pH Dependence of Tritium Exchange with the C-2 Protons of the Histidines in Bovine Trypsin†

Monty Krieger,† Roger E. Koeppel II,§ and Robert M. Stroud*
trypsin: His-57 at the active site and His-40, which are also present in chymotrypsin, and His-91. The observed rates for each of the three histidines are slower than any previously reported rates for tritium exchange into imidazole.

Materials and Methods

**Materials.** p-Nitrophenyl p'-guanidinobenzoate was obtained from Cyclo Chemical Co., and tritiated water, 1 Ci/ml, was from New England Nuclear. Trypsin (Lot 73M339, 3x recrystallized, sterile), chymotrypsin (Lot 340698), and soybean trypsin inhibitor (Lots 54J338 and 1AA) were purchased from Worthington Biochemical Corp.

**Protein Purification and Assay.** Trypsin of high activity was isolated by Sephadex G-25 or G-75 chromatography (column dimensions: 4.91 cm x 90 cm; buffer: HCl, pH 2.5, with and without 40 mM CaCl₂). Soybean trypsin inhibitor (STI)² was purified by chromatography on Sephadex G-75 (pH 7.8, 4 °C) to remove lower and higher molecular weight contaminants. The concentration of active trypsin was determined by active-site titration with p-nitrophenyl p'-guanidinobenzoate (Chase and Shaw, 1967), and by absorbance at 280 nm (ε = 1.54 mg⁻¹ cm⁻¹; mol wt 23 891 (Robinson et al., 1971)).

A Gilford Model 240 spectrophotometer was used for all spectrophotometric measurements. All experiments were conducted at room temperature unless otherwise indicated.

The number of active sites in the purified trypsin was usually about 95% of the theoretical value. Control incubations without tritium showed that the benzamidine included in the incubation buffer completely prevented autolysis during incubations of 0, 7, 10, and 14 days.

**Tritium Exchange.** The procedure used was based on the method described by Ohe et al. (1974). Incubation buffers with pH below 4 were prepared by adding concentrated HCl to buffer A (50 mM benzamidine, 50 mM calcium acetate, 50 mM Pipes, 100 mM HCl, pH 4.1). Incubation buffers with pH above 4 were prepared by adding the appropriate amount of buffer B (50 mM calcium acetate, 50 mM benzamidine, 50 mM Pipes, 50 mM NaOH, 50 mM NaCl, pH 10.6) to buffer A. 50 mM Benzamidine, a competitive reversible inhibitor of trypsin (Mares-Guia and Shaw, 1965), was included to prevent autolysis. Benzamidine binds to trypsin in the specificity binding pocket, but does not affect solvent accessibility to histidine in the catalytic site (Krieger et al., 1974). Trypsin (10 mg) and tritiated water (25 μl) were added to 200 μl of incubation buffer and incubated at 37 °C. The final specific activity of the water was 186 cpm/nmol. A Beckman Model LS-350 scintillation counter was used for all tritium counting. After 38 h, the reaction was quenched with 25 μl of glacial acetic acid. Protein was separated from the incubation buffer and the bulk of the tritiated water by Sephadex G-25 chromatography (column dimensions: 4.91 cm x 90 cm; buffer: HCl, pH 2.5). Whenever possible, samples were maintained at pH 2-3 at room temperature or below, where back exchange of tritium was quenched.

Following Sephadex G-25 chromatography, the protein was prepared for proteolytic digestion to separate the histidine-containing peptides. Each sample was lyophilized twice from the pH 2.5 buffer and redissolved in 0.5 ml of HCl, pH 2.0. To improve the results of the subsequent enzymatic digestion, each sample was denatured by immersion in boiling water for 5 min. The heat-treated sample was lyophilized and redissolved in 250 μl of 20 mM ammonium bicarbonate buffer (pH 7.8). This solution was incubated at 37 °C, and 10-μl aliquots of chymotrypsin (1 mg/ml in 20 mM NH₄HCO₃) were added successively at 0, 45, and 85 min. (The chymotrypsin was less than 3% of the sample by weight.) After 180 min, the digestion was quenched by addition of one drop of glacial acetic acid. The digestion mixture was lyophilized, and redissolved in 50 μl of water.

The histidine-containing peptides were then separated on two-dimensional peptide maps. The entire sample was spotted on chromatographic paper, and the peptides were resolved by two-phase descending chromatography (acetic acid:butanol:water, 1:3.375:5, v/v/v, pH 3.5) followed by electrophoresis (pyridine:acetic acid:water, 1:2:20, v/v/v, pH 3.5; 53 V/cm; 80 min) (Bennett, 1967). The locations of the ninhydrin-staining spots containing histidine peptides were first identified by amino acid analysis of peptides from control maps using similarly digested trypsin. All analyses were run on a Beckman Model 120-C amino acid analyzer. Regions of the map containing histidine peptides were cut out and eluted with 10% formic acid. In order to quantitate amounts of histidine in each sample, 25 nml of norleucine was added to each sample as an internal standard. Seventy-five percent of each sample (375 μl in 10% formic acid) was added to 7 ml of Aquasol and counted for a minimum of 300 min. The remaining 25% of each sample was lyophilized and analyzed for amino acids. Peptides that did not contain histidine were treated identically and used to determine the background counts. The origin of each peptide map was found to have the amino acid composition of intact trypsin and was, therefore, analyzed to quantitate total tritium incorporation into trypsin.

The specific activity (cpm/nmol) of each sample was used to calculate a first-order rate constant for exchange using the expression:

\[ k = -\frac{1}{t} \ln \left( \frac{e - sa}{e} \right) \]

where sa is the observed specific activity after an incubation of time t (38 h), and e is the specific activity of the tritiated water used in the incubation (186 cpm/nmol). e corresponds to the expected specific activity of the histidines in the protein when equilibrium with the buffer is reached.

The rates of tritium exchange for the imidazoles in trypsin were found to be much lower than expected. Therefore, additional experiments were carried out to ensure that the tritium was incorporated into fully-active trypsin. In a longer exchange, using water of higher specific activity, 10 mg of trypsin was incubated at 37 °C in pH 7.4 buffer for 14 days. The specific activity of the incubation solution (225 μl) was 1840 cpm/nmol. The exchange was quenched and the protein was separated from the buffer and from the bulk of the tritiated water as described above. Four milligrams of the tritiated trypsin was divided into two portions. Eighty percent of the sample (3.2 mg) was mixed with 30 mg of purified STI in 1 ml of 0.1 M NH₄HCO₃, pH 7.8, and the STI-trypsin complex was purified on a Sephadex G-75 column (4.91 cm x 120 cm; buffer: 0.1 M NH₄HCO₃, pH 7.8) at 4 °C. The complex was then hydrolyzed in 6 N HCl and the individual amino acids were separated by high-voltage paper electrophoresis (pH 1.7, 7600 V, ~350 mA, 2 h (Dreyer and Bynum, 1967)). The histidine sample was eluted from the paper with 10% formic acid and its specific activity was determined. Arginine from the hydrolysate was treated similarly and used to determine the background counts.

The remaining 0.8 mg (20%) of the tritiated trypsin was not
complexed with STI. The sample was hydrolyzed and the specific activity of the isolated histidine was determined as described above for the STI-trypsin complex.

NMR Spectroscopy. In view of the low rates of tritium incorporation, nuclear magnetic resonance was used to determine the extent of possible labeling at the C-4 position. Deuterium exchange with the protons of L-histidine (0.05 M in 99% D₂O, pH 8.2, at 37 °C) was measured with a Varian HR-220 spectrometer. The proton resonances were assigned according to Schutte et al. (1966). The relative extent of exchange was measured by comparing the areas of the observed peaks and the C₄ proton resonance was used as an internal standard. Both continuous wave and Fourier transformed NMR spectra were observed after incubation times of 0.1, 0.6, 2, 3, 5, 8, 15, and 115 days.

Results

After incubation for 38 h in various buffer solutions containing tritiated water between pH 2 and 9, each sample was enzymatically digested, and the peptides were separated by chromatography and electrophoresis. Figure 1 is a photograph and a tracing of the ninhydrin-staining pattern that was typical of all of the two-dimensional peptide maps. The isolated peptides were products of digestion by both the added chymotrypsin and the fraction of the trypsin in the sample that was still active after boiling. The three histidines were found in different peptides: one containing His-40, one containing His-57, and two peptides of overlapping sequence containing His-91. The amino acid compositions of these peptides were used to determine their locations in the known sequence of bovine trypsin (Table 1).

Part of each histidine peptide sample was used to count the incorporated tritium, and part was used to quantitate the amount of histidine present. The specific activities determined from these measurements were then used to calculate pseudo-first-order rate constants for the exchange, k, according to eq 1. The rate constants for each histidine at each incubation pH are listed in Table II. These rate constants were used to extract pK₄ values for the imidazole side chains by least-squares fitting to a theoretical curve based on the exchange mechanism proposed by Vaughan et al. (1970). This mechanism involves the rate-determining formation of an ylide intermediate through abstraction of a proton from an imidazo-

<table>
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<th>TABLE I: Relative Molar Ratios of Amino Acids in Chymotryptic-Tryptic Peptides of Trypsin.</th>
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<td>Peptide</td>
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<td>Asx</td>
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<td>Phe</td>
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<td>His</td>
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| Sequence | Assignment | His40 to | Val57 to | Ser88 to | Leu99 to |
|----------|------------|----------|----------|----------|
| 91A | 40 | Ser88 | Val57 | His40 |

| a The numbers in parentheses are the values expected from the known sequence of bovine trypsin (Titani et al., 1975). b The N-terminal residue is partially destroyed by ninhydrin.

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<th>TABLE II: Pseudo-First-Order Rate Constants for the Incorporation of Tritium into the Histidines of Bovine Trypsin.</th>
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<td>pH</td>
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<tr>
<td>1.73</td>
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<tr>
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<tr>
<td>7.27</td>
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<td>8.98</td>
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</table>

a The pseudo-first-order rate constants, k, were calculated using eq 1 with t = 38 h and e = 186 cpn/mmol. All incubations were at 37 °C. b These values reflect the exchange of an unknown number of protons and, therefore, are not pseudo-first-order rate constants.
HISTIDINES IN BOVINE TRYPsin

\[
R\text{ Histidine} + H_2O \xrightarrow{k_a} R\text{ Histidine} + H_2O^+ \\
R\text{ Histidine} + OH^- \xrightarrow{k_b} R\text{ Histidine} + H_2O \\
R\text{ Histidine} + THO \xrightarrow{\text{fast}} R\text{ Histidine} + OH^-
\]

**Figure 2:** The tritium mechanism of tritium exchange into the C-2 position of imidazole (Vaughan et al., 1970).

The tritium cation by water \(k_a\) or by hydroxide ion \(k_b\) (Figure 2), and leads to the following rate equation:

\[
k = \frac{k_a[H^+][OH^-] + k_b[H_2O][H^+]}{K_a + [H^+]} \tag{2}
\]

where \(k\) is the pseudo-first-order rate constant and \(K_a\) is the ionization constant for imidazole.

The experimental data for the pH dependence of the exchange rates for histidine-40, -57, and -91, together with the least-squares fits to the above rate equation, are illustrated in Figure 3. The least-squares determined values of \(k_a\), \(k_b\), and \(pK_a\) for each histidine in Table III. \(k_b\) is the rate constant for the reaction pathway catalyzed by \(OH^-\), and determines the maximum exchange rate that will be observed at high pH. The values of \(k_b\) for the histidines of trypsin are much smaller than for the histidines in ribonuclease or lysozyme, or for small molecules that contain imidazole (Table III). The best fit to the data for His-57, the active site histidine, gives an apparent \(pK_a\) of 6.55. Because the specific activities for histidine-40 and -91 were only slightly above background, the \(pK_a\) assignments for these groups are tentative. The data suggest that exchange into His-40 may depend on two \(pK_a\)’s.

Tritium exchange rate data can frequently be fit by a simple function that describes the ionization of a single group; for example,

\[
k' = \frac{C \times 10^{(pH-pK_a)}}{1 + 10^{(pH-pK_a)}} \tag{3}
\]

or

\[
k' = C \left[1 - \frac{10^{(pH-pK_a)}}{1 + 10^{(pH-pK_a)}}\right] \tag{4}
\]

where \(C\) is a constant. The \(pK_a\)’s determined from such a fit will be similar, but not necessarily identical, to the apparent \(pK_a\)’s calculated by fitting eq 2 to the rate data (Table III).

The extent of tritium incorporation into the intact molecule (isolated from the origins of the peptide maps) was somewhat greater than the sum of the counts incorporated into all three histidines. The difference is presumably due to incorporation of tritium into other groups in the intact molecule that also back exchange slowly. The “rate constants” calculated using eq 1 (Table II) do not in this case reflect a single process.

Because the tritium-exchange rates for the histidines in trypsin are very low, lower than any previously observed, only a maximum of 1% of the trypsin had undergone exchange during the 38-h incubation. This low incorporation raised the question of whether only denatured trypsin had undergone exchange at the histidines because the trypsin used in the experiment was only \(\sim 95\%\) active. To eliminate this possibility, a second exchange experiment was performed. Water with higher specific activity (1840 cpm/nmol) and a longer incubation time (14 days) were used to increase the amount of label incorporated into the histidines. Soybean trypsin inhibitor was used to isolate “active” trypsin from inactive protein.

The trypsin labeled during the 14-day incubation at pH 7.14 was split into two portions. (Control experiments showed that the benzamide in the incubation buffer completely inhibited autolysis.) One portion of the trypsin was converted to a trypsin–soybean trypsin inhibitor complex and was purified by gel filtration (Figure 4). Complex formation was used as an assay for the “active” or inhibitor-binding trypsin in the sample. A comparison of the specific activities of the histidines in the uncomplexed and complexed trypsin (Table IV) shows that approximately 80% of the tritiated histidine was in “active” trypsin.

A comparison of the observed rate constant for the uncomplexed trypsin at pH 7.14 with the expected value based on the histidine exchange rates during the 38-h incubation (Table IV) reconfirms the very slow rate of exchange seen in the 38-h experiment, although the rates are not identical. The difference may be due to back-exchange of tritium out of imidazole during the 20-h acid hydrolysis (110 °C). The samples in the 14-day experiment were acid hydrolyzed before scintillation counting, whereas the samples that were counted in the 38-h experiment had not been acid hydrolyzed. An “observed” rate constant for back exchange during the acid hydrolysis step can be calculated from the expected value of the specific activity, 193.1 cpm/nmol (Table IV), and the observed value after acid hydrolysis, 87.5 cpm/nmol, according to

\[
k_{back} = (-1/r) \ln \left(\frac{87.5}{193.1}\right) \tag{5}
\]

where \(r\) is the time of hydrolysis (20 h). This “observed” rate
constant is $39.6 \times 10^{-3}$ h$^{-1}$.

Alternatively, the rate constant for back-exchange during acid hydrolysis can be roughly estimated from the rate of deuterium exchange at 65 °C and pH 0.26 (0.090 h$^{-1}$, average value from Vaughan et al., 1970) by applying corrections for the tritium isotope effect ($k_H/k_T \approx 18.2$) and the temperature dependence of the rate. The estimated value based on this calculation was $46 \times 10^{-3}$ h$^{-1}$. Because of the similarity between the observed and estimated rates of back exchange, it is reasonable to assume that the difference between the rate constants for the 14-day and 38-h experiments (Table IV) is due to back exchange during the acid hydrolysis of the 14-day sample. If this is the case, then the rate constants for the histidines in ribonuclease and lysozyme (Table III) may be too low because the samples were acid hydrolyzed before counting (Matsuo et al., 1972; Ohe et al., 1974).

Because of the slow-exchange rates, there was some question as to which proton on His-57 was exchanging. Although $^3$H or $^3$H exchange for the C-2 proton of imidazole is normally faster than exchange with the proton in the C-4 position (Matsuo et al., 1972), the C-2 proton of His-57 in trypsin is relatively solvent inaccessible, while the C-4 proton is solvent accessible (Stroud et al., 1971). Therefore, it seemed possible that the C-4 rather than the C-2 protons of His-57 might have exchanged during the incubations at 37 °C. Isotope exchange at the C-4 position of imidazole at 180 °C had previously been studied (Vaughan et al., 1970). To determine the relative rates of exchange at the C-2 and -4 positions under conditions similar to those used in the tritium experiment, deuterium exchange into L-histidine at 37 °C was investigated using NMR spectroscopy. The first-order rate constant for C-2 exchange was calculated by fitting an exponential to the data. The associated half-time of exchange, 2.8 days, is compared to the exchange rates for other histidines in Table II. In contrast to the C-2 exchange, there was no C-4 exchange for up to 115 days of incubation. Thus, the tritium exchange into His-57 of trypsin, which occurs with an apparent $pK_a$ of 6.55 and a half-time of 73 days, takes place at the C-2 position.

**Discussion**

All previously observed pH-rate profiles for $^3$H exchange at the C-2 of histidine show an increase in the rate of exchange as the pH is raised in the region of the $pK_a$ of the imidazole (Vaughan et al., 1970; Matsuo et al., 1972; Ohe et al., 1974). These profiles look similar to a pH titration curve and were so treated by Ohe et al. (1974). According to the zide mechanism, the exchange rate depends on the imidazolium ion and
<table>
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<th>Incubation Time (days)</th>
<th>Specific Activity (cpm/nmol)</th>
<th>Relative Tritium Incorporation (%)</th>
<th>$k$ ($h^{-1} \times 10^5$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total trypsin sample</td>
<td>14</td>
<td>87.5$^a$</td>
<td>100.0</td>
<td>14.5</td>
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<tr>
<td>ST1 binding component</td>
<td>14</td>
<td>72.5$^c$</td>
<td>85.7</td>
<td>12.0</td>
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<tr>
<td>His-57 (expected value)$^d$</td>
<td>193.1</td>
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$^a$ "Active" trypsin is operationally defined as that portion that is recovered complexed with ST1 after chromatography on Sephadex G-75 (see Figure 4). $^b$ This value is per nmol of trypsin, assuming three histidines per molecule. $^c$ This value is per nmol of trypsin–ST1 complex, assuming five histidines in the complex. $^d$ These values were calculated from the data for the 38-h incubation (Figure 3, Table II). $k$ was calculated using eq 2, a pH of 7.14, and the kinetic parameters in Table III. The specific activity ($\alpha$) was determined from eq 1 assuming $t = 14$ days and $e = 1840$ cpm/nmol.

Hydroxide ion concentrations. Therefore, the pseudo-first-order rate constant, $k$, can either increase, decrease, or remain unchanged as the imidazole titrates. Its behavior will depend upon the ratio of the hydroxide-mediated ($k_b$) and the water-mediated ($k_w$) rates of ylide formation. The water-mediated exchange rate controls the low-pH region of the profile, while the high-pH region depends on the hydroxide-mediated exchange. The $k_b$ for His-40 and His-91 are sufficiently small that the decrease in imidazolium ion concentration dominates the overall exchange. His-57 follows the trend set in previous studies and $k$ increases as the pH increases from 6 to 8.

The data for the exchange into His-40 (Figure 3, Table II) suggest that a second $pK_a$ near 2.7 affects the exchange of this group. Either the high $pK_a$ (6.9) or the low $pK_a$ (2.7) could be that of the imidazole ring of His-40, while the other apparent $pK_a$ could be due to a group or groups that perturb the exchange reaction. The curve representing the pH dependence of tritium exchange into His-40 in Figure 3 was calculated by fitting the data to an equation for the ylide exchange mechanism (eq 2) that includes a term to account for a perturbation by an ionizable group ($pK_a$):

$$k = \frac{10^{(pH-pK_a)} k_b[H^+][OH^-] + k_w[H_2O][H^+]}{K_a + [H^+]^2}$$

The histidines in trypsin exhibit the slowest imidazole tritium exchange rates yet reported. The kinetic parameters for exchange into the histidines of trypsin, lysozyme, ribonuclease, $N$-acetyl-$L$-histidine and $L$-histidine are listed in Table III. These parameters were derived by fitting eq 2 to the rate data. The rate constants for the hydroxide-mediated exchange ($k_b$) at the imidazoles of trypsin are approximately one to four orders of magnitude smaller than those of the other examples. The rate constants for the water-mediated exchange ($k_w$) listed in the table are based on the slow exchange that occurs in the low-pH region, and are too uncertain to be used for a detailed comparison. The slow exchange rate for His-40 can be explained by the relative inaccessibility of the C-2 proton to solvent in the trypsin structure. The side chain of His-91 lies in a depression on the surface of trypsin; however, there is no obvious explanation for why the exchange is very slow.

The slow exchange rate of His-57 can be explained by considering its local environment in the active site of benzamidine-inhibited trypsin (Krieger et al., 1974). The plane of the imidazole ring is approximately normal to the surface of the protein. The N-1 and N-3 nitrogen are hydrogen bonded to the carboxylate group of Asp-102 and the side chain of Ser-195, respectively. The C-2 proton points toward the center of the molecule and is sequestered from solvent by the Cys-42-$L$-disulfide bridge and the backbone of residues 57–58 above the ring and by Leu-99 and the backbone of 214–215 from below. One might expect that tritium exchange at the buried C-2 position of His-57 in the active conformation of the enzyme would be much slower than the exchange of His-91 because of greater steric hindrance. Exchange into the solvent-accessible C-4 position of the ring is very unlikely because there was no observable incorporation of deuterium into the C-4 position of $L$-histidine in $D_2O$ after a 115-day incubation at $37 ^\circ C$. pD 8.2.

The imidazole of His-57, however, can swing out into solution by disrupting the hydrogen bonds to Asp-102 and Ser-195 and by rotating about the $C_\alpha-C_\beta$ bond. Precedence for such a conformational change can be found in the structure of silver diisopropylphosphoryltrypsin. The silver ion induces a rotation of the imidazole about the $C_\alpha-C_\beta$ bond with little perturbation of the rest of the structure (Chambers et al., 1974). If the side chain of His-57 is involved in a conformational equilibrium between the active "in" position and an inactive "out" position, it might only exchange when it is in the out conformation. The tritium exchange would then detect the $pK_a$ of His-57 in the out position rather than in the active conformation. The exchange rate in the out position could be similar to that for small molecules containing imidazole, and, if this were so, the overall slow rate of exchange could be explained by an "in-out" equilibrium in which about 2% of the trypsin would have His-57 populating the out conformation at any one time. Because of the long duration of the tritium exchange experiment, the exchange due to the "in-out" swinging of the histidine imidazole ring cannot be distinguished from the slow exchange of the in form. This ambiguity is a characteristic drawback of a reaction that is as slow as tritium-hydrogen exchange at the C-2 position of histidine, and is an especially serious problem for the case of His-57 of trypsin, which exchanges at a very slow rate.

In addition to the ylide mechanism, there are other possible explanations for the tritium exchange into His-57. Of particular interest are mechanisms in which the ionization of a neighboring group affects the rate of tritium exchange of the imidazole. These mechanisms could be of two types: (1) those in which the "primary" pH dependence at pH 6.55 is due to the titration of the neighboring group and the titration of the imidazole itself is either not seen at all or is seen merely as a small perturbation on top of the "primary" effect, and (2) those in which the ylide mechanism is operative with the "primary" titration at pH 6.55 due to the imidazole titration, but with an additional perturbation in the pH dependence due to the titration of a neighboring group.

An example of the first type of mechanism would be one in
which neutral imidazole, rather than positively charged imidazolium, is the reacting species and the neighboring Asp-102 carboxylic acid side chain catalyzes the proton transfers. The form of the rate equation for such a mechanism is identical to that predicted by the ylide mechanism. However, the rate-determining step would no longer be proton abstraction.

To test whether the data were consistent with the second possibility, eq 2 was modified to incorporate a simple additional positive or negative titration curve (see eq 3, 4, and 6). No acceptable fit was found when the perturbations were included in the least-squares analysis.

In summary, the tritium exchange data for His-57 in bovine trypsin can be explained by the titration of only one ionizable group that affects the exchange rate. This ionization could be due either to His-57 with the in or with the out conformation, or to Asp-102 if the mechanism of exchange were different.

Conclusion

Several studies have provided evidence that Asp-102 is the group at the active site of the serine proteases which has a pK_a near 6.7 (Hunkapiller et al., 1973; Koepppe and Stroud, 1976). The pH dependence of the kinetics of substrate hydrolysis by the serine proteases shows that only the single pK_a of 6.7 affects the rate of hydrolysis between pH 2 and 8 (Kezdy and Bender, 1964; Fersht and Renard, 1974). This would lead to the conclusion that His-57 of trypsin has a pK_a below 2 in the active conformation. The tritium-exchange data would be consistent with this conclusion if histidine in the active form had a low pK_a and did not measurably exchange, but had a pK_a of 6.55 when swung out into solution in the exchangeable form. This interpretation seems to be the most reasonable one because the in conformation of His-57 appears completely inaccessible to proton exchange at the C-2 position, and because the imidazole ring can rotate out into solution with minimal disruption of the rest of the structure.

Acknowledgments

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References


