where K_s, k_2, and k_3 are the binding, acylation, and deacylation constants, respectively, the decline of the curve may be attributed to the weakening of substrate binding (increase in K_s). The other possibility, the decrease in k_2 relative to k_3, is not probable because $k_{cat} = k_2 k_3/(k_2 + k_3)$ does not change in the alkaline pH range. As expected, the value of the specificity rate constant, k_{cat}/K_m, calculated from the Michaelis kinetics is identical, within experimental error, with the second-order rate constant derived from the data obtained under first-order conditions.

The electrostatic effects are even more pronounced with the dipeptide substrate having the lysine residue at position P_2 (Figure 3 and Table IV). The maximum is sharpened and shifted to the more alkaline pH values, indicating that the positively charged substrates prefer the high pH form of prolyl oligopeptidase. The effects of salt on the rate constants were not studied in this case because Z-KP-(4M)Nap exhibited the lowest K_m among the substrates examined, so that the more precise measurements under first-order conditions were difficult to perform even with the sensitive spectrofluorometric method. The K_m at neutrality was about 1 μM which increased with increasing pH up to 3 μM at pH 9.

While a positive charge on the substrate enhances $k(\text{limit})_2$ relative to $k(\text{limit})_1$, a negative charge leads to the opposite result, as illustrated by the pH dependence of the reaction of Z-DGP-Nap substrate (Figure 4). It is clearly seen from the curves and from Table IV that the low pH form displays higher activity compared to the physiologically competent high pH form. This finding most convincingly demonstrates the existence of two active enzyme forms and the importance of electrostatic effects in the catalysis of prolyl oligopeptidase. The results can be rationalized in terms of a negative charge(s) existing in the active site region, whereby the rections are facilitated with positively charged substrates and inhibited with negatively charged substrates. Most interestingly, in the presence of salt there is a dramatic change in the pH–rate profile, so that a curve having a simple ionizing group with pK_1 (eq 3) may not be fitted to the experimental points due to the abrupt decrease at low pH. This unexpected finding is not due to the denaturation of the protein, as preincubation of the enzyme under the same conditions had no effect on the rate constants. Moreover, the enzyme does not exhibit such a behavior in the reactions with other substrates (see Figures 1 and 2). Precipitation of the substrate may also be excluded because it is soluble even at a concentration considerably higher than that employed here.

DISCUSSION

Prolyl oligopeptidase has several interesting features not shared by the simple serine proteases. Thus, it exhibits unusual secondary specificity inasmuch as it hydrolyzes more effectively the dipeptide substrate Z-GP-Nap than its elongated derivatives (compounds II, V, and VI in Table III). In this respect, prolyl oligoptidase is different from endopeptidases which usually hydrolyze tri-, tetra-, and pentapeptides faster, relative to dipeptides and single amino acid derivatives, even by 1–2 orders of magnitude. Hence, the oligopeptidase may permit a limited access to its binding site, just as the exopeptidases dipeptidyl peptidase IV and acylaminoacyl hydrolase, the members of the same family of serine peptidases (Rawlings et al., 1991).

An additional feature of prolyl oligopeptidase is that it has two active forms which interconvert with changing pH. This is most lucidly seen when the pH dependence of a basic substrate (Figure 3) is compared to that of an acidic substrate (Figure 4). The basic compound reacts almost excusively with the high pH form, whereas the acidic one clearly prefers the low pH form. The neutral compounds react with the two

forms at similar rates so that the activity contributions of the two forms merge to provide an apparent bell-shaped curve. This is the first experimental evidence showing that, in contrast to the usual assumption, a simple bell-shaped curve may not represent a single enzyme form whose activity depends on an acidic and a basic group but that it may hide a more complicated system arising from the coincidence of certain events. Two factors may be considered in this respect: the activities and the pK_a values of the two reacting forms. If the reactivities of the two forms are similar, they may provide an apparent bell-shaped curve even if the two pK_a values (pK_1 and pK_2 of eq 3) are not very close. In such a case, the two pK_a values for a carboxy and an imidazole group can provide a single apparent pK_a. If the two pK_a values become closer, a larger difference in the activities is permitted.

The pK_a values extracted from pH–rate profiles are in most cases characteristic of the ionization of the catalytically competent groups of enzymes. Thus, in serine proteases an imidazole base displaying a pK_a of about 7 is essential for catalysis. Although pK_2 (Table IV) can be a candidate for the ionization of the essential histidine of prolyl oligopeptidase, this pK_a is not characteristic of the catalytic step. Specifically, the rate-limiting step in the prolyl oligopeptidase catalysis is a conformational change rather than a chemical step involving general base catalysis by the histidine (Polgár, 1991, 1992). This has been confirmed in this work by studying kinetic deuterium isotope effects on the reactions with Z-AAQGP-Nap and Z-AKQGP-Nap substrates. The lack of any significant isotope effects on the $k(\text{limit})_2$ values (not demonstrated) is consistent with the previous results obtained with Z-GP-Nap (Polgár, 1991). Thus, around pK_2 some configurational change may occur, and this, of course, may be coupled with the ionization of a histidine residue that is significantly perturbed by other charged groups.

The alkaline limbs of the pH-dependence curves may arise from a conformational change as well. A similar phenomenon was demonstrated in chymotrypsin reactions, where the dissociation of a proton with increasing pH from the α-ammonium group of Ile-16 disrupts the salt bridge between Ile-16 and Asp-194, thereby transforming the active site structure into an inactive form [cf. Polgár (1987, 1989)].

The most intriguing question concerns the structural basis of the specificity of prolyl oligopeptidase. It can be anticipated that oligopeptidases must have a strategy for substrate binding, which is fundamentally different from that of endopeptidases. This renders it possible to exclude large peptides from their active site, so that the native proteins are not attacked by oligopeptidases in the cell. Unfortunately, X-ray crystallographic data have not yet been available. However, some kinetic results allows us to speculate about the binding site of the enzyme. Prolyl oligopeptidase can hydrolyze oligopeptides containing 25–30 residues but not longer peptides and proteins (Camargo et al., 1979, Wilk, 1983; Moriyama et al., 1988). The cleavage products of several tryptic peptides of 10–20 residues long have been determined (Moriyama et al., 1988). Perusal of the cleavage sites clearly shows that the enzyme can cut 3–4 residues from either the C- or the N-terminus, but it cannot split the oxidized insulin B-chain (30 residues) at the middle of the molecule. These data are consistent with a binding site covered by a "flap" or "lid" rather than with an open cleft common to many endopeptidases. Since the residues from P_3 to P_2' determine the specificity of the enzyme (Wilk, 1983), the tunnel-like binding site, which also holds the catalytic groups, may accommodate five residues.

It may be assumed that the substrate can approach the tunnel through either opening. For longer peptides (10–30 residues), the binding will only be productive if the substrate approaches one entrance with its N-terminus or the other with its C-terminus, depending on which terminus is to be cut. Large structured peptides cannot be hydrolyzed. Small peptides not having definite structures can penetrate into the enzyme with either the carboxy or the amino end. If only one end of the tunnel holds the negative charge, the positively charged substrate can get into the tunnel through this opening, and this is facilitated by a high concentration of salt. The negatively charged substrate enters the tunnel through the noncharged opening, thereby evading the repelling power.

It is reasonable to assume that only a few residues can get through the tunnel. A longer peptide with a rest, say 10 residues outside the tunnel, will more probably withdraw rather than get through, so that cutting in half a peptide of 30 residues is less probable. This assumption is further supported by the case of flagellin. This protein has a long random C-terminal peptide chain which is readily hydrolyzed by several endopeptidases. However, prolyl oligopeptiase is ineffective although the eighth residue from the C-terminus is proline (Dr. F. Vondervist, personal communication).

The sensitivity of the catalysis to various thiol reagents is also consistent with this model. Specifically, it was pointed out that a large reagent, like *p*-(chloromercuri)benzoate or *N*-ethylmaleimide, inhibits the enzyme virtually completely but the small iodoacetamide only partially (Polgár, 1991). Bearing a large compound, the thiol group, existing either inside or at the entrance of the tunnel, can completely block the reaction, whereas the small iodoacetamide leaves enough space for the substrate to bind, even though the binding is somewhat hindered.

A rate-limiting conformational change (Polgár, 1991, 1992) is consistent with this unique binding model. Moving a substrate into the tunnel is a more complex process than its accommodation in a cleft. Due to the enzyme–substrate interactions, the structure of the tunnel may be altered relative to that of the free enzyme, and the resulting changes may vary from one substrate to the other. This can explain the diverse kinetics obtained with different substrates. Structural changes caused by the high ionic strength may affect the two entrances of the tunnel to different extents. This might offer an explanation for the inconceivable, abrupt decrease of the rate constants for the negatively charged Z-Asp-Gly-Pro-Nap (Figure 4) if the opening through which this compound enters becomes impaired as a consequence of the structural change.

The proposed model suggests a new approach for the oligopeptidases to metabolize regulatory and other peptides without the attack of native proteins. Besides serine endopeptidases, oligopeptidases also extend to metallopeptidases (Barrett & Rawlings, 1992) which presumably operate with the use of the same strategy.

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