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Assignment of Histidine Resonances in the ^{1}H NMR (500 MHz) Spectrum of Subtilisin BPN′ Using Site-Directed Mutagenesis†

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ABSTRACT: A spin-echo pulse sequence has been used to resolve the six histidine C-2H protons in the 500-MHz NMR spectrum of subtilisin BPN′. Five of these residues have been substituted by site-directed mutagenesis, and this has enabled a complete assignment of these protons to be obtained. Analysis of the pH titration curves of these signals has provided microscopic pK_a's for the six histidines in this enzyme. The pK_a's of the histidine residues in subtilisin BPN′ have been compared with the values obtained for the histidines in the homologous enzyme from *Bacillus licheniformis* (subtilisin Carlsberg). Four of the five conserved histidines titrate with essentially identical pK_a's in the two enzymes. It therefore appears that the assignments made for these residues in subtilisin BPN′ can be transferred to subtilisin Carlsberg. On the basis of these assignments, the one histidine that titrates with a substantially different pK_a in the two enzymes can be assigned to histidine-238. This difference in pK_a has been attributed to a Trp to Lys substitution at position 241 in subtilisin Carlsberg.

Nuclear magnetic resonance spectroscopy is a powerful method for studying protein structure and function in solution (Jardetzky & Roberts, 1981). Interpretation of NMR data is, however, hindered in many cases by a lack of sequence-specific resonance assignments. For small proteins of less than 150 amino acids the introduction of methods based on 2-D NMR has largely overcome this problem (Wüthrich, 1986). Unfortunately, the application of these methods to larger proteins is limited by rapid spin–spin relaxation and broad resonances.

One promising new approach to the problem of resonance assignment in NMR spectroscopy is the use of site-directed mutagenesis (Prigodich et al., 1986; Clore et al., 1987). Naturally occurring protein variants have been used for resonance assignments for many years [reviewed in Jardetzky and Roberts (1981)], but this technique has been limited by the availability of suitable proteins. The advances in recombinant DNA techniques now allow the introduction of any desired mutation into proteins for which the cloned gene is available (Zoller & Smith, 1983). Provided that the mutation does not produce a large conformational change, the spectra of the mutant and wild-type proteins should be essentially identical, except for the absence of the peak of interest, making assignment straightforward.

We report the use of site-directed mutagenesis in the assignment of the histidine C-2H protons in the 500-MHz NMR spectrum of subtilisin BPN′, a bacterial serine protease containing 275 amino acids (Markland et al., 1971). This protein is currently being used as a model system in which to study electrostatic effects in proteins by protein engineering (Thomas et al., 1985; Russell et al., 1987; Russell & Fersht, 1987; Sternberg et al., 1987). In these studies, the effect of mutations on the kinetically determined pK_a of the active site histidine is being investigated. NMR can be used to determine the microscopic pK_a's of histidine residues in proteins by following the pH-dependent chemical shifts of the C-2H protons (Markley, 1975). By use of the assignments described here, the pK_a's of all the histidines in subtilisin BPN′ can be determined. This will enable the effects of mutations on the pK_a's of any of the histidines in this protein to be investigated.

Materials and Methods

Reagents were obtained from Sigma (London), Aldrich, Amersham International, and New England Nuclear. Oligonucleotides were synthesized on an Applied Biosystems DNA synthesizer.

Mutagenesis and Protein Purification. The mutations His → Gln-17, His → Gln-39, His → Gln-67, His → Gln-226, and His → Gln-238 were constructed by site-directed mutagenesis of the subtilisin BPN′ gene cloned into M13mp9, with the primers 5′CTTGAGAT*TGCAGAGC3′, 5′AATCAGT*-

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TGAGAAG3′, 5′CGGCAAC*CTGAGTTC3′, 5′CGGCAACT*TGCGGAG3′, and 5′TCCAGTT*CGGGTGCTTAGA3′ (where * denotes a mismatch), as described previously (Thomas et al., 1985). Wild-type and mutant proteins were purified from cultures of *Bacillus subtilis* DB104 harboring the wild-type or mutant gene ligated into plasmid pUB110 as described, except that 10 mM $CaCl_2$ was included in the culture media to reduce losses from autolysis. After purification, the proteins were dialyzed against water and then freeze-dried. Purified proteins were found to be greater than 95% active as determined by active site titration with *N-trans*-cinnamoylimidazole (Bender et al., 1966).

Subtilisin Carlsberg was obtained from Sigma and was purified as described for subtilisin BPN′.

NMR Methods. The lyophilized protein was dissolved in 0.5 mL of 99.98% D_2O containing 5 mM EDTA and 250 mM kCl to give a 1–1.5 mM solution. Proton NMR spectra were recorded at 25 °C on a Bruker AM-500 spectrometer using a spin-echo pulse sequence to minimize the background of signals from undeuteriated N-H protons (Campbell et al., 1975). A total of 128 transients were averaged for each spectrum with a 6200-Hz sweep width. Prior to Fourier transformation, the free induction decay was multiplied by an exponential equivalent to a line broadening of 2 Hz. Chemical shifts are quoted relative to 4,4-dimethyl-4-silapentane-1-sulfonate (DSS). The pH was systematically changed between spectra by the addition of microliter aliquots of NaOD or DCl. pH measurements were made with a microelectrode directly in the NMR tube and are uncorrected and denoted by pH*. The variation of the observed chemical shift of a histidine resonance δ_{obsd} with pH was analyzed with

$$\delta_{obsd} = \delta_A + (\delta_{AH} - \delta_A)\frac{[H^+]}{K_a + [H^+]}$$

where K_a is the dissociation constant, δ_A is the chemical shift of the resonance of the unprotonated histidine, δ_{AH} is the chemical shift of the resonance of the protonated histidine, and $[H^+]$ is calculated from pH*. Experimental data were fitted to this equation using ENZFITTER, a nonlinear least-squares data analysis program (Leatherbarrow, 1987).

Determination of Kinetic pK_a. The pH dependence of k_{cat}/K_m for the hydrolysis of succinyl-L-alanyl-L-prolyl-L-phenylalanine *p*-nitroanalide (su-A-A-P-Fp-NA) in D_2O containing 250 mM KCl was determined according to the methods described in Russell et al. (1987).

RESULTS

Observation of Histidine Resonances in Subtilisin BPN′. The signals from histidine C-2H protons in large proteins are often obscured by backbone N-H resonances. In a previous NMR study of subtilisins, this problem was overcome by performing N-H to N-D exchange (Jordan et al., 1985). This required overnight incubation at high pH which results in substantial loss of enzyme activity from autolysis. We have found that excellent suppression of the signals from N-H protons in subtilisin can, instead, be obtained with a spin-echo pulse sequence which exploits the differences in relaxation time between N-H and histidine C-2H protons (Campbell et al., 1975) (Figure 1). This method does not require a preexchange step and so represents a considerable improvement on the previously reported method for resolving these resonances.

pH Titrations of the Histidine C-2H Resonances in Wild-Type Subtilisin. Figure 2 shows spectra of subtilisin BPN′ at five different values of pH*. The peaks that titrate with the characteristics of histidine C-2H protons are labeled A–F.

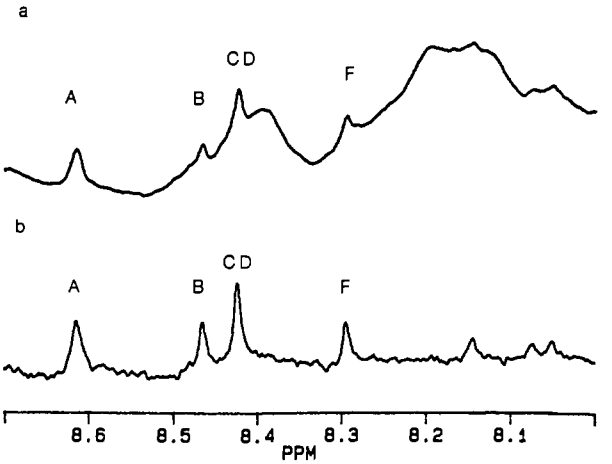

FIGURE 1: 500-MHz ^1H NMR spectra (25 °C, pH 6.32, 250 mM KCl, 3 mM EDTA) of 1 mM subtilisin BPN′ His → Gln-39, acquired with (a) a 75° pulse and (b) a 90°–*t*–180°–*t*–collect spin-echo pulse sequence (Campbell et al., 1975). The labeling of the peaks is discussed in the legend to Figure 2.

Table I: ^1H NMR pH Titration Parameters for Histidine Residues in Subtilisin BPN′

histidine peak[a]	pK_a[b]	low-pH chemical shift (ppm)	high-pH chemical shift (ppm)
A	7.45	8.73	7.73
B	7.23	8.61	7.66
C	7.12	8.60	7.71
D	7.04	8.61	7.71
E	6.95	8.57	7.71
F	6.72	8.61	7.74

[a] The labels refer to the peak designation in Figure 2. [b] Uncertainty less than ± 0.05 in all determinations.

Table II: Comparison of the pK_a's of Native Subtilisin and the His → Gln-17 Mutant

	pK_a	
histidine resonance	native	His → Gln-17
A	7.45	7.47
B	7.23	7.25
C	7.12	7.13
D	7.04	
E	6.95	6.94
F	6.72	6.76

Fitted pH titration curves for these peaks are shown in Figure 3, and the titration parameters obtained are summarized in Table I.

Spectra of Mutant Subtilisins. The histidine C-2H regions of the NMR spectra of the mutants His → Gln-39, His → Gln-238, His → Gln-17, and His → Gln-226 are shown in Figures 4 and 5. A spectrum of each mutant is presented together with a spectrum of the native enzyme at a similar pH. Although only one representative spectrum is shown for each mutant, assignments are based on full titrations of the histidines in the mutant proteins. Inspection of Figure 4 clearly shows that, on substitution of His-39 by Gln, peak E disappears while substitution of His-238 results in the disappearance of peak A. Spectra of the His → Gln-17 and His → Gln-226 mutants are shown in Figure 5. For both of these mutants only one resonance is observed to titrate with a pK_a between 7.00 and 7.15 whereas for the wild-type enzyme both peaks C and D titrate in this region. The pK_a's obtained from the titration of the histidine C-2H resonances in the His → Gln-17 mutant are shown in Table II. Comparison of these values with those for the native enzyme shows that peak D is absent in the spectrum of this mutant. The peak that disappears on

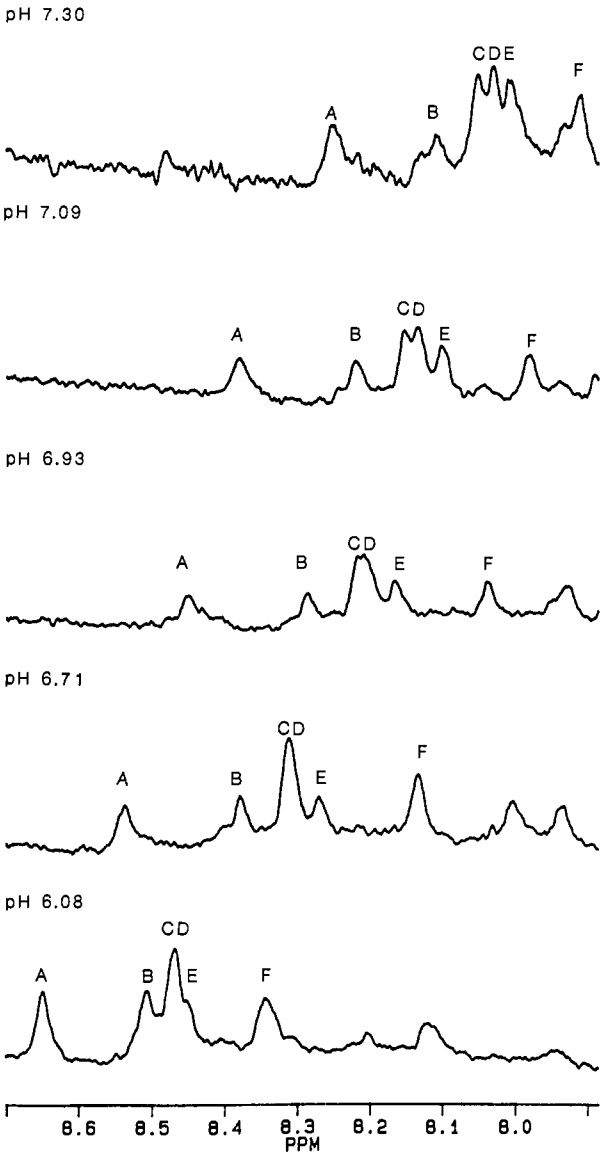

FIGURE 2: ^{1}H NMR spectra at 500 MHz of subtilisin BPN′ (histidine C-2H region) at selected pH* values. Conditions are described in the legend of Figure 1. The six peaks that titrate with the characteristics of histidine C-2 protons are denoted A–F.

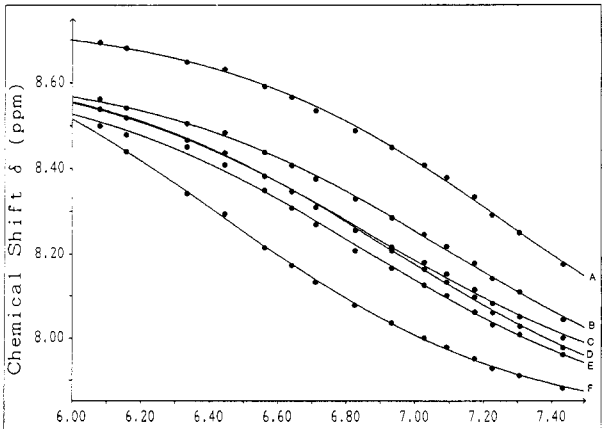

FIGURE 3: ^{1}H-monitored pH titration of the histidine residues of substilisin BPN′. Solid lines are theoretical curves to the experimental points.

substitution of His-226 is therefore resonance C. These results lead to the unequivocal assignment of resonance A to His-238, resonance C to His-226, resonance D to His-17, and resonance E to His-39.

Table III: Summary of Histidine Assignments

histidine resonance[a]	residue no.
A	238
B	64
C	226
D	17
E	39
F	67

[a] The labels refer to the peak designation in Figure 2.

Table IV: pK_a's of Histidines in Subtilisins BPN′ and Carlsberg

histidine	pK_a in BPN′	pK_a in Carlsberg[a]
17	7.04	absent
39	6.95	6.94
64	7.23	7.22
67	6.72	6.80
226	7.12	7.16
238	7.45	6.36

[a] Data from Jordan et al. (1985).

Assignment of the Active Site Histidine. Analysis of the pH dependence of k_{cat}/K_m for the hydrolysis of su-A-A–P-Fp-NA in D_2O containing 250 mM KCl gives a pK_a of 7.20 ± 0.02. This value corresponds to the pK_a of the active site histidine (His-64) in the free enzyme (Russell et al., 1987) and may therefore be directly compared with the pK_a's obtained from the NMR measurements. Of the unassigned resonances only resonance B titrates with a similar pK_a, and this may thus be tentatively assigned to histidine-64. It is not, however, possible to confirm this assignment directly by mutagenesis, because mutation of this residue has been shown to result in a large reduction in the level of protein expression (Carter & Wells, 1987) and it is not feasible to obtain sufficient material for NMR studies.

Spectra of the His → Gln-67 Mutant. Only resonance F remains unassigned, and by a process of elimination it must correspond to histidine-67. A complete assignment has thus been obtained which is summarized in Table III. The histidine C-2H region of the NMR spectrum of the His → Gln-67 mutant is shown in Figure 6. Simple inspection of this spectrum shows that this mutation has produced substantial changes in this region. Analyses of the titration data for this mutant give pK_a values of 7.48, 7.34, 7.11, 6.81, and 6.64. Comparison with the values obtained for the wild-type enzyme indicates that the pK_a's of histidines-17 and -238 appear to be unaffected by the mutation but that the pK_a of histidines-64, -226, and -39 are perturbed. Kinetic analysis of this mutant shows a lowering of k_{cat} and a substantial rise in K_m (M. Bycroft and A. R. Fersht, unpublished data). It therefore appears that mutation of this residue produces some conformational change in the enzyme.

Comparison of pK_a's of the Histidine Residues in Subtilisins BPN′ and Carlsberg. Subtilisins BPN′ and Carlsberg are approximately 70% homologous. Both enzymes have histidines at positions 39, 64, 67, 226, and 238. Subtilisin Carlsberg, however, has a Gln at position 17 instead of a histidine. Jordan et al. (1985) have reported a comparison of histidine C-2H regions of the 200-MHz spectra of subtilisins BPN′ and Carlsberg. Since the publication of that work, it has been shown that the subtilisin BPN′ used by Jordan et al. was not the authentic enzyme because of mislabeling by the manufacters (Russell & Fersht, 1986). This has cast doubt on a number of their conclusions. Figure 7 shows a comparison of the histidine C-2H regions of the 500-MHz spectra of subtilisins Carlsberg and BPN′. pH titration of the five histidine resonances in subtilisin Carlsberg gives pK_a's similar

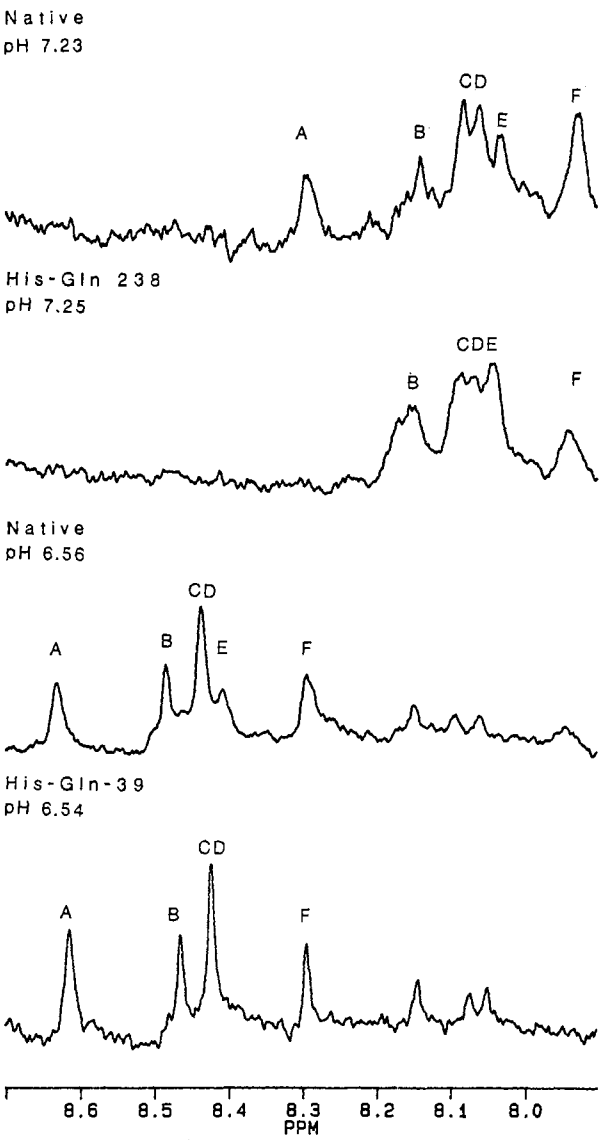

FIGURE 4: 500-MHz ^1H NMR spectra of histidine C-2 proton resonances of the mutants His → Gln-238 and His → Gln-39. The labeling of the His C-2H resonances is the same as in Figure 2.

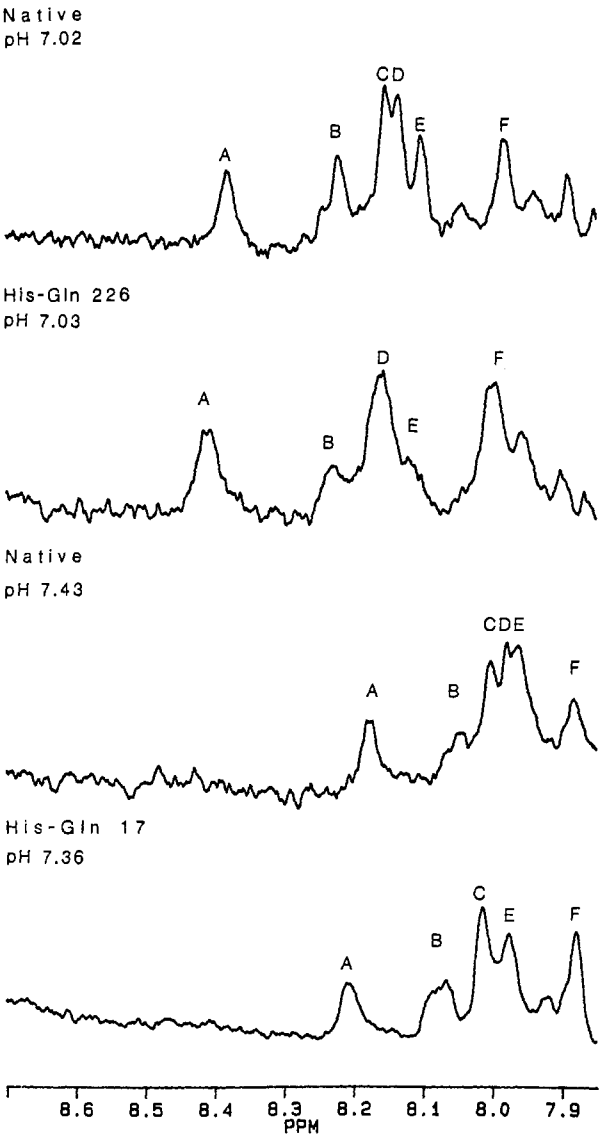

FIGURE 5: 500-MHz ^1H NMR spectra of histidine C-2 proton resonances of the mutants His → Gln-226 and His → Gln-17. The labeling of the His C-2H resonances is the same as in Figure 2.

to the values reported by Jordan et al. (1985). Comparison of the titration data for the two enzymes (Table IV) shows that the resonance that has been assigned to His-17 in subtilisin BPN′ is absent in the spectrum of subtilisin Carlsberg, thus confirming the assignment made by mutagenesis. Four of the five conserved histidines titrate with essentially identical pK_a's in the two enzymes. The assignments made for these residues in subtilisin BPN′ are, therefore, likely to correspound to those in subtilisin Carlsberg. If this is so, histidines-39, -64, -67, and -226 in subtilisin Carlsberg can be assigned, and the peak titrating with a pK_a of 6.3 will then correspond to His-238.

Discussion

The use of site-directed mutagenesis has allowed the assignment of all the histidine C-2H protons in subtilisin BPN′. Assignment of these resonances would be extremely difficult to achieve by any other method, and these results demonstrate the power of this technique for resonance assignment.

Four of the five mutants studied give spectra that are identical with those of the native protein except for the absence of one of the histidine resonances. The similarity of mutant and wild-type spectra suggests that the mutation of these residues does not produced any large conformational change. These results are consistent with the findings of a number of

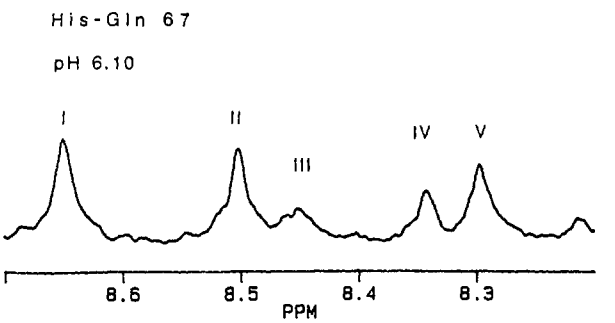

FIGURE 6: ^1H 500-MHz spectrum of the histidine C-2H resonances of the mutant His → Gln-67. Resonances that titrate with the characteristics of histidine protons are indicated.

X-ray crystallographic studies on mutant proteins that have indicated that, in many cases, point mutations do not produce major perturbations in protein structure (Howell et al., 1986; Brown et al., 1987). In contrast to the other mutants, mutation of His-67 does produce a number of changes in the His C-2H region of the spectrum, other than the removal of one of the peaks, suggesting that a conformational change has occurred. Assignment of a histidine resonance based on this mutant was, accordingly, difficult. Since in this study a series of mutations

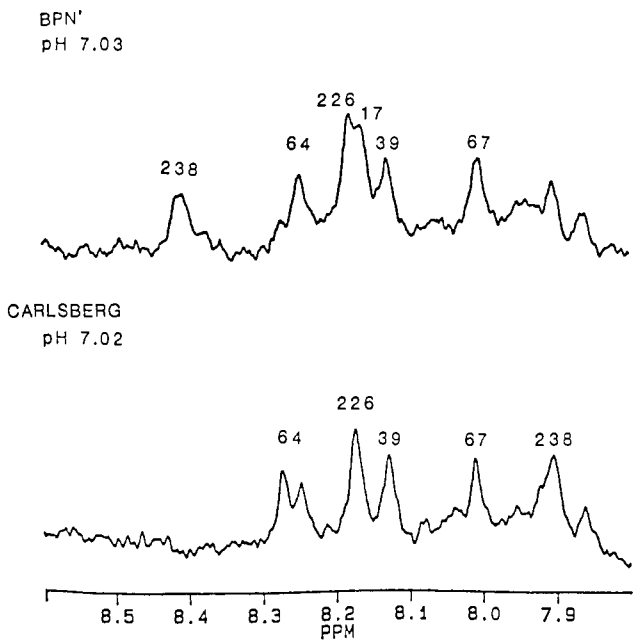

FIGURE 7: Comparison of the ^{1}H NMR spectra of subtilisins BPN′ and Carlsberg (histidine C-2H region). The labels refer to the assignments discussed in the text. Conditions are described in the legend of Figure 1. The peak corresponding to His-64 in subtilisin Carlsberg is found to be split, indicating that there are two environments for this proton in this enzyme.

were analyzed, it is still possible to obtain a complete assignment of all of the histidines in this protein. This result does, however, indicate that problems may be encountered when site-directed mutagenesis is used to assign structurally important residues.

Comparison of the pK_a's of histidines in homologus enzymes such as subtilisins BPN′ and Carlsberg provides an oppoortunity to study the effects of sequence changes on the pK_a's of histidine residues. Four of the five histidines that are conserved between subtilisin BPN′ and subtilisin Carlsberg titrate with essentially the same pK_a's in both enzymes. This is perhaps not too surprising since examination of the crystal structures of the two proteins (Drenth & Holl, 1967; Wright et al., 1969) indicates that the environments of these residues are similar in both enzymes. In addition, the pK_a's have been determined in the presence of 250 mM KCl, and at this ionic strength many electrostatic interactions, which could potentially perturb the pK_a's of histidine residues, will be masked by counterions.

The histidines at positions 238 do, however, have substantially different pK_a's in the two enzymes. This residue has a pK_a of 7.4 in subtilisin BPN′ and a pK_a of 6.3 in subtilisin Carlsberg. Comparison of the sequences around this residue shows that there is a Trp → Lys substitution at position 241 in subtilisin Carlsberg which introduces a positive charge within a few angstroms of histidine-238. This substitution would be expected to produce the observed decrease in the pK_a of this residue because of electrostatic destabilization of the protonated form of the imidazole ring.

Kinetic analysis of mutant subtilisins in which charged residues have been systematically altered has provided a number of insights into the electrostatic properties of proteins (Russell & Fersht, 1987). It is gratifying that both NMR and kinetic measurements give a very similar value for the pK_a of the active site histidine. With the availability of the assignments described herein, NMR can now be used to determine the effects of mutations on the pK_a's of histidines other than that at the active site.

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