Prolyl endopeptidase catalysis

A physical rather than a chemical step is rate-limiting

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Prolyl endopeptidase represents a new family of serine proteases, and it has a mechanistic feature distinct from that of the enzymes of the extensively studied chymotrypsin and subtilisin families. The rate-determining step in the catalysis of serine proteases is a general base/acid-catalysed chemical step. For prolyl endopeptidase, however, the chemical step is not rate-limiting, as demonstrated by using substrates with different leaving groups. It is known that the acylation of chymotrypsin and subtilisin proceeds faster by several orders of magnitude with the activated nitrophenyl ester than with the corresponding amide substrates. In contrast, for the acylation of prolyl endopeptidase similar rate constants were obtained with nitrophenyl ester and several amide substrates. This result, combined with kinetic isotope studies [Polgár (1991) Eur. J. Biochem. **197**, 441–447], offers strong evidence that a physical step, presumably a conformational change associated with substrate binding, is the rate-determining step in the prolyl endopeptidase catalysis.

INTRODUCTION

Prolyl endopeptidase, a large (76 kDa) intracellular serine protease, preferentially hydrolyses proline-containing peptides at the carboxy end of proline residues [1,2]. 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 chymotrypsin or subtilisin family of enzymes, but represents a new family of serine proteases [5,6]. Its kinetic behaviour is also different from that of the extensively studied small (25–30 kDa) extracellular serine proteases such as chymotrypsin and subtilisin. These enzymes exhibit a simple pH–rate profile controlled by a single ionizing group of pK_a about 7, and display kinetic deuterium isotope effects of 2–3, which is indicative of rate-determining general base/acid catalysis [7,8]. In contrast, prolyl endopeptidase shows a doubly sigmoidal curve and has practically no kinetic isotope effect [9]. The lack of kinetic deuterium isotope effects suggests, but does not prove, that the rate-determining step for prolyl endopeptidase catalysis is a conformational change. Therefore in the present work a new approach was employed, which utilized substrates having different leaving groups. Specifically, a substrate with a better leaving group is known to react with the enzyme at a higher rate provided that the chemical step is rate-limiting. On the other hand, the leaving group effect is not significant if a physical step, e.g. conformational change or substrate binding, controls the catalysis.

MATERIALS AND METHODS

Prolyl endopeptidase was prepared as described previously [9]. Z-Gly-Pro-NH-Nap, Z-Gly-Pro-NH-Mec, Z-Gly-Pro-NH-Np and Z-Gly-Pro-O-Np were purchased from Bachem. The rate constants were measured under first-order conditions, i.e. at substrate concentrations below the K_m values (Table 1). The K_m values were obtained from Michaelis–Menten kinetics by non-linear-regression data analysis. The second-order rate constants were obtained by dividing the first-order rate constant by the enzyme concentration in the reaction mixture [9]. The hydrolysis of the 4-nitroanilide substrate was monitored spectrophotometrically at 410 nm [10]. The reaction of Z-Gly-Pro-O-Np was measured at 400 nm. Under the conditions employed spon-

taneous hydrolysis of this substrate was less than 15% of the enzymic hydrolysis. The reactions of Z-Gly-Pro-NH-Nap and Z-Gly-Pro-NH-Mec were monitored spectrofluorimetrically. The excitation and emission wavelengths were 340 nm and 410 nm [9] respectively for the former and 370 nm and 440 nm [11] respectively for the latter compound. The enzyme and substrate concentrations employed are shown in Table 1. The acetonitrile concentration in the reaction mixture was less than 0.1%, which did not appreciably affect the rate constants.

RESULTS AND DISCUSSION

Hydrolysis by serine proteases proceeds through the formation of an acyl-enzyme intermediate according to eqn. (1), where E, S, ES, ES', P_1, P_2, K_s, k_2 and k_3 are the free enzyme, the substrate, the enzyme–substrate complex, the acyl-enzyme, the leaving group of the substrate (alcohol or amine), the acyl moiety of the substrate, the dissociation constant of the ES complex, the first-order acylation constant and the deacylation constant respectively [7,8]:
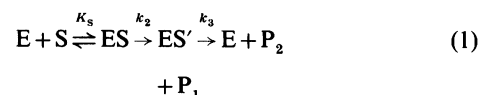
$$E + S \overset{K_s}{\rightleftharpoons} ES \overset{k_2}{\rightarrow} ES' \overset{k_3}{\rightarrow} E + P_2 \qquad (1)$$

$$+ P_1$$

$$E + S \overset{k}{\rightarrow} ES' + P_1 \qquad (2)$$

Table 1. Reaction conditions for measuring the pseudo-first-order rate constants

K_m values were determined in 0.05 M-Hepes buffer, pH 7.5–8.0, containing 0.5 M-NaCl and 0.2–0.5% acetonitrile.

Substrate	[Enzyme] (nM)	[Substrate] (μM)	K_m (μM)
Z-Gly-Pro-O-Np	8–16	4–8	80 ± 25
Z-Gly-Pro-NH-Nap	3–6	1–2	14 ± 1.4
Z-Gly-Pro-NH-Mec	3–6	0.2–0.4	3.4 ± 0.4
Z-Gly-Pro-NH-Np	10–20	2–4	24 ± 4.7

Abbreviations used: Z-, benzyloxycarbonyl-; -NH-Nap, 2-naphthylamide; -NH-Np, 4-nitroanilide; -O-Np, 4-nitrophenyl ester; -NH-Mec, 7-(4-methylcoumaryl)amide.

Table 2. Effects of the leaving group on the rate constant for prolyl endopeptidase

Measurements were made in 0.05 M-Hepes buffer, pH 8.0, at 25 °C.

	k (mM$^{-1}\cdot$s^{-1})		
Substrate	NaCl absent	0.5 M-NaCl present	k_{NaCl}/k_0
Z-Gly-Pro-O-Np	1180 ± 100	2570 ± 200	2.2
Z-Gly-Pro-NH-Nap	1240 ± 80	3050 ± 280	2.5
Z-Gly-Pro-NH-Mec	990 ± 70	1980 ± 70	2.0
Z-Gly-Pro-NH-Np	460 ± 35	1320 ± 70	2.9

The second-order acylation rate constant (k in eqn. 2) involves both binding and acylation. This rate constant, also called specificity constant, is usually dependent on the nature of the leaving group of the substrate. Thus acylation of chymotrypsin is faster by five orders of magnitude with a 4-nitrophenyl ester substrate than with the corresponding amide [12]. The nitrophenyl esters are activated compounds and thus they are hydrolysed spontaneously even at neutrality. This deleterious effect has to be taken into account when measuring the enzymic hydrolysis of the nitrophenyl ester substrates, such as Z-Gly-Pro-O-Np shown in Table 2. On the other hand, the corresponding amide derivatives are not hydrolysed even at pH 11 (results not shown).

Table 2 shows the reactions of prolyl endopeptidase with different derivatives of Z-Gly-Pro in the absence and in the presence of 0.5 M-NaCl. It has previously been demonstrated that the rate constants for the prolyl endopeptidase reactions increase considerably with increasing ionic strength. This is a further difference from the chymotrypsin- and subtilisin-catalysed reactions. It is apparent from Table 2 that the 4-nitrophenyl ester substrate has a similar rate constant to those of the amide derivatives, regardless of the ionic strength of the reaction mixture. Only the 4-nitroanilide derivative has a significantly lower rate constant relative to the nitrophenyl ester, but even this difference is rather small compared with the several orders of magnitude expected from the very different reactivities of these compounds. The similarity between the ester and amide substrates is also manifested in the K_m values (Table 1), which only extend over one order of magnitude, the K_m for the nitrophenyl ester being the highest. In contrast, with chymotrypsin the nitrophenyl ester relative to the amide substrates displays a lower

K_m by several orders of magnitude. In this case the chemical step is rate-limiting and thus the higher reactivity of the nitrophenyl ester can prevail. Specifically, the much greater k_2 relative to that of the amide substrates results in a much lower K_m, with $K_m = K_s k_3/(k_2+k_3)$. It may be further noted that the substrate binding (K_s) may not exert a compensatory effect of many orders of magnitude because the nitrophenyl ester and the amide substrates, in particular the nitroanilide, have very similar structures. Accordingly, prolyl endopeptidase, the representative of a new family of serine proteases [5,6], displays a distinct mechanistic feature when compared with the members of the chymotrypsin and subtilisin families. This is consistent with the earlier observation that general base catalysis is not seen in the acylation reaction [9]. The results imply that a physical step rather than a chemical step is rate-limiting. As to the nature of the physical step, two possibilities may be considered: (1) diffusion-controlled binding of the substrate, and (2) conformational change associated with the catalytic process. As the first possibility has previously been excluded by kinetic studies [9], the second possibility seems to be the most probable explanation. The conformational change may be associated with the enzyme–substrate interaction. Indeed, prolyl endopeptidase is much larger than chymotrypsin and subtilisin, and in addition to a protease domain it may also contain other domains of unknown function. The relative movements of these domain structures may have catalytic consequences.

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