THE SIGNIFICANCE OF AN UNIQUE pH-PROFILE FOR TRYPSIN CATALYSIS*

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Summary--The pH-profiles for the trypsin-catalyzed hydrolysis of non-N-acylated α-amino substrates were found to be complex. The profile for L-lysine methyl ester gave two rate constants and four acid-base equilibrium constants. The rate constants arise from two different ionic forms (structures) of the site which are reactive. The pKa values calculated from the profile are indicative of an ionizable amino, imidazoyl and carboxyl group at the site, and the free α-amino group in the substrate. A negative heat of ionization was obtained for the group with the smallest pKa, which is kinetic evidence for the existence of a free carboxyl at or near the site.

The pH-optima of the rate-controlling step for trypsin systems varies somewhat with different substrates, but it is usually reported to be near 8 (1). A notable exception to this is the pH-optimum at 5.8 for L-arginine methyl ester as reported by Goldenberg and Goldenberg (2). Work by others (3,4) and in this laboratory with substrates containing a free α-amino group confirm this result. To date, the significance of this shift in pH-optimum for substrates with a free α-amino group has been ignored, and it is not too easy to reconcile this result with proposed mechanisms (5). The purpose of this communication is to present the experimental pH-profile for the L-lysine methyl ester (LME) -trypsin system and to illustrate how a general mechanism based on acid-base equilibrium theory can be used to analyze the result.

Materials- Trypsin 2X crystallized and L-lysine methyl ester (LME) dihydrochloride were purchased from Sigma Chemical Company. Deionized,

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boiled, distilled water was employed throughout and all chemicals used were the best reagent grade available.

Kinetic Measurements- The steady state kinetics of trypsin with LME were studied by automatic titration. The equipment and procedure used have been described previously (6). Since the pKa for the α-amino group of an amino acid is 2-3 units greater than the same pKa of the ester, automatic titration methods could not be used effectively beyond pH 6.5. Thus, the hydroxamic acid method (7) for the determination of esters was employed. This was done removing aliquots from the reaction mixture at given times and treating them with hydroxylamine-ferric chloride reagent. Standards were prepared using known amounts of LME and the pH of the reaction mixture was held constant by automatic titration. The final concentration of trypsin was held constant at 2×10^{-7} M, and the final concentrations of substrate ranged from 2 to 0.1×10^{-2} M.

Theoretical - A general scheme for ionizable systems of the trypsin-LME type is illustrated in Figure 1. The nomenclature used is given in the caption and has been described previously (8). The corresponding equation is:

$$k_i' = (k_{i21} + k_{i31}\, a_H/Ka_{i\underline{3}1} + k_{i11}\, Ka_{i\underline{2}1}/a_H + k_{i10}\, Ka_{i1\underline{1}}\, Ka_{i\underline{2}1}/a_H^2 + k_{i00}\, Ka_{i10}\, Ka_{i1\underline{1}}\, Ka_{i\underline{2}1}/a_H^3) \Big/ (1 + a_H/Ka_{i\underline{3}1} + Ka_{i\underline{2}1}/a_H + Ka_{i1\underline{1}}\, Ka_{i\underline{2}1}/a_H^2 + Ka_i10\, Ka_{i1\underline{1}}\, Ka_{i\underline{2}1}/a_H) \quad (1)$$

This expression is valid for any elementary stage i, where i = 1, 2, 3, etc. If k_i' is a steady state rate constant, it can be obtained from:

$$\text{rate} = k_i'\, E^o S^o / (K_m' + S^o) \quad (2)$$

as outlined before (6). Under saturated conditions this equation becomes:

$$\text{rate} = k_i'\, E^o. \quad (3)$$

The heat of ionization is given by (9):

$$\ln Ka = -\Delta H_{ion}/RT + \text{constant}. \quad (4)$$

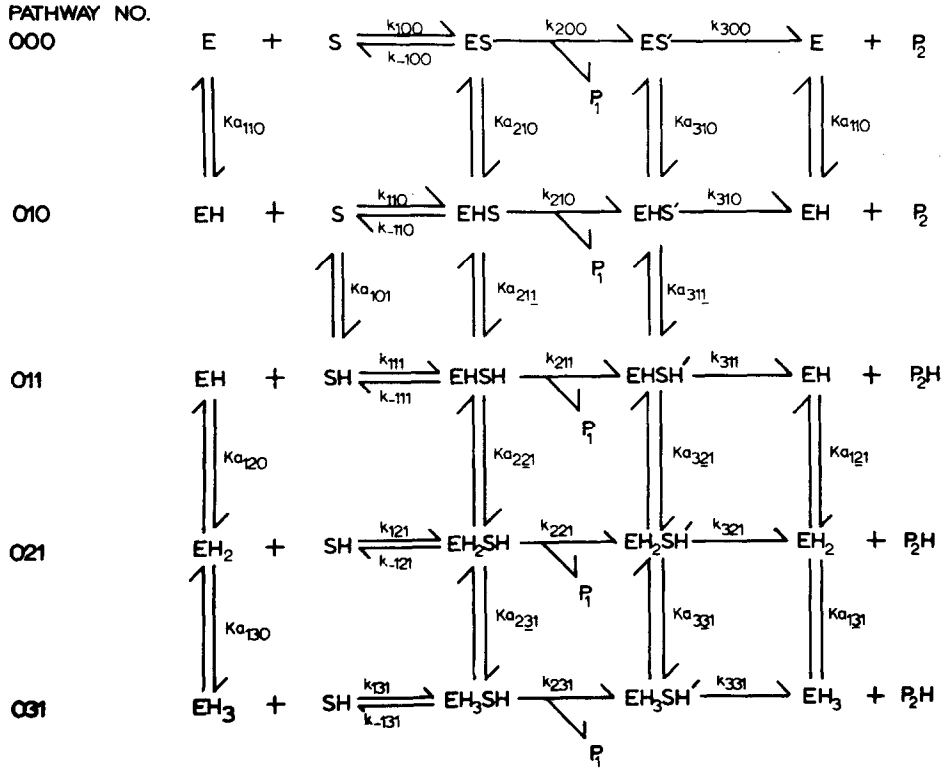

Figure 1. A general three-stage mechanism for the enzyme-catalyzed hydrolysis of an ionizable substrate. E, EH, EH_2, and EH_3 are the different protonated forms of the enzyme site; S and SH represent dissociated and associated substrate; ES, EHS, EH_2S, EH_3S, and ESH, EHSH, EH_2SH and EH_3SH are the different protonated forms of the enzyme-substrate complex, with and without protonated substrate, respectively; ES', EHS', EH_2S', EH_3S', ESH', EHSH', EH_2SH', and EH_3SH' are the different protonated forms of the acylated or alkoxylated, etc., enzyme intermediates; and P_1, P_2 and P_2H are the products of hydrolysis, where H is a functional proton. The specific rate constants k_{000} are coded by the use of three-digit subscripts; the first digit represents the stage of catalysis, the second digit the number of ionizable protons concerned with the active site of the enzyme, and the third digit represents the number of ionizable protons on the substrate which enter into the catalytic mechanism. The acid-base equilibrium constants Ka_{000} are also identified by three-digit subscripts; the first digit indicates the stage of catalysis, the second digit the number of ionizable protons on the site in its associated form, and the third digit the number of ionizable protons on the substrate in its associated form. The equilibrium constant's subscript is barred, when necessary, to designate whether it is the enzyme or the substrate dissociating. Thus for example, $Ka_{12\bar{1}}$ refers to the substrate and $Ka_{1\bar{2}1}$ the enzyme.

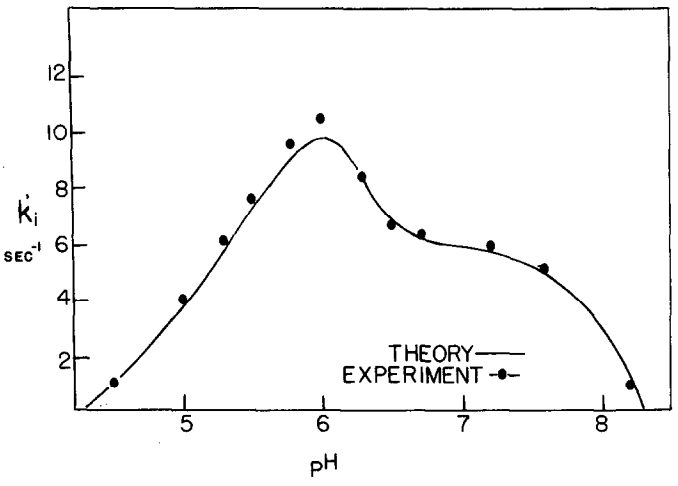

Figure 2. A plot of k_i' vs pH for the trypsin- L-lysine methyl ester (LME) system. The solid points are experimental and were calculated using equation 3. The solid curve is theoretical and was obtained by substituting the constants in Table I into equation 5.

Table I

True Rate Constants and Equilibrium Constants for LME-Trypsin System as Calculated from Equation 5.

Constant	Value	Classification
$k_{i21}(sec^{-1})$	13.50	rate-controlling at low pH
$k_{i10}(sec^{-1})$	6.65	rate-controlling at high pH
$pKa_{i\underline{3}1}$	5.42	carboxyl group
$pKa_{i\underline{2}1}$	6.57	imidazoyl group
pKa_{i10}	8.21	amino group
$pKa_{i\underline{1}1}$	7.60	α-amino substrate
pKa(LME)	7.10	actual value for α-amino

Results- The pH-profile for LME is shown in Figure 2. Table I contains the constants obtained from a theoretical 'fit' of equation 1. This was accomplished by a comparison of the experimental with hypothetical profiles as described for non-ionizable substrates (8). Equation 5 describes the profile in Figure 2,

$$k_i' = (k_{i21} + k_{i10}\, Ka_{i\underline{1}1}\, Ka_{i\underline{2}1}/a_H^2)/(1 + a_H/Ka_{i\underline{3}1} + Ka_{i\underline{2}1}/a_H + Ka_{i\underline{1}1}\, Ka_{i\underline{2}1}/a_H^2 + Ka_{i10}\, Ka_{i\underline{1}1}\, Ka_{i\underline{2}1}/a_H^3) \quad (5)$$

and the corresponding mechanism is:

$$\begin{array}{ll} & (E + S)i \\ Ka_{i10} & \uparrow\downarrow \\ & (EH + S)i \xrightarrow{k_{i10}} \\ Ka_{i11} & \uparrow\downarrow \\ & (EH + SH)i \\ Ka_{i21} & \uparrow\downarrow \\ & (EH_2 + SH)i \xrightarrow{k_{i21}} \\ Ka_{i31} & \uparrow\downarrow \\ & (EH_3 + SH)i \end{array} \qquad (6)$$

TABLE II

The pH and Temperature Dependent Constants from Eadie Plots of Equation 2

pH	k_i' (sec^{-1})	K'm x 10^2 (M.)
	Temperature 12.50°C.	
4.74	3.22 ± .58	5.28 ± .93
4.83	3.56 ± .51	4.57 ± .63
5.01	3.65 ± .41	2.82 ± .29
5.11	4.21 ± .39	2.46 ± .21
5.21	4.86 ± .32	2.23 ± .14
	Temperature 15.0°C.	
4.49	2.51 ± .43	5.48 ± .90
4.58	2.95 ± .23	4.71 ± .35
4.68	3.10 ± .41	3.72 ± .47
4.81	4.22 ± .15	3.42 ± .12
4.99	4.31 ± .39	1.96 ± .16
5.09	5.42 ± .41	2.21 ± .15
5.18	5.32 ± .89	1.32 ± .19
	Temperature 20.0°C.	
4.62	4.37 ± .94	4.39 ± .90
4.79	5.40 ± .34	3.22 ± .19
4.96	6.67 ± .62	2.65 ± .23
5.11	7.49 ± .45	1.18 ± .06
5.20	8.29 ± .39	1.71 ± .07
	Temperature 25.0°C.	
4.58	4.96 ± .27	4.65 ± .25
4.68	6.11 ± .39	4.41 ± .27
4.81	7.31 ± .45	3.27 ± .19
4.91	8.84 ± .52	3.08 ± .17
5.07	8.66 ± .58	1.82 ± .11
5.21	10.90 ± .73	1.75 ± .10
	Temperature 27.50°C.	
4.57	5.80 ± .61	5.05 ± .51
4.69	7.85 ± .49	4.42 ± .26
4.82	8.40 ± .24	3.06 ± .08
4.99	11.94 ± 1.42	2.91 ± .32
5.10	11.26 ± .72	1.97 ± .11
5.19	14.36 ± 1.82	2.30 ± .27

In order to determine whether or not $pKa_{i\underline{3}1}$ arises from a carboxyl group, its heat of ionization was calculated using equation 4 and 7. In acidic media equation 5 reduces to:

$$k_i' = k_{i21}/(1 + a_H/Ka_{i\underline{3}1}). \quad (7)$$

Several duplicate runs were made at each pH listed in Table II employing a range of substrate concentrations and temperatures. An appropriate computer program was written and the experimental rates were analyzed according to the method of Eadie(10) using a least squares regression. The results given in Table II were substituted into equation 7 and values for k_{i21} and $pKa_{i\underline{3}1}$ were found at several temperatures. This data and the heat of ionization of $pKa_{i\underline{3}1}$ calculated from equation 4 are shown in Table III.

TABLE III

The pH Independent k_{i21} and Ionization Constants from Equation 7.

Temp. °C.	$k_i(sec^{-1})$	pKa	$\Delta H_{ion}(kcal\ M^{-1})$
12.5	5.66 ± 0.69	4.63	
15.0	7.71 ± 0.80	4.80	
20.0	11.85 ± 0.33	4.86	-8.8 ± 1.6
25.0	16.61 ± 2.32	4.93	
27.5	23.24 ± 4.25	5.03	

Discussion- When one examines the results in Table I, it becomes evident that trypsin is capable of hydrolyzing LME by two kinetic pathways as illustrated by mechanism 6. This is a reasonable finding, since both the enzyme and the substrate contain ionic groups. In essence they become new 'chemicals' when they change ionic form through the loss or gain of a proton. If these ionic groups are not catalytic, then changes in ionic form may hinder but not necessarily prevent catalysis. Two of the pKa's calculated from the pH-profile (Figure 2) can readily be assigned to ionic groups associated with tryptic activity. $pKa_{i\underline{2}1}$ and pKa_{i10} correspond to an imidazoyl and α-amino group, respectively, and they have been

assigned previously to His-46 and Ile-7 (11). But neither pKa_{i2l} or pKa_{i3l} have appeared before in the rate-controlling step of catalysis. The value 7.6 found for pKa_{i2l} is in agreement with 7.1 the pKa for the free α-amino group in LME. The pKa of this group in lysine acid is 9.18 (12). Thus it is likely complex formation with the enzyme would shift this pKa to a higher value.

The assignment of pKa_{i3l} is not entirely straightforward. Its value is low for an imidazoyl and high for a carboxyl. However, the location of three aspartyl residues near the site (13) and recent modification experiments (14) would tend to suggest that pKa_{i3l} is due to a carboxyl. Also, kinetic evidence for a functional carboxyl in trypsin has been reported, but its pKa was much smaller and intrinsic in Km (6). The thermodynamic data in Table III is direct evidence for a carboxyl. Carboxyl groups possess small or negative heats of ionization (12), and ΔH_{ion} was estimated to be -8.8 ± 1.6 kcal for pKa_{i3l}. To help verify this conclusion, the heat of ionization of the carboxyl group which acts as an intramolecular general base catalyst in aspirin hydrolysis was determined kinetically and found to be -3.7 kcal (15). In summary it can be stated that different ionic forms of the active site of trypsin are capable of being catalytic. With substrates possessing a free α-amino group the pH-optimum is shifted to acidic values and an unidentified carboxyl group is involved in the rate-controlling step.

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