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Proton Magnetic Resonance Studies of the States of Ionization of Histidines in Native and Modified Subtilisins†

Frank Jordan,*,‡ Laszlo Polgar,§ and Guillermo Tous‡

Department of Chemistry, Rutgers University, Newark, New Jersey 07102, and Institute of Enzymology, Biological Research Center, Hungarian Academy of Sciences, Budapest H-1502, Hungary

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ABSTRACT: A technique was developed to exchange the backbone –N–H protons in D_2O in the native subtilisins Carlsberg and BPN (Novo) that resulted in clearly resolved proton resonances in the aromatic region of the nuclear magnetic resonance spectrum. pH titration curves for four of the five histidine C2–H resonances in subtilisin Carlsberg and five of the six in subtilisin BPN between 7.5 and 8.8 ppm downfield from 4,4-dimethyl-4-silapentane-1-sulfonic acid sodium salt provided microscopic pK_a's between 6.3 and 7.2 for both sources of the enzyme at ambient (~22 °C) probe temperature. A resonance that titrated with a pK_{app} of 7.35 ± 0.05 was observed in the ^1H spectra only of the diisopropylphosphoryl derivatives of the subtilisins from both sources. The ^{31}P NMR pH titration of the same preparations under identical conditions of solvent (D_2O) and temperature gave a pK_{app} = 7.40 ± 0.05 of the single titratable resonance. Both observations must pertain to His-64 at the active center. A resonance smaller than the others and titrating with a pK_{app} of 7.2 could also be observed in the native enzymes. This resonance was assigned to the catalytic center histidine since its pK corresponded to that derived from kinetic studies. No major perturbations in the chemical shifts or the pK's derived from the pH dependence of the observed resonances were apparent in the presence of saturating concentrations of the two putative transition-state analogues phenylboronic acid and bis[3,5-(trifluoromethyl)phenyl]boronic acid and in monoisopropylphosphorylsubtilisin. It can be concluded that the C2–H resonance corresponding to His-64 in native subtilisins is difficult to observe perhaps on account of the limited mobility of this side chain compared to its mobility in the diisopropylphosphoryl derivative.

A number of nuclear magnetic resonance (NMR) approaches have been applied to serine proteases [see review in Steitz & Shulman (1982)]. An early study by Robillard & Shulman (1972, 1974a,b) on chymotrypsin and other serine proteases found a ^1H resonance at 15–18 ppm downfield from 4,4-dimethyl-4-silapentane-1-sulfonic acid sodium salt (DSS)[1] that was attributed to the hydrogen located between the Asp and His residues at the catalytic center, residing very likely on the nitrogen (Bachovchin & Roberts, 1978; Kossiakoff & Spencer, 1981). Surprisingly, no such resonance was found in the spectra of native subtilisins (Jordan & Polgar, 1981; Jordan et al., 1982). The resonance, however, was observed when there was located a second negative charge in the catalytic center so as to create an $Asp^-His^+X^-$ charge distribution (where X^- represents a thiolate anion produced by conversion of the serine enzyme to its chemically mutated thiol form, or of a covalently attached putative transition-state analogue phenylboronic acid on the serine enzyme, or even a noncovalent interaction between the serine enzyme and the reversible competitive inhibitor *N*-acetyl-L-tryptophan). To explain these results, it was suggested that the His imidazole is more mobile in the native enzyme than in the transition-state analogue-like –+– charge distribution state. The resonance corresponding to the N–H proton located between Asp and His is subject to exchange broadening, and apparently the rate of exchange with solvent slows down by placing a negative charge on both sides of the histidinium ion. An independent technique to enable observation of the His imidazoles directly is to monitor the resonance of the C2–H in the aromatic region of the ^1H spectrum. Extensive results were published by Markley and co-workers on this spectral region of trypsin, chymotrypsin, and α-lytic protease (Markley & Porubcan, 1976; Markley & Ibanez, 1978; Markley, 1979). Since exchange of the C2–H with solvent is very slow ($t_{1/2}$ of several days), these resonances are not subject to exchange broadening with solvent and

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‡ Rutgers University.
§ Hungarian Academy of Sciences.

[1] Abbreviations: DSS, 4,4-dimethyl-4-silapentane-1-sulfonic acid sodium salt; MES, 2-(*N*-morpholino)ethanesulfonic acid; Tris, tris(hydroxymethyl)aminomethane; DIFP, diisopropyl fluorophosphate; PMSF, phenylmethanesulfonyl fluoride; EDTA, ethylenediaminetetraacetic acid.

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provide insight about the catalytic center environment.

We report that we have succeeded at clearly resolving four of five His C2–H resonances in the native form of subtilisin Carlsberg and five of six in subtilisin Novo (BPN). We also detected another resonance that probably corresponds to His-64 at the catalytic center in the native enzyme. The pH-dependent chemical shifts allowed us to determine the microscopic pK of each His. Studies were also performed on the diisopropylphosphoryl derivative as well as on the phenylboronate and the monoisopropylphosphoryl derivatives of the enzyme. Interestingly, the catalytic center His C2–H resonance is readily resolved in the diisopropylphosphoryl derivative but not in the native enzyme or in the transition-state analogue-bound enzyme.

Experimental Procedures

Materials. Subtilisins Carlsberg and BPN (the latter equivalent to Novo), leupeptin, and Sephadex G25-80 were purchased from Sigma, St. Louis, MO; phenylboronic acid was from Aldrich, Milwaukee, WI; bis[3,5-(trifluoromethyl)-phenyl]boronic acid was from Alfa Inorganics. The pH measurements were made on Radiometer pH m62 or pH m26 instruments employing an Ingold combination electrode directly in the NMR tubes. The pH readings were uncorrected.

Preparation of Native Enzymes for Experiments in D_2O. In a typical experiment, 500 mg of enzyme was dissolved in 5 mL of D_2O that contained 0.01 M Tris base and 5 mM EDTA, and the pH_{app} was adjusted to 9.0. The solution was incubated at 28 °C for 16 h. Preliminary control experiments had indicated that at least 12 h were required at this initial pH for exchange of the backbone N–H's to proceed substantially. After the incubation, the solutions were gel-filtered on a 28-mL bed volume Sephadex G25-80 column that had been prewashed with either D_2O with 0.005 M Tris, pH 9.0, or D_2O with 0.005 M P_i, pH 6.0, depending on whether storage was desirable at pH 9 or 6. The protein was eluted in the void volume and was detected at 280 nm. The combined protein fractions were freeze-dried. Recovery of enzyme (that contained 80–85% active enzyme according to active-site titration) was 20–30%. Better resolved spectra were obtained if after incubation the sample was purified near pH 6 rather than at pH 9.0.

Preparation of Monoisopropylphosphorylsubtilisin in D_2O. Subtilisin BPN (250 mg) was dissolved in 2.5 mL of 0.1 M phosphate, pH 6.89. Fifty microliters of 0.5 M diisopropyl fluorophosphate (DIFP) dissolved in isopropyl alcohol was added to result in a 10 mM final concentration of DIFP. The solution was incubated at 37 °C for about 60 h. Under these conditions, the diisopropylphosphorylsubtilisin is converted to the monoisopropylphosphoryl derivative (Van der Drift, 1983). This was confirmed in this laboratory on both subtilisins. The ^{31}P chemical shift of the diisopropylphosphoryl derivative is pH dependent; that of the aged, presumably monoisopropylphosphoryl derivative is pH independent and downfield from the former in both subtilisins by about the same amount. After the incubation, the backbone NH's were exchanged as follows. The solution was filtered through a 28-mL G25-80 column that had been equilibrated with 5 mM Tris and 5 mM EDTA, pH 8.9, in D_2O. The D_2O solution of the eluted protein was next incubated at pH_{app} 10 for 16 h at 28 °C and freeze-dried or was used immediately.

Preparation of Diisopropylphosphorylsubtilisins in D_2O. The enzyme solution (200 mg) was dissolved in 3 mL of D_2O (0.01 M Tris and 5 mM EDTA, pH 8.0) and was first treated with 20 μL of 0.5 M DIFP (in isopropyl alcohol) for 6 h; then another 20 μL of 0.5 M DIFP was added, and the solution was maintained at 28 °C for 16 h more. At this time, the enzyme was more than 99.6% inactivated. The pH was adjusted to 10, and the incubation was continued for another 24 h. The yield was about 54 mg of enzyme after purification on Sephadex G25-80 and elution of the protein with 0.01 M Tris and 1 mM EDTA, pH 8.05, in D_2O.

Enzyme Assay and Active-Site Titrations. Native subtilisin activity was determined by using *p*-nitrophenyl acetate as substrate. In a typical assay, 10 μL of enzyme solution was added to a solution that was 10^{-4} M in *p*-nitrophenyl acetate and 0.02 M Tris, pH 7.0, 25 °C. The release of *p*-nitrophenoxide was monitored at 400 nm.

The concentration of active subtilisin after the D_2O exchange and subsequent purification on Sephadex was determined by active-site titration against *N-trans*-cinnamoylimidazole (Bender et al., 1966; Polgar, 1968).

Sample Preparation and pH Titration for NMR Measurements. The lyophilized purified enzyme preparations were dissolved (usually 1–1.5 mM) in 99.8% D_2O containing DSS, 3–5 mM EDTA, and 0.25 M KCl. pH titration was performed by the addition of microliter amounts of 0.1 or 1 M MES in D_2O, pH 6.0, or of 0.1 or 1 M Tris in D_2O, pH 8.5. For ^1H NMR, 0.4–0.5-mL samples were used, and for ^{31}P NMR, 1.3–1.5-mL samples were employed.

The ^1H NMR measurements were performed mostly at 200 MHz (IBM WP 200 SY). Water suppression was achieved by gated decoupling of the residual HOD signal. Most experiments were performed at 22–23 °C. All chemical shifts were measured with reference to internal DSS and are reproducible to 0.02 ppm from experiment to experiment. On the highly purified samples (preexchanged according to the protocols discussed above), 1 mM concentration of the native enzyme gave excellent spectra in ca. 250–400 transients at 200 MHz (where most of the data here presented were collected) with a 45° pulse width, a 4000-Hz sweep width, and a 3–4-s total recycle time. The pH of the sample during the ^1H NMR experiment (typically less than 30 min) remained unchanged within 0.03 unit. Usually a 0.5-Hz line broadening was applied to the free induction decay prior to Fourier transformation.

^{31}P NMR was always performed at 22 °C at 81.026 MHz on the IBM WP 200 SY instrument. Typically, a 90° pulse width with a total recycle time of 4 s and a spectrum width of 4000 Hz (50 ppm) was employed on 0.5–1 mM samples. Inverse gated decoupling was used to minimize the effects of dielectric heating. Typically, 2000 transients gave satisfactory signal to noise ratios (5/1 to 10/1) so that each point on a pH titration curve took approximately 2 h. While the diisopropylphosphoryl enzyme was slightly "aging" during the pH titration, this introduced no problems as the ^{31}P signals corresponding to the monoisopropylphosphoryl and diisopropylphosphoryl enzymes could always be differentiated.

Results and Discussion

Protocol for Observing Aromatic Resonances in Native Subtilisins. One of the most difficult problems in observing the aromatic region of the ^1H NMR of serine proteases is the presence in the same region of peptide backbone N–H resonances that exchange to N–D only very slowly.

The elegant methods employed by Markley and his coworkers, e.g., exchange the proenzyme in D_2O and then cleave to the active enzyme (on chymotrypsin; Markley & Ibanez, 1978) or exchange at pH 3 at high temperature (on trypsin; Markley & Porubcan, 1976), were not applicable to subtilisins. Subtilisins do not possess a zymogen form and undergo denaturation below pH 5.5 (Polgar & Bender, 1967). We therefore had to resort to exchange of the N–H to N–D at

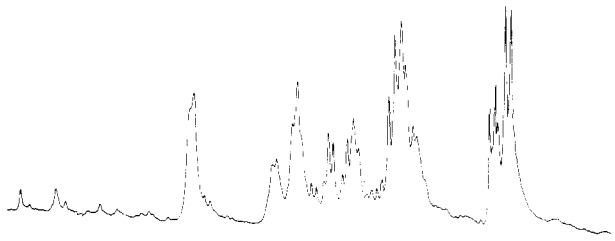

FIGURE 1: 500-MHz ^{1}H NMR spectrum of subtilisin BPN in D_2O between 6.7 and 8.5 ppm downfield from DSS near pH 7.0, 22 °C.

high pH, at which a very significant loss of active enzyme occurred due to autolysis. The small peptides were completely separated from the symmetrical protein peak by gel filtration. Recovery of the protein was 20–30%, but the protein contained 80–85% active enzyme according to active-site titrations.

It was also observed that Ca^{2+} (presumably present in all of these serine proteases), while stabilizing the enzyme against autolysis, also slowed down the N–H to N–D exchange rate very significantly. We therefore employed 3–5 mM EDTA during the incubation leading to exchange. As adventitious metal ions also degrade spectral resolution, EDTA was also employed during the recording of the spectra. It was observed that the presence of higher salt concentrations (0.2–0.5 M KCl) improved spectral resolution. Other qualitative observations were equally important. For example, subtilisin BPN underwent N–H to N–D exchange faster than subtilisin Carlsberg as evidenced by the spectral appearance on samples prepared under the same conditions. Also, any active-site modifications, including treatment with phenylmethanesulfonyl fluoride, phenylboronic acid, and diisopropyl fluorophosphate, led to much slower N–H to N–D exchange rates. These results imply less accessibility of the N–H's to solvent.

It is also worth mentioning that pulse techniques exist (Carr–Purcell and Carr–Purcell–Meiboom–Gill modifications for determining T_2's) that can emphasize the sharper C2–H resonances while broadening further the N–H resonances. Such techniques were not useful on subtilisins, unless preceded by extensive incubation in D_2O.

Figure 1 demonstrates the 500-MHz spectrum of the aromatic region of subtilisin BPN that had undergone extensive alkaline N–H to N–D exchange and was purified further on Sephadex G25-80. The aromatic region is quite well resolved for an enzyme of this size (ca. 275 amino acids), and the protocol should be useful for preparing samples even for two-dimensional or difference spectroscopic experiments. The aliphatic region (not shown) also possessed a large number of well-resolved resonances.

pH Titrations of the Histidine C2–H Resonances in Native Subtilisins. Figure 2 presents some spectra of subtilisin BPN at various pH values. First, it should be pointed out that there are two doublets present in the spectra of subtilisins from both sources, between 7.50 and 7.70 ppm. These two doublets have pH-independent chemical shifts between pH 5.7 and 8.5. By homonuclear decoupling techniques, it was demonstrated that these resonances are both coupled to resonances near 6.7 ppm. The doublet appearance is characteristic of the phenolic C–H's of tyrosines. On the basis of comparison of the areas of these doublets to the areas of C2–H resonances, it appears that both doublets belong to tyrosines whose phenol rings flip slowly on the ^{1}H NMR time scale. In fact, these tyrosines must be two among the seven that are conserved between the two primary sequences of the BPN and Carlsberg varieties of the enzyme. The doublet further downfield with peaks at 7.64 and 7.68 ppm is so remarkably constant in chemical shift between pH 5.7 and 8.5 that it can well serve as an internal chemical shift standard for the aromatic region.

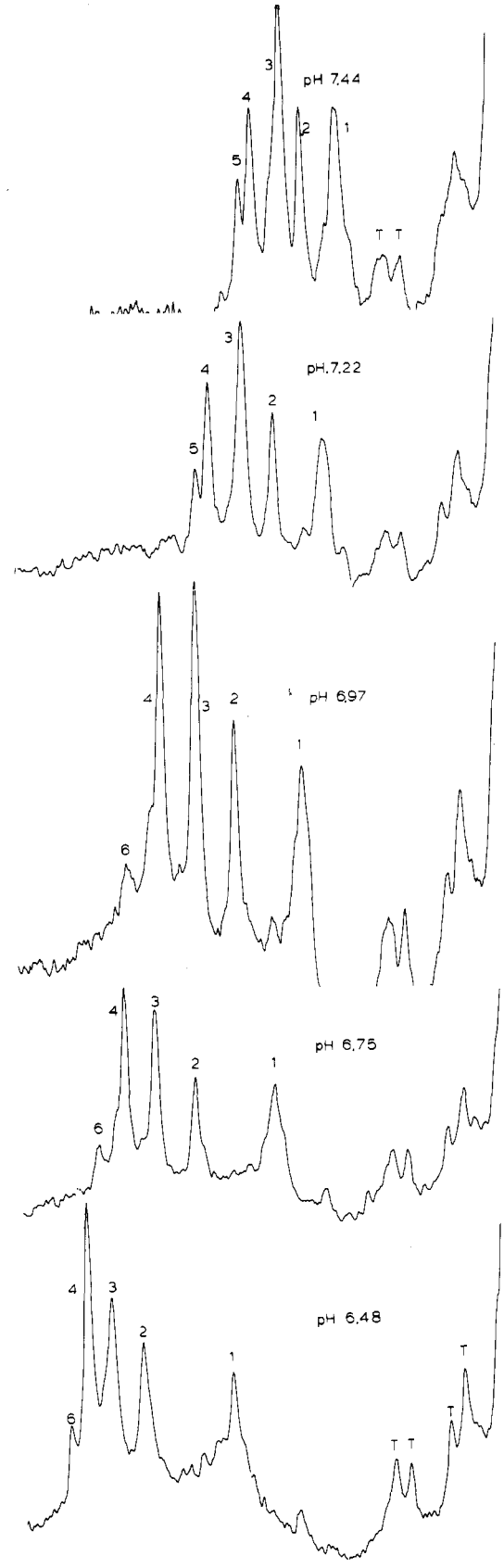

FIGURE 2: 200-MHz ^{1}H NMR spectra of subtilisin BPN in D_2O between 7.4 and 8.8 ppm downfield from DSS at 22 °C and the indicated pHs. T denotes tyrosine; the numbered peaks are pH-dependent His C2–H resonances.

Figure 2 illustrates that depending on the pH four or five His C2–H resonances are very clearly observed in the spectrum

Table I: Microscopic pK's[a] and Chemical Shift Parameters[b] Derived from Histidine C2-H Resonances in Native Subtilisins and under the Influence of Catalytic Center Perturbation

	subtilisin BPN				subtilisin Carlsberg		
peak	native pK (Δ,[b] ppm)	MIP[c] pK (Δ,[b] ppm)	DIP[d] pK (Δ,[b] ppm)	DIP(^{31}P)[e] pK (Δ,[b] ppm)	native pK (Δ,[b] ppm)	DIP[d] pK (Δ,[b] ppm)	DIP(^{31}P)[e] pK (Δ,[b] ppm)
1	6.40 (0.75)	6.54 (0.95)	6.32 (0.65)		6.36 (0.95)	6.38 (0.90)	
2	6.76 (0.90)	6.88 (0.90)	6.82 (0.90)		6.80 (0.85)	6.64 (0.90)	
3	7.00 (0.85)	7.12 (0.90)	7.00 (0.80)		6.94 (0.85)	7.02 (0.9)	
4	7.14 (0.90)	7.34 (1.00)	7.13 (0.80)		7.16 (0.90)	7.20 (0.9)	
5	7.20[f] (0.90)	7.32 (0.90)					
6	7.22[f] (0.90)				7.22[f] (0.90)		
			7.36 (0.90)	7.40 (1.5)		7.35 (0.95)	7.44 (1.5)

[a] All at 22–23 °C, ca. 0.2 ionic strength (KCl), uncertainty less than ±0.05; all determinations in D_2O; the values are uncorrected. [b] Difference in chemical shift between the extrema of the titration curves, i.e., between totally protonated and neutral species; the alkaline extremum is 7.70 ± 0.02 ppm for all C2-H's. [c] Monoisopropylphosphoryl enzyme obtained by aging the diisopropylphosphoryl form. [d] Diisopropylphosphoryl enzyme. [e] Diisopropylphosphoryl enzyme, pH titration of the ^{31}P NMR pH-dependent resonance. [f] Only partial pH titration curves were employed; see data in Figures 2 and 3.

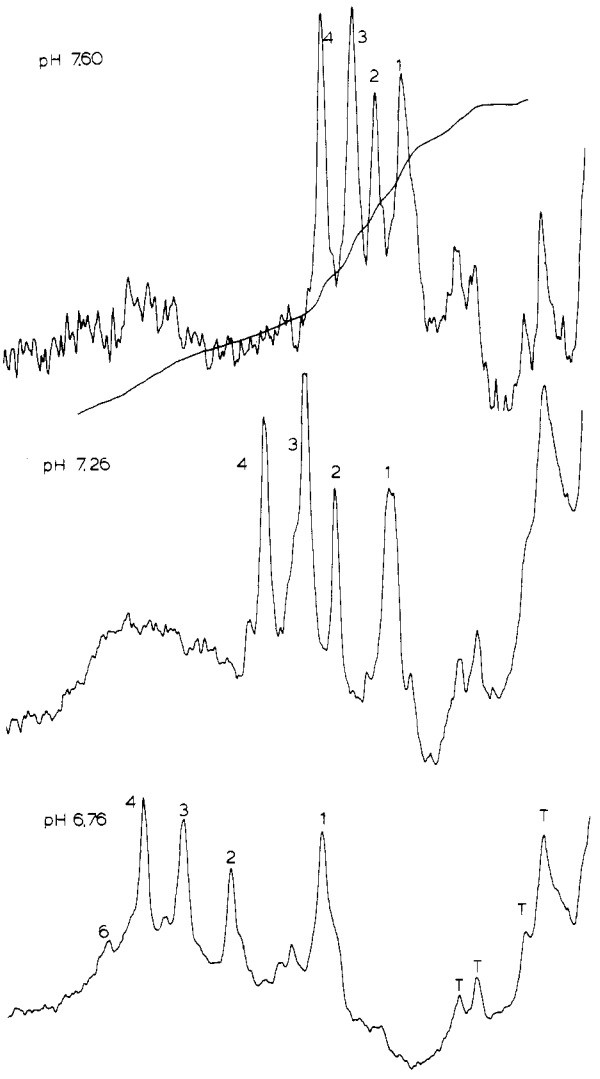

FIGURE 3: 200-MHz ^{1}H NMR spectra of subtilisin Carlsberg in D_2O between 7.4 and 8.8 ppm downfield from DSS at 22 °C and the indicated pHs. T denotes tyrosine; the numbered peaks are pH-dependent His C2-H resonances.

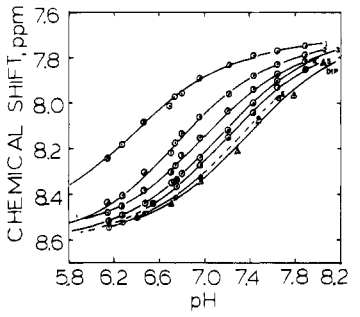

FIGURE 4: pH dependence of the chemical shifts of resonances shown in Figure 2 at 22 °C (subtilisin BPN). The solid lines are theoretical curves; the broken portions indicate regions over which no resonance was observed.

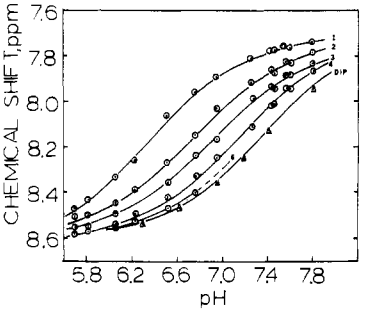

FIGURE 5: pH dependence of the chemical shifts of resonances shown in Figure 3 at 22 °C (subtilisin Carlsberg). The solid lines are theoretical curves to the experimental points.

of the BPN enzyme, of the six histidines reported to exist in subtilisin BPN (Smith et al., 1966). Figure 3 illustrates the appearance of the same chemical shift region at three pH values for subtilisin Carlsberg in which five histidines are reported in the primary sequence. Although at pH 7 or below the spectra from the two sources of the enzyme are superimposable, above pH 7, peak 4 in the spectrum of the BPN enzyme clearly splits into two peaks (4 and 5) that persist throughout the alkaline titration. At several pH values below pH 7, peak 5 is a downfield shoulder on peak 4. This peak 5 is totally absent in the spectrum of subtilisin Carlsberg. We assign this resonance to His-17 in the primary sequence of subtilisin BPN since that is the only His missing in subtilisin Carlsberg.

It should be pointed out that further downfield in a number of spectra especially at pH values below 7 there was found a small peak that also underwent pH-dependent chemical shift changes, characteristic of histidines. This peak is referred to as peak 6, and an approximate pK for this peak could be estimated (Table I) from a partial titration curve.

The chemical shift vs. pH titration curves are virtually superimposable for the two native enzyme sources as seen in Figures 4 and 5 and in Table I. The microscopic pK's obtained for peaks 1–4 in spectra of the Carlsberg enzyme and peaks 1–5 in spectra of the BPN enzyme appear to be perfectly normal. This is an important result when compared with two previous reports in the literature. In one of these, Ottesen and

Ralston reported studies on phenylmethanesulfonyl fluoride inhibited subtilisin BPN (Ottesen & Ralston, 1974). On the basis of potentiometric and electrophoretic studies, it was claimed that four histidines are buried in subtilisin BPN between pH 3.8 and 10.7 and do not change their states of ionization but remain neutral. However, the interpretation of their experimental data in terms of microscopic pK's for histidines appears to be incorrect in light of the present NMR results. This points out once again that one should be cautious when deducing microscopic pK's from macroscopic measurements on a protein. In another study, Omar et al. (1979) reported ^{1}H NMR studies on phenylmethanesulfonylsubtilisin BPN in which they observed four resonances between pH 4.0 and 7 that they attributed to His C2–H's. As our results pertaining to the native enzyme (Figures 4 and 5) were not affected by the addition of PMSF (not shown), both their reported pK's and their Δ values—the difference in chemical shift corresponding to the fully protonated and neutral His—are in discord with our results. According to their protocol, phenylmethanesulfonylsubtilisins underwent N–H → N–D exchange exceedingly slowly, and the resulting ^{1}H NMR spectra in general were of very poor quality.

Search for the Catalytic Center (His-64) C2–H Resonance. The data at hand did not allow us to assign a particular C2–H resonance to His-64 at the catalytic center. On the basis of kinetic experiments in D_2O, the uncorrected pK of His-64 is about 7.3 (Polgar, 1973). Therefore, the C2H resonance pertaining to His-64 is likely to be among peaks 3, 4, and 6. Several methods were tried to introduce a selective catalytic center perturbation that may affect only one of the observed resonances. As mentioned above, treatment of a sample of native BPN enzyme with a 2-fold molar excess of phenylmethanesulfonyl fluoride gave a spectrum essentially identical with that of the unperturbed enzyme at pH 7.0. Addition of leupeptin (at pH 7.0), a reasonably potent reversible inhibitor [cf. Philipp & Bender (1983)] of subtilisins, did not induce any chemical shift perturbation. That binding had taken place was evident, since all well-resolved C2–H resonances suffered some line broadening.

In transition-state analogue-like structures, the active center His should be protonated in the entire pH range of 6–8 (Robillard & Shulman 1972, 1974a,b; Jordan & Polgar, 1981; Kossiakoff & Spencer, 1981). We therefore expected to observe one C2–H resonance that not only is pH independent between pH 6 and 8 but also has a chemical shift that is characteristic of protonated His, i.e., downfield from ca. 7.9 ppm. There is good evidence that both phenylboronic acid (Kraut, 1977) and monoisopropylphosphoryl derivatives (Kossiakoff & Spencer, 1981) of serine proteases exist as transition-state-like tetrahedral geometries at the catalytic center.

First, the two reversible inhibitors phenylboronic acid and bis[3,5-(trifluoromethyl)phenyl]boronic acid were added to give final concentrations (at pH 7 or below) that would lead to greater than 90% inhibition as determined by activity measurements [K_i's are reported in the review by Philipp & Bender (1983)]. In the spectra of enzyme from either source, the four or five observed peaks suffered negligible, i.e., less than 0.03 ppm, chemical shift change upon saturation with the boronic acids. Peak 4 was somewhat diminished; peak 6 essentially disappeared. No additional peak at any pH between 6 and 8 appeared anywhere in the spectrum, i.e., ca. downfield from 7.9 ppm (Figure 6). There were pK perturbations of less than 0.1 unit upon saturation with phenylboronic acid (Figure 7).

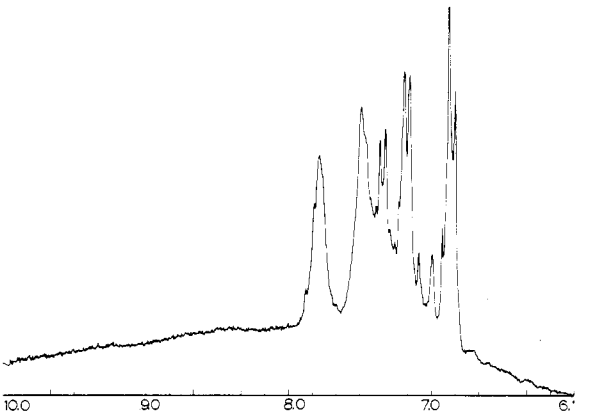

FIGURE 6: 200-MHz ^{1}H NMR spectrum of subtilisin BPN (~1 mM) in the presence of 3.5 mM phenylboronic acid at pH 7.96.

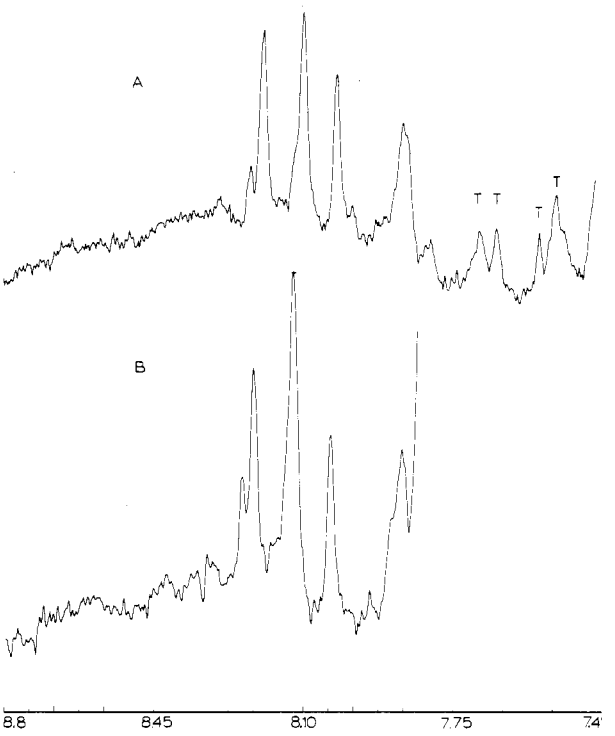

FIGURE 7: 200-MHz ^{1}H NMR spectrum of subtilisin BPN (~1 mM) in the absence (A) and in the presence (B) of 7.0 mM phenylboronic acid at pH 7.00.

The next transition-state analogue studied was monoisopropylphosphorylsubtilisin BPN which was prepared by conversion of the diisopropylphosphoryl derivative. Formation of the monoisopropylphosphoryl derivative was confirmed by ^{31}P NMR spectroscopy [see previous work on other serine proteases by this technique in Gorenstein & Findlay (1976), Reeck et al., (1977), and Porubcan et al. (1979); on subtilisin BPN by Van der Drift (1983)]. No unique perturbations were found in the ^{1}H NMR spectra recorded at different pHs. Peaks 1–5 shifted almost uniformly by about 0.15 ppm. Peak 4 underwent a larger change in pK and Δ than the other four peaks upon formation of the monoisopropylphosphoryl enzyme (Table I). Peak 6 was never observed in any of the spectra of monoisopropylphosphoryl enzyme nor was, however, the additional pH-independent peak apparent between pH 6.0 and 7.6 with a chemical shift characteristic of His H$^+$.

Finally, we turned to the diisopropylphosphoryl derivative. We had recently learned that the ^{31}P chemical shift of diisopropylphosphorylsubtilisin is pH dependent, describing a pK that presumably reflects the pK of His-64 in the catalytic

center (Van der Drift, 1983). Therefore, we prepared diisopropylphosphorylsubtilisin, also preexchanging N–H to N–D, and performed ^{31}P and ^{1}H experiments on the same sample under identical conditions. It was found that the pH dependence of the ^{31}P resonance and of only one of the C2–H resonances gave identical pK's within experimental error. Specifically, the active center had a pK of 7.40 ± 0.05 in D_2O according to ^{31}P NMR chemical shift measurements of the diisopropylphosphoryl derivative (both Carlsberg and BPN). The ^{1}H NMR pH titration curves of the same samples indicated that there was an additional prominent resonance further downfield than peaks 1–4 in Carlsberg, or peaks 1–5 in BPN, a peak (DIP in Figures 4 and 5) that titrated with a pK of 7.35 ± 0.05. Peaks 1–4 had essentially identical pK's in the diisopropylphosphoryl and native enzymes (Table I), and peak 6 was missing in the former. Therefore, in the diisopropylphosphoryl subtilisins, the active center His-64 has a pK of ca. 7.40. At pH 8.10, no ^{1}H resonance was detected below 7.8 ppm.

Peak 6 is tentatively assigned to His-64 in the native enzyme for the following reason. Of all peaks that were observed to undergo pH-dependent chemical shift changes, only peaks 3, 4, and 6 have pK's near the 7.3 value observed kinetically (Polgar, 1973). However, peaks 3 and 4 can be observed in *all* preparations, including the diisopropylphosphoryl enzymes, in which peaks 3 and 4 clearly *are not* the resonance corresponding to the active center.

Why does one observe different relative areas of peak 6 to peaks 1–5 in the native enzyme (<1), in the diisopropylphosphoryl enzyme (~1), and in the transition-state analogues (≪1)? Several possible explanations exist. One is that the C2–H corresponding to peak 6 exchanges with solvent D_2O at different rates in the three different environments. In fact, there is some evidence that the various C2–H's exchange at somewhat different rates (see Figures 2 and 3). The fact that the area of peak 6 compared to the areas of peaks 1–5 appears to be nearly the same in a large number of native enzyme preparations argues against this explanation. Also counter to this explanation is the observation that peak 6 disappears as soon as either phenylboronic acid derivative is added to a D_2O solution of the native enzyme.

A second likely explanation is that the mobility of the His-64 imidazole is different in the three environments; hence, the size of peak 6 is variable. For a given set of spectral acquisition parameters, the size of some of the peaks may diminish if the corresponding nucleus is partially relaxed. The peak may be too broad to be observed if the imidazole ring is immobilized. Yet a third possibility is that there is a slow exchange among various environments in which His-64 may exist in the native enzyme, and we only monitor one of them. When we observed the His imidazole N^3–H with a different technique (Jordan & Polgar, 1981), the exchange of this hydrogen with H_2O as solvent was fast enough in the native enzymes so as to make this resonance too broad to observe. On the basis of several experiments, it was concluded that the active center imidazole is more accessible to solvent (and likely more mobile) in the native state than in the transition-state-bound analogues. Therefore, if peak 6 does correspond to His-64, in the transition-state analogue-bound forms peak 6 should be even broader than in the native enzyme, as is found. Because of the difficulty in correctly integrating the peak areas (there is always a residual N–H background), we cannot choose among the three choices (or among any others not mentioned). Yet the collective evidence suggests that the mobility/relaxation argument is at least partly responsible for the observations here reported. Of all the active center perturbations introduced, only diisopropylphosphorylation was large enough to allow rapid averaged motion for the imidazole of His-64.

The tentative assignment of peak 6 to His-64 in the native enzyme suggests that this His is the most basic one in subtilisins. This enhanced basicity is perhaps due to the presence of the Asp-32 carboxylate that would stabilize the histidinium ion by electrostatic forces. The fact that peak 6 is better resolved below the pK, than above it, may imply that above pH 7 there is a hydrogen bond between Ser-221 as donor and His-64 as acceptor, thus rendering His-64 less mobile and the corresponding resonances more difficult to observe. While we are aware of the fact that current crystallographic evidence is against such a hydrogen bond in the native subtilisin (Kraut, 1977; Steitz & Shulman, 1982), we believe that such a hydrogen bond is not yet excluded in solution.

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Registry No. Ca, 7440-70-2; subtilisin, 9014-01-1; L-histidine, 71-00-1; diisopropyl fluorophosphate, 55-91-4.

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Naturally Occurring Porcine Relaxins and Large-Scale Preparation of the B29 Hormone†

Erika E. Büllesbach* and Christian Schwabe
Department of Biochemistry, Medical University of South Carolina, Charleston, South Carolina 29425
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ABSTRACT: Porcine ovaries were collected from pregnant sows under conditions designed to keep autolysis to an absolute minimum. During the extraction the tissues were never allowed to warm up to 0 °C until submerged in 1.6 N HCl. Isolation and fractionation of the various relaxin forms became possible by application of CM-cellulose chromatography at pH 5.5 and pH 7.8, gel filtration, and high-performance liquid chromatography. The new isolation procedure has made it possible to isolate and identify Leu^{B32} relaxin. Also, [Leu-Phe^{A0}]B29 relaxin was identified and the existence of a [Leu-Phe^{A0}]B32 relaxin may be deduced from our data. Controlled digestion of B-chain-extended relaxins with carboxypeptidase A led to the large-scale production of homogeneous B29 relaxin, a suitable starting material for controlled chemical modification of porcine relaxin.

The peptide hormone relaxin causes the softening and lengthening of the symphysis pubis through significant connective tissue changes in the target region, as well as the inhibition of the uterine muscle contraction [for review, see Schwabe et al. (1978)]. Porcine relaxin (Figure 1) (Schwabe et al., 1976, 1977; Schwabe & McDonald, 1977; James et al., 1977) consists of two chains linked by disulfide bonds. The relative position of the one intrachain and the two interchain disulfide links is identical with the disulfide bond location in the pancreatic hormone insulin (Schwabe & McDonald, 1977). One significant property of porcine relaxin is the lack of homogeneity of the biologically and immunologically active material when isolated by the method of Sherwood & O'Byrne (1974), who obtained three equipotent relaxins in comparable amounts via ion-exchange chromatography. Sequence analysis of these different forms of relaxin have elucidated a constant A chain of 22 amino acids and variations in length at the C terminus of the B chain from 28 to 31 amino acid residues (Niall et al., 1980). Walsh & Niall (1980), using a small-scale extraction procedure, described B31 relaxin as the predominant form of the hormone in pig ovaries; only minimal contaminants of B29 relaxin were observed. They postulated that shortened forms are generated during the isolation procedure by proteolysis.

Using a different isolation procedure, we have recently obtained the first relaxin variant involving the A chain (Büllesbach & Schwabe, 1985). A [Phe^{A0}]relaxin was observed in considerable amounts (about 10%), and we have postulated that this [Phe^{A0}]relaxin is an intermediate in prorelaxin–relaxin conversion. We have furthermore predicted that a [Leu-Phe^{A0}]relaxin should be occurring in porcine ovaries.

Neither the variability in length of the C terminus of the B chain (Sherwood & O'Byrne, 1974; Tregear et al., 1983; Anderson, 1984) nor the extension at the N terminus of the A chain (Büllesbach & Schwabe, 1985; Schwabe, 1983) caused differences in biological activities. However, the chemical modification of relaxin and the semisynthesis of relaxin analogues would be severely hampered by the variability of available material.

The ultimate aim of the experiments presented in this paper was the production of a highly purified porcine relaxin on a preparative scale. The homogeneous relaxin could serve as defined starting material for chemical modifications for studies of the structure–function relationship of porcine relaxin and of the relaxin receptor.

Experimental Procedures

Materials

Porcine ovaries from late pregnant sows were collected in liquid nitrogen and stored at −70 °C before use. Immature ICR mice (20–25 g) were used for bioassay. Chemicals for chromatography were HPLC[1]-grade (J. T. Baker Chemicals Co., Phillipsburg, NJ) or distilled in glass quality (Burdick & Jackson, Muskegon, MI). All other chemicals were analytically pure.

Urea stock solution (7 M) was stored over a mixed-bed ion exchanger at 4 °C. Carboxypeptidase A (bovine pancreas, EC 3.4.17.1) was obtained from Schwarz Bioresearch Inc. Dialysis membrane tubing (Spectrophor 3, exclusion limit M_r 3500) was purchased from American Scientific Products (McGaw Park, IL). CM-cellulose (Whatman CM-52) was supplied by Reeve Angel (Clifton, NJ), and Sephadex products

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[1] Abbreviations: CP-A, carboxypeptidase A; HPLC, high-performance liquid chromatography; Tris, tris(hydroxymethyl)aminomethane; PTH, phenylthiohydantoin.

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