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INTERPRETATION OF RATE PROFILES OF THE pH-DEPENDENT TRYPSIN- AND α-CHYMOTRYPSIN-CATALYZED HYDROLYSIS OF ESTERS WITH A FREE α-AMINO GROUP

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SUMMARY

An hypothesis is postulated to interpret the anomalous pH dependence of the rate of hydrolysis of esters with a free α-amino group catalyzed by trypsin (EC 3.4.4.4) and α-chymotrypsin (EC 3.4.4.5). This involves the supposition of a hybrid system composed of $E \cdot S\alpha NH_3^+$ (I) + $E \cdot S\alpha NH_2$ (II), each of these having distinct constants for the three steps of the reaction. The optimum pH of System I, about 6–6.5, maybe accounted for in terms of an effect upon the enzyme of the α-ammonium group of the substrate species prevailing at low pH. It is possible that when near the enzyme this cationic group perturbs ionization equilibria of groups within the "charge relay system" of the active center. This effect appears as an acid shift of the rate profiles and apparently reinforces the catalytic activity: the rates observed in the acid region are faster in spite of high K_m values. Consequently the fall of rate around pH 7 observed in reactions of α-aminoacyl esters with trypsin and α-chymotrypsin, seems to be due to the conversion of substrate from the more reactive α-ammonium species to the less reactive α-amino species. The rate dependence for System II is apparently like that observed with "normal" substrates without a free α-amino group.

INTRODUCTION

The rate profiles of either the pH-dependent hydrolysis of L-lysine methyl ester and L-arginine methyl ester[1,2] catalyzed by trypsin (EC 3.4.4.4), or the pH-dependent hydrolysis of L-phenylalanine ethyl ester and L-tyrosine ethyl ester[3] catalyzed by α-chymotrypsin (EC 3.4.4.5), show maxima at pH 6–6.5. Contrariwise, reactions of these enzymes with protein and synthetic α-N-substituted substrates show optimal rates about pH 8. According to present views on the mechanism of action of trypsin and α-chymotrypsin[4,5], the two ionizable groups associated with rate profiles are: (1) an imidazole of a histidine residue with pK_a 6–7, for which an important catalytic role is invoked. In order for this function imidazole is required as the unprotonated

form, and (2) a group of pK_a 8.5–9 for α-chymotrypsin or one of pK_a approx. 10 for trypsin which must be protonated for full activity and which presumably plays a conformational role. Then, the fall of rate at the alkaline side above pH 6.5 and the occurrence of an inflection in the descending profile about pH 7, observed in reactions of the two enzymes with susceptible α-aminoacyl esters, raised the question whether or not the mechanism of action of both trypsin and α-chymotrypsin with these substrates is distinct from that which operates with "normal" substrates[1]. The present investigation was conducted in the search of a feasible interpretation of the anomalous pH dependence of rate in the aforementioned reactions.

MATERIALS AND METHODS

Trypsin (bovine) and α-chymotrypsin (bovine) were twice-crystallized salt-free preparations from Nutritional Biochemicals Corp. The titration of active centers of these enzymes with *p*-nitrophenyl acetate according to the procedure of BENDER *et al.*[6], yielded the following values: 82 ± 2% and 55 ± 2% for α-chymotrypsin and trypsin, respectively. Substrates were of Mann Research Corp. All the chemicals employed were of analytic reagent grade.

The rates of hydrolysis were measured by the method of titration to a constant pH at 25 ± 0.01° as previously described[1]. Even though this procedure may produce low rate values because of a shift of pK_a of the α-ammonium group upon hydrolysis of the substrate, it was preferred on account of its reproducibility and, what is more important, the possibility of recording the time-course since very early stages of the reaction. In order to ascertain the correct rate values, the titration method was previously calibrated by running parallel measurements under identical conditions of titration values and residual substrate concentration. The latter was determined by means of the alkaline hydroxylamine procedure of HESTRIN[7] which is not affected by pK_a shifts. As shown earlier[2], the fraction of protons liberated by hydrolysis which can be titrated is a function of H^+ concentration and the dissociation constants K_a and K_a' of the α-ammonium group of either the amino acid set free upon hydrolysis or the ester substrate, respectively:

$$\text{Fraction titrated} = 1 + \frac{1}{1 + \frac{K_a'}{[H^+]}} - \frac{1}{1 + \frac{K_a}{[H^+]}} \qquad (1)$$

A plot of the fraction titrated *versus* pH results in an inverted bell-shaped curve with a minimum at pH = (pK_a + pK_a')/2. Employing the values of p$K_a \simeq 9$ (ref. 8) and 9.2 (ref. 9) for the α-ammonium group of lysine and phenylalanine, respectively, the figures 7 and 7.2 were obtained for pK_a' of the corresponding group in L-lysine methyl ester and L-phenylalanine ethyl ester from the theoretical curves which best fitted the experimental ratios of titration to colorimetric values as a function of pH. In a previous investigation[2], the pK_a' value of 7.2 had been found for the α-ammonium group of L-lysine methyl ester upon comparison of the titration procedure with the chromatographic determination of residual substrate and product concentration in hydrolyzates. However, the conditions of the former and present work were not entirely the same. In view that the rate of trypsin- and α-chymotrypsin-catalyzed hydrolysis of esters is modified by an increase of the medium ionic

strength[10], in the present study NaCl was added to make the total ionic strength up to a constant value 0.5. This was done in order to compensate for one of the effects of increasing the concentration of ionic substrates. The value of 7.2 for pK_a' was obtained in the absence of added salt. To check further the validity of the procedure employed, the titration values were compared with those obtained with the aid of HESTRIN[7] colorimetric method in the way described above, but now in reaction media which did not contain added NaCl. The value of pK_a' calculated for the α-ammonium group of L-lysine methyl ester under these conditions was 7.2, in full agreement with that reported previously.

The initial rates v_0 were calculated from the slopes of tangents to initial portions of automatically recorded plots of titrant volume *vs.* time and then corrected dividing by the fraction titrated at the pH of the measurement (Eqn. 1). These slopes were always plotted within the first 5% of the total reaction. The units of v_0 are moles/l per sec. For the determination of k_{cat} and K_m (app) at every pH value from 6 to 8 different substrate concentrations were used and from 8 to 10 measurements run for each $[S_0]$. The concentration of L-lysine methyl ester ranged from 1 to 8 mM with 1.72 μM trypsin within the entire pH range of the present study. The concentration of α-chymotrypsin was 2.48 μM from pH 5 to 7 and 4.96 μM from pH 7.5 to 9. The concentration of L-phenylalanine ethyl ester varied from 2 to 20 mM at the pH values of faster rate and from 8 to 80 mM at those of slower rate. In all cases prior to calculation, it was checked that plots of either $1/v_0$ *vs.* $1/[S_0]$ or $[S_0]$ *vs.* $[S_0]/v_0$ yielded straight lines within the selected range of substrate concentration.

The values of catalytic rate constant ($k_{cat} = v_{max}/[E_0]$) and apparent Michaelis

TABLE I

VALUES OF CATALYTIC RATE CONSTANTS AND MICHAELIS CONSTANTS AS A FUNCTION OF pH FOR THE TRYPSIN-CATALYZED HYDROLYSIS OF L-LYSINE METHYL ESTER AND THE α-CHYMOTRYPSIN-CATALYZED HYDROLYSIS OF L-PHENYLALANINE ETHYL ESTER AT 25 ± 0.01° AND IONIC STRENGTH 0.5 (WITH NaCl)

pH	L-*Lysine methyl ester–trypsin*				L-*Phenylalanine ethyl ester–α-chymotrypsin*			
	$k_{cat} \pm$ S.D. (sec^{-1})	$K_m \pm$ S.D. (*mM*)	$\frac{k_{cat}}{K_m}$		$k_{cat} \pm$ S.D. (sec^{-1})	$K_m \pm$ S.D. (*mM*)	$\frac{k_{cat}}{K_m}$	
4.50	12.37 ± 0.51	28.94 ± 2.86	0.43		— —	— —	—	
5.00	12.42 ± 1.61	8.82 ± 2.71	1.41		10.08 ± 1.60	140.65 ± 40.25	0.07	
5.50	19.82 ± 1.65	4.50 ± 0.43	4.40		11.32 ± 0.98	54.06 ± 8.72	0.21	
5.75	— —	— —	—		11.50 ± 0.80	29.02 ± 2.25	0.40	
6.00	27.58 ± 1.14	2.45 ± 0.26	11.26		12.99 ± 0.77	15.56 ± 1.31	0.83	
6.25	— —	— —	—		14.12 ± 0.73	11.72 ± 1.31	1.20	
6.50	35.35 ± 2.35	3 05 ± 0.95	11.59		12.93 ± 0.61	11.00 ± 0.76	1.17	
6.75	— —	— —	—		11.35 ± 0.80	6.59 ± 0.69	1.72	1.43**
7.00	34.13 ± 1.95	1.86 ± 0.33	18.35		10.38 ± 1.01	14.28 ± 2.86	0.73	1.05**
7.50	21.19 ± 1.88	1.29 ± 0.34	16.43		8.48 ± 1.68	9.44 ± 2.78	0.86	1.28**
8 00	13.18 ± 0.78	0.72 ± 0.25	18.30	12.67*	5.53 ± 0.23	2.87 ± 0.21	1.93	1.39**
8.50	9.56 ± 0.89	1.68 ± 0.17	5.69	9.19*	4.93 ± 0.36	5.41 ± 0.87	0.91	1.33**
9.00	7.40 ± 0.15	0.54 ± 0.07	13.70	7.11*	3.46 ± 0.25	2.76 ± 0.67	1.25	1.27*

* Values obtained dividing k_{cat} by the weighted average of K_m when this constant levels off (*cf.* Fig. 1), that is from pH 8 to 9, $K_m = 1.04 \pm 0.20$.

** Values calculated by averaging ratios k_{cat}/K_m for individual experiments.

constant (K_m (app)), as well as their respective standard deviations, were estimated statistically with the aid of WILKINSON[11] method which takes into account the distinct variability of measurements at the various substrate concentrations. The calculations were carried out by means of an IBM digital computer Model 1130. The program was elaborated by Fortran method and data worked out with "Extended Precision".

RESULTS AND DISCUSSION

In Table I are included all the values obtained with their respective standard deviations. Figs. 1a and 1b show the variation of K_m (app) with pH for the system trypsin–L-lysine methyl ester (a) and for the one formed by α-chymotrypsin and L-phenylalanine ethyl ester (b). At the lowest pH values, K_m is very high, especially

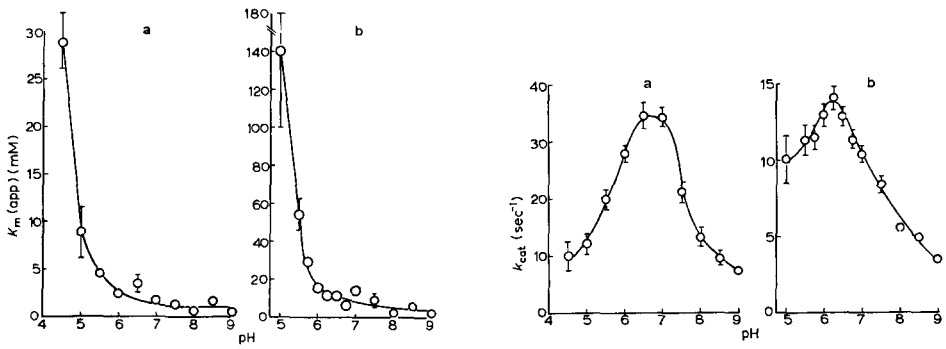


Fig. 1. Dependence of pH of apparent Michaelis constant for the trypsin-catalyzed hydrolysis of L-lysine methyl ester (a), and α-chymotrypsin-catalyzed hydrolysis of L-phenylalanine ethyl ester (b) .Temperature, 25°; ionic strength constant at all substrate concentrations by addition of NaCl to make $I = 0.5$. No buffer added. Vertical lines across points represent standard deviations when greater than the radius of the circle.

Fig. 2. Catalytic rate constant ($v_{max}/[E_0]$) as a function of pH for the trypsin-catalyzed hydrolysis of L-lysine methyl ester (a), and α-chymotrypsin-catalyzed hydrolysis of L-phenylalanine ethyl ester (b). Conditions as in Fig. 1.

with α-chymotrypsin, then decreases steeply and finally levels off from about pH 7 to 9.

Figs. 2a and 2b show the plots of k_{cat} as a function of pH for the aforementioned systems. Both of them present optima at pH values below 7 and decrease markedly at more alkaline pH's. This behavior differs, *e.g.* from that of α-chymotrypsin-catalyzed hydrolysis of methyl and ethyl *N*-acetyl L-tryptophanate which possesses a k_{cat} independent of pH from 8 to 11.6 (ref. 12).

Fig. 3 shows the plots of the ratio k_{cat}/K_m for the two enzyme systems under investigation. These are asymmetrical with inflection points of the ascending profiles at pH 5–6. The plot for the trypsin-catalyzed hydrolysis of L-lysine methyl ester reaches a maximum at pH 7 and has a descending branch with an inflection about pH 8, but that of α-chymotrypsin appears rather independent of pH from approx. pH 7 to 9. Anyhow, as can be seen in Table I, the ratio k_{cat}/K_m becomes more un-

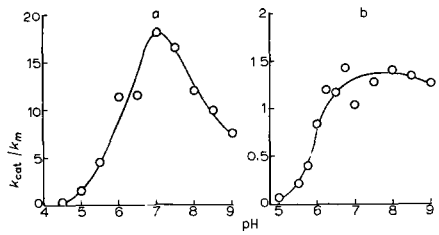


Fig. 3. Plot of the ratio k_{cat}/K_m as a function of pH for the trypsin-catalyzed hydrolysis of L-lysine methyl ester (a), and α-chymotrypsin-catalyzed hydrolysis of L-phenylalanine ethyl ester (b) at 25° and $I = 0.5$.

certain at the alkaline side of the range. It is thought that a plot of k_{cat}/K_m *versus* pH renders the dissociation constants of functional groups of the free enzyme irrespective of the number of intermediate steps of the reaction[13,14]. If substrate ionizes in the pH range under study, this ratio must vary with pH in proportion to $1/f_e \cdot f_s$ (see Eqn. 9 below), in which f_e and f_s are the Michaelis pH functions of the free enzyme and substrate, respectively. Hence, in order to ascertain pK_a's of groups involved in f_e, the ratio k_{cat}/K_m must be multiplied by f_s. This can be done in two ways: (a) considering f_s as that of the α-ionized form, or (b) taking f_s as that of the uncharged form. In the first case, the ascending line of the profiles remains practically unaltered because $f_s \simeq 1$ within this pH range. Above pH 6.5 f_s increases rapidly so that the profile of the product *vs.* pH is converted to a sigmoid curve. In the second case the values which do not change sensibly upon multiplication by f_s, are those in the range of pH 7.5 or 8 to 9, while those of the acid side increase up to a limit value, in such a way that the resulting curve is again a sigmoid one but in the descending direction. Thus, it can be considered that the ascending portion of profiles k_{cat}/K_m of Fig. 3 represent the behavior of the enzyme in the presence of ionized substrate and that points within the alkaline side would indicate the behavior with the uncharged substrate.

The pH dependence of the reactions we are dealing with is distinct from that observed in trypsin- and α-chymotrypsin-catalyzed reactions involving α-N-acylated substrates. For instance, BENDER *et al.*[14] report that chymotrypsin reactions which follow Eqn. 2 show pH dependence of k_2/K_s (or the equivalent k_{cat}/K_m) like a bell-shaped curve with pK_a values of 6.8 ± 0.3 and 8.9 ± 0.3.

$$E + S \underset{}{\overset{K_s}{\rightleftarrows}} ES \xrightarrow{k_2} ES' \xrightarrow{k_3} E + P_2 \qquad (2)$$
$$\downarrow$$
$$P_1$$

The ionizable group of pK_a of 6.8 is usually identified as the imidazole group of histidine 57 of chymotrypsin[4,15], while that of pK_a 8.9 has been assigned to the α-ammonium group of the N-terminal isoleucine residue[16]. Histidine has also been involved in trypsin action by dependence of the reaction rate upon a basic group of pK_a 6–7 (ref. 17) and specific inhibition of this enzyme by 5-L-amino 1-tosyl amino pentyl chloromethyl ketone[18]. In addition to this, from the effect of pH on K_m (app) and k_{cat} of tryptic hydrolysis of α-*N*-benzoyl-L-arginine amide, WANG AND CARPEN-

TER[19] deduced that the enzyme possessed an ionizable group with a pK_a of 10.4 which must be protonated in order to have a fully active enzyme.

It had been assumed earlier that the acylation step (k_2) depended on the two ionizable groups which appear in the rate profiles of chymotrypsin and trypsin, and that deacylation (k_3) depended only on the group of pK_a approx. 7, namely imidazole[4,14]. The group of alkaline pK_a in chymotrypsin was thought to control a conformational change of the enzyme in the k_2 step. Upon further investigations involving kinetic and conformational studies, the conclusion was reached that this group of chymotrypsin[12,20], or that of pK_a 10.4 of trypsin[19], does play a conformational role not in the k_2 step but in substrate binding. It has been suggested that the native enzyme exists in two forms, only one of which is capable to bind substrate, and that a rapid equilibrium is established between them which is controlled by the ionization of the already mentioned group[12]. By X-ray diffraction studies, MATTHEWS *et al.*[21] have found that this α-amino group of terminal isoleucine in chymotrypsin forms an internal ion pair with aspartic acid 194, which is adjacent to the active serine 195. These authors suggest that the maintenance of such ion pair is essential for catalytic activity and substrate binding. At present it seems to prevail the opinion that the same catalytic group, unprotonated imidazole, is operative in both acylation and deacylation steps, which is more in accordance with the principle of microscopic reversibility. By means of electron-density maps of α-chymotrypsin which have thrown light on the stereochemistry of the active center, it has been possible to postulate a "charge relay system" constituted by histidine 57 hydrogen bonded to serine 195 on the one hand, and to aspartic acid 102, on the other. This system affords a mechanism of greatly enhanced transfer of protons from serine to histidine in acylation or *vice versa* in deacylation[22].

After various attempts to conciliate the results presented in this paper with the currently accepted ideas about the mechanism of action of trypsin and chymotrypsin, a working hypothesis was elaborated which seems to account for the apparently anomalous pH dependence of the reactions of these enzymes with substrates having a free α-amino group. The lack of symmetry of the pH profiles of k_{cat}/K_m suggest that these do not represent a unique system but rather a hybrid one. Within the pH range of the measurements, the population of substrate molecules is composed of two distinct species which differ in the presence or absence of a positive charge in α-position:

(I) $H_3N^+-(CH_2)_4-CH(NH_3^+)-COOCH_3$ or $C_6H_5-CH_2-CH(NH_3^+)-COOC_2H_5$

(II) $H_3N^+-(CH_2)_4-CH(NH_2)-COOCH_3$ or $C_6H_5-CH_2-CH(NH_2)-COOC_2H_5$

The relative proportion of each form varies with pH from almost pure I at pH 4.5 or 5 to nearly pure II at pH 9. The K_m values of Fig. 1 would indicate the distinct affinity of the enzyme for the two species of substrate. If we assume that these are hydrolyzed independently by the enzyme and that the values of K_s, k_2 and k_3 are different for the two substrates, the observed rate of hydrolysis would be:

$$v = \left(\frac{k_{cat\,I}\,[S_I]}{[S_I] + K_{m\,I}} + \frac{k_{cat\,II}\,[S_{II}]}{[S_{II}] + K_{m\,II}}\right)[E_0] \qquad (3)$$

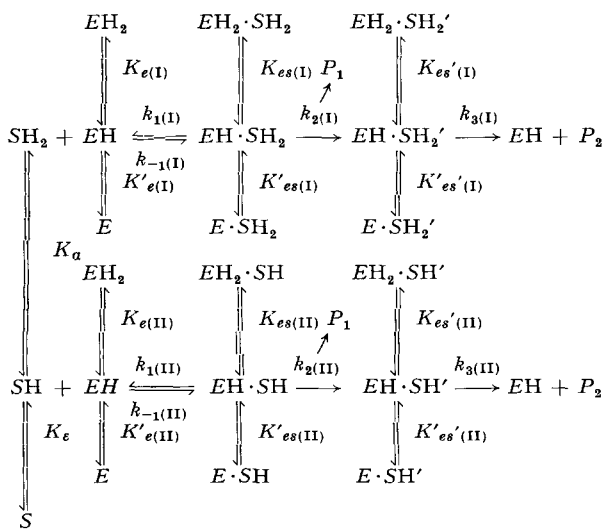
The equivalence of the respective k_{cat} and K_m (app) values for Systems I and II in terms of K_s, k_2 and k_3 would be[23]:

$$k_{cat\ I} = \frac{k_{2(I)}k_{3(I)}}{k_{2(I)} + k_{3(I)}} \qquad k_{cat\ II} = \frac{k_{2(II)}k_{3(II)}}{k_{2(II)} + k_{3(II)}} \tag{4}$$

$$K_{m\ I} = \frac{K_{s(I)}k_{3(I)}}{k_{2(I)} + k_{3(I)}} \qquad K_{m\ II} = \frac{K_{s(II)}k_{3(II)}}{k_{2(II)} + k_{3(II)}} \tag{5}$$

In relation to pH dependence of the various constants included it can be noted in Fig. 3 that an apparent shift of pK_a occurs from the value 6–7 usually observed with α-*N*-acyl substrates to 5–6. Even though pK_a values obtained from rate profiles should be taken cautiously, especially at the light of the finding of the "charge relay system"[22], it is possible to suggest, on a comparative basis, that the positive charge in α-position of substrate perturbs the ionization equilibrium of imidazole within the hydrogen-bonded system. In support of this idea we may refer that pK_a of imidazole group in free histidine is 1.5 units lower than that of methyl imidazole "presumably owing primarily to the effect of the charged ammonium group"[24]. The effect of a near positive charge resulting in increased acidity of imidazole can be observed also in histidylhistidine or histidylglycine where the imidazole pK_a is 5.6 or 5.7, respectively[25,26].

If the trypsin-catalyzed hydrolysis of L-lysine methyl ester is formulated according to the three-step mechanism involving the formation of acyl-enzyme intermediate, we may represent our hypothesis by means of the following scheme:



(6)

In Scheme 6, *E* stands for enzyme, *S* for substrate, and H for hydrogen ion. The constants k_1, k_2, *etc.* are the rate constants for each of the corresponding steps of the reaction; K_e, K'_e, K_{es}, K'_{es} $K_{es'}$ and $K'_{es'}$ are the first and second dissociation constants for the free enzyme, Michaelis complex and acyl-enzyme, respectively, *e.g.*

$$K_e = \frac{[EH]\,[H]}{[EH_2]} \qquad K'_e = \frac{[E][H]}{[EH]},\ etc.$$

In the same way K_a and K_ε represent the two dissociation constants for α- and ε-ammonium groups of L-lysine methyl ester. It is assumed that only the two protonated forms SH and SH_2 carrying a positive charge in ε-position are productive in the enzyme reaction. An identical scheme, except as far as the ionization of substrate is concerned, would represent the reaction of α-chymotrypsin and L-phenylalanine ethyl ester. For this, only K_a and the two forms of substrate S and SH would be involved.

With the aid of the usual approximation of steady state the following relationships may be obtained:

$$v_{0(I)} = \frac{\dfrac{k_{2(I)}k_{3(I)}}{k_{2(I)}fes'_{(I)} + k_{3(I)}fes_{(I)}}[E_0]\,[S_0]}{\left(\dfrac{k_{-1(I)} + k_{2(I)}}{k_{1(I)}}\right)\left(\dfrac{k_{3(I)}}{k_{2(I)}fes'_{(I)} + k_{3(I)}fes_{(I)}}\right)fe_{(I)}fs_{(I)} + [S_0]} \tag{7}$$

$$v_{0(II)} = \frac{\dfrac{k_{2(II)}k_{3(II)}}{k_{2(II)}fes'_{(II)} + k_{3(II)}fes_{(II)}}[E_0]\,[S_0]}{\left(\dfrac{k_{-1(II)} + k_{2(II)}}{k_{1(II)}}\right)\left(\dfrac{k_{3(II)}}{k_{2(II)}fes'_{(II)} + k_{3(II)}fes_{(II)}}\right)fe_{(II)}fs_{(II)} + [S_0]} \tag{8}$$

and

$$v_0\ (\text{total}) = v_0(I) + v_0(II) \tag{9}$$

in which f_e, f_{es}, and $f_{es'}$ are the Michaelis pH functions of the enzyme, ES complex and acyl-enzyme intermediate compound (ES') respectively, *e.g.*

$$f_s = 1 + \frac{[H^+]}{K_e} + \frac{K_e'}{[H^+]}$$

and the like.

The Michaelis pH functions of substrate L-lysine methyl ester are:

$$f_{s(I)} = 1 + \frac{K_a}{[H^+]} + \frac{K_aK_\varepsilon}{[H^+]^2} = 1 + \frac{10^{-7}}{[H^+]} + \frac{10^{-17.3}}{[H^+]^2}$$

and

$$f_{s(II)} = 1 + \frac{[H^+]}{K_a} + \frac{K_\varepsilon}{[H^+]} = 1 + \frac{[H^+]}{10^{-7}} + \frac{10^{-10.3}}{[H^+]}$$

in which pK_a of ε-ammonium group was taken as 10.3, value estimated by the following reasoning: the pK_a of ε-ammonium group is 10.75 in ε-amino caproic acid and 10.53 in the respective ethyl ester[27]. Assuming a similar shift upon esterification of lysine, the pK_a of ε-ammonium would change of 10.53 (ref. 8) to approx. 10.3. Anyhow, the exact value of this pK_a is not essential because either term $K_\varepsilon/[H^+]$ or $K_aK_\varepsilon/[H^+]^2$ of $f_{s(II)}$ or $f_{s(I)}$ are quite small even at pH 9.

The pH functions of L-phenylalanine ethyl ester are:

$$f_{e(I)} = 1 + \frac{K_a}{[H^+]} \quad \text{and} \quad f_{e(II)} = 1 + \frac{[H^+]}{K_a} = \frac{[H^+]}{10^{-7.2}} + 1$$

We ignore the rate constants of the individual steps k_1, k_{-1}, *etc.*, but if for the sake of simplicity we assume that pK_a values of functional groups are approximately the same in ES and ES', namely that $f_{es} \simeq f_{es}'$, each of the previous Eqns. 7 or 8 can be reduced to:

$$v_0 = \frac{\left(\frac{1}{f_{es}}\right)\left(\frac{k_2 k_3}{k_2 + k_3}\right)[E_0]\,[S_0]}{\left(\frac{k_{-1} + k_2}{k_1}\right)\left(\frac{k_3}{k_2 + k_3}\right)\left(\frac{f_e \cdot f_s}{f_{es}}\right) + [S_0]}$$

or, for the total rate in terms of K_m (app) and k_{cat}:

$$\frac{v_0}{[E_0]} = [S_0]\left\{\frac{\frac{k^\circ_{cat(I)}}{f_{es(I)}}}{K^\circ_{m(I)}\frac{f_e \cdot f_s}{f_{es}}_{(I)} + [S_0]} + \frac{\frac{k^\circ_{cat(II)}}{f_{es(II)}}}{K^\circ_{m(II)}\frac{f_e \cdot f_s}{f_{es}}_{(II)} + [S_0]}\right\} \qquad (10)$$

In Eqn. 10 $k^\circ_{cat(I)}$ and $k^\circ_{cat(II)}$ stand for limiting values of the catalytic rate constant for either System I or II, while $K^\circ_{m(I)}$ and $K^\circ_{m(II)}$ represent limiting values of K_m for the respective system. Since at extreme pH values of the range studied, the proportion of either one species of substrate is nearly 100%, k_{cat} and K_m determined at the lowest pH were assigned to System I, and those calculated at pH 9 were attributed to System II. From these data various theoretical curves were calculated assuming different values of pK_e, pK'_e, pK_{es} and pK'_{es} for each system. In all of them the supposition was made that pK_a values of System I were about 1 unit less than the corresponding ones of System II.

Fig. 4a shows some of the theoretical curves computed for $v_0/[E_0]$ as a function of pH, both for individual systems ES_I and ES_{II} (broken lines) and overall rate (smooth lines). All of these were calculated for $[E_0] = 1.72$ μM and $[S_0] = 8$ mM. Curves a_1, a_2 and a_3 resulted from the assumption that $f_e = f_{es} = f_{es}'$ with pK_a values of 5.5, 5.6 and 5.7, respectively, for the first ionizable group, and 10.4 for the second group in any of the three cases. Curves b_1 and b_2 were calculated assigning pK and pK' values of 7 and 10.4, respectively, to System II. The difference between them lies in the values of K_m and k_{cat} utilized for the computation: b_1 from those obtained at pH 9 and b_2 from the values determined at pH 4.5. No significant difference was observed whether curves of System I were calculated from data of pH 4.5 or 5.

Fig. 4b shows the theoretical curve which best fitted experimental values $v_0/[E_0]$ *vs.* pH for the conditions above stated. This is the one computed attributing the following values to system ES_I: pK_e = 5.6, pK_{es} = pK_{es}' = 5.5, and pK'_e = pK'_{es} = pK'_{es}' = 10.4. For the ES_{II} system, Curve b is the average of Curves b_1 and b_2 of Fig. 4a. When instead of average values of k_{cat} and K_m, the statistical upper and lower limits were utilized for the computation, the pK_a values required for best fit of curves were the following: pK_e of 5.65 for the combination yielding the lower rate (upper K_m and lower k_{cat}), and of 5.52 for the one resulting in the higher velocity (smaller K_m and higher k_{cat}). Similar results were attained starting from the supposition that $f_e = f_{es} = f_{es}'$ with pK of 5.55 ± 0.07 and pK' of 10.4 ± 0.1.

Similarly, Fig. 5a presents a selection of theoretical curves computed for the α-chymotrypsin-catalyzed hydrolysis of L-phenylalanine ethyl ester for values of $[E_0]$ and $[S_0]$ of 2.48 μM and 12.5 mM, respectively. In Curve a_1 the values 5.6 and 9 were

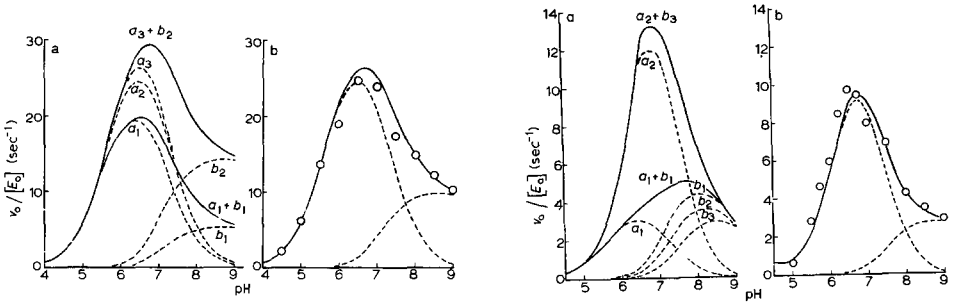


Fig. 4. a. Some theoretical rate profiles of the pH-dependent trypsin-catalyzed hydrolysis of L-lysine methyl ester calculated with the aid of Eqn. 10 in this way: a_1 through a_3 from experimental k_{cat} and K_m values at pH 4.5 assuming that the Michaelis pH functions of the free enzyme, ES complex and acyl-enzyme ES' are equal, *i.e.* $f_e = f_{es} = f_{es}'$. The values assigned to the two ionizable groups involved were: a_1, $pK = 5.5$ and $pK' = 10.4$; a_2, $pK = 5.6$ and $pK' = 10.4$; a_3, $pK = 5.7$ and $pK' = 10.4$. Curve b_1, $f_e = f_{es} = f_{es}'$, the three functions of $pK = 7$ and $pK' = 10.4$. Values computed from k_{cat} and K_m (app) obtained at pH 9. Curve b_2, the same as in Curve b_1 but from experimental values determined at pH 8.5. All the calculations were done considering $[E_0] = 1.72$ μM and $[S_0] = 8$ mM. b. Curve a was computed from experimental k_{cat} and K_m at pH 4.5. In order for best fit it was necessary to ascribe the following values to pK_a values of functional groups: $pK_e = 5.6 \pm 0.07$, $pK_e' = 10.4 \pm 0.1$, $pK_{es} = pK_{es}' = 5.5 \pm 0.07$; and $pK_e' = pK'_{es} = pK'_{es}' = 10.4 \pm 0.1$. For Curve b, the average values of b_1 and b_2 were taken. Circles are experimental rates determined for $[E_0] = 1.72$ μM and $[S_0] = 8$ mM. Ionic strength = 0.5.

Fig. 5. a. Selected theoretical curves of rate *versus* pH computed for the α-chymotrypsin-catalyzed hydrolysis of L-phenylalanine ethyl ester from Eqn. 10 for $[S_0] = 12.5$ mM and $[E_0] = 2.48$ μM. Curve a_1 was calculated from experimental k_{cat} and K_m (app) at pH 5, assigning pK_a values of 5.6 and 9 to the groups involved in the Michaelis pH functions of the enzyme f_e, and pK_a values of 5.5 and 9 to those belonging to functions of ES complex and acyl-enzyme ES', f_{es} and f_{es}'. Curve a_2 was computed from the same values as a_1 but with the following pK_a values: 6.5 and 8.8 for f_e; and 6 and 8.8 for f_{es} and f_{es}'. Curves b were calculated from experimental k_{cat} at pH 9 and weighted average of K_m at pH 8, 8.5 and 9 when this became sensibly constant (3.24 ± 0.40): for b_1 it was assumed that $f_e = f_{es} = f_{es}'$, the three functions of pK 7 and pK' 9; for b_2 the same equality of functions was supposed but the pK_a values considered were 7.5 and 9.2. In the case of b_3, the pK_a values assigned to the groups in the three Michaelis functions were 7.5 and 9.5. b. Experimental rate values (circles) of rate of the α-chymotrypsin-catalyzed hydrolysis of L-phenylalanine ethyl ester for the above stated conditions and theoretical curve (smooth line) which best fitted them. For this line the values required for pK_e and pK_e' of System I (broken line, a) were 6.3 ± 0.3 and 8.7 ± 0.2, respectively. For pK and pK' of either f_{es} or f_{es}' of System I, the values 6.0 ± 0.3 and 8.7 ± 0.2 were found to fit the curve. The rate profile of System II (broken line, b) was computed in terms of pK_a values of 7.5 ± 0.2 and 10 ± 0.2 for each of the Michaelis functions f_e, f_{es} and f_{es}'.

given to the pK_a values of groups involved in f_e, and 5.5 and 9 to those of f_{es} and f_{es}'. In the case of Curve a_2, it was considered that 6.5 and 8.8 were the pK_a values of f_e, and those of f_{es} and f_{es}' of 6 and 8.8. The following assumptions were made for the plot of curves of System II (α-chymotrypsin neutral form of L-phenylalanine ethyl ester): $f_e = f_{es} = f_{es}'$, all of them functions of ionizable groups of pK_a values 7 and 9; b_2 the same as b_1 with pK_a values of 7.5 and 9.2; and b_3 also with the three functions of equal pK_a values, 7.5 and 9.5.

Fig. 5b shows the theoretical curve which was nearer the experimental points of $v_0/[E_0]$ for the α-chymotrypsin-catalyzed hydrolysis of L-phenylalanine ethyl ester. For this graph, the values required for pK_e and pK_e' of System I were 6.3 ± 0.3 and 8.7 ± 0.2, respectively. The pK and pK' values considered for Michaelis functions of ES and ES' of System I were 6.0 ± 0.3 and 8.7 ± 0.2. Another way of getting a

good fit was taking $f_e = f_{es} = f_{es}'$ with pK_a values of 6.25 ± 0.3 and 8.7 ± 0.2. The line which represents the pH dependence of System II was computed in terms of pK_a values of 7.5 ± 0.2 and 10 ± 0.2 for each f_e, f_{es} and f_{es}'.

The idea that different substrates may affect in a distinct manner the ionization of functional groups of the enzyme is not new. ALBERTY *et al.*[28] reported that the pK_a of the first ionizable group of fumarase saturated with malate is about 7.3, while under the same conditions the corresponding value in the presence of fumarate is 5.8. These authors also observed a similar shift of pK_a of the second ionizable group from 8.5 with malate to 7.3–7.7 with fumarate.

Summing up, the hypothesis proposed to account for the apparently abnormal rate profiles of the pH-dependent trypsin- and α-chymotrypsin-catalyzed hydrolysis of α-aminoacyl esters is the following:

(1) The positive charge in α-position may influence the "charge relay system" of the active center of these enzymes in some way leading to an apparent shift of the ionization equilibrium of imidazole which results in a greater acidity and enhanced catalytic activity. In addition to the assumed effect on imidazole, the proximity of a cationic group may also increase the reactivity of serine in enzymes[29]. Obviously with these substrates the reactivity is greater when they exist as the α-cationic species. In spite of the low affinity, the maximum rates occur at the acid side of the pH range.

(2) As pH increases, the proportion of α-ammonium species of substrates decreases logarithmically, so that the system is progressively converted from ES_I to ES_{II}, the latter having a greater affinity (lower K_m) but less reactivity (smaller k_{cat}). Thus, the descending profiles with apparent inflection about pH 7 may be explained in terms of increased dissociation of the substrate α-ammonium group.

(3) In a previous work of this laboratory[2], about the nucleophilic competition of water and ethanol in the reaction of trypsin and L-lysine methyl ester, it was observed a linear relationship between log k_4/k_3 and log [α-cationic substrate]/[α-neutral substrate], k_4 and k_3 being the respective deacylation rate constants with either ethanol or water as the acceptor of the acyl group. This relationship may be better understood on the basis of the double enzyme–substrate system $ES_I + ES_{II}$ proposed in this paper.

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