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Cysteine proteases: the S_2P_2 hydrogen bond is more important for catalysis than is the analogous S_1P_1 bond

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High hydrophobicity of the second amino acid N-terminal to the scissile bond (P_2 residue) is generally considered to be the major factor in the specificity of the substrates for cysteine proteases of the papain family. To examine the catalytic contribution of the S_2P_2 hydrogen bond apparent from X-ray crystallographic studies, the kinetics of Z-Phe-Gly-OEt and its thiono derivative were compared. The thiono compound contains a sulfur atom in place of the carbonyl oxygen of the phenylalanine residue. It was found that the specificity rate constants for the reactions of the thiono substrate with various cysteine proteases are lower by 2–3 orders of magnitude as compared to the corresponding rate constants for the oxo substrate. This remarkable effect is not expected in the light of previous studies indicating that the change from oxygen to sulfur in the P_1 residue was without an appreciable effect. The results are interpreted in terms of a distorted binding of the thiono substrate.

Cysteine protease; Papain; Protease mechanism; Substrate binding

1. INTRODUCTION

Interactions between the subsites of a protease and the amino acid residues of its peptide substrates can significantly contribute to catalytic rate enhancement [1]. In the case of papain and the related cysteine proteases, the S_2 subsite governs the specificity by accepting a hydrophobic amino acid residue, in particular phenylalanine, from the P_2 position of the substrate. The scissile bond is between the P_1 and P_1' residues (for notation see [2]). X-ray crystallographic and model building studies have suggested that besides the S_2P_2 hydrophobic interaction, the S_1P_1 and S_2P_2 hydrogen bonds are also required for catalysis [3–5]. A possible way of studying the importance of the -CO...NH- hydrogen bond formation has recently been introduced [6,7]. This approach involved the replacement of the carbonyl oxygen of the scissile bond by a sulfur atom, which implied the study of the thiono derivatives of natural substrates. The similarity of the hydrolyses of the oxo and the corresponding thiono substrates has unexpectedly shown that the S_1P_1 hydrogen bond may not be essential in cysteine protease catalysis [6,7]. In the present study, by the use of a similar approach it is demonstrated that the S_2P_2 hydrogen bond, though not directly associated with the scissile bond, is required for an efficient catalysis.

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Abbreviations: Z, benzyloxycarbonyl; Phe^t, thiophenylalanine

2. MATERIALS AND METHODS

2.1. *Enzymes*

Papain was purchased from Sigma (Type III, 2×crystallized), purified and assayed as described earlier [6]. Chymopapains were isolated from Sigma papaya latex (Papain: Type I, crude), as described [8]. Two chromatographically homogeneous fractions which displayed different basicity were used as chymopapains. Papaya peptidase A was also isolated from papaya latex [8] and purified as described [9]. The

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preparation and purification of cathepsin B is given elsewhere [10].

2.2. *Substrates*

The syntheses of Z-Phe-Gly-OEt [11] and Z-Phe^t-Gly-OEt [12] were as described.

2.3. *Kinetic measurements*

The hydrolysis by cysteine proteases proceeds according to eqn 1 [13].

$$E + S \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} ES \xrightarrow{k_2} \underset{+P_1}{ES'} \xrightarrow{k_3} E + P_2 \quad (1)$$

where ES, ES', k_2 and k_3 represent the enzyme-substrate complex, the acyl-enzyme, the acylation rate constant and the deacylation rate constant, respectively. All reactions were followed in 0.2 M KCl at pH 6.0 in a pH-stat apparatus at 25°C as described [11]. Because of the poor solubility of the thiono compound, its K_m value could not be determined and all experiments were carried out at low substrate concentrations (50–300 μM). The fact that all reactions were apparently first-order at least 90% of substrate consumption indicated that the concentrations were below K_m. These apparent first-order rate constants were divided by the enzyme concentration, which gave rise to the apparent second-order acylation rate constants ($k_{cat}/K_m = k_2/K_s$ [13]). The components of this specificity constant are defined by eqns 2 and 3.

$$k_{cat} = \frac{V}{[E_0]} = \frac{k_2 \cdot k_3}{k_2 + k_3} \quad (2)$$

$$K_m = K_s \frac{k_3}{k_2 + k_3} \quad (3)$$

3. RESULTS AND DISCUSSION

The kinetics of the hydrolyis of Z-Phe-Gly-OEt and Z-Phe^t-Gly-OEt by various cysteine proteases have been studied. The former compound is a highly specific substrate of papain, and the latter is its thiono derivative holding a sulfur atom in place of the carbonyl oxygen of the P_2 (phenylalanine) residue. The sulfur atom is known to form a much weaker hydrogen bond than the oxygen atom. In addition, the former is a significantly larger and more hydrophobic atom than the latter. Therefore, if the enzyme-substrate interaction during the catalysis by papain is determined primarily by the formation of a precise S_2P_2 hydrogen bond, then Z-Phe^t-Gly-OEt should be a much poorer substrate than the corresponding oxo compound. The effects of substitution of sulfur for oxygen in the catalysis by serine proteases [6] and in the conversion of the serine protease subtilisin into thiolsubtilisin [4] are indeed consonant with the above considerations.

Table 1 shows the second-order rate constants for acylation of papain and of other cysteine proteases. These rate constants are characteristic of the kinetic specificity of enzymes and are not affected by non-productive binding [13]. It is seen from the data that Z-Phe^t-Gly-OEt is cleaved by papain at a rate lower by more than three orders of magnitude than Z-Phe-Gly-OEt. Essentially similar results were obtained with the other enzymes shown in table 1. The enzymes investigated include not only plant proteases but also the mammalian enzyme, cathepsin B. The fact that the ratios of the rate constants for the oxo and thiono substrates are similar for all cysteine proteases of table 1 clearly indicates the importance of the stringent S_2P_2 hydrogen bond interaction in the catalysis by cysteine proteases.

The decrease in rate constants upon the substitution of sulfur for oxygen may not simply be a consequence of the loss in binding energy of the hydrogen bond, but it may mostly be due to the steric strain caused by the large sulfur atom which hinders the substrate on the way towards the transition state. This can be rationalized in stereochemical terms. Specifically, residues Cys-25 and His-159, which form the catalytically essential ion-pair of papain [15], are situated on the opposite walls of the cleft accommodating the substrates of

Table 1

Second-order rate constants for acylation of cysteine proteases with Z-Phe-Gly derivatives[a]

Enzyme	k (M^{-1} s^{-1})	
	Z-Phe-Gly-OEt	Z-Phe^t-Gly-OEt
Papain	120 000 ± 10 000	60 ± 5
Chymopapain, less basic	5 200 ± 500	< 30
Chymopapain, more basic	47 000 ± 4 000	90 ± 10
Papaya peptidase A	29 000 ± 300	7 ± 1
Cathepsin B	2 000 ± 200	20 ± 2

[a]At pH 6.0, 0.2 M KCl, 25°C, in 20% (v/v) acetonitrile. The substrate concentrations for both the oxo and the thiono compound were 50–300 μM. The enzyme concentrations with the oxo substrate were 0.02–0.05 μM, 0.2–0.7 μM, 0.03–0.08 μM, 0.05–0.11 μM and 0.8–1.6 μM, and with the thiono substrate were 2–6 μM, 10 μM, z–5 μM, 20–30 μM and 8–16 μM for papain, the less basic chymopapain, the more basic chymopapain, papaya peptidase A and cathepsin B, respectively

papain [4]. The P_2-P_1 peptide bond of the substrate cross-links this cleft by forming hydrogen bonds with the main chain peptide groups on either wall [4]. Accordingly, the large sulfur atom may dislocate both the substrate main chain and the catalytic residues from the appropriate positions, and this leads to inefficient catalysis. It appears that the S_2P_2 hydrogen bond primarily accounts for the proper alignment of the substrate whereas the S_2P_2 hydrophobic interaction yields the major portion of the S_2P_2 binding energy. These two effects together confer the principal part of specificity in the catalysis by the proteases of the papain family.

REFERENCES

[1] Fruton, J.S. (1976) Adv. Enzymol. 44, 1-36.
[2] Schechter, I. and Berger, A. (1967) Biochem. Biophys. Res. Commun. 27, 157-162.
[3] Drenth, J., Kalk, K.H. and Swen, H.M. (1976) Biochemistry 15, 3731-3738.
[4] Baker, E.N. and Drenth, J. (1987) Biological Macromolecules and Assemblies (Jurnak, F.A. and McPherson, A. eds) vol. 3, pp. 313-368, John Wiley and Sons, New York.
[5] Lowe, G. and Yuthavong, Y. (1971) Biochem. J. 124, 107-115.
[6] Asbóth, B. and Polgár, L. (1983) Biochemistry 22, 117-122.
[7] Asbóth, B., Stokum, É., Khan, I.U. and Polgár, L. (1985) Biochemistry 24, 606-609.
[8] Khan, I.U. and Polgár, L. (1983) Biochim. Biophys. Acta 760, 350-356.
[9] Polgár, L. (1981) Biochim. Biophys. Acta 658, 262-269.
[10] Polgár, L. and Csoma, C. (1987) J. Biol. Chem. 262, 14448-14453.
[11] Asbóth, B. and Polgár, L. (1977) Arch. Biochem. Biophys. Acad. Sci. Hung. 12, 223-230.
[12] Kajtár, M., Hollósi, M., Kajtár, J., Majer, Zs. and Kövér, K.E. (1986) Tetrahedron 42, 3931-3942.
[13] Bender, M.L. and Kézdy, F.J. (1965) Annu. Rev. Biochem. 34, 49-76.
[14] Polgár. L. and Bender, M.L. (1970) Adv. Enzymol. 33, 381-400.
[15] Polgár, L. (1974) FEBS Lett. 47, 15-18.