Kinetic Studies of the Effect of pH on the Trypsin-Catalyzed Hydrolysis of N-α-benzyloxycarbonyl-L-lysine-p-nitroanilide: Mechanism of Trypsin Catalysis

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ABSTRACT: The pH dependence of the trypsin-catalyzed hydrolysis of N-α-benzyloxycarbonyl-L-lysine-p-nitroanilide has been studied at 25 °C. $k_{cat}/K_M$ was maximal at alkaline pH values but decreased with decreasing pH. $k_{cat}/K_M$ was dependent on free enzyme pKₐ values of 6.75 ± 0.09 and 4.10 ± 0.13, which were assigned to the ionization of the active site histidine-S7 and aspartate-189, respectively. Protonation of either group abolished catalytic activity. $k_{cat}$ is shown to equal the acylation rate constant $k_2$ over the pH range studied. $k_2$ decreased on the protonation of two groups with pKₐ values of 4.81 ± 0.15 and 4.23 ± 0.19. We assign the pKₐ of 4.23 to the ionization of the aspartate-189 residue and the pKₐ of 4.81 to the oxyanion of the tetrahedral intermediate formed during acylation. We conclude that during acylation, breakdown of the catalytic tetrahedral intermediate is rate-limiting and that there is a strong interaction between the imidazolium ion of histidine-S7 and the oxyanion of the catalytic tetrahedral intermediate, which perturbs their pKₐ values. From the pH dependence of $k_3$, we conclude that deacylation depends on a pKₐ of 6.41 ± 0.22 and that the ionization of the carboxylate group of aspartate-189 does not have a significant effect on the rate of deacylation ($k_3$). A catalytic mechanism is proposed to explain the pH dependence of catalysis.

1. INTRODUCTION

Trypsin and trypsin-like serine proteases specifically catalyze the hydrolysis of peptide bonds involving the carbonyl carbon of the α-carboxylate group of the positively charged amino acid residues lysine or arginine. Trypsin is a serine protease involved in protein digestion. Due to its high specificity for positively charged amino acid residues trypsin is widely used for peptide sequencing in proteomics.1,2 Trypsin-like serine proteases are involved in a range of biological processes and diseases, e.g., the protease responsible for fibrinolyis3 and cancer progression.4,5 In this study, we utilize pH studies to investigate the catalytic mechanism of trypsin.

Catalysis by the serine proteases can be described by the minimal three-step kinetic mechanism (eq 1) below

$$E + S \overset{k_1}{\rightarrow} ES \overset{k_2}{\rightarrow} ES^+ \overset{k_3}{\rightarrow} E + P_2$$

where ES is the Michaelis complex and ES' is the acyl intermediate. $K_2$ is the dissociation constant of the Michaelis complex ($K_2 = k_{cat}/k_{cat} = [E][S]/[ES]$). $k_2$ and $k_3$ are the first-order rate constants for acylation and deacylation, respectively. Catalysis obeys the Michaelis–Menten equation (eq 2).

$$[P] = k_{cat}K_2/[E]_0/[S]_0 + K_M$$

where $[P]$ is the concentration of substrate (P), $k_{cat}$ is the turnover number, and $K_M$ is the Michaelis constant. The Michaelis parameters are complex assemblies of rate constants with $k_{cat} = k_2k_3/(k_2 + k_3)$ and $K_M = k_3/[k_{cat}]/(k_2 + k_3)$, and if $k_{cat} >> k_2$, then $K_M = k_3/[k_{cat}]/(k_2 + k_3)$. Therefore, the mechanistic significance of the pH dependence of these Michaelis parameters is often not clear. However, pioneering studies with chymotrypsin at pH 7.95 have shown that with ester substrates with good leaving groups (P₁), deacylation ($k_3$) is rate limiting and so

$$k_2 \gg k_3 \text{ and } k_{cat} = k_3 + K_M = K_s/k_2$$

while with amide and anilide substrates with poor leaving groups (P₁), acylation ($k_2$) can be rate limiting with

$$k_3 \gg k_2 \text{ and } k_{cat} = k_2 + K_M = K_s$$

Therefore, with highly reactive ester substrates, it should be possible to determine $k_3$ from $k_{cat}$ values, while with less reactive amide or anilide substrates, it should be possible to determine $k_2$ and $K_s$ values from $k_{cat}$ and $K_M$, respectively.

At pH 2.66, the reaction of trypsin with the reactive ester substrate Z-lys-pnp was slow enough for both $k_2$ and $k_3$ to be measured and it was shown that acylation was much more rapid

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than deacylation \( \left( \frac{k_2}{k_3} = 27.6 \right) \). However, it was found that at higher pH values, the ratio \( \frac{k_2}{k_3} \) decreased and \( k_2 \) was not much greater than \( k_3 \). Therefore, it cannot be assumed that \( k_2 \) is always very much greater than \( k_3 \) with reactive ester substrates and so pH studies are essential if we are to fully understand the kinetics of catalysis at different pH values. The less reactive para-nitroanilide substrates are thought to be better models than the more reactive para-nitrophenol ester substrates for natural peptide substrates. However, a detailed kinetic analysis of the trypsin-catalyzed hydrolysis of the equivalent less reactive para-nitroanilide substrate Z-Lys-pna has not been carried out.

Therefore, in the present study a detailed study of the trypsin-catalyzed hydrolysis of the anilide substrate Z-Lys-pna has been undertaken. The effect of pH on the ratio \( \frac{k_2}{k_3} \) and on values of \( K_M \) and \( K_s \) has been quantified. Also, the effect of pH on \( k_m/K_M \) and on the rates of acylation \( (k_2) \) and deacylation \( (k_3) \) has been determined. From these studies, the pK_\alpha values affecting catalysis in the free enzyme as well as during acylation and deacylation have been determined. In any pH study, it is difficult to dismiss the possibility that ionizations outside the active site can also affect catalytic activity. However, in this study, there is no evidence of this and all observed ionizations are assigned to active site groups.

The mechanistic significance of these results are discussed and a catalytic mechanism is proposed, which explains the pH dependence of catalysis. The mechanistic proposals and their background are briefly summarized in the following paragraph.

Some earlier studies \(^{10-14} \) have suggested that the pK_\alpha of the catalytic histidine is decreased during acylation (pK_\alpha < 7), while more recent studies \(^{5-23} \) have suggested that the histidine pK_\alpha must be raised (pK_\alpha > 11) so that it can be an effective general base catalyst during acylation and that the pK_\alpha of the oxanyanion of

Figure 1. Determination of the catalytic parameters for the trypsin-catalyzed hydrolysis of Z-Lys-pna at different pH values. Initial rates (d[P]/dt) were fitted to eq 1 \( (d[P]/dt = V_m \cdot [S_0]/(S_0 + K_M)) \). The pH, trypsin concentration, and the fitted values of \( V_m, V_m/K_M \) and \( K_M \) were: (A) pH 3.13, 83.5 μM, 0.0768 ± 0.0207 μM s\(^{-1}\), 2.22 ± 0.9 μs\(^{-1}\), 34.6 ± 10.5 mM; (B) pH 3.82, 25.6 μM, 0.388 ± 0.094 μM s\(^{-1}\), 15.8 ± 7.0 μs\(^{-1}\), 24.5 ± 9.2 mM; (C) pH 4.40, 4.68 μM, 0.444 ± 0.037 μM s\(^{-1}\), 32.3 ± 5.7 μs\(^{-1}\), 13.8 ± 2.1 mM; (D) pH 5.98, 0.39 μM, 0.344 ± 0.029 μM s\(^{-1}\), 52.8 ± 8.9 μs\(^{-1}\), 6.51 ± 0.95 mM; (E) pH 6.94, 0.427 μM, 0.202 ± 0.005 μM s\(^{-1}\), 395 ± 32 μs\(^{-1}\), 0.512 ± 0.039 mM; (F) pH 9.05, 0.379 μM, 0.182 ± 0.005 μM s\(^{-1}\), 463 ± 34 μs\(^{-1}\), 0.394 ± 0.027 mM.

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the catalytic tetrahedral intermediate (THI) is lowered so that trypsin can be an effective enzyme at physiological pH values. In the present work, $pK_a$ values of 4.8 and 4.2 were detected from the pH studies of the acylation step of catalysis ($k_c$ in eq 1). The $pK_a$ of 4.8 is assigned to the oxygenation of the catalytic tetrahedral intermediate formed during acylation and not to the $pK_a$ of the catalytic histidine that is assigned a $pK_a > 11$ in ES and the tetrahedral intermediate. Aspartate-189 that binds the lower pHs (Figure 1).

2. RESULTS

2.1. Determination of the Catalytic Parameters $k_{cat}$, $k_{cat}/K_M$, and $k_{cat}/K_M$ at Different pH Values. The Michaelis parameters $k_{cat}$, $K_M$, and $k_{cat}/K_M$ for the trypsin-catalyzed hydrolysis of Z-Lys-pna were determined by computer-fitting the initial rate data obtained at a given pH to the hyperbolic form of the Michaelis–Menten equation ($\Delta[P]/\Delta t = V_{max}[S] / ([S] + K_M)$) using the method of Wilkinson.

Examples of the experimental data and the fitted lines are given in Figure 1. The trypsin concentrations were increased at lower pH values to compensate for the decreases in $k_{cat}$ and $k_{cat}/K_M$. $K_M$ values increased as the pH decreased; and so higher substrate concentrations were used for determining $K_M$ values at lower pHs (Figure 1).

2.2. Effect of pH on $k_{cat}/K_M$ for the Trypsin-Catalyzed Hydrolysis of Z-Lys-pna. $k_{cat}/K_M$ values were determined by dividing $V_{max}/K_M$ values by the enzyme concentration in the reaction mixture. $k_{cat}/K_M$ values were dependent on the sequential ionization of two groups ($k_{cat}/K_M = (k_{cat}/K_M)_\text{max}/(1 + [H^+]/K_a + [H^+]^2/K_b)$): one with a $pK_a$ of 6.75 ± 0.09 and the other $pK_a$ is 4.10 ± 0.13 (Figure 2). The pH dependence of $k_{cat}/K_M$ reflects ionizations in either the free enzyme or the free substrate. Previous studies with other substrates have detected similar free enzyme $pK_a$ values of ~7 and ~4.9,25–30

2.3. Calculation of $k_3$ for the Trypsin-Catalyzed Hydrolysis of Z-Lys-pnp and Z-Lys-pna. The trypsin-catalyzed hydrolysis of both Z-Lys-pna and Z-Lys-pnp proceeds via a common acyl intermediate (Z-Lys-trypsin, ES' in eq 1 and structure d1 in Scheme 1). Therefore, both will have the same deacylation rate constant ($k_d$). Using pre-steady-state kinetics, the $k_3$ values for the trypsin-catalyzed hydrolysis of Z-Lys-pnp have been determined.23 Steady-state kinetics have been used to determine $k_{cat}$ values for Z-Lys-pnp,25 $k_a = k_{cat}/(k_3 + k_d)$, and this equation can be rearranged to give the equation, $k_3 = k_{cat}/k_a = (k_3 - k_d)$. The experimental data was fitted to give the equation, $k_3 = k_{cat}/(1 + [H^+]/K_a + [H^+]^2/K_b)$, which has been used to calculate the $k_3$ values (line 1 in Figure 3A) for Z-Lys-pna and Z-Lys-pnp from the experimentally determined values of $k_d$ and $k_{cat}$ for Z-Lys-pnp.25 The experimentally determined values of $k_d$ and $k_{cat}$ for Z-Lys-pnp are given in Table 1. Therefore, $k_{cat}$ values for the trypsin-catalyzed hydrolysis of Z-Lys-pna in the present work have been determined for Z-Lys-pna from pH 3.1–9.8 (Figure 3A, line 2). $k_{cat} = k_{cat}/(k_3 + k_d)$, which can be rearranged to give $k_3 = k_{cat}/k_a$. As $k_3$ is the same for both Z-Lys-pnp and Z-Lys-pna, we can use the fitted parameters obtained from the pH dependence of $k_3$ for Z-Lys-pnp to calculate the value of $k_3$ for Z-Lys-pna for each value of $k_{cat}$ determined for Z-Lys-pna from pH 3.1–9.8. Therefore, as $k_d$ and $k_{cat}$ are known for Z-Lys-pna, $k_3$ values could be calculated for Z-Lys-pna. However, this calculation was not necessary to calculate $k_3$ because $k_3$ was 2–3 orders of magnitude greater than $k_d$ (Figure 3B) and so within the experimental error (<2%) $k_{cat} = k_3$. Therefore, $k_{cat}$ values for the trypsin-catalyzed hydrolysis of Z-Lys-pna can be assumed to equal $k_3$ (Figure 3A, line 2). Experimental $k_{cat}$ data ($k_{cat} = k_3$) was fitted to the equation $k_{cat} = (k_{cat})_\text{max}/(1 + [H^+]^2/K_a + [H^+]^4/K_b)$ assuming $k_3$ was constant on the sequential ionization of two ionizing groups (line 2 in Figure 3A). We conclude that $k_3$ has a maximal value of 0.517 ± 0.014 s⁻¹ and is dependent on the sequential ionization of two ionizing groups (line 2 in Figure 3A) with $pK_a$ values of 4.81 ± 0.15 and the 4.23 ± 0.19 (Table 1).

2.5. Effect of pH on the Ratio $k_3/k_2$ for the Trypsin-Catalyzed Hydrolysis of Z-Lys-pna. The fitted values of $k_2$ and $k_3$ (lines 1 and 2 in Figure 3A) were used to calculate the ratio of $k_2/k_3$ from pH 2.6 to 9.8 (Figure 3B). The ratio $k_2/k_3$ was dependent on pH having a minimal value of ~55 at pH 4.5 but reaching values of 1200 and 1080 at pH 2.6 and 9.8, respectively (Figure 3B). Therefore, for the trypsin-catalyzed hydrolysis of Z-Lys-pna, $k_3 \gg k_2$ from pH 2.6 to 9.8 and so $k_2$ is rate limiting for Z-Lys-pna and the expression for $K_M$ ($K_M = K_M = k_{cat}/(k_3 + k_3)$) simplifies to $K_M = K_3$ from pH 2.6 to 9.8. Therefore, for Z-Lys-pna, $K_M$ values are equal to $K_3$ values.

2.6. Determination of $K_3$ and its pH Dependence. Analyzing the $K_M$ versus pH data for dependence on one $pK_a$ (equation $K_M = K_{cat}/(1 + [H^+]^2/K_a)$ gives a minimum $K_a$ value of 0.333 ± 0.025 mM and a maximum $K_a$ value of 23.5 ± 1.9 mM, with a $pK_a$ of 5.12 ± 0.17 (Figure 4A). For optimal accuracy when determining $K_M$ using the Michaelis–Menten equation, substrate concentrations should ideally be in the range $K_M/5$ to $5 \times K_M$ to optimize accuracy. However, the maximum concentration of Z-Lys-pna was ~20 mM and so at low pHs, substrate concentrations were similar to the $K_M$ values and therefore accurate $K_M$ values could not be determined (Figure 4A). In contrast, the experimental data for $k_{cat}/K_M$ (Figure 2) and $k_{cat}/K_M$ (Figure 3) were both in good agreement with the fitted lines. This is because both $k_{cat}$ and $k_{cat}/K_M$ decrease rapidly and do not level off like $K_M$ values to a fixed value. Consequently, $k_{cat}$ and $k_{cat}/K_M$ values cover a larger range.
of values than $K_M$ values. The log plots of $k_{cat}/K_M$ and $k_{cat}$ will reflect two $pK_a$ values, $pK_{E1}$ and $pK_{E2}$ for $k_{cat}/K_M$ and $pK_{E31}$ and $pK_{E32}$ for $k_{cat}$. In contrast, a plot of $K_M$ versus pH should give $pK_a$ values of 4.2 and 4.8 (Table 1). However, the experimental $K_M$ data (Figure 4A) is not good enough to resolve these two $pK_a$ values and determine the pH-independent values of $K_a$.

To determine the pH-independent $K_a$ values ($K_{a1}, K_{a2},$ and $K_{a3}$ in Scheme 1), $K_a$ values ($K_a = K_{aM}$) were fitted (Figure 4B) to the equation for a doubly ionizing system ($K_{a(obs)} = K_{a1}(H^+)^2 + K_{a2}(H^+)^3 + K_{a3}(H^+)^4 + K_{a4}(H^+)^5 + K_{a5}$), and this gave three pH-independent $K_a$ values of 24 mM ($K_{a1}$), 32.2 mM ($K_{a2}$), and 0.373 mM ($K_{a3}$) (Scheme 1 and Table 1). The fitted values of $pK_a$ and $pK_b$ determined from the pH dependence of $K_a$ had values of 4.23 and 4.82, in good agreement with the $pK_a$ values obtained from the pH dependence of $K_a$ (Table 1). This is expected as the pH dependence of $K_a$ (or $K_{aM}$) like $k_{cat}$ reflects ionizations within the ES complex or THI intermediate (Scheme 1). The pH dependence of $1/K_a$ like that of $k_{cat}/K_M$ reflects ionizations in the free enzyme. Fitting the $1/K_a$ values (Figure 4C) to a doubly ionizing system gave $pK_a$ values of 6.75 and 4.1, which, as expected, were the same as observed for the pH dependence of $k_{cat}/K_M$ (Table 1), which also reflects ionizations in the free enzyme. The agreement of the $pK_a$ values determined from the pH dependence of $K_a$ and $1/K_a$ with the $pK_a$ values obtained from the pH dependence of $k_{cat}$ and $k_{cat}/K_M$ respectively, confirms that this approach should allow the determination of estimates of the pH-independent $K_a$ values $K_{a1}, K_{a2}$ and $K_{a3}$ that are consistent with the experimentally determined values of $k_{cat}$ and $k_{cat}/K_M$ used to calculate the $K_a$ values used. This is supported by the fact that the pH-independent $K_a$ values $K_{a1}, K_{a2}$ and $K_{a3}$ obtained from the pH dependence of $K_a$ and $1/K_a$ were essentially the same (Table 1).

### 3. DISCUSSION

The pH dependence of $k_{cat}/K_M$ showed that the active group was $A^\ddagger$ in eq 2 and that it was inhibited by protonation to form $AH^+$ and $AH_2$ (eq 2)

$$\text{AH}_2 = \text{AH}^+ + \text{H}^+$$

The free enzyme $pK_a$ values 6.75 ± 0.09 and $pK_{b1}$ = 4.10 ± 0.13 obtained from the pH dependence of $k_{cat}/K_M$ and $1/K_a$ (Table 1) have been attributed to the trypsin residues histidine-57 and aspartate-189, respectively (9,25–30). In both cases, it is the ionized form of these groups that are catalytically active (structure a1 in Scheme 1). Histidine-57 is part of the catalytic triad and its ionized form acts as a general base catalyst during catalysis (b1 in Scheme 1). Once it is protonated (AH+, eq 2), it can no longer act as a general base catalyst for tetrahedral intermediate formation (b1 in Scheme 1) and so catalysis is inhibited. However, if the histidine $pK_a$ is raised in the ES complex to a value >11 (structures b1 and b2 in Scheme 1) so that it can be an effective general base catalyst for tetrahedral intermediate formation (structures b1 to c1 in Scheme 1), then this $pK_a$ will not be observed in our pH studies from pH 3–10. Protonation of histidine-57 in the free enzyme (structures a1 to a2 in Scheme 1) is also known to decrease substrate and inhibitor binding and so this should also contribute to substrate catalysis being inhibited with a $pK_a$ of 6.75 (31–32).

The negatively charged side chain carboxylate group of the aspartate-189 residue is located at the bottom of the S1 specificity site where it can form an ion pair with the positively charged side chains of lysine or arginine substrates. $pK_a$ values of 7.1 and 4.55 have been obtained from the pH dependence of $k_{cat}$ for the $p$-nitrophenol substrate Z-Lys-pnp with trypsin. With the neutral substrate Z-Ala-pnp, no substrate interaction with aspartate-189 is expected and so the fact that only one $pK_a$
value of 6.9 was obtained from the pH dependence of $k_{cat}/K_M$ with this neutral substrate, which confirms the assignment of $pK_a$ value of $\sim 4$ to aspartate-189 in the free trypsin. Likewise, the fact that pH studies of $k_{cat}$ showed that the $pK_a$ of $\sim 4$ was detected with the lysine substrate Z-Lys-pnp but not with the neutral substrate Z-Ala-pnp also confirms the assignment of $pK_a$ of $\sim 4$ to aspartate-189 in the trypsin ES complex with Z-Lys-pnp. Therefore, the $pK_a$ values of 4.1 and 4.23 obtained from the pH dependence of $k_{cat}/K_M$ and $k_1$, respectively (Table 1), are consistent with the assignment of aspartate-189 to activate trypsin and has a primary role of lysine and arginine substrates binding to aspartate-189 and the positively charged side chains of lysine or arginine substrates mainly used to activate trypsin.39 Therefore, these results show that the binding energy between the carboxylate group of aspartate-189 and the positively charged side chains of lysine or arginine substrates is mainly used to activate trypsin and has a minimal effect on $K_c$. This explains why protonation of aspartate-189 ($pK_a, 4.1-4.2$) has such a small effect on $K_c$ (42.4-24.0 mM, Table 1) and yet such a large effect on $k_{cat}$, resulting in the stoichiometric inhibition of trypsin (line 2 in Figure 3A).

With the positively charged Z-Lys-pna substrate, an additional $pK_a$ of 4.81 was obtained from the pH dependence of the acylation rate constant $k_1$ (eq 1). Protonation of this group led to an 86-fold increase in $K_b$ (eq 3). A similar 86-fold increase in $K_b$ was observed when histidine-57 was protonated in the free enzyme with a $pK_a$ of 6.75 (Table 1), which would appear to suggest that the $pK_a$ of histidine-57 changes from 6.75 in the free enzyme to 4.82 in the tetrahedral intermediate adduct ($pK_a$thi in Scheme 1) and that the 86-fold decrease in $K_b$ is due to binding energy being used to lower the $pK_a$ of histidine-57 from 6.75 to 4.82. It also shows that the protonation state of histidine-57 has a major role in substrate binding. An 86-fold increase in $K_b$ below pH 7 was also observed with N-benzoxycarbonyl-L-arginine-p-toluidide and trypsin,40 but in this case, the decrease in histidine $pK_a$ was much smaller ($pK_a, 6.38$). Larger 13-fold increases in $K_b$ values have also been observed when histidine-57 in chymotrypsin is protonated with hydrazide substrates.41 It has been suggested

### Table 1. Catalytic Parameters for the Trypsin-Catalyzed Hydrolysis of Z-Lys-pna

| parameter | $k_{cat}$/M$^{-1}$s$^{-1}$ | $pK_a$ | $pK_b$ | $K_b$ | $K_{cat}$ | $K_{cat}^{\text{ES}}$ |
|-----------|------------------|--------|---------|------|----------|-----------------|
| $k_{cat}$ | $1390 \pm 72$ | $6.75 \pm 0.09$ | $4.10 \pm 0.13$ | $24.0$ | $32.2$ | $0.373$ |
| $k_1$ (s$^{-1}$)$^{\text{eq}}$ | $0.517 \pm 0.014$ | $4.23 \pm 0.19$ | $4.81 \pm 0.15$ | $24.0$ | $32.2$ | $0.373$ |
| $k_2$ (s$^{-1}$)$^{\text{eq}}$ | $561 \pm 104$ | $6.41 \pm 0.22$ | $6.41 \pm 0.22$ | $24.0$ | $32.2$ | $0.373$ |
| $K_b$ (mM)$^{\text{eq}}$ | $4.23$ | $4.82$ | $6.75$ | $24.0$ | $32.2$ | $0.373$ |
| $1/K_b$ (mM$^{-1}$)$^{\text{eq}}$ | $4.10$ | $6.75$ | $24.0$ | $32.4$ | $0.372$ |

*Errors are standard errors of the fitted parameters. $^{\text{eq}}$The $K_b$ values used to determine $pK_a$, $pK_b$, $K_{cat}$, $K_{cat}^{\text{ES}}$, and $K_{cat}^{\text{thi}}$ were calculated from the fitted values of $k_{cat}$ and $k_{cat}/K_{cat}$ (see text for details).
The solid line was calculated using the equation for $k$ (circles) were calculated at 0.1 pH intervals by dividing the using the equation (1/\(K_s\)) values determined by fitting initial rate values to the Michaelis–Menten equation, as described in the Experimental Section. The solid line was calculated using the equation $K_{\text{obs}} = (K_a/[H^+]) + K_{\text{obs}}/(1 + [H^+]/K_a)$ and the fitted values of $K_{\text{obs}} = 23.5 \pm 1.9$ mM, $K_a = 0.333 \pm 0.025$ mM, and $pK_a = 5.12 \pm 0.17$. (B) $K_a$ values (solid circles) were calculated at 0.1 pH intervals by dividing the fitted values for $k_a$ (Figure 3A) by the fitted values for $k_{cat}/K_{cat}$ (Figure 2), $k_{cat}/K_{cat} = k_a/K_a$. The solid line was calculated using the equation $K_{\text{obs}} = (K_a/[H^+]) + K_{\text{obs}}/[1 + [H^+]/K_a]$ and the fitted values $K_{\text{obs}} = 24.0$ mM, $K_a = 32.4$ mM, $K_{\text{obs}} = 0.372$ mM, $pK_a = 6.75$, $pK_b = 4.10$. that these decreases in binding were due to the positively charged histidine-57 interacting with the leaving group amine. As expected with trypsin and neutral p-nitrophenol substrates that do not have a leaving group amine, there is no increase in $K_a$ when histidine-57 is protonated. However, with chymotrypsin, decreases in inhibitor binding at low pH have also been observed with 2-p-toluidinylaphthalene-6-sulfonate, flavin, and peptide-derived glyoxal inhibitors. This suggests that a neutral imidazol group of the histidine-57 residue is required for optimal binding of these substrates and inhibitors. There is a considerable body of evidence that shows that the serine proteases stabilize zwitterionic tetrahedral adducts that mimic the catalytic zwitterionic tetrahedral intermediate. These zwitterionic tetrahedral adducts refer to the negatively charged oxyanion of the tetrahedral intermediate and the positively charged imidazolium ion of histidine-57 and ignore all other charged groups on the enzyme. In these zwitterionic tetrahedral adducts, the $pK_a$ of the majority of histidine-57 (analogous to $pK_{THI}$ in Scheme 1) is raised$^{16,18,20,22,42}$ and the $pK_b$ (analogous to $pK_{THII}$ in Scheme 1) of the majority of the oxyanion is lowered.$^{16,18,19,22,43,44}$ Specific peptide-derived glyoxal inhibitors are tightly bound as neutral zwitterionic tetrahedral complexes (structure c1 in Scheme 1), which are thought to mimic the catalytic tetrahedral intermediate.$^{22,43,45}$ In these zwitterionic glyoxal complexes, $^1H$ NMR and $^{13}C$ NMR have been used to show that the $pK_a$ of histidine-57 is >11 and the oxyanion $pK_a$ is ~4.$^{22,44}$ The increase in $K_v$ values at low pH was dependent on a $pK_v$ of ~4, and this $pK_v$ was assigned to protonation of the oxyanion in the glyoxal-chymotrypsin tetrahedral adduct.$^{22}$ Therefore, if the breakdown of the zwitterionic tetrahedral intermediate in the acylation step of the trypsin-catalyzed hydrolysis of Z-Lys-pna (species c1 in Scheme 1) is rate-limiting, then the $pK_b$ of 4.81 (Table 1) in trypsin catalysis should be assigned to protonation of the oxyanion in the catalytic zwitterionic tetrahedral intermediate ($pK_{THII}$ in Scheme 1). It also shows that tight binding of inhibitors and substrates is possible when histidine-57 is protonated provided the oxyanion is also present to neutralize the charge on the imidazolium ion of histidine-57 (ImH+ O−). If the oxyanion is protonated (ImH+ OH−), the charge on the protonated imidazolium ion will no longer be neutralized by the oxyanion and so the positively charged imidazolium ion of histidine-57 will inhibit binding. Therefore, $K_a$ values will increase as the oxyanion is protonated, as observed with peptide glyoxal inhibitors that bind to chymotrypsin as zwitterionic tetrahedral adducts mimicking the catalytic tetrahedral intermediate.$^{22}$ This also explains why $K_a$ values with trypsin increase by essentially the same amount when histidine-57 is protonated (1/$K_a$ in Table 1) in the free enzyme (a1 to a2 in Scheme 1) and also in the acylation complex ($K_a$ in Table 1) when the oxyanion of the zwitterionic tetrahedral intermediate is protonated (structures c1 to c2 in Scheme 1).

It is generally accepted that the nucleophilicity of the hydroxyl group of serine-195 is enhanced by the imidazole group of histidine-57 acting as a general base catalyst (structure b1 in Scheme 1). It has been argued that as the $pK_b$ of the serine hydroxyl group is ~15,$^{15,16,22,23,25,42}$ then for general base catalysis by histidine to be effective (structure b1 in Scheme 1), its $pK_b$ should raised to a similar value of ~15 on forming the enzyme substrate complex (ES in Scheme 1)$^{15,16,22,23}$ and not lowered to a value <7.

Earlier studies on the pH dependence of chymotrypsin catalysis appeared to contradict these results as it appeared that
the pK_a of histidine-57 had been decreased to a value <7 within the ES complex. The reassignment of this pK_a to the oxyanion in the present work resolves this contradiction. As protonation of the oxyanion inhibits catalysis, it is essential that the oxyanion pK_a is reduced to ensure the enzyme is catalytically active at physiological pH values. This therefore explains why the serine proteases have evolved to lower the oxyanion pK_a so effectively.

When an interaction occurs between two ionizing groups, four species would be formed with four microscopic pK_a values (c_1, c_2, c_3, c_4 in Scheme 1). So, for example, if in Scheme 1 the concentrations of all species (c_1, c_2, c_3, c_4 in Scheme 1) are equal, then 50% of histidine-57 (pK_THI in Scheme 1) and 50% of the oxyanion (pK_THII in Scheme 1) will both have pK_a values of 4.8. Likewise, 50% of histidine-57 (pK_THII in Scheme 1) and 50% of the oxyanion (pK_THII in Scheme 1) will have pK_a values of >11. So this interaction could explain how histidine-57 could have microscopic pK_a values of both ~4.8 (pK_THII) and >11 (pK_THII) in the THI (Scheme 1). Likewise, the oxyanion could have microscopic pK_a values of ~4.8 (pK_THII) and >11 (pK_THII) in the THI (Scheme 1). However, the serine proteases preferentially stabilize zwitterionic tetrahedral intermediates (structure c1 in Scheme 1) and so it is expected that at least 99% of the oxyanion and the imidazolium ion of histidine-57 will have pK_a values of ~4.8 and >11, respectively.

The deacylation rate constant (k_d in eq 1) was dependent on a singly ionizing group (AH = A^-) with a pK_a of 6.4 ± 0.22, which we assign to histidine-57. Therefore, we can conclude that the ionization of aspartate-189 does not appear to have a significant effect on the pH dependence of deacylation.

### 3.1. Mechanism for the pH Dependence of the Trypsin-Catalyzed Hydrolysis of Z-Lys-pna

The proposed mechanism is summarized in Scheme 1. In the free enzyme (a1, a2, and a3 in Scheme 1), the imidazole group of histidine-57 has a pK_a of 6.75 and the carboxy group of aspartate-189 (Scheme 1) that binds the side chains of the lysine or arginine residues of substrates or inhibitors has a pK_a of 4.1. Binding of the substrate to form the enzyme-substrate complex (ES in Scheme 1) causes a strong interaction between the carboxy group of aspartate-102 of the catalytic triad and the imidazolium group of histidine-57 (structure c1 in Scheme 1) and so it is expected that at least 99% of the oxyanion and the imidazolium ion of histidine-57 will have pK_a values of ~4.8 and >11, respectively.

The deacylation rate constant (k_d in eq 1) was dependent on a singly ionizing group (AH = A^-) with a pK_a of 6.4 ± 0.22, which we assign to histidine-57. Therefore, we can conclude that the ionization of aspartate-189 does not appear to have a significant effect on the pH dependence of deacylation.

### 4. EXPERIMENTAL SECTION

#### 4.1. Materials

Trypsin (type III, 2X crystalized, salt free from bovine pancreas) and all other reagents were obtained from Sigma-Aldrich Chemical Co., Gillingham, Dorset, U.K. Trypsin was 72% fully active by active site titration with p-nitrophenyl-p-guanidobenzoate, as described by Malthouse et al. Z-Lys-pna was synthesized as described by Mackenzie et al.

#### 4.2. Kinetic Studies

The trypsin-catalyzed hydrolysis of Z-Lys-pna was studied at 25 °C in 3 mL volumes and 0.1 M ionic strength buffers. The buffers used were pH 3.1–4.38 (sodium formate), pH 4.40–5.49 (sodium acetate), 5.98–7.72 (potassium phosphate), 7.76–8.68 (Tris–HCl), pH 9.05 (sodium borate), and pH 9.76 (sodium carbonate). pH measurements were made either with a Radiometer combination electrode (GK 2401C) or by using a Beckman combination electrode model number 39522. Stock solutions of Z-Lys-pna were made up in 1 mM HCl (maximum solubility ~18 mM) and quantified using ε_{314} = 13 900 M\(^{-1}\)cm\(^{-1}\), as described by Mackenzie et al. Primary stock solutions of ~1 mM fully active trypsin were prepared in 1 mM HCl, and this solution was diluted in 1 mM HCl to prepare appropriate concentrations for the pH studies. A typical assay contained 1 mL of buffer (I = 0.3 M), 1.9–x mL of 1 mM HCl, and x mL of substrate in 1 mM HCl. Different substrate concentrations were obtained by adding different amounts of the stock substrate (x mL). Catalysis was initiated by adding 0.1 mL trypsin in 1 mM HCl. The concentration of trypsin was kept constant when determining the catalytic parameters at a given pH. However, fully active enzyme concentrations were increased (0.35–83.6 μM) as the pH was decreased to help compensate for the decrease in k_d and k_2/K_M as the pH decreased. The fully active trypsin concentrations used in the assays were 11–84 μM (pH 3.1–5.0), 3.7–4.4 μM (pH 4.4–5.5), and 0.35–0.39 μM (pH 6.0–9).
9.8. Initial rates of hydrolysis of Z-Lys-pna were followed by determining the amount of \( p \)-nitroaniline (\( E_{410} = 8800 \text{ M}^{-1} \cdot \text{cm}^{-1} \)) released over a 5–15 min period. It was ensured that there was always at least a 15-fold excess of the substrate over enzyme when initial rates were determined. \( k_{\text{cat}}/K_M \text{ data at pHs 3.56 and 4.38 were determined from initial rates obtained when } S_0 \ll K_M. \text{ The effect of pH on the catalytic parameters was determined by fitting experimental data to the appropriate function as described by Cleland.}^{33}

**ASSOCIATED CONTENT**

Accession Codes
The UniProtKB accession ID for bovine trypsin is P00760.

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**ABBREVIATIONS**
Z, benzyloxy carbonyl; pna, \( p \)-nitroanilide; pnp, \( p \)-nitrophenyl ester

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