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A Proposal for the Mechanism of Chymotrypsinogen Activation†

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ABSTRACT: Carboxymethylation of Met-192 of chymotrypsin with iodoacetate inhibits the hydrolysis of acetyltyrosine-*p*-nitroanilide and of acetyltyrosine ethyl ester but has no effect on the hydrolysis of carbobenzyloxytyrosine *p*-nitrophenyl ester or on the reaction with active site titrants. In chymotrypsinogen, Met-192 is largely unreactive toward iodoacetate, whereas Met-180 reacts slowly. Prior acylation of Ser-195 has little effect on the rates of subsequent alkylation of the zymogen or the enzyme. These results, taken together with previous kinetic, spectral, and crystallographic data, lead to a model of zymogen activation which entails not only an enhancement of substrate binding but also a mechanism for stabilization of the transition state during catalysis. In this model, the absence of the "oxyanion hole" in the zymogen precludes effective catalysis even when the substrate is bound.

It has been recently demonstrated that, contrary to traditional views, certain zymogens are not enzymatically inert but possess an intrinsic activity which upon activation becomes magnified by several orders of magnitude (Morgan et al., 1972; Kassell and Kay, 1973; Gertler et al., 1974a). Chymotrypsinogen and trypsinogen react stoichiometrically with active site titrants, such as Dip-F[1] or CH_3SO_3F, and the hydrolysis of the pseudosubstrate *p*-nitrophenyl-*p'*-guanidinobenzoate (NPGB) by chymotrypsinogen proceeds via the same type of acyl intermediates as does the chymotrypsin-catalyzed reaction. Kinetic analyses, including competitive inhibition studies, as well as spectral probes have lead to the conclusion that the principal impairment of the catalytic effectiveness of these zymogens, as compared with the corresponding enzymes, is a diminished binding of the substrate rather than a deficiency in the catalytic elements per se (Gertler et al., 1974a,b; Kerr et al., 1975). This view is in accord with the interpretations of the differences between the electron density maps derived from x-ray structure analysis of chymotrypsinogen and α-chymotrypsin (Freer et al., 1970; Matthews et al., 1967; Steitz et al., 1969). The most significant difference appears to be a distortion in the zymogen of the substrate binding pocket (also referred to as tosyl hole) which in the enzyme accommodates the side chain of the amino acid which contributes the carbonyl group to the susceptible peptide bond. The formation of the specificity pocket results from the repositioning of Met-192 from a deeply buried position in the zymogen to the surface in the enzyme, but the distortion of this pocket alone may not be sufficient to explain the enormous difference in catalytic activity between zymogen and enzyme (Wright, 1973a,b).

We have recently reported that the intrinsic enzymatic activity of chymotrypsinogen toward NPGB was increased approximately sevenfold upon oxidation of Met-192, due to increased affinity of the enzyme for the substrate (Gertler et al., 1974b). While this change is minute, when compared with that accompanying the normal zymogen activation, it is at least in the same direction. Attempts to extend these studies to another type of modification of Met-192, i.e., by carboxymethylation (Koshland et al., 1962), lead us to the conclusion that the rate of this reaction is a sensitive probe of the topography of the region of the substrate binding site and of its involvement in the catalysis by chymotrypsinogen.

Materials and Methods

α-Chymotrypsin and chymotrypsinogen A were obtained from Worthington Biochemicals. [1-^{14}C]Iodoacetic acid was a product of New England Nuclear Corp. Substrates and active-site titrants were obtained as described previously (Kerr et al., 1975).

Carboxymethylation of chymotrypsin, chymotrypsinogen, and their derivatives was carried out by incubation of the proteins (54 μM) at 37 °C with radioactive iodoacetate (54 mM) in 0.2 M acetate buffer, pH 4.5. Incorporation of radioactivity into the proteins was measured, after desalting over Sephadex G-25 in 1 mM HCl, using a Packard 574 liquid scintillation counter. Protein concentrations were calculated from the absorbance at 280 nm or by the method of Lowry et al. (1951).

Amino acid analysis of the radioactive products of acid hydrolysis of the proteins was carried out on a Beckman 120B

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[1] Abbreviations used: NPGB, *p*-nitrophenyl-*p'*-guanidinobenzoate; pGB, *p*-guanidinobenzoyl; NPSA, *p*-nitrophenyl-*p'*-(dimethylsulfonioacetamido)benzoate; pSA, *p*-(dimethylsulfonioacetamido)benzoyl; MUTMAC, methylumbelliferyl-*p'*-trimethylammonium cinnamate; pTMAC, *p'*-trimethylammoniumcinnamoyl; CD, circular dichroism; Dip-F, diisopropyl fluorophosphate; CH_3SO_3F, methylsulfonyl fluoride.

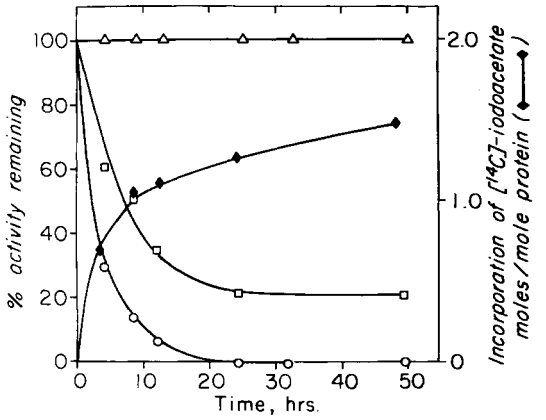

FIGURE 1: The reaction of chymotrypsin with [^{14}C]iodoacetate. Experimental details are given in the Materials and Methods section. Incorporation of radioactivity into chymotrypsin (♦). The effect of the reaction with iodoacetate on the enzymic activity of chymotrypsin toward *N*-acetyltyrosine *p*-nitroanilide (○) and *N*-acetyltyrosine ethyl ester (□). The reaction with iodoacetate did not inhibit enzymic activity toward *N*-carbobenzyloxytyrosine nitrophenyl ester or the active site titrants (NPGB, NPSA, and MUTMAC (△)).

TABLE I: Carboxymethylation of Chymotrypsin, Chymotrypsinogen, and Their Derivatives at pH 3.0.

	Incorp of [^{14}C]Iodoacetate (mol/mol of protein)				
	4 h	8 h	12 h	24 h	48 h
Chymotrypsin					
Native	0.7	1.1	1.2	1.3	1.6
Denatured[a]				2.0	
Sulfoxide of Met-192				0.3	
p-GB-chymotrypsin	0.7	0.9	1.1	1.2	1.4
p-TMAC-chymotrypsin					1.6
p-SA-chymotrypsin					1.6
Chymotrypsinogen					
Native	0.1	0.3	0.3	0.4	0.7
Denatured				2.0	
Sulfoxide of Met-192				0.3	
p-GB-chymotrypsinogen	0.1	0.1	0.2	0.2	0.4
p-TMAC-chymotrypsinogen					0.5
p-SA-chymotrypsinogen					0.6

[a] Denatured by inclusion of 8 M urea in the reaction mixture.

analyzer by the method of Goren et al. (1968) using two temperatures.

Fractions (2 ml) were collected directly from the column and counted for radioactivity. Quantitative amino acid analyses were performed on the same samples using a Durrum D-500 analyzer.

The oxidation of Met-192 in chymotrypsin and chymotrypsinogen was carried out as described by Wasi and Hofmann (1973). The preparation and analysis of acyl-enzymes and acyl-zymogens have been described in an earlier publication (Kerr et al., 1975; Gertler et al., 1974a).

Chymotrypsinogen was converted to chymotrypsin by an acid protease purified from *A. oryzae* as described previously (Gertler et al., 1974a).

Results

The Reaction of α-Chymotrypsin with Iodoacetate. The time course for the reaction of chymotrypsin with radioactive iodoacetate is shown in Figure 1. The reaction was characterized by the initial incorporation of 1 mol of radioactivity per mol of enzyme followed by the much slower incorporation of a second mole. The reaction can be analyzed in terms of two independent first-order reactions with rate constants of 32 and 3.0×10^{-3} min^{-1}, respectively. Amino acid analysis of the products of the reaction showed that the incorporation of radioactivity was due solely to the carboxymethylation of methionyl residues. Ion-exchange chromatography of hydrolyzed samples of labeled protein, desalted at various time intervals, showed in all cases four radioactive peaks, three of which emerged before aspartate and one shortly after proline. Such a distribution is characteristic of the breakdown products of the *S*-carboxymethylmethionine sulfonium salt which is unstable under the conditions of acid hydrolysis (Goren et al., 1968). No carboxymethylhistidine derivatives were seen, and no loss of histidine was observed upon quantitative amino acid analyses of the same samples. Chymotrypsin, denatured by incubation with 8 M urea, reacted rapidly with iodoacetate resulting in the alkylation of two methionyl residues (Table I).

The reactivity of native chymotrypsin with iodoacetate is analogous to the reactivity of the native enzyme with hydrogen peroxide at pH 3.0. It has been shown that only methionyl residues are oxidized and that one residue (Met-192) reacts more rapidly than the other (Met-180) (Wasi and Hofmann, 1973). By choice of suitable reaction time, chymotrypsin can be oxidized with hydrogen peroxide almost exclusively at Met-192. When this oxidized derivative is then treated with [^{14}C]iodoacetate, the incorporation of the first mole of radioactive label is abolished. Koshland et al. (1962) also reported that the two reactions are mutually exclusive.

The alkylation of the first methionyl residue upon reaction of native chymotrypsin with iodoacetate was accompanied by a marked decrease in the enzymic activity toward certain specific substrates (Figure 1). The rate of hydrolysis of *N*-acetyltyrosine-*p*-nitroanilide was decreased to an almost undetectable level while that of *N*-acetyltyrosine ethyl ester was decreased to about 20% of that of a control sample. The rates of hydrolysis of carbobenzyloxytyrosine *p*-nitrophenyl ester and of the nonspecific substrate NPGB were not decreased. In no case did the modification of the second methionyl residue decrease the activity further. The total number of active sites of the enzyme remained constant throughout the alkylation reaction. Reaction of the alkylated derivatives with the active site titrants NPSA[1] and MUTMAC showed in each case rapid acylation and very slow deacylation. The "burst" of released *p*-nitrophenol or methylumbelliferol was identical for each sample.

The circular dichroic spectra of the acyl-enzyme derivatives, formed by reaction of NPGB, NPSA, and MUTMAC with carboxymethylchymotrypsin and oxidized chymotrypsin, were the same as those of the corresponding acyl-chymotrypsins. pSA-chymotrypsins and pTMAC-chymotrypsins showed induced negative ellipticities due to the acylating group whereas pGB chymotrypsins did not (Kerr et al., 1975).

The Reaction of Chymotrypsinogen with Iodoacetate. In contrast to chymotrypsin, chymotrypsinogen reacted only slowly with [^{14}C]iodoacetate (Table I). Incorporation of 1 equiv of radioactivity required more than 48 h. Amino acid analysis again showed that only methionyl residues had been modified. Chymotrypsinogen which had been specifically oxidized at Met-192 (Wasi and Hofmann, 1973) showed only slightly less incorporation, equivalent to that of similarly oxidized chymotrypsin. Denatured chymotrypsinogen reacted

rapidly, two methionine residues per molecule being alkylated.

The intrinsic activity of chymotrypsinogen was slightly increased by reaction with iodoacetate for 48 h. The acylation rate constant with NPGB was 30 min^{-1} M^{-1} compared with 25 min^{-1} M^{-1} for the control. Oxidation of Met-192 increased the activity of the zymogen five- to sevenfold (Gertler et al., 1974b). The activity of methionine-oxidized chymotrypsinogen remained five- to sevenfold higher than that of native chymotrypsinogen after treatment with iodoacetate.

When chymotrypsinogen was first alkylated with iodoacetate for 48 h and then converted to chymotrypsin, the enzyme had the same specific activity as native chymotrypsin toward *N*-acetyltyrosine-*p*-nitroanilide, *N*-acetyltyrosine ethyl ester, and *N*-carbobenzyloxytyrosine *p*-nitrophenyl ester. The number of active sites titrable by NPSA or MUTMAC was identical. The residue that was modified rapidly in chymotrypsin, resulting in a decrease in enzymic activity, was therefore not available in native chymotrypsinogen. Thus, the incorporation of radioactivity into chymotrypsinogen was due to the modification of Met-180.

The CD spectra of the acyl-zymogens formed by reaction of NPGB, NPSA, and MUTMAC with oxidized and partially carboxymethylated zymogen were identical with those of acyl-chymotrypsinogens, which differ significantly from the corresponding acyl-enzymes (Kerr et al., 1975).

The Reaction of Acyl-enzymes and Acyl-zymogens with Iodoacetate. Since chymotrypsin and chymotrypsinogen react with certain pseudosubstrates to form acyl intermediates which are stable at acid pH, these intermediates could be treated with [^{14}C]iodoacetate under the same conditions as the native proteins. The rates of incorporation of radioactivity into pGB-chymotrypsin and pGB-chymotrypsinogen were slightly slower than those of incorporation into the native proteins, respectively (Table I). No significant deacylation of the acyl proteins occurred during the reaction. Similarly, the pTMAC and pSA intermediates were alkylated to the same extent in 48 h as the native enzyme and zymogen.

Discussion

The present results show that the reaction of chymotrypsin with iodoacetate at acid pH results in the modification of one methionine residue (Met-192) followed by the much slower modification of the second (Met-180). The alkylation of Met-192 is accompanied by a significant decrease in activity toward certain specific ester and amide substrates, although the total number of active sites and the activity toward nonspecific substrates remain unaltered. If chymotrypsin is first denatured by treatment with 8 M urea, both methionines can be carboxymethylated. The data are therefore consistent with the crystallographic studies which showed Met-192 to be on the surface of the enzyme whereas Met-180 is buried in the hydrophobic core.

The reaction of chymotrypsinogen with iodoacetate is slower. Since Met-192-oxidized chymotrypsinogen and native chymotrypsinogen react at almost the same rate and since the incorporation of radioactivity into chymotrypsinogen does not affect the activity of the enzyme formed from the modified zymogen, it is concluded that Met-192 reacts extremely slowly with iodoacetate. Similar results have been obtained in studies of the oxidation of the same methionyl residue. Wasi and Hofmann (1973) showed that the rate of oxidation of Met-192 in chymotrypsin approximates that of free methionine in solution, whereas the same residue in chymotrypsinogen reacts some 20 times slower. Both studies are consistent with the crystal structure analyses of chymotrypsin and chymotrypsinogen (Matthews et al., 1967; Freer et al., 1970) which indicate that the repositioning of Met-192 from inside the molecule to the surface is a major feature of zymogen activation in solution.

This repositioning of Met-192 is accompanied by a relocation of the adjacent residue Gly-193 (Freer et al., 1970). It has been proposed that this residue facilitates stabilization of the tetrahedral intermediate formed during acylation and deacylation of chymotrypsin (Henderson et al., 1971; Robertus et al., 1972; Robillard et al., 1972). It would, therefore, be expected that the catalytic efficiency as well as the substrate binding affinity of the zymogen would be decreased. From analysis of the hydrolysis of ester pseudosubstrates by chymotrypsinogen, it was concluded that the substrate binding affinity of the zymogen is much lower than that of the enzyme (Gertler et al., 1974a). As a result, the rate of acylation of the zymogen is much slower than that of the enzyme. However, the rates of deacylation of several acyl-zymogens have been shown to be similar to those of the corresponding acyl-enzymes (Kerr et al., 1975). The rates of deacylation were considerably greater than could be explained by simple alkaline hydrolysis of the ester bond, implying that for these pseudosubstrates the catalytic efficiency of the zymogen and enzyme are comparable.

One possible explanation of the similarity of deacylation rates is that acylation of the zymogen results in a conformational change toward a more enzyme-like state. The CD spectra at shorter wavelengths suggested that a change resembling zymogen to enzyme transformation might occur upon acylation of the zymogen, but the spectra at longer wavelengths showed that the acyl group in each acyl-zymogen was in a different environment from that in the corresponding acyl-enzyme (Kerr et al., 1975; Gertler et al., 1974a). Since the rate of carboxymethylation of Met-192 in chymotrypsin differs greatly from that in chymotrypsinogen, the rate difference can be interpreted as a probe of the topography of the molecule in the binding site region. The slow rates of modification of Met-192 in the three acylzymogens suggest that in each case the residue remains buried. The residue is able to react in the corresponding acyl-enzyme and CD spectra suggest that modification of this residue does not alter the mode of binding of the acyl group. It appears, therefore, that completion of the specificity pocket is not obligatory for catalysis of the deacylation reaction.

Catalysis by Chymotrypsin. In order to explain the catalytic activity of chymotrypsinogen, it is desirable to first consider the nature of catalysis by chymotrypsin. The pathway of catalysis can be represented by:

$$\begin{array}{ccccc} E + \text{Ac-NP} & & & & \\ \quad \uparrow\downarrow K_s & & \text{nitrophenol} & \text{HOH} & \\ \text{(E·Ac-NP)} & \xrightarrow{k_2} & \text{Ac-E} & \xrightarrow{k_3} & E + \text{AcOH} \end{array}$$

The rate of catalysis depends on the strength of binding ($1/K_s$) of the acyl-nitrophenylate substrate (AcNP) and on the rate constants for the transacylation reactions (k_2 and k_3) which form and hydrolyze the acyl-enzyme intermediate (AcE). These reactions are thought to be promoted by the concurrent involvement of three basic components:

(1) The specificity pocket which binds the hydrophobic side chain of the substrate in a manner which permits the acylamido group of the substrate to interact with Ser-214, orienting the susceptible ester bond (Steitz et al., 1969).

(2) The "charge relay system" which elevates the nucleo-

TABLE II: Comparison of Parameters for Catalysis by Chymotrypsin and by Chymotrypsinogen (25 °C).

Acyl Group of Substrate	Acylation by *p*-Nitrophenyl Esters: k_2 (s^{-1})	K_s (M)	k_2/K_s (s^{-1} M^{-1})	Deacylation Rates, k_3 (s^{-1})	pH	Rel Rate Constants: k_2[b]	k_3[b]	k_2/k_3	Ref
(A) Chymotrypsin									
Specific substrates									
N-AcTrp	38 300	2.5×10^{-3}	1.52×10^{7}	30.5	7.0	3.5×10^{8}	277 000	1250	*c*
Pseudosubstrates									
Cinnamoyl-	45	4.1×10^{-3}	1.1×10^{4}	1.1×10^{-2}	8.3	4.1×10^{5}	100	4100	*d*
GB-			$>10^{4}$	1.1×10^{-2}	7.2		100		*e, f, h*
Active-site titrants									
TpTMAC-			$\geq 2 \times 10^{4}$[a]	1.8×10^{-4}	8.2		1.6		*f, g*
pSA-	0.56	2.6×10^{-5}	2.1×10^{6}	7.8×10^{-5}	8.3	5.1×10^{3}	0.71	7200	*h*
(B) Chymotrypsinogen									
GB-		$>10^{-3}$	0.33	8.3×10^{-5}	7.2		0.75		*e, i*
pTMAC-			28[a]	1.1×10^{-4}	8.2		1.0		*f*
pSA-		$\geq 10^{-3}$	0.14	1.5×10^{-4}	8.2		1.4		*f*

[a] Methylumbelliferyl ester. [b] Setting arbitrarily 1.1×10^{-4} s^{-1} as 1.0. [c] Zerner et al. (1964). [d] Bender and Zerner (1962). [e] Gertler et al. (1974a). [f] Kerr et al. (1975). [g] Jameson et al. (1973). [h] Wang and Shaw (1972). [i] Gertler et al. (1974b).

philicity of Ser-195, promoting its attack on the oriented substrate during the acylation reaction and also elevates the nucleophilicity of a water molecule during the deacylation reaction (Blow et al., 1969).

(3) Hydrogen bonds from the -NH functions of Gly-193 and Ser-195 (the "oxyanion hole") to the properly oriented carbonyl oxygen of the substrate, stabilizing the tetrahedral intermediates (transition states) and thereby promoting both acylation and deacylation of the enzyme (Henderson et al., 1971; Robertus et al., 1972).

With an ideal substrate for chymotrypsin, the geometries of the enzyme and of the substrate are compatible to promote binding in a productive mode which stabilizes transition states during both the acylation and the deacylation reactions. With poor substrates or pseudosubstrates, the complex is less productive because of misalignment of the susceptible bond relative to the idealized transition states. This contention is supported by the data in Table II which show that the magnitudes of the relative intrinsic acylation rates (k_2) for chymotrypsin vary over five orders of magnitude and that similar variations occur in the deacylation rates (k_3). The simplest explanation of the concordant variations of these two rates is that the transition states in the two reactions are similar and that restrictions on productive orientation of the acyl moiety in these transition states are of a similar kind. Thus, a good substrate may be defined not only by its tight binding but also by the productivity of that mode of binding. Although the binding of pseudosubstrates and of active-site titrants appear to approximate those of specific substrate (as expressed by K_s values), misalignment of binding sites may retard subsequent acylation *and* deacylation reactions by as much as five orders of magnitude.

Since neither the substrate nor the enzyme is a rigid molecule, the rate of hydrolysis of a substrate may depend on the ratio of productive and nonproductive modes of binding of the acyl group. While good substrates must bind in a productive mode, active-site titrants may be stabilized in a nonproductive mode, and pseudosubstrates (whose reactivities are intermediate between the two) may be able to bind in either mode. This view is supported by evidence from studies of the CD spectra of acyl-enzymes formed by reaction of chymotrypsin with NPGB, a pseudosubstrate, and NPSA or MUTMAC, two active-site titrants. The latter, which deacylate very slowly, show an induced negative ellipticity in the region of absorption of the acyl groups (Kerr et al., 1975) which can be attributed to tight binding of the group in such nonproductive mode. p-GB-chymotrypsin, on the other hand, shows no induced negative ellipticity (Gertler et al., 1974a) suggesting rotational freedom of the acyl group.

Catalysis by Chymotrypsinogen. Catalysis by chymotrypsinogen has been shown to proceed via an acyl-zymogen intermediate just as chymotryptic catalysis proceeds via an acyl-enzyme intermediate (Gertler et al., 1974a). One may therefore inquire whether the inefficiency of the zymogen reflects misalignment of certain components of the binding site or of the catalytic apparatus. As discussed above, both x-ray crystallographic studies and chemical studies reveal that Met-192 is buried in the specificity pocket of the zymogen and that Gly-193 is relocated to a position which precludes its participation in the stabilization of the transition states during catalysis. These two alterations may well be sufficient to account for the observed differences between zymogen and enzyme. The binding of NPSA to chymotrypsinogen ($1/K_s$) is at least 100 times weaker than that to chymotrypsin, and the binding of *p*-aminobenzamidine to trypsinogen is about 6000-times weaker than that to trypsin (Gertler et al., 1974a,b). Yet binding alone is only the first step in catalysis, whereas the degree of stabilization of the transition states in the subsequent acylation and deacylation reactions determines the rate of catalysis. With the enzyme, satisfactory catalysis occurs when the structure of the substrate permits interaction of its carbonyl oxygen with the -NH functions of Gly-193 and Ser-195. With the zymogen, the interaction with Gly-193 is not possible. With the enzyme, the structure of a pseudosubstrate or active-site titrant precludes favorable interaction with the functional groups of the active site; with the zymogen, the misalignment of Gly-193 precludes favorable interaction. Thus, Table II shows that the acyl-enzymes formed by reaction

with active-site titrants (pTMAC-chymotrypsin and pSA-chymotrypsin) deacylate at virtually the same rates as do the corresponding acyl-zymogens. In the acyl-enzymes the slow rate is determined by the unfavorable geometry of the substrate; in the acyl-zymogens the unfavorable geometry of the substrate precludes observation of the unfavorable geometry of the protein. Chymotrypsinogen must be considered as an unsatisfactory catalyst for *all* substrates because even a good substrate for the enzyme cannot utilize the distorted oxyanion hole of the zymogen. At best, the catalytic efficiency can only be comparable with the poorest substrates for chymotrypsin, e.g., NPSA or MUTMAC. In contrast, NPGB is a better substrate for chymotrypsin and the acyl-enzyme, pGB-chymotrypsin, deacylates almost two orders of magnitude faster than the corresponding acyl-zymogen. Yet, the rate of deacylation of pGB-chymotrypsinogen is equivalent to that of pTMAC or pSA-chymotrypsinogen.

In summary, comparison of kinetic data for the hydrolysis of ester pseudosubstrates by chymotrypsin and chymotrypsinogen allows a model of activation consistent with crystallographic, circular dichroic, and chemical modification data. In this model, binding of pseudosubstrates is consistently weaker to chymotrypsinogen than to chymotrypsin. More importantly, the mode of binding to the zymogen is less productive. In the zymogen the improper orientation of Gly-193 may preclude the stabilization of the transition states characteristic of the enzyme-catalyzed reaction; in the enzyme, improper orientation of pseudosubstrates relative to Gly-193 may approach the same operational nonproductivity. The charge-relay system appears to be preformed in the zymogen and the nucleophilicity of Ser-195 may well be similar, if not identical with that in the enzyme.

In this model, the two key features of zymogen activation are (a) the improvement of specificity pocket; and (b) the reorientation of Gly-193 to facilitate stabilization of the transition states. Together, these two changes allow a productive orientation of tightly bound substrates in the enzyme.

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