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THE EFFECT OF PRE-INCUBATION ON TRYPSIN KINETICS AT LOW pH

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Summary

A possible source of discrepancy between kinetic and spectroscopic studies of the active site ionizations in the enzyme trypsin (EC 3.4.21.4) could arise if a slow pH-dependent conformational change affected the rates at low pH. No such effect is observed within the time range of 1 min– 3 h when pre-incubation of trypsin at pH 2.0 or at pH 6.9 precedes the enzymatic hydrolysis of N_α-carbobenzoxy-L-lysine-*p*-nitrophenyl ester. The deacylation rate of this hydrolysis depends on a single pK_a on the enzyme between pH 3 and pH 7.

Introduction

The active site in the trypsin-like enzymes contains both an imidazole (His-57) and a carboxylic acid (Asp-102). Because the normal pK_a values of the side-chains of histidine and aspartic acid in solution are about 6.0 and 3.6 [1], respectively, it is surprising that there is only one group (of pK_a 6.8) whose ionization affects the rate of catalysis by chymotrypsin or trypsin (EC 3.4.21.4) in the pH range 2—7 [2—4].

It has sometimes been assumed that if individual pK_a values could be assigned to the two groups, then His-57 would most likely be the group of p$K_a \approx 7$. However, some spectroscopic studies provide evidence that the group ionizing at pH $\approx$ 7 is Asp-102 [5,6]. Because only one pK_a associated with the active site is detected in the rate profile, the second group (His-57) may have a remarkably low pK_a (less than 2.0), although there is as yet no precedent for such a large perturbation of imidazole ionization in proteins.

Both kinetic and spectroscopic techniques are used to determine the ionization constants of catalytic groups in enzymes. A possible source of discrepancy

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between rapid kinetic experiments and spectroscopic studies [7] could arise if a slow pH-dependent conformational change took place during the long incubations required for most spectroscopic studies, and affected the pK_a values of groups at the active center.

One way to approach the question of whether a slow conformational change affects the pH dependence of serine protease hydrolysis would be to conduct kinetic experiments with enzyme pre-incubated at different pH values. Using this method, Rodriguez and Hollaway [8] detected a pH-dependent conformational change for phosphofructokinase having a half-time of about 4 min.

We measured the rate of hydrolysis of N_α-carbobenzoxy-L-lysine-*p*-nitrophenyl ester by trypsin which had been pre-incubated for up to 3 h at pH 2, or at pH 6.9.

Materials and Methods

Materials

Bovine trypsin (three times crystallized, lyophilized, salt-free; Lot 73M339) was purchased from Worthington Biochemical Corporation, and N_α-carbobenzoxy-L-lysine-*p*-nitrophenyl ester (Z-Lys-ONph) was obtained from Cyclo Chemicals (Lot D-1308).

Kinetics

Stock solutions of trypsin (28 μM active sites [9]) were pre-incubated in low pH (HCl, pH = 2) or high pH (2 mM piperazine-*N*,N'-bis(2-ethanesulfonic acid) · HCl (PIPES), pH = 6.9) buffers at 0°C for up to 3 h. Reaction buffers in the pH range 2.5—5.0 were prepared by mixing the appropriate amounts of buffer A (200 mM glacial acetic acid/12 mM HCl, pH = 2.01) with buffer B (200 mM sodium acetate/12 mM HCl, pH = 5.91).

The rates of Z-Lys-ONph hydrolysis were followed by observing the rate of liberation of *p*-nitrophenol in a Gilford Model 240 spectrophotometer at 340 nm. The reaction were run at 30.6 ± 0.1°C, and the pH of the reaction solutions remained constant throughout the reaction. In a typical run, 2 ml of reaction buffer and 20 μl of pre-incubated enzyme solution were mixed in a 3-ml cuvette, and the reaction was initiated by the addition of 100 μl of Z-Lys-ONph (2.19 mM) in ethanol. (In control experiments, the spontaneous hydrolysis of the substrate was measured by omitting the enzyme. All data were corrected for this low level, pH-dependent spontaneous hydrolysis.) The rate of hydrolysis (k) was determined by calculating the slope of the best straight line (determined by least squares) relating the change in absorbance at 340 nm to time.

Results

The rate constants for the hydrolysis of Z-Lys-ONph by trypsin are listed in Table I. There is essentially no difference between the results for enzyme pre-incubated at pH 2 and at pH 6.9; therefore, a slow conformational change with a time constant between 1 min and 3 h does not affect the pH dependence of Z-Lys-ONph hydrolysis by trypsin. Fig. 1 is a Dixon plot [10] of the rate data together with overall standard deviations.

TABLE I

RATES OF Z-Lys-ONph HYDROLYSIS BY TRYPSIN WHICH WAS PRE-INCUBATED AT pH 2.0, OR AT pH 6.9

Reaction pH	Pre-incubation pH	No. of observations	Average k ($\Delta A_{340} \cdot min^{-1}$)	Overall k * ($\Delta A_{340} \cdot min^{-1}$)	Overall σ *
2.5	2.0	1	0.0023	0.00245	$2.2 \cdot 10^{-4}$
	6.9	1	0.0026		
3.08	2.0	2	0.0142	0.0126	0.0012
	6.9	7	0.0121		
3.95	2.0	2	0.0597	0.0571	0.0063
	6.9	2	0.0613		
4.2	2.0	2	0.1008	0.1014	0.0008
	6.9	2	0.1019		
4.39	2.0	4	0.1206	0.1256	0.0086
	6.9	5	0.1295		
4.6	2.0	2	0.2090	0.2060	0.0052
	6.9	2	0.2029		
4.81	2.0	2	0.3148	0.3065	0.020
	6.9	2	0.2981		
5.05	2.0	3	0.491	0.479	0.049
	6.9	3	0.466		

* The "overall" rate is the average value of the rates observed at both conditions of pre-incubation.

$$\sigma = \left(\frac{\sum_{i=1}^{N} (k_r(\text{overall}) - k_{r_i})^2}{N - 1} \right)^{1/2}$$

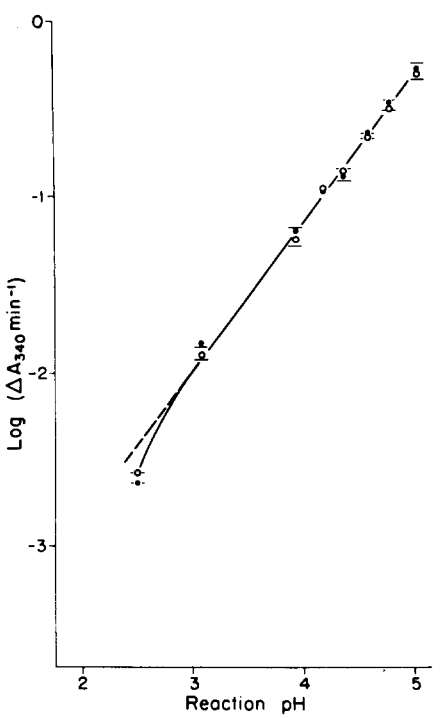

Fig. 1. pH dependence of the rate of Z-Lys-ONph hydrolysis by trypsin which was pre-incubated at pH 2.0 (●——●) or at pH 6.9 (○——○). The error bars represent the overall standard deviations listed in Table I.

The data in Fig. 1 fall on a straigth line between pH 5 and pH 3. The slope of this line is 0.83, whereas a slope of 1.0 would be expected if the rate depended on a single ionizing group [10]. A decreased slope such as this may be an effect of the low ionic strength of the solution, and the overall charge on the protein [11]. Data for the hydrolysis of N_α-acetyl-D,L-tryptophan-*p*-nitrophenyl ester by α-chymotrypsin at ionic strength 0.05 are reported by Kezdy et al. [2] in their Table III. A least-squares fit of a straight line to their data yields a slope of 0.86. Between pH 3 and 2.5, the slope of the Dixon plot in Fig. 1 changes.

Discussion

After pre-incubation of enzyme, the tryptic hydrolysis of Z-Lys-ONph was run at several pH values between 2.5 and 5.05. The rates of hydrolysis by trypsin pre-incubated at pH 2 or at pH 6.9 were identical. Thus, there is no slow pH-dependent conformational change which affects activity in this pH range. This means that equilibrium and kinetic methods of detecting ionization changes in the active site should be equivalent in this range.

The hydrolysis of Z-Lys-ONph by trypsin has been shown to proceed via an acyl enzyme (ES′) intermediate [12]. Thus the reaction may be represented by:

$$E + S \rightleftharpoons ES \overset{k_2}{\rightarrow} \underset{+}{ES'} \overset{k_3}{\rightarrow} E + P_2$$
$$P_1$$

where E is the free enzyme, S the substrate, ES the Michaelis complex, P_1 is the *p*-nitrophenol released, and P_2 is the free acid. Bender and Kezdy [13] and Bender et al. [3] have shown that for this reaction, under conditions of enzyme saturation, the steady state rate of release of *p*-nitrophenol is proportional to k_3, the deacylation rate constant. Over the pH range 3—5.05 in our experiment, Z-Lys-ONph was apparently at an enzyme saturating concentration of $1.03 \cdot 10^{-4}$ M ($K_m = 1 \cdot 10^{-5}$ M at pH 5.8 [13]). Under these conditions, pK_a values determined from the Dixon plot can be assigned to groups in the acyl enzyme.

Our results show that in the acyl enzyme there is no group with a pK_a between 3 and 5 which affects the deacylation rate, k_3. Furthermore, since the rate has been shown to depend on a group of pK_a 6.8 [3] and since the slope between pH 3 and 5 in Fig. 1 is characteristic of a single ionizing group, there can be no second rate-affecting pK_a between 3 and 7. For the same reaction Bender et al. [3] reported that k_{cat} depends on a single basic group between pH 2 and 7.4. These results are also consistent with the findings of Stewart and Dobson [14] who showed that a Dixon plot was linear between pH 3.6 and 4.4, and had a slope near unity for the deacylation of N_α-benzoyl-L-arginyl trypsin. Because the group titrating with a pK_a of 6.8 is Asp-102 [6], the pK_a of His-57 must be below 3 in trypsin. Although the structure of the active site [15] must somehow be involved in stabilizing the neutral form of the imidazole ring, there is as yet no satisfactory explanation as to why the pK_a of His-57 should be so low.

The most likely explanation for the curvature in the Dixon plot between pH 3 and 2.5 is that the substrate concentration was not saturating ($K_S = 7.95 \cdot 10^{-4}$ M at pH 2.66) and that the overall reaction rate in this range depended not only on deacylation, but on other steps in the reaction pathway (e.g. substrate binding, acylation). Bender et al. [3] stated that their analysis of tryptic hydrolysis of Z-Lys-ONph showed no such curvature above pH 2. Their substrate concentration was presumably higher, and their report again suggests that a lower than saturating substrate concentration was responsible for the curvature in Fig. 1. It is highly unlikely that a rate-controlling ionization or a pH-dependent structural change account for the departure from linearity.

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