Binding of Competitive Inhibitors to δ-Chymotrypsin in the Alkaline pH Region. Competitive Inhibition Kinetics and Proton-Uptake Measurements*

Pablo Valenzuela† and Myron L. Bender

ABSTRACT: The binding of the three competitive inhibitors benzyl alcohol, tryptophol, and *N*-acetyl-D-tryptophanamide to α- and δ-chymotrypsins was studied over the pH range 7 to 11 by competitive inhibition kinetics using *N*-furylacryloyl-L-tryptophan methyl ester as substrate. The results indicate that the binding of these inhibitors to δ-chymotrypsin exhibits a pH dependence significantly different from the pH dependence obtained with α-chymotrypsin. Analysis of K_i *vs.* pH profiles for the interaction of benzyl alcohol, tryptophol, and *N*-acetyl-D-tryptophanamide with δ-chymotrypsin indicates that the pK_a of an ionizing group of the enzyme (9.2, 9.5, and 9.2, respectively) is shifted to a pK_a of 10.0, 10.1, and 9.8, respectively, in the enzyme–inhibitor complex. This behavior differs from that of α-chymotrypsin, where, in agreement with previous reports, the binding of the three inhibitors was found to be strictly dependent on the ionization of a group in the enzyme with a pK_a of 9.0 that apparently shifts upon binding of inhibitor to a value of 11.5 or higher. The proton uptake that accompanies the binding of the three inhibitors to δ-chymotrypsin was also measured as a function of pH. A good agreement between the measured proton uptake and the predicted values from the corresponding pK_a shifts was found. It is concluded that the different behavior of α- and δ-chymotrypsins at high pH reflects a difference in the binding abilities of the enzymes. The present hypotheses regarding the conformation and activity of α-chymotrypsin at alkaline pH are discussed in the light of this particular behavior of δ-chymotrypsin.

δ-Chymotrypsin is one of the catalytically active proteins obtained by the activation of chymotrypsinogen A (Desnuelle, 1960). It is specifically obtained by the trypsin-catalyzed "rapid activation" of chymotrypsinogen (Jacobsen, 1947). δ-Chymotrypsin differs from α-chymotrypsin in that it has a dipeptide threonyl-asparagine which covalently ties together the tyrosine 146 residue of the B chain with the alanine 149 residue of the C chain. The remainder of the primary structure is identical in the two proteins.

The amino-terminal residue of both enzymes is the isoleucine 16. The ionization state of this group has been proposed to control the substrate binding ability of α-chymotrypsin and in more precise terms, to be responsible for the reversible inactivation of the enzyme at alkaline pH. There are three main pieces of evidence for this proposal. First, the splitting of the single bond arginine[15]-isoleucine[16] in the zymogen is associated with the appearance of enzymatic activity (Dreyer and Neurath, 1955; Rovery *et al.*, 1955). Second, the enzyme is completely inactivated when this amino group is blocked by acetylation (Oppenheimer *et al.*, 1966). Third, the pH dependence of K_m or K_s for specific substrates shows a dependence on an acid group which ionizes with a pK_a around 9.0 (Himoe and Hess, 1966; Bender *et al.*, 1966; Johnson and Knowles, 1967). The pH dependence of δ-chymotrypsin-catalyzed reactions has not been fully investigated and usually it has been assumed to be similar to that of α-chymotrypsin.

We have recently studied the alkaline pH dependence of K_m for the δ-chymotrypsin-catalyzed hydrolysis of *N*-acetyl-L-tryptophan methyl ester and *N*-furylacryloyl-L-tryptophanamide (Valenzuela and Bender, 1969). It was found that K_m for both these specific substrates depends on pH in a way that is markedly different from the pH dependence of K_m shown by α-chymotrypsin with specific substrates. The K_m values for the α-chymotrypsin-catalyzed hydrolysis of *N*-acetyl-L-tryptophan methyl ester increase sharply above pH 8.8, but the K_m values for the δ-chymotrypsin-catalyzed hydrolysis only increase by a factor of three from pH 8 to pH 11. The K_m values seem to be pH independent at pH higher than 11. The difference in K_m between α- and δ-chymotrypsin was suggested to be due to a difference in K_s, the dissociation constant of the enzyme–substrate complex.

In this paper we extend our initial studies on the high pH mechanism of δ-chymotrypsin-catalyzed hydrolyses. We have measured the pH dependence of the binding constants of the competitive inhibitors benzyl alcohol, tryptophol, and *N*-acetyl-D-tryptophanamide by the use of competitive inhibition kinetics. As recent studies indicate that the binding of substrates and competitive inhibitors to α- and δ-chymotrypsin causes an uptake of protons from the medium (Bender and Wedler, 1967; McConn *et al.*, 1968; Wedler and Bender, 1969; Garel and Labouesse, 1970), we have also measured the pH dependence of the proton uptake observed upon binding of the above three inhibitors to δ-chymotrypsin.

The results obtained give rise to a meaningful interpretation of the mechanism of binding of specific substrates and inhibitors to δ-chymotrypsin in the alkaline pH region and

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† On leave from the School of Chemistry and Pharmacy, University of Chile, Santiago, Chile.

establish a clear difference between the binding abilities of α- and δ-chymotrypsins in this pH region. Preliminary data from this study have been briefly reported (Valenzuela and Bender, 1970).

Materials and Methods

Enzymes. Salt-free, three-times-crystallized, chromatographically homogeneous α-chymotrypsin (Worthington Biochem. Corp., lot CDS 6602) was used. Quantitative N-terminal group analysis performed by the method of Sanger (1945) as described by Labouesse and Gervais (1967) gave 0.85 ± 0.10 mole of isoleucine and 0.65 ± 0.10 mole of alanine per mole of active enzyme. Salt-free, three-times-crystallized δ-chymotrypsin (Worthington Biochem. Corp., lot CDD 6032) was employed. This protein contains 0.85 ± 0.10 mole of isoleucine and 0.0 mole of alanine as α-amino-terminal residues per mole of active enzyme. Salt-free, five-times-crystallized chymotrypsinogen A (Worthington Biochem. Corp. lot CGC 8CC) was used. This protein was shown to contain less than 1% intrinsic chymotryptic activity measured by a specific substrate assay (Zerner and Bender, 1964). Stock solutions of enzymes were prepared in 0.1 M KCl just before use and the concentration of protein determined spectrophotometrically at 280 nm using an extinction coefficient of 4.7×10^{-4} M^{-1} cm^{-1} (Dixon and Neurath, 1957). The normality of active enzyme solutions was determined by spectrophotometric titration with *N-trans*-cinnamoylimidazole (Schonbaum *et al.*, 1961).

Substrates and Inhibitors. *N*-Acetyl-L-tryptophan methyl ester (Cyclo Chem Corp., lot 3-4735) was recrystallized twice from acetonitrile before use, mp 153°. *N-trans*-Cinnamoylimidazole (Cyclo Chem Corp.) was crystallized three times from hexane before use, mp 132°. *N*-2-Furylacryloyl-L-tryptophan methyl ester (Cyclo Chem Corp., lot M-3685) was recrystallized twice from ethyl acetate–petroleum ether before use, mp 146°. *N*-Acetyl-D-tryptophanamide (Cyclo Chem Corp., lot K-5723), mp 193°, was used as delivered. Tryptophol was obtained from Cyclo Chem Corp. or from Sigma and was crystallized two times before use, mp 59°. Benzyl alcohol (Fisher Certified Reagent) was purified by three distillations over Na_2CO_3, under nitrogen and was kept under nitrogen before use.

Stock solutions of substrates and inhibitors were prepared in either acetonitrile (Mallinckrodt, nanograde, without further purification) or dimethyl sulfoxide (Matheson Coleman and Bell, without further purification).

All melting points were determined by the capillary tube method and are uncorrected.

Kinetic Runs. The kinetics of hydrolysis were determined using a Cary 14 PM recording spectrophotometer equipped with a thermostatted cell compartment at 25.0 ± 0.2°. The hydrolysis of *N*-acetyl-L-tryptophan methyl ester was followed at 300 nm as described before (Zerner and Bender, 1964). The hydrolysis of *N*-furylacryloyl-L-tryptophan methyl ester (λ_{max} 304 nm (ϵ 26.400)) was followed at 335 nm; the absorbance data were converted into rate data using $\Delta\epsilon$ 1086 as the difference in molar absorptivities between the ester and the acid (Miller, 1968), The results were analyzed using a one-run digital computer program based on a least-squares analysis of v *vs.* v/S (Eadie, 1942).

The pH of each reaction was determined at the beginning and at the end of the reaction using a Radiometer 4c pH meter with type B glass electrode. The buffers used in the kinetic runs were: potassium phosphate buffer from pH 7.0 to 8.0; Tris-HCl from pH 8.0 to 9.0; ammonia–ammonium chloride or sodium carbonate buffers were used with identical results between pH 9.0 and 10.0. Sodium carbonate buffers were used in all runs above pH 10.0. All buffer solutions were of ionic strength 0.1 and were prepared from analytical reagent grade materials.

Determination of K_i. Benzyl alcohol, tryptophol, and *N*-acetyl-D-tryptophanamide were found to be competitive inhibitors with the substrates used at all pH values studied. The same V_{max} was obtained with or without inhibitor, within the experimental error. The reported values of K_i were obtained using *N*-furylacryloyl-L-tryptophan methyl ester as substrate but the same values of K_i were obtained in some cases in which *N*-acetyl-L-tryptophan methyl ester was used as substrate. K_i values were determined from the K_m values obtained from Eadie plots of kinetic runs in the presence of inhibitor (K_{mi}) and in the absence of inhibitor (K_{mo}) according to eq 1 (Dixon and Webb, 1964). The concentrations

$$K_i = \frac{[I]}{\frac{K_{mi}}{K_{mo}} - 1} \quad (1)$$

of inhibitors used in the pH–K_i profiles were benzyl alcohol: 39 mM (δ-chymotrypsin) and 80 mM (α-chymotrypsin); *N*-acetyl-D-tryptophanamide: 8 mM; and tryptophol; 8 mM.

Proton-Uptake Measurements. The proton absorption studies were performed at 25° using a Sargent Model SR recorder attached to a Corning Model 12 Research pH meter. A semimicro Ag/AgCl combination electrode (Corning) was used. A detailed description of this technique has been previously reported (Wedler and Bender, 1969). The protein was dissolved in 0.1 M KCl and a volume of 5 ml was used in each experiment. The inhibitors were added by means of a Hamilton gas-tight syringe. Chymotrypsinogen was used as a "blank reaction." Benzyl alcohol was added as a 1:1 solution in methanol in order to achieve a faster mixing of the benzyl alcohol with the protein solution. Tryptophol and *N*-acetyl-D-tryptophanamide were added as 0.5 M solutions in dimethyl sulfoxide. Methanol does not induce proton uptake. A small proton absorption (10%) produced by dimethyl sulfoxide when used in large amounts (more than 3%) was corrected for after adequate controls using dimethyl sulfoxide alone.

Results

Inhibition Experiments. Benzyl alcohol, tryptophol, and *N*-acetyl-D-tryptophanamide were assayed at different concentration as inhibitors of α- and δ-chymotrypsin-catalyzed hydrolyses of *N*-furylacryloyl-L-tryptophan methyl ester. It was found that the three compounds behave as competitive inhibitors at all concentrations assayed up to 80 mM (benzyl alcohol) and 8 mM (tryptophol and *N*-acetyl-D-tryptophanamide). No assays were carried out at higher inhibitor concentration.

Figure 1 shows typical Eadie plots used to calculate the values of K_i. It can be seen that the same V_{max} is obtained

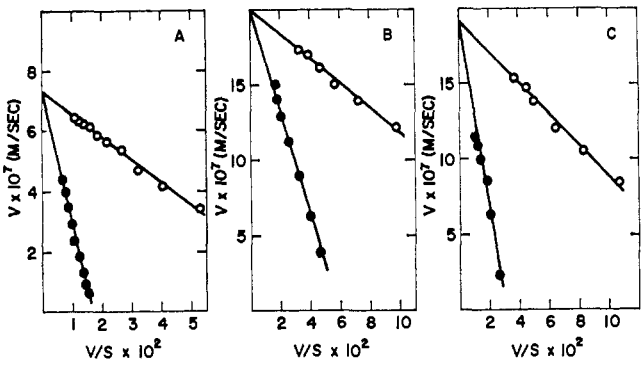

FIGURE 1: Typical plots for the inhibition by benzyl alcohol, tryptophol, and *N*-acetyl-D-tryptophanamide of the δ-chymotrypsin-catalyzed hydrolysis of *N*-furylacryloyl-L-tryptophan methyl ester: (●) with inhibitor; (○) without inhibitor; A: 39 mM benzyl alcohol, pH 9.02; B: 8 mM tryptophol, pH 9.38; C: 8 mM *N*-acetyl-D-tryptophanamide, pH 9.20. All runs were performed at 25° in 3.1% dimethyl sulfoxide.

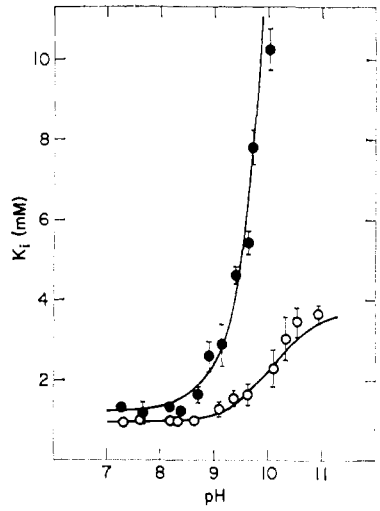

FIGURE 3: The pH dependence of K_i for the tryptophol inhibition of the α-chymotrypsin-catalyzed (●), and δ-chymotrypsin-catalyzed (○) hydrolysis of *N*-furylacryloyl-L-tryptophan methyl ester. All experiments were performed at 25° in 3.1% dimethyl sulfoxide. For buffer composition see Materials and Methods. Each experimental point represents the average of at least three K_i determinations. The solid lines are calculated from eq 3 (see text) using the values $pK_H^E = 9.0$, $pK_H^{EI} = 11.5$ for α-chymotrypsin and $pK_H^E = 9.5$, $pK_H^{EI} = 10.1$ for δ-chymotrypsin.

with and without inhibitor. Similar results were obtained at other pH values. The fact that the inhibitors show strictly competitive inhibition permit us to consider K_i as the dissociation constant of the enzyme–inhibitor complex.

The pH dependences of K_i for the three compounds as inhibitors of α- and δ-chymotrypsin-catalyzed hydrolyses of *N*-furylacryloyl-L-tryptophan methyl ester are shown in Figures 2, 3, and 4. The shapes of the K_i–pH profiles appear to be essentially the same for the three inhibitors used in this study. The profiles for α-chymotrypsin show a pH-independent region between pH 7 and 8.5. Above pH 8.5 the enzyme binds inhibitor progressively less well and a sharp increase in the K_i values is noted. When these profiles are analyzed by the method of Dixon (1953), it is found that K_i is dependent on the ionization of a group in the enzyme with a pK_a of 9.0 for the three inhibitors, in good agreement with the very well known behavior of α-chymotrypsin at high pH (Bender *et al.*, 1966; Himoe and Hess, 1966; Himoe

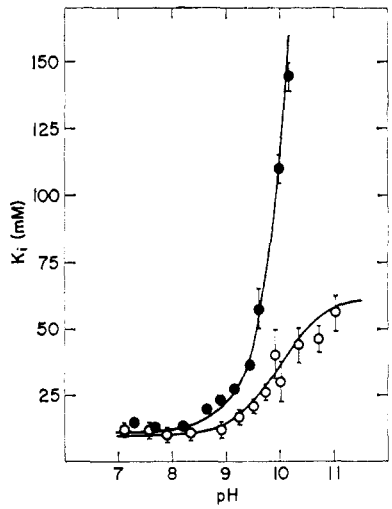

FIGURE 2: The pH dependence of K_i for the benzyl alcohol inhibition of the α-chymotrypsin-catalyzed (●) and δ-chymotrypsin-catalyzed (○) hydrolyses of *N*-furylacryloyl-L-tryptophan methyl ester. All experiments were performed at 25° in 3.1% dimethyl sulfoxide. For buffer composition see Material and Methods. Each experimental point represents the average of at least three K_i determinations. The solid lines are calculated from eq 3 (see text) using the values $pK_H^E = 9.0$, $pK_H^{EI} = 11.5$ for α-chymotrypsin and values $pK_H^E = 9.2$, $pK_H^{EI} = 10.0$ for δ-chymotrypsin.

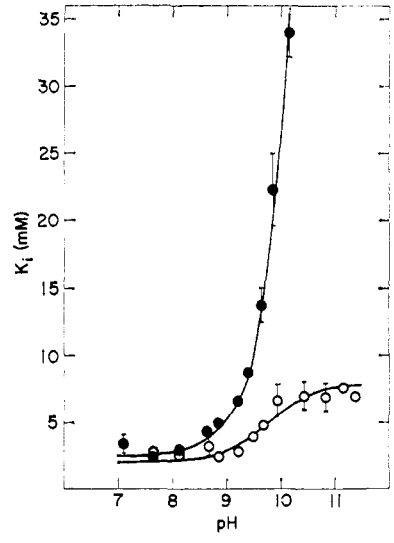

FIGURE 4: The pH dependence of K_i for the *N*-acetyl-D-tryptophanamide inhibition of the α-chymotrypsin-catalyzed (●) and δ-chymotrypsin-catalyzed (○) hydrolysis of *N*-furylacryloyl-L-tryptophan methyl ester. All experiments were performed at 25° in 3.1% dimethyl sulfoxide. For buffer composition see Materials and Methods. Each experimental point represents the average of at least three determinations of K_i. The solid lines are calculated from eq 3 (see text) using the values $pK_H^E = 9.0$, $pK_H^{EI} = 11.5$ for α-chymotrypsin and $pK_H^E = 9.2$, $pK_H^{EI} = 9.8$ for δ-chymotrypsin.

TABLE I: pK_a Values and Corresponding pK_a Shifts for the Interaction of Some Specific Substrates and Inhibitors with δ-Chymotrypsin.[a]

Substrates	pK_H^E	pK_H^{ES}	pK_a Shift
N-Acetyl-L-tryptophanamide[b]	9.00	9.60	0.60
N-Furylacryloyl-L-tryptophanamide[c]	9.50	10.00	0.50
N-Furylacryloyl-L-tryptophanamide, ionic strength 0.5[c]	9.50	10.00	0.50
N-Acetyl-L-tryptophan methyl ester[c]	9.25	9.75	0.50
N-Acetyl-L-tryptophan methyl ester, ionic strength 0.5[c]	9.25	9.55	0.30
N-Acetyl-L-tryptophan methyl ester, ionic strength 1.0[c]	9.25	9.50	0.25
Inhibitors	pK_H^E	pK_H^{EI}	
Benzyl alcohol[d]	9.20	10.00	0.80
Tryptophol[d]	9.50	10.10	0.60
N-Acetyl-D-tryptophanamide[d]	9.20	9.80	0.60
Indole[e]	9.00	10.50	1.50

[a] Ionic strength 0.1, otherwise indicated. [b] Approximate values from proton-uptake experiments. (McConn *et al.*, 1968). [c] Values from Valenzuela and Bender (1969). [d] This research. [e] Garel and Labouesse (1970).

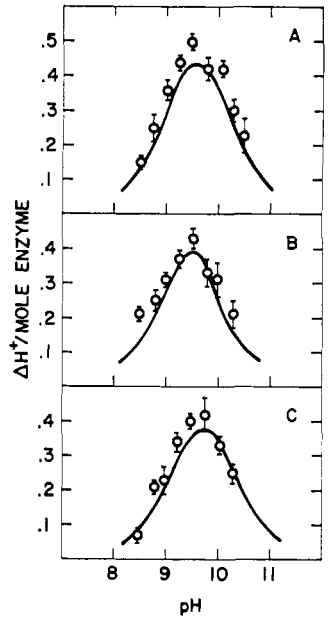

FIGURE 5: The pH dependence of the proton absorption, ΔH^+ per mole of enzyme, by 8×10^{-5} M δ-chymotrypsin upon binding of benzyl alcohol (A), *N*-acetyl-D-tryptophanamide (B), and tryptophol (C) at 25°. Each experimental point represents the average of three ΔH^+ determinations. The solid lines are calculated from eq 4 (see text) using the corresponding pK_a values obtained in the competitive inhibition experiments.

et al., 1967). Our results with *N*-acetyl-D-tryptophanamide show a reasonably good agreement with the results obtained by Johnson and Knowles (1967) for the binding of the same inhibitor to α-chymotrypsin using the technique of equilibrium dialysis.

The K_i–pH profiles obtained for the δ-chymotrypsin-catalyzed reactions were found to be quite different from those of the α-chymotrypsin-catalyzed reactions. The K_i's are independent of the pH between pH 7 and 9, above pH 9, K_i rises but the values start leveling off again at pH values near 10.5–11.0, the exact value apparently depending on the nature of the inhibitor. The shape of the profiles for δ-chymotrypsin indicates that the binding of inhibitor is dependent on an acid group of the enzyme and that the binding of the inhibitor produces a shift in the pK_a of this group to a higher value in the enzyme–inhibitor complex. A similar observation has been made before regarding the dependence on pH of the K_m values for the δ-chymotrypsin-catalyzed hydrolysis of some specific substrates (Valenzuela and Bender, 1969). Table I shows the pK_a values and the corresponding shifts for the present and previous studies on the interaction of δ-chymotrypsin with specific substrates and inhibitors. It can be seen that in all cases already studied, except in the interaction with indole (Garel and Labouesse, 1970), the shift is less than one pK_a unit and the values depend on the nature of the substrate or inhibitor. In the cases of *N*-acetyl-L-tryptophan methyl ester the shift decreases as the ionic strength increases. The same ionic strength effect has been observed with other ester substrates but not with amide substrates (P. Valenzuela and M. L. Bender, unpublished preliminary results).

Proton-Uptake Experiments. The nature of the pK_a shifts (difference between pK_H^{EI} and pK_H^E) indicates that the protonation of a group in the enzyme is aided by the binding of substrate or inhibitor and indicates also that saturation of the enzyme with substrate or inhibitor should result in a proton uptake from the medium. This proton uptake depends on the pH of the medium and on the magnitude of the pK_a shift.

The addition of saturating amounts of benzyl alcohol, tryptophol, and *N*-acetyl-D-tryptophanamide to unbuffered solutions of δ-chymotrypsin resulted in an immediate increase in the pH of the solution. No change in the protonation state of chymotrypsinogen was noted upon the addition of saturating amounts of any of these inhibitors.

The pH dependence of the proton uptake upon addition was determined separately for the three inhibitors. The results are shown in Figure 5. Bell-shaped profiles with a maximal proton uptake of 0.4 to 0.5 proton per mole of enzyme were found, as expected from the shifts of about 0.6 pK_a unit found in the above competitive inhibition experiments. The data agree reasonably well with the theoretical curves calculated using the pK_a values obtained in the kinetic experiments.

In another series of experiments, the observed ΔH^+ per mole of enzyme or "proton signal" was measured as a function of the inhibitor concentration at the pH value of maximal "proton signal." The saturation curves shown in Figure 6 were obtained for each inhibitor. From these data, the dissociation constants K_d were calculated from the slopes of plots of ΔH^+ *vs.* $\Delta H^+/[I]$. In Table II the K_d values obtained are compared with the corresponding values of K_i determined by inhibition kinetics at the same pH. It can be seen that the

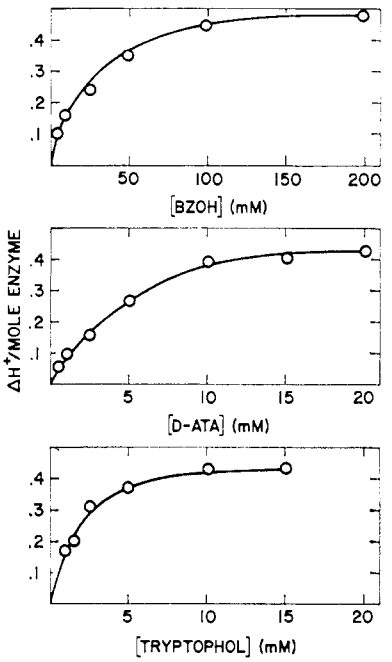

FIGURE 6: The dependence of ΔH^+ per mole of δ-chymotrypsin upon the concentration of added inhibitor at 25° pH 9.50, 8×10^{-5} M enzyme in 0.1 M KCl. Each experimental point represents the average of three determinations of ΔH^+. The solid lines are the estimated best fit to the data.

dissociation constants obtained from equilibrium measurements (proton uptake) agree quite well with the inhibition constant obtained from kinetic measurements indicating again a clear link between the proton binding equilibria and the inhibitor binding equilibria.

Discussion

The simplest model that would account for the data on the alkaline pH dependence of binding of inhibitors to δ-chymotrypsin is given by eq 2.[1]

$$\begin{array}{ccc} E + H^+ & \overset{K_H^E}{\rightleftharpoons} & EH^+ \\ + & & + \\ I & & I \\ K_I^E \uparrow\downarrow & & \downarrow\uparrow K_I^{EH} \\ EI + H^+ & \overset{K_H^{EI}}{\rightleftharpoons} & EH^+I \end{array} \qquad (2)$$

Equation 2 represents the equilibria between the protonated and the unprotonated forms of the enzyme in the free state and in the form of a complex with the competitive inhibitor. The binding constants K_H^E, K_H^{EI}, K_I^E, and K_I^{EH} have been evaluated in this study using the competitive inhibition experiments. In accordance with eq 2, the pH dependence of the observed K_i in the alkaline pH region will be given by eq 3.

Equation 3 was used in the calculation of the theoretical

[1] Similar schemes to that of eq 2 have been used previously to account for pH dependence data of chymotrypsin (Bender *et al.*, 1964; Kaplan and Laidler, 1967; McConn *et al.*, 1968; Garel and Labouesse, 1970).

TABLE II: Comparison of the Values of the Dissociation Constant of the Enzyme–Inhibitor Complex, Obtained from Competitive Inhibition Kinetics and from Proton-Uptake Measurements, for the Interaction of Benzyl Alcohol, Tryptophol, and *N*-Acetyl-D-tryptophanamide with δ-Chymotrypsin.[a]

Inhibitor	Competitive Inhibition K_i (mM)	Proton Uptake K_d (mM)
Benzyl alcohol	22.0	20.0
Tryptophol	1.5	1.5
N-Acetyl-D-tryptophanamide	4.0	4.6

[a] pH 9.50, 25°. Other conditions are given in Materials and Methods.

$$K_i(\text{obsd}) = K_I^{EH} \times \frac{1 + K_H^E/[H^+]}{1 + K_H^{EI}/[H^+]} \qquad (3)$$

lines of Figures 2, 3, and 4 using the values of K_H^E and K_H^{EI} obtained by the method of Dixon (1953).

The model of eq 2 shows that the inhibitor binding equilibria are coupled to the proton binding equilibria. As binding of inhibitor occurs 3 to 5 times better to the protonated form of the enzyme, saturation with inhibitor displaces the protonation equilibria toward the protonated form of the enzyme, shifting the observed equilibrium constant from K_H^E to a lower value K_H^{EI}. This shift causes an uptake of protons from the medium upon binding of inhibitor to the enzyme. This uptake is pH dependent and related to the difference between K_H^{EI} and K_H^E according to eq 4.

$$\Delta H^+/\text{mole of enzyme} = \frac{[H^+]}{[H^+] + K_H^{EI}} - \frac{[H^+]}{[H^+] + K_H^E} \qquad (4)$$

Equation 4 is derived from the reaction sequence of eq 2, assuming that the inhibitor is present in saturating amounts so that [EI] = [E]. Equation 4 is used to calculate the theoretical lines of Figure 5 using the values of K_H^E and K_H^{EI} obtained from the corresponding competitive inhibition experiment.

The mechanism of α- and δ-chymotrypsin-catalyzed hydrolyses can be represented by eq 5. The data presented

$$E + S \overset{K_s}{\longleftarrow} E \cdot S \overset{k_2}{\longrightarrow} \underset{P_1}{E\text{–}S} \overset{k_3}{\longrightarrow} E + P_2 \qquad (5)$$

indicate that the difference in behavior at alkaline pH between α- and δ-chymotrypsin is due to a different pH dependence of the K_s term of eq 5. This was suggested previously to account for the pH dependence of K_m for the δ-chymotrypsin-catalyzed hydrolysis of specific substrates (Valenzuela and Bender, 1969).

The binding of substrates and inhibitors to α-chymotrypsin is known to be strictly dependent on the ionization state of an acidic group with a pK_a around 8.8–9.0 in the free enzyme (Himoe and Hess, 1966; Bender *et al.*, 1966; Johnson and Knowles, 1967). The analysis of the pH–K_s profiles as well

as the results from proton-uptake measurements upon binding of substrates or inhibitors to α-chymotrypsin are consistent with shift of this pK_a to a much higher value in the enzyme–substrate or enzyme–inhibitor complex (Himoe *et al.*, 1967; Bender and Wedler, 1967). Our results obtained in this research with α-chymotrypsin indicate that the binding of benzyl alcohol, tryptophol, and *N*-acetyl-D-tryptophanamide to the enzyme is dependent on a group with a pK_H^E around 9.0 and the K_i–pH profiles fit quite well with a theoretical line calculated using a pK_H^{EI} equal to 11.5.

The results that were obtained with δ-chymotrypsin are significantly different. The binding of the inhibitors is only partially dependent on the pH in the alkaline region. An acidic group with a pK_H^E of 9.2–9.5 in the free enzyme that shifts to a higher value of 9.8–10.0 in the enzyme–inhibitor complex is observed with the three inhibitors used in this study.

The pH dependence of the proton uptake upon binding of benzyl alcohol, tryptophol, and *N*-acetyl-D-tryptophanamide to δ-chymotrypsin is different from that previously found with α-chymotrypsin (Wedler and Bender, 1969). Bell-shaped pH–ΔH^+ profiles were found for the binding of the three inhibitors to δ-chymotrypsin. Maximal uptakes were of the order of 0.4 to 0.5 proton per mole of enzyme as expected for the smaller pK_a shift. This situation contrasts with the sigmoidal profile with a maximal proton uptake of one proton (per mole of enzyme) found previously with α-chymotrypsin as expected for a pK_a shift of the order of 2.0 or more pK_a units (Wedler and Bender, 1969). Qualitatively similar differences between the enzymes have been reported by McConn *et al.* (1968) who studied the proton uptake upon binding of *N*-acetyl-D-tryptophanamide and *N*-acetyl-L-tryptophanamide to α-chymotrypsin and δ-chymotrypsin, respectively. Our proton uptake data on δ-chymotrypsin also agree with the results reported recently by Garel and Labouesse (1970) who used indole as a competitive inhibitor.

Although there is at present no direct proof available, the acid group involved in the binding ability of δ-chymotrypsin is probably the α-amino group of the isoleucine 16 residue. This group has been suggested to be responsible for the sharp increase in K_m of the α-chymotrypsin-catalyzed hydrolysis of specific substrates (Himoe *et al.*, 1967; Bender *et al.*, 1966; Johnson and Knowles, 1967).

Several hypotheses have been formulated to explain the mechanism of inactivation of α-chymotrypsin at alkaline pH. The X-ray data (Matthews *et al.*, 1967; Sigler *et al.*, 1968) show that in crystalline α-chymotrypsin the ammonium group of the isoleucine 16 and the carboxylate group of aspartic acid residue 194 (which is adjacent to the serine residue 195 at the active site) form an internal ion pair in a nonpolar region of the protein away from the solvent. The disruption of this ion pair upon deprotonation of the isoleucine amino group is postulated to produce a conformational change in the enzyme that affects its activity and binding ability. Two mechanisms have been suggested to explain the disruptive effect of this conformational change. (a) When the isoleucine 16 amino group is deprotonated, the nonpolar B chain terminal residue binds to the hydrophobic pocket of the active site producing a intramolecular competitive inhibition (Bender *et al.*, 1966). (b) When the positive charge is removed from the isoleucine amino group, the carboxylate group of aspartate 194 seeks an alternative orientation into a more polar environment, producing a disruption in the active site of the enzyme (Sigler *et al.*, 1968).

δ-Chymotrypsin is structurally different from α-chymotrypsin. The C chain amino-terminal alanine 149 and the B chain carboxyl-terminal tyrosine 146 residues of α-chymotrypsin are covalently bound in δ-chymotrypsin through the dipeptide threonyl-serine. It is safe to assume that in solution, at least in this region of the protein, δ-chymotrypsin has a more rigid structure than α-chymotrypsin.

With these considerations in mind the special behavior of δ-chymotrypsin at high pH in comparison with that of α-chymotrypsin may be tentatively explained by one of the following: (a) the isoleucyl-valine dipeptide of the B chain is not able to compete effectively with the substrate for the active site of δ-chymotrypsin as it does in the case of α-chymotrypsin; (b) the rotation of aspartate 194 after disruption of the salt bridge produces only a minor affect in δ-chymotrypsin compared with the large effect in α-chymotrypsin; (c) α-chymotrypsin is a special case in which a direct involvement of the residues alanine 149 or tyrosine 146 takes place in the inactivation at high pH. This has been suggested previously from the results of the δ-chymotrypsin-catalyzed hydrolysis of some specific substrates (Valenzuela and Bender, 1969).

An interesting problem that arises from the studies on the behavior of δ-chymotrypsin at high pH is the mechanism of, inactivation of α- and δ-chymotrypsins upon acetylation of the isoleucine 16 amino group (Oppenheimer *et al.*, 1966). This phenomenon as well as the inactivity of chymotrypsinogen has been generally explained as being due to the fact this group when modified in the form of an amide linkage can no longer be protonated. The unprotonated enzyme is considered to be in the inactive conformation as discussed above. Our results with δ-chymotrypsin seems to challenge this explanation. δ-Chymotrypsin is active and binds substrates and inhibitors to a significant extent when isoleucine 16 is not protonated. For example, from the data in this paper, it is possible to show that δ-chymotrypsin at pH 11.5 binds *N*-acetyl-D-tryptophanamide with a K_s equal to 8×10^{-3} M. At this pH less than 1% of the free enzyme and less than 2% of the enzyme–inhibitor complex have a protonated isoleucine 16 amino group, as calculated from the pK_H^E and pK_H^{EI} values. Similar conclusions can be made from the results of studies on the pH dependence of the δ-chymotrypsin-catalyzed hydrolysis of specific substrates (Valenzuela and Bender, 1969). The lack of a positive charge on the isoleucine may indeed explain the lack of activity of polyacetylated α-chymotrypsin but does not explain that of polyacetylated δ-chymotrypsin. Further research along this line is necessary to clarify this important point.

In conclusion, the particular behavior found in δ-chymotrypsin at high pH and the fact that may be considered a structurally simpler enzyme than α-chymotrypsin, suggest that δ-chymotrypsin should be regarded as an adequate model in studying the nature of the conformational changes that seem to affect the active site of α-chymotrypsin at high pH.

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