Proton Magnetic Resonance Titration Curves of the Three Histidine Residues of Staphylococcal Protease†

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ABSTRACT: Proton magnetic resonance spectra of staphylococcal protease, a serine protease from *Staphylococcus aureus*, strain V8, are presented. Initial proton spectra were obtained at 220 MHz, and more detailed studies of the aromatic region were carried out by correlation spectroscopy at 250 MHz. The overall spectrum bears a close resemblance to one calculated from the sum of spectra of the component amino acids. Chemical shifts of the three tyrosine, four phenylalanine, and three histidine residues appear to be equivalent at pH 3.7 and 8.5 indicating that they are all in normal chemical environments in the enzyme. The staphylococcal protease contains a large number of slowly exchanging protons. In fact, interpretable spectra of the aromatic region were obtained only after extensive exchange of N–H groups with deuterium from the D_2O solvent. Proton magnetic resonance titration studies of the three histidine residues indicate that these have normal chemical shifts and pK' values. When the data are fitted to single noninteracting titration curves, the histidine pK' values are 7.19 ± 0.02, 6.85 ± 0.03, and 6.69 ± 0.02. The titration curves of two of the histidine residues indicate negative cooperativity. A possible explanation for this is a direct electrostatic interaction between the two histidines. The titration data for these histidines give a significantly better fit to such a mutual interaction model than to noninteracting titration curves. The component microscopic dissociation constants have been calculated. Mutual interaction leads to pK' displacements of 0.31 unit, which indicates a distance of approximately 7 Å between the two interacting histidine rings according to the model of Tanford and Roxby. The proton resonances of the two interacting histidines are doubled in the pH region 6.7–7.0 suggesting the presence of two forms of the enzyme having lifetimes in excess of 30 msec.

The purification of an extracellular proteolytic enzyme from culture filtrates of *Staphylococcus aureus*, strain V8, has been reported (Drapeau *et al.*, 1972). The protease from the V8 strain is composed of a single polypeptide chain of molecular weight 12,000. Amino acid analysis indicates an unusually high content of dicarboxylic amino acids or amides (over 30%) and of proline residues (10%) and the complete absence of cysteine (Table I). The protease is active in the pH range of 3.5–9.5 and exhibits two activity maxima at pH 4.0 and 7.8 with hemoglobin as substrate. The enzyme is believed to be a serine protease since it is inhibited by diisopropyl fluorophosphonate (Dip-F),[1] an organophosphorus inhibitor specific for enzymes with a serine residue at the catalytic site and is not inhibited by EDTA. It has also been shown to react specifically with a spin-label analog of Dip-F at only one serine residue (Dugas and Gaudet, 1974). This protease has also been shown to have an unusual specificity since it cleaves peptide bonds specifically on the carboxyl-terminal side of either aspartic or glutamic acid in phosphate buffer (pH 7.8). Subsequent studies (Houmard and Drapeau, 1972) have demonstrated that the specificity is further restricted to only glutamyl bonds in either ammonium bicarbonate (pH 7.8) or ammonium acetate buffer (pH 4.0).

More recently, several proteases having a similar specificity have been isolated from other strains of *Staphylococcus aureus*. Some of these have molecular weights similar to that of the protease from the V8 strain (Beaudet *et al.*, 1974) while others have molecular weights in the range 21,500–24,000 and 29,000–30,000 (Ryden *et al.*, 1974). The amino terminal residue of all these proteases is valine, whereas the carboxyl-terminal has proved to be glutamic acid in all cases studied. It has been proposed (Beaudet *et al.*, 1974) that the variations in molecular weight are the result of various degrees of autodigestion at the carboxyl-terminal. For this reason, the protease from the V8 strain used in this study will be referred to as the staphylococcal protease active fragment of molecular weight 12,000.

The low molecular weight and stability (Drapeau *et al.*, 1972) of the staphylococcal protease active fragment make it ideally suited for nuclear magnetic resonance (nmr) studies. The histidine region of the spectrum is of particular interest because histidine appears to play a catalytic role in all serine proteases homologous with trypsin and chymotrypsin and in thiol proteases related to papain. We report here proton magnetic resonance spectra of the staphylococcal protease active fragment that reveal some general aspects of its structure in solution. A detailed ^1H nmr titration study of the three histidine residues of the enzyme has yielded information about their local environments and individual proton dissociation constants.

Experimental Procedures

Materials. Staphylococcal protease was prepared from culture filtrates of *Staphylococcus aureus*, strain V8, as de-

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[1] Abbreviations used are: Dip-F, diisopropyl fluorophosphonate; DSS, sodium 2,2-dimethyl-2-silapentane-5-sulfonate.

scribed previously (Drapeau *et al.*, 1972) and was homogeneous on polyacrylamide gel. The enzyme (8–10% w/v) was lyophilized once in 0.1 or 0.2M phosphate buffer of appropriate pH and three times in D_2O (99.7% Merk, Sharp & Dohme, Canada, or Bio-Rad, or 100% Diaprep, Aldrich). No autodigestion was found to occur and no obvious changes in viscosity were observed at these high concentrations. Those enzyme samples that were preexchanged with D_2O were heated in the 0.1M phosphate buffer (pH 5.0) to 80° for 3 min. Previous studies (Drapeau *et al.*, 1972) have shown that the staphylococcal protease active fragment is resistant to heat inactivation.

Methods. pH measurements of solutions in D_2O were made at 25° using a Corning digital 112 pH meter with an Ingold 6030 micro combination electrode. The meter was calibrated with two standard pH buffers in H_2O that bracketed the measurement. All pH values given are actual meter readings uncorrected for the deuterium isotope effect at the glass electrode (−0.40 unit) (Glascoe and Long, 1960). The symbol pH* is used to indicate the uncorrected pH meter reading of the D_2O solution using a glass electrode standardized with H_2O buffers. (It has been found (Roberts *et al.*, 1969) that histidine pK' values determined directly from uncorrected glass electrode readings in D_2O agree well with those determined in H_2O. The isotope effect on the imidazole pK (Li *et al.*, 1961) is apparently equal and opposite to glass electrode effect.) The error introduced by the systematic drift to the emf when the electrode is transferred from an H_2O to a D_2O solution caused by replacement of H_2O by D_2O in the gel layer of the electrode (0.03 unit over 3 hr) (Bates, 1968; Lowe and Smith, 1973) was minimized by making the measurements in D_2O as rapidly as possible and by extrapolating the readings back to the time the electrode was transferred to D_2O. The pH was measured before and after nmr spectra were taken; the spectral data were used only if these measurements agreed within 0.03 pH unit. Adjustment of pH was made by adding 1M NaOD or 1M DCl by means of a micrometer syringe (Gilmont) and fine Teflon needle (Hamilton). No significant denaturation appears to take place if the acid or base is added slowly with rapid mixing; 5-mm precision nmr samples tubes (Wilmad Glass Co., no. 528 PP) were used.

The 220-MHz ^{1}H nmr spectra were taken with a Varian HR-220 (Canadian 220 MHz Nmr Center, Ontario Research Foundation, Sheridan Park, Ont.) with sample temperatures of about 18°; 250-MHz ^{1}H nmr spectra were obtained using a superconducting correlation spectrometer (Dadok and Sprecher, 1974) (Nmr Facility for Biomedical Studies, Carnegie-Mellon University, Pittsburgh, Pa.) with sample temperatures of 30°. The 250-MHz spectra reported are averages of 500 1.5-sec scans of a 1.5-kHz region. A filtering factor of 0.5 Hz was used resulting in an instrumental broadening of all peaks by 0.5 Hz. Reported line widths have been corrected for this.

Sodium 2,2-dimethyl-2-silapentane-5-sulfonate (DSS) at a concentration of 0.5–1% was employed as an internal reference in the 220-MHz spectra. The small resonances at −0.64, −1.77, and −2.90 ppm (Figure 1) belong to the reference but do not interfere to any appreciable extent with the rest of the spectrum. The 250-MHz spectrometer was locked on the HDO peak. A coaxial capillary insert (Wilmad) containing 5% $(CH_3)_4Si$ in CCl_4 was used to check the calibration of spectra, and chemical shifts are given relative to this reference.

The nmr titration data were fitted by a nonlinear least-squares program to the Hill equation where δ_{H^+}, δ_{H^0}, and

$$\frac{\delta_{H^+} - \delta_{obsd}}{\delta_{H^+} - \delta_{H^0}} = \frac{Ka^n}{Ka^n + [D_3O^+]^n} \quad (1)$$

δ_{obsd} are respectively the chemical shift of the histidine residue in the fully protonated, neutral, and observed intermediate forms, Ka is the dissociation constant of the histidine, n is the Hill coefficient, and $[D_3O^+]$ is the hydrogen ion concentration as determined from pH* measurement. Both four-parameter fits (Ka, δ_{H^+}, δ_{H^0}, and n free) and three-parameter fits (n fixed equal to 1) were tried (Markley, 1973). The notation pK' is used throughout the text to represent the apparent pK obtained from the nmr titration curves.

Results

General Features of the ^{1}H Nmr Spectrum. Single scan 220-MHz ^{1}H nmr spectra of the staphylococcal protease active fragment are shown in Figure 1a–c. The protein α C–H peaks are obscured by the strong line from residual HDO solvent at −4.8 to −4.9 ppm. The spectrum of native enzyme at pH* 5.6 (Figure 1a) is compared with those of partially denatured enzyme at pH* 10.6 (Figure 1b) and fully denatured enzyme in 0.1M NaOD (Figure 1c). The amino acid composition of the staphylococcal protease fragment (Drapeau *et al.*, 1972) is given in Table I. The enzyme contains approximately 525 nonexchangeable protons. A calculated spectrum of the staphylococcal protease based in ^{1}H nmr spectra of the component amino acids at neutral pH at 40° is shown in Figure 1d. The α C–H peaks have been omitted in the calculated spectrum. With the exception of the α CH region such a calculated spectrum is expected to be a good approximation to that of the denatured random-coil protein at this pH and temperature (McDonald and Phillips, 1969).

It can be seen in the *single scan* 220-MHz spectrum of the staphylococcal protease fragment determined at pH* 5.6 that the majority of proton types are fairly well resolved. It is particularly striking to note that the chemical shifts of the main peaks of the native enzyme are very close to the values of the peaks for the partially and fully denatured enzyme and to those of the individual amino acids shown in the computed spectrum. Table II summarizes the spectral assignments and only relevant features of the assignment are presented below.

The benzylic protons of phenylalanime and tryosine, the proline (δ-CH), the arginine (δ-CH_2), and lysine (ϵ-CH_2) protons expected to appear in the −3.00 to 3.30-ppm region are not observed in the protein spectra. They are probably shifted upfield under the methylene peaks of aspartic acid and asparagine around −2.75 ppm. This latter peak is resolved into at least two sharp and two broad peaks in 0.1M NaOD. The resonance at −2.00 ppm exhibits a strong intensity with a narrow half-line width of 35 Hz indicating that a sizable fraction of these residues are exposed to solvent, and therefore are probably at the surface of the enzyme molecule. Higher resolution of these protons is observed in the spectrum of the denatured enzyme in 0.1M NaOD. The single methionine methyl proton of the enzyme also is predicted to appear at this position, but it could not be resolved from the contributions of the other proton types. The intensity of the methyl resonance at −0.93 ppm (line width 50 Hz) is much weaker at pH* 5.6 than in the spectra of the partially or completely denatured enzyme suggesting either dipolar broadening or displacements of the chemical

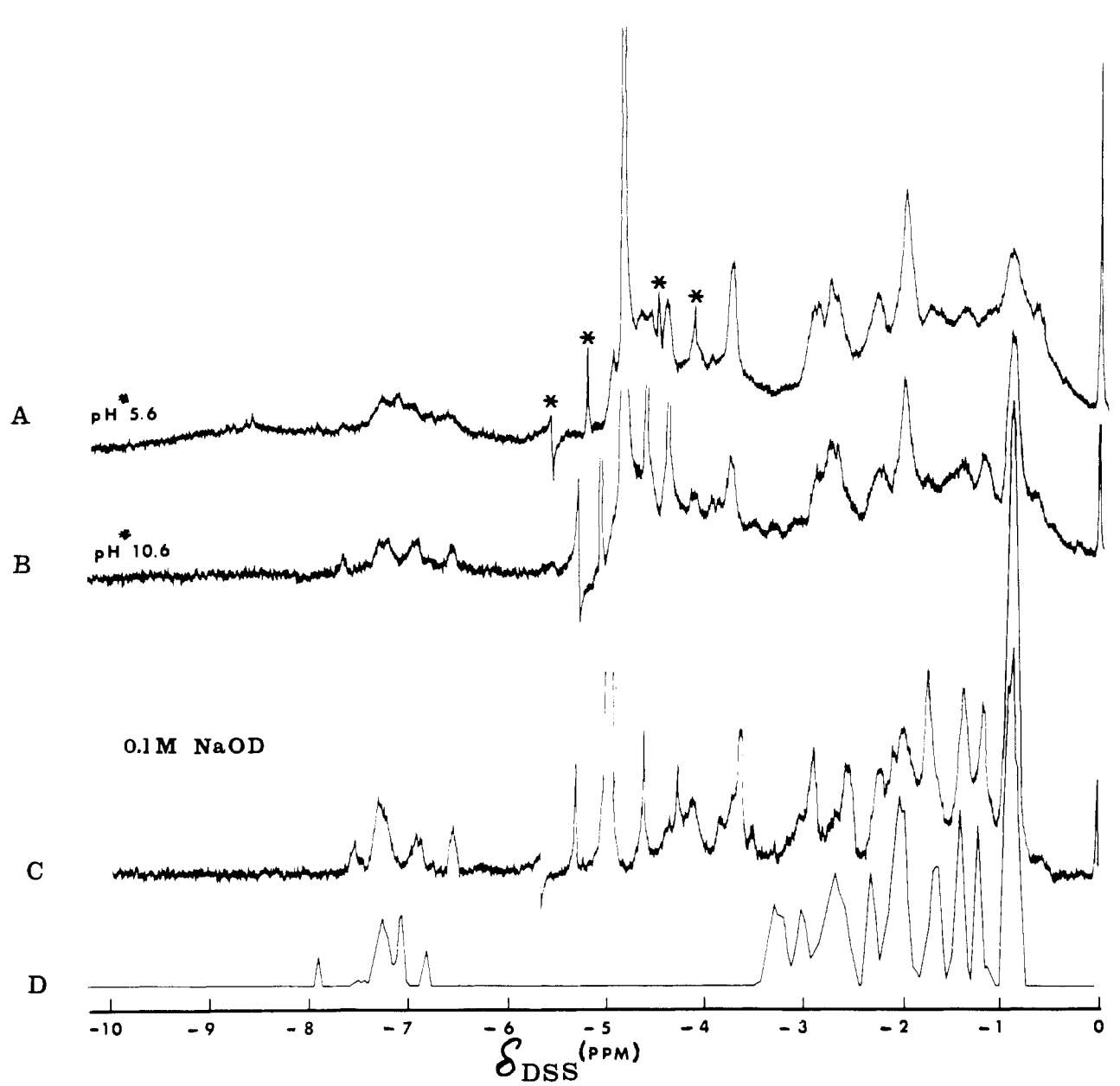


FIGURE 1: The 220-MHz ^1H nmr spectra of the staphylococcal protease active fragment at 18° in 0.2M phosphate buffer: (A) pH* 5.6; (B) pH* 10.6; (C) in 0.1M NaOD; (D) computer simulated spectrum based on spectra of the component amino acids at neutral pH. Spinning side bands from the sharp HDO signal at −4.8 to −4.9 ppm are marked with asterisks. The sharp peak at 0 ppm and the small sharp peaks at −0.64, −1.77, and −2.90 ppm in spectra A-C come from the internal reference DSS.

shifts resulting from the tertiary structure of the enzyme. The sharpening of the methyl peaks as the enzyme is partially and fully denatured suggests that the methyl groups become more equivalent or have more freedom of movement as the protein unfolds. As expected, resonances appear better resolved in the computed spectrum than in the actual spectrum of the denatured enzyme because by simulating the resonances by triangles, the intensity in the wings is neglected (McDonald and Phillips, 1969). No high-field resonances were detected above DSS.

Resonances from the ring protons of the aromatic amino acids (phenylalanine, tryptophan, and tyrosine) and from the C(4) ring proton of histidine are located in the −6.6 to 7.3-ppm region. The tyrosine ring proton resonances in the enzyme spectra taken at pH* 10.6 and in 0.1M NaOD lie upfield of the corresponding peaks in the computed spectrum because of titration shifts.

By varying the temperature from 10 to 45° a gradual narrowing of the line widths of all resonances, particularly the methyl peak at −0.93 ppm, was observed, but in no cases were any appreciable chemical shifts observed, and no resonances emerged from the background.

Unfortunately, it was not possible to obtain spectra of intact enzyme at temperatures higher than 45° due to extensive autodigestion (Drapeau *et al.*, 1972). Attempts in using Dip-F-inhibited enzyme in order to obviate this difficulty were not successful because aggregation was found to occur at higher temperatures. In a parallel experiment, the addition of 6M urea to an enzyme solution at pH* 6.2 resulted in a narrowing of line widths. The narrowing was less marked than in the spectrum taken at pH* 10.6 with the exception of the aromatic proton region. No chemical shift displacements were observed.

Analysis of the Aromatic Region, Preexchange with D_2O. Correlation ^1H nmr spectra of the staphylococcal protease active fragment exchanged under normal conditions

Table I: Amino Acid Composition of the Staphylococcal Protease Active Fragment.[a]

Neutral amino acids		Basic amino acids	
Glycine	9	Arginine	1
Serine	4	Lysine	6
Proline	11	Aromatic amino acids	
Alanine	7	Histidine	3
Threonine	8	Phenylalanine	4
Valine	7	Tryptophan	1
Leucine	4	Tyrosine	3
Isoleucine	6	Sulfur-containing amino acids	
Acidic amino acids		Cysteine	0
Aspartic acid	29	Methionine	1
Glutamic acid	10		

Total: 114 amino acids

[a] This table is an adaption from Drapeau *et al.*, 1972.

Table II: Summary of the General Features of the ^{1}H-Nmr Spectrum of Staphylococcal Protease Active Fragment.

Chemical Shifts (ppm from DSS)	Assignments
−0.93 (50-Hz line width)	CH_3 of Ile, Leu, and Val
−1.00 to −2.00	CH_3 of Ala and Thr (well resolved in 0.1 M NaOD)
−2.00 (35-Hz line width)	β-CH_2 of Glu, Pro, and Arg
−2.30	β-CH_2 of Gln (better resolution in 0.1 M NaOD)
−2.75	CH_2 of Asp, Asn; benzylic CH_2 of Phe and Tyr; δ-CH_2 of Pro, Arg; ϵ-CH_2 of Lys (better resolution in 0.1 M NaOD)
−3.80	CH_2 of Gly and Ser (not present in the computed spectrum)
−7.3	Aromatic protons of Phe
−7.7 (at pH* 10.6)	H(2) protons of His
−6.6	Ortho protons of Tyr
−7.0	Meta protons of Tyr

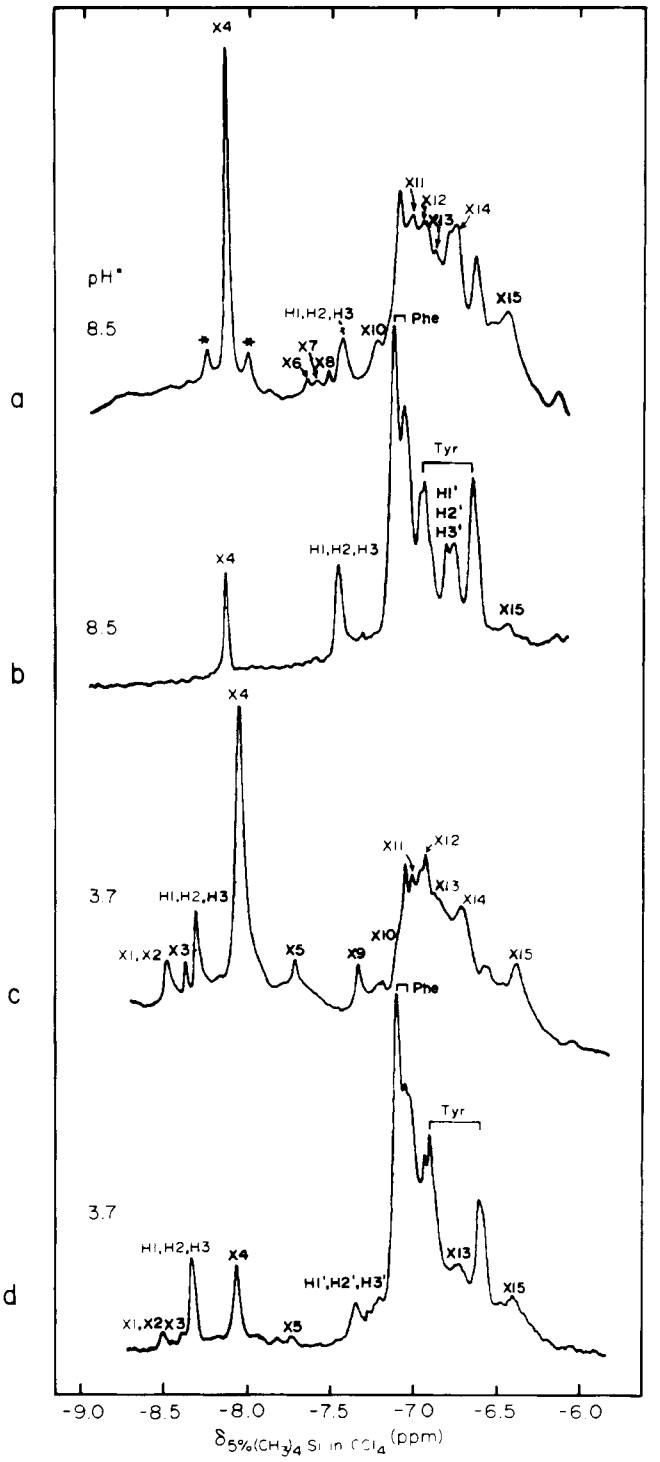


FIGURE 2: Simplification of the aromatic ^{1}H nmr spectrum of the staphylococcal protease active fragment obtained by preexchange of the enzyme: (a and c) enzyme lyophilized three times from D_2O; (b and d) enzyme preexchanged by heating in D_2O 3 min at 80° at pH* 5.0. pH* values of D_2O solutions in 0.1M phosphate buffer: (a) 8.51; (b) 8.45; (c) 3.72, (d) 3.69. Assignments H(1)-H(3), His C(2)-H; H(1')-H(3'), His C(4)-H; X(1)-X(15) slowly exchanging N-H groups; *, spinning side bands. (Spectra a and c are shown at a lower gain than spectra b and d.)

at room temperature are compared with spectra of the enzyme preexchanged 3 min at 80° at pH* 5.0 in Figure 2. Considerable simplification of the aromatic region is achieved by the preexchange procedure.

Peaks corresponding to slowly exchanging protons are readily identified by comparing spectra of the normally exchanged and preexchanged protease. These peaks are labeled (X(1)-X(15)) in Figure 2. Several of these peaks (X1-X4, X13, X15) are not removed completely by the preexchange procedure and show up at low pH (Figure 2d). The largest slowly exchangeable peak is X4 which has an intensity of approximately 18 protons in the normally exchanged protease and 3 protons in the preexchanged enzyme.

Spectra were taken in parallel of both normally and preexchanged protease at all pH values. An example of two such parallel spectra of pH* 6.30 is shown in Figure 3. In all cases, spectra of the normally exchanged protease contained peaks with chemical shifts corresponding to all those in spectra of the preexchanged enzyme. In particular, the data indicate that the positions of the histidine peaks are very similar in both preparations.

Spectra of the preexchanged enzyme are relatively simple at pH* 8.5 (Figure 2b) and pH* 3.7 (Figure 2d) where the histidine C(2)-H (H(1)-H(3)) and C(4)-H peaks (H(1')-H(3')) are equivalent and do not overlap other aro-

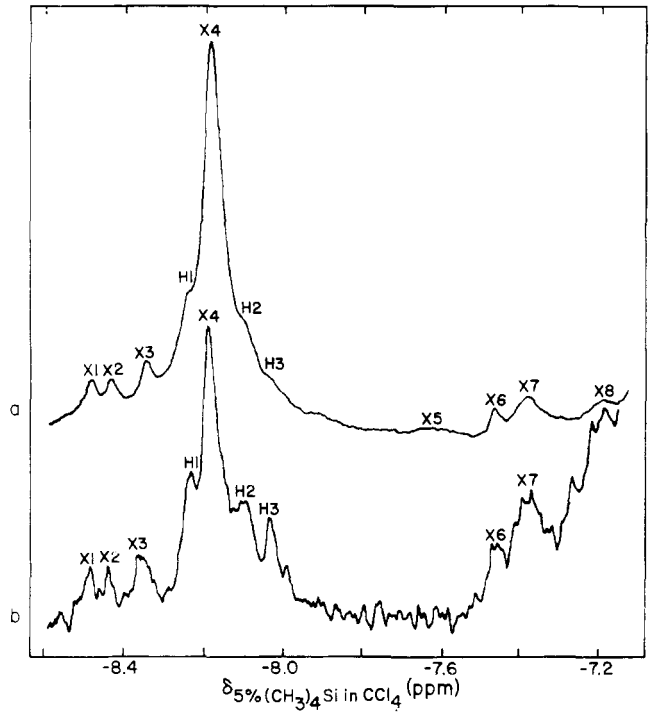


FIGURE 3: Comparison of the lower aromatic ^1H nmr spectral region of normal and preexchanged staphylococcal protease active fragment: (a) enzyme lyophilized three times from D_2O, pH* 6.30; (b) enzyme preexchanged by heating in D_2O 3 min at 80° at pH* 5.0, pH* 6.30. (Spectrum a is shown at a lower gain than spectrum b.)

matic peaks. Resonances from the single tryptophan residue do not show up and apparently are buried beneath peaks from the four phenylalanines and three tyrosines. Single sharp peaks appear for the various proton types of phenylalanine and tyrosine indicating that the side chains of the residues of each amino acid type are in equivalent environments.

Correlation ^1H nmr spectra of the His C(2)-H region of preexchanged staphylococcal protease active fragment at several pH values are shown in Figure 4. The C(2)-H peaks of the histidine residues of the protease are clearly visible except at pH values where they overlap the large exchangeable NH peak (X4). The spectra indicate the presence of three histidines in the protease in agreement with the amino acid analysis. In the pH* region 6.7–7.0, histidine peaks H(2) and H(3) each are resolved as two peaks of approximatively half-proton intensity. The maximum splitting observed is 7 Hz for H(2) and 12 Hz for H(3) at pH* 6.76. Since the doubled peaks do not appear to be exchange broadened, this suggests that two forms of the enzyme exist that have lifetimes in excess of 30 msec and that have different environments for histidine H(2) and H(3).

Chemical shifts of the His C(2)-H peaks are plotted as a function of pH* in Figure 5. The open circles show the chemical shifts of four very slowly exchanging N-H peaks (X1-X4). Peak X4 shifts upfield below pH* 6, and minor shifts in peaks X1-X3 also occur in this region. The chemical shifts of the three histidine residues are nearly identical below pH* 5 and above pH* 8.5.

The results of least-squares analysis of the histidine titration data are presented in Table III. There is no significant difference in the fitted pK' values obtained by either fixing the Hill coefficient at 1 (Table IIIa) or allowing it to vary (Table IIIb). The fit of the data for histidines H(2) and

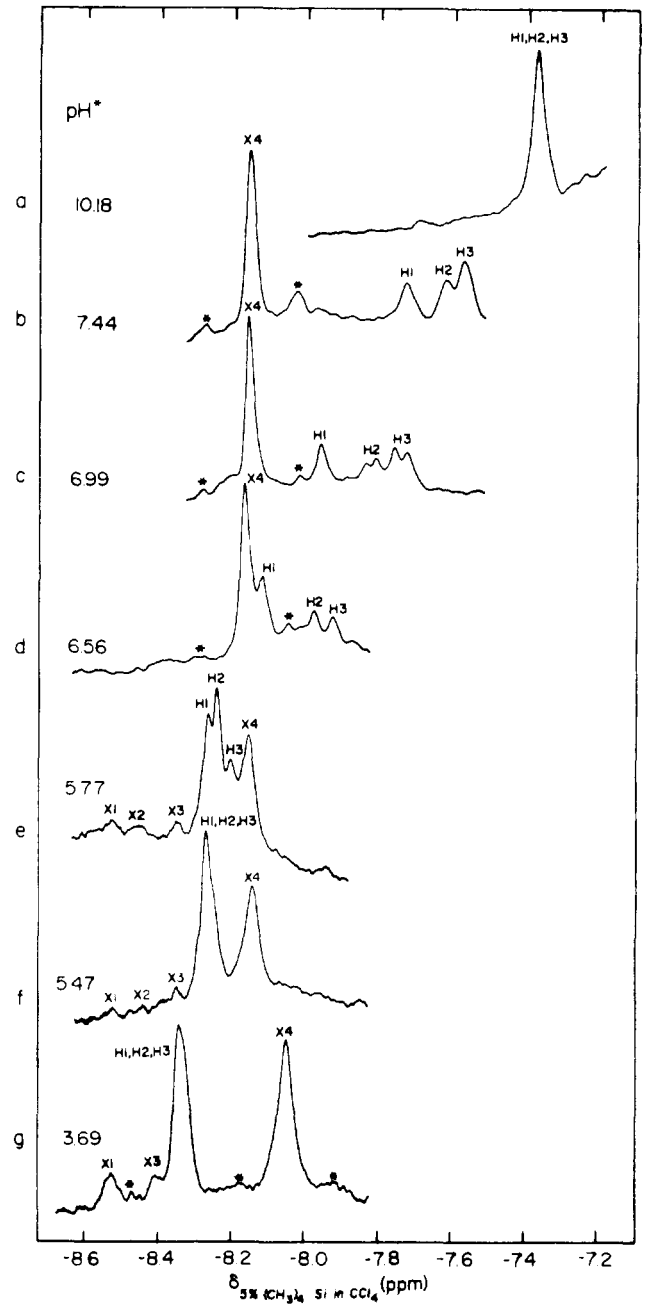


FIGURE 4: ^1H nmr spectra of the histidine C(2)-H region of preexchanged staphylococcal protease at different pH values in 0.1 M phosphate buffer in D_2O. Assignments: H(1)-H(3), His C(2)-H; X(1)-X(4), N-H peaks from groups not deuterated by the preexchange; *, spinning side bands. Note the doubling of histidine peaks H(2) and H(3) at pH* 6.99 (spectrum c).

H(3) is significantly better with a variable Hill coefficient. The fitted pK' values for histidines H(1), H(2), and H(3) are respectively 7.20 ± 0.02, 6.87 ± 0.02, and 6.69 ± 0.02. Histidine H(1) has a Hill coefficient of unity within experimental error while histidines H(2) and H(3) have nearly identical Hill coefficients of 0.85 ± 0.02 and 0.86 ± 0.02.

A Hill coefficient of less than unity is expected if the net positive charge in the vicinity of the titrating group decreases as the pH is raised. Perhaps the simplest mechanism for this would be the titration of a nearby group in the pH region. Thus either histidines H(2) and H(3) are interacting with one another or they interact with one or more different groups having pK values around 6.7. Since histidine

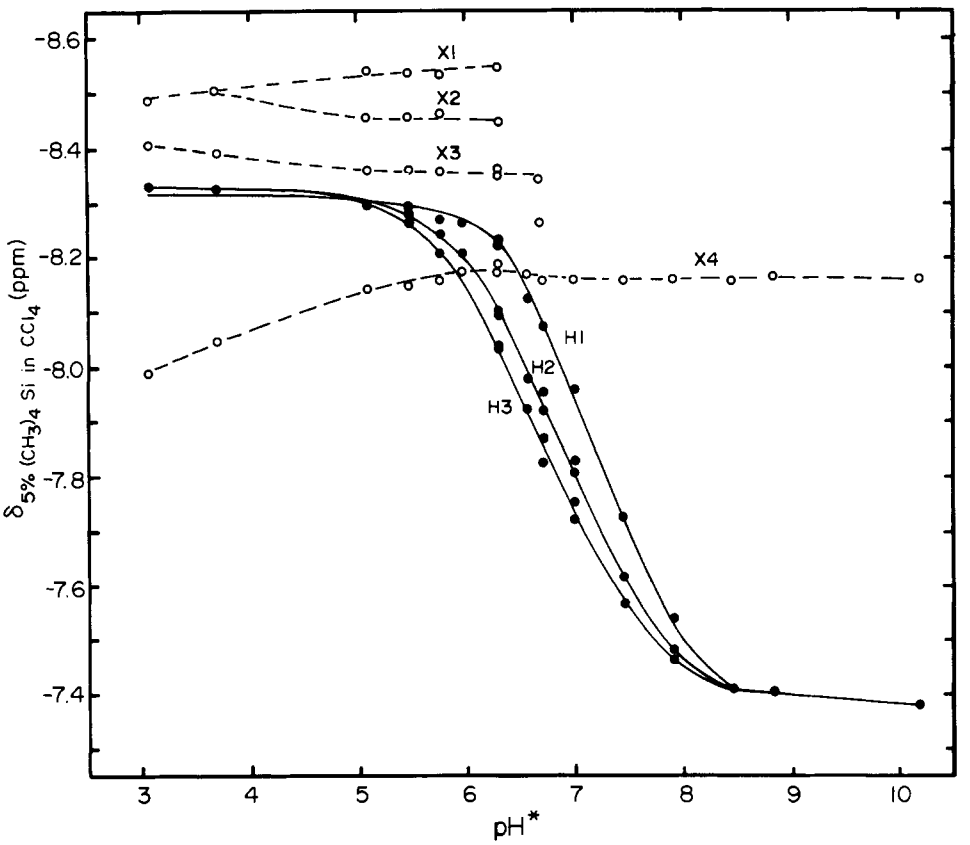
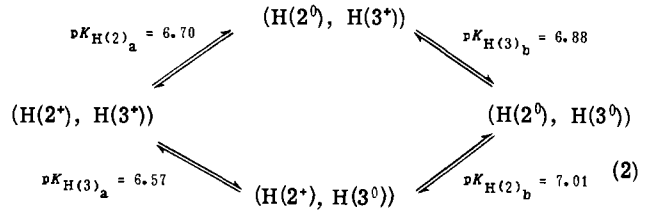


FIGURE 5: ^{1}H nmr pH titration curves of the three histidine residues (H(1)-H(3), C(2)-H peaks) of staphylococcal protease in 0.1 M phosphate buffer in D_2O. The chemical shifts of four very slowly exchangeable N-H groups (X(1)-X(4)) are indicated by dashed lines. Histidine pK' values, Hill coefficients, and other titration parameters are given in Table III. The titration curves are least-squares computer fits to the data; curves for H(2) and H(3) are based on a mutual interaction model (see text). The ^{1}H nmr peaks of histidines H(2) and H(3) are doubled in the pH* region 6.7-7.0 which indicates the presence of two forms of the enzyme having lifetimes in excess of 30 msec.

is the only amino acid having this value as a normal pK, mutual interaction of histidines H(2) and H(3) appears to be a likely possibility. The nmr titration data for these two residues have been fitted to a mutual interaction model. The results are shown in Table IIIc. According to the variance of the fitted and experimental values, the mutual interaction model gives a fit significantly better than even that involving variable Hill coefficients. Microscopic dissociation constants have been obtained for the various protonation states shown in eq 2. More work should be done on this particular point to see if there is any relation between the pK's reported here and the pH-rate profile of the enzyme (Drapeau *et al.*, 1972) where activity maxima at pH 4.0 and 7.8 with hemoglobin as substrate were found.



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Discussion

Two general characteristics of the staphylococcal protease active fragment are revealed by the ^{1}H nmr spectra. First, the peaks are sharp and present very little evidence of side chain chemical shift displacements resulting from tertiary structure. This would suggest that the majority of side chains are in equivalent environments and are probably exposed to the solvent. Separate peaks corresponding to the three histidine residues are resolved only in their titration region where they become distinct because of small differences in their pK' values. Second, the enzyme contains a large number of very slowly exchangeable N-H groups whose resonances show up in the aromatic region. These hydrogens do not exchange with D_2O at room temperature over a period of several hours. Only partial exchange was achieved by heating protease solutions in D_2O to 80° for 3 min at pH* 5.0. The results imply that these N-H groups are rigidly held and are not exposed to the solvent.

These two characteristics of the staphylococcal protease active fragment appear somewhat contradictory. On the one hand, sharp, equivalent spectra are obtained for many side chains suggesting a loose structure; and, on the other hand, numerous hydrogens exchange very slowly with D_2O implying a very rigid structure. The high content of proline residues in the protease (Drapeau *et al*, 1972) suggests that it would have little helical structure. Current circular dichroism studies tend to support this interpretation. The large sedimentation coefficient of 2.9 S for the protease suggests an extended rather than compact structure. Thus, on the basis of all present data, the protease would be expected to have an open, extended structure about a rigid hydrogen-bonded core that is shielded from the solvent.

Large numbers of very slowly exchanging N-H groups have also been observed in ^{1}H nmr spectra of trypsin inhibitors (Karplus *et al.*, 1973; J. L. Markley, unpublished data) and trypsins (J. L. Markley and M. A. Porubcan, unpublished data). In contrast, far fewer very slowly exchanging

Table III: Least-Squares Analysis of Nmr Titration Data for the Histidine Residues of Staphylococcal Protease Active Fragment.

a. Hill Coefficient Fixed at 1

Nmr peak	pK'	Variance $\times 10^4$	Chemical Shift δ(ppm) from 5% $(CH_3)_4Si$ in CCl_4		
			δ_{H^+}	δ_{H^0}	$\Delta\delta_{H^+,H^0}$
H(1)	7.19 ± 0.02	1.8	−8.217 ± 0.006	−7.378 ± 0.008	0.94
H(2)	6.85 ± 0.03	3.8	−8.313 ± 0.009	−7.398 ± 0.010	0.92
H(3)	6.69 ± 0.02	2.9	−8.317 ± 0.008	−7.403 ± 0.009	0.91

b. Hill Coefficient Variable

Nmr peak	pK'	Hill Coefficient	Variance $\times 10^4$	Chemical Shift δ(ppm) from 5% $(CH_3)_4Si$ in CCl_4		
				δ_{H^+}	δ_{H^0}	$\Delta\delta_{H^+,H^0}$
H(1)	7.20 ± 0.02	0.97 ± 0.04	1.9	−8.320 ± 0.007	−7.373 ± 0.010	0.95
H(2)	6.87 ± 0.02	0.85 ± 0.02	1.1	−8.332 ± 0.006	−7.376 ± 0.007	0.96
H(3)	6.69 ± 0.02	0.86 ± 0.02	0.6	−8.335 ± 0.005	−7.384 ± 0.005	0.95

c. Model Assuming Direct Interaction between H(2) and H(3)

Nmr peak	pK_a'	pK_b'	Variance $\times 10^4$	Chemical Shift δ(ppm) from 5% $(CH_3)_4Si$ in CCl_4		
				δ_{H^+}	δ_{H^0}	$\Delta\delta_{H^+,H^0}$
H(2)	6.70 ± 0.02	7.01 ± 0.03	0.6	−8.329 ± 0.004	−7.382 ± 0.005	0.95
H(3)	6.57 ± 0.01	6.88 ± 0.02		−8.331 ± 0.004	−7.386 ± 0.005	0.95

N-H groups appear in spectra of other enzymes such as lysozyme (McDonald and Phillips, 1967), ribonuclease (Wishnia and Saunders, 1962), and staphylococcal nuclease (J. L. Markley, unpublished data). This would indicate that the proteinases and proteinase inhibitors are more rigid toward "breathing motions" of the protein leading to hydrogen exchange. Such stability should also protect these molecules from proteolysis.

The large (18 proton intensity) exchangeable peak X4 (Figure 2) is a unique feature of spectra of the staphylococcal protease.[2] A peak with similar chemical shift but with intensity of only 1 proton has been observed in spectra of trypsin-modified soybean trypsin inhibitor (Kunitz) (J. L. Markley, unpublished data). On the basis of its chemical shift and in view of the very high composition of Asx and Glx in the protease (Table I), peak X4 is assigned tentatively to approximately nine buried amide $N-H_2$ groups. Since peak X4 is so sharp (line width 10 Hz at pH* 7.45) all the groups contributing to its intensity must be in nearly equivalent environments. Thus, there is probably some regularity in the bonding interactions of the buried amide groups.

Since the chemical shifts of the carbon-bound and residual N-H protons in the aromatic region are not changed when the buried N-H groups are deuterated, deuteration of these groups (and the heat treatment) apparently does not alter the structure of the enzyme significantly.

The ^1H nmr data for histidines H(2) and H(3) fit the mutual interaction model (Sachs *et al.*, 1971) remarkably well (Table IIIc). The agreement would be only fortuitous if the two histidines interacted with one or two additional groups having a similar pK. Aside from histidine the only other likely group would be the amino terminal with an abnormally low pK or possibly a buried carboxyl group with an abnormally high pK (Hunkapiller *et al.*, 1973). The remaining histidine H(1) may be ruled out because it has a normal Hill coefficient. In the absence of contradictory data, mutual interaction of the two histidines appears to be the simplest assumption.

The $\Delta pK'$ resulting from mutual interaction of histidines H(2) and H(3) is 0.31 unit. Tanford and Roxby (1972) have published a relationship between $\Delta pK'$ and the distance between the perturbing groups based on the Tanford–Kirkwood theory. At the ionic strength used in the present study, this relationship predicts a distance of approximately 7Å between histidines H(2) and H(3). As Tanford and Roxby have pointed out, the reliability of their relationship is critically dependent on a rather arbitrary assumption concerning the depth of charges below the cavity surface at which they are located. As a test of the model, a similar calculation has been carried out (J. L. Markley and W. R. Finkenstadt, to be published) for the two active site histidines of bovine pancreatic ribonuclease A (His^{12} and His^{119}). The 250-MHz ^1H nmr correlation data on these histidines give a best fit to a mutual interaction model that also presupposes a perturbation of His^{119} outside the titration region at pH 3.7. The $\Delta pK'$ is 0.28 at an ionic strength of 0.3. Based on this, the calculated His^{12}–His^{119} distance (using Tanford and Roxby relationship) is 7.5 Å. This distance is in excellent agreement with the recent X-ray data for inhibited ribonuclease A (Carlisle *et al.*, 1974) which yield a distance of 7 Å between the two active site histidine residues.

The histidine titration curves of staphylococcal protease are of particular interest because the enzyme appears to be a serine protease. Serine proteases (Blow *et al.*, 1969) that are evolutionarily homologous to trypsin invariably have three residues conserved (an aspartic acid, serine, and histidine) as part of the active site. Subtilisin (Kraut *et al.*,

[2] The presence of this peak is very surprising but since it does exchange with D_2O and does also shift with pH, it cannot be an antifact of the 250-MHz correlation technique.

1972), a bacterial enzyme with no sequence homology to the chymotrypsin family, seems to have developed the identical set of three residues in its active site by convergent evolution. The X-ray structures of chymotrypsin, trypsin, and elastase indicate that the aspartic acid, serine, and histidine residues form a hydrogen-bonded system which has been named the "charge relay" system (Blow *et al.*, 1969). The existence of the hydrogen-bonded charge relay system has received support from nmr studies in solution (Robillard and Shulman, 1972). A recent ^{13}C nmr experiment (Hunkapiller *et al.*, 1973) with enrichment at C(2) ring carbon of the single histidine residue of α-lytic protease, which has a sequence homologous to that of elastase, suggests an inversion of the usual pK values of the carboxyl and imidazole residues in the charge relay. However, it remains to be demonstrated that the present protease has a charge relay system.

The present ^1H nmr evidence on staphylococcal protease indicates that all three histidines have normal pK values. Furthermore, the chemical shifts of the protonated and dissociated forms of all three histidines are normal. Thus there is no evidence that any of the histidines of the staphylococcal protease active fragment is located in a hydrophobic environment, or that any one acts as a hydrogen-bond donor or acceptor. In chymotrypsin (Brown and Hartley, 1966) the active site histidine is residue 57 and the active site serine is residue 195. Thus the distance between these residues is 138 amino acids which is greater than the total of 114 present in the staphylococcal protease fragment being studied. If the active site serine sequence of staphylococcal protease is homologous to that of chymotrypsin, the protein would have to contain a sizable deletion in order for the chain to contain a histidine homologous to His^{57}. The present evidence suggests that the staphylococcal protease active fragment of molecular weight 12,000 is an example of an active diisopropyl phosphofluoridate inhibitable protease that does not have a partially buried histidine with a low pK in its active site.[3] Further discussion of this must await the results of sequencing studies of staphylococcal protease now in progress, and nmr studies of inhibited protease. Since the enzyme used in these studies appears to be an active fragment of a larger active precursor it will be interesting to learn the environments and pK' values of the histidines in the longer chain forms of staphylococcal protease.

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[3] It may be relevant to note here that recent ^1H nmr studies of the four histidine residues of porcine trypsin reveal the existence of one broad histidine C(2)-H peak that has an abnormally high chemical shift in its protonated state (δ_{H^+} −7.97) which is consistent with its being hydrogen bonded in a hydrophobic environment, an abnormally low chemical shift in its dissociated state (δ_{H^0} −7.65), and a pK' value of 5.0 (J. L. Markley and M. A. Porubcan, unpublished data).

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