Effects of ionic strength on the catalysis and stability of prolyl oligopeptidase

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Prolyl oligopeptidase is the prototype of a new serine protease family, unrelated to trypsin and subtilisin. In contrast with these proteases, prolyl oligopeptidase is remarkably sensitive to ionic strength, being more active in the presence of high concentrations of salt. The enzyme has two catalytic forms, which interconvert with changing pH. To reveal the structural bases of these phenomena, the effects of 0.5 M NaCl on the stability of the enzyme were investigated by studying its denaturation as a function of pH, temperature, and urea concentration. The three independent methods have unequivocally demonstrated that denaturation of the enzyme is promoted in the presence of NaCl. Furthermore, destabilization of the low-pH form by urea is more

significant than that of the high-pH form. Examination of the fluorescence emission spectra of various denatured forms indicates that the enzyme is not fully unfolded in 8 M urea, nor at acidic pH. The tryptophan residues in the acid-denatured state are mainly buried. The results are interpreted in terms of the decay of the protective water shell at the higher ionic strength. The higher enthalpy and entropy of activation for heat denaturation provide further evidence that a more ordered water structure stabilizes the protein in the absence of salt. The biphasic kinetics obtained with denaturation by heat and urea suggest that the enzyme has two domains of different stabilities.

INTRODUCTION

Prolyl oligopeptidase, a large (molecular mass 80 kDa) intracellular serine peptidase, preferentially hydrolyses proline-containing peptides at the carboxy end of proline residues [1]. It may be involved in the maturation and degradation of peptide hormones and neuropeptides [1,2], as well as in amnesia [3] and Alzheimer's disease [4]. The enzyme is not related to the trypsin and subtilisin families, but belongs to a new family of serine peptidases [5,6]. Its kinetic behaviour is also different from that of the small (25–30 kDa) extracellular serine peptidases. These enzymes exhibit a simple pH–rate profile controlled by a single ionizing group of pK_a about 7 and are only slightly, if at all, affected by the ionic strength of the reaction medium [7,8]. In contrast, prolyl oligopeptidase catalysis conforms to a doubly sigmoidal curve, and is rather sensitive to ionic strength [9]. The doubly sigmoidal pH–rate profile indicates the existence of two pH-dependent enzyme forms, one being predominant at pH 6, and the other at pH 8. Kinetic investigations have also shown that the rate-determining step for prolyl oligopeptidase catalysis is a conformational change [9,10] rather than the chemical step characteristic of the chymotrypsin reactions.

Increasing the NaCl concentration frequently stabilizes protein structure by preferential hydration [11]. To establish whether or not the enhanced catalytic activity of prolyl oligopeptidase is associated with a more stable structure, the stability of the enzyme has been examined with regard to pH, heat, and urea.

EXPERIMENTAL

Enzyme and activity assay

Prolyl oligopeptidase was prepared from pig muscle as described [1]. Its activity was determined fluorimetrically with Z-Gly-Pro-2-naphthylamide (Nap) [1], by using a Jasco FP 777 spectrofluorimeter. The excitation and emission wavelengths were, respectively, 340 and 410 nm, with slit widths of 1.5 and

5 nm. Cells with excitation and emission path-lengths of 1.0 and 0.4 cm, respectively, were used. The concentration of the enzyme was determined at 280 nm [1].

Kinetics

Rate constants were measured under first-order conditions, i.e. at substrate concentrations lower than the K_m [1,9] and were calculated by non-linear regression data analysis. The second-order rate constants (k_{cat}/K_m) were obtained by dividing the first-order rate constant by the total enzyme concentration present in the reaction mixture.

pH dependence of inactivation and unfolding

Inactivation and unfolding were measured in the same reaction mixture by using a four-component buffer system of constant ionic strength consisting of 25 mM glycine, 25 mM acetic acid, 25 mM Mes, 75 mM Tris, 1 mM EDTA and 1 mM dithioerythritol (DTE). The fluorescence intensity of the enzyme (0.14 μ M) was monitored in 600 μ l of reaction mixture at 25 °C, using 280 and 340 nm excitation and emission wavelengths, respectively. Other settings were the same as used in the activity assay. By taking a 20 μ l aliquot from each sample after 40 min incubation, the remaining activity was determined from initial rate measurements in 1.0 ml of assay mixture at 25 °C. The fluorescence intensity values were read after 60 min incubation, and were corrected for the fluorescence of the corresponding buffers.

Denaturation in urea

Unfolding of prolyl oligopeptidase at increasing urea concentrations was determined as described [12,13]. Fluorescence intensities (I_{obs}) were measured in 50 mM Hepes buffer containing 1 mM EDTA and 1 mM DTE at 25 °C. The measuring conditions were as above. Unfolding measurements were carried out on 555 μ l prolyl oligopeptidase solutions (0.15–0.20 μ M) after in-

cubation with indicated final concentrations of urea for 90 min at 25 °C. For each value of I_{obs} , the fraction of unfolded protein (f_u) was calculated from

$$f_u = (I_t - I_{\text{obs}}) / (I_t - I_u) \quad (1)$$

where I_u and I_t are the respective fluorescence intensities of the fully unfolded protein obtained at high concentrations of urea and of the fully folded protein acquired in the absence of the denaturant. The values for I_u and I_t were obtained at the baselines of the transition curves, at which I_{obs} became invariant with changing urea concentration. The I_{obs} values were corrected by subtraction of the fluorescence of the buffer solution in the absence of protein.

Many globular proteins have been found to follow a two-state mechanism [eqn. (2)], in which only the folded and unfolded states are present at significant concentrations in the transition region. On this model, the change in f_u with increasing urea concentration conforms to eqn. (3) (GraFit computer program, R. J. Leatherbarrow, Erithacus Software), where m is a measure of the dependence of ΔG on the denaturant concentration, and ΔG_0 represents the conformational stability of the protein at zero concentration of denaturant ($[D] = 0$).

$$[\text{Folded}] \rightleftharpoons [\text{Unfolded}] \quad (2)$$

$$f_u = 1 - \exp\{(m[D] - \Delta G_0)/(RT)\} / (1 + \exp\{(m[D] - \Delta G_0)/(RT)\}) \quad (3)$$

Heat Inactivation

The enzyme (0.36 μM) was incubated in 1.0 ml of 50 mM Hepes buffer containing 1 mM EDTA and 1 mM DTE in the temperature range of 46–50 °C. A 20 μl sample of this reaction mixture was added at appropriate times to a final volume of 1.0 ml of 50 mM phosphate buffer, pH 8.0, containing 1 mM EDTA and 1 mM DTE, and initial rates were measured for 1 min at 25 °C. The assay mixture contained 7 μl of 0.3 mg/ml substrate.

Thermodynamic parameters were calculated from Eyring plots [eqn. (4)], where k is the rate constant, R is the gas constant (8.314 J/mol per K), T is the absolute temperature, N_A is the Avogadro number (6.022×10^{23} /mol), h is the Planck constant (6.626×10^{-34} J·s), the enthalpy of activation $\Delta H^* = -(\text{slope}) \times 8.314$ J/mol, and the entropy of activation $\Delta S^* = (\text{intercept} - 23.76) \times 8.314$ J/mol per K. The free energy of activation, ΔG^* , was calculated from eqn. (5).

$$\ln(k/T) = \ln(R/N_A h) + \Delta S^*/R - \Delta H^*/RT \quad (4)$$

$$\Delta G^* = \Delta H^* - T\Delta S^* \quad (5)$$

RESULTS

pH-Induced unfolding and inactivation

Denaturation curves are especially useful for measuring the differences in conformational stabilities of a protein under different conditions. Because native protein commonly has a much greater fluorescence than the denatured protein [12], the unfolding of prolyl oligopeptidase at extreme pH or at increasing urea concentration can be monitored fluorimetrically. Figure 1 shows that the enzyme is stable over a relatively narrow pH range, and unfolds at moderate acidic and alkaline pH values. Addition of salt promotes unfolding at alkaline pH, but not at acidic pH. In the acidic pH range the denaturation state is different from that observed at alkaline pH values, as seen from the higher level of fluorescence intensity below pH 4.5 compared with that found above pH 10.

Figure 1 pH dependence of unfolding of prolyl oligopeptidase

The fluorescence intensities are shown in the absence (○) and presence (△) of 0.5 M NaCl.

The transition midpoints between the native and acid-unfolded forms and the native and alkali-denatured forms, respectively (Figure 1), are pH 5.1 and 9.4 without added salt, and pH 5.1 and 9.0 in the presence of 0.5 M NaCl. This clearly indicates that the protein is destabilized by higher ionic strength above neutrality, but not below. Similar transition midpoints are observed when prolyl oligopeptidase unfolding is followed by enzyme activity (Figure 2), indicating that pH-induced inactivation correlates with protein stability. The transition midpoints for unfolding are pH 5.1 and 9.8, and pH 5.1 and 9.3, respectively, in the absence and presence of 0.5 M NaCl.

Unfolding in urea

Denaturation curves can be constructed by plotting the unfolded fraction, f_u [eqn. (1)], against the urea concentration. The results of the unfolding experiment fit well to a two-state transition model (Figure 3). However, under the refolding conditions employed, unfolding is irreversible and the free energies could

Figure 2 pH dependence of inactivation of prolyl oligopeptidase

The activities are from initial rate measurements after incubation for 40 min in the absence (○) and presence (△) of 0.5 M NaCl.

Figure 3 Urea denaturation of prolyl oligopeptidase

Protein unfolding was monitored at 25 °C, at pH 6.0 (a) and pH 8.0 (b) in the presence of 0.5 M NaCl (+). The data points are taken from two independent experiments. The curves represent best fits to the experimental points by non-linear regression from eqn. (5) (GraFit computer program, Sigma Co.). The unfolding curves in the absence of salt are illustrated by thin lines.

Table 1 Differences in stability of prolyl oligopeptidase determined by urea denaturation

The data are extracted from the curves of Figure 3. $[D]_{1/2}$ represents the transition midpoint for urea denaturation.

pH	[NaCl] (M)	$[D]_{1/2}$ (M)
8.02	—	2.87
8.03	0.5	2.50
6.00	—	2.56
6.01	0.5	2.08

not be calculated. The relative stability of the protein under various conditions is therefore estimated by calculating the midpoint of denaturation. The results are shown in Table 1, in which it is seen that the enzyme is more stable at pH 8 than at pH 6. Furthermore, in the presence of salt the transition midpoints of the denaturation are significantly diminished.

The study of the kinetics of denaturation reveals two processes, one fast and the other slow. The unfolding in 8 M urea, as

Figure 4 Kinetics of unfolding of prolyl oligopeptidase

The decrease in fluorescence intensity (F) was measured in 8 M urea at pH 7.5. The points were fitted by non-linear regression analysis to a double-exponential curve, $F = 2924e^{-k(1)t} + 2214e^{-k(2)t} + 1680$, where $k(1) = 0.1239 \text{ s}^{-1}$ and $k(2) = 0.0049 \text{ s}^{-1}$. The thin line represents a single-exponential decay $F = 2907e^{-kt} + 1855$, where $k = 0.0111 \text{ s}^{-1}$.

Figure 5 Fluorescence emission spectra of native and denatured prolyl oligopeptidases

The spectra of 0.14 μM enzyme samples were recorded at a speed of 200 nm/min at pH 7.0 (+), pH 1.8 ($\times$), pH 7.0 and 8 M urea ($\circ$), pH 1.8 and 8 M urea ($\square$), and pH 12.3 ($\triangle$) at 25 °C. The fluorescence intensities of buffers (25 mM glycine, 25 mM acetic acid, 25 mM Mes and 75 mM Tris) were subtracted from the protein spectra.

measured by the decrease in fluorescence, follows a double-exponential decay, well separated from the single-exponential curve (Figure 4). This strongly suggests that two domains in the enzyme have different stabilities, their unfolding rate constants differing by more than an order of magnitude.

Fluorescence spectra

On denaturation, the emission maximum of the spectrum of proteins containing tryptophan is usually shifted from shorter wavelengths to about 350 nm, which corresponds to the fluorescence maximum of tryptophan in aqueous solution [14]. It can be seen in Figure 5 that the emission maximum of native prolyl

Figure 6 Kinetics of heat inactivation of prolyl oligopeptidase

The enzyme was incubated at 48 °C without (a) and with (b) added salt at pH 8.0. The activity was determined at 25 °C with initial rate measurements in arbitrary units ($\Delta F/\text{min}$).

Table 2 Activation parameters for heat inactivation of prolyl oligopeptidase

The reactions were carried out in the temperature range 46–50 °C. The standard errors in the activation parameters are less than 2%.

pH	[NaCl] (M)	ΔG^* (kJ/mol)	ΔH^* (kJ/mol)	ΔS^* (J/mol per K)
8.02	—	130	526	1328
7.97	0.5	119	418	1003
6.05	—	124	462	1132
6.05	0.5	116	395	936

oligopeptidase is at 332 nm, indicating that most tryptophans are located in the interior of the enzyme. Interestingly, the acid-induced unfolded state has an emission maximum identical to that of the native state (332 nm) whereas the enzyme denatured at high pH (12.3) shows a broad maximum at 350 nm. The urea-denatured forms also display red-shifted emission maxima both at pH 7 (350 nm) and pH 1.8 (350 nm), indicating that the tryptophans experience more hydrophilic environments.

Thermal inactivation

The effect of salt on heat inactivation of prolyl oligopeptidase has also been studied. The inactivation of most proteins follows first-order kinetics. In contrast, two phases can be observed in Figure 6, indicating that the denaturation of prolyl oligopeptidase is more complex. The first phase cannot be seen clearly under all conditions. Whether or not both phases are seen depends upon pH, temperature and ionic strength. Generally, the first reaction is dependent on the temperature to a smaller extent than the second one. Because the first rate constant cannot be determined with sufficient accuracy in all cases, the thermodynamic parameters are calculated only for the rate-limiting, second, reaction. It can be seen from Table 2 that the Gibbs free energy of activation (ΔG^*) is lower in the presence of NaCl, i.e. the enzyme is less stable, in particular at pH 6. This structure is also less ordered, probably a looser species, as suggested by the smaller ΔS^* .

DISCUSSION

Kinetic analysis of the hydrolysis of Z-Gly-Pro-Nap by prolyl oligopeptidase has previously shown that the reaction is remarkably sensitive to the ionic strength of the medium, and that the pH-rate profile has a doubly sigmoidal form. It has therefore been concluded that the enzyme has two forms that exhibit different activities and interconvert with changing pH [9]. The structural bases of the salt effects may be explored by studying the denaturation of the protein with various methods.

The intrinsic fluorescence of the tryptophan residues of proteins is a sensitive probe for protein denaturation. Prolyl oligopeptidase contains twelve tryptophan residues distributed throughout the molecule. The protease domain, which amounts to one-third of the molecule, has four tryptophan residues. The unfolding of the protein with changing pH is shown in Figure 1. It can be seen that the two forms of the enzyme, existing at pH 6 and 8, respectively, have similar conformations with respect to the positions of the tryptophan residues, and these structures are not affected by the addition of salt. However, a conformational change occurs in the alkaline pH region, and this starts at a lower pH in the presence of 0.5 M NaCl, indicating that the salt destabilizes the protein. It is also seen that the acidic limbs of the curves are practically independent of the presence of salt, and that the unfolding is less complete at acidic than at alkaline pH.

The pH-induced denaturation may be interpreted in terms of the different stabilities of the low-pH and high-pH enzyme forms. Because the isoelectric point of prolyl oligopeptidase is in the slightly acidic region, pH 4.8–4.9 [2], but is 5.71 calculated from the amino acid sequence, the high-pH form possesses a considerable amount of net negative charge (-26.96 , calculated at pH 8.0), which repels the hydroxide ion. Electrostatic shielding by NaCl facilitates the attack of hydroxide ion and the consequent unfolding. The low-pH form is close to the isoelectric point, where the positive and negative charges are balanced to a great extent (the calculated values being -3.45 at pH 6.0, and $+2.43$ at pH 5.5). This could explain why the unfolding of the low-pH form is virtually unaffected by the ionic strength.

Unfolding of prolyl oligopeptidase in the presence of urea is significantly affected by the presence of NaCl (Figure 3 and Table 1). As a weak salting-out agent, NaCl often stabilizes the protein structure by a mechanism of preferential hydration of the protein and preferential exclusion of NaCl from the water shell formed around the protein [11]. In contrast, prolyl oligopeptidase is destabilized in the presence of NaCl, as shown in Figure 3 and Table 1. This implies that NaCl penetrates into the zone of

preferential hydration and binds to the enzyme at specific sites. The stability of the protein depends on the balance between the binding and exclusion of NaCl. Apparently, prolyl oligopeptidase possesses sufficient ionizing groups on its surface, which shifts the balance towards the binding of the salt. In addition, the binding of NaCl can weaken possible attractive electrostatic forces that may stabilize the protein conformation.

The kinetics of urea denaturation (Figure 4) and heat inactivation (Figure 6) indicate that prolyl oligopeptidase is composed of two domains of different stabilities. This is reasonable for a large peptidase containing an unknown domain in addition to the catalytic one.

The heat inactivation experiment is consistent with the importance of the hydration shell inferred from the denaturation by urea. The breakdown of the larger array of water molecules around the protein in the absence of salt requires a higher enthalpy of activation, and the decomposition of such an ordered state results in a more positive entropy of activation. This is clearly seen from Table 2, showing higher ΔH^* and ΔS^* in the absence of salt, in agreement with a more ordered, more compact structure. It therefore appears that the reduced water shell in the presence of 0.5 M NaCl can account for the increased catalytic activity.

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