Cleavage of the Lys196–Ser197 Bond of Prolyl Oligopeptidase: Enhanced Catalytic Activity for One of the Two Active Enzyme Forms†

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ABSTRACT: Prolyl oligopeptidase, a representative of a new family of serine proteases, is remarkably sensitive to ionic strength and has two catalytically active forms, which interconvert with changing pH [Polgár, L. (1991) *Eur. J. Biochem. 197*, 441–447]. To reveal whether conformational changes are associated with these effects, prolyl oligopeptidase was digested with trypsin. SDS gel electrophoresis studies demonstrated that tryptic digestion of the 75-kDa native protein generated two fragments, one having a molecular mass of 51 kDa and the other of 26 kDa. The digestion was markedly dependent on the ionic strength. Specifically, the digestion proceeded more rapidly in 0.05 M Hepes buffer than in 0.05 M Hepes buffer containing 0.5 M NaCl. Moreover, the nicked enzyme formed at low ionic strength was not stable but degraded and inactivated during an extended incubation. The digestion experiments suggested that alteration in the ionic strength elicits conformational changes in native prolyl oligopeptidase, and this may account for the enhanced catalytic activity observed at higher ionic strength. The two fragments of the nicked prolyl oligopeptidase did not separate during size-exclusion chromatography under nondenaturing conditions on a Superose 12 column and eluted in place of the native enzyme, indicating that they were strongly associated. The reactive serine residues of the nicked enzyme was labeled with tritiated diisopropyl phosphofluoridate, and the fragments were separated by size-exclusion chromatography in urea. The radioactive label was found in the large fragment. N-terminal sequencing showed that the small fragment, as well as native prolyl oligopeptidase, did not have a free N-terminal amino group, while the large fragment displayed an N-terminal sequence corresponding to residues 197–205 of native prolyl oligopeptidase. The residues around the cleaved bond may represent a linkage between two unknown domains of prolyl oligopeptidase. The nicked protease had a specificity rate constant slightly more active than the native enzyme (1.3-fold) at pH 8.0 where the high-pH form of the enzyme exists. However, at pH 6.0 where the low-pH form is predominant, the increase in the rate constant was 3–4-fold with the substrates Z-Gly-Pro-2-naphthylamide and Z-Lys-Pro-2-(4-methoxy)naphthylamide. As a consequence of the tryptic cleavage, the increase in k_{cat} was even higher, in particular with Z-Lys-Pro-2-(4-methoxy)naphthylamide at pH 6, where the increase was more than 1 order of magnitude. These results are consistent with the existence of two catalytically active enzyme forms.

Prolyl oligopeptidase, also called prolyl endopeptidase or post-proline cleaving enzyme, is a serine protease that may be involved in the maturation and degradation of peptide hormones and neuropeptides (Walter et al., 1980; Wilk, 1983; Mentlein, 1988). It may be a key enzyme in the degradation of phosphoproteins containing a Pro-Ser(P) bond (Rosén et al., 1991). Recent data have also suggested that the enzyme is implicated in amnesia (Yoshimoto et al., 1987) and Alzheimer's disease (Ishiura et al., 1990). Prolyl oligopeptidase does not exhibit an evolutionary relationship to the extensively studied trypsin and subtilisin families, but along with dipeptidyl peptidase IV and *N*-acylaminoacyl hydrolase, it forms a new family of serine peptidases (Rawlings et al., 1991). Indeed, there are significant mechanistic differences between prolyl oligopeptidase and the members of the trypsin and subtilisin families. Specifically, prolyl oligopeptidase has two active forms which interconvert with changing pH, and the physiologically competent high-pH form exhibits a physical rather than a chemical rate-limiting step (Polgár, 1991, 1992a). Also, prolyl oligopeptidase is distinct from the well-known serine peptidases in that it is remarkably sensitive to ionic strength. For instance, addition of 0.5 M sodium chloride to the reaction mixture results in a 2.5-fold increase in the specificity rate constant (Polgár, 1991). An additional interesting feature of prolyl oligopeptidase is its large molecular mass (75 kDa), which may result from a multidomain structure comprising a simple protease domain attached covalently to some noncatalytic domains. This is the case with many large serine proteases, such as those involved in blood coagulation, fibrinolysis, and complement activation.

In the present work, the structural differences of the putative enzyme forms and the possible domain structure of prolyl oligopeptidase have been probed by means of proteolytic digestion. This is a sensitive method for detecting subtle alterations in native protein conformations since a looser structure is digested more readily compared to a tighter one. Proteolytic digestion was also used to study the possible domain structure of prolyl oligopeptidase since proteases preferentially cleave the relatively accessible interdomain stretches. For example, after a specific cleavage of plasminogen, the smaller trypsin-like protease domain could be separated from the larger noncatalytic structure simply by gel chromatography (Wu et al., 1987; Shi & Wu, 1988). Here we have found that (1) ionic strength strongly affected the digestibility of the native prolyl oligopeptidase, (2) digestion under controlled conditions resulted in the cleavage of the Lys196–Ser197 bond, a possible domain border of the enzyme, and (3) the nicked enzyme, in

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particular its low-pH form, displayed enhanced catalytic activity.

EXPERIMENTAL PROCEDURES

Chemicals. Bz-DL-Arg-Nan·HCl[1] and Z-Gly-Pro-Nap were from Serva Feinbiochemica GmbH and Co., and Bachem, Inc., respectively. [1,3-^{3}H]DIPF was purchased from NEN as a 0.25 mM solution in propylene glycol, specific activity 148 GBq/mmol (4 Ci/mmol). Scintol 5 and toluene were from Koch-Light Ltd. TPCK-treated trypsin, clostripain, and subtilisin were products of Sigma Chemical Co.

Prolyl Oligopeptidase. The enzyme was prepared from pig muscle as described (Polgár, 1991). Its activity was measured fluorometrically with Z-Gly-Pro-Nap (Polgár, 1991), using a Jasco FP 777 spectrofluorometer. The excitation and emission wavelengths were 340 and 410 nm, respectively. The second-order rate constants (k_2) were measured under first-order conditions, i.e., below the K_m values as described (Polgár, 1991). The reactions with Z-Lys-Pro-(4M)Nap were measured at the above-mentioned wavelengths, but at a lower substrate concentration (58 nM), because of the low K_m associated with this compound. The k_{cat} and K_m values were determined from initial rates, using substrate concentrations below and above the K_m values. The data were calculated by fitting the experimental points to the Michaeleis–Menten equation by using a nonlinear regression analysis. The ratio of k_{cat} to K_m is equal to the second-order rate constant (Bender & Kézdy, 1965). The errors in k_2, k_{cat}, and K_m were 5–10%, 8–15%, and 20–40%, respectively, being higher at pH 6 than at pH 8. Because of the poor solubility of Z-Gly-Pro-Nap, the relatively high K_m values of its reactions could only be determined in the presence 0.22% acetonitrile.

Purification of Trypsin Inhibitor. Commercial soybean trypsin inhibitor (30 mg in 1 mL of 10 mM phosphate buffer, pH 7.6) was chromatographed on a Whatman DE32-cellulose column (1 × 6.5 cm) equilibrated with 10 mM phosphate buffer, pH 7.6. The inhibitor was eluted with a linear NaCl gradient (0–0.4 M) in the same buffer. The protein concentration of trypsin inhibitor was calculated by using A_{280} = 0.91 for the 1 mg/mL solution (Laskowski, 1955).

Tryptic Digestion. Prolyl oligopeptidase (0.2–0.5 mg/mL) was digested at 25 °C in 0.05 M Hepes buffer, pH 8.0, with trypsin (0.05–0.1 mg/mL). The concentration of trypsin was determined by active site titration with *p*-nitrophenyl *p*-guanidinobenzoate (Chase & Shaw, 1970). The reaction mixtures always contained 2 mM DTE and 1 mM $CaCl_2$. Aliquots were taken at appropriate times, and the reactions were stopped with soybean trypsin inhibitor (0.15 mg/mL). The activities were measured with Z-Gly-Pro-Nap. Alternatively, the aliquots were immediately diluted into the assay mixtures. The samples were also analyzed by SDS gel electrophoresis. They were treated with trypsin inhibitor for 2 min and then with the sample cocktail containing SDS and DTE. Each sample was immediately heated to boiling.

Digestion with Clostripain and Subtilisin. These experiments were similar to that performed with trypsin. The reaction mixture contained 2 mM DTE, 5 mM $CaCl_2$, and 0.3 mg/mL clostripain in 0.05 M Hepes buffer, pH 8.0, or 2 mM DTE, 1 mM $CaCl_2$, and 0.01 mg/mL subtilisin in 0.05 M Hepes buffer, pH 8.0, containing 0.5 M NaCl.

[1] Abbreviations: Bz, benzoyl; Z, benzyloxycarbonyl; Nap, 2-naphthylamide; (4M)Nap, 2-(4-methoxy)naphthylamide; Nan, 4-nitroanilide; DTE, dithioerythritol; DIPF, diisopropyl phosphofluoridate; Hepes, 2-[4-[2-(hydroxyethyl)-1-piperazinyl]]ethanesulfonic acid.

Isolation of the Nicked Enzyme. After digestion of prolyl oligopeptidase (10 mL, 0.2 mg/mL) with trypsin (0.1 mg/mL) in 0.05 M Hepes buffer, pH 8.0, containing 0.5 M NaCl, 2 mM DTE, and 1 mM $CaCl_2$ for 30 min, the reaction was stopped by the addition of 1 mM benzamidine, 5 mM DTE, and 2 mM EDTA, and the pH was adjusted to 6.5 with 0.2 M KH_2PO_4. The resulting solution was concentrated on an Amicon YM 10 membrane to 0.20 mL in an ice bath and loaded onto a Superose 12 column equilibrated with 50 mM phosphate buffer, pH 6.5, containing 2 mM DTE and 1 mM EDTA. Elution of the proteins was effected by the same buffer at a flow rate of 0.4 mL/min. The first peak (1.9 mL), eluting at 33.3 min, contained the nicked enzyme (1.4 mg). The second major peak, eluting at 40 min (2.5 mL), exhibited trypsin activity.

The nicked enzyme obtained from the Superose column was further purified on a Mono Q HR 5/5 column as follows. The enzyme solution was diluted 3-fold with water containing 2 mM EDTA and concentrated on an Amicon YM 10 membrane. A 0.25-mg sample (in 0.2 mL) was applied to the column 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 between 18 and 30% was developed during 22 min with a flow rate of 0.5 mL/min.

An appropriate purification of the nicked enzyme was also achieved when the reaction mixture, without chromatography on the Superose column, was directly applied to the Mono Q column. In this case, the elution of trypsin was effected by washing the column with elution buffer (a mixture of 82% solvent A and 18% solvent B).

Separation of the Fragments in Urea. The nicked enzyme, purified in the preceding step, was concentrated to 200 μL on an Amicon membrane YM 10, freeze-dried, and dissolved in 20 mM phosphate buffer, pH 6.5, containing 6 M urea, 2 mM DTE, and 1 mM EDTA. A 200-μL aliquot of this solution (5 mg/mL) was kept at 55 °C for 10 min and then applied to a Superose 12 column (1 × 30 cm) equilibrated with the same buffer. The chromatogram was developed at a flow rate of 0.4 mL/min. The large fragment was eluted at 20 min, the small one at 31 min. The fragments were identified by means of SDS gel electrophoresis. The urea was immediately removed from the solution by ultrafiltration through an Amicon YM 10 membrane. To this end, the solution was repeatedly (five times) diluted with an equal volume of 2 mM phosphate buffer, pH 6.5, containing 1 mM EDTA and 2 mM DTE, and concentrated by ultrafiltration to the original volume before each dilution. The fragments were lyophilized and stored. From 0.95 mg of nicked enzyme (190 μL, 5 mg/mL) were obtained 0.56 mg of large fragment (0.4 mL, 1.4 mg/mL) and 0.1 mg of small fragment (0.2 mL, 0.5 mg/mL).

Preparation of Labeled Nicked Enzyme. Prolyl oligopeptidase was digested and purified as described above, except that the chromatography on Superose column was carried out at pH 8.0. The enzyme obtained from the Superose column was concentrated on an Amicon YM 10 membrane (250 μL, 10 μM) and reacted with [1,3-^{3}H]DIPF (10 μL, 250 μM) in 0.1 M phosphate buffer, pH 8.0, containing 1 mM DTE and 1 mM EDTA. After 20-min incubation at room temperature, the reaction mixture was gel-filtered on a Sepharose 12 column at pH 8.0 to eliminate nonreacted radioactive material. The solution was concentrated by ultrafiltration to 150 μL, and after the addition of 72 mg of urea (6 M), the fragments were separated on the Superose column in 20 mM phosphate buffer,

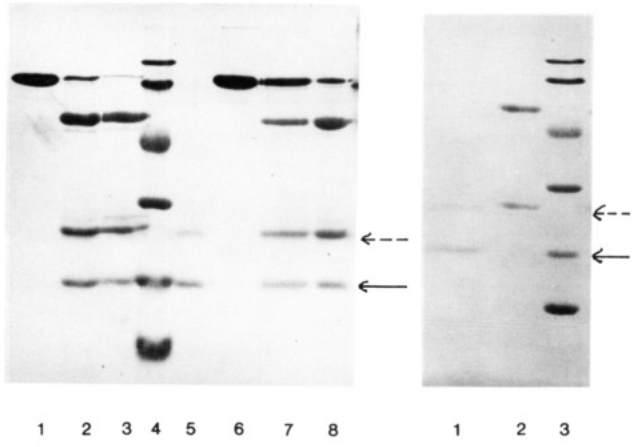

FIGURE 1: SDS–PAGE of prolyl oligopeptidase digested with trypsin. (a, left) Hydrolysis of 0.5 mg/mL prolyl oligopeptidase was performed with 0.05 mg/mL trypsin in 0.05 M Hepes buffer, pH 8.0, for 0, 5, and 30 min (lanes 1, 2, and 3, respectively) and in the same buffer containing 0.5 M NaCl and incubated for the same periods (lanes 6, 7, and 8, respectively). The molecular masses of marker proteins (lane 4) were 14.4, 20.1, 30, 43, 67, and 94 kDa. Trypsin and trypsin inhibitor (dashed and solid arrow, respectively) were employed at the same concentrations as in the reaction mixtures (lane 5). (b, right) Digested prolyl oligopeptidase: after removal of trypsin (lane 2); trypsin and trypsin inhibitor (lane 1); marker proteins (lane 3).

pH 8.0, containing 6 M urea, 2 mM DTE, and 1 mM EDTA. The chromatographic conditions were the same as above.

Determination of Radioactivity. The tritium content of labeled protein was measured in a cocktail containing 250 mL of toluene, 125 mL of Triton X-100, and 20 mL of Scintol 5. To 9.5 mL of cocktail was added 50 μL of sample and the resultant mixture was measured in an LKB 1211 Rackbeta liquid scintillation counter.

SDS–Polyacrylamide Gel Electrophoresis. The electrophoresis was performed on 15% slab gels (1 × 150 × 100 mm) by using a discontinuous buffer system (Laemmli, 1970). Gels were stained for 2 h in 0.05% (w/v) Coomassie Brillant Blue R250 in methanol/acetic acid/water (5:1:4 by volume) and destained in methanol/acetic acid/water (1:1:8 by volume).

N-Terminal Sequencing. The fragments of the nicked enzyme were separated by reverse-phase HPLC. A 0.5-nmol sample of lyophilized protein was dissolved in 50 μL of water and the resultant mixture chromatographed on an Aquapore OD300 column (2.1 × 200 mm) using a linear gradient of acetonitrile (0–90%) in 0.1% trifluoroacetic acid over 30 min at 0.3 mL/min. The fragments were detected at 220 nm and sequenced on a pulsed liquid-phase sequencer (Model 471A; Applied Biosystems, Foster City, CA).

RESULTS AND DISCUSSION

Proteolytic Digestion. Prolyl oligopeptidase is a representative of a new family of serine proteases. Its structure and activity have been tested here by digestion with trypsin in 0.05 M Hepes buffer, pH 8.0, and in the same buffer containing 0.5 M NaCl. It can be seen from Figure 1 that the proteolysis generated two major components. The larger fragment had a molecular mass of 51 kDa. The smaller fragment that appeared at 26 kDa moved slightly slower than trypsin. The sum of the molecular masses of the fragments amounts to the molecular mass of the native enzyme (75 kDa) within experimental error. This indicates that prolyl oligopeptidase was specifically cleaved at a single site.

Although their molecular masses are very similar, the smaller fragment cannot be confused with trypsin, which is at a lower concentration and gives a much fainter band than the fragment does (Figure 1a). The formation of the small fragment is also demonstrated in Figure 1b, where the trypsin was removed by gel chromatography from the sample prior to the gel electrophoresis. This experiment also demonstrates that the proteolysis by trypsin does occur with the native prolyl oligopeptidase, and not with the denatured enzyme while being heated with SDS.

The effects of ionic strength on proteolysis are also illustrated by Figure 1a. It is clearly seen that the breakdown of the native protein is virtually complete at 5 min at low ionic strength, whereas a significant amount of native enzyme still exists at the same time in the presence of 0.5 M NaCl. Although the initially formed two fragments constitute the major bands in 0.05 M Hepes buffer, additional faint bands can be seen at 47 and 27 kDa, indicating that proteolysis can occur at an additional site, too. However, this is negligible relative to the primary cleavage. The secondary cleavage is even less important at high ionic strength. Upon a longer digestion at low ionic strength, a new band appears at 17 kDa (not shown), which is a further indication that the enzyme is less stable in the absence of salt.

It should be noted that the addition of DTE to the reaction mixture, while it protected prolyl oligopeptidase, facilitated the autolysis of trypsin in the absence of $CaCl_2$. However, the trypsin activity was practically constant during digestion in the presence of 1 mM $CaCl_2$, as measured with Bz-Arg-Nan at 410 nm. This rules out the possibility that the apparent stability of prolyl oligopeptidase at high ionic strength is a consequence of the breakdown of trypsin. It was also examined whether or not the activity of trypsin toward the small synthetic substrate Bz-Arg-Nan is affected by the ionic strength. It was found that the reaction rate was independent of the presence of 0.5 M NaCl. Hence, an increase in the ionic strength may alter the conformation of prolyl oligopeptidase, which becomes more active and less accessible to trypsin.

Trypsin is a rather specific enzyme which cleaves the peptide bond at lysine and arginine residues. Clostripain is even more specific, hydrolyzing only at arginines. No cleavage could be detected in prolyl oligopeptidase by SDS gel electrophoresis during incubation for 150 min with clostripain although the incubation was carried out in 0.05 M Hepes buffer, which favors proteolytic digestion of prolyl oligopeptidase. Furthermore, the activity of clostripain did not change during the incubation, as checked with Bz-Arg-Nan substrate. These results suggested that the limited proteolysis of prolyl oligopeptidase occurred at a lysine rather than arginine residue.

The less specific subtilisin, on the other hand, degraded the prolyl oligopeptidase into small peptides even in the presence of 0.5 M NaCl, which stabilized its structure. Limited proteolysis could not be elicited with either chymotrypsin or papain.

The change in the activity of prolyl oligopeptidase during digestion is shown in Figure 2. Unexpectedly, the activity increased upon digestion, slower in the presence than in the absence of 0.5 M NaCl. The significance of the slight rate enhancement is apparent from Figure 2, which represents a typical case of six repeated experiments. When a large excess of trypsin (1 mg/mL) over prolyl oligopeptidase (0.2 mg/mL) was employed, the enzyme was inactivated even in the presence of 0.5 M NaCl. A similar degree of inactivation of prolyl oligopeptidase (0.2 mg/mL) was observed in the absence of salt and at lower trypsin concentration (0.1 mg/mL), but in the presence of 0.5 M NaCl, the inactivation was negligible (not shown). The inactivation can be attributed to degradation of the nicked enzyme. The comparison of the results of Figures

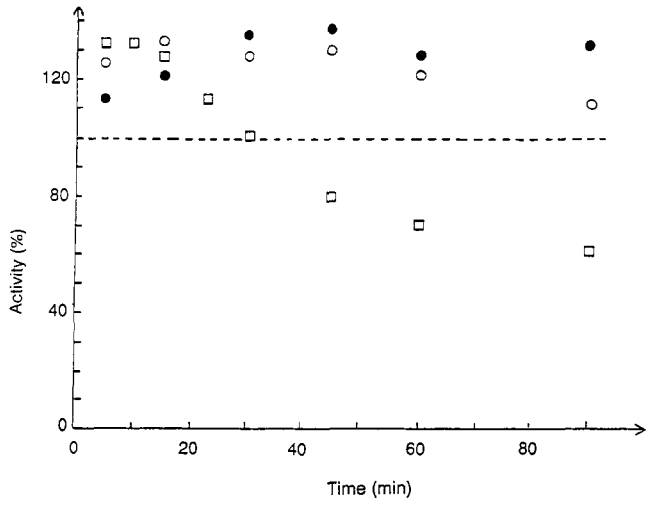

FIGURE 2: Activity dependence of prolyl oligopeptidase upon tryptic digestion. Hydrolysis of 0.5 mg/mL prolyl oligopeptidase with 0.05 mg/mL trypsin in 0.05 M Hepes buffer, pH 8.0, containing 2 mM DTE (○) and in 0.05 M Hepes buffer, pH 8.0, containing 0.5 M NaCl and 2 mM DTE (●) and hydrolysis of 0.2 mg/mL prolyl oligopeptidase with 1 mg/mL trypsin in 0.05 M Hepes buffer, pH 8.0, containing 0.5 M NaCl and 2 mM DTE (□). The dashed line represents the enzyme activity in the absence of trypsin in 0.05 M Hepes buffer, pH 8.0, containing 2 mM DTE. The activity of each aliquot was measured fluorometrically in 0.1 M phosphate buffer, pH 8.0, containing 1 mM DTE and 1 mM EDTA. The zero time activity of the reaction mixture (100%) was measured prior to the addition of trypsin.

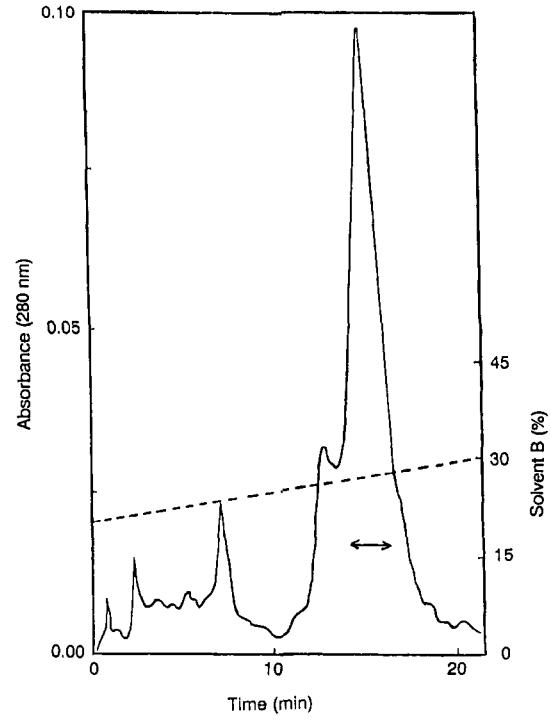

FIGURE 3: Purification of nicked prolyl oligopeptidase on a Mono Q column. Chromatographic conditions are described in Experimental Procedures. The double arrow indicates the pooled fractions.

1 and 2 clearly shows that prolyl oligopeptidase can be converted by limiting proteolysis into a relatively stable, modified form that displays an enhanced catalytic activity.

Isolation of the Nicked Enzyme. Limited proteolysis of prolyl oligopeptidase generates two fragments, the smaller one being about of the size of trypsin, as found with the catalytic domain of several multidomain serine proteases. Separation of the smaller domain from the rest of the molecule, however, was not successful under nondenaturing conditions with the use of either ion-exchange or size-exclusion chromatography. The elution times from a Superose 12 column for the native and nicked enzymes were practically identical, indicating that the two fragments were intimately associated and that the hydrodynamic volume of prolyl oligopeptidase was not seriously affected by the limited proteolysis. Indeed, the nicked enzyme had a specific activity even higher by about 30% than that of the native enzyme. The strong association of the fragments was apparently not changed under different chromatographic conditions where the enzymic activity was preserved. Thus, the nicked enzyme eluted from the Superose column at the same place between pH 5.6 and 8.2, in the presence and in the absence of 0.5 M NaCl, and with or without 1.5 M urea. In 6 M urea the fragments were separated, but we were not able to renature the large fragment in a catalytically active form even if the small fragment was added to the refolding mixture. The refolding of the noncleaved enzyme was also unsuccessful.

Chromatography on the Superose column proved to be a useful method for the preparation of nicked enzyme free of trypsin. Not surprisingly, it was much easier to separate the prolyl oligopeptidase from the free trypsin than from the trysin–trypsin inhibitor complex. Therefore, for preparative purposes, the digestion was stopped by benzamidine instead of soybean trypsin inhibitor.

Separation of the native, single-chain prolyl oligopeptidase from the nicked enzyme could be achieved by ion-exchange chromatography on a Mono Q column (Figure 3). The nicked enzyme was eluted at 25.8% solvent B, slightly after the small peak of the native protease (24.4% solvent B). SDS gel electrophoresis confirmed that the nicked enzyme did not contain the parent protein (Figure 1b).

When the nicked prolyl oligopeptidase labeled with tritiated DIPF was chromatographed on a Superose 12 column in 6 M urea, the fragments were clearly separated (see Experimental Procedures). The large fragment eluting first contained the radioactivity. The other protein peak had no radioactivity. Consequently, the active site serine residue was associated with the large fragment, ruling out the possibility that the 26-kDa fragment is the protease domain. As the active site serine residue is located at the C-terminal region (Rennex et al., 1991), it can be concluded that the small fragment of the nicked enzyme belongs to the N-terminus, whereas the large fragment constitutes the C-terminal region.

Site of Limited Proteolysis. The recently published amino acid sequence of prolyl oligopeptidase (Rennex et al., 1991) rendered it possible to identify the peptide bond cleaved by the trypsin. To this end, the nicked enzyme was purified as described above and lyophilized from 50 mM ammonium hydrocarbonate solution. This sample provided two major peaks on HPLC chromatography. The first peak could not be sequenced, similarly to the noncleaved protein. This indicated that the N-terminus of the first fragment was blocked and that this fragment was actually the N-terminal portion of the molecule. This finding is consistent with the recent observation (Rennex et al., 1991) that the N-terminal amino group of prolyl oligopeptidase is blocked. The second peak gave rise to the following amino acid sequence: Ser-Asp-Gly-Thr-Glu-Thr-Ser-Thr-Asn-, which is identical with the segment between residues 197 and 205. Residue 196 is a lysine residue, in agreement with the finding that limited proteolysis occurs with trypsin, but not with clostripain. From inspection of the amino acid sequence, it is immediately apparent that the peptide segment only contains hydrophilic

Table I: Effect of Limited Proteolysis on the Activity of Prolyl Oligopeptidase[a]

enzyme	k_{cat} (s^{-1})	K_m (μM)	k_{cat}/K_m ($\mu M^{-1} s^{-1}$)	k_2 ($\mu M^{-1} s^{-1}$)
		Z-Gly-Pro-Nap[b]		
native, pH 6	2.1	1.7	1.2	1.1
cleaved, pH 6	10.5	3.5	3.0	2.6
cleaved/native	5.0	2.1	2.5	2.4
native, pH 8	12.0	5.5	2.2	2.6
cleaved, pH 8	16.7	6.0	2.8	3.5
cleaved/native	1.4	1.1	1.3	1.4
		Z-Lys-Pro-(4M)Nap		
native, pH 6	0.26	0.29	0.90	0.80
cleaved, pH 6	3.65	0.90	4.05	3.46
cleaved/native	14.0	3.1	4.5	4.3
native, pH 8	4.2	1.6	2.6	2.9
cleaved, pH 8	6.2	1.9	3.3	4.0
cleaved/native	1.5	1.2	1.3	1.4

[a] Measured in 0.05 M Hepes buffer containing 0.5 M NaCl. For more details see Experimental Procedures. [b] In the presence of 0.22% acetonitrile.

amino acids that may constitute a surface loop. This is supported by perusal of the other side of the Lys196–Ser197 bond, which is -Pro-Gln-Gln-Asp-Gly-Lys-. Indeed, the 15-residue-long segment contains several α-helix-breaking (Thr, Ser, Gly, Pro) and β-sheet-breaking (Glu, Gln, Lys, Asp, Asn, Pro) residues [cf. Levitt (1978)].

The molecular masses of the fragments as calculated from their amino acid sequences (23 and 57 kDa) are slightly different from those determined by SDS gel electrophoresis (26 and 51 kDa). This difference is, however, not significant if it is considered that the molecular masses obtained with the two methods for the whole prolyl oligopeptidase molecule are also different: 75 and 80 kDa from SDS gel electrophoresis and from amino acid sequencing, respectively (Rennex et al., 1991).

The above results show that one-third of the molecule, the N-terminal region, is linked to the rest of prolyl oligopeptidase through a relatively large surface loop, which presumably represents a domain border. This is supported by preliminary digestion of prolyl oligopeptidase at a low concentration of urea (2 M) which resulted in complete breakdown of the large fragment, while the small fragment remained intact, indicating that the small fragment is a compact structural domain. Comparative studies of lipases and the enzymes of the prolyl oligopeptidase family have shown that they are structurally related, having a catalytic domain of about 30 kDa, in which the sequential order of the triad residues is Ser, Asp, and His (Polgár, 1992b). Hence, only the C-terminal moiety of the large fragment may represent the protease domain; the rest of the fragment is a noncatalytic domain that cannot be split off by digestion with trypsin. Preliminary trials with other proteases were also unsuccessful, as mentioned above. The noncatalytic portions distinguish prolyl oligopeptidase from simple digestive enzymes and strengthen the idea that it functions as a regulatory enzyme.

Activity of the Nicked Enzyme. Previous kinetic investigations suggested that prolyl oligopeptidase exhibits two active forms which interconvert with changing pH (Polgár, 1991). If there is some conformational difference between the two forms, the activity of the low-pH form may be affected by the cleavage of the Lys196–Ser197 bond to a different extent than observed with the high-pH form. The slight increase in the activity of the high-pH form was demonstrated by digestion of prolyl oligopeptidase in the presence of 0.5 M NaCl at pH 8 (Figure 2). Table I shows the kinetic parameters of the nicked enzyme measured at pH 6.0, where the low-pH form is predominant, and at pH 8.0, where the high-pH form exists. In addition to the classic substrate Z-Gly-Pro-Nap, the activities were also measured with Z-Lys-Pro-(4M)Nap, an analogous substrate showing similar activity in the presence of 0.5 M NaCl. As compared with the increase found at pH 8.0, the specificity rate constants ($k_2 = k_{cat}/K_m$) increased considerably at pH 6.0, particularly with Z-Lys-Pro-(4M)-Nap. In the rate enhancement of the specificity constant, the increase in k_{cat} was of crucial importance. It increased more than 1 order of magnitude in the case of Z-Lys-Pro-(4M)-Nap, but this was partly compensated by the higher K_m value. The results clearly indicate that prolyl oligopeptidase exists in two different forms, one of which is activated by limited proteolysis to a great extent, whereas the other form is affected only slightly. Apparently, a proteolytic event occurring in the polypeptide chain far from the catalytic domain exerts serious effects on the enzymic reaction. This is consistent with the finding of the present work that the two fragments of the nicked enzyme are strongly associated.

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