Transition-State Stabilization at the Oxyanion Binding Sites of Serine and Thiol Proteinases: Hydrolyses of Thiono and Oxygen Esters†

Bence Asbóth and László Polgár*

ABSTRACT: X-ray diffraction studies suggested that the tetrahedral intermediate formed during the catalysis by serine and thiol proteinases can be stabilized by hydrogen bonds from the protein to the oxyanion of the intermediate [cf. Kraut, J. (1977) *Annu. Rev. Biochem. 46*, 331–358; Drenth, J., Kalk, K. H., & Swen, H. M. (1976) *Biochemistry 15*, 3731–3738]. To obtain evidence in favor or against this hypothesis, we synthesized thiono substrates (the derivatives of *N*-benzoylglycine methyl ester and *N*-acetylphenylalanine ethyl ester) containing a sulfur in place of the carbonyl oxygen atom of the scissile ester bond. We anticipated that this relatively subtle structural change specifically directed to the oxyanion binding site should produce serious catalytic consequences owing to the different properties of oxygen and sulfur if transition-state stabilization in the oxyanion hole is indeed important. In fact, while in alkaline hydrolysis the chemical reactivities of oxygen esters and corresponding thiono esters proved to be similar, neither chymotrypsin nor subtilisin hydrolyzed the thiono esters at a measurable rate. This result substantiates the crucial role of the oxyanion binding site in serine proteinase catalysis. On the basis of the similar values of the binding constants found for oxygen esters and their thiono counterparts, it can be concluded that the substitution of sulfur for oxygen significantly influences transition state stabilization but not substrate binding. The thiol proteinases papain and chymopapain react with the oxygen and thiono esters of *N*-benzoylglycine at similar rates. Apparently, in these reactions the above stabilizing mechanism is absent or not important, which is a major mechanistic difference between the catalyses by serine and thiol proteinases.

Serine proteinases, like chymotrypsin and subtilisin, are among the most extensively studied enzymes. The basic features of their mechanism of action are illustrated in Figure 1 (cf. Bender & Kézdy, 1965). It can be seen that the nucleophilic attack by the serine hydroxyl group on the carbonyl carbon atom of the substrate is catalyzed by a histidine residue as a general base. This leads to the formation of a tetrahedral intermediate and an imidazolium ion. The intermediate breaks down by general acid catalysis to an acyl-enzyme, an imidazole base, and alcohol or amine. The acyl-enzyme is hydrolyzed through the reverse reaction pathway of acylation. The tetrahedral adduct formed during catalysis by serine proteinases is a transition-state-like species (Robertus et al., 1972; Polgár, 1972a). The stabilization of its negatively charged oxygen by hydrogen bonds from the protein was postulated on the basis of low Hammett ρ values (Williams, 1970). X-ray diffraction studies established that in chymotrypsin the backbone NH groups of glycine-193 and serine-195 (Henderson, 1970), whereas in subtilisin the backbone NH of serine-221 and the amide group of asparagine-155 (Robertus et al., 1972), may form hydrogen bonds with the oxyanion. The oxyanion hole was observed in all serine proteinases whose three-dimensional structure has been determined (Kraut, 1977; James, 1980). Recently molecular mechanic calculations pointed to the importance of these hydrogen bonds in the stereospecificity of chymotrypsin (DeTar, 1981).

Thiol proteinases operate in many respects similarly to serine proteinases (cf. Bender & Kézdy, 1965). They form an acyl-enzyme intermediate, a thiol ester, which builds up and breaks down through a tetrahedral adduct. On the basis of X-ray diffraction studies, an oxyanion binding site similar to that found with serine proteinases was proposed to be involved also in the catalysis by thiol proteinases (Drenth et al., 1975, 1976).

In this work we present experimental evidence that transition-state stabilization at the oxyanion binding site is indeed important in the catalysis by serine proteinases but may not be a critical factor in thiol proteinase catalysis. We assumed that stabilization of the tetrahedral adduct in the oxyanion hole demands a precise alignment of the groups involved. Thus, replacement of the oxygen by a sulfur atom, which is larger and has reduced hydrogen-bonding ability, may affect the enzymatic reaction. Therefore, we synthesized thiono ester substrates containing a sulfur atom in place of the carbonyl oxygen and compared the second-order rate constants of acylation of chymotrypsin, subtilisin, papain, and chymopapain with these thiono ester compounds to those of the corresponding oxygen ester substrates.

Experimental Procedures

Materials. Chymotrypsin was purchased from Sigma and subtilisin Carlsberg from Novo Industri, and both were used without further purification. The operational molarities were determined by titrating the enzymes with *N-trans*-cinnamoylimidazole according to Bender et al. (1966); in the case of subtilisin, the titration procedure was modified as described earlier (Polgár, 1968). Papain was purchased from Sigma, purified on an agarose–mercurial column according to Sluyterman & Wijdenes (1970), and assayed as described previously (Asbóth & Polgár, 1977). Chymopapain was a Sigma product, purified, and assayed as described (Polgár, 1981).

N-Benzoylglycine methyl ester was synthesized by esterification of glycine with thionyl chloride in methanol (Brenner & Huber, 1953), and a 0.2 M suspension of the resulting ester was acylated in methylene chloride with a 1.5 molar excess of benzoyl chloride in the presence of a 3 molar excess of triethylamine. The crude product was recrystallized from aqueous methanol (50% v/v). The final product had mp 80–81 °C (lit. mp 81 °C; Lowe & Williams, 1965) and was shown to be a single component by TLC.[1]

† From the Institute of Enzymology, Biological Research Center, Hungarian Academy of Sciences, Budapest, Hungary, H-1502. *Received April 8, 1982.*

[1] Abbreviations: ATEE, *N*-acetyl-L-tyrosine ethyl ester; EDTA, ethylenediaminetetraacetic acid; TLC, thin-layer chromatography.

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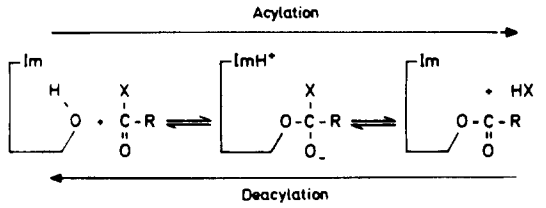

FIGURE 1: Scheme of the reaction mechanism for serine proteinases. X stands for an OR′ or an NHR′ group in acylation and for an OH group in deacylation.

N-Benzoylthioglycine *O*-methyl ester was synthesized as described by Lowe & Williams (1965) except that the conversion of nitrile to imino ester was modified as follows: *N*-benzoylglycine nitrile (1200 mg) was dissolved in 14 mL of methanol 3 N in HCl, incubated at room temperature for 20–25 min, and then poured on ice-cold ether (ca. 5 volumes). The crystals so obtained were filtered off, dried in vacuo, and used for the thiono ester preparation without further purification. The thiono ester was recrystallized several times from ether–hexane (1:3); the final product was homogeneous as tested by TLC and gas chromatography and had mp 80–81 °C (lit. mp 83–84 °C; Lowe & Williams, 1965).

N-Acetyl-L-phenylalanine ethyl ester was synthesized by esterification of phenylalanine in ethanol with the thionyl chloride method (Brenner & Huber, 1953). The resulting ester was then acylated with acetyl chloride in the presence of triethylamine in methylene chloride similarly to that described for the glycine ester. After three subsequent extractions with 0.1 M HCl, water, and 0.1 M $NaHCO_3$, the organic phase was dried on molecular sieves (Serva, 4 Å) and evaporated to dryness. After recrystallization from ethyl acetate–ether (1:4), the product proved to be homogeneous on TLC and had mp 90–91 °C (lit. mp 90–91 °C; Huang et al., 1952).

The synthesis of *N*-acetylthiophenylalanine *O*-ethyl ester was carried out as follows. Acetyl-L-phenylalanine ethyl ester was amidated according to Greenstein & Winitz (1961), and then the amide was dehydrated to the nitrile according to Woolley et al. (1963). However, the product after recrystallization had mp 133–134 °C and $[\alpha]^{25}_D = -56.8°$, markedly different from those reported previously [lit. mp 106–108 °C and lit. $[\alpha]^{25}_D = -10.2°$, as given by Woolley et al. (1963)]. Because of this discrepancy, the product was further analyzed: IR (ν_{max}) 3280 (NH), 2240 (CN), 1655 (CO–NH), 1540 (NH), and 750 and 704 cm^{-1} (benzene ring); mass spectrum, *m/e* 43 (9.5), 65 (3.5), 91 (100), 92 (7), 129 (67), 130 (6), 188 (17); ^1H NMR ($CDCl_3$) 2.0 (3 H, CH_3), 3.1 (2 H, CH_2), 5.1 (1 H, CH), 6.9 (1 H, NH), and 7.36 (5 H, C_6H_5). Anal. Calcd for $C_{11}H_{12}N_2O$: C, H, N. These data confirmed that our material is acetylphenylalanine nitrile. The nitrile was then treated according to Woolley et al. (1963) in chloroform with 8 M hydrochloric acid in ethanol. The resulting imino ester was not isolated, but the reaction mixture was evaporated in vacuo, and the residue was dissolved in anhydrous ethanol. In the presence of 2% (v/v) pyridine, equimolar to the starting nitrile, dry hydrogen sulfide was bubbled through the solution at room temperature. After about 30 min, when thiono ester formation was completed as shown by TLC, the introduction of H_2S was stopped, and the solvent was removed under reduced pressure. The residue was dissolved in ethyl acetate and extracted with water, then with 0.1 M $NaHCO_3$, and finally with water. The organic phase was dried on molecular sieves and then evaporated to dryness in vacuo. The oily residue was extracted with boiling hexane. The hexane solution was evaporated to dryness; the residue contained about 15% oxygen ester as shown by HPLC. The mixture was resolved by preparative thin-layer chromatography (Reanal PF-254 silica gel, 2 mm thickness) run repeatedly in ethyl acetate–hexane, 1:1. The thiono ester eluted from the gel with ethyl acetate, after evaporation was crystallized from hexane (mp 77–78 °C) (Anal. Calcd for $C_{13}H_{17}NO_2S$: C, H, N, S). The pure thiono ester showed practically no optical activity (*c* 1.7, ethanol). Thus, although racemization is not supported by earlier data (Woolley et al., 1963), it appears to take place during synthesis.

Analytical Methods. Thin-layer chromatography was run on precoated Polygram-Sil G-UV 254 sheets (Macherey-Nagel) in three systems: chloroform–methanol, 9:1; methanol–water, 75:25; and ethyl acetate–hexane, 1:1. Spots were first detected under a short-wave UV lamp and then by exposure to iodine vapor, which selectively showed sulfur-containing spots. Deacylated compounds formed during preparation could be identified with ninhydrin after iodine was removed by a hot air stream.

High-pressure liquid chromatography was run on a LiChroSorb (20 cm) column in heptane–2-propanol–methanol, 7:2:1, by using an Altex apparatus.

Gas chromatography was run on a 2 m × 2 mm glass column filled with 3% OV-17 (Applied Science) on 100–120 mesh Gas-ChromQ (Applied Science) at 200 °C by using a Hewlett-Packard 5720 A apparatus.

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; in reactions with thiol enzymes, solutions also contained 10^{-3} M EDTA. Stock solutions of the substrates were prepared in acetonitrile. The final concentration of the organic solvent in the reaction mixtures was 3%, except in the determinations of binding constants with subtilisin, where 8% acetonitrile was employed.

Reactions were followed under pseudo-first-order conditions, i.e., $[S]_0 \ll K_m$. The apparent first-order rate constant was divided by the active enzyme concentration to obtain the second-order rate constant of acylation, $k_2/K_s = k_{cat}/K_m$ (cf. Bender & Kézdy, 1965).

Determination of Binding Constants. Binding of poor substrates (the thiono esters and *N*-benzoylglycine methyl ester) to chymotrypsin and subtilisin was studied by measuring their inhibitory effect on the hydrolysis of a highly specific substrate, *N*-acetyl-L-tyrosine ethyl ester (ATEE). This inhibition proved to be purely competitive as shown by Lineweaver–Burk and Dixon plots. Therefore, for calculation of K_i, the formula of Hunter & Downs (1945) was used:

$$K_i = \left(\frac{[I]}{v/v_i - 1}\right)\left(\frac{K_m}{[S] + K_m}\right) \quad (1)$$

where v is the velocity in the absence of the inhibitor and v_i is the velocity in the presence of inhibitor.

The enzymatic hydrolysis of ATEE was followed under pseudo-first-order conditions ($[ATEE]_0 \ll K_{m,ATEE}$) at fixed enzyme and substrate (ATEE) concentrations in the presence of different concentrations of the esters (I). Under this condition, eq 1 simplifies to

$$K_i = \frac{[I]}{v/v_i - 1} \quad (2)$$

Since under pseudo-first-order conditions

$$v = k[ATEE] \quad (3)$$

and

$$v_i = k_i[ATEE]_i \quad (4)$$

where k and k_i are the observed rate constants in the absence

Table I: Second-Order Rate Constants for the Base-Catalyzed Hydrolysis of Some Oxygen and Corresponding Thiono Esters

	k_O (M^{-1} s^{-1})	k_S (M^{-1} s^{-1})	k_O/k_S
methyl *N*-benzoylglycinate[a]	1.9 ± 0.2	2.7 ± 0.4	0.7
ethyl *N*-acetylphenylalaninate[a]	0.4 ± 0.1	0.4 ± 0.1	1
ethyl benzoate[b]	0.0055	0.0057	1
benzoylcholine[c]	0.56	0.30	1.9
2-(dimethylamino)ethyl benzoate[c]	0.058	0.086	0.7

[a] In 0.2 M KCl and 3% v/v acetonitrile at 25 °C; determined at least at three different pH values between pH 10.0 and 11.3; ester concentrations were in the range (0.3–3) × 10^{-3} M. [b] Smith & O'Leary (1963). [c] Bruice & Mautner (1973).

and presence of inhibitor, respectively, and [ATEE] = $[ATEE]_i$, substitution of eq 3 and 4 into eq 2 leads to eq 5:

$$K_i = \frac{[I]}{k/k_i - 1} \quad (5)$$

The values for binding constants determined according to eq 5 did not alter when the concentration of ATEE and/or enzyme was changed within a factor of 3. As this method is based on the determination of first-order rate constants, its experimental error is less than that of Lineweaver–Burk plots.

The K_s of the complex of subtilisin and *N*-acetyl-L-phenylalanine ethyl ester was determined by the 1,4-butanediol method of Berezin et al. (1971).

Results and Discussion

Alkaline Hydrolysis of Esters and the Corresponding Thiono Esters. Before the enzymatic hydrolysis of substrates is studied, the effect of oxygen–sulfur exchange on the chemical reactivity of the bond to be split should be clarified. To this end the alkaline hydrolyses of esters and thiono esters, which also take place through the formation of a tetrahedral adduct (Smith & O'Leary, 1963), were examined. Table I shows the second-order rate constants of the specific base-catalyzed hydrolysis of the substrates of this study, as well as literature data of some other thiono–oxygen pairs. The fact that the k_O/k_S ratios range from 0.7 to 1.9 indicates that the inherent chemical reactivities of the ester and thiono ester bonds are similar. Thus, large k_O/k_S ratios in the enzymatic reactions should primarily reflect different interactions of thiono and oxygen esters with the oxyanion binding site.

Reactions of Esters and Thiono Esters with Serine Proteinases. The effect upon catalysis of substitution of sulfur for the substrate carbonyl oxygen atom was studied by determining the acylation rate constants for chymotrypsin and subtilisin with the ester and thiono ester derivatives of *N*-benzoylglycine and *N*-acetylphenylalanine, the latter a specific substrate of both enzymes. As seen from Table II, thiono esters are not hydrolyzed by either serine proteinase at a measurable rate. Owing to the autolysis of chymotrypsin and subtilisin at the high enzyme concentrations employed, only upper limits for rate constants of the thiono ester reactions could be determined. It should be noted that lack of reactivity of the thiono ester of acetylphenylalanine cannot be assigned to a possible inhibition by the D enantiomer present in the substrate. This follows from the fact that the thiono ester, in the concentration range where the rate constants were determined, does not inhibit significantly the hydrolysis rate of a good substrate, acetyl-L-tyrosine ethyl ester. Due to the slight inhibition, values in the last column of Table II may be higher by at most 15%. Nevertheless, the increased values are still

Table II: Second-Order Rate Constants for Acylation of Serine Proteinases by the Oxygen and Thiono Esters[a]

	methyl *N*-benzoylglycinates		ethyl *N*-acetylphenylalaninates	
	k_O (M^{-1} s^{-1})[b]	k_S (M^{-1} s^{-1})[c]	k_O (M^{-1} s^{-1})[d]	k_S (M^{-1} s^{-1})[e]
subtilisin[f]	18 ± 0.5	<0.6	25 000 ± 1000	<2
chymotrypsin[g]	31 ± 1	<1	15 000 ± 1000	<1

[a] In 0.2 M KCl and 3% v/v acetonitrile, at 25 °C. [b] Substrate concentration range (3–6) × 10^{-4} M; enzyme concentration range 5 × 10^{-5}–1 × 10^{-4} M. [c] Substrate concentration range 5 × 10^{-5}–5 × 10^{-4} M; enzyme concentration range 5 × 10^{-4}–1 × 10^{-3} M. [d] Substrate concentration range (2–5) × 10^{-4} M; enzyme concentration range (1–3) × 10^{-7} M. [e] Substrate concentration range (1–3) × 10^{-3} M; enzyme concentration range 5 × 10^{-4}–1 × 10^{-3} M. [f] At pH 8.5. [g] At pH 7.5.

negligible as compared to the rate constants obtained with the oxygen ester.

Two explanations may be offered for the inertness of serine proteinases toward thiono esters: (1) The first is the steric hindrance by the larger sulfur atom in place of the oxygen. As deduced from the active-site models of the enzymes, the larger sulfur atom cannot fit into the oxyanion hole, and thus hydrogen bonds cannot be formed; or if formed are strongly distorted; or if properly oriented hydrogen bonds are formed, some other part of the tetrahedral intermediate will necessarily be misaligned, and thus, for example, proton transfer to the leaving group will not occur. (2) The second explanation is the chemical consequences of the oxygen to sulfur change; namely, sulfur is less prone to accept hydrogen bonds than is oxygen. Hence, transition-state stabilization via hydrogen bonding is expected to be less effective with thiono substrates. In the present case, the chemical reason appears to be more important than the steric one, since with subtilisin a part of the oxyanion hole is formed by the side chain of asparagine-155, which is expected to be sufficiently flexible to accommodate the larger sulfur atom, and nevertheless subtilisin is inactive toward the thiono esters.

In a study on trypsin catalysis, Stapf et al. (1974) found that the dithio derivative of ethyl 6-aminohexanoate was hydrolyzed 70 times slower than the corresponding oxygen ester. With dithio esters, however, bond cleavage takes place at a C–S bond, and the enzymatic hydrolyses of alkyl thiol esters are 20–80 times as fast as those of the corresponding oxygen esters when measured with chymotrypsin and subtilisin (Polgár, 1972b). Thus, the effect of the change of carbonyl oxygen to sulfur can be estimated to cause a 1400–5600-fold decrease in rate; i.e., oxyanion binding, even with a very poor substrate, should be important in trypsin catalysis as well.

The above interpretation implies that the sulfur for oxygen change does not basically alter the binding mode of the substrate. This is a reasonable assumption since substrate binding is governed by several interactions most of which are presumably unaffected by this single atom replacement. Such interactions revealed by kinetic and X-ray data are covalent bond formation between the essential serine oxygen and the substrate carbonyl carbon atom, nonpolar interactions with the substrate side chain, and hydrogen bonds with the α-amino group and the leaving group of the substrate (cf. Kraut, 1977). Accordingly, it can be expected that changes in reactivity arise primarily from alterations in oxyanion binding. The comparable values for the binding constants of the oxygen and corresponding thiono esters, as it is seen in the next section, is a further important argument for the similar binding mode.

Table III: Binding Constants of Substrates to Serine Proteinases[a]

	methyl *N*-benzoylglycinates		ethyl *N*-acetylphenylalaninates	
	oxygen	thiono	oxygen	thiono
subtilisin	9 ± 3^b	7 ± 3^b	20 ± 4^c	11 ± 4^d
chymotrypsin	4 ± 0.5^e	4 ± 0.5^e	7.4^f	4 ± 0.5^g

[a] The values are given in 10^{-3} M and were determined in 0.2 M KCl, at 25 °C. [b] In 8% v/v acetonitrile at pH 9.0; ATEE concentrations (0.5–2) × 10^{-3} M; substrate concentration (2–15) × 10^{-3} M; enzyme concentration (1–3) × 10^{-7} M. [c] This K_s value was determined by the method of Berezin et al. (1971) in 8% v/v acetonitrile at pH 9.0. Substrate concentration (5–15) × 10^{-3} M; enzyme concentration 2 × 10^{-7} M; 1,4-butanediol concentration 0.11 and 0.22 M. [d] As in footnote *b* but substrate concentration is (1–6) × 10^{-3} M. [e] In 3% v/v acetonitrile at pH 7.0. ATEE concentration (2–5) × 10^{-4} M; substrate concentration (1–10) × 10^{-3} M; enzyme concentration (1–3) × 10^{-7} M. [f] A K_s value from Zerner et al. (1964). [g] As in footnote *e* but substrate concentration is (0.2–2) × 10^{-3} M.

Binding of Esters and Thiono Esters to Serine Proteinases. In the previous section we have shown that there is a considerable difference in the second-order rate constants of acylation (k_2/K_s) with the ester and corresponding thiono ester substrates. Since this composite rate constant involves K_s, the question has emerged whether or not the difference observed in rate may be due to binding. Table III shows the binding constants for the substrates of this study. In the case of the *N*-benzoylglycine, *N*-benzoylthioglycine, and *N*-acetylthiophenylalanine *O*-esters, these constants were calculated from the inhibition of the hydrolysis of a highly specific substrate, *N*-acetyl-L-tyrosine ethyl ester, as described under Experimental Procedures. Obviously, this method could not be used for *N*-acetyl-L-phenylalanine ethyl ester, a good substrate of both serine proteinases. Therefore, its binding to chymotrypsin has been characterized by a K_s value taken from the literature (Zerner et al., 1964), whereas the K_s for subtilisin was determined by the method of Berezin et al. (1971). It is seen from Table III that binding is not significantly affected by the oxygen to sulfur change, as the respective constants are within a factor of 2. Accordingly, the substantial differences in the acylation rate constants may be attributed to poor catalysis with thiono esters rather than to the impediment of the formation of the enzyme–substrate complex.

There is no consensus (Robertus et al., 1972; Fersht et al., 1973; Campbell & Nashed, 1978) as to whether the hydrogen bonds at the oxyanion binding site are already present in the Michaelis complex or they are formed parallel with the building up of the tetrahedral intermediate. The similar binding constants of esters and thiono esters with their extremely different behaviors in enzymatic reactions strongly suggest that substrate binding in the oxyanion hole is critical in the transition state (tetrahedral adduct) while it is less important in the ground state (Michaelis complex) of the reaction.

Stereochemical Consequences of Oxyanion Binding. The present study clearly indicates that the interaction between the oxyanion and its binding site should be considered as a crucial factor in building of the model of the tetrahedral adduct. Previous model building studies indicated that there were two positions of serine O_G atom of chymotrypsin where formation of true intermediates appeared to be possible: one position found in the native enzyme and the other in its various derivatives (Polgár & Asbóth, 1974; Bizzozero & Dutler, 1981, and references therein). The models have indicated that the hydrogen bonds to the oxyanion are optimal both in orientation and in distance if the tetrahedral adduct is constructed in the "native enzyme" position, whereas the orientation, particularly in the case of the hydrogen bond from the backbone NH group of the essential serine residue, is much less favorable with the other geometry. Furthermore, the steric incompatibility of the larger sulfur atom with the oxyanion hole is obvious in the former but is not so in the latter position. Although this consideration seems to support the native enzyme position, we feel bound to emphasize that the oxyanion hole criterion alone is not sufficient to distinguish between the two possible geometries.

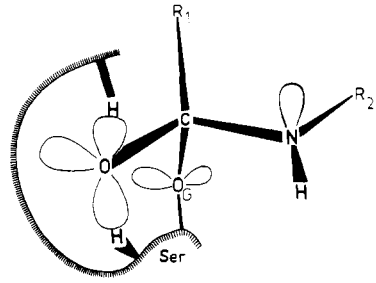

FIGURE 2: Orientation of the lone pair orbitals of the tetrahedral intermediate in the active site of serine proteinases. C is the tetrahedral carbon atom; O_G represents the serine oxygen atom. The C–N bond to be cleaved is between amino acids R_1 and R_2. The oxyanion situated on the left side of the figure has three electron pairs: two are hydrogen bonded to the protein, and the one pointing to the left is the "free" lone pair, which is anti periplanar to the C–N bond but not to the C–O_G bond.

Stereoelectronic Control and Oxyanion Binding. Stereoelectronic control of the breakdown of the tetrahedral intermediates of small organic compounds (Deslongchamps et al., 1975) has been invoked in serine proteinase catalysis (Bizzozero & Zweifel, 1975; Petkov et al., 1978; Dugas & Penney, 1981; Bizzozero & Dutler, 1981). The stereoelectronic theory imposes restrictions on the orientation of the lone pair orbitals of the three heteroatoms of the tetrahedral intermediate: the leaving nitrogen of a peptide substrate, the oxyanion, and the serine O_G. According to this concept, cleavage of a C–O or C–N bond requires the assistance of anti periplanar lone pair orbitals on each of the two remaining heteroatoms. In accordance with the principle of microscopic reversibility, this should also be valid for the formation of a new bond. Thus, when the tetrahedral intermediate builds up, the lone pair orbitals of the leaving nitrogen and of the oxyanion must be anti periplanar to the new C–O bond, and when the tetrahedral intermediate decomposes, each of the two oxygen atoms geminal to the leaving group (i.e., serine O_G and oxyanion) should have a lone pair orbital anti periplanar to the C–N bond which is to be broken.

Although the above requirements prescribe the orientation of two lone pairs of the oxyanion in the tetrahedral intermediate, it has been suggested that since the oxyanion has three lone pairs, two of them should easily adopt the required orientation (Bizzozero & Dutler, 1981). However, our results indicate that the oxyanion is hydrogen bonded to the oxyanion binding site, most probably through two electron pairs. Since an electron pair involved in hydrogen bonding cannot be regarded as a real lone pair, only one free electron pair remains, the orientation of which is necessarily defined by the two hydrogen bonds. If models of the tetrahedral intermediates at the active sites of the two serine proteinases are constructed on the basis of X-ray data (cf. Kraut, 1977), a conformation depicted in Figure 2 is obtained. It is seen from Figure 2 that the only real lone pair is actually anti periplanar to the C–N bond in both chymotrypsin and subtilisin.

Table IV: Second-Order Rate Constants for Acylation of Thiol Proteinases by the Oxygen and Thiono Esters of Benzoylglycine[a]

	k_O (M^{-1} s^{-1})	k_S (M^{-1} s^{-1})	k_O/k_S
papain	240 ± 10[b]	540 ± 30[b]	0.45
	20.5[c]	22.8[c]	0.9
chymopapain	13 ± 1[d]	31 ± 2[d]	0.42

[a] In 0.2 M KCl and 3% v/v acetonitrile, at 25 °C, pH 6.5, in the presence of 10^{-3} M EDTA. [b] Substrate concentration range (1–5) × 10^{-4} M; enzyme concentration range 2 × 10^{-6}–1 × 10^{-5} M. [c] Lowe & Williams (1965). [d] Substrate concentration range (2–5) × 10^{-4} M; enzyme concentration 2 × 10^{-5} M.

It follows that stereoelectronic control cannot exclusively prevail in serine proteinase catalysis; hydrogen bonding and other interactions with the protein environment may endow the tetrahedral intermediate with properties different from those in simple solution reactions, and therefore these energetically potent interactions may overrule stereoelectronic effects.

Hydrolysis of Oxygen and Thiono Esters by Thiol Proteinases. In an earlier study acyl-enzyme formation was demonstrated in papain catalysis by using *O*-methyl *N*-benzoylthioglycinate (Lowe & Williams, 1965). The k_{cat}/K_m value obtained with this substrate was similar to that found with methyl *N*-benzoylglycinate. The implications of this result with respect to a possible oxyanion binding site were not grasped at that time. A stereochemical mechanism based on X-ray diffraction and kinetic investigations (Wolthers et al., 1970; Lowe & Yuthavong, 1971) suggested that the oxyanion was in a sterically not restricted environment, i.e., without hydrogen bonding to papain. In contrast, from the three-dimensional structure of a papain derivative formed with a specific chloromethyl ketone inhibitor, it was inferred that the backbone NH group of the essential cysteine and the NH_2 group of glutamine-19 constitute an oxyanion binding site (Drenth et al., 1975, 1976). In order to clarify this contradiction we compared the reactions of papain and chymopapain, because the latter contains a valine residue in place of glutamine-19 (Tsunoda & Yasunobu, 1966), and thus, in contrast to papain, it presumably does not possess an oxyanion binding site.

The second-order rate constants for papain and chymopapain reactions are shown in Table IV. It is seen that the rate constants obtained with papain are much higher than those determined by Lowe & Williams (1965) in the presence of 30% acetone, but the k_O/k_S ratios are similar in both cases. Namely, the thiono ester reaction is not inhibited but rather is slightly promoted compared to the oxygen ester, which is in agreement with their relative reactivities in the alkaline hydrolysis (see Table I). This can be interpreted in terms of similar solvation of the oxyanion in water and in the protein environment; i.e., it appears that a special oxyanion binding site does not exist in papain, but the oxyanion might point toward the solvent.

An alternative interpretation emerges in the light of the data of Lowe & Williams (1965) showing that the similarity of the composite rate constant $k_{cat}/K_m = k_2/K_s$, with the oxygen and thiono esters of benzoylglycine is the result of compensation: both k_{cat} and K_m are about 10 times as high with the oxygen as with the thiono ester. A compensation between k_2 and K_s may also be possible in the present case. Such a compensation implies that an inhibited acylation with thiono esters is compensated by tighter binding as compared to oxygen esters. This would be a remarkable difference from the catalysis by serine proteinases. However, this interpretation is not supported by the results obtained with chymopapain lacking glutamine-19, a building stone of the oxyanion hole. As it is seen from Table IV, chymopapain exhibits virtually the same k_O/k_S ratio as does papain. Since an identical rate-binding compensation with a different enzyme seems highly improbable, the results with chymopapain seem to devaluate the importance of the oxyanion binding site. On the other hand, although benzoylglycine methyl ester is a fairly good substrate of papain, which should in most respects conform to the general mechanism, it cannot be ruled out that more specific substrates, like those related to the inhibitors used in the X-ray diffraction studies (Drenth et al., 1976), may interact with an oxyanion binding site on their reaction pathway.

We have pointed out earlier (Polgár, 1973) that there is a major difference between the mechanisms of serine and thiol proteinases inasmuch as the nucleophilic attack on the substrate is a general-base-catalyzed process with serine proteinases whereas it is not catalyzed with the thiol proteinases possessing a mercaptide ion as nucleophile. By comparing the reactions of thiono and oxygen esters in this study, we observed an important difference in the oxyanion binding, too, at least in the case of benzoylglycine derivatives.

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Polarization of Substrate Carbonyl Groups by Yeast Aldolase: Investigation by Fourier Transform Infrared Spectroscopy†

Joel G. Belasco‡ and Jeremy R. Knowles*

ABSTRACT: The infrared spectrum of the complex of D-fructose 1,6-bisphosphate bound to yeast aldolase displays three spectral features between 1700 and 1800 cm^{-1}. One of these (at 1730 cm^{-1}) corresponds to the carbonyl group of enzyme-bound D-fructose 1,6-bisphosphate and/or dihydroxyacetone phosphate. The frequency of this band, which is unaffected by the removal of the intrinsic zinc ion from the enzyme, demonstrates that this carbonyl group is not significantly polarized when the substrate binds to the enzyme. In contrast, the spectral band assigned to the carbonyl group of enzyme-bound D-glyceraldehyde 3-phosphate (at 1706 cm^{-1}) appears at a frequency 24 cm^{-1} lower than when this substrate is in aqueous solution. This shift indicates considerable polarization of the carbonyl group when D-glyceraldehyde 3-phosphate is bound at the active site. The third spectral feature (at 1748 cm^{-1}), which is observed only in the presence of potassium ion, probably corresponds to an enzymic carboxyl group in a nonpolar environment.

Enolization is a common enzyme-catalyzed reaction, yet a detailed understanding of how enzymes accelerate this process is still lacking. One means that an enzyme might employ is the polarization of the substrate's carbonyl group by a strong electrophile. Such an interaction would facilitate proton abstraction from the adjacent carbon atoms by electronic distortion of the substrate toward the structure of the transition state for enolization and by stabilization of the developing negative change on the incipient enolate oxygen in this transition state.

While attractive as a hypothesis, direct evidence for carbonyl polarization exists for only one enzyme. This evidence is based on the knowledge that the polarization of a carbonyl group weakens the carbon-oxygen double bond, resulting in a reduction in the carbonyl stretching frequency. The detection of such a change in the vibrational character of a single carbonyl group among the hundreds present in an enzyme-substrate complex requires an exceptionally sensitive spectroscopic method, such as Fourier transform infrared spectroscopy. By this means, we have earlier shown that most molecules of dihydroxyacetone phosphate experience a substantial reduction in their carbonyl stretching frequency upon binding to triosephosphate isomerase, which can be interpreted as catalytically productive polarization induced by an enzymic electrophile (Belasco & Knowles, 1980).

Fructose-1,6-bisphosphate aldolase from yeast catalyzes the abstraction of an α proton from dihydroxyacetone phosphate and the condensation of the resulting carbanionic species with D-glyceraldehyde 3-phosphate to yield D-fructose 1,6-bisphosphate. The enzyme is a dimer, and each subunit bears one tightly bound zinc ion which is absolutely required for catalytic activity (Kobes et al., 1969; Harris et al., 1969). In addition, high concentrations of potassium ion elicit a roughly 7-fold rate enhancement (Richards & Rutter, 1961). Unlike the aldolases of higher eukaryotes, the mechanism of the yeast enzyme appears not to involve an enamine intermediate (Rutter, 1964; Stribling & Perham, 1973).

The catalytic role of the essential divalent metal ion has been a matter of special interest. On the basis of NMR studies using the Mn^{2+}-substituted enzyme, Smith & Mildvan (1981) have concluded that while the carbonyl group of dihydroxyacetone phosphate bound to aldolase is oriented toward the divalent metal ion, the distance between them (>6 Å) is too great for direct, inner-sphere, coordination (Smith et al., 1980). Nevertheless, they have argued that the zinc ion might still serve as an electrophile for the carbonyl group of dihydroxyacetone phosphate (and, presumably, that of fructose 1,6-

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‡ Present address: Department of Genetics, Stanford University School of Medicine, Stanford, CA 94305.

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