Mechanism of Action of Cysteine Proteinases: Oxyanion Binding Site Is Not Essential in the Hydrolysis of Specific Substrates

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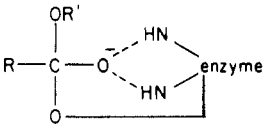
ABSTRACT: To study the possible stabilization of the oxyanion of the tetrahedral intermediate formed in the course of the catalyses by cysteine proteinases, papain, chymopapain, papaya peptidase A, and ficin, we synthesized *N*-(benzyloxycarbonyl)phenylalanylthioglycine *O*-ethyl ester and compared its hydrolysis with that of the corresponding oxygen ester, a highly specific substrate of the above enzymes. It was found that the substitution of sulfur for the carbonyl oxygen hardly affected the second-order rate constant of acylation and diminished catalytic activity by about 1 order of magnitude in deacylation. These results contrast with those obtained with serine proteinases [Asbóth, B., & Polgár, L. (1983) *Biochemistry 22*, 117-122], where the hydrolysis of thiono esters could not be detected. From the results the following conclusions can be drawn. Stabilization of the tetrahedral intermediate at an oxyanion binding site is not essential with cysteine proteinases. Therefore, and because of the lack of general base catalysis, cysteine proteinases have a less constrained transition-state structure than serine proteinases.

The basic features of the mechanism of action of serine and cysteine proteinases are common inasmuch as both involve an acyl-enzyme intermediate that is formed and hydrolyzed via tetrahedral adducts [for a review see Polgár & Halász (1982)]. In the case of serine proteinases it has been shown by X-ray diffraction measurements [cf. Kraut (1977)] that two hydrogen bonds from the enzyme to the negative oxygen atom of



the tetrahedral adduct can stabilize the intermediate, which resembles the transition state (Robertus et al., 1972; Polgár, 1972). This mechanism has been corroborated by kinetic investigations with thiono substrates (Asbóth & Polgár, 1983) containing a sulfur in place of the carbonyl oxygen atom. Since sulfur is not only larger than oxygen but is less prone to hydrogen bonding, catalytic competence of thiono substrates should be significantly altered if the interaction of the oxyanion with its binding site is indeed essential. In fact, thiono esters are not hydrolyzed by chymotrypsin and subtilisin, although both the chemical reactivities and the binding to the enzyme of the corresponding oxo and thiono esters are similar (Asbóth & Polgár, 1983). This clearly shows the importance of the oxyanion binding site in the catalysis by serine proteinases.

In the case of cysteine proteinases the available data pertinent to an oxyanion binding site are contradictory. On the basis of X-ray diffraction (Wolthers et al., 1970) and kinetic (Lowe & Yuthavong, 1971) investigations, the stereochemical mechanism proposed for papain action implied that the oxyanion was in a sterically not restricted environment, i.e., without hydrogen bonding to an oxyanion binding site. Studies on papain with the thiono derivative of methyl *N*-benzoylglycinate showed that replacement of oxygen by sulfur did not influence significantly the acylation rate and thus supported the above mechanism not involving an oxyanion binding site (Asbóth & Polgár, 1983). However, from the three-dimensional structure of papain reacted with specific chloromethyl ketone inhibitors, the existence of an oxyanion binding site, which is composed of the side chain NH_2 group of Gln-19 and the backbone NH of Cys-25, was deduced (Drenth et al., 1976). This site can be as flexible as in the case of subtilisin, where also a side chain (Asn-155) constitutes a part of the oxyanion binding site (Robertus et al., 1972).

The above apparent contradiction may be resolved if the oxyanion binding site of papain only functions with specific substrates. Therefore, we synthesized the thiono derivative of a specific substrate, ethyl Z-phenylalanylglycinate.[1] The chloromethyl ketone derivative of this compound was used in the X-ray diffraction study that suggested the existence of an oxyanion binding site in papain (Drenth et al., 1976). We compared the hydrolysis rate of the dipeptide oxo ester with that of the corresponding thiono ester in the case of papain, as well as in the cases of some other cysteine proteinases: chymopapain, papaya peptidase A, and ficin. The specificity of these enzymes resembles that of papain, though the active sites were suggested to differ in some respects (Brocklehurst et al., 1981). From the results we conclude that stabilization of the tetrahedral adduct by an exquisite oxyanion binding site is not significant with cysteine proteinases, in marked contrast to the mechanism of action of serine enzymes.

Experimental Procedures

Materials. Papain was purchased from Sigma, purified on an agarose–mercurial column according to Sluyterman & Wijdenes (1970), and assayed as described earlier (Asbóth & Polgár, 1977). Chymopapain was isolated from commercial papaya latex (Sigma) and purified as described elsewhere (Khan & Polgár, 1983). Papaya peptidase A was also isolated from papaya latex (Khan & Polgár, 1983) and purified as described earlier (Polgár, 1981). This enzyme exhibited higher activity than the one isolated from commercial chymopapain (Polgár, 1981). Ficin was purchased from Sigma and purified on an agarose–mercurial column similarly to papain except that the eluent contained 0.05 M phosphate, pH 6.6 (Anderson & Hall, 1974), instead of 0.05 M acetate, pH 5.0. All thiol-enzymes were stored in the inactive mercury form and

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[1] Abbreviations: PDS, 2,2'-dipyridyl disulfide; Z, benzyloxycarbonyl; EDTA, ethylenediaminetetraacetic acid; TLC, thin-layer chromatography.

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were activated prior to use with cysteine (Asbóth & Polgár, 1977). Routinely the activities of the enzymes were checked by rate assays using Z-glycine *p*-nitrophenyl ester (10^{-4} M) in 0.1 M acetate buffer, pH 5.5, containing 1 mM EDTA at 25 °C. Active enzyme concentrations were determined by active site titration with PDS at pH 4.0 according to Brocklehurst & Little (1970) except that a molar absorption value of 7700 was used.

Preparation of *N*-Z-phenylalanylglycine ethyl ester was described earlier (Asbóth & Polgár, 1977).

Synthesis of Z-phenylalanylthioglycine O-Ethyl Ester. To a cooled (−10 °C) solution of 3.5 g (11.7 mmol) of *N*-Z-phenylalanine and 1.7 mL (12.1 mmol) of triethylamine in 25 mL of methylene chloride was added dropwise 2 g (14.7 mmol) of isobutyl chlorocarbonate in 5 mL of methylene chloride under vigorous stirring. After a 10-min stirring, an aqueous solution (7 mL) of 2 g (13 mmol) of aminoacetonitrile sulfate neutralized with 4 N NaOH was added, and the mixture was stirred at −10 °C for an additional 30 min. The reaction mixture was allowed to warm up to room temperature with constant stirring for about 4 h. The organic phase was evaporated in vacuo, and the oily residue was dissolved in 15 mL of methylene chloride. On slow addition of an equal volume of cyclohexane, *N*-Z-phenylalanylglycine nitrile crystallized immediately. The crystalline material (1.3 g, 39%) was homogeneous on TLC and had a melting point of 137–139 °C.

A portion of the above nitrile (250 mg, 0.74 mmol) was suspended in anhydrous ethanol (2.3 mL) and then treated with an equal volume of 4.7 N hydrochloric acid in anhydrous ethanol. The suspension slowly cleared up on stirring with a Vortex-Genie mixer. After being allowed to stand at room temperature for 1 h, the solution was evaporated under reduced pressure. The residue was dissolved in 4 mL of ice-cold dry pyridine previously saturated with hydrogen sulfide. Then 0.5 mL of anhydrous ethanol was added, and hydrogen sulfide was bubbled through the solution at 0 °C for about 1 h. After evaporation the residue was extracted twice with 10 mL of boiling ethyl acetate. The ethyl acetate solution was filtered and evaporated to dryness. The crude product was purified by column chromatography on silica gel (1:50 weight ratio) with ethyl acetate–*n*-hexane (1:1) as eluent. The fractions, which contained the pure product, as tested by TLC, were combined, evaporated under reduced pressure, and crystallized from ethyl acetate–cyclohexane (1:7): yield 50 mg (17%); mp 113–115 °C. Anal. Calcd for $C_{21}H_{24}N_2O_4S$: C, H, N, S.

Thin-Layer Chromatography. The samples were run on precoated Polygram-Sil G-UV 254 sheets (Macherey-Nagel) in three systems: chloroform–methanol, 9:1; methanol–water, 75:25, ethyl acetate–*n*-hexane, 1:1. The spots were first detected under a short-wave UV lamp and then by exposure to iodine vapor, which selectively showed the sulfur-containing compounds.

Kinetic Measurements. Hydrolysis of substrates was followed in a Radiometer pH-stat apparatus as described earlier (Asbóth & Polgár, 1977). All kinetic measurements were made in 0.2 M KCl solution at 25 °C at pH 6.0. Substrate stock solutions were prepared in acetonitrile. The final concentration of the organic solvent in the reaction mixtures was 1%.

The reactions catalyzed by cysteine proteinases proceed according to eq 1 where E is the enzyme, S is the substrate,

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

Table I: Kinetic Parameters of the Reactions of Cysteine Proteinases with the Oxo and Thiono Ethyl Esters of *N*-Z-phenylalanylglycine[a]

	k_{cat}/K_m (M^{-1} s^{-1})	k_{cat} (s^{-1})	K_m (μM)
papain			
oxo ester[b]	430 000 ± 40 000	9.1 ± 1	21 ± 8
thiono ester[c]	240 000 ± 25 000	0.77 ± 0.1	3.2 ± 1
oxo/thiono	1.8	12	
chymopapain			
oxo ester[d]	10 000 ± 1800	0.75 ± 0.15	75 ± 30
thiono ester[e]	4400 ± 500	0.05 ± 0.01	12 ± 5
oxo/thiono	2.3	15	
papaya peptidase A			
oxo ester[b]	320 000 ± 30 000	16 ± 4	50 ± 20
thiono ester[d]	100 000 ± 15 000	1 ± 0.2	10 ± 7
oxo/thiono	3.2	16	
ficin			
oxo ester[c]	44 000 ± 4000	6.6 ± 2	150 ± 70
thiono ester[d]	61 000 ± 6000	0.2 ± 0.06	3.3 ± 1
oxo/thiono	0.7	33	

[a] In 0.2 M KCl, at 25 °C, at pH 6.0, in 1% v/v acetonitrile. The concentration of oxo and thiono esters was 20–200 and 10–50 μM, respectively. Data are given with ±SD. [b] Enzyme concentration 0.06 μM. [c] Enzyme concentration 0.2 μM. [d] Enzyme concentration 0.5 μM. [e] Enzyme concentration 5 μM.

ES is the noncovalent enzyme–substrate complex, ES′ is the acyl-enzyme intermediate, P_1 is the leaving group, and P_2 is the acid portion of the substrate; k_1 and k_{-1} are the association and dissociation rate constants, respectively, of the ES complex, and k_2 and k_3 are the first-order rate constants of acylation and deacylation, respectively.

Kinetic parameters, $k_{cat} = k_2k_3/(k_2 + k_3)$ and $K_m = k_3K_s/(k_2 + k_3)$ were determined from the analysis of entire progress curves of the reactions by use of an S/v vs. S linearization and also by fitting directly the experimental points to the integrated Michaelis–Menten equation by a procedure to be described elsewhere. The two methods of data treatment gave the same results within experimental error. The progress curves were recorded at different initial substrate concentrations, each in triplicate. If a tendency of changes in K_m with initial substrate concentration indicated a slight product inhibition, the parameters were determined from initial rate studies. For the determination of k_2, k_3, and K_s, the added nucleophile method (Bender et al., 1964) was used with ethanol as nucleophile.

Results

Acylation of Cysteine Proteinases. The first column in Table I shows k_{cat}/K_m values, the apparent second-order rate constants of acylation [cf. Bender & Kézdy (1965)], for the reactions of Z-phenylalanylglycine derivatives with the cysteine proteinases papain, chymopapain, papaya peptidase A, and ficin. It is seen that the ratios of the rate constants (oxo/thiono) are between 0.7 and 3.2, which is a negligible difference, when compared to the more than 10 000-fold rate decrease found in the reactions of serine proteinases (Asbóth & Polgár, 1983). It should be kept in mind, however, that the apparent second-order rate constant, $k_{cat}/K_m = k_2/K_s$ [cf. Bender & Kézdy (1965)], is a composite constant that involves binding. Therefore, we attempted to use the added nucleophile method (Bender et al., 1964). This method provides values of the individual constants, k_2, k_3, and K_s, if the difference between k_2 and k_3, the first-order acylation and deacylation rate constants, respectively, is not greater than 1 order of magnitude. In the hydrolyses of both the oxo and thiono esters of Z-phenylalanylglycine we found that the ratios of k_2/k_3 were more than 10 with all enzymes studied. Therefore, k_2 and K_s

could not be determined separately. Thus, it can only be stated that since there is not significant decrease in k_{cat}/K_m values, "acylation-and-binding" is not inhibited with the thiono substrate.

Deacylation Reactions. The above finding that the ratio of k_2/k_3 is more than 10 in all cases implies that the rate-limiting step of catalysis is deacylation and, thus, $k_{cat} \simeq k_3$ [cf. Bender & Kézdy (1965)]. Consequently, a comparison of the k_{cat} values (second column in Table I) provides information on the stabilization of the oxyanion in the deacylation step. It can be seen from Table I that the oxo/thiono ratios for k_{cat} are between 12 and 33. These values are apparently higher than the corresponding ratios in the k_{cat}/K_m values (0.7–3.2), but the thiono ester is still a good substrate of the cysteine proteinases, a marked difference from that observed with serine proteinases. The meaning of changes in K_m values (Table I) is not discussed here, because they do not represent true binding constants [$K_m = [k_3/(k_2 + k_3)]K_s$].

Discussion

Role of Oxyanion Binding Site. We have found that the acylation constant (k_{cat}/K_m) for cysteine proteinases is not significantly affected by the substitution of sulfur for the carbonyl oxygen of the substrate. On the other hand, deacylation (k_{cat}) decreased by about 1 order of magnitude. The somewhat different change in acylation and deacylation may result from the different mechanisms of the two steps: acylation is a simple nucleophilic attack, whereas deacylation is a general base-catalyzed process (Polgár, 1973, 1974). An alternative possibility may be that, on substituting sulfur for oxygen, the first-order rate constant, k_2, may decrease to a greater extent than the second-order rate constant, $k_{cat}/K_m = k_2/K_s$, provided that a similar decrease in K_s compensates for the change in k_2. The decrease in k_2, however, may not be significantly more than an order of magnitude, since the difference between k_2 and k_3 is generally 1 order of magnitude (Whitaker & Bender, 1965; Bender & Brubacher, 1966). Consequently, if, in the case of the thiono substrate, the decrease in k_2 had been considerably greater than in k_3, we could have determined the ratio of k_2/k_3 by the added nucleophile method, contrary to our finding that $k_2/k_3 > 10$. Thus, it cannot be established whether acylation is affected to a lesser extent than deacylation or they are affected similarly. Nonetheless, the above data indicate that, even with a specific substrate, an oxyanion binding site cannot play a significant role in the catalysis by the cysteine proteinases examined here. If a critical interaction existed between the oxyanion and an oxyanion binding site, on substituting sulfur for the oxygen atom the catalysis would be affected more seriously than we actually observed: (1) In serine proteinases, chymotrypsin and subtilisin, such a substitution virtually abolished the catalytic activity (Asbóth & Polgár, 1983). It should be emphasized that it may not be the flexibility of the oxyanion binding site that accounts for the activity of cysteine proteinases hydrolyzing the thiono substrates, because subtilisin has a similarly flexible active site, and it still does not catalyze the hydrolysis of thiono substrates. (2) The first site-specific mutation, conversion of subtilisin into thiosubtilisin, inactivated the enzyme toward natural substrates (Polgár & Bender, 1970). Both examples show that the flexibility of an enzyme, contrary to the common anticipation, may not compensate for the replacement of a catalytically essential oxygen atom.

It should be noted, that recently a ^{13}C NMR study was published on the complex formed between papain and *N*-acetylphenylalanyl[1-^{13}C]glycinal (Gamcsik et al., 1983). In the enzyme–aldehyde complex there were observed two very closely spaced ^{13}C signals that were attributed to the formation of two diastereomeric tetrahedral hemithioacetals. Although so speculated, the observations do not require the interaction of one of the diastereoisomers with an oxyanion hole.

Stringency of the Mechanism. The mechanism of action of serine and that of cysteine proteinases are usually considered to be similar. An important difference between the two mechanisms, however, has already been pointed out: the nucleophilic attack on the substrate by serine proteinases is a general base-catalyzed process, whereas the same step is not catalyzed in the case of cysteine proteinases where a mercaptide–imidazolium ion pair is the nucleophile (Polgár, 1973, 1974). In the present paper we provided evidence that the transition state stabilization is also different: with serine proteinases, interaction between the oxyanion binding site and the oxyanion leads to a constrained transition state, whereas with cysteine proteinases, the lack of a similarly strong interaction may permit the formation of a transition state with more freedom of motion. This implies that a gain in enthalpy at the expense of entropy contributes to the driving force in the catalysis by serine proteinases, whereas the opposite is true in the cysteine proteinase catalysis. By virtue of the less stringent geometries in the nucleophilic attack and in transition-state stabilization, the catalysis by cysteine proteinases is simple as compared with the more sophisticated mechanism of action of serine proteinases. This may be worth considering when designing relatively simple catalysts with enzymic activity, which is one of the greatest challenges to the present day organic chemists. Furthermore, as a relatively simple mechanism is expected to be realized readily during evolution, cysteine proteinases, as compared with serine proteinases, might represent a more ancient family of enzymes.

Registry No. Z-phenylalanylthioglycine, 94137-27-6; *N*-Z-phenylalanylglycine ethyl ester, 2778-34-9; *N*-Z-phenylalanine, 1161-13-3; isobutyl chlorocarbonate, 543-27-1; aminoacetonitrile sulfate, 5466-22-8; *N*-Z-phenylalanylglycinenitrile, 76318-94-0; papain, 9001-73-4; chymopapain, 9001-09-6; papaya peptidase A, 39307-22-7; ficin, 9001-33-6; cysteine proteinase, 37353-41-6.

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Kinetic Mechanism for Stimulation by Monovalent Cations of the Amidase Activity of the Plasma Protease Bovine Activated Protein C†

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ABSTRACT: A study of the effect of monovalent cations on the steady-state kinetic parameters for the hydrolysis of the synthetic substrate N^{α}-benzoyl-L-arginine-*p*-nitroanilide by activated bovine plasma protein C (APC) has been undertaken. The enzyme displayed a strict requirement for monovalent cations in its expression of amidolytic activity toward this substrate. Analysis of the variation in initial hydrolytic reaction rates, as a function of metal ion concentrations, suggested that at least two cation sites, or classes of sites, were necessary for catalysis to occur. After examination of the rate equations consequential to many different enzymic mechanisms that could account for these kinetic data, a mechanism was developed that fit the great majority of the experimental observations. In this mechanism it is postulated that cations bind to the enzyme in pairs, with a kinetically observable single binding constant, either preceded by or followed by binding of substrate. Catalysis occurs only after the enzyme–(metal cation)$_2$–substrate complex is assembled. Some physical support for this mechanism was obtained upon the discovery that the binding (dissociation) constant for a competitive inhibitor of APC, *p*-aminobenzamidine, as determined by kinetic methodology, was independent of the concentration of Na^+ and Cs^+.

Protein C (PC) is a vitamin K dependent plasma glycoprotein (Stenflo, 1976), which serves as the zymogen of the serine protease activated protein C (APC) (Kisiel et al., 1976). Bovine plasma PC contains two disulfide-linked polypeptide chains of known amino acid sequence. Its light chain consists of 155 amino acid residues and possesses all 11 of the Gla residues of the protein, which have been placed within the first 35 residues from the amino terminus (Fernlund & Stenflo, 1982). A single glycosylation site in this chain is present at residue Asn_{97}. The heavy chain of PC comprises 260 amino acids and contains the latent active site residues His_{56}, Asp_{102}, and Ser_{201}, as well as three points of glycosylation at residuess Asn_{93}, Asn_{154}, and Asn_{170} (Stenflo & Fernlund, 1982). It is believed that Cys_{122} represents the heavy chain residue that covalently binds the two chains of PC (Stenflo & Fernlund, 1982). The sequence position of its companion residue in the light chain is not known.

PC is converted to APC as a consequence of cleavage of the Arg_{14}–Ile_{15} peptide bond in the heavy chain of PC (Kisiel et al., 1976), and cations play a significant role in this process. The coagulant protein from the venom of Russell's viper (RVV-X) is able to catalyze the cleavage required for activation (Kisiel et al., 1976), in a step accelerated by Ca^{2+} (Amphlett et al., 1981). Thrombin also catalyzes this same reaction (Kisiel et al., 1977), but this process is inhibited by Ca^{2+} (Amphlett et al., 1981). The thrombin-catalyzed activation of PC is greatly accelerated by a protein cofactor, thrombomodulin, present in endothelial cells (Esmon & Owen, 1981; Owen & Esmon, 1981). The resultant protease, APC, possesses esterolytic (Steiner et al., 1980) and amidolytic (Kisiel et al., 1976, 1977) activities toward synthetic substrates.

The physiological role of APC centers around its anticoagulant activity (Kisiel et al., 1977). This activity may be explained by the observations that APC inactivates an important cofactor for prothrombin activation, factor Va (Kisiel et al., 1977; Walker et al., 1979), and an important cofactor for factor X activation, factor VIIIa (Vehar & Davie, 1980). APC is also believed to function in fibrinolysis, since increased levels of activators of plasminogen are found in plasma upon infusion of APC (Comp & Esmon, 1981). The resultant effect of all of the above activities of APC is to maintain the fluid state of blood. The importance of APC in this regard is punctuated by the finding that members of a family with a history of recurrent thrombotic episodes possessed abnormally low levels of plasma PC (Griffin et al., 1981).

Our laboratory had originally demonstrated that the amidolytic and esterolytic activities of APC were dependent upon the presence of cations (Steiner et al., 1980; Steiner & Castellino, 1982). While monovalent cations exerted the greatest influence in this regard, divalent cations, such as Ca^{2+}, also served this function (Steiner et al., 1980). Although certain monovalent cations have been shown to enhance, to a small extent, the amidolytic activities of α-thrombin (Orthner & Kosow, 1980) and factor Xa (Orthner & Kosow, 1978), our observations (Steiner et al., 1980) that the amidolytic acitvity of APC was absolutely dependent upon monovalent (and to a lesser degree, divalent) cations and was progressively enhanced by monovalent cations through the Hofmeister series

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