Mechanism of Hydrolysis by Serine Proteases: Direct Determination of the pK_a's of Aspartyl-102 and Aspartyl-194 in Bovine Trypsin Using Difference Infrared Spectroscopy†

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ABSTRACT: The pK_a of aspartyl-102 in trypsin is shown to be 6.8 ± 0.2 by difference infrared titration. All but 2.5 of the carboxyls in bovine trypsin were first modified with semicarbazide. The modified enzyme still retains full activity toward nonspecific substrates. The remaining free carboxyls include one equivalent each of Asp-102 and Asp-194. The absorbances associated with the C⁼⁼O and C=O stretching modes at 1570 and 1710 cm^{-1} were used to monitor the proportion of ionized or protonated carboxyl present in the enzyme as a function of pD. The pK_a of 6.8 was assigned to Asp-102 using copper ions that bind to trypsin between Asp-102 and histidine-57, so lowering the pK_{app} of Asp-102. The implication of this result for the ionization of the active site, and for the mechanism of serine proteases, is discussed. Asp-194 and the C terminus are shown to titrate with an average pK_a of 2.9.

The crystallographically determined structures of all of the pancreatic serine proteases (chymotrypsin, elastase, and trypsin) studied so far show that the catalytic site contains the hydroxymethyl group of Ser-195, the imidazole side chain of His-57, and the carboxyl of Asp-102.[1] Subtilisin of different evolutionary heritage contains the same arrangement of side chains at the catalytic site (Alden et al., 1970), thus emphasizing the seemingly universal efficacy of this particular arrangement of side chains in peptide hydrolysis by serine proteases.

Early studies showed that activity depended on a single group of pK_a between 6 and 7 for trypsin and chymotrypsin (Hammond and Gutfreund, 1955). More detailed results demonstrated that a pK_a near 7 controls both acylation and deacylation (Gutfreund and Sturtevant, 1956; Bender et al., 1962). This has usually been attributed to His-57 and, since reaction occurs best above pH 7, most mechanistic schemes have used His-57 first as a general base. One notable exception is the charge relay mechanism of Blow et al. (1969), which first gave functionality to Asp-102 as the base in the Asp-102–His-57 system. Jencks (1969), however, pointed out that the group with apparent pK_a of 7 could represent another group in the enzyme controlling conformation, or effecting a change in rate-determining step.

The assumption that His-57 was the catalytic group having a pK_a of 7 seemed to indicate a mainly structural role for the carboxyl group of Asp-102—perhaps in maintaining the orientation of the imidazole of His-57, or in stabilizing an imidazolium ion during the catalysis. Hunkapiller et al. (1973) presented strong NMR evidence that His-57 in α-lytic protease is not the group which titrates at pH 6.8, and assigned the titration to a neighboring group that they presumed to be Asp-102. We report direct evidence that Asp-102 is the group of pK_a 6.8 that controls catalysis.

Timasheff and Rupley (1972) demonstrated that infrared difference spectroscopy could be used to study the overall ionization of all of the carboxyl groups in lysozyme. Bovine trypsin contains eleven carboxyl groups (Titani et al., 1975), of which three are most easily identified as being specifically linked to substrate binding (Asp-189), conformation (Asp-194), and catalytic function (Asp-102). Trypsin (rather than any other serine protease) was chosen for this study because it contains fewer carboxyl residues than many of the other extracellular serine proteases, because its structure has been well-characterized (Stroud et al., 1971, 1974) and refined at 1.5-Å resolution (J. L. Chambers and R. M. Stroud, submitted), and because it is the most specific of the pancreatic enzymes. As a result, ionization of the active site in the many well known trypsin-inhibitor complexes can also be characterized.

Materials and Methods

Materials. Bovine trypsin from Worthington was 2× crystallized, salt free, lots 2JB and 33P685. D_2O was from Stohler. Semicarbazide hydrochloride (Matheson) was recrystallized from ethanol/water before use. 1-Ethyl-3-(3-dimethylaminopropyl)carbodiimde from Sigma and benzamidine hydrochloride (Aldrich) were used without further purification.

[^{14}C]SCZ[2] was prepared according to the procedure of Fersht and Sperling (1973), starting with potassium [^{14}C] cyanate (ICN) and aqueous hydrazine at pH 7. The reaction yielded 96% (of starting OCN^-) semicarbazide based on the 1,2-naphthoquinone-4-sulfonic acid assay (Fersht and Re-

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[1] The numbering system referred to is that of chymotrypsin (Walsh and Neurath, 1964).

[2] Abbreviations used are: [^{14}C]SCZ, [^{14}C]semicarbazide hydrochloride; [^{14}C]Gly-Et, [^{14}C]glycine ethyl ester hydrochloride; DTT, dithiothreitol; BzArgOEt, *N*-benzoyl-L-arginine ethyl ester; NPGB, *p*-nitrophenyl *p*′-guanidinobenzoate; Ac-Gly-NPE, *N*-acetylglycine *p*-nitrophenyl ester; Pipes, piperazine-*N*,*N*′-bis(2-ethanesulfonic acid); trypsin-SCZ, trypsin in which accessible carboxyl groups have been blocked with semicarbazide by means of a carbodiimide coupling procedure; DIP-trypsin, diisopropylphosphoryltrypsin.

quena, 1971); 4.3% of the initial OCN^- was recovered as bis(urea), a side product which after recrystallization had a specific activity of 215 ± 2.1 cpm/nmol. [^{14}C]Gly-Et (50 μCi) from New England Nuclear was diluted with cold carrier to a specific activity of 285 ± 6.8 cpm/nmol. All scintillation counting was done using "Aquasol" (New England Nuclear) in a Beckman Model LS-250 liquid scintillation counter. Reference solutions of known concentrations of [^{14}C]Gly-Et and bis([^{14}C]urea) were used to correct for counting efficiency.

Schwarz/Mann ultrapure urea was freshly dissolved to 10 M concentration, deionized on a column of Dowex AG501-X8D, and then immediately diluted to 8 M in the reaction mixture. Dithiothreitol, histamine, thyrotropin-releasing hormone (Calbiochem), ethylenimine (Eastman), L-histidine, glycine, and glycyl-L-histidylglycine (Sigma) were used without further purification. Normal solutions of "DCl," "DNO_3," and "NaOD" were prepared as described by Timasheff and Rupley (1972) by dissolving the appropriate reagent in D_2O.

Substrates used to assay trypsin activity were BzArgOEt (Schwarz/Mann), NPGB (Cyclo), and Ac-Gly-NPE (Cyclo, recrystallized before use). Pipes, used in buffers, was from Calbiochem. All other chemicals were of reagent grade.

Preparation of Trypsin–SCZ. It can be difficult to determine individual pK_a values from a titration curve that is the sum of eleven separate overlapping curves. The problem was simplified by modifying accessible carboxyls with semicarbazide using the carbodiimide reaction of Hoare and Koshland (1967) and the procedure of Fersht and Sperling (1973). The reaction has been shown to modify surface carboxyl groups while not affecting those which are buried inside trypsin (Eyl and Inagami, 1971) or chymotrypsin (Carraway et al., 1969; Fersht and Sperling, 1973).

Special precautions were taken to stabilize the active conformation of the enzyme and to prevent autolysis during the semicarbazide labeling procedure: (a) 12 mM benzamidine was present throughout, and (b) the time of reaction was 3 h, somewhat shorter than used by Fersht and Sperling for chymotrypsin. Semicarbazide hydrochloride (11.1 g; 1 M) and benzamidine hydrochloride (190 mg; 12 mM) were dissolved in 100 ml of H_2O containing enough NaOH to bring the pH to 3.7. Six-hundred milligrams of trypsin was added. The reaction mixture was stirred for 3 h at room temperature and 1.2-g portions of the carbodiimide reagent were added at time zero and again after 1 h. At the end of the 3-h reaction period, the solution was extensively dialyzed against 12 mM benzamidine at 4 °C, and then lyophilized to yield 583 mg of white powder.

Infrared Spectra. Trypsin (400 mg) or trypsin-SCZ (400 mg) was dissolved in 8 ml of D_2O containing 12 mM benzamidine and left overnight at 4 °C for solvent hydrogen exchange. The sample was then lyophilized, reconstituted in 8 ml of D_2O containing 6 mM NaCl, or 6 mM $Cu(Cl_2)$, or 2 mM $Cu(Cl_2)$ with 4 mM NaCl, as appropriate, adjusted to pD 7.8 with 1 M NaOD, and filtered through a Millipore membrane (0.45 μ). The pD of the bulk solution was adjusted in stepwise fashion from 7.8 to 1.5 using 1 M DCl, and 400-μl aliquots were removed at each step change in pD. Unfortunately, trypsin is not soluble enough to give good ir spectra above pD ~ 7.8; therefore, the concentration of enzyme was that of a saturated solution (native or modified enzyme) at pD 7.8 (~1.5 mM), samples of which were pH adjusted and used throughout the entire pD range. Protein concentration was determined using the extinction coefficient for trypsin at 280 nm (A_{280} = 1.54 for 1 mg/ml, and molecular weight of 23 891 (Robinson et al., 1971)), and was always near 36 mg/ml (1.5 mM).

The pD values referred to above are 0.4 unit higher than the ones actually read on a Beckman pH meter (Glasoe and Long, 1960). This effect is almost exactly compensated by the fact that the pK_a's of carboxyls are observed about 0.5 unit higher in D_2O than in H_2O (Glasoe and Long, 1960). Therefore, we are omitting both corrections in reporting our results; that is, we are using direct pH meter readings and unaltered pK_{app}'s. These values are too low for the enzyme in D_2O, but accurately reflect the situation in H_2O.

Difference spectra were recorded from pairs of accurately balanced solutions of benzamidine trypsin (native and semicarbazide modified) of different pD on a Perkin-Elmer Model 225 infrared spectrophotometer, or on a Beckman Model 4240 infrared spectrophotometer interfaced to a Fabri-Tek Model 1062 computer, to permit averaging of repetitive scans. Samples were contained in matched sealed Perkin-Elmer cells of path length 0.125 or 0.150 mm, or in a variable path cell, all equipped with BaF_2 windows. Baseline controls were run using solutions of identical pD in the two cells. The temperature of the samples in the infrared beam was kept somewhat below room temperature by blowing chilled dry air onto the windows and by circulating ice-water through copper coils that were attached to the cells.

Enzymatic Activity. The activity of trypsin-SCZ was determined for specific and nonspecific substrates, and compared to "native" (Worthington) enzyme under the same conditions. One specific assay used the difference in absorbance at 253 nm between *N*-benzoyl-L-arginine and its ester (Schwert and Takenaka, 1955). Another assay involved use of NPGB in a specific titration of active sites (Chase and Shaw, 1967).

Nonspecific activity was determined by following the increase in absorbance at 412 nm due to hydrolysis of Ac-Gly-NPE. The substrate was dissolved in ethanol, the enzyme in 1 mM HCl at pH 3, and aliquots of each were injected into the Pipes buffer. Final conditions were: 2.1×10^{-6} M enzyme, 1 mM substrate, 50 mM Pipes, pH 7.0, 25.0 °C. Correction was made for the spontaneous hydrolysis of substrate in the absence of enzyme. The effect of 0.01 M benzamidine on both native and modified enzyme activities was tested.

Identification of Free Carboxyls in Trypsin-SCZ. In order to identify those buried carboxyl groups that had not reacted in the coupling of semicarbazide to native trypsin, trypsin-SCZ (70 mg) was unfolded in 8 M urea and newly exposed carboxyls were labeled with [^{14}C]SCZ. To assure complete unfolding, the six disulfide bridges of trypsin were first reduced according to the procedure of Konigsberg (1972), and then aminoethylated by the method of Raftery and Cole (1963). Part of the sample was saved for a control thermolysin digestion (see below) and part was used for the radioactive labeling of previously unmodified carboxyls.

In order to assay for the amounts of each carboxyl modified, [^{14}C]SCZ or [^{14}C]Gly-Et was coupled to the remaining free carboxyls in reduced *S*-aminoethyltrypsin-SCZ by procedures similar to those of Carraway et al. (1969) and Fersht and Sperling (1973). An aliquot of each labeled protein solution was saved for scintillation counting and A_{280} measurement. The remaining samples were lyophilized prior to digestion with thermolysin.

Three batches of modified *S*-aminoethyl trypsin-SCZ were then available: a "cold" sample that still had free those carboxyl groups that are buried in native trypsin, and two radioactively-labeled samples, one containing [^{14}C]SCZ and the other containing [^{14}C]Gly-Et attached to the previously buried

carboxyls. Ten milligrams of each protein sample was digested by 60 μg of thermolysin (Matsubara and Sasaki, 1968).

To determine which peptides contained the label, each sample of thermolytic peptides was subjected to two-dimensional chromatography and electrophoresis at pH 6.5 (Bennett, 1967). At least three maps were run for each sample. Autoradiography was used to locate spots with labeled peptides, on both ninhydrin-stained maps and unstained maps from the same sample. Radiograms were made using Kodak RP-54 medical x-ray film placed in contact with the maps for 5–18 days.

In order to define the location of labeled residues in the trypsin sequence, peptides were eluted from the paper using 10% aqueous NH_4OH. The specific activity of each peptide was determined by scintillation counting and by quantitative amino acid analysis on a Beckman Model 120-C or a Durrum Model D-500 amino acid analyzer.

Results

Catalytic Activity of Semicarbazide-Modified Trypsin. The modified enzyme (trypsin–SCZ) was tested for activity toward specific and nonspecific substrates. The NPGB (initial burst) results showed that there were 11 ± 3% of the theoretical number of active sites, and the BzArgOEt (overall rate) results showed an activity of 8 ± 0.2% of the activity of unmodified enzyme. The agreement between the two results indicates that the action of trypsin–SCZ toward specific substrates can be completely accounted for by the modification of Asp-189 in 88–92% of the molecules, and that any other carboxyl groups that had been modified have no effect on activity. The use of the active site titrant NPGB shows that the 8–12% residual activity is in fact due to molecules having normal binding pockets, and is not merely nonspecific hydrolysis by blocked trypsin.

The catalytic activity of trypsin–SCZ toward the nonspecific substrate Ac-Gly-NPE was 50% greater than the activity of native trypsin. This result is not surprising for trypsin, when a small molecule is bound in the specific side chain binding pocket. Inagami and York (1968) have observed similar enhancements of activity upon binding of small side chain analogues such as methylguanidine to trypsin. The activity of trypsin–SCZ toward Ac-Gly-NPE was diminished only 10% by the presence of 10 mM benzamidine, a fact which further confirms that the binding pocket has been blocked in 90% of the molecules. The pH–rate profile for hydrolysis of Ac-Gly-NPE was measured and depended on a pK_a of 6.8.

The blocking of Asp-189 in the trypsin binding pocket was also observed by Eyl and Inagami (1971) when they coupled glycinamide to accessible carboxyl groups using the carbodiimide procedure at pH 4.75. At that pH the modification of Asp-189 was shown to be inhibited by 0.4 M benzamidine, which binds in the specificity pocket (Krieger et al., 1974). In our hands, a much lower concentration of benzamidine (12 mM) did not prevent reaction of Asp-189 during carbodiimide coupling of semicarbazide at pH 3.9. These results are expected and consistent, since benzamidine binding has been shown to depend on a pK_a of 4.6 (East and Trowbridge, 1968). In our case, both the lower inhibitor concentration and the neutrality of Asp-189 act to reduce benzamidine binding. The size and geometry of the semicarbazide group in the binding pocket of trypsin were estimated by building the blocking group into a wire model of the active site region. The minimum distance from the blocking group to the active-site serine hydroxyl is 7.3 Å in the model. Therefore, no close contact with the catalytic groups or with nonspecific substrates is anticipated.

Identification of Remaining Free Carboxyl Groups in Trypsin–SCZ. A sample of trypsin–SCZ was denatured to expose free carboxyls that had escaped modification in the blocking reaction. The reduced *S*-aminoethylated trypsin–SCZ was dissolved in 8 M urea. The carbodiimide reaction was again used to couple [^{14}C]Gly-Et or [^{14}C]SCZ to the remaining free carboxyls. Each protein molecule incorporated 2.57 ± 0.09 molecules of SCZ label, or 2.53 ± 0.12 molecules of Gly label. Thus, the two labeling tests are in agreement and show that 2.5 carboxyl groups remained unblocked in trypsin–SCZ. However, the number 2.5 could be too low, since trypsin–SCZ could contain contaminating material that absorbs at 280 nm. The amount of possible contaminant is nevertheless probably quite small, since the trypsin–SCZ was extensively dialyzed, and since the number of active sites in native trypsin from Worthington was found to increase from 70 to 95% of the theoretical value after chromatography on Sephadex G-25.

Thermolysin was used to digest *S*-aminoethylated trypsin–SCZ, both before and after incorporation of each of the two radioactive labels. The peptides were separated on two-dimensional peptide maps. The analyses of the critical peptide spots are summarized in Tables I and II.

Negatively charged peptides at pH 6.5 released from digestion of trypsin–SCZ necessarily contain a free carboxylate side chain. Only two such peptides were obtained (C-1 and C-2), and they are unambiguously identified as peptides containing Asp-102.

The maps generated from the digest of [^{14}C]SCZ-labeled trypsin–SCZ (shown in Figure 1) were autoradiographed and used to quantitate the extent of labeling of Asp-102 and Asp-194. The results showed the peptide Leu_{99}–Asp_{102} (Z-102) incorporated 1.02 ± 0.1 moles of [^{14}C]SCZ. The peptide Leu_{185}–Val_{199} (Z-194) incorporated 0.97 ± 0.1 mole of [^{14}C]SCZ. This implies that one equivalent of Asp_{102} and one of Asp_{194} were ir titratable in trypsin–SCZ.

The peptide Leu_{185}–Val_{199} actually contains three acidic side chains that are potential candidates for carrying the [^{14}C]SCZ label. However, the studies of substrate specificity of trypsin–SCZ (see section above) exclude Asp-189 as a possibility. The three-dimensional structure of trypsin that shows Glu-186 exposed to solvent and Asp-194 buried, and the fact that Carraway et al. (1969) and Fersht and Sperling (1973) have shown that Asp-194 in chymotrypsin will not react with carbodiimide unless the enzyme is denatured, indicate that the assignment of Asp-194 as the radiolabeled group is correct.

The peptides generated from [^{14}C]Gly-Et-labeled trypsin–SCZ were treated similarly and confirmed that Asp_{102} and Asp_{194} incorporated the majority of the label. The only remaining radioactive spot (G-245) was assigned to the carboxyl terminal sequence of trypsin, Ile_{242}–Asn_{245}. This peptide did not appear as a single spot after [^{14}C]SCZ-labeling; therefore, it was not possible to quantitate the extent of labeling at this site. Nevertheless, Asn-245 must be responsible for most of the remaining 0.5 mol of radioactivity, although Asp-189 presumably contains ~0.1 mol of label, and other groups could have been labeled to small extents. Thus, the modified enzyme trypsin–SCZ contains 2.5 free carboxyl groups, and these are identified as Asp-102, Asp-194, and about 50% of the molecules that have the C-terminus unblocked.

Infrared Spectra. A complete set of infrared spectra are available as supplementary material (see paragraph at the end of the paper). They are also available from the authors, or in the Ph.D. Thesis of Koeppe (1976).

Table I: Relative Molar Ratios of Amino Acids in Thermolytic Peptides of Trypsin–SCZ.[a]

Amino Acid	Peptides						
	C-1	C-2	G-102	G-194	G-245	Z-102	Z-194
Asp	3.00 (3)	2.30 (3)	3.00 (3)	2.00 (2)	1.00 (1)	3.66 (3)	2.00 (2)
Thr	0.05	0.02			0.14		
Ser	0.07	0.11	1.01	2.25 (2)	0.89 (1)		2.27 (2)
Glu	0.09	0.11	2.11	2.05 (2)	0.25	1.38	1.82 (2)
Pro			0.79	1.01 (1)	0.26		0.86 (1)
Gly	0.07	0.11	2.02 (1)	5.96 (6)	0.93 (1)	0.95	5.35 (5)
Ala			0.63		0.99 (1)		
Val			0.69	1.61 (1) or (2)	0.35	0.34	1.39 (1) or (2)
Met		1.14 (1)					
Ile		1.00 (1)	0.57		0.34 (1^b)	0.18	
Leu	0.26 (1^b)	1.23 (2^b)	0.91 (1^b)	0.56 (1^b)	0.38	1.00 (1)	0.79 (1)
Tyr			0.61		0.14		
Phe					0.12		
His					0.22		
Lys				1.03 (1)	0.14		1.17 (1)
Arg					0.21		

[a] The codes for the peptides refer to: C, peptides of trypsin–SCZ having a net negative charge from a pH 6.5 map stained with ninhydrin; G, radioactive peptides from trypsin–SCZ that had been denatured and labeled with [^{14}C]Gly-Et, pH 6.5 map stained with ninhydrin; Z, trypsin–SCZ, denatured and labeled with [^{14}C]SCZ, radioactive peptides from an unstained pH 6.5 map. The numbers in parentheses are the expected values for amino acid composition based on the sequence assignments for the peptide fragments as listed in Table II. [b] N-terminal residue, partially destroyed by ninhydrin.

Table II: Peptide Assignments Based on the Data in Table I and the Sequence of Titani et al. (1975)

	Peptide	Contaminant
C-1	Leu_{99}-Asn-Asn-Asp_{102}	None
C-2	Leu_{99}-Asn-Asn-Asp_{102}-Ile-Met-Leu	None
G-102	Leu_{99}-Asn-Asn-Asp_{102} | *Gly-Et	Yes
Z-102	Leu_{99}-Asn-Asn-Asp_{102} | *SCZ	Val_{76}-Glu-Gly-Asn-Glu_{80} | | SCZ SCZ
G-194 or Z-194	Leu_{185}-Glu-Gly-Gly-Lys-Asp_{189}-Ser-Cys-Gln-Gly-Asp_{194}-Ser-Gly-Gly-Pro-Val-(Val) | *(Gly-Et or SCZ)	None
G-245	Ile_{242}-Ala-Ser-Asn_{245}-*Gly-Et	Yes

(*a*) *Benzamidine Trypsin.* The most notable features of the direct spectrum of trypsin dissolved in D_2O are the broad band centered at 1640 cm^{-1} due to absorbance by peptide backbone amide carbonyl groups, and the band at 1450 cm^{-1} that shows extensive deuteration of the peptide N–H groups. The absorbance values at the frequencies that will be of greatest interest in the difference spectra below are 0.6 at 1600 cm^{-1} and 0.8 at 1680 cm^{-1}.

Difference spectra were recorded in the range from 1800 to 1500 cm^{-1} using pairs of samples with equivalent protein concentration from a series of protein solutions of varying pD.[3] One example is shown in Figure 2A. At 1710 cm^{-1} there is a peak representing the excess population of protonated carboxyl groups present in the low-pH sample, while at 1570 cm^{-1} there appears a peak of opposite sense representing the corresponding excess of ionized carboxyls in the high-pH sample. By plotting the heights of the peaks at either frequency from a series of spectra obtained using solutions of various different pD's, one can obtain a titration curve that is the sum of the titration curves of all of the carboxyl groups in the enzyme, as was done by Timasheff and Rupley (1972) for lysozyme. Because of variations in the baselines, the peak at 1710 cm^{-1} was easier to measure than the peak at 1570 cm^{-1}. Differences in baselines are attributed to small differences in protein concentrations and in HOD content between the reference and sample cells. Measurements were made by converting the values of percent transmittance to absorbance at the baseline and at the maximum of each 1710-cm^{-1} peak. The differences in absorbance were plotted on a graph in which the absorbance at pD 1.5 was arbitrarily set equal to zero (Figure 3). The graph is the sum of the titration curves of the carboxyl groups of bovine trypsin.

(*b*) *Infrared Spectra of Trypsin–SCZ.* Difference spectra were recorded in the same way as for unmodified trypsin.[3] Benzamidine was present in all solutions to prevent autolysis by the 10% of molecules that still had binding pockets containing unblocked Asp-189. Difference infrared spectra recorded without a variable path length reference cell often do not allow quantitation of the COO^- peak, since any small difference in path length will be reflected in the amide I band, centered at 1645 cm^{-1}, and magnified by the total number of amides (~220) in the trypsin molecule.

In the low-pH region, an absorption due to COOH appears

[3] Spectra referred to in text are available as supplementary material or with explanatory text from the authors upon request.

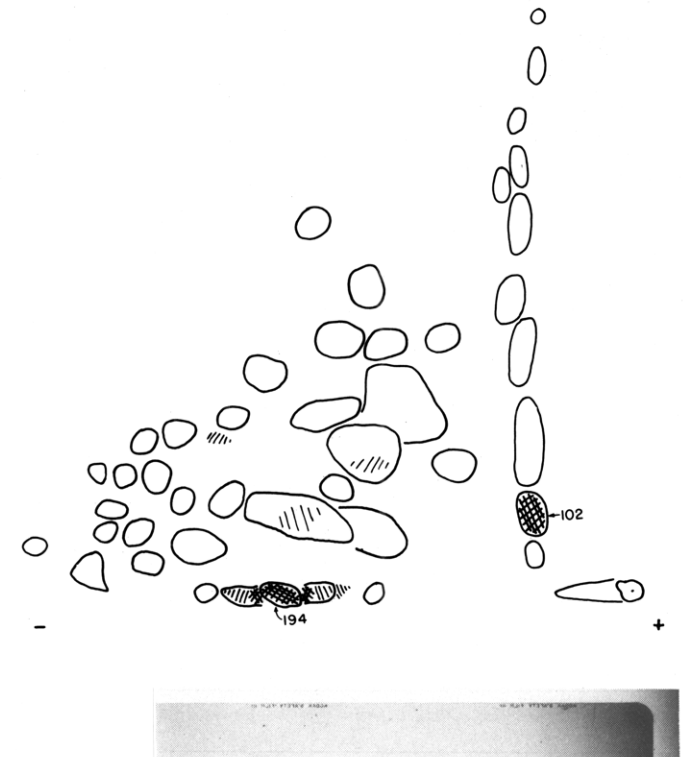


FIGURE 1: Autoradiogram and drawing of peptide map of [^{14}C]SCZ labeled *S*-aminoethyltrypsin-SCZ. The origin is in the lower right-hand corner of the map. Descending chromatography in the vertical direction was followed by electrophoresis in the horizontal direction. The two major radioactive peptides contain 1 mol of ^{14}C-label per mole of Asp-102 and Asp-194, respectively. In this case the C terminus, which was identified by labeling with [^{14}C]Gly-Et, did not appear in a single cleanly digested peptide, but was scattered over the map.

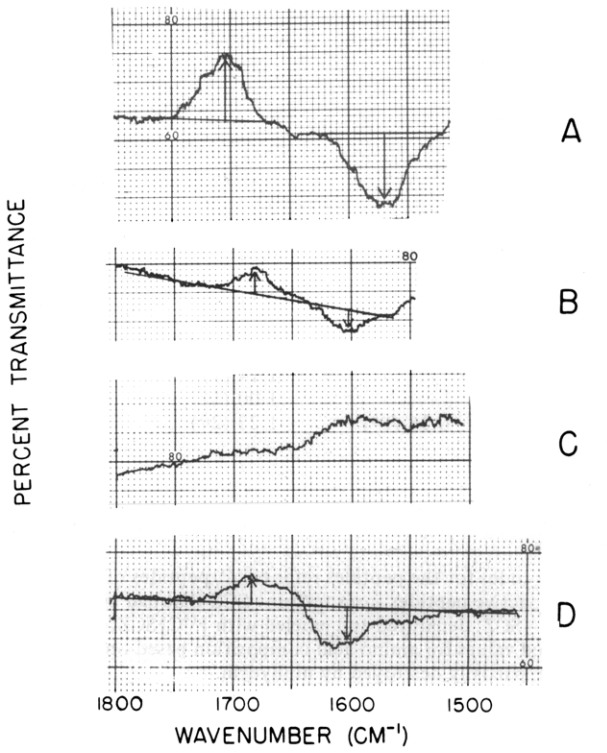
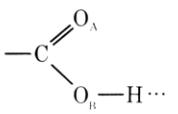


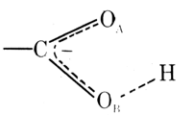
FIGURE 2: Difference infrared spectra recorded on a Perkin-Elmer Model 225 spectrophotometer. (A) 1.5 mM Trypsin, 12 mM benzamidine, 6 mM NaCl. Path length 0.125 mm. Sample pD 6.4, reference pD 3.4. (B) 1.5 mM trypsin-SCZ, 12 mM benzamidine, 6 mM NaCl. Path length 0.15 mm. Sample pD 7.13, reference pD 6.50. (C) 1.5 mM trypsin-SCZ, 12 mM benzamidine, 6 mM $CuCl_2$. Path length 0.15 mm. Sample pD 7.5, reference pD 6.1. (D) 1.5 mM trypsin-SCZ, 12 mM benzamidine, 6 mM $CuCl_2$. Path length 0.15 mm. Sample pD 5.4, reference pD 4.8.

at the usual frequency (1710 cm^{-1}) and disappears between pH 2 and 3.5 as the titration occurs (Figure 4, left). Between pH 3.5 and 6, the infrared spectrum of trypsin-SCZ contains no pH-dependent peaks in the region from 1800 to 1500 cm^{-1} (Figure 4, center). Between pH 6 and 7.5, difference peaks appear at frequencies that are shifted by 30 cm^{-1} from the usual frequencies observed for carboxyl groups. An example of a difference spectrum in this region showing peaks at 1600 and at 1680 cm^{-1} is shown in Figure 2B.

Frequencies of 1680 and 1600 cm^{-1} are reasonable for a carboxylic acid that is strongly hydrogen bonded. If the protonated acid were in a position to act as an unusually strong hydrogen bond donor, then the H-O_B bond distance would be a little longer than normal, the C-O_B bond distance would be a little stronger than a single bond, and the C=O_A bond



(whose stretching is normally observed at 1710 cm^{-1}) would be a little weaker than a normal double bond and would absorb radiation of a lower energy, namely 1680 cm^{-1}. By the same token, a carboxylate base which was hydrogen bonded would experience an uneven distribution of charge on the two oxygens and a nonequivalence of bonds C-O_A and C-O_B. In this case,



the symmetric stretch would be expected at higher energy than the normal 1570-1590-cm^{-1} range, and the asymmetric stretch at lower energy than the normal 1410 cm^{-1}. Frequency shifts of 30-40 cm^{-1} from the usual positions are common for carbonyl groups which are hydrogen bonded (Susi, 1972).

The data from a number of difference spectra of solutions of trypsin-SCZ in D_2O at various pD's from 1.7 to 7.3 were combined to obtain the titration curve shown in Figure 4. The data from peaks at 1710 and at 1680 cm^{-1} were plotted on the same scale after independent conversion to absorbance units. Two titrations can be seen, and both are steeper than would be expected for single or noninteracting groups. Nevertheless, the slope of the titration having its midpoint at pH 2.9 is 1.5 times the slope of the titration at high pH. The curve cannot be extended beyond pH 7.5 because of the decreased solubility

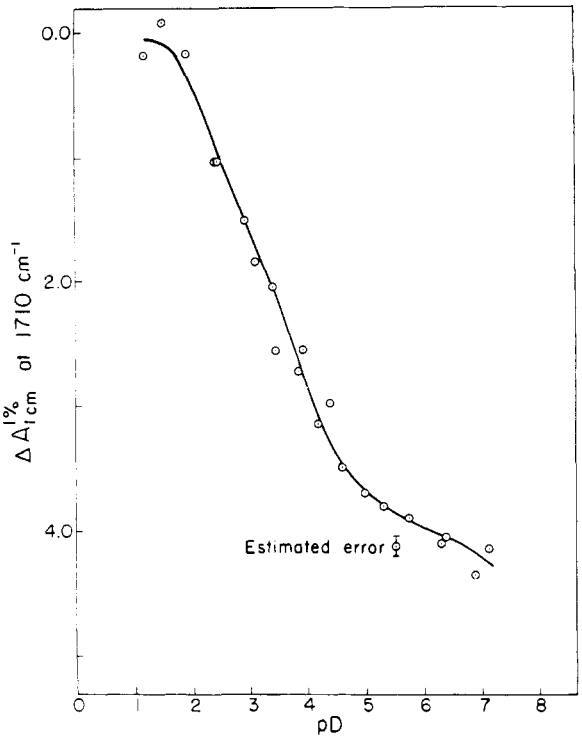


FIGURE 3: Titration curve for the carboxyl groups of bovine trypsin. Data are taken from the heights of the peaks in a series of difference infrared spectra that are available upon request (see paragraph at end of paper)

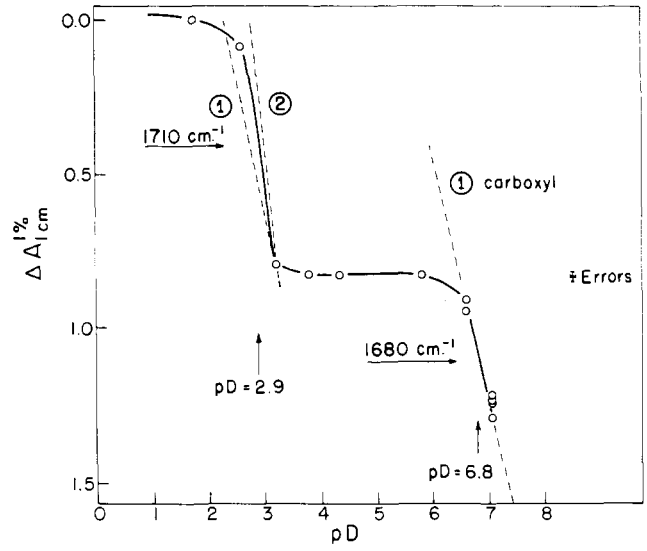


FIGURE 4: Titration curve for the carboxyl groups of trypsin–SCZ. Data are taken from the peak heights in a series of difference infrared spectra which are available upon request (see paragraph at end of paper), or from the authors. Of the total of 2.5 carboxyl groups/molecule, 1.5 titrate with an average pK_a of 2.9, while one carboxyl titrates with a pK_a of 6.8.

of trypsin at high pH, so one cannot see the endpoint of the second titration. However, the ratio of the slopes of the two titrations, 1:1.5, and the total of 2.5 carboxyl groups in the trypsin–SCZ molecules suggest that the low-pH titration represents 1.5 carboxyl groups, while the titration at high pH is due to a single carboxyl group. In addition, the observation of an absorption at an unusual frequency (1680 cm^{-1}) can most reasonably be assigned to a single group in an unusually strong hydrogen-bonded environment. If the low-pH titration is assumed to represent the titration of 1.5 groups, the expected endpoint (and therefore the midpoint) of the pH 6.8 titration can be estimated. The midpoint occurs at pH 6.8.

The average intensity of the absorption by one carboxyl group in trypsin can be determined from Figure 3 using the total of eleven carboxyl groups (ten of which absorb at 1710 cm^{-1}) in the entire molecule (Titani et al., 1975). When this average intensity is compared with Figure 4, the low-pH limb of the titration curve is found to correspond to 2.0 carboxyl groups of "average" intensity. Thus, the extinction coefficients for carboxyls in different environments are not directly comparable, a conclusion also proven by the control experiments discussed below.[3]

(*c*) *Trypsin–SCZ in the Presence of Copper (Cu^{2+}) ions.* The use of copper ions as a specific probe permits one to assign the pK_a of 6.8 to Asp-102 rather than to either Asp-194 or Asn-245. In the presence of 2 or 6 mM $CuCl_2$ the difference infrared spectrum of 1.5 mM trypsin–SCZ no longer shows peaks between pH 6 and 7.5 (Figure 2C), but instead the peaks at 1680 and 1600 cm^{-1} appear between pH 4 and 5.5 (Figure 2D). The titration of pK_a 6.8 is shifted to lower pH due to competition between Cu^{2+} ions and protons. The observed pK_{app} depends on the binding constant for copper and the concentration of copper, as well as upon the intrinsic pK_a in the absence of copper. If the system is described by the following equilibria:

$$E^- + Cu^{2+} = (E^-)(Cu^{2+}),\ pK_1 = 4.0 \text{ (Berezin et al., 1967)}$$

$$E^- + H^+ = E\text{-}H,\ pK_a = 6.8$$

then the observed pK_{app} will be the pH at which the ratio

$$\frac{E\text{-}H}{(E^-) + (E^-)(Cu^{2+})}$$

is equal to $0.5\{K_a \text{ (apparent)} = K_a \text{ (true)}(1 + [(Cu^{2+})/K_1])\}$. This apparent p$K_a$ will be 5.5 for the above ionization constants with 2 mM Cu^{2+}. This formulation assumes that Cu^{2+} does not bind to the E–H form, since Cu^{2+} binding has been shown to depend upon a group of pK_a of 6.8 (Martinek et al., 1969).

The location of the Cu^{2+} binding site is derived from crystallographic studies of Ag^+ or Cu^{2+} ion binding to DIP-trypsin (Chambers et al., 1974) or to trypsinogen (A. Kossiakoff, L. Kay, and R. Stroud, in preparation), and from the fact that Cu^{2+} and Ag^+ are inhibitors of trypsin and chymotrypsin that compete with each other (Martinek et al., 1969). An Ag^+ ion binds between His-57 and Asp-102 in two crystalline forms of DIP-trypsin and in trypsinogen. Cu^{2+} (17 mM) binds at a single major site in crystalline DIP-trypsin at pH 6.9, and this site is the same as was found for silver binding (J. Chambers, R. Koeppe, L. Kay, and R. Stroud, unpublished). Therefore, Cu^{2+} presumably inhibits trypsin by binding between Asp-102 and His-57.

(*d*) *Spectra of Control Compounds.* Since 1680 and 1600 cm^{-1} are not the most common frequencies for carboxyl group absorption, one may question whether an imidazole side chain could give rise to these bands in the infrared spectra. Being an aromatic ring, imidazole does indeed absorb between 1600 and 1700 cm^{-1}, although the extinction coefficient for this absorption is much lower than for carbonyl absorption. Therefore, several compounds containing imidazole were used to test for the presence of any histidine-dependent titrations that would be seen in this region of the infrared spectrum.[3]

Neither imidazole nor histamine at 20 mM concentration produces any peaks in a difference spectrum over the pH range 4.5–10.5 between the frequencies 1800 and 1500 cm^{-1}. This observation rules out the possibility that infrared absorption

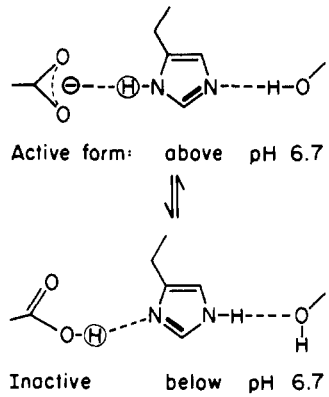


FIGURE 5: State of ionization of the active-site residues above and below pH 6.8.

by a titrating imidazole could give rise to the peaks at 1680 and 1600 cm^{-1} in difference spectra of trypsin–SCZ. Therefore, a carbonyl group of trypsin–SCZ is the only species that could give peaks between 1600 and 1700 cm^{-1} that are strong enough to be seen with the concentration of enzyme being used.

The question of whether a carbonyl absorption can be perturbed by titration of a neighboring group is a more interesting one. This phenomenon was studied using glycine and/or histidine in D_2O solutions. For the case of glycine, titration of the amino group does affect the position and intensity of the glycine carboxylate infrared absorption. This is presumably an electron-withdrawing effect of the ammonium group, on the carboxyl of glycine. With histidine the same effect is seen when the amino group titrates. In addition, there is a further perturbation of the α carboxyl due to titration of the imidazole, although this situation does not occur in the protein where there is no α carboxyl.

Free histidine in solution is not a good model for histidine in a protein because the amino and carboxyl groups are not involved in amide bonds. The tripeptides Gly-His-Gly and pyro-Glu-His-Pro-amide (thyrotropin-releasing hormone) provide examples of histidine in peptides. Gly-His-Gly contains two peptide carbonyl groups that absorb at 1655 cm^{-1} and the carboxyl terminal that absorbs at 1585 cm^{-1} at pD 4.5 in a direct spectrum with D_2O in the reference cell. Difference spectra show that titration of the imidazole of Gly-His-Gly changes the intensity of the amide carbonyl absorption band. The ionization state of neither the amino terminal nor the imidazole ring has any effect on the infrared absorption of the carboxyl terminal of Gly-His-Gly. The intensity changes that are seen in the control compounds such as glycine and Gly-His-Gly indicate that absolute extinction coefficients are meaningless for carboxyl or carbonyl groups in unusual environments such as proteins.

The infrared peaks due to the amide carbonyl groups of pyro-Glu-His-Pro-amide in which imidazole is the only ionizable species are also affected by imidazole titration, but only to a very small extent. The difference spectrum for the entire pH range from 3.0–10.3 is biphasic—unlike a simple difference spectrum. The intensities of the two larger peaks (at 1675 and 1620 cm^{-1}) are less than 5 and 22% of the intensities seen for trypsin–SCZ. This low intensity would seem to rule out a consideration of histidine perturbation of a neighboring carbonyl being responsible for the difference infrared spectrum of trypsin–SCZ.

The most telling control experiment is the enzyme itself. In the presence of 2 mM Cu^{2+} ions the peaks disappear from the pH 6–7 region and reappear at lower pH. Two of the histidines (His-40 and His-91) titrate with apparently normal pK_a's between 6 and 7 (Markley and Porubcan, 1976; Krieger et al., 1976). Yet no difference peaks are seen between pH 6 and 7 for 2 mM Cu^{2+} with 1.5 mM trypsin–SCZ. The mole ratio of copper to enzyme is 1.3:1, so since Cu^{2+} binds between Asp-102 and His-57 with $K_I = 10^{-4}$ M, there is no possibility of copper binding stoichiometrically to the other histidines, although some nonspecific copper binding has been observed (Berezin et al., 1967). Thus, the infrared difference peaks seen at 1680 and 1600 cm^{-1} for trypsin–SCZ must be due to a single group on the enzyme that binds copper. The intensities of the peaks require that they be due to a protonated and an ionized carboxylic acid, respectively, and the frequencies of the peaks are the ones expected for a strongly hydrogen-bonded carboxylic acid such as Asp-102.

Discussion

The slopes of both limbs of the titration curve of trypsin–SCZ are steeper than would be expected for single or noninteracting groups. The explanation could involve difficulties in obtaining the intensities of the infrared peaks—such as the use of peak heights rather than areas, or the insensitivity of the instruments to the very small peaks that should be present between pH 3.5 and pH 6, but which were not observed.

The pK_a's of the 1.5 groups (Asp-194 and the carboxyl terminal of Asn-245) contributing to the low-pH portion of the curve were not resolved, although both titrate close to pH 2.9. A pK_a near 2.9 is in agreement with previous suggestions of a pK_a of 3 for Asp-194 that accompanies a pH-dependent conformation change in δ-chymotrypsin having a pK_a of 3 (Garel et al., 1974). A lower than normal pK_a would be expected for a carboxyl group in a buried salt bridge. pH 2–3 is the normal range for a pK_a of an α-carboxyl group, such as the C terminal of Asn-245, so again the assignment is expected.

The pK_a of Asp-102 in trypsin is shown to be 6.8 and this pK_a, rather than that of His-57, is responsible for the low limb of the pH–activity profile. The enthalpy of ionization of the rate-controlling group of pK_a 6.8 has been determined and is 5.0 kcal/mol for acylation and 4.1 kcal/mol for deacylation of α-chymotrypsin (Rajender et al., 1971). These values are intermediate between the expected enthalpies of ionization in water solution for an imidazole ring (7 kcal/mol) and a carboxylic acid (1 kcal/mol, Greenstein and Winitz, 1961). The enthalpy of ionization of Asp-102 in trypsin is therefore 3–4 kcal/mol higher than that of a free carboxyl group in solution. There is some precedent for this, since a similar value of ΔH_{ioniz} = 3.5 kcal/mol has been reported for Asp-52 of lysozyme (Parsons and Raftery, 1972).

The pK_a of His-57 in trypsin is still unknown, although there is evidence that the imidazole ring of His-57 in a bacterial serine protease remains neutral between pH 8 and 4 (Hunkapiller et al., 1973). Kinetic measurements show that the pH–rate profile for acylation and deacylation of trypsin or chymotrypsin can be satisfactorily explained by a single pK_a of 6.8 between pH 8 and 2–3 (Kezdy et al., 1964; Stewart and Dobson, 1965; Fersht and Renard, 1974). Therefore, His-57 must have a pK_a below 2–3 in chymotrypsin and trypsin. One can only conclude that it is exceedingly difficult to protonate the solvent-inaccessible δ nitrogen of His-57, and that the high stability of the active center structure simply prevents this from occurring above pH 2–3.

The ionization of the active site residues at pH 6.8 is as described in Figure 5. Below pH 6.8 the system as a whole takes

up a single proton, and this is relayed via the imidazole to Asp-102. The proportion of imidazolium ion at pH 7.0 is probably less than 10^{-4}. Thus, Asp-102 is the base of $pK_a \sim 7.0$ responsible for the low limb of the pH–activity profile. The mechanistic implications of this assignment for Asp-102 have been discussed by Hunkapiller et al. (1973) and by Stroud et al. (1975).

During formation of the tetrahedral intermediate, a proton is transferred from the hydroxyl group of serine 195 to the ϵ nitrogen of His-57. The expectation then is that the proton in the hydrogen bond to Asp-102 and attached to the δ nitrogen of His-57 will be in fast exchange and consequently will be immediately transferred to Asp-102. Two proton transfers would then be associated with the formation of the tetrahedral intermediate. These transfers could take place in either a stepwise or a concerted manner. A concerted scheme would seem to involve intermediates of lower energy, for then at no stage would there be a significant amount of positive charge anywhere in the system. The usual entropic disadvantage of concerted events would in this case be overcome by the precise alignment of the residues on the enzyme. Such concerted proton transfer in solution would require a simultaneous four-body encounter (substrate, Ser, His, and Asp), and therefore could not occur. By bringing all of the reactants together in the proper orientation, the enzyme can provide access to an energetically favorable reaction pathway that would otherwise be impossible due to an enormous entropy of activation.

A small but apparently primary deuterium isotope effect, k_H/k_D in the range of 2–3, has been observed for both the acylation and deacylation steps during hydrolysis by chymotrypsin (Bender and Hamilton, 1962; Pollock et al., 1973). The isotope effects so far reported are consistent with either stepwise or concerted transfers of the two protons, since contributions of secondary isotope effects to the rates obtained using mixed isotopic solvents are unknown (Kresge, 1973).

Henderson (1971) has shown that methyl chymotrypsin has about $1/5000$ of the chymotrypsin activity, and that activity depends on a group of $pK_a \sim 7.0$. As the pK_a of 6.8 normally belongs to Asp-102, one wonders what the group of pK_a 7.0 could be in the methyl enzyme where the ϵ nitrogen is no longer available to transfer the proton. Henderson (1971) proposed that residual activity could be retained if the imidazole were to swing out into the solvent, or even to reverse its orientation in the active site, leaving the δ nitrogen free to participate in catalysis. Either way, the pK_a of Asp-102 can no longer be relayed to the active site via histidine because the hydrogen bonds are necessarily broken, and the pK_a of 7.0 is not unreasonable for methylimidazole swung out into aqueous solution.

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Supplementary Material Available

Infrared spectra of the compounds studied are available (8 pages). Ordering information is given on any current masthead page.

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

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ABSTRACT: At pH 8.9 and 37 °C the half-times for tritium exchange with the C-2 protons of the histidines of trypsin are 73 days for His-57, and greater than 1000 days for His-40 and His-91. These half-times are much longer than the half-life of exchange for the C-2 proton of free histidine (2.8 days at pD 8.2), and longer than any previously reported half-time of exchange at pH > 8. These very low rates of exchange are discussed with reference to the refined structure of trypsin. The tritium exchange of His-57 depends on an apparent pK_a of 6.6. This pK_a may represent the pK_a of the imidazole of His-57 in an inactive conformation of the enzyme.

The catalytic sites of all serine proteases contain three amino acid side chains that are essential for enzymatic activity: Ser-195, His-57, and Asp-102.[1] The mechanism by which these enzymes hydrolyze peptides, amides, or esters involves nucleophilic attack by the serine hydroxyl group on the susceptible carbonyl carbon of the substrate. The histidine and aspartic acid side chains may be regarded as a coupled hydrogen-bonded system that promotes the reaction by general base catalysis (Bender and Kezdy, 1964; Inward and Jencks, 1965). The base facilitates proton transfers among the reacting species, first accepting the serine hydroxyl proton during the nucleophilic attack, and later donating a proton to one of the products of hydrolysis.

This paper reports an attempt to determine the pK_a's of the three histidines of bovine trypsin using the isotope exchange method of Ohe et al. (1974). This technique involves incubating the enzyme in tritiated water at various pH's, digesting the protein, separating the histidine-containing peptides, and determining the extent of the isotope incorporation into each histidine.

The kinetics of deuteration of imidazole (Vaughan et al., 1970) and of *N*-acetylhistidine (Matsuo et al., 1972) in aqueous solutions have been studied as a function of pH. The pH dependence of the exchange rate can be explained by a mechanism that involves a rate-determining abstraction of the C-2 proton by OH^- or by H_2O, followed by a fast protonation (Vaughan et al., 1970). Markley and Cheung (1973) showed that proton abstraction is involved in the rate-determining step during tritium exchange into the tripeptide Gly-His-Gly. The pK_a of the imidazole ring can be determined by fitting the pH-exchange data to the rate equation derived from the mechanism of Vaughan et al. (1970).

The rate of exchange at the C-2 of imidazole is intermediate between the rates for fast-exchanging O-H and N-H protons, and nonexchanging C-H protons. Therefore, the uniqueness of the rate of C-2 exchange allows the specific labeling of the histidine rings of a protein in tritiated water (Matsuo et al., 1972). The tritium can be incorporated under mild, nondenaturing conditions (37 °C, pH 2–10), and the labile protons can be back-exchanged, thus leaving only histidines labeled. Studies of the pH dependence of tritium incorporation into the histidines of lysozyme (Matsuo et al., 1972) and ribonuclease (Ohe et al., 1974) have been used to determine the pK_a's of the histidines in these proteins.

Our experiments involved measuring the pH dependence of the rate of tritium incorporation into each histidine of bovine

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[1] The numbering system referred to is that of chymotrypsinogen.