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CONFORMATIONAL STATES OF CHYMOTRYPSIN AT HIGH pH SUBSTRATE ACTIVATION AND SIDECHAIN INTERACTIONS

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SUMMARY

A cross-over from specific to non-specific behavior occurs with amino acid substrate sidechain groups equal to or smaller than that of L-alanine, as revealed by the pH dependence of δ- and α-chymotrypsin-catalyzed hydrolysis of L-leucine and L-alanine derivatives These data suggests that the $-CH_3$ sidechain group, properly aligned, is large enough to sterically induce an active site conformation such that k_{cat} is pH independent above pH 7, in contrast to behavior observed with non-specific substrates, e g hippurates Increased catalytic efficiency is apparently induced primarily by filling the remainder of the sidechain binding site with an appropriate aromatic moiety The parameters k_{cat} and K_m were determined, plus the individual constants k_2 and k_3, separated by the technique of added nucleophiles With the alanine derivative, substrate activation was observed, and is interpreted in terms of binding to a separate interacting non-catalytic site

INTRODUCTION

Since the earliest studies of primary specificity of chymotrypsin (EC 3 4 21 1) by Niemann and coworkers [1–3], the various structural features essential to optimal catalysis by the enzyme have been the subject of intense and continued interest With virtually all types of substrates, the pH dependence of the specificity constants, (k_{cat}/K_m), is a bell-shaped curve, predicted by the equation

$$\frac{k_{cat}}{K_m} = (k_{cat}\ \bar{K}) = \frac{k_{cat}\,(\lim)}{1 + \frac{[H^+]}{K_{-1}}} \frac{\bar{K}\,(\lim)}{1 + \frac{K_{-2}}{[H^+]}} \qquad (1)$$

With the discovery of a conformational change in the enzyme at high pH [4, 5], it was found that this phenomenon involved the apolar sidechain binding region and thus mainly the K_m value [6], but could also effect catalysis (k_{cat}), depending on the nature of the substrate used With large, "specific" site-filling substrates such as *N*-acyl-L-tyrosine derivatives, the ionization of a group of pK_a (apparent) about 8 8

Abbreviations Ac-Leu-OMe, *N*-acetyl-L-leucine methyl ester, Bz-Ala-OMe, *N*-benzoyl-L-alanine methyl ester

alters mainly K_m, but with small, "non-specific" substrates such as hippurates or acetate esters, the change at high pH decreases k_{cat}, not K_m [7] Phenomenologically, the high pH conformational change induces an intramolecular competitive inhibition for specific substrates, but non-competitive inhibition for non-specific ones

This raises the question, at what size of sidechain group does a cross-over from specific to non-specific behavior occur, i e what steric size of sidechain group, upon binding, can produce a catalytically active enzyme? In this study, we report data consistent with the view that the basic juxtapositioning of catalytic groups, primarily Ser 195, in the active site can be accomplished by even the small ($-CH_3$) alanine sidechain group Also, observations on substrate activation with the alaine substrate are discussed in terms of secondary binding sites on the protein

MATERIALS AND METHODS

α-Chymotrypsins and δ-chymotrypsins were products of Worthington Biochemical Corp *N*-Acetyl-L-leucine methyl ester (Ac-Leu-OMe) was synthesized from *N*-acetyl-L-leucine (Cyclo Chemical Co) by use of diazomethane, m p 43 5–45 °C, lit m p 43–44 5 °C [8] *N*-benzoyl-L-alanine methyl ester (Bz-Ala-OMe) was obtained in a similar manner from *N*-benzoyl-L-alanine (Cyclo Chemical Co), m p 58–59 °C, lit m p 57–58 °C [2, 3]

The kinetics of hydrolysis was followed by pH stat (Radiometer) at 25 °C, both as initial velocity and as complete reaction curves Complete reaction curve analysis to obtain k_{cat} and K_m (apparent) values was accomplished by a one-run computer program kindly supplied by Dr M L Bender Kinetic parameters were calculated by a weighted least squares subprogram Each data point reported is the result of five or more experiments, each with a confidence factor of 0 95 or better

Of the various *N*-acyl-alanine esters, Bz-Ala-OMe was chosen, since its (k_{cat}/K_m) ratio is relatively high and it is thus presumed to bind primarily in a productive rather than a "wrong-way" mode, as shown by Rapp et al [9]

RESULTS AND DISCUSSION

The kinetics of chymotrypsin-catalyzed hydrolysis of leucine and alanine substrates were examined at 25 °C over the pH range of 6 0–10 0, shown in Fig 1 These data indicate that the leucine substrate (top) behaves essentially as do other "specific" substrates with α- and δ-chymotrypsins, namely that k_{cat} depends only on an ionizable group of pK about 7 in its deprotonated form, but that K_m increases ($\overline{K}$ decreases) at high pH, dependent on a group of pK about 8 8 This latter behavior is not quite as pronounced with δ-enzyme, for reasons discussed previously [2, 3, 10] Thus, "specific" behavior by chymotrypsin at high pH is not strictly limited to aromatic sidechain amino acids

With the alanine substrate (Fig 1, bottom), again k_{cat} behaves with almost purely sigmoidal pH dependence and $\overline{K}$ decreases at high pH This strongly indicates that the high pH conformational change includes in a critical way the region filled by the methyl (or methylene) side chain, and that forced binding into this region can completely revert the enzyme to a catalytically active state This is not true for substrates lacking a sidechain group, e g hippurates [7] Thus, under dynamic conditions

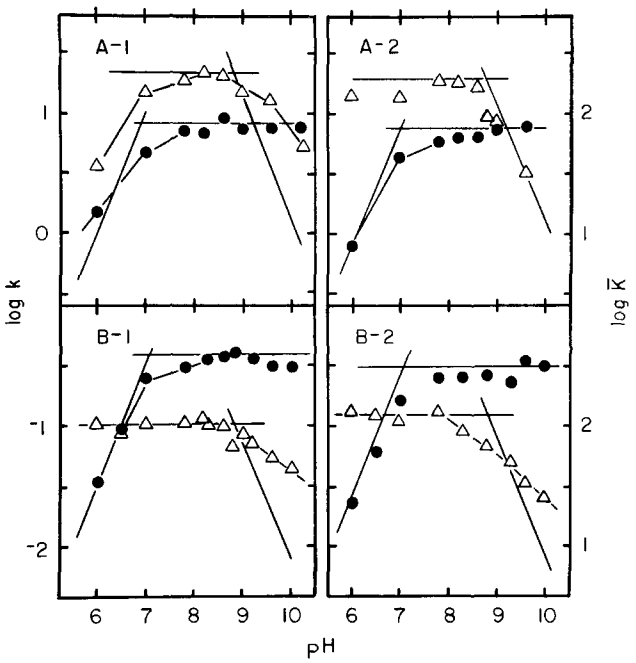

Fig 1 pH dependence of log k_{cat} (●) and log $\bar{K}_m$ (△) for the hydrolysis of *N*-acetyl-L-leucine methyl ester (A) and *N*-benzoyl-L-alanine methyl ester (B), catalyzed by δ-chymotrypsin (1) and α-chymo trypsin (2), 25 °C $I = 0.2$ M

the transition between specific and non-specific behaviour occurs with loss of the β carbon group

In terms of $\bar{K}$, because binding with α-chymotrypsin does not decrease as drastically with alanine (Fig 1B(2)) as with leucine (Fig 1A(2)), alanine or the methyl sidechain may represent the cross-over point in behavior Since k_{cat} values for the alanine substrate are obviously much smaller than those for leucine, it seems reasonable to propose that catalytic specificity at high pH is expressed in at least two separate ways (1) the β-methylene group makes k_{cat} pH independent above pH 7 8, and (2) aliphatic or aromatic groups at the γ-position cause optimal orientation of catalytic groups in the active site The first of these is associated with the salt bridge between Asp 194 and Ile 16, the second with Ser 195, and the two effects are doubtless somewhat coupled

For both substrates, the technique of added nucleophiles was used to separate and determine k_2 and k_3 values that contribute to k_{cat} [11] at 25 °C according to the equation

$$k_{cat} = \frac{k_2 k_3}{k_2 + k_3}$$

With the leucine substrate, deacylation is definitely rate limiting, or $k_2 \gg k_3$, so $k_{cat} \simeq k_3$ at pH 7 8 and 9 6 Also, with the alanine substrate, at pH 7 8 it was found that $k_2 > k_3$ for both forms of the enzyme, which is in conflict with data reported earlier [11] The explanation for differences in relative values of k_2 and k_3 may lie in

activation effects (reported below) at levels of alanine substrate above 10 mM It was also found that at pH 9 6 with the alanine substrate, $k_2 > k_3$ for α-chymotrypsin, however, but that $k_2 \simeq k_3$ for δ-chymotrypsin This suggests a pH-dependent change in rate-limiting step at high pH for the δ-enzyme

In studying the kinetic binding of the alanine substrate, it became apparent that an activation effect occurs above and below the pK_a (app) for the enzyme conformational change Fig 2 shows the biphasic nature of the kinetic binding curves with both forms of the enzyme at pH 7 8 and 9 6 The data of Figs 1B(1) and 1B(2) for k_{cat} and K were taken from extrapolated reciprocal plots of the first portion of these curves, with $[S] < 10$ mM The data of Fig 2 indicate that the K_m values from each portion

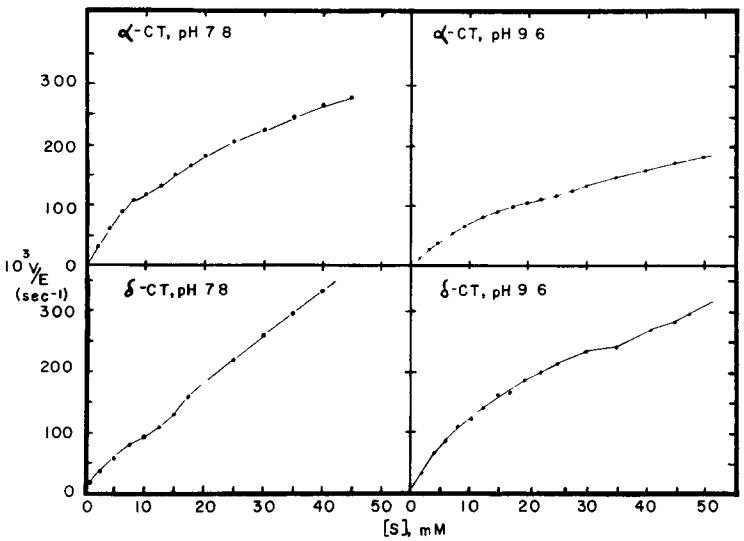

Fig 2 Substrate activation phenomena in kinetic binding curves with *N*-benzoyl-L-alanine methyl ester at pH 7 8 and 9 6 with α- and δ-chymotrypsin (α- and δ-CT), 25 °C Velocity data points were obtained as initial velocity at each initial substrate concentration, as indicated

of the curve are increased at pH 9 6 relative to those at pH 7 8 The activation above 20 mM substrate at pH 7 8 with δ-chymotrypsin is notably more pronounced than with α-chymotrypsin These data are consistent with binding of a second molecule of substrate to acyl–enzyme as in Eqn 2, where $k_3^* > k_3$

$$\begin{array}{l} E + S \overset{K_s}{\rightleftarrows} ES \xrightarrow{k_2} ES' \xrightarrow{k_3} E + P_2 \\ \qquad\qquad \searrow \\ \qquad\qquad P_1 \end{array} \qquad \begin{array}{c} \\ \uparrow\downarrow S \\ k_3^* \\ SES' \longrightarrow SE + P_2 \end{array} \qquad (2)$$

Similar behavior has been reported for certain glycine substrates [13] but not for the alanine moiety [9, 14]

The site and mode of activation binding is not certain The relatively high

(k_{cat}/K_m) value for Bz-Ala-OMe makes it unlikely that binding of the second substrate molecule results in blocking out or decreasing "wrong-way binding" modes [9] Other possibilities include (a) binding of the benzoyl group to the unfilled portion of the apolar sidechain site, or (b) binding to an apolar allosteric site, perhaps that observed by others with non-substrate activator molecules [5, 16] Subsites on the protein normally occupied by residues of a bound polypeptide adjacent to the catalytic site should be considered as well, i e the influence of "secondary specificity" sites [1, 15]

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