Newm to one of the proposed subgroups of λ chains (Smith *et al.* 1971; Baczko *et al.*, 1970) appears difficult and will be postponed for a future report. The correlation of the amino acid sequence with the three-dimensional structure of the molecule has been discussed before (Poljak *et al.*, 1973) and will be reexamined in the near future using information derived from a 2-Å Fourier map of Fab' New which is currently under study in this laboratory.

Acknowledgment

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Catalysis by Chymotrypsinogen. Demonstration of an Acyl-Zymogen Intermediate†

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ABSTRACT: Bovine chymotrypsinogen reacts stoichiometrically with diisopropyl phosphorofluoridate (DFP) and catalyzes the hydrolysis of the ester *p*-nitrophenyl *p*-guanidinobenzoate (NPGB). These reactions are mutually exclusive, indicating that the same residue on the active site (Ser-195) is involved in both cases. Although the second-order rate of ester hydrolysis by chymotrypsinogen is 10^6–10^7 times slower than by chymotrypsin, the reaction proceeds in both cases *via* the formation of acyl intermediates. The deacylation rate of the isolated acyl-enzyme is only 70 times slower than that of acyl-enzyme and is dependent upon the ionization of a single group, presumably His 57. The hydrolysis of NPGB by the homologous zymogen–enzyme pair, trypsinogen–trypsin, is competitively inhibited by *p*-aminobenzamidine. The apparent inhibition constant K_i is almost four orders of magnitude higher for the zymogen than for the enzyme. These data suggest that the inferior catalytic properties of the zymogens of the pancreatic serine proteases are primarily due to an undeveloped binding site and only secondarily to a less efficient catalytic apparatus. Circular dichroic spectra of acyl-chymotrypsinogen at pH 4.0 indicate that the changes in ellipticity in the 220–250-nm range induced by acylation resemble those induced in the enzyme. In the range of 260–290 nm, however, the spectrum of the acyl-zymogen is significantly different, indicating a perturbed environment of the *p*-guanidinobenzoyl group and suggesting a different and possibly less efficient mode of binding. These spectral changes are completely reversible upon deacylation.

The zymogens of several proteolytic enzymes react slowly with site-specific reagents and catalyze certain zymogen–enzyme transformations. Foltmann (1966) first showed that the activation of prorennin at low pH was catalyzed by the zymogen itself. Lacko and Neurath (1970), Reeck and Neurath (1972), Uren *et al.* (1972), and Behnke and Vallee (1972) demonstrated an intrinsic activity of procarboxypeptidases A and B toward ester and peptide substrates of the respective

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enzymes. Kay and Kassell (1971) demonstrated enzymatic activity in trypsinogen and Bustin and Conway-Jacobs (1971) in pepsinogen. Evidence of active sites in zymogens of serine proteases was provided by Morgan *et al.* (1972) and Robinson *et al.* (1973a), who demonstrated that trypsinogen reacts with a single equivalent of DFP or *p*-nitrophenyl *p*-guanidinobenzoate (NPGB).[1] The site of phosphorylation of trypsinogen by DFP is identical with the site in trypsin. These various observations indicate that the conversion of a zymogen to an enzyme is not the activation of an inert zymogen, but the potentiation of catalytic activity intrinsic to the zymogen.

Although structural changes accompanying "activation" of several zymogens have been investigated (Neurath, 1957), the detailed nature of the structural rearrangements in the protein molecule and their functional relationship to enzyme activity remain conjectural. The question also remains whether the zymogen–enzyme conversion involves primarily an improvement of the substrate binding site, of the catalytic apparatus, or of both.

Chymotrypsinogen was chosen for a more detailed inquiry into the mechanism of zymogen activation since the amino acid sequence and the detailed three-dimensional structure are known for the zymogen and for the enzyme (Walsh and Neurath, 1964; Matthews *et al.*, 1967; Freer *et al.*, 1970). Both proteins react with DFP (Morgan *et al.*, 1972) and as will be shown herein, also with NPGB. This paper presents a comparison of the characteristics of the hydrolysis of NPBG by chymotrypsinogen and chymotrypsin, respectively.

Materials and Methods

Materials. Once-crystallized bovine trypsinogen, three-times-crystallized α-chymotrypsin and salt-free lyophilized δ-chymotrypsin were products of Worthington Biochemical Corp. Crystalline porcine trypsin was obtained from Novo Industri A/S and seven-times-crystallized bovine chymotrypsinogen A containing only 0.02% of active enzyme was a gift from Dr. K. Kurachi.

Purified *Aspergillus oryzae* acid protease was prepared according to Davidson *et al.* (1973). This enzyme appears to be identical with that described by Robinson *et al.* (1973b).

p-Nitrophenyl *p'*-guanidinobenzoate hydrochloride (NPGB), *p*-aminobenzamidine dihydrochloride, and acetyl-L-tyrosine ethyl ester were purchased from Cyclo Chemical Co. *p*-Nitrophenyl *p'*-(ω-dimethylsulfonioacetamido)benzoate bromide (NPSA), a specific active-site titrant of chymotrypsin (Wang and Shaw, 1972), was a gift of Dr. E. Shaw. *p*-Nitrophenyl acetate was obtained from Eastman Organic Chemicals.

A "stock solution" of radioactive DFP containing 0.1 mCi was prepared by mixing 1 g of pure DFP (Pierce Chemical Co.) with 0.167 mg of [^{14}C]DFP (New England Nuclear) in 0.5 ml of propylene glycol. The specific radioactivity was determined as described by Robinson *et al.* (1973a).

Activation of Chymotrypsinogen. To 1 ml of a solution of 1 mM HCl containing 0.5–1.0 g of chymotrypsinogen was added 1 ml of 0.2 M Tris-HCl buffer (pH 8.0) containing 0.1 M $CaCl_2$ and sufficient porcine trypsin to yield a zymogen : enzyme ratio of 10:1. Porcine rather than bovine trypsin was used because its activity toward AcTyrOEt is negligibly low. The solution was incubated for 3 hr at 0° and chymotryptic activity determined in a pH-Stat using 0.01 M AcTyrOEt as a substrate.

Preparation of DIP-chymotrypsinogen and DIP-trypsinogen. Chymotrypsinogen or trypsinogen (200 mg) was dissolved in 37 ml of 0.1 M Tris-HCl buffer (pH 7.6). After adding 3 ml of 1 M DFP in isopropyl alcohol, the solution was stirred at room temperature with occasional adjustment to pH 7.6. After 18 hr the pH was lowered to 3.5 by the addition of 1 M formic acid and the insoluble material removed by centrifugation. The supernatant was dialyzed at 4° against distilled water and lyophilized. The potential activity of the DIP-zymogens toward AcTyrOEt or TosArgOMe was less than 0.5% as compared to the native zymogens.

Reaction of Chymotrypsinogen or Trypsinogen with NPGB and NPSA. The reaction mixture (2.5 ml) contained 0.5 ml of chymotrypsinogen (0.1 mM) in 1 mM HCl, 1.95 ml of 0.1 M Pipes and Hepes buffer (pH 6.0–8.2) which included 0.04 M $CaCl_2$, and 50 μl of 0.02 M NPGB (or 100 μl of 0.01 M NPSA) dissolved in dimethylformamide. The initial substrate : zymogen ratio was therefore 20:1. The reaction was followed in a Cary 14 spectrophotometer at 25° which recorded the release of *p*-nitrophenol at 400 or 340 nm for reactions carried out above and below pH 6.4, respectively. In control reactions chymotrypsinogen was replaced by DIP-chymotrypsinogen. The extent of acylation was calculated at various times from the difference in absorbance between the reaction and the control. The change in absorbance resulting from the release of 1 mol of *p*-nitrophenol/mol of chymotrypsinogen was determined from the concentration of chymotrypsinogen and from the specific molar absorbance of *p*-nitrophenol in 0.1 N NaOH (ϵ_{400} = 18,000) or in 0.1 N HCl (ϵ_{340} = 6300) (Kezdy and Kaiser, 1970). Since the reaction was carried out at different pH values, the absorbance at each pH was measured by mixing an aliquot of *p*-nitrophenol with the corresponding buffer. The exact concentration of NPGB in each experiment was calculated from the absorbance at 400 nm after complete hydrolysis in 0.1 N NaOH.

The reaction with trypsinogen was similarly followed at different concentrations of NPGB both in the presence and in the absence of the competitive inhibitor *p*-aminobenzamidine. The pH was carefully adjusted to 7.2 prior to the addition of the zymogen.

Preparation of Acyl-chymotrypsinogen and Acyl-chymotrypsins. To 5 ml of 0.5% chymotrypsinogen in 1 mM HCl were added 19.5 ml of 0.1 M Pipes buffer (pH 7.2), containing 0.04 M $CaCl_2$, and 0.5 ml of 0.035 M NPGB in dimethylformamide. The highest possible concentration of NPGB was chosen to ensure maximal acylation of chymotrypsinogen. A small precipitate of the reagent occasionally appeared but did not interfere with the acylation. The mixture was incubated for 4 hr and the reaction was terminated by adjusting to pH 3.5 with 1 M formic acid and removing any insoluble material by centrifugation. The protein fraction was separated from the reagents by gel filtration on a column (2.5 × 38 cm) of Sephadex G-25 previously equilibrated with 1 mM HCl, and lyophilized.

The acyl derivatives α- and δ-chymotrypsins were similarly prepared but the reaction time was limited to 1 min.

The yield, on a weight basis, was greater than 90%. The corresponding acyl derivatives were designated *p*-guanidinobenzoyl-(*p*-GB-)chymotrypsinogen and *p*-GB-chymotrypsins, respectively. Since the acyl derivatives have a higher specific

[1] Abbreviations used are: NPGB, *p*-nitrophenyl *p'*-guanidinobenzoate; *p*-GB-, *p*-guanidinobenzoyl-; DFP, diisopropyl phosphorofluoridate; DIP, diisopropylphosphoryl; Pipes, piperazine-*N*,*N'*-bis(2-ethanesulfonic acid); Hepes, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid; ZTNE, *N*-carbobenzoxy-L-tyrosine nitrophenyl ester; NPSA, *p*-nitrophenyl *p'*-(ω-dimethylsulfonioacetamido)benzoate bromide.

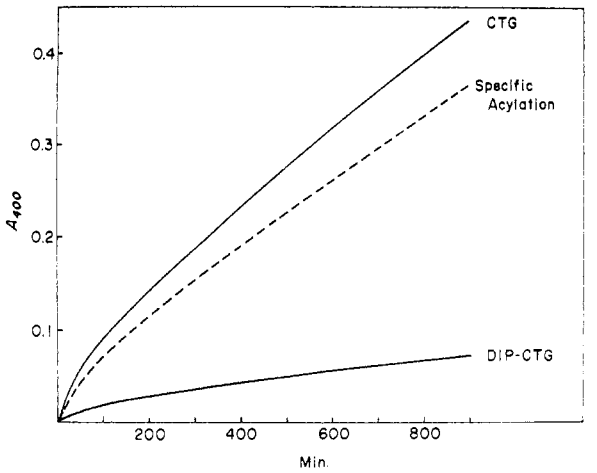

FIGURE 1: Reaction of chymotrypsinogen (1.5×10^{-5} M) with NPGB (3.5×10^{-4} M) in 0.08 M Pipes buffer (pH 7.2) containing 0.032 M $CaCl_2$. The release of 1 mol of *p*-nitrophenol/mol of chymotrypsinogen at this pH should result in a "burst" of 0.165 A_{400}. Specific acylation is calculated from the difference between the two solid lines.

absorbance at 280 nm than the native proteins, the specific absorbance of each derivative was calculated from the protein concentrations determined by the Lowry method (Lowry *et al.*, 1951) and standardized with chymotrypsinogen and chymotrypsins. The acetyl derivatives of α- and δ-chymotrypsins were prepared in a similar way but the concentration of *p*-nitrophenyl acetate was 0.1 M. The isolated proteins were 95% acetylated.

Ultraviolet Spectra. The uv spectra of the proteins and their acyl derivatives were measured with a Cary 15 spectrophotometer at room temperature. The proteins were dissolved in 1 mM HCl to yield approximately 0.03% solutions. The protein concentrations were calculated from absorbance at 280 nm and by the Lowry method (Lowry *et al.*, 1951).

Circular dichroic spectra were recorded at room temperature using a Cary 60 spectropolarimeter equipped with a circular dichroism attachment. Chymotrypsinogen, α-chymotrypsin, δ-chymotrypsin, and their acyl derivatives were dissolved in 0.02 M sodium acetate buffer (pH 4.0) containing 0.1 M NaCl. In the range of 250–330 nm, protein concentrations of 0.5 mg/ml were used in cells of 1.00-cm path length. Below 250 nm, cells of 0.1-cm path length were used. The results were calculated as mean residue ellipticity ((deg cm^2) $dmol^{-1}$), using 115 as the mean residue weight.

Estimation of p-GB-chymotrypsinogen in Mixtures with Chymotrypsinogen. To 0.5-ml aliquots of *p*-GB-chymotrypsinogen (2–3 mg/ml) containing chymotrypsinogen (in 1 mM HCl or in dilute buffer) were added 50 μl of 1 N sodium formate buffer, pH 3.0–3.5 (to ensure a final pH of 3.5), and 50 μl of purified *A. oryzae* acid protease dissolved in water (2.5 mg/ml). Preliminary experiments indicated that about 95% of the zymogen was activated in 90–120 min and that the activity remained constant for at least 3–4 hr. Activation was usually terminated after 2.5 hr at 25°. Aliquots of 0.1–0.2 ml were then withdrawn and mixed with 3.0 ml of ZTNE (1.67×10^{-5} M, in 0.05 M citric acid containing 1.67% dioxane), and the esterolytic activity of chymotrypsin was measured at 25° by recording the increase of absorbance at 340 nm. Simultaneously, 0.2 ml of the activation sample was mixed with 0.2 ml of 0.4 N Tris-HCl buffer (pH 8.2), incubated for 15 min at 25° to ensure full deacylation of *p*-GB-chymotrypsin and assayed again with ZTNE.

Since the activation product of *p*-GB-chymotrypsinogen is *p*-GB-chymotrypsin which is inactive but deacylates rapidly at pH 8.0 and recovers full activity, and since the rates of activation of chymotrypsinogen and *p*-GB-chymotrypsinogen by *A. oryzae* acid protease are identical, the relative increase in specific activity after deacylation is a measure of the relative amount of *p*-GB-chymotrypsinogen in the initial material. Equation 1 was therefore used to calculate the percentage of chymotrypsinogen in the acylated form.

$$\% \; p\text{-GB-chymotrypsinogen} = [1 - (A_1/A_2)] \times 100 \quad (1)$$

where A_1 is the specific activity after activation at pH 3.5 and A_2 is the specific activity after activation at pH 3.5 followed by 15-min incubation at pH 8.0–8.3. Control experiments established that the specific activity of native δ-chymotrypsin toward ZTNE did not change during 1 hr at pH 8.2.

Deacylation of p-GB-chymotrypsinogen and p-GB-chymotrypsins. To deacylate the acyl-zymogen, 2.5 ml of 0.5% *p*-GB-chymotrypsinogen was mixed with an equal volume of 0.04 M Pipes and Hepes buffers (pH 5.6–8.0), containing 0.08 M $CaCl_2$, and incubated at 25° for 3–4 hr. Aliquots of 0.5 ml were withdrawn, acidified by adding 50 μl of 1 M sodium formate buffer (pH 3.0–3.5) to stop the deacylation and the amount of *p*-GB-chymotrypsinogen was determined as described above. Deacylation of *p*-GB-α-chymotrypsin and *p*-GB-δ-chymotrypsin was similarly followed during a 5-min period after which the reaction was completed. The aliquots were immediately assayed for chymotrypsin activity at low pH using ZTNE as described before. Pseudo-first-order constants of deacylation (k_3) were calculated from semilogarithmic plots.

Reaction of Chymotrypsinogen with [^{14}C]DFP at Various pH Values. To a jacketed vessel (25°) of a Radiometer TTT-1 autotitrator were successively added 2 ml of 0.25 N KCl, 1 ml of 0.2 M $CaCl_2$ and 75 μl of [^{14}C]DFP (stock solution). The pH was adjusted to the desired value and the volume was increased to 4 ml. The reaction was then started by the addition of 1 ml of 1.75% chymotrypsinogen in water. Aliquots of 0.7 ml were removed during the 3–4-hr period, mixed with 0.1 ml of 1 M sodium formate buffer (pH 3.5), and desalted on a column (0.9 × 25 cm) of Sephadex G-25 equilibrated with 1 mM HCl. The protein fraction was analyzed for radioactivity, content of unmodified zymogen, and protein concentration. The latter was estimated at 280 nm using a molar extinction coefficient of $\epsilon = 50{,}000$ M^{-1} cm^{-1} (Wilcox, 1970).

Second-order rate constants (k_{II}) were calculated from semilogarithmic plots using an integrated equation that corrects for spontaneous hydrolysis and for progressive dilution (Morgan *et al.*, 1972). The reactions were carried out within the range of pH 6.1–10.0.

Radioactivity was measured with a Packard Model 3003 Tri-Carb scintillation counter using an Aquasol (New England Nuclear) scintillant.

Results

Reaction of Chymotrypsinogen with NPGB. A typical course of reaction of chymotrypsinogen with NPGB is shown in Figure 1. The specific acylation rate (dashed line) was calculated after correcting for nonspecific hydrolysis by DIP-chymotrypsinogen and for traces of chymotrypsin (0.02%) present in the zymogen. It will be noted that unlike the analogous reaction of trypsinogen (Robinson *et al.*, 1973a), the specific release of *p*-nitrophenol exceeded 1 mol/mol of zymogen, thus indicating that not only acylation but also deacylation occurred. The reaction slowly reached a steady state in which most of the zymogen was acylated. When the

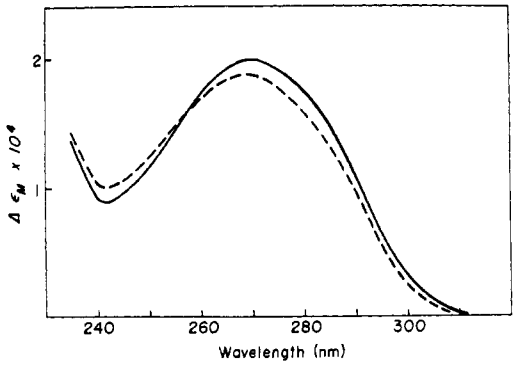

FIGURE 2: Difference between spectra of *p*-GB-chymotrypsinogen and chymotrypsinogen (- - - - -) and of *p*-GB-α-chymotrypsin and α-chymotrypsin (—) in 1 mM HCl. The $\Delta\epsilon_M$ was calculated after correction for incomplete acylation (64% in the case of chymotrypsinogen and 89% in the case of α-chymotrypsin).

initial concentration of NPGB was 0.7 mM, the steady state was reached after 3–4 hr and the protein fraction isolated by gel filtration after adjustment to pH 3.5 contained 65% acylated and 35% free chymotrypsinogen. Prolonged incubations (up to 7 hr) did not change this ratio.

Uv Spectra of p-GB-chymotrypsinogen and p-GB-α-chymotrypsin. The contribution of the *p*-GB-chromophore to the uv spectrum of the acyl-zymogen was determined as the difference between the spectra of *p*-GB-chymotrypsinogen and chymotrypsinogen (Figure 2). A broad absorption band was found, similar to those of *p*-GB-chymotrypsin and of *p*-GB-trypsin (Chase and Shaw, 1969). The absorbance changes of chymotrypsinogen were reversed by deacylation (Table I), except for a small residual increase, probably due to nonspecific acylation (see rows 4 and 5 of Table I).

Circular Dichroic Spectra of p-GB-chymotrypsinogen, p-GB-chymotrypsins, and Acetyl-chymotrypsins. Circular dichroic spectra of chymotrypsinogen, α-chymotrypsin, δ-chymotrypsin, and their acyl derivatives (see Figure 3) revealed two essential features. (1) Upon acylation of both enzymes and of the zymogen, a significant decrease of ellipticity at 228–229 nm occurs but the ellipticity of the acylated zymogen is less negative as compared to the acylated enzymes. However, after correction for incomplete acylation (65% in the case of chymotrypsinogen, over 90% in the case of the enzymes), the absolute change in ellipticity is about the same for all three proteins. Since the change in ellipticity is similar for *p*-GB-chymotrypsin and for acetyl-chymotrypsin, the change cannot be due to the absorption of the *p*-GB-group but probably signals a conformational change during acylation. (2) In the 260–290-nm range, the circular dichroic spectrum of *p*-GB-chymotrypsinogen is very different from that of *p*-GB-chymotrypsin while the spectra of various acyl-chymotrypsins are quite similar to each other. The small difference between the acyl derivatives of α- and δ-chymotrypsins at 262 nm is difficult to interpret, since the ellipticity of α-chymotrypsin changes upon dimerization (Dorrington and Hofmann, 1973).

The change in the spectrum of *p*-GB-chymotrypsinogen at 260–290 nm definitely results from specific acylation since the spectra of DIP-chymotrypsinogen or DIP-chymotrypsinogen treated with NPGB were almost identical with that of chymotrypsinogen. It should be further noted that both changes at both 260–290 nm and at 228–229 nm were completely reversed by deacylation.

Reaction of Chymotrypsinogen and p-GB-chymotrypsinogen with DFP. Comparison of the loss of activatability with the incorporation of [^{14}C]DFP shows that *p*-GB-chymotrypsinogen reacts with DFP much more slowly than does the native zymogen (Figure 4). This observation suggests that the reaction with DFP occurs only after deacylation of the acyl-zymogen. Conversely, DIP-chymotrypsinogen releases only a fraction of 1 equiv of *p*-nitrophenol from NPGB (Figure 1). Thus it appears that DFP and NPGB react with the same site on the zymogen.

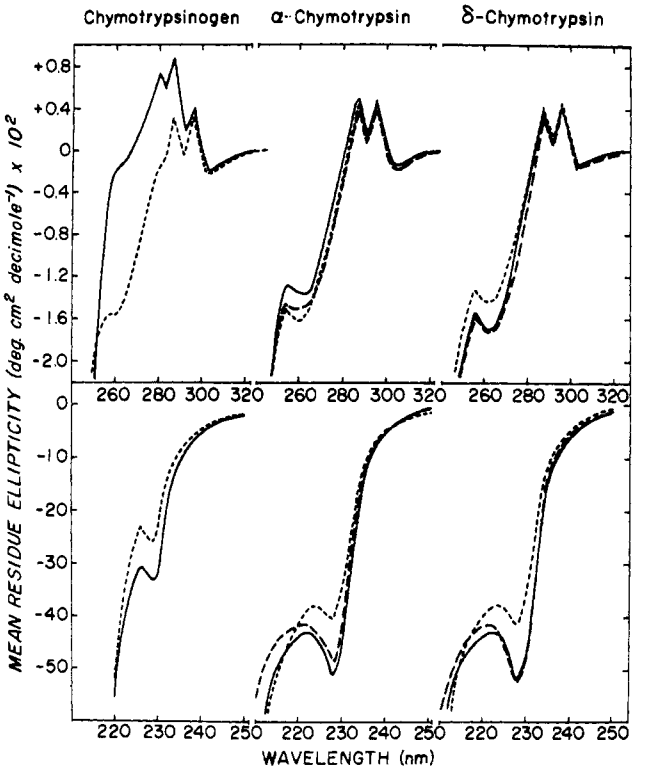

FIGURE 3: Circular dichroic spectra of native chymotrypsinogen or chymotrypsin (- - - - -), *p*-GB-chymotrypsinogen or *p*-GB-chymotrypsin (—), and acetyl-chymotrypsins (— — —) in 0.1 N NaCl–0.02 N sodium acetate (pH 4.0).

Effect of pH on Deacylation of p-GB-chymotrypsinogen and p-GB-chymotrypsins. Incubation of *p*-GB-chymotrypsinogen

TABLE I: Relative Absorbance (280 nm) of Native, Acylated, and Deacylated Chymotrypsinogen and α-Chymotrypsin.

No.	Fraction	Rel Absorbance at 280 nm[a]	% Acylated
1	Native chymotrypsinogen	100	0
2	DIP-chymotrypsinogen	101	0
3	No. 1 after 4-hr reaction with NPGB[b]	124	64
4	No. 2 after 4-hr reaction with NPGB[b]	103	0
5	No. 3 after 20 hr at 25° at pH 7.2[c]	103	0
6	No. 3 after 20 hr at 25° in 1 mM HCl	122	61
7	Native α-chymotrypsin	100	0
8	No. 7 after 1-min reaction with NPGB[b]	133	89
9	No. 8 after 60 min at 25° at pH 7.2[c]	101	0
10	No. 8 after 60 min at 25° in 1 mM HCl	133	88

[a] The absorbance and the protein concentration (by the Lowry method) were measured after gel filtration on Sephadex G-25 in 1 mM HCl. [b] pH 7.2, 0.7 mM NPGB. [c] In 0.02 M Pipes buffer (pH 7.2) containing 0.05 M $CaCl_2$.

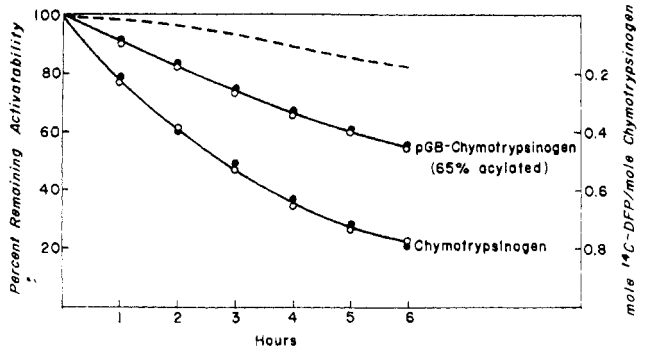

FIGURE 4: Inactivation of chymotrypsinogen and *p*-GB-chymotrypsinogen by [^{14}C]DFP at pH 7.0, 25°: (○) loss of activity; (●) incorporation of [^{14}C]DFP. The upper solid line gives the experimental results for *p*-GB-chymotrypsinogen and the dashed line represents calculated values corrected for partial (65%) acylation.

with *A. oryzae* acid protease yields *p*-GB-chymotrypsin which is stable at pH 3.5 but deacylates rapidly at pH 8 ($t_{1/2}$ approximately 1 min). This result provides confirmatory evidence that the same site is acylated in the zymogen and in the enzyme.

The rates of deacylation of *p*-GB-chymotrypsinogen, *p*-GB-α-chymotrypsin, and *p*-GB-δ-chymotrypsin were measured in the pH range 5.5–8.0. The apparent first-order rate constants k_3 at each pH were multiplied by the respective (H^+) and plotted against k_3 according to the linear equation (2).

$$k_3(H^+) = K_a k_{max} - K_a k_3 \quad (2)$$

In all cases a linear plot was obtained using a least-squares program, thus indicating that deacylation was dependent on the ionization of a single group. The ionization constant K_a was calculated from the slope of the line and k_{max} from the ordinate intercept (see Table II). These values were used for calculation of the theoretical dissociation curves shown in Figure 5.

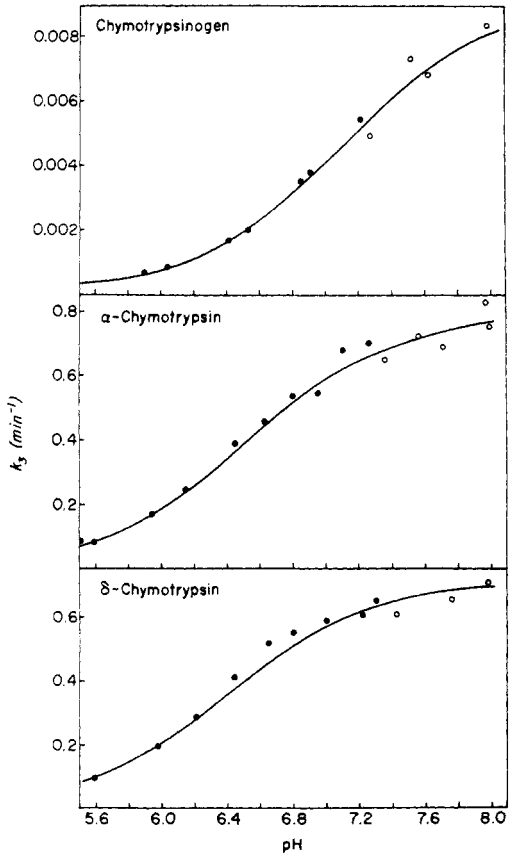

FIGURE 5: Deacylation of *p*-GB-chymotrypsinogen and *p*-GB-chymotrypsins at various pH values: (●) Pipes buffer; (○) Hepes buffer. The solid lines represent the theoretical dissociation curves calculated from the results given in Table II.

TABLE II: Deacylation Rates of *p*-GB-chymotrypsinogen and *p*-GB-chymotrypsin.[a]

Acyl Intermediate	$k_{3,max}$ (min^{-1})	pK_a
p-GB-chymotrypsinogen	0.0089	7.07
p-GB-α-chymotrypsin	0.617	6.51
p-GB-δ-chymotrypsin	0.565	6.41

[a] In all three cases the reaction is dependent on the ionization of a single group.

Effect of pH on Inactivation of Chymotrypsinogen by DFP. The reaction was studied in the pH range of 6.1–10. Between pH 6.1 and 8.2, a single equivalent of [^{14}C]DFP was incorporated and the rate of incorporation closely paralleled the rate of inactivation. At higher pH values the rate of incorporation progressively increased and exceeded 1 mol/mol of protein, presumably due to alkyl phosphorylation of tyrosine residues. The apparent second-order rate constants (k_{II}) for the incorporation of [^{14}C]DFP (pH 6.1–8.2) and for inactivation (pH 6.1–10.0) were multiplied by the respective (H^+) and plotted as a function of k_{II}, as described above. Since a good linear fit was obtained, it was concluded that this reaction, too, was dependent on the ionization of a single group. The calculated values of pK_a and k_{max} were 7.15 and 0.244 min^{-1} M^{-1}, respectively. Virtually identical results were obtained when the measurements of incorporation of [^{14}C]-DFP and of inactivation were separately analyzed. The theoretical dissociation curve and the experimental data are shown in Figure 6. These observations differ qualitatively from similar measurements with chymotrypsin (Mounter *et al.*, 1957; Moon *et al.*, 1965). In the case of the zymogen, the inactivation rate is practically independent of pH in the range of pH 8–10, indicating that the reaction is solely dependent on the ionization of a group having a pK = 7.15. In the case of the enzyme, the inactivation rate decreases between pH 8 and 10, as does the catalytic activity, suggesting the participation of a second ionizable group (Bender and Killheffer, 1973).

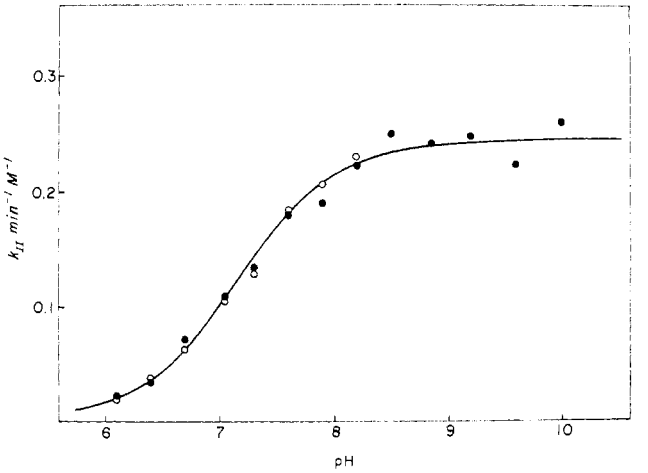

FIGURE 6: Inactivation of chymotrypsinogen by DFP at different pH values: (●) loss of potential activity; (○) incorporation of [^{14}C]DFP. The solid line represents the theoretical curve for pK_a = 7.15 and k_{max} = 0.244 min^{-1} M^{-1}.

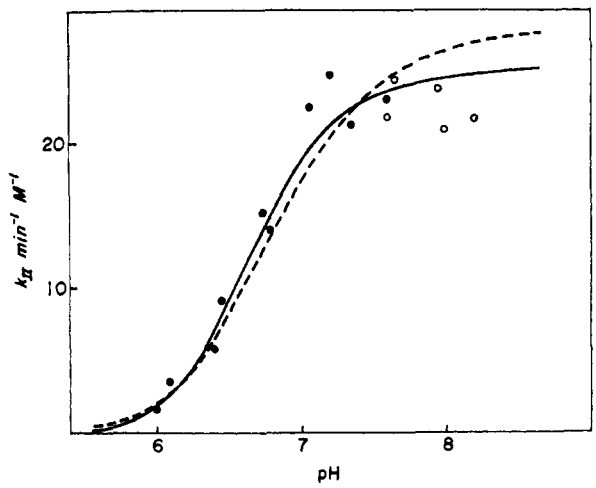

FIGURE 7: Effect of pH on the acylation of chymotrypsinogen by NPGB: (●) Pipes buffer; (○) Hepes buffer. The solid line represents the theoretical dissociation curve assuming two 'cooperative" groups and the dashed line assuming two "noncooperative" groups (see the text).

Effect of pH on the Acylation of Chymotrypsinogen by NPGB. The acylation rate of chymotrypsinogen was examined between pH 6 and 8.2. Attempts to extend this study above pH 8.2 were unsuccessful due to the instability of the reagent and a sharp increase in the rate of nonspecific acylation as indicated by the control reaction of DIP-chymotrypsinogen.

The pseudo-first-order rate constants for acylation were calculated from the initial portion of the semilogarithmic plot assuming that the initial "burst" releases 1 mol of *p*-nitrophenol/mol of zymogen. During this initial reaction less than 20% of chymotrypsinogen is acylated and the release of free deacylated zymogen has a negligible effect on the calculated rate.

Calculated second order rate constants are given in the legend to Figure 7. Attempts to plot the results in a linear form (eq 2) failed, indicating that ionization of more than one group affects this reaction. Theoretical dissociation curves were therefore calculated, assuming that the ionizations of two groups control reaction rates.

If these two groups are assumed to be independent of each other, the best "noncooperative" model (see dashed line in Figure 7) results in values of pK_a of 6.3 and 6.5, respectively, and of $k_{3,max}$ = 27.9 min^{-1} M^{-1}. These pK_a values seem to be rather low as compared to the pK_a of 7.15 obtained from the reaction with DFP and the experimental data fit the curve poorly, especially above pH 7.

Alternatively, if it is assumed that the ionization of one group affects that of the other in a "cooperative model" a family of approximate solutions can be derived. One of these curves is depicted in Figure 7 (solid line) where pK_{x_1} = 7.10, pK_{x_2} = 6.77, pK_{y_1} = 6.77, pK_{y_2} = 5.90, and $k_{3,max}$ = 25.2 min^{-1} M^{-1}.

Competitive Inhibition of Acylation of Trypsinogen. It was not possible to measure accurately the affinity of chymotrypsinogen for substrates or inhibitors because preliminary experiments indicated that K_i or K_s for the zymogen is approximately three to four orders of magnitude higher than for the enzyme and no known competitive inhibitor of chymotrypsin is sufficiently soluble to permit the estimation of K_i. However, the experiment could be performed with the homologous zymogen, trypsinogen, because *p*-aminobenzamidine is an effective inhibitor and is soluble up to 50 mM.

Rates of acylation of trypsinogen by NPGB (six different substrate concentrations) followed pseudo-first-order kinetics in the presence and the absence of *p*-aminobenzamidine (Figure 8). The results indicate that the inhibition is competitive (as in the case of trypsin) and that K_i = 88 mM. In contrast, when the same inhibitor was tested on trypsin, using the same conditions except that benzoyl-DL-arginine-*p*-nitroanilide served as a substrate, a value of K_i = 0.0142 mM was obtained.

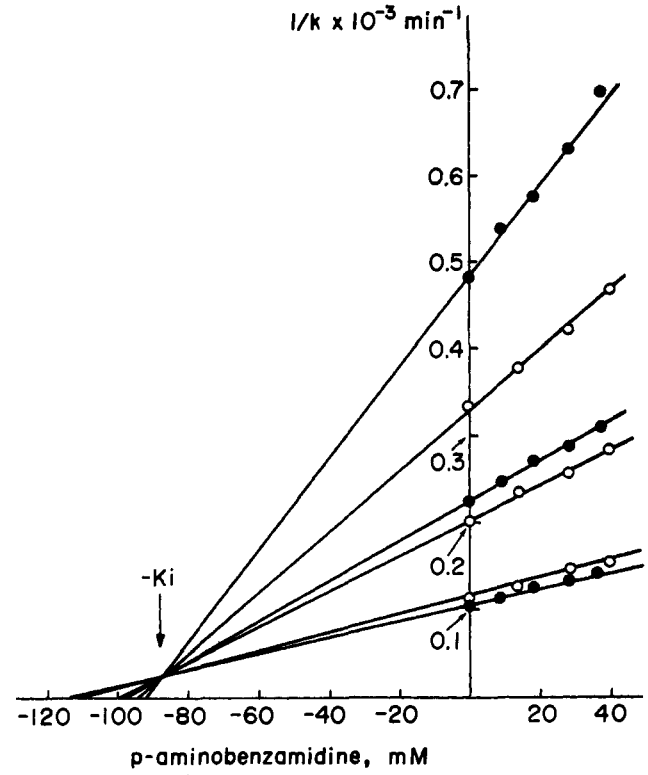

FIGURE 8: Acylation of trypsinogen by NPGB at pH 7.2, 25°, in the presence and absence of *p*-aminobenzamidine. The final concentrations of NPGB were respectively 0.09, 0.11, 0.16, 0.19, 0.32, and 0.38 mM and the final concentrations of inhibitor varied from 0 to 40 mM. The full and open circles indicate two independent experiments.

The results given in Figure 8 also enable the calculation of K_s and k_2 for the acylation of trypsinogen by NPGB. The values are K_s = 1.6 mM and k_2 = 0.04 min^{-1}, but the standard deviation exceeds 50% of the average. Nonetheless they are similar to the corresponding values for the reaction of α-carbamyl-ϵ-guanidinated trypsin with NPGB (Robinson *et al.*, 1973a). The second-order rate constant for the reaction of NPGB with trypsinogen, given by the ratio k_2/K_s, is 25 min^{-1} M^{-1}, almost identical with that obtained for the reactions with chymotrypsinogen (see Figure 7).

Discussion

Recent work from our laboratory has indicated that chymotrypsinogen and trypsinogen react with DFP and that in each case the serine residue of the active site of the corresponding enzymes becomes alkyl-phosphorylated (Robinson *et al.*, 1973a; Morgan *et al.*, 1972). This preferential reactivity of Ser-195 (chymotrypsinogen numbering system) in the zymogen is consistent with X-ray data which indicate that the "charge-relay system" is partially, if not fully, developed prior to activation (Freer *et al.*, 1970).

The present experimental results extend this concept by proving that the chymotrypsinogen-catalyzed hydrolysis of NPGB proceeds in a manner analogous to the chymotrypsin-

catalyzed hydrolysis of *p*-nitrophenyl acetate (Bender and Killheffer, 1973). The progress curve reveals an initially fast reaction followed by a linear turnover which can be terminated at low pH (pH 3.5) to yield an acyl-zymogen intermediate. Since DIP-chymotrypsinogen does not significantly catalyze the hydrolysis of NPGB and *p*-GB-chymotrypsinogen reacts much more slowly with DFP than does the native enzyme, it is concluded that DFP and NPGB react with the same residue on the active site, *i.e.*, Ser-195. Procedures which convert chymotrypsinogen to chymotrypsin similarly convert *p*-GB-chymotrypsinogen to *p*-GB-chymotrypsin. Thus activation of *p*-GB-chymotrypsinogen by an acid protease (from *A. oryzae*) at pH 3.5 yields an inactive product which is stable at low pH but at pH 8 spontaneously recovers full activity at a rate identical with that of *p*-GB-chymotrypsin.

The reaction of NPGB with chymotrypsin is not fully understood. The acyl moiety fulfills the specificity requirements of trypsin and since its reaction with trypsin stops at the acyl intermediate stage, this ester was proposed as an active site titrant for this enzyme (Chase and Shaw, 1967).

Ethyl *p*-guanidinobenzoate is neither a substrate nor an inhibitor of chymotrypsin, indicating that the *p*-GB group does not bind well to this enzyme whereas the *p*-nitrophenyl group might (Mares-Guia and Shaw, 1967). On the other hand, acetyl-D-tryptophan methyl ester and indole, both competitive inhibitors of chymotrypsin (Cunningham, 1965), inhibit the hydrolysis of NPGB by chymotrypsin, indicating that the binding sites overlap. Although no binding site for the *p*-nitrophenyl moiety has so far been identified in chymotrypsin, trypsin, plasmin, or thrombin, kinetic data strongly indicate that the *p*-nitrophenyl group is not only a better "leaving" group which increases the acylation rates (Chase and Shaw, 1969) but also contributes to binding. Thus inclusion of the nitrophenyl moiety reduces the dissociation constant (K_s) by three orders of magnitude, especially of weakly bound substrates (Silver and Matta, 1972; Chase and Shaw, 1969). Since the binding site in chymotrypsinogen is at least partly distorted relative to chymotrypsin (Freer *et al.*, 1970), the *p*-nitrophenyl moiety could contribute significantly to the binding to the zymogen.

Because the reaction of NPGB with chymotrypsinogen involves both acylation and deacylation, and the limited solubility of the substrate precludes enzyme saturation, the acyl-zymogen intermediate was obtained in a yield of only 65–68%. If the reaction is assumed to consist of *two* principal and experimentally measurable steps, *i.e.*

$$\text{CTG} + \text{NPGB} \xrightarrow{k_{II}} p\text{-GB-CTG} \xrightarrow{k_3} \text{CTG} + p\text{-GB}\cdot\text{OH} \quad (3)$$

so that

$$\text{d}(p\text{-GB-CTG})/\text{d}t = k_{II}(\text{CTG}) - k_3(p\text{-GB-CTG}) \quad (4)$$

in the steady state

$$(p\text{-GB-CTG})/(\text{CTG}) = k_{II}/k_3 \quad (5)$$

In these equations CTG denotes chymotrypsinogen, *p*-GB·OH, *p*-guanidinobenzoic acid, and quantities in brackets are concentration terms.

It is possible to calculate the steady-state concentration of the acyl intermediate from the respective rate constants and initial concentrations of the reactants. At pH 7.2, the second-order rate constant (k_{II}) for acylation is approximately 21 $\text{min}^{-1}\ \text{M}^{-1}$ (Figure 7), and the deacylation constant k_3 is approximately $0.51 \times 10^{-2}\ \text{min}^{-1}$ (Figure 5). At an initial concentration of 0.7 mM NPGB, the steady state is reached in 3–4 hr when the concentration of substrate has become 0.55 mM. Substituting these numbers into eq 5 the per cent of zymogen acylated is calculated as 70% as compared to the experimentally observed value of 65–68%.

Measurements of the rate and pH dependence of deacylation of *p*-GB-chymotrypsinogen (Figure 5) yield a maximum deacylation rate constant of 0.0089 min^{-1} for the acyl-zymogen as compared to 0.56–0.61 min^{-1} for the acyl-enzyme (Table II). The ratio of these constants is approximately 70 whereas the ratio of the second-order rate constants for acylation (which is a product of both acylation and binding constants) is 10^6–10^7. The pH dependence of deacylation is governed by the ionization of a single group having a pK of 7.1 as compared to 6.5 for the deacylation of acyl-chymotrypsin. It is significant that the pH dependence of deacylation of *p*-GB-chymotrypsin is similar to the pH dependence of the alkyl phosphorylation by DFP, suggesting that the same group (probably His-57) controls these reactions and that this group is probably in a more negative microenvironment in the enzyme than in the zymogen. In contrast, acylation of the zymogen by NPGB appears to be dependent on the ionization of two groups having similar if not identical dissociation constants. The present data are not sufficient to identify the nature of the second group but considering that the second-order rate constant includes terms of both binding and acylation, it is very likely thatth is group is involved in the binding of the positively charged *p*-GB group.

A significant difference between zymogen and enzyme was observed in the shape of the pH profile during alkyl phosphorylation by DFP. The second-order rate constant for the reaction of the enzyme with DFP has a bell-shaped pH-dependence curve, as does the hydrolysis of substrates (Mounter *et al.*, 1957; Moon *et al.*, 1965). In the latter case, the acid limb is controlled by the deprotonation of His-57 and the alkaline limb by the protonation of Ile-16 (Bender and Killheffer, 1973). In the case of the zymogen, a sigmoidal curve is obtained and the reaction is independent of pH in the range of pH 8–10. Since the rate constants for acylation and deacylation of the enzyme (Bender and Killheffer, 1973) and for deacylation of the zymogen have a similar dependence upon pH, the independence of pH above pH 8 is probably related to the absence of the free α-amino group of Ile-16 in the zymogen. This conclusion is consistent with the various studies which have correlated the binding site of chymotrypsin with the protonation of Ile-16 (Hess, 1971).

The present data can account, at least in part, for the difference in catalytic efficiency between chymotrypsinogen and chymotrypsin. While the second order rate constant for acylation (which is a product of both acylation and binding constants) is five to seven orders of magnitude lower for the zymogen than for the enzyme, the rate constants of deacylation differ only by less than two orders of magnitude (Table III). Experiments with the homologous zymogen–enzyme pair, trypsinogen–trypsin, indicate that the affinity of the zymogen for the competitive inhibitors is three to four orders of magnitude lower than that of the enzyme, suggesting that the principal defect in the zymogen is a distorted binding site. Since the zymogen-catalyzed hydrolysis of the ester substrate NPGB proceeds *via* the same type of acyl intermediate as the enzyme-catalyzed reaction of *p*-nitrophenyl acetate, the low efficiency of catalysis by the zymogen may be primarily attributed to a defective binding site and only secondarily to a distorted catalytic site. Since it appears that the "charge-relay system" preexists in the zymogen (Freer *et al.*, 1970; Morgan

TABLE III: Reactions of Zymogens and Enzymes.

	k (Enzyme)/k (Zymogen)	Reference
TG[a] + DFP → DIP-TG	(k_{II}) 6.5×10^4	Morgan *et al.* (1972)
CTG + DFP → DIP-CTG	(k_{II}) 7.8×10^4	This work; Moon *et al.* (1965)
TG + NPGB → *p*-GB-TG	(k_{II}) 7.7×10^6	This work; Chase and Shaw (1969)
CTG + NPSA → acyl-CTG	(k_{II}) 1.6×10^7 [b]	This work; Wang and Shaw (1972)
p-GB-CTG → CTG + *p*-GB-COOH	(k_3) 7.4×10^1	This work
PABA·TG ⇌ PABA + TG	($1/K_i$) 6.2×10^3	This work

[a] Abbreviations used are: TG, trypsinogen; CTG, chymotrypsinogen; PABA, *p*-aminobenzamidine. [b] No data for the second-order rate constant for the reaction of chymotrypsin with NPSA are available, but the enzyme:zymogen rate ratio is probably similar to that for NPGB.

et al., 1972) a different orientation of the acyl group may account in part for the 70-times slower deacylation rate of the acyl-zymogen as compared to the acyl-enzyme. Actually, the separation of enzymatic action into a binding step and a catalytic step is an oversimplification which results from kinetic analysis. It is more likely that the binding of a substrate or inhibitor induces a conformational change which increases the reactivity of the catalytic center (Wootton and Hess, 1962; Himoe *et al.*, 1967; Koshland, 1970). Yet the differentiation between binding and catalytic sites suggests additional experimental approaches to the problem of zymogen activation.[2]

If the major difference between the zymogen and the enzyme is indeed in binding rather than in the bond-breaking mechanism, one would expect that the substrate NPGB would be in a different microenvironment in the two proteins. This is in fact indicated by the significant differences in the circular dichroic (CD) spectra of the *p*-GB derivatives in the range of 260–290 nm. Since perturbation of many residues (tyrosyl, tryptophanyl, phenylalanyl, and cystinyl) could contribute to the CD spectra in this range, it is difficult to interpret these changes in terms of specific intramolecular events. However, if one calculates the difference between the spectra of *p*-GB-chymotrypsinogen and chymotrypsinogen (Figure 3) an assymmetric bell-shaped curve is obtained with a maximum at 260 nm. This difference spectrum corresponds to the uv absorption spectrum of *p*-guanidinobenzoate, indicating a perturbed environment of this group in the *p*-GB-zymogen but not in the *p*-GB-enzyme. Thus the mode of binding of the *p*-GB group in the zymogen appears to be different from that in the enzyme. In contrast, comparison of the CD spectra in the 220–250-nm range reveals that the changes in ellipticity induced by acylation of the zymogen closely resemble those induced in the enzyme. Furthermore, similar changes result from acetylation (Figure 3) or from trimethylacetylation (Volini and Tobias, 1969) of chymotrypsin. Changes in the CD spectrum at these wavelengths have been reported to result from activation of chymotrypsinogen (Fasman *et al.*, 1966) or from exposure of chymotrypsin to solutions of high ionic strength (Cuppett *et al.*, 1971; Dorrington and Hofmann, 1973). These changes have been associated with the increased reactivity of the amino-terminal Ile-16 and with a conformational change during zymogen activation. Thus acylation of Ser-195 in the zymogen may favor a protein conformation resembling that of the acyl-enzyme intermediate.

[2] Morgan *et al.* (1974) examined the relative reaction rates of trypsinogen and trypsin with methanesulfonyl fluoride, an active site titrant which lacks the potential for interaction with the binding pocket of trypsin. The relative rates of reaction with the enzyme and the zymogen were of the same order (48:1) as the relative deacylation rates of the *p*-GB derivatives of chymotrypsin and chymotrypsinogen (70:1).

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