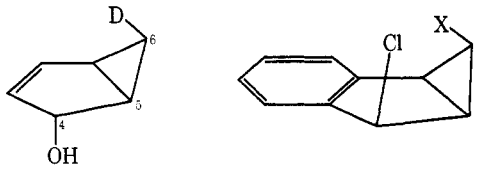

IV Va, X = D
b, X = H

A related reaction of naphthvalene, with DCl gas in CCl_4, stereospecifically[21] gives Va (mp 53.5–56.5°) in 98% yield.[22] The structure is proven by its proton nmr spectrum[23] and that of the epimer of Vb formed upon reaction with LiCl in acetone,[24] and by reaction of Vb with triphenyltin hydride,[25] which gives quantitatively benzobicyclo[3.1.0]hex-2-ene (57%),[26] 1-methylindene (40%),[27] and 1,2-dihydronaphthalene (3%).[28] The stereospecificity of the chloride attack seems remarkable[29] although that of the protonation is anticipated by two other results[30] and by theories.[31]

The abundance of benzvalenes should allow extensive studies of their properties.

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(21) Within 5% (nmr analysis).
(22) The parent peaks in the mass spectrum, and the carbon, hydrogen, and chlorine analyses are those required.
(23) HCl adduct: τ 2.89 (3.97 H), 4.40 (6.5-Hz doublet, 0.98 H), 7.72 (multiplet, 2.00 H), 8.91 (multiplet, 1.03 H), 9.31 (4.2-Hz quartet, 1.03 H). DCl adduct: no τ 9.31 (endo H)[17] resonance; τ 8.92 is an 8.1-Hz triplet.[17c]
(24) τ 9.31 → τ 9.88;[17,20] 6.5-Hz doublet → 1.7-Hz doublet.[18,20]
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The Difference between α- and δ-Chymotrypsins. Preparation and Alkaline pH Dependence of α_1-Chymotrypsin-Catalyzed Hydrolysis of *N*-Acetyl-L-tryptophan Methyl Ester (ATME). The Involvement of Alanine-149 in α-Chymotrypsin Catalysis

Sir:

We wish to report evidence which strongly implicates the amino terminus of alanine-149 as a participant in catalysis by the enzyme α-chymotrypsin. The ionization state of this amino acid leads to some structural change at the active site which determines the kinetic behavior of the enzyme. It is known that α-chymotrypsin loses its ability to bind specific substrates or inhibitors in the alkaline pH region.[1-3] Although it has not been proved, this reversible inactivation has been associated with the disruption of an ion pair between the carboxyl group of aspartate-194 and the N-terminal amino group of isoleucine-16, triggered by the deprotonation of this last residue.[4]

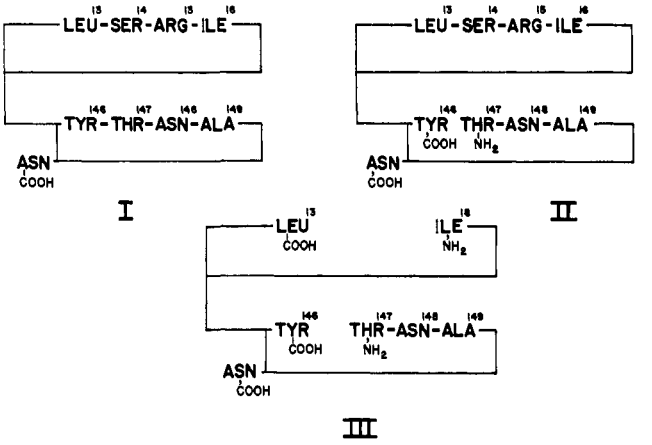

Figure 1. Schematic representation of the structures of chymotrypsinogen A (I), *threo*-neochymotrypsinogen (II), and α_1-chymotrypsin (III).

Recent studies from this laboratory on the pH dependence of δ-chymotrypsin-catalyzed reactions[5,6] indicated that the binding ability of this enzyme is remarkably less dependent on pH when compared with α-chymotrypsin. Although there is evidence for the existence of the same ionic bond in crystals of phenylmethanesulfonyl-δ-chymotrypsin,[7] our results clearly indicated that the deprotonation of the isoleucine-16 amino group causes only a minor effect on the binding ability of this enzyme. This led us to suggest[5] that the peculiar behavior of α-chymotrypsin at alkaline pH may be caused by the ionization of the phenolic group of tyrosine-146 or the amino group of alanine-149, which are present as chain termini in α-chymotrypsin but not in δ-chymotrypsin.

In this communication we wish to report preliminary results on the preparation and the alkaline pH dependence of another active form of chymotrypsin, α_1-chymotrypsin (III). This enzyme, whose existence was first recognized by Desnuelle and coworkers, differs from α-chymotrypsin because it has threonine-147 instead of alanine-149 as the N-terminal amino acid of the C chain (Figure 1).[8]

III was prepared by enzymatic activation of *threo*-neochymotrypsinogen[9] (II), according to the following procedure: chymotrypsinogen A (I) was treated with 5% (w/w) purified δ-chymotrypsin and 2% (w/w) crystalline soybean trypsin inhibitor in 0.1 *M* phosphate

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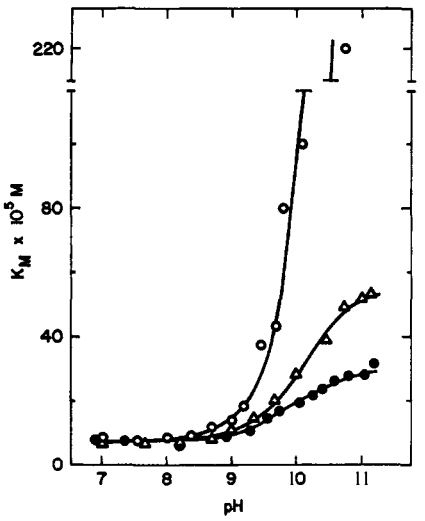

Figure 2. pH-K_m(app) profiles for the α-chymotrypsin- (○), α_1-chymotrypsin- (△), and δ-chymotrypsin-catalyzed (●) hydrolyses of ATME. Runs were performed at 25° in 1.6% (v/v) dimethyl sulfoxide. Each point is the average of three determinations which agreed within 10%. The solid lines are calculated from the equation $K_m(app) = K_m^{lim}(app)\{(1 + K_a^E)/[H^+]\}/\{(1 + K_a^{ES})/[H^+]\}$ using the values $pK_a^E = 9.0$, $pK_a^{ES} > 11$ for α-chymotrypsin; $pK_a^E = 9.3$, $pK_a^{ES} = 10.2$ for α_1-chymotrypsin; and $pK_a^E = 9.25$, $pK_a^{ES} = 9.75$ for δ-chymotrypsin.

buffer, pH 7.6, 0.3 M in $(NH_4)_2SO_4$. After 12 hr at 25°, the solution was made 4×10^{-4} M in diisopropyl fluorophosphate (DFP), incubated 2 hr at 25°, and dialyzed extensively against 1×10^{-4} M HCl. The protein obtained is inactive (assayed with ATME prior to the addition of DFP). It contains 1.0 mol of tyrosine/mol of protein as C-terminal[10] and 0.75 mol of threonine and 0.10 mol of alanine/mol of protein as N-termini,[11] indicating that the preparation consists mainly of II, a protein called *threo*-neochymotrypsinogen after Rovery, *et al.*[9]

Treatment of II with 5% (w/w) trypsin results in a very rapid activation, to the extent of 85–90%. The enzyme obtained after removal of trypsin and unreacted zymogen by affinity chromatography[12] contains 1.0 mol of tyrosine and 0.90 mol of leucine/mol of enzyme as C-terminal residues. N-Terminal residues were 0.80 mol of isoleucine, 0.72 mol of threonine, and 0.10 mol of alanine/mol of enzyme, indicating that the preparation consists of about 90% α_1-chymotrypsin and 10% α-chymotrypsin.

The hydrolysis of ATME was followed in a Cary-14 recording spectrophotometer as described previously.[5] K_m(app) and k_{cat} values were obtained from Eadie plots[13] of three consecutive runs at each pH. The pH dependence of K_m(app) for the α_1-chymotrypsin-catalyzed hydrolysis of ATME is presented in Figure 2 where a comparison is made with the values obtained with α- and δ-chymotrypsins. The k_{cat} values and their pH dependences were found to be the same for the three enzymes.

It can be seen that the K_m(app) values for the α_1-chymotrypsin-catalyzed reaction increase significantly less above pH 9 compared to α-chymotrypsin. The data for α_1-chymotrypsin are consistent with a dependence on a group of the enzyme with an apparent pK_a of 9.3 which shifts upon binding to 10.2. This pH dependence of K_m resembles very closely the behavior of δ-chymotrypsin.[5] Similar results have been obtained with other specific ester substrates such as *N-trans*-(2-furyl)acryloyl-L-tryptophan methyl ester and *N-trans*-(2-furyl)acryloyl-L-phenylalanine methyl ester.[14]

This result together with those reported previously on the kinetic properties of δ-chymotrypsin lead us to conclude tentatively that the ionization state of the amino group of alanine-149 is a key factor in determining the behavior of chymotrypsins at high pH. Thus, it is conceivable that the loss of the binding ability of α-chymotrypsin in the alkaline pH region is due to two, apparently unrelated, causes: (a) a major disruption or blocking of the binding site, triggered by the deprotonation of the alanine-149 amino group; (b) a minor disruptive effect caused by the deprotonation of the isoleucine-16 amino group. In the case of δ- and α_1-chymotrypsins, where the alanine-149 amino group is not free to ionize, only (b) is operative. The evidence relating the ionization state of the isoleucine-16 amino group with the decrease in k_{cat}/K_m is indirect. Furthermore much of this evidence was obtained using δ-chymotrypsin rather than α-chymotrypsin. This extrapolation is tacitly based on two questionable assumptions: (1) that the kinetic behaviors of the two enzymes at high pH are the same; (2) that the structures of both enzymes are identical, but they are not. Further work exploring the postulated involvement of alanine-149 is in progress.

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(10) Quantitative N-terminal group determinations were performed by the method of F. Sanger, *Biochem. J.*, **39**, 507 (1945). Dinitrophenyl amino acids were measured spectrophotometrically after separation by thin-layer chromatography.

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Substitutionally Labile Chromium(III)

Sir:

We have previously discovered that the porphyrin ligand labilizes Co(III) in its substitution reactions.[1] Recently, labilization of Co(III) has been found to occur in other types of complexes[2] and a similar mechanism proposed for substitution in a macrocyclic complex.[3] Our previous interpretation of the labilized cobalt was based either on an internal redox reaction[4]

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