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Cleavage of peptide bonds bearing ionizable amino acids at P_1 by serine proteases with hydrophobic S_1 pocket

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ABSTRACT

Enzymatic hydrolysis of the synthetic substrate succinyl-Ala-Ala-Pro-Xxx-pNA (where Xxx = Leu, Asp or Lys) catalyzed by bovine chymotrypsin (CHYM) or *Streptomyces griseus* protease B (SGPB) has been studied at different pH values in the pH range 3–11. The pH optima for substrates having Leu, Asp, and Lys have been found to be 7.5–8.0, 5.5–6.0, and ~10, respectively. At the normally reported pH optimum (pH 7–8) of CHYM and SGPB, the substrate with Leu at the reactive site is more than 25,000-fold more reactive than that with Asp. However, when fully protonated, Asp is nearly as good a substrate as Leu. The p*K* values of the side chains of Asp and Lys in the hydrophobic S_1 pocket of CHYM and SGPB have been calculated from pH-dependent hydrolysis data and have been found to be about 9 for Asp and 7.4 and 9.7 for Lys for CHYM and SGPB, respectively. The results presented in this communication suggest a possible application of CHYM like enzymes in cleaving peptide bonds contributed by acidic amino acids between pH 5 and 6.

1. Introduction

Serine proteases are ubiquitous and exist in a wide spectrum of specificities [1,2] with the capability of hydrolyzing virtually any peptide bond. Serine proteases that preferentially hydrolyze peptide bonds contributed by hydrophobic (Ala, Val, Leu, Ile, Met, Phe, Tyr, Trp), cationic (Lys, Arg), anionic (Asp, Glu), and polar (Ser, Gln) side chains at P_1 (Schechter–Berger nomenclature [3]) as well as proline at P_1 are known to exist [1]. Of these specific classes of serine proteases, the ones that hydrolyze at hydrophobic and cationic amino acids at P_1 are the best characterized.

A great deal has been learned about the size, shape, and chemistry of the primary specificity pocket (S_1 pocket) of serine proteases by studying their interaction with Standard Mechanism protein inhibitors [4–6]. The inhibitor–protease complex mimics the substrate protease transition state complex, and, therefore, the good correlation often found [7,8] between association equilibrium constant (K_a) values for a set of inhibitor variants and the corresponding k_{cat}/K_m values for substrates is not surprising. In Standard Mechanism inhibitors the reactive site peptide bond never shifts, even if the P_1 residue is deleterious for the target enzyme [9,10]. This is not the case with substrates, which can readily shift their scissile peptide bond (their reactive site) so as to undergo cleavage according to the specificity of the serine protease. Thus serine proteases with a hydrophobic S_1 pocket, such as bovine chymotrypsin (CHYM) and *Streptomyces griseus* protease B (SGPB), have not been reported to hydrolyze peptide bonds in which the P_1 residue is contributed by Asp or Glu. In contrast to substrates, Standard Mechanism inhibitors with a P_1 Asp or Glu are able to bind, albeit weakly, with CHYM or SGPB [4]. Our investigations on the binding of serine proteases to variants of turkey ovomucoid third domain (OMTKY3) containing all possible ionizable residues at P_1 led to the finding of large shifts of the pK_a values of these P_1 residues when in the inhibitor–protease complex [11]. We ask a similar question here for the hydrolysis of synthetic substrates bearing Asp and Lys at the reactive site by two serine proteases that have a neutral hydrophobic S_1 pocket, CHYM and SGPB. The pH-dependence of the hydrolysis of these substrates reveals interesting results that are discussed in this paper.

2. Materials and methods

TPCK treated bovine chymotrypsin was obtained from Sigma. SGPB was purified from a commercial (Sigma) preparation of Pronase as described earlier [12]. The purity of SGPB was confirmed by ion exchange and size exclusion column chromatographies, while its identity was confirmed by amino acid composition determination.

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Synthetic protease substrates, such as Suc-Ala-Ala-Pro-Leu-pNA (Suc-AAPL-pNA), Suc-Ala-Ala-Pro-Asp-pNA (Suc-AAPD-pNA), and Suc-Ala-Ala-Pro-Lys-pNA (Suc-AAPK-pNA), were purchased from BACHEM. Various buffers and solutions used with their pH ranges in parentheses were: 0.1 M NaCl–NaOH (pH 9.5–11.0), 0.1 M Tris–HCl (pH 7.0–9.0), 0.1 M Bis–Tris (pH 6.0–7.0), 0.1 M sodium acetate–acetic acid (pH 4.0–5.5), 0.1 M glycine buffer (pH 3.0–3.5). Stock solutions of 100 mM substrates were prepared in dimethyl sulfoxide (DMSO).

Kinetics of enzymatic hydrolysis of substrates were performed at different pH values and at 22 °C by following the change in absorbance over a period of 120–300 s in the wavelength range 380–410 nm on a Hewlett–Packard HP8453 diode array spectrophotometer. The kinetic data were automatically corrected for any light scattering by continuously subtracting the absorbance values in the wavelength range 650–700 nm during the kinetic run. The rates of hydrolysis of substrates obtained as change in absorbance per second were converted to moles of product (p-nitroaniline) produced per second (V_o) by using a molar extinction coefficient of 8800 M^{-1} cm^{-1} [13] for p-nitroaniline. K_m and k_{cat} values were calculated from the rates of hydrolysis of substrates at different substrate concentrations using the Lineweaver–Burk equation. The expression of the P_1Q variant of OMTKY3 and the procedure for the measurement of the association equilibrium constant are described in an earlier publication [4].

3. Results and discussion

Hydrolysis of Suc-AAPL-pNA by CHYM and SGPB was studied at different pH values between pH 4.0 and pH 10.0 (Fig. 1A). CHYM and many other serine proteases show maximum enzymatic activity between pH 7 and 8. The decline in enzyme activity below pH 7 is well known and has been attributed to protonation of catalytic histidine 57 (chymotrypsinogen numbering). The decrease in activity above pH 8 can be attributed to the deprotonation of the α-amino group of Ile16 and the disruption of charge–charge interaction between Ile16 and the side chain carboxyl group of Asp194 [14]. The salt bridge between Ile16 and Asp194 is characteristic of CHYM like enzymes but is either absent in other serine proteases or is substituted by a different type of salt bridge. For example, SGPB, a bacterial serine protease, has a salt bridge involving Asp194 and the side chain of Arg138 [14]. Because the arginine side chain has a very high pK_a value (>12), the interaction between Asp194 and Arg138 in SGPB is unlikely to be disrupted significantly below pH 11. This is consistent with the observation that the pH-dependence of k_{cat}/K_m for Suc-AAPL-pNA with SGPB shows a flat region between pH 8 and pH 10 (Fig. 1A). Substrates with other neutral hydrophobic side chains (such as Ala, Phe, Val) in place of Leu gave results similar to those shown in Fig. 1A.

The pH-dependence of the association equilibrium constant, K_a, of inhibitors with non-ionizable residues at P_1 has the same shape as that for the pH-dependence of the hydrolysis of Suc-AAPL-pNA (Fig. 1A). The pH-dependence of K_a of P_1 variants of OMTKY3 with different serine proteases has been extensively studied in our laboratory at Purdue. As an example, the pH-dependence of K_a of P_1Q variant of OMTKY3 with CHYM and SGPB is shown here (Fig. 1B). The strong correlation between free energy of association of inhibitors ($\log K_a$) and transition state free energies of substrates ($\log k_{cat}/K_m$) has been observed by us as well as by others [4,8,15,16].

The two serine proteases used here have a hydrophobic S_1 pocket and therefore have a preference for amino acids with hydrophobic side chains at P_1. Thus OMTKY3 variants with Asp, Glu, Lys or Arg at P_1 bind weakly to these serine proteases [4]. We performed pH-dependent measurements of association equilibrium constants of such inhibitors with different serine proteases and found that when a charged side chain is present at P_1, it undergoes large pK shifts in the direction that makes it neutral in the S_1 pocket. Thus Asp and Glu undergo large increases in their pK_a values whereas Lys undergoes a decrease in its pK_a value [11,15].

The pH-dependence of k_{cat}/K_m determined for Suc-AAPD-pNA and Suc-AAPK-pNA with CHYM and SGPB (Fig. 2) shows a large shift in the optimum pH with both enzymes. When Asp is present at the scissile peptide bond, the optimum shifts to pH 5.0–5.5, whereas when Lys is present, the pH optimum is 10 or higher. These shifts are consistent with the binding of Asp and Lys in their neutral state. Suc-AAPD-pNA is an extremely poor substrate for CHYM and SGPB at pH 8 but becomes a moderately good substrate (~100-fold better) at pH 5.5. The pH-dependence of Asp and Lys substrates can be used to determine the pK_a values of these two residues when they are inserted in the S_1 pocket of the enzyme (designated as pK_c) in the transition state complex. We have used a procedure we initially described for inhibitors [11] to determine the pK_c values of Asp and Lys in the substrates. In this procedure an assumption is made that the difference in pH-dependence of $\log k_{cat}/K_m$ of two similar substrates, one with an ionizable side chain at P_1 and the other with a non-ionizable side chain at P_1, can solely be explained by the pK_a value of ionizable group in free-state (pK_f) and in bound state (pK_c). Free and bound states refer, respectively, to the substrate that is not bound to a protease and to the substrate that is bound to a protease. We used Suc-AAPL-pNA with Leu at P_1 as our representative non-ionizable P_1 residue. As an example, plots obtained with CHYM for Suc-AAPD-pNA and Suc-AAPK-pNA are shown in Fig. 3. In these plots, the difference in $\log k_{cat}/K_m$ values between Suc-AAPL-pNA and Suc-AAPD-pNA (or Suc-AAPK-pNA) designated as $\log R$ is plotted against pH. The pK_f values for

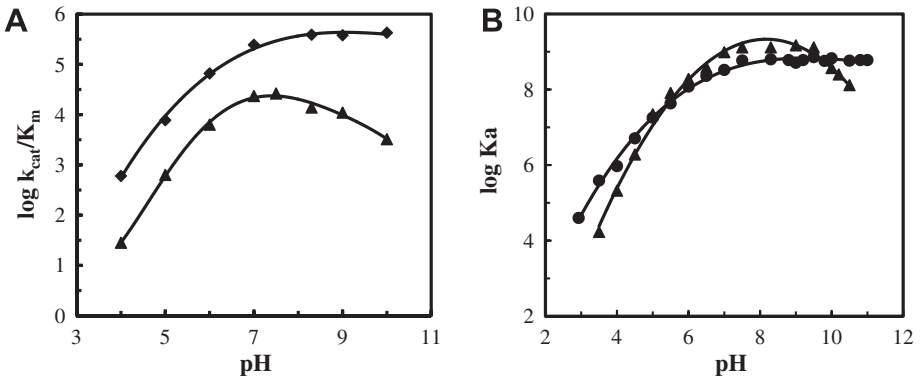

Fig. 1. (A) $\log k_{cat}/K_m$ plot for Suc-AAPL-pNA as a function of pH for CHYM (▲) and for SGPB (■). (B) $\log K_a$ plot for P_1Q variant of OMTKY3 for CHYM (▲) and SGPB (●).

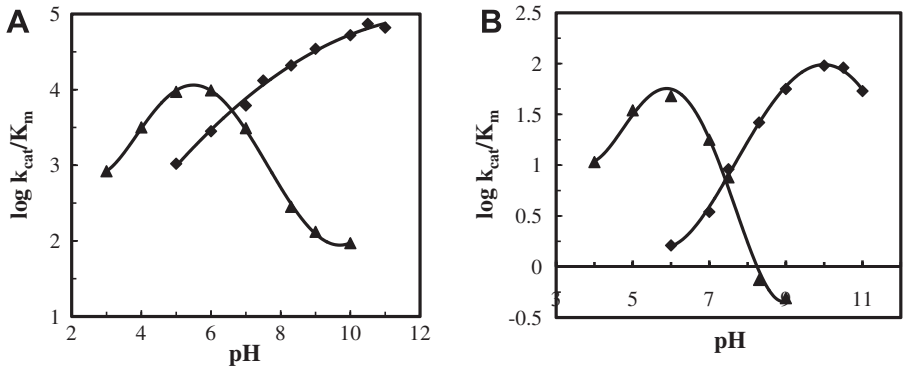

Fig. 2. pH-dependence of log k_{cat}/K_m for Suc-AAPD-pNA (▲) and Suc-AAPK-pNA (■) for SGPB (A) and CHYM (B).

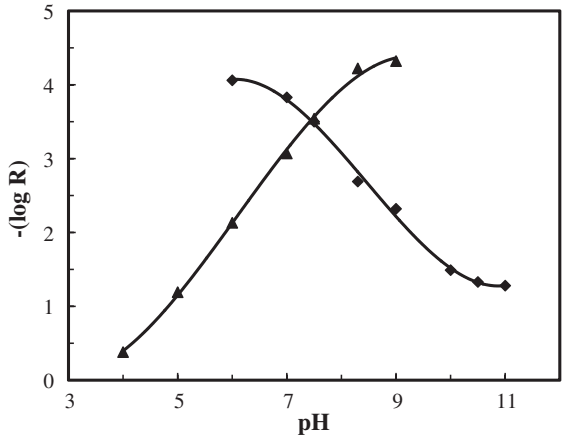

Fig. 3. pH-dependence of log R for Suc-AAPD-pNA (▲) and Suc-AAPK-pNA (■) for CHYM. For details, see text and Eq. (1).

Suc-AAPD-pNA and Suc-AAPK-pNA were determined by NMR spectroscopy [17]. The plot is fitted to the equation we developed earlier [11] for the pH-dependence of association equilibrium constants for the interaction of P_1 variants of OMTKY3 with serine proteases:

$$\log R = \log R^0 + \log(1 + 10^{(\mathrm{pH}-\mathrm{p}K_c)}) - \log(1 + 10^{(\mathrm{pH}-\mathrm{p}K_f)}) \quad (1)$$

In this equation, R^0 is the ratio of k_{cat}/K_m values for an ionizable residue to a non-ionizable residue at a pH where the ionizable residue is completely protonated and pK_f and pK_c are the pK_a values of the ionizable group in the substrate and enzyme–substrate transition state, respectively.

Fitting the data according to the relation given above allowed us to calculate the pK_c values for the ionizable residues, Asp and Lys, in the transition state complex of the substrates with the enzymes (Table 1). It is interesting to note that in the transition state complex with CHYM, Lys is a stronger acid (pK_c = 7.44) than Asp (pK_c = 8.92). The pK shifts of the Asp side chain were nearly identical for CHYM and SGPB (pK_c − pK_f = ΔpK = 5.0 ± 0.1) and represent one of the largest shifts reported for acidic amino acid side chains in proteins [11,18]. The pK shifts for the Lys side chain were quite different for CHYM and SGPB (see Table 1). The probable cause of this difference is the flexibility of the lysine side chain and its ability to adopt different conformations in the S_1 pocket of proteases. In an earlier study, we found that the pK shift for the P_1 lysine of BPTI is much smaller than that for the P_1 Lys variant of OMTKY3 in complex with CHYM [15]. The molecular explanation emerged from comparison of the X-ray crystallographic structures of the complexes with CHYM of BPTI [19,20] and P_1 Lys–OMTKY3 (1hja, unpublished). In the P_1 Lys-OMTKY3–CHYM complex, the P_1 Lys side chain is inserted deep into S_1 pocket of CHYM, whereas in the BPTI–CHYM complex, the P_1 Lys side chain goes into the S_1 pocket but bends back in such a manner that the ε-amino group is able to make a couple of hydrogen bonds with the backbone oxygens of Ser^{217} of the enzyme and Pro^{13} (P_3) of the inhibitor. The difference in the microenvironment of Lys in the BPTI–CHYM complex and in the P_1 Lys–OMTKY3–CHYM complex accounts for the difference in the pK_c value of these two lysine side chains. Thus it is likely that a similar mechanism accounts for the difference in the pK_c values of Lys in Suc-AAPK-pNA in its transition state complexes with CHYM and SGPB.

Why chymotrypsin-like enzymes are not reported to hydrolyze peptide bonds contributed by Asp and Glu is obviously due to extremely poor activity of these enzymes towards such peptides in the pH range 7–8. For example, at pH 8, k_{cat}/K_m for the hydrolysis of Suc-AAPD-pNA by CHYM was found to be 0.74 M^{-1} s^{-1} compared to 25,000 M^{-1} s^{-1} for Suc-AAPL-pNA. This is because in order bind to the S_1 pocket of CHYM, the Asp side chain must become protonated—energetically an expensive process at pH above 7. However, at low pH values (pH 4), k_{cat}/K_m for Suc-AAPD-pNA approaches that of Suc-AAPL-pNA (10.7 M^{-1} s^{-1} vs 28.2 M^{-1} s^{-1}). This is also indicated by a near zero log R value at low pH values (log R value of zero means that k_{cat}/K_m for Suc-AAPD-pNA and Suc-AAPL-pNA are equal).

The pK_c values reported here are very similar to the one found for P_1 Asp and Lys variants of OMTKY3 in complex with SGPB, CHYM, porcine pancreatic elastase and subtilisin Carlsberg [11,15] (Qasim and Laskowski, unpublished). Thus, the strong correlation reported between association equilibrium constants in inhibitors and k_{cat}/K_m values in substrates at pH 8.3 [4,8,15,16] for P_1 variants extends over the whole ionizable range of pH 3–11.

The results presented here clearly show that the pH optimum of a neutral serine protease is dependent on the nature of the side chain at the scissile peptide bond. The large preferential increase in the catalytic activity of neutral serine proteases for acidic amino

Table 1
pK_c values of Asp and Lys side chains in the S_1 pockets of CHYM and SGPB.

Substrates	pK values	CHYM	SGPB
Suc-AAPD-pNA	pK_f[a] = 3.84		
	pK_c	8.92	8.72
	ΔpK	5.08	4.88
Suc-AAPK-pNA	pK_f[a] = 10.51		
	pK_c	7.44[b]	9.71
	ΔpK	3.07	0.80

[a] pK_f values were determined by NMR as reported earlier [17].
[b] Qasim et al. [15] determined pK_c using Suc-AAPA-pNA as reference and found it to be 7.35. In this study, Suc-AAPL-pNA was used as reference.

acid side chains at pH 5.0–5.5 may have a physiological role. For example, granulocytes are rich in chymotrypsin-like serine proteases and have a pH between 5.0 and 6.0 [21]—a pH range well suited for cleavage at peptide bonds contributed by Asp and Glu. Regardless of any physiological role of the action of serine proteases on acidic amino acids at low pH, the results presented here suggest that such proteases can be used for cleavage at acidic amino acids by performing the cleavage at an appropriate low pH.

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