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Prolyl oligopeptidase catalysis

Reactions with thiono substrates reveal substrate-induced conformational change to be the rate-limiting step

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Prolyl oligopeptidase, a member of the new family of serine proteases, exhibits significant mechanistic differences compared with the enzymes of the chymotrypsin and subtilisin families. Our kinetic study using the thiono substrate, benzyloxycarbonyl-Gly-Pro[CS-NH]-2-naphthylamide suggests that the putative oxyanion binding site is important in prolyl oligopeptidase catalysis, although to a lesser extent than in the chymotrypsin- and subtilisin-catalyzed reactions. By using another thiono substrate, benzyloxycarbonyl-Gly[CS-NH]Pro-2-naphthylamide, it is demonstrated that the distant S2P2 hydrogen bond (formed between the S2 subsite and P2 peptide residue) makes a greater contribution to catalysis than does stabilization by the oxyanion binding site involved directly in the bond cleavage. In contrast to the reactions catalyzed by chymotrypsin and subtilisin, no kinetic deuterium isotope effect is apparent in the acylation of prolyl oligopeptidase measured either with the specific benzyloxycarbonyl-Gly-Pro-2-naphthylamide, or with the very poor substrate, benzyloxycarbonyl-Gly-Pro[CS-NH]-2-naphthylamide. This indicates that the rate-limiting conformational change is induced by the substrate.

Enzyme mechanism; Rate-limiting step; Conformational change; Subsite specificity; Thiono substrate

1. INTRODUCTION

Proline, as an imino rather than an amino acid, does not possess the free hydrogen atom that is required to form an S1P1 hydrogen bond with a backbone carbonyl oxygen during the catalysis performed by chymotrypsin and subtilisin (cf. [1,2]). Indeed, these serine enzymes are unable to hydrolyze the peptides at proline residues. On the other hand, prolyl oligopeptidase, a protagonist of the new family of serine peptidases [3–5], readily cleaves peptide bonds at the carboxamido group of proline residues. In the absence of the S1P1 interaction (a hydrogen bond between the backbone -NH at subsite S1 and the carbonyl oxygen of the P1 residue of a peptide substrate), the neighboring S2P2 hydrogen bond might help prolyl oligopeptidase in assuming the proper conformation for reaction. Such an S2P2 interaction can be probed with the use of thiono substrates containing a sulfur atom in place of the carbonyl oxygen of residue P2 [6].

Thiono substrates having a sulfur atom in the P1 residue have proved to be helpful in studying the oxyanion binding site of serine proteases [7]. The oxyanion binding site stabilizes the tetrahedral intermediate by providing hydrogen bonds to its charged oxygen atom, as indicated by X-ray crystallographic analysis [1,2,8]. Since the tertiary structure of prolyl oligopeptidase is unknown, the study of its reaction with thiono substrates appears to be a useful approach to reveal the importance of the oxyanion binding site in the catalysis. Indeed, the very low rate found in the present study using 2-Gly-Pro^t-Nap has shown that the oxyanion binding site is catalytically competent.

Prolyl oligopeptidase has been shown to exhibit a different rate-limiting step compared with chymotrypsin and subtilisin [9,10]. Whereas with these enzymes the rate-limiting step is a general base-catalyzed chemical process, it is probably a substrate-induced conformational change with prolyl oligopeptidase.

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Abbreviations: Z, benzyloxycarbonyl; Boc, *t*-butyloxycarbonyl; Nap, 2-naphthylamide; DTE, dithioerythritol; Gly^t and Pro^t, represent Gly and Pro, respectively, containing a sulfur atom in place of the carbonyl oxygen.

2. MATERIALS AND METHODS

2.1. *Substrates*

Z-Gly-Pro-Nap was purchased from Bachem Inc.

Z-Gly^t-Pro-Nap was synthesized as follows. Z-Gly^t-Pro-OMe was prepared as described earlier [11] and hydrolyzed with 1.5 equivalents

of sodium hydroxide (1 M, in a 1:1 mixture of methanol/water). The resulting Z-Gly^t-Pro-OH was coupled with 2-naphthylamine by the mixed anhydride method [12], using isobutyl chloroformate and *N*-methyl morpholine in tetrahydrofuran. The crude product was purified by flash chromatography on a silica gel (Kieselgel 60, E. Merck) column using dichloromethane-ethyl acetate (9:1) as eluent. Calculated: C 67.08, H 5.63, N 9.39, S 7.16; found: C 66.77, H 5.76, N 9.38, S 7.02.

The synthesis of Z-Gly-Pro^t-Nap started with Boc-Pro-OH (Bachem Inc.) which was coupled with 2-naphthylamine [12] as described above. The thiono derivative of Boc-Pro-Nap was prepared in dry toluene with 0.5 equivalents of Lawesson's reagent [13]. The crude product (Boc-Pro^t-Nap) was purified by flash chromatography as above. The Boc group was removed with HCl/dioxane, and the product (HCl.H-Pro^t-Nap) was coupled with Z-Gly-OH by the mixed anhydride method [12] to yield Z-Gly-Pro^t-Nap. The purity was checked by TLC (DC Alufolien, Kieselgel 60, E. Merck) in a variety of eluents. Using the chlorine/tolidine or iodine test, no detectable impurities were found. Calculated: C 67.08, H 5.63, N 9.39, S 7.16; found: C 66.27, H 5.66, N 9.08, S 7.40.

2.2. *Prolyl oligopeptidase*

The enzyme was prepared from pig muscle as described [14]. Its activity was determined fluorometrically with Z-Gly-Pro-Nap [9], using a Jasco FP 777 spectrofluorometer. The excitation and emission wavelengths were 340 and 410 nm, respectively, with a slit of 1.5 nm at each wavelength. The photomultiplier gain was set to 'high'. The hydrolyses of the thiono derivatives were measured in the same way, but at much higher enzyme concentrations (2–6 μM). Cells with emission and excitation path-lengths of 0.4 and 1.0 cm, respectively, were used. The concentration of the enzyme was determined at 280 nm [9].

2.3. *Kinetics*

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

The k_{cat} and K_m values were determined from initial rates, using substrate concentrations below and above the K_m value. The data were calculated by fitting the experimental points to the Michaelis–Menten equation using non-linear regression analysis.

The pH dependence of the rate constants were measured in 50 mM HEPES buffer or in a three-component buffer system [15] containing 0.5 M NaCl, 1 mM EDTA and 1 mM DTE. At the end of the reaction, the pH of each sample was determined and found to be identical with the initial value. The theoretical curve for a bell-shaped pH rate profile was calculated by non-linear regression analysis according to

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

where k(limit) stands for the pH-independent maximum rate constant and K_1 and K_2 represent the acidic ionization constant of the base and acid, respectively.

Rate-limiting general base catalysis was tested in heavy water (99.9%). Values of p^2H of deuterium oxide solutions can be obtained from pH meter readings according to the relationship p^2H = pH(meter reading) + 0.4 [16].

3. RESULTS AND DISCUSSION

3.1. *Oxyanion binding site*

By accommodating the charged oxygen atom of the tetrahedral intermediate, the oxyanion hole, or preferably the oxyanion binding site, makes an important contribution to the catalysis of chymotrypsin and subtilisin. This is apparent from X-ray crystallographic studies [8] and from the finding that these enzymes are practically unable to hydrolyze thiono substrates having a sulfur atom in place of the carbonyl oxygen [7]. Compared with the oxo substrates, the rate constants for the thiono substrates are lower by more than four orders of magnitude, which is the limit of the determinations [7]. The low rate may not be attributed to a lower chemical reactivity because the reactivities of the oxo and the corresponding thiono compounds are similar [7]. The existence of an oxyanion binding site in the enzymes of the prolyl oligopeptidase family has not yet been investigated. To obtain information about the catalytic importance of oxyanion binding site, we have measured the specificity rate constant of the reaction of prolyl oligopeptidase with a thiono substrate, Z-Gly-Pro^t-Nap. The parent compound of this thiono substrate is one of the best substrates of prolyl oligopeptidase, and its kinetic behavior has already been analyzed [9]. Fig. 1 shows the pH dependence of the rate constant for the thiono derivative. The points, within experimental error, conform to a bell-shaped pH-rate profile, and the calculated value of k(limit) (514 ± 22 $M^{-1} \cdot s^{-1}$) is 5,622 times lower than that found with the oxo substrate (2,890 ± 126 $mM^{-1} \cdot s^{-1}$, [9]). Although less than those found with chymotrypsin and subtilisin, this decrease is sufficiently large to demonstrate the importance of the oxyanion binding site in the catalysis performed by prolyl oligopeptidase, too.

Since the second-order rate constant is equal to k_{cat}/K_m [17], we tried to determine how these two parameters are affected by the change of oxygen to sulfur. For reasons of solubility the K_m for the Z-Gly-Pro-Nap reaction could not be measured under the standard condition, i.e. at 0.03% acetonitrile. Therefore, the acetonitrile concentration was increased up to 0.24%, which inhibited the enzyme, but no more than by 10%. Under this condition a K_m of 10 μM was obtained. On the other hand, the K_m for the thiono derivative was much higher, by about 1 order of magnitude. However, it was not possible to determine the precise value because even the highest substrate concentration employed was below the K_m value. This indicated that the increase in the K_m alone cannot account for the almost 4 orders of magnitude decrease in k_{cat}/K_m, but a significant reduction in k_{cat} should also contribute to the effect. It appears then that the substitution of sulfur for oxygen leads to destabilization of both the enzyme–substrate complex and the transition state, but the latter to a much higher extent. This may be visualized as a weaker and improper substrate binding at the oxyanion site, and this may be attributed to the larger size of the sulfur atom.

3.2. *Substrate-induced conformational change*

It has previously been shown that in the catalysis of prolyl oligopeptidase, conformational change rather than a chemical step is the rate-limiting process with

Z-Gly-Pro-Nap [9,10]. The very low rate constant obtained with Z-Gly-Pro^{t}-Nap offers a possibility to examine two basic mechanisms whereby the rate-limiting conformational change may occur. First, the enzyme may have two conformations (E and E′), but only one (E′) can bind the substrate (eqn. 2). Secondly, the enzyme has one inactive conformation (E), which becomes active (E′) only upon substrate binding (eqn. 3).

$$E \rightleftharpoons E' + S \rightleftharpoons E'S \rightarrow E'S' + P \quad (2)$$

$$E + S \rightleftharpoons ES \rightleftharpoons E'S \rightarrow E'S' + P \quad (3)$$
$$\updownarrow$$
$$E'$$

The rate-limiting conformational change according to the first mechanism is independent of substrate binding, whereas it is a substrate-induced process in the second case. To distinguish between the two mechanisms, we determined the rate-limiting step when turning from a specific (Z-Gly-Pro-Nap) to a poor substrate (Z-Gly-Pro^{t}-Nap). Since the hydrolysis of the specific substrate is very fast [9], the conformational change in the free enzyme must also be a rapid process. Consequently, the conformational change cannot be rate-limiting with the slow thiono substrate provided that eqn. 2 holds. In this case the chemical step, i.e. the general base-catalyzed acyl-enzyme formation, should be rate-limiting. On the other hand, if the physical step remains rate-limiting with the slow substrate (eqn. 3), general base catalysis may not be observed kinetically. General base catalysis can be detected by performing the reaction in deuterium oxide, where it becomes 2–3 times slower (cf. [1,2]). However, kinetic deuterium isotope effect was not detected with the specific oxo substrate [9]. As seen from Fig. 1, a similar result was obtained with the poor thiono substrate, the ratio of $k(H_2O)$ to $k(^2H_2O)$ being about 1.1. We did not observe a kinetic deuterium isotope effect with a slow oxo substrate either (Polgár, L., unpublished result). These findings rule out the existence of a rate-limiting equilibrium between E and E′ (eqn. 2), and support a substrate-induced conformational change as the rate-limiting step with both the specific and the non-specific substrates (eqn. 3).

3.3. *Subsite binding*

Chymotrypsin and subtilisin have extended binding sites that form β-pleated sheets with polypeptide substrates [1,2]. This involves several hydrogen bonds between the enzyme and substrate, such as that formed between the backbone carbonyl oxygen of S1 and the amide nitrogen of P1. However, there is no S2P2 hydrogen bond in chymotrypsin because the -NH group of P2 points to the solvent rather than to the protein. An S2P2 hydrogen bond may be important in the prolyl oligopeptidase catalysis, where the formation of S1P1 hydrogen bond is impossible. This was tested by using Z-Gly^{t}-Pro-Nap, having a sulfur atom in place of the

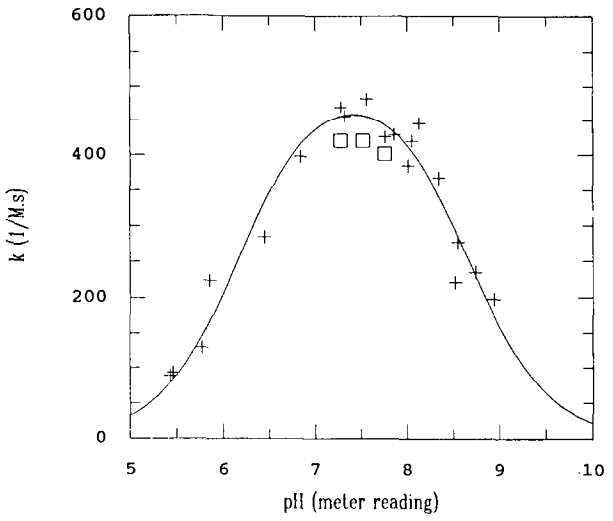

Fig. 1. pH dependence of acylation of prolyl oligopeptidase with Z-Gly-Pro^{t}-Nap. The rate constants were measured at 25°C in water (+) and in heavy water (□). The continuous line represents the theoretical curve calculated using eqn. 1 with parameters k(limit) = 514 $M^{-1} \cdot s^{-1}$, pK_1 = 6.17, and pK_2 = 8.65. Curve fitting was performed by non-linear regression analysis.

glycine carbonyl oxygen. An earlier study with cysteine proteases of the papain family has shown that substitution of sulfur for the carbonyl oxygen of the P2 residue resulted in about 200-fold decrease in the specificity rate constant [6], in accordance with X-ray crystallographic studies indicating the formation of S2P2 hydrogen bond. In the reaction of Z-Gly^{t}-Pro-Nap with prolyl oligopeptidase the rate decrease was much greater, even greater than with Z-Gly-Pro^{t}-Nap. Actually, no reaction was observed, and this indicated that the reduction in rate was at least five orders of magnitude. Consequently, the S2P2 hydrogen bond appears to be even more important than the interaction between oxyanion binding site and the tetrahedral intermediate. It is not possible that such a large effect would be duc to different *cis-trans* equilibria with the oxo and thiono compounds. This would require the thiono substrate to be practically in the *cis* conformation, in contrast to the experimental finding that the equilibrium is shifted to the *trans* conformer both with the oxo and the thiono compound [18]. It should also be noted that, contrary to repeated assertions, the sulfur atom of a thioamide can readily form hydrogen bond with the peptide -NH group [18]. The lack of reaction with the thiono substrate may be best explained in terms of the relatively large size of the sulfur atom, which affects the S2P2 interaction, so that the resulting slight distortion is transmitted to the scissile bond. The catalysis of serine proteases exhibits very stringent stereochemistry [1,2], and unfavourable binding of the substrate may not be compensated for by the restricted freedom of motion of the catalytic groups.

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