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Probes of the Mechanism of Zymogen Catalysis†

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ABSTRACT: Trypsinogen and chymotrypsinogen hydrolyze *p*-nitrophenyl esters of several peptides and *tert*-butyloxycarbonyl amino acids. The best substrate found for chymotrypsinogen was Boc-Ala-ONp and for trypsinogen Z-Gly-Hyp-Gly-ONp. Comparison of the kinetic parameters indicates that in the zymogens the catalytic site is distorted and reduced in effectiveness by about two orders of magnitude, in addition to a 10 000-fold decrease in catalysis due to a distortion of the primary substrate binding site (Kerr, M. A., Walsh, K. A., & Neurath, H. (1976) *Biochemistry 15,* 5566). Using Boc-Ala-ONp as substrate and certain aldehydes and borates as inhibitors, the zymogens were tested for the integrity of the "oxyanion hole", but these results were largely inconclusive. Probes for the secondary binding sites indicated their presence in trypsinogen and their absence in chymotrypsinogen.

Kinetic studies of the catalytic activity of zymogens have indicated that the major change in the zymogen/enzyme transformation involves the development of a primary binding site (also called the tosyl hole or P_1 binding site; Gertler et al., 1974; Kerr et al., 1975). Since the zymogen reacts more slowly than the enzyme with methanesulfonyl fluoride (Morgan et al., 1972), and since acyl-zymogens deacylate more slowly than acyl-enzymes (Kerr et al., 1975), it was suggested that in the zymogen the oxyanion hole is also distorted (Kerr et al., 1976). Similar conclusions have been drawn from studies employing a variety of physical and chemical techniques (Robillard & Schulman, 1974; Freer et al., 1970; Birktoft et al., 1976; Hanai, 1976; Fehlhammer et al., 1977; Reeck et al., 1977; Porubcan et al., 1977). Past kinetic studies have been restricted to the use of pseudo-substrates or active-site titrants, e.g., *p*-nitrophenyl-*p'*-guanidinobenzoate, and consequently may be called into question because the peculiar molecular geometry of these compounds may render them unsuitable for the detection of the subtle rearrangements which have been observed by x-ray studies (see Kerr et al., 1976). To overcome this possible restriction, we have explored in the present work aminoacyl esters as potential substrates for trypsinogen and chymotrypsinogen. The effect of the length of both the P_1 side chain and the peptide chain itself upon the catalytic activity of these zymogens was studied. In addition, by utilizing a new substrate to monitor catalysis, we have compared zymogens and their enzymes in their reactivity toward various ligands which interact with the secondary binding sites and/or the oxyanion hole.

Materials and Methods

Once recrystallized bovine trypsinogen, lyophilized bovine trypsin, 3× crystallized bovine chymotrypsinogen, and α-chymotrypsin were products of Worthington Biochemical Corp. The esters Boc-Ala-ONp[1] and Ac-Ala-Ala-Ala-OMe and the inhibitor phenylboronic acid were products of Sigma. The compounds Ac-Ala-Ala-AzaAla-ONp and Z-Gly-Leu-Ala chloromethyl ketone were gifts from Dr. J. C. Powers (Georgia Institute of Technology). The esters Z-Ser-Gly-Gly-ONp and Z-Gly-Hyp-Gly-ONp were prepared for us by Professor T. Wieland (Heidelberg). Elastatinal, a peptide al-

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[1] Abbreviations used: Boc, *tert*-butyloxycarbonyl; Z, benzyloxycarbonyl; Abu, α-aminobutyric acid; Nva, norvaline; Hyp, hydroxyproline; AzaAla, azaalanine (*N*[1]-methyl carbazate); Dip-F, diisopropyl fluorophosphate; DIP, diisopropyl phosphoryl; NPGB, *p*-nitrophenyl *p'*-guanidinobenzoate; -ONp, *p*-nitrophenyl ester; -OMe, methyl ester; Pipes, piperazine-*N*,*N'*-bis(2-ethanesulfonic acid).

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TABLE I: Kinetic Parameters of the Hydrolysis of Several Substrates by Chymotrypsinogen, Trypsinogen, and Their Respective Enzymes (in 16.7% Dioxane).[a]

	Boc-Gly-ONp		Boc-Ala-ONp		Boc-Abu-ONp		Boc-Nva-ONp	
	k_{cat} (s^{-1})	K_m (M)	k_{cat} (s^{-1})	K_m (M)	k_{cat} (s^{-1})	K_m (M)	k_{cat} (s^{-1})	K_m (M)
chymotrypsinogen	7.7×10^{-3}	2.7×10^{-3}	28.1×10^{-3}	0.4×10^{-3}	4.1×10^{-3}	1.2×10^{-3}	8.4×10^{-3}	1.2×10^{-3}
chymotrypsin	1.2	1.3×10^{-3}	2.3	2.1×10^{-3}	0.9	0.1×10^{-3}	2.2	0.06×10^{-3}
trypsinogen	3.6×10^{-3}	3.4×10^{-3}	3.9×10^{-3}	3.5×10^{-3}	1.8×10^{-3}	2.3×10^{-3}	0.68×10^{-3}	0.6×10^{-3}
trypsin	1.2	1.45×10^{-3}	0.68	2.2×10^{-3}	0.15	0.03×10^{-3}	1.4	0.5×10^{-3}
	specificity constant, k_{cat}/K_m (M^{-1} s^{-1})							
chymotrypsinogen	2.9		72		3.3		7.1	
chymotrypsin	0.9×10^3		1.1×10^3		8.4×10^3		34.8×10^3	
trypsinogen	1.1		1.1		0.8		1.1	
trypsin	0.9×10^3		0.3×10^3		4.7×10^3		2.6×10^3	

[a] Kinetic parameters for the hydrolysis by chymotrypsinogen, chymotrypsin, trypsinogen, and trypsin of several *p*-nitrophenol esters of Boc-amino acids. The upper panel lists the kinetic constants k_{cat} and K_m and the lower panel the specificity constant (k_{cat}/K_m) (Brot & Bender, 1969). The assay system consisted of 2.0 mL of Pipes (0.1 M, pH 7.2, $CaCl_2$, 0.04 M), 0.5 mL of substrate in dioxane, and 0.5 mL of enzyme or zymogen in 1 mM HCl. The control solution (automatically subtracted in reference beam sample) contained DIP-enzyme or DIP-zymogen in place of pure enzyme or zymogen.

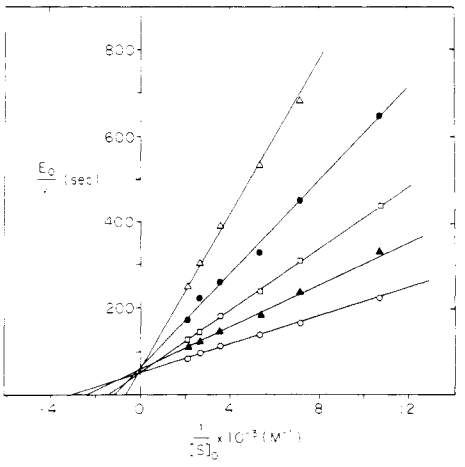

FIGURE 1: The competitive inhibition by borate of the chymotrypsinogen (5×10^{-6} M) hydrolysis of Boc-Ala-ONp. The concentrations of borate were (○) zero, (▲) 18.7 mM, (□) 37.4 mM, (●) 74.8 mM, and (△) 149.5 mM in 0.093 M Pipes, pH 7.2, containing 0.037 M $CaCl_2$ and 1.9% dimethylformamide. The reaction was followed at 400 nm, 25 °C.

dehyde (*N*-(1-carboxyisopentyl)carbamoyl-α-(2-iminohexahydro-4-pyrimidyl)glycylglutaminylalaninal), was a gift from Dr. W. Troll (New York University Medical Center).

The *p*-nitrophenyl esters of Boc-Nva and Boc-Abu were prepared in dry ethyl acetate at 0 °C employing a 5% molar excess of *p*-nitrophenol and dicyclohexylcarbodiimide over the respective Boc-amino acids. The mixtures were stirred overnight at 4 °C. The precipitate of dicyclohexylurea was removed by filtration, the solution volume reduced by rotary evaporation, and the product precipitated by the addition of light petroleum ether (30–60 °C). Recrystallization was performed from a dilute solution of chloroform/light petroleum ether at −20 °C. Boc-Abu-ONp (yield 67%): mp 76 °C. Anal. Calcd: C, 55.6; H, 6.2; N, 8.6. Found: C, 54.6; H, 6.1; N, 8.4. Boc-Nva-ONp (yield 25%): mp 69 °C. Anal. Calcd: C, 56.8; H, 6.6; N, 8.3. Found: C, 56.8; H, 6.6; N, 8.0. No attempt was made to maximize the yields.

DIP-trypsinogen, DIP-trypsin, DIP-chymotrypsin, and DIP-chymotrypsinogen were prepared as described by Gertler et al. (1974). Trypsinogen and chymotrypsinogen were activated by an acid protease from *A. orzyae* as previously described (Gertler et al., 1974).

Human plasminogen was prepared from fresh human plasma by the procedure of Deutsch & Mertz (1970). The plasminogen was then rechromatographed on a fresh column of lysine-Sepharose (30 × 2.5 cm) by applying a 2-L linear gradient (from 0 to 0.04 M) of ε-aminocaproic acid in 0.05 M sodium phosphate, 0.02 M benzamidine, pH 7.5. Two plasminogen fractions were eluted by 0.005 M and 0.0062 M ε-aminocaproic acid, respectively. Gel filtration on Sephadex G-150 (5 × 47.5 cm) in 0.05 M sodium phosphate, 1 mM benzamidine (pH 7.6) gave essentially one fraction in an elution volume corresponding approximately to a molecular weight of 90 000.

Reaction kinetics were monitored on a Cary Model 16 double beam spectrophotometer by following the change in optical density at 400 nm, pH 7.2, produced by the release of *p*-nitrophenol from the respective substrates (E_{400} = 12 200). The substrate concentrations ranged from 0.02 mM to a maximum of 0.5 mM. DIP-zymogen was added to the reference beam sample to correct for any nonspecific hydrolysis of the substrate by the zymogen. Reaction rates were calculated from the slopes of the curves after a steady state had been achieved. The kinetic parameters, K_m and k_{cat}, were computed on a Wang 720C calculator by least-square methods.

Results

Primary Binding Sites. Several *p*-nitrophenyl esters of N^{α}-acylated amino acids of increasing side chain length were tested as substrates for chymotrypsinogen and trypsinogen. Preliminary experiments with Z-Gly-ONp and Ac-Gly-ONp showed that these potential substrates were unsuitable due to their limited solubility and high rate of nonspecific hydrolysis. The ester Boc-Gly-ONp appeared to be suitable as it was sufficiently soluble and stable to permit a study of the integrity of the active sites of the zymogens. The individual rate constants, k_{cat} and K_m, as well as the second-order rate constant, k_{cat}/K_m, were calculated from Lineweaver-Burk plots (cf. Figure 1) derived from the steady-state portions of the hydrolysis curves. In the case of chymotrypsinogen, the steady state was achieved after 1–2 min and, in the case of trypsinogen, after 10–15 min. The results are included in Table I.

It should be noted that these assays were performed in 16.7% *p*-dioxane, a competitive inhibitor of chymotrypsin which binds in the tosyl hole (Applewhite et al., 1958; Clement & Bender, 1963; Steitz et al., 1969). When the solvent was changed to 1.9% dimethylformamide (see below), the K_m for the reaction of chymotrypsin with Boc-Ala-ONp decreased dramatically

TABLE II: Hydrolysis of a Glycyl Ester and Tripeptide Glycyl Esters by Zymogens and Their Enzymes.[a]

	k_{cat} (s^{-1})	K_m ($M \times 10^4$)	k_{cat}/K_m (M^{-1} (s^{-1})
trypsinogen			
Boc-Gly-ONP	6.1×10^{-3}	26.4	2.3
Z-Ser-Gly-Gly-ONp	4.0×10^{-3}	6.3	6.4
Z-Gly-Hyp-Gly-ONP	7.6×10^{-3}	1.8	41.5
trypsin			
Boc-Gly-ONp	0.22	5.2	0.43×10^3
Z-Ser-Gly-Gly-ONp	0.47	5.8	0.81×10^3
Z-Gly-Hyp-Gly-ONp	0.99	5.0	1.97×10^3
chymotrypsinogen			
Boc-Gly-ONp	6.6×10^{-3}	6.3	10.5
Z-Ser-Gly-Gly-ONp	3.7×10^{-3}	7.1	5.2
Z-Gly-Hyp-Gly-ONp	9.3×10^{-3}	3.9	23.8
chymotrypsin			
Boc-Gly-ONp	0.52	0.22	23.6×10^3
Z-Ser-Gly-Gly-ONp	0.32	0.47	6.8×10^3
Z-Gly-Hyp-Gly-ONp	1.88	1.75	10.7×10^3

[a] Measurements were performed in 0.093 M Pipes, pH 7.2, 0.037 M $CaCl_2$, 1.9% dimethylformamide at 25 °C.

from 2.1×10^{-3} to 5×10^{-5} M, while that for the reaction of chymotrypsinogen (3×10^{-4} M) was little affected.

Secondary Binding Sites. The integrity of the secondary binding sites, P_2 and P_3, in chymotrypsinogen (notation of Schechter & Berger, 1967) was tested by utilizing substrates or inhibitors of elastase. These all have small P_1 side chains; consequently their interactions with trypsinogen and chymotrypsinogen at P_1 should be minimal.

The first approach employed potential substrates with extended peptide chains as shown in Table II for a series of glycine derivatives. As in the experiments shown in Table I, the zymogen is in each case a less efficient catalyst than the corresponding enzyme. In the case of trypsinogen/trypsin, elongation of the simple glycyl ester to a tripeptide ester increases the specificity constant (k_{cat}/K_m), whereas the chymotrypsinogen/chymotrypsin system remains unaffected.

An alternative approach to test the integrity of secondary binding sites in chymotrypsinogen utilized compounds which might be expected to bind at these sites and so inhibit the intrinsic activity of the zymogen; however, none was found to do so. Under conditions where Ac-Ala-Ala-Ala-OMe (4 mM, pH 7.2) caused inhibition (K_I, ca. 6 mM) of the chymotryptic hydrolysis of Boc-Ala-ONp, no inhibition of chymotrypsinogen, trypsinogen, or trypsin was observed. Similarly, elastatinal (4 mg/mL) had no effect upon the activity of chymotrypsinogen, but in this instance, the inhibitor also failed to affect the activity of chymotrypsin. Z-Gly-Leu-Ala chloromethyl ketone (2.5×10^{-3} M) failed to inhibit chymotrypsinogen even after incubation at room temperature (pH 7.2) for 98 h, while chymotrypsin was inhibited by 50% within 10 s. The ester Ac-Ala-Ala-AzaAla-ONp (90 μM) reacted with chymotrypsin, releasing a 3-min burst of nitrophenol, but failed to do so with the zymogen.

Transition-State Probes. Boronic acids have been employed as probes of the tetrahedral transition state in the catalysis by chymotrypsin and subtilisin (Koehler & Lienhard, 1971; Phillip & Bender, 1971; Lindquist & Terry, 1974). In the present work, both boric acid (Figure 1) and phenylboronic acid were found to inhibit competitively the chymotrypsinogen catalyzed hydrolysis of Boc-Ala-ONp. Boric acid also competitively inhibited trypsinogen but less so than trypsin (Table III). The inhibition of trypsin[illegible]en by phenylboronic acid (10 mM) was so weak that K_I cou[illegible] not be reliably calculated.

TABLE III: Competitive Inhibition Constants (K_I) for Phenylboronic Acid and Boric Acid.[a]

	phenylboronic acid (M)	boric acid (M)
chymotrypsinogen	4×10^{-3}	35×10^{-3}
chymotrypsin	0.2×10^{-3} [b]	70×10^{-3}
trypsinogen		140×10^{-3}
trypsin	11×10^{-3}	70×10^{-3}

[a] Measurements of hydrolysis of Boc-Ala-ONp in 0.093 M Pipes, pH 7.2, at 25 °C. [b] Phillip & Bender (1971).

Aliphatic, site-specific aldeh[illegible]les have also been suggested as transition-state analogues of [illegible]erine proteases (Thompson, 1973; Leinhard, 1973; Rawn & [illegible]ienhard, 1974; Schultz & Cheerva, 1975). Benzaldehyde (20 mM) had no effect upon chymotrypsinogen and *p*-nitrobenzaldehyde (2.8 mM), which should more readily form the hemiacetal, also failed to inhibit chymotrypsinogen; *p*-hydroxybenzaldehyde (10 mM) caused noncompetitive inhibition. Although these aldehydes resemble in structure phenylboronic acid, they may not be satisfactory probes of the oxyanion hole (Schultz & Cheerva, 1975; Gorenstein et al., 1976). While they inhibit competitively the chymotrypsin catalyzed hydrolysis of Boc-Ala-ONp at pH 7.2 (benzaldehyde K_I = 3.2 mM; *p*-hydroxybenzaldehyde K_I = 2.7 mM; *p*-nitrobenzaldehyde K_I = 1.1 mM), the K_I values are not as low as might be anticipated for good transition-state analogues of chymotrypsin.

Plasminogen. Under the conditions normally employed for the demonstration of the intrinsic activity of trypsinogen or chymotrypsinogen, human plasminogen was found to be inactive toward Boc-Ala-ONp (0.5 mM), NPGB (0.7 mM), and Dip-F (0.075 M). Activity toward benzoylarginine ethyl ester was not induced in plasminogen by 0.1 M isoleucylvalinamide, whereas the free dipeptide induces conformational changes indicative of the activation of trypsinogen (Bode & Huber, 1976).

Discussion

Chymotrypsinogen. Kerr et al. (1976) proposed that chymotrypsinogen should be considered a poor catalyst for *all* substrates because even those which bind well would not be able to utilize the distorted oxyanion hole of the zymogen. However, this view was based on evidence obtained from distorted substrate analogues and active site titrants such as NPGB, *p*-nitrophenyl *p'*-(dimethylsulfonioacetamido)benzoate and methylumbelliferyl-*p'*-trimethylammonium cinnamate. The present study extends this line of inquiry by utilizing α-aminoacyl esters which lack such distortions and are potentially better substrates to probe for components of the active sites in the zymogens.

Table I shows that the new substrates are sufficiently compatible with the active site of chymotrypsinogen to permit kinetic analysis of the differences between zymogen and enzyme and, more importantly, to permit a distinction between effects on K_m from those on k_{cat}. The k_{cat} values for chymotrypsinogen are in the range 10^{-2} s^{-1}, which is approximately 100-fold greater than prior estimates using distorted substrates. Nevertheless, for each new substrate chymotrypsin still has a larger k_{cat} than chymotrypsinogen by 2–3 orders of magni-

tude. Since any difference in binding affinity for the substrate would be expected to affect K_m rather than k_{cat} (as is observed with chymotrypsin and the longer side-chain substrates), the large differences in k_{cat} between enzyme and zymogen indicate that there is indeed a significant lesion in some element of the catalytic apparatus of the zymogen as observed by Morgan et al. (1974) with trypsinogen and as proposed by Kerr et al. (1976) for chymotrypsinogen. Since the *p'*-(dimethylsulfonioacetamido)benzoyl derivatives of chymotrypsin and chymotrypsinogen deacylate at the same rate (Kerr et al., 1975), it seems unlikely that the lesion involves the charge relay system since serine-195 of the charge relay is obviously involved in the deacylation of both zymogen and enzyme. Consequently Kerr et al. (1976) postulated that this lesion was a result of the misalignment of the oxyanion hole in the zymogen. Aldehydes and borates have been reported to be analogues of the transition-state intermediates of chymotrypsin-catalyzed hydrolyses which utilize the oxyanion hole in their binding. We therefore tested the postulate of Kerr et al. (1976) with these compounds but with only limited success. For instance, there is no need to invoke the concept of transition-state analogues to explain the moderate inhibition of chymotrypsin by aldehydes. Binding at the P_1 site is probably sufficient and it is therefore not surprising that these aldehydes failed to inhibit chymotrypsinogen. On the other hand, the inhibition of chymotrypsin and chymotrypsinogen by boric acid may be in accord with the concept of a distorted oxyanion hole in the zymogen but the very weak binding makes interpretation hazardous. Furthermore, it seems incongruous that the zymogen should bind boric acid more tightly than the enzyme for which it is supposed to be a transition-state analogue. The nature of the moderate binding of phenylboronic acid to chymotrypsinogen also remains obscure. Unlike chymotrypsin (and subtilisin, Matthews et al., 1975) where the tight binding of phenylboronic acid can be equated with the concerted interactions of the phenyl group with the tosyl hole and the boronic group with the catalytic site, the tosyl hole is unavailable in chymotrypsinogen. Possibly the phenyl group binds in the hydrophobic P' site observed by Scofield et al. (1977). Benzaldehyde, however, gave no evidence for such a binding site.

Previous studies have shown that both chymotrypsinogen and trypsinogen have less effective primary binding sites than their enzymes (Gertler et al., 1974). In order to probe these differences more fully, we studied the influence of the length of the side chain (P_1) upon catalysis by chymotrypsinogen. A comparison of the data for the probes of this binding site (Table I) shows that chymotrypsinogen exhibits a marked preference for Boc-Ala-ONp over the other substrates with improvements in both k_{cat} and K_m. This observation suggests that the restricted tosyl hole in the zymogen may accept the methyl group. In this respect, it differs from chymotrypsin which shows a progressive increase in catalysis of the longer side-chain substrates as expressed by improved K_m values. Since these substrates are all *p*-nitrophenyl esters, it is not possible to equate K_m with the binding constant, K_s, although it is probable that deacylation is involved in the rate-determining step as shown by the induction period necessary for the attainment of the steady state. Nevertheless, these substrates and, in particular, Boc-Ala-ONp are useful probes of the various aspects of the intrinsic activity of chymotrypsinogen.

In order to probe for a possible lesion in the secondary binding sites of chymotrypsinogen, we utilized both elongated peptide substrates and peptides as potential inhibitors. The elongated substrates (Table II) showed no marked increase in susceptibility to catalysis by either chymotrypsin or chymotrypsinogen and are consequently ineffective probes for these secondary binding sites. The inhibitors, however, gave more definitive results. The failure of Ac-Ala-Ala-Ala-OMe, Ac-Ala-Ala-AzaAla-ONp, and Z-Gly-Leu-Ala chloromethyl ketone to inhibit the hydrolysis of Boc-Ala-ONp by chymotrypsinogen while inhibiting the hydrolysis by chymotrypsin suggests that the secondary binding sites are absent in this zymogen.

Trypsinogen. Similar studies of trypsinogen show several features both in common with and different from chymotrypsinogen. For instance trypsinogen, like chymotrypsinogen, has a lower k_{cat} than its enzyme for each of the substrates tested, suggesting an impaired catalytic apparatus. The nature of this impediment may be slightly different from that of chymotrypsinogen since the K_I for boric acid inhibition of trypsinogen is slightly higher than that of trypsin, whereas that for chymotrypsinogen is lower than for chymotrypsin. Furthermore, while the primary binding sites of both zymogens are distorted (Gertler et al., 1974; Kerr et al., 1975; Birktoft et al., 1976; Fehlhammer et al., 1977; Kossiakoff et al., 1977) trypsinogen shows no marked preference for Boc-Ala-ONp or for any of the other α-aminoacyl substrates employed. In parallel with trypsin, trypsinogen shows improvements in catalysis when elongated peptide substrates (Z-Ser-Gly-Gly-ONp and Z-Gly-Hyp-Gly-ONp) replaced Boc-Gly-ONp. However, with trypsin the effect is expressed in k_{cat} and with trypsinogen in K_m. The difference could be the result of an induced fit in trypsin which is precluded in trypsinogen or a result of the requirement for precise alignment of substrate by trypsin to elicit the cooperative interaction of the oxyanion hole with the charge relay system. If the oxyanion hole is unavailable, then the improved binding would not be reflected in k_{cat} as indeed is the case with trypsinogen.

Mechanisms of Zymogen Activation. In an oversimplified view, three basic features could account for the suppressed activity of the zymogens of serine proteases: (1) a distortion of the binding sites (primary and/or secondary); (2) a distortion of the catalytic site (charge relay system and/or oxyanion hole); or (3) a blocking of these sites by the activation peptide (Neurath & Walsh, 1976). Since, in principle, it is only necessary that a zymogen should be susceptible to a conformational correction by limited proteolysis, there is no a priori reason why any one method of suppression should have been selected during biological evolution. While the catalysis by trypsinogen or chymotrypsinogen appears to be inhibited by a combination of distortions of both the binding and the catalytic sites, the inhibition may differ in detail in each case. Other combinations may also exist. For instance, among the zymogens of the plasma serine proteases, factor X catalyzes the hydrolysis of NPGB and is susceptible to inhibition by Dip-F (Kerr et al., 1978), while plasminogen does not react with these agents (see also Wohl et al., 1977). In contrast, factor VII appears to react with Dip-F as well as does the active enzyme, factor VII_a, suggesting that the charge relay system, the oxyanion hole, and the P_1 binding site may be largely intact in this zymogen (Nemerson, 1976).

In conclusion, probes for the individual substructures of trypsinogen and chymotrypsinogen now reveal that during activation the catalytic sites, as well as the primary binding sites, become realigned. These realignments may differ in detail in each case and may yet be different in other zymogens which react with Dip-F relatively rapidly (factor VII) or not at all (plasminogen). Thus, despite analogies in charge relay system, oxyanion hole and binding site, each enzyme/zymogen pair appears to have evolved with a characteristic and unique mode of activation.

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