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Effect of pH on Substrate and Inhibitor Kinetic Constants of Human Liver Alanine Aminopeptidase. Evidence for Two Ionizable Active Center Groups†

Charles W. Garner and Francis J. Behal*

ABSTRACT: The presence of at least two ionizable active center groups has been detected by a study of the effect of pH upon catalysis of hydrolysis of L-alanyl-β-naphthylamide by human liver alanine aminopeptidase and upon the inhibition of hydrolysis by inhibitors and substrate analogs. Octanoic acid, octylamine, and peptide inhibitors have been found to be competitive inhibitors and are therefore thought to bind the active center. L-Phe was previously shown to bind the active center since it was found to be a competitive inhibitor of the hydrolysis of tripeptide substrates (Garner, C. W., and Behal, F. J. (1975), *Biochemistry 14,* 3208). A plot of pK_m vs. pH for the substrate L-Ala-β-naphthylamide showed that binding decreased below pH 5.9 and above 7.5, the points at which the theoretical curve undergoes an integral change in slope. These points are interpreted as the pK_a either of substrate ionizable groups or binding-dependent enzyme active center groups. Similar plots of pK_m vs. pH for L-alanyl-*p*-nitroanilide (as substrate) and pK_i vs. pH for L-Leu-L-Leu-L-Leu and D-Leu-L-Tyr (as inhibitors) gave pairs of pK_a values of 5.8 and 7.4, 6.0 and 7.5, and 5.7 and 7.5, respectively. All the above substrates (and D-Leu-L-Tyr) have pK_a values near 7.5; therefore, the binding-dependent group with a pK_a value near 7.5 is possibly this substrate group. Similar plots of pK_i vs. pH for the inhibitors L-Phe, L-Met, L-Leu, octylamine, and octanoic acid had only one bending point at 7.7, 7.6, 7.4, 6.3, and 5.9, respectively. Amino acid inhibitors, octylamine, and octanoic acid have no groups with pK_a values between 5 and 9. These data indicate that there are two active center ionizable groups with pK_a values of approximately 6.0 and 7.5 which are involved in substrate binding or inhibitory amino acid binding but not in catalysis since V_{max} was constant at all pH values tested.

Human liver alanine aminopeptidase is an important member of that class of aminopeptidases which act upon peptides as well as chromogenic substrates like aminoacyl-β-naphthylamides. This enzyme cleaves those aminoacyl-β-naphthylamides having nonpolar side chains. L-Alanyl-β-naphthylamide is the substrate most rapidly hydrolyzed. The enzyme has been purified and some of its properties have been reported (Little, 1970; Little and Behal, 1971; Starnes and Behal, 1974; Garner and Behal, 1974). The enzyme contains 17.5% carbohydrate and has a monomeric molecular weight of 118000. One atom of zinc is present per monomer.

Attempts to identify active center residues of this aminopeptidase have recently been initiated in an effort to understand its mechanism of action. Similar studies have been performed with other aminopeptidases. Through a study of the kinetics of hydrolysis and chemical modifications, a cooperative tyrosine–histidine system has been proposed for aminopeptidase M (Pfleiderer and Femfert, 1969; Femfert and Pfleiderer, 1969; Femfert, 1971; Femfert et al., 1972). A hydrophobic region was also proposed (Femfert and Cochocki, 1974). Cysteine and histidine have been proposed as active center residues in aminopeptidase B (Makinen and Hopsu-Havu, 1967a,b).

Since several inhibitors and substrates of the human liver enzyme are available, a study of the effect of pH on binding was made to detect and possibly identify active center ionizable groups. Two enzyme ionizable groups are indicated with pK_a values of 6.0 and 7.5.

Experimental Section

Materials. Human liver alanine aminopeptidase was prepared by a procedure described previously (Garner and Behal, 1975). The preparations used were greater than 92% pure by polyacrylamide gel electrophoresis. Peptides, substrates, and inhibitors were obtained from ICN, Sigma, or Aldrich as the highest purity available and were used without further purification. All were shown to be homogeneous on an amino acid analyzer or by thin-layer chromatography (TLC). Water was doubly deionized on a mixed-bed ion exchange column before use. Buffers varying in pH from 5.0 to 9.5 were prepared from a mixture of potassium phosphate, maleic acid, and boric acid (0.1 *M* each). The pH

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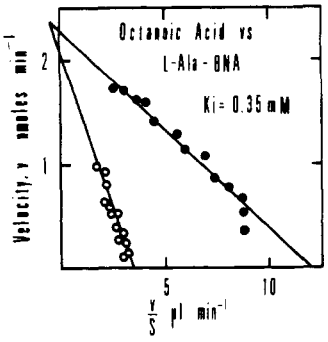

FIGURE 1: Kinetics of inhibition of aminopeptidase (0.3 μg) by octanoic acid. The reaction rate in the absence (●) and presence (○) of octanoic acid, 1.0 mM, was determined from the increase in absorbance at 340 nm at pH 6.8 with 0.05 M potassium phosphate, as described in the Experimental Section.

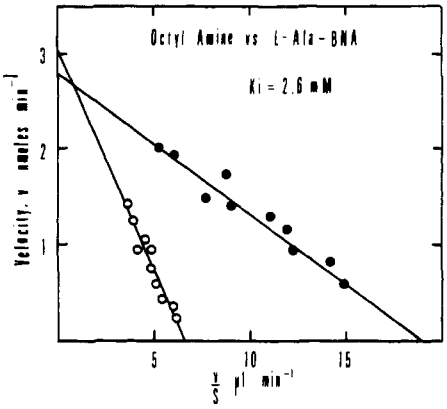

FIGURE 2: Kinetics of inhibition of aminopeptidase by octylamine. The reaction rate was determined in the absence (●) and presence (○) of 5.0 mM octylamine. Other conditions are as in Figure 1.

was adjusted with 1.0 N sodium hydroxide. The ionic strength of each solution was maintained at 0.6 by the addition of sodium chloride. No specific buffer effects were observed.

Aminopeptidase Assays. Two assay methods were used to measure aminopeptidase activity. A procedure based on that of Goldbarg and Rutenburg (1958) was developed to measure the amount of β-naphthylamine released during an incubation for 10 min at 37°. Enzyme (0.2–0.5 μg) was added to a 1-ml reaction mixture containing 0.05 M buffer, described above, and 1.0 mM L-Ala-BNA.[1] At the end of incubation, trichloroacetic acid (0.5 ml of 2.5 M) was added followed by 0.5 ml of 0.1% (w/v) sodium nitrite. After an incubation of 5 min at 37°, ammonium sulfamate (0.5 ml of 0.5% [w/v]) was then added. Color was developed during an incubation at 37° for 15 min after the addition of 1.25 ml of N-(1-naphthyl)ethylenediamine (0.1% in 95% ethanol [w/v]). Thorough mixing after each addition is essential. Absorbance was determined at 580 nm. An absorbance of 1.0 represents the release of 0.1 μmol of β-naphthylamine.

Data for kinetic experiments were collected by monitoring the increase in absorption at 340 nm at 37° with time due to the release of β-naphthylamine (ϵ 1780, Lee et al., 1971). A Beckman Acta III uv/visible spectrophotometer with recorder span set at 0.1 A was used. In certain experiments L-Ala-pNA was used as substrate. Its hydrolysis was monitored by the continuous release of p-nitroaniline at 405 nm (ϵ 9620, Pfleiderer, 1970); K_m, V_{max}, and K_i values were calculated from plots of $1/v$ vs. $1/S$, v vs. v/S, or $I(v/v_c)/(1 - v/v_c)$ (Hunter and Downs, 1945), where v is the velocity of an enzyme-catalyzed reaction and v is the velocity of a control. Inhibitors were added in 10–50-μl aliquots after the initial reaction velocity was established. Thus v and v_c were measured on the same reaction mixture. The reaction mixture was otherwise the same as above.

Results

Inhibitors of Human Liver Alanine Aminopeptidase. Several inhibitors of alanine aminopeptidase have been investigated in an effort to probe for and identify ionizable groups at the active center. The inhibitors studied vs. the hydrolysis of L-Ala-BNA as substrate include octanoic acid, octylamine, hydrophobic L-amino acids, substrate analogs such as D-Leu-L-Tyr, and other substrates such as L-Leu$_3$ and L-Ala-pNA. The inhibition type was determined in each case from plots of v vs. v/S. Peptide substrates and substrate analogs were found to be competitive inhibitors, as expected. L-Phe has been shown previously to be a competitive inhibitor of the hydrolysis of tripeptide substrates such as L-Leu$_3$ suggesting that hydrophobic amino acids bind at the active center. When the substrate was L-Ala-BNA, inhibition by L-Phe was of the noncompetitive type. Thus it was concluded that hydrophobic amino acids may bind at the third residue subsite, i.e., the subsite into which the third residue of a tripeptide binds (Garner and Behal, 1975). Octanoic acid and octylamine both were found in this investigation to be competitive inhibitors of L-Ala-BNA hydrolysis, shown in Figures 1 and 2.

All of the above inhibitors appear to bind the active center since classical competitive inhibition has been demonstrated in each case. The effect of pH on the value of kinetic constants was then measured for each substrate, substrate analog, and inhibitor in order to determine the pK_a value and possibly the identity of the enzyme groups involved.

The Effect of pH on Kinetic Constants. When the extent of activity or the extent of inhibition of activity increases with increasing pH, the data can be represented by

$$K_x = K^*(1 + [H^+]/K_a) \quad (1)$$

where K_x is an experimentally determined K_i or K_m and K^* is the minimum value of K_x. In the linear form, the equation becomes:

$$[H^+]/K_x = -K_a/K_x + K_a/K^* \quad (2)$$

By plotting $[H^+]/K_x$ vs. $1/K_x$, the slope of the line is $-K_a$. Values of pK_a were determined from the left limbs of plots of pK_m vs. pH for L-Ala-BNA and L-Ala-pNA and plots of pK_i vs. pH for L-Leu$_3$, octylamine, and D-Leu-L-Tyr (Figures 3 and 4). The pK_a values so obtained correspond to the bending points of the pK vs. pH plots.

When the extent of activity or the extent of inhibition of activity decreases with increasing pH, the data can be represented by

$$K_x = K^*(1 + K_a/[H^+]) \quad (3)$$

In the linear form the equation becomes:

$$1/K_x = -K_a/K_x[H^+] + 1/K^* \quad (4)$$

By plotting $1/K_x$ vs. $1/[H^+]K_x$, the slope of the line is $-K_a$.

[1] Abbreviations used are: L-Ala-BNA, L-alanyl-β-naphthylamide; L-Ala-pNA, L-alanyl-p-nitroanilide; L-Leu$_3$, L-Leu-L-Leu-L-Leu.

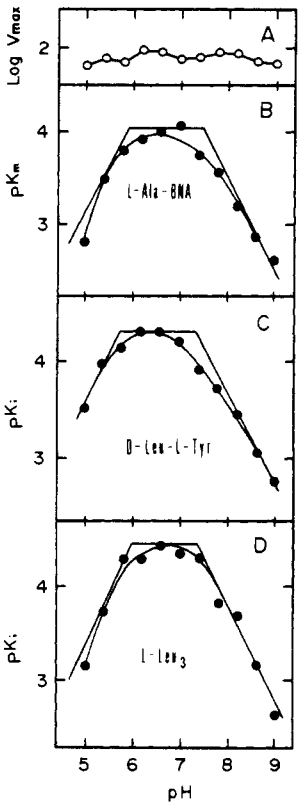

FIGURE 3: Effect of pH on the values of kinetic constants of substrates of substrate analogs. Rates were determined from the increase in absorbance at 340 nm as described in the Experimental Section. (A) Log V_{max} for hydrolysis of L-Ala-BNA calculated from plots of v vs. v/S is shown in arbitrary units. (B) K_m for L-Ala-BNA was calculated from plots of v vs. v/S. (C and D) K_i for D-Leu-L-Tyr and L-Leu$_3$ were calculated from plots described by Hunter and Downs (1945).

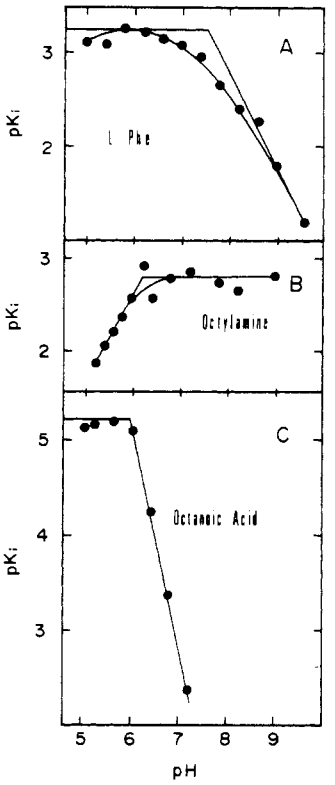

FIGURE 4: Effect of pH on kinetic constants of inhibitors of aminopeptidase. K_i values for L-Phe, octylamine, and octanoic acid were calculated from plots described by Hunter and Downs (1945). Experimental details same as in Figure 3.

Values of pK_a were determined from the right limbs of plots of pK_m for L-Ala-BNA and L-Ala-pNA and plots of pK_i vs. pH for L-Met, L-Phe, L-Leu, D-Leu-L-Tyr, and L-Leu$_3$.

The effect of pH on V_{max} for hydrolysis of the substrate L-Ala-BNA was determined. The data, plotted as log V_{max} vs. pH (Figure 3A), show that V_{max} does not vary with pH between pH 5 and 9. Therefore any enzyme ionizable group shown to be involved in the action of this aminopeptidase will likely be affecting binding and not the catalytic step. A plot of pK_m vs. pH for the substrate L-Ala-BNA (Figure 3B) indeed shows that pH affects binding. A curved line connects the points while theoretical straight lines have been drawn with integral slopes (Dixon, 1953) with the help of eq 2 and 4. Bending points (i.e., pK_a) occur at pH 5.9 and 7.5. The substrate L-Ala-pNA displayed the same behavior and has bending points at pH 5.8 and 7.4.

The inhibition constant K_i for several inhibitors was determined with L-Ala-BNA as substrate. The plot of pK_i vs. pH for D-Leu-L-Tyr (Figure 3C) has the same shape as that for the substrate, having bending points at pH 5.7 and 7.5. Shown in Figure 3D is a similar plot for the inhibitor L-Leu$_3$, which also is a substrate. In this case bending points are seen at pH 6.0 and 7.5. It can be seen that all three curves described above have the same features.

The plot of pK_i vs. pH for L-Phe (Figure 4A), while differing from that of substrate, retains the right limb of the substrate plot, having a bending point at pH 7.7. L-Met and L-Leu had bending points at 7.6 and 7.4, respectively. Some similarity was expected since L-Phe does bind a portion of the active center. The plot of pK_i vs. pH for octylamine (Figure 4B) bears similarity with the plot for substrate only on the left limb, having a bending point at 6.3. Octanoic acid behaves radically different from substrate. The plot of pK_i vs. pH (Figure 4C) shows that octanoic acid binds strongly only at pH values below 5.9, the bending point. All the pK_a values detected in these plots have been collected in Table I in two columns, pK_a (I) and pK_a (II).

A calculation of pK_m for L-Ala-BNA by the method of Alberty (1956) based on an evaluation of half-maximal velocity values taken from v vs. pH curves gave values of pK (I) and pK (II) as 5.7 and 7.8, in reasonable agreement with the values obtained above.

The Heat of Ionization of the Enzyme Group with pK_a = 7.5. The pK_a value of enzyme group upon which L-Leu inhibition is dependent was measured at 21.6 and 43.6°. The pK_a values were calculated from pK_i values with the use of eq 4. Values of pK_i were collected at eight different pH values between pH 7.0 and 8.4 (triplicate experiments) yielding a pK_a value of 7.60 ± 0.04 at 21.6°. The pK_a value at 43.6° was 7.28 ± 0.07, using values of pK_i collected at eight different pH values between pH 6.8 and 8.2 (pentuplicate experiments). The heat of ionization was computed with the following equation (Barnard and Stein, 1958):

$$\Delta H = -\Delta pK_a[2.3RT_1T_2/(T_2 - T_1)] \quad (5)$$

where ΔpK_a is the difference in pK_a values at two temperatures T_1 (294.7 K) and T (316.7 K). The heat of ionization was found to be 6.2 ± 2.1 kcal mol^{-1}.

Discussion

In order to gain a better understanding of the role of ionizable groups in the mechanism of action of human liver alanine aminopeptidase, an investigation of the effect of pH

on binding constants of several substrates and inhibitors has been performed. This enzyme displays a pH profile with a maximum rate of hydrolysis near pH 7. The activity diminishes sharply on each side of the maximum. This behavior alone suggests that there are two (or more) ionizable groups important to activity. These two groups affect binding (K_m) but not the catalytic step (V_{max}). This behavior would be expected also if the substrate had ionizable groups which were important to binding. Indeed, L-Ala-BNA, L-Ala-pNA, and L-Leu_3 all have pK_a values near 7.5

Bending points in plots of pK_m vs. pH for a substrate are interpreted as the pK_a values of essential ionizable groups (Dixon, 1953), in this case, one on the enzyme near pK_a = 6.0 and one possibly on the substrate near pK_a = 7.5. This treatment has been extended to inhibitors and their plots of pK_i vs. pH (Webb, 1963). Amino acids inhibit L-Ala-BNA or L-Ala-pNA hydrolysis noncompetitively while inhibiting tripeptide hydrolysis competitively (Garner and Behal, 1975). Thus inhibition of L-Ala-pNA hydrolysis by L-Phe, L-Met, or L-Leu should occur without regard to substrate concentration or substrate ionizable groups. The binding-dependent group detected with these amino acids thus is an enzyme group. These amino acids have a pK_a value above 9, too high to account for the pK_a value of 7.5 observed on pK_i vs. pH plots. Octylamine and octanoic acid inhibition are both dependent on a group of pK_a values near 6.0. The group appears to be an enzyme group since even-carbon carboxylic acids from acetic to decanoic acid had a pK_a value of 4.7, too low to account for the pK_a = 6.0 group. Also octylamine has a pK_a value above 10, also too high to account for the pK_a = 6.0 group. Determinations of pK_a values of substrates and inhibitors were made by titration. Values were essentially independent of ionic strength.

The pK_a values of binding-dependent groups have been collected in Table I. These data suggest the presence of two enzyme ionizable groups having pK_a values of about 6.0 and 7.5.

The simplest interpretation of the data presented herein is that all the inhibitors and substrates bind to sites all in close proximity, since (a) the binding of all inhibitors and substrates (except L-amino acids) is dependent on the ionization of an enzyme group with a pK_a value of approximately 6.0, and (b) all inhibitors and substrates (except L-amino acids) are competitive inhibitors of L-Ala-BNA hydrolysis. Amino acids are also thought to bind to a site near the active center since the mechanism of inhibition progresses from noncompetitive to competitive as the complexity of the substrate increases from a simple amino acid amide to a tripeptide (Garner and Behal, 1975).

The group affecting substrate binding having a pK_a value of 7.5 may be the substrate ammonium group or an enzyme group. If it is an enzyme group, then substrate apparently binds in both the protonated and unprotonated form.

The slope of the pK_i vs. pH curve can give an indication as to the number of groups involved in the binding of the inhibitor. A curve with a slope of one indicates the involvement of one enzyme or substrate (inhibitor) ionizable group. A curve with a slope of two indicates the involvement of two groups. A slope of two could be demonstrated only in the case of octanoic acid. The meaning of the implication that two ionizable groups are involved in the binding of octanoic acid is not clear. It is possible that only the unionized form of octanoic acid binds the enzyme above pH 6.0. This would make binding dependent on the enzyme ionization and the inhibitor ionization. Or it is possible that there are two enzyme groups with pK_a values of 6.0 involved.

Table I: pK_a of Binding-Dependent Groups Involved in Substrate or Inhibitor Binding.[a]

Substrate or Inhibitor	pK_a (I)	pK_a (II)
1. L-Ala-BNA	5.9 ± 0.3	7.5 ± 0.2
2. L-Ala-pNA	5.8 ± 0.2	7.4 ± 0.2
3. L-Leu_3	6.0 ± 0.2	7.5 ± 0.3
4. D-Leu-L-Tyr	5.7 ± 0.3	7.5 ± 0.2
5. L-Phe		7.7 ± 0.2
6. L-Met		7.6 ± 0.2
7. L-Leu		7.4 ± 0.1
8. Octylamine	6.3 ± 0.3	
9. Octanoic acid	5.9 ± 0.1	

[a] All pK_a values in column I, except that for octanoic acid, were determined from plots based on eq 2. All pK_a values in column II were determined from plots based on eq 4. Straight lines were fitted by a least-squares program on a Wang 500 calculator. Linear correlation coefficients were greater than 0.95. Standard errors are given. pK_a values (I) and (II) for L-Ala-BNA and L-Ala-pNA were computed from pK_m. All other pK_a were computed from pK_i values. Reaction mixtures contained in 1.0 ml, 0.2–0.5 μg of enzyme and 0.05 *M* buffer at 37°. L-Ala-BNA was the substrate in lines 1, 3, 4, 8, and 9. L-Ala-pNA was substrate in all others. Data were collected by monitoring the change in absorbance at 340 or 405 nm. Other procedures are described in the Experimental Section.

The identity of an enzyme ionizable group often can be deduced from the value of its pK_a. A pK_a value of 6.0 is diagnostic for histidine imidazole. A pK_a value of 7.5, however, suggests several possible residues: histidine, tyrosine, sulfhydryl, and α-amino. Sulfhydryl can be excluded since the enzyme contains only trace amounts of sulfhydryl (Starnes and Behal, 1974) and is not sensitive to sulfhydryl reagents (Garner and Behal, 1974). The α-amino group seems to be excluded since the heat of ionization of the group in question, 6200 ± 2100 cal mol^{-1}, is too low for an α-amino group, which is approximately 12000 cal mol^{-1} (Barnard and Stein, 1958). Tyrosine and histidine residues cannot be distinguished by heat of ionization data (Greenstein and Winitz, 1961). An additional possibility for the identity of the pK_a = 7.5 group is the coordinated water molecule of the active site zinc.

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Catalysis by Serine Proteases and Their Zymogens. A Study of Acyl Intermediates by Circular Dichroism†

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ABSTRACT: *p*-Nitrophenyl *p'*-guanidinobenzoate and methylumbelliferyl *p'*-guanidinobenzoate, which are active site titrants for trypsin, and *p*-nitrophenyl *p'*-dimethylsulfonioacetamidobenzoate and methylumbelliferyl *p'*-trimethylammoniocinnamate, which are active site titrants for chymotrypsin, are also hydrolyzed by the respective zymogens. Hydrolysis in each case proceeds via the formation of acyl-zymogens. The acylation rates for the zymogens are 10^3–10^7 times slower than for the enzymes whereas the deacylation rates of acyl-enzymes and acyl-zymogens are comparable. These findings are consistent with the idea that the diminished catalytic activity of these zymogens is due primarily to their distorted substrate binding sites. The circular dichroic spectra of the acyl-enzymes show induced negative ellipticities in the region of absorption of the acyl group, due to binding of the group in an asymmetric environment. The circular dichroic spectra of the acyl-zymogens do not, but conversion of the acyl-zymogens to acyl-enzymes changes the circular dichroic spectra to those characteristic of the acyl-enzymes. α-Carbamyl-ε-guanidinated trypsin is a derivative which resembles trypsinogen in lacking activity toward specific ester substrates but possessing low activity toward *p*-nitrophenyl *p'*-guanidinobenzoate. The circular dichroic spectrum of the acyl-enzyme formed during hydrolysis of *p*-nitrophenyl *p'*-guanidinobenzoate by this derivative resembles that of guanidinobenzoyltrypsinogen, and not that of guanidinobenzoyltrypsin. These circular dichroism studies confirm that the same serine residue is involved in catalysis by both enzymes and zymogens. They demonstrate directly that the acylating group is in a different environment in each and indicate that this specific environment is a determinant in the catalytic activity of each. Thus the circular dichroic spectra of these acyl intermediates provide a sensitive probe of the subtle conformational changes which occur on zymogen activation. The results support the previous conclusion that the major feature of the activation of trypsinogen and chymotrypsinogen is the rearrangement of the substrate binding site and that the appearance of a new amino terminus causes this rearrangement.

Several zymogens, including trypsinogen and chymotrypsinogen, display toward certain substrates or pseudosubstrates a weak intrinsic activity prior to activation (Neurath et al., 1973). An extensive study of the hydrolysis of the ester *p*-nitrophenyl *p'*-guanidinobenzoate by chymotrypsinogen and trypsinogen demonstrated that the same functional groups are involved in the catalysis by the enzymes and their parent zymogens, and that the inferior activities of the zymogens are due primarily to an underdeveloped binding site and only secondarily to a less efficient catalytic apparatus (Gertler, 1973; Gertler et al., 1974a,b). This conclusion was supported by the observation that methanesulfonyl fluoride, an active site titrant which lacks the potential for interacting with the binding pocket of the enzyme, reacted with trypsin and trypsinogen at comparable rates (Morgan et al., 1974). Since hydrolysis of NPGB[1] by chymotrypsin and chymotrypsinogen proceeds via the formation of a *p*-guanidinobenzoyl intermediate which decomposes only slowly at acid pH, this acyl intermediate could be isolated and studied. The circular dichroic (CD) spectrum of *p*-guanidinobenzoylchymotrypsinogen differed significantly from that of *p*-guanidinobenzoylchymotrypsin

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[1] Abbreviations used are: NPGB, *p*-nitrophenyl *p'*-guanidinobenzoate; pGB, *p*-guanidinobenzoyl; Dip, diisopropylphosphoryl; NPSA, *p*-nitrophenyl *p'*-(dimethylsulfonioacetamido)benzoate; pSA, *p'*-(dimethylsulfonioacetamido)benzoyl; MUGB, methylumbelliferyl guanidinobenzoate; MUTMAC, methylumbelliferyl *p'*-trimethylammoniocinnamate; TMAC, *p'*-trimethylammoniocinnamoyl; Hepes, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid.