Proton Nuclear Magnetic Resonance Studies on Bacitracin A and Its Interaction with Zinc Ion†

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ABSTRACT: Bacitracin A is a cyclic dodecapeptide antibiotic produced by *Bacillus licheniformis*. A divalent metal cation, such as Zn^{2+}, is required for biological activity. Previous studies have shown one metal ion bound per bacitracin A molecule in solution. A model has been proposed [Scogin, D. A., Mosberg, H. I., Storm, D. R., & Gennis, R. B. (1980) *Biochemistry* (preceding paper in this issue)] in which the metal is bound to the histidine, thiazoline, and glutamic acid residues of the peptide. The aspartic acid and N-terminal amino group are not directly involved in metal binding, but the p*K* of the latter group is lowered in the presence of the bound metal cation. In this paper, high-resolution ^1H NMR is used to examine Zn^{2+} binding to bacitracin A. Assignments were made by monitoring the pH dependence of the ^1H NMR chemical shifts and also by using homonuclear decoupling at 270 MHz. Resonances were assigned to the N-terminal isoleucine, the thiazoline ring, the histidine, the glutamic acid, and the aspartic acid residues. In the presence of 0.025 M Zn^{2+} at pH 4.8, a number of resonances are perturbed. Analysis indicates Zn^{2+} binding to the thiazoline ring, the histidine imidazole, and the glutamic acid carboxyl. The aspartate group is not involved in metal binding, as previously postulated. Data pertaining to the involvement of the N-terminal amino are equivocal but are consistent with the suggested model. All the ^1H NMR data are compatible with this model. To a large extent, this defines the conformation of the zinc–bacitracin A complex in solution.

Bacitracin A is a dodecapeptide antibiotic that requires a divalent metal ion for biological activity. Commonly, zinc portrays this role, although several other metal ions are equally effective (Weinberg, 1965). It has been shown (Scogin et al., 1980; Craig et al., 1969; Chornock, 1957; Gross, 1954) that the stoichiometry of the metal binding is 1:1. There are several groups on the polypeptide that can participate in this binding. Previous work from other laboratories has implicated many of these groups, but the results have been conflicting. We have chosen to reexamine the divalent metal ion binding properties of bacitracin A via high-resolution nuclear magnetic resonance (^1H NMR).[1]

The ^1H NMR spectrum of bacitracin A has been only partially assigned (Campbell et al., 1974; Cornell & Guiney, 1970). Although the complete assignment of the bacitracin A spectrum was not a goal of this study, the assignment of groups likely to interact with divalent metal ions was vital. To this end, assignments were made at 220 MHz based on pH-dependent chemical shifts indicative of nearby titrating groups. In addition, homonuclear decoupling experiments were performed at 270 MHz to confirm assignments.

Materials and Methods

Commercial bacitracin, a mixture of similar peptides, was donated by IMC Chemical Group, Terre Haute, IN. The active form, bacitracin A, was isolated by countercurrent distribution, and inorganic phosphate was removed by anion-exchange chromatography as described previously (Scogin et al., 1980).

The purified bacitracin A was lyophilized and dissolved in 99.8% D_2O (Columbia Organic Chemicals) which had been treated with Chelex 100 resin to remove polyvalent metal ions. The bacitracin A was then relyophilized and dissolved in 100.0% D_2O which is low in paramagnetic impurities (Stohler Isotope Chemicals) to decrease the proton resonance of the solvent. This procedure minimizes complications due to divalent metal ions. The pH was adjusted with DCl or NaOD. All reported pH values are uncorrected meter readings. Bacitracin A ^1H NMR samples were ~10 mM in antibiotic and usually contained a small amount of 2,2-dimethyl-2-silapentane-5-sulfonate (DSS) as an internal chemical shift standard. All ^1H NMR samples were unbuffered. Gel filtration chromatography on bacitracin A, both in the presence and in the absence of Zn^{2+} under conditions similar to those used for ^1H NMR experiments, showed no peptide aggregation.

For zinc titrations, solutions of ultrapure $ZnCl_2$ (Alfa Products, Danvers, MA) were used. A constant pH was maintained by using an autoburet after successive zinc additions.

^1H NMR spectra at various pH values and zinc concentrations were obtained on a Varian HR220 spectrometer modified for pulsed, Fourier transform experiments with a Nicolet Instruments Corp. NIC-80 system. Probe temperature was maintained at 26 °C by a Varian temperature control unit. The still dominant HDO resonance was reduced by using a two-pulse, 180°–τ–90° experiment (Patt & Sykes, 1972) in which τ is adjusted such that the water signal is approximately nulled while the faster relaxing bacitracin A resonances are fully relaxed. The absence of any appreciable paramagnetic impurity was indicated by the long values of the HDO longitudinal relaxation time inferred from the τ values required for nulling the solvent resonance.

^1H NMR spectra at 270 MHz were obtained at the Department of Chemistry, University of Wisconsin, Madison, on a Bruker WH270 spectrometer with homonuclear double-resonance capability. Spectra were obtained on a sample in

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[1] Abbreviations used: DSS, 2,2-dimethyl-2-silapentane-5-sulfonate; ^1H NMR, proton nuclear magnetic resonance.

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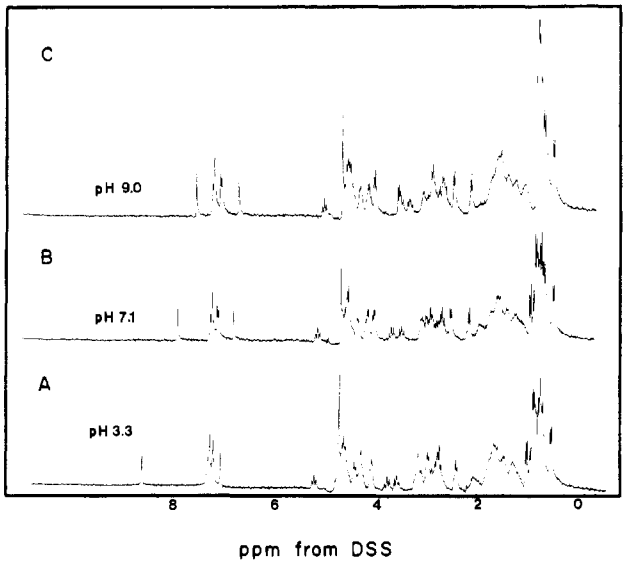

FIGURE 1: Effect of pH on the 220-MHz ^{1}H NMR spectrum of bacitracin A. Chemical shifts are relative to DSS.

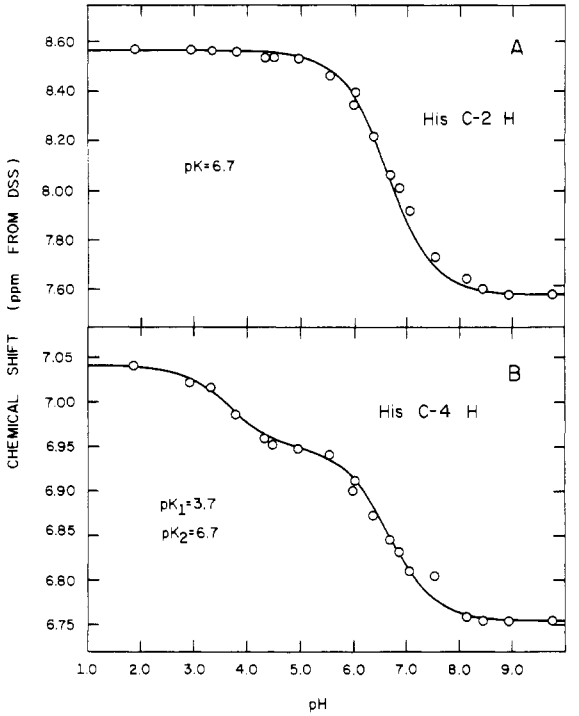

FIGURE 2: Effect of pH on the chemical shifts of the histidine imidazole protons.

D_2O at pH 5.8 without attempting to diminish the solvent resonance.

Results and Discussion

pH Dependence of Chemical Shifts. For a polypeptide like bacitracin A with a known structure and several titratable groups, the pH dependence of the ^{1}H NMR chemical shifts can greatly aid the spectral assignment. As Figure 1 shows, there is a large pH effect on the spectrum and many of the affected resonances are sufficiently isolated to be easily followed. Figures 2 and 3 depict the pH dependence of the chemical shifts of some of the protons most affected and hence, most probably, proximal to titrating groups.

Figure 2 describes the pH effect on the chemical shifts of the easily assigned histidine C-2 and C-4 protons. Both protons titrate with a pK of 6.7, well within the range expected for the histidine imidazole. In addition, the C-4 proton undergoes

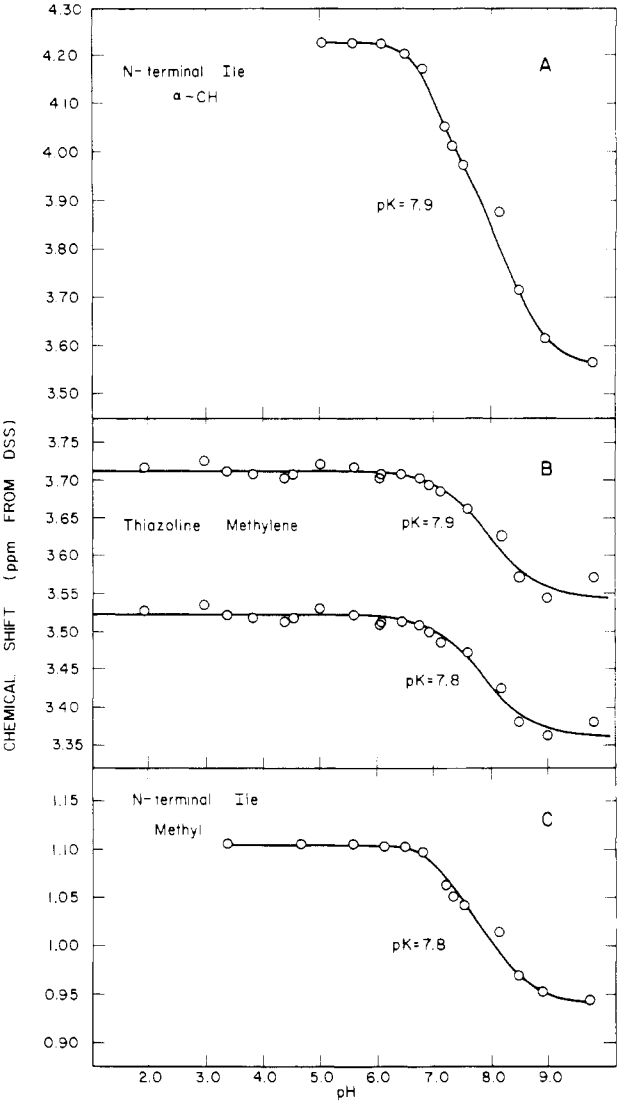

FIGURE 3: Effect of pH on the chemical shifts of the (A) N-terminal isoleucine α-carbon proton, (B) ring methylene protons of the thiazoline moiety, and (C) N-terminal Ile methyl protons.

a second inflection with a pK of 3.7 presumably due to titration of the neighbor aspartate carboxyl group.

The pH-dependent chemical shift of two multiplets in the chemical shift range expected for the β-methylene protons of aspartate and the γ protons of glutamate (McDonald & Phillips, 1969) was also observed (data not shown). The more downfield multiplet titrates with a pK of 4.0 while the upfield multiplet has a pK of 4.3. The pK values and the relative chemical shifts are consistent with an assignment of the aspartate β-methylene protons giving rise to the more downfield multiplet and the glutamate γ-methylene protons being responsible for the upfield multiplet (McDonald & Phillips, 1969). Unequivocal assignment was possible from homonuclear decoupling experiments (vide infra).

Figure 3 details the pH dependence of the chemical shift of four resonances influenced by a group titrating with a pK of 7.8–7.9. Of the possible groups in bacitracin A, this is almost certainly the N-terminal isoleucine amino group as the pK expected for the δ amino of the ornithine residue would be much higher.

Figure 3A is an unresolved doublet in a group of resonances ~4 ppm downfield of DSS (pH <6). Increasing the pH results in a profound upfield shift. The chemical shift range (which is within the region expected for α-carbon protons),

Table I: Comparison of pK Values from NMR and Direct Titration of Bacitracin A[a]

group	pK_{NMR}	pK_{DT}
Asp COO^-	4.0	4.1
Glu COO^-	4.3	4.5
His imidazole	6.7	6.7
Ile NH_3^+	7.9	7.7
Orn δ-NH_3^+		10.0

[a] The direct titration was performed as previously described (Scogin et al., 1980) in H_2O. The pK values were not significantly altered when the titration was performed in D_2O.

the multiplicity, and the pronounced sensitivity to pH all lead to the conclusion that the resonance monitored in Figure 3A is due to the N-terminal isoleucine α-carbon proton.

The pH dependence of a pair of low-intensity apparent triplets upfield of the α-carbon proton region is shown in Figure 3B. The multiplets titrate with a pK of 7.8–7.9 and are obviously influenced by the titration of the isoleucine amino group. The chemical shifts and multiplicities are inconsistent with any protons on the isoleucine, however. The chemical shifts are similar to those seen for the ring protons of 2-methyl-2-thiazoline (Weinberger & Greenhalgh, 1963). Since the two methylene protons of the thiazoline are inequivalent (Weinberger & Greenhalgh, 1963), one would expect a complex and not easily predicted splitting pattern for these two protons and the ring methine proton to which they are also coupled (Emsley et al., 1965). While this pattern might not be readily apparent, the assignments can be made by reducing the spectrum to a simpler one via decoupling (vide infra).

Figure 3C depicts the effect of pH on the chemical shift of the most downfield doublet in the methyl region of the spectrum. This resonance, too, reflects the titration of the terminal amino group. The pH dependence implies that the resonance arises from a methyl group of the terminal isoleucine. The chemical shift, downfield of the other methyl resonances at pH ≲8, is consistent with this assignment since the protonated amino group has a deshielding effect. The multiplicity allows the methyl group to be identified as bonded to the methine carbon. The remaining methyl group, which gives rise to a triplet, is also influenced by the amino group titration but, due to interference from other overlapping resonances, the effect is not easily followed.

Direct pH Titration. The pK values determined from the ^{1}H NMR chemical shifts can be compared to those obtained from a direct pH titration. Bacitracin A has five titratable groups. The pK values have been determined (Scogin et al., 1980) and are listed in Table I. Also listed in Table I are the pK values determined from the ^{1}H NMR studies.

Homonuclear Double Resonance. Homonuclear decoupling experiments were performed at 270 MHz to aid in the elucidation of several important spectral assignments. The ^{1}H NMR spectrum at this frequency is shown in Figure 4 in which some resonances, important for the discussion below, are labeled.

Figure 5 depicts the portion of the 270-MHz ^{1}H NMR spectrum from about 2.5 to 4.5 ppm under various decoupling conditions. Figure 5A shows the region in the absence of decoupling for comparison. Figure 5B demonstrates the effect of irradiating the multiplet centered at 5.24 ppm (resonance a, Figure 4). This spectrum indicates that the two multiplets affected (resonances c and d, Figure 4) are unresolved doublets of doublets, consistent with their assignment as the methylene protons of the thiazoline moiety. Figure 5C, in which the irradiation is at 3.58 ppm (resonance d, Figure 4), shows that the residual splitting in Figure 5B of resonances c and d is due

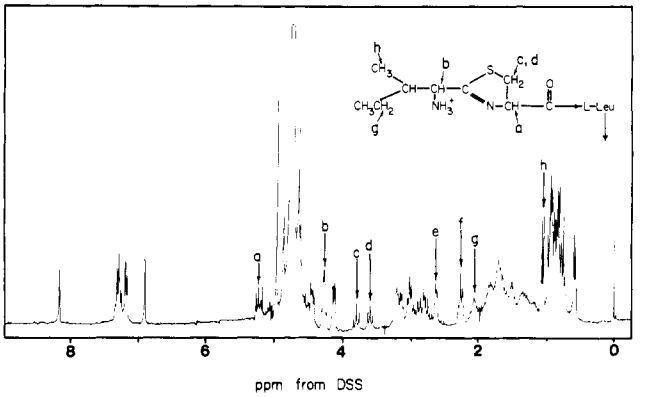

FIGURE 4: 270-MHz ^{1}H NMR spectrum of bacitracin A at pH 5.8. Letters denote multiplets of interest in decoupling experiments.

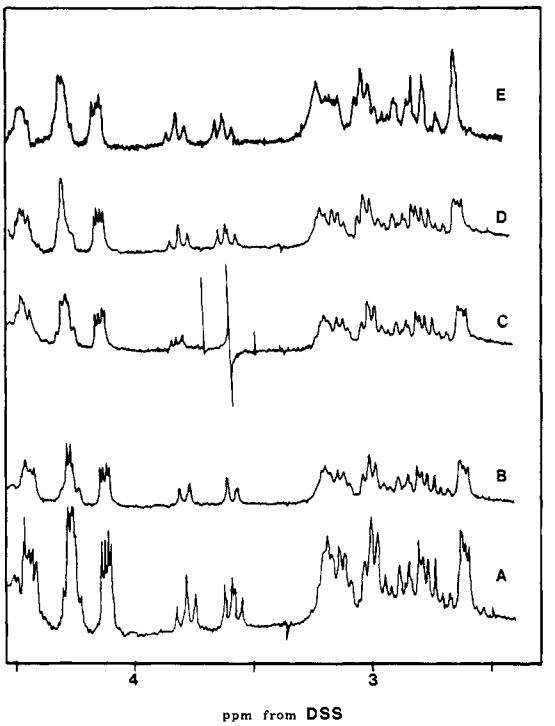

FIGURE 5: 2.5–4.5-ppm region of the 270-MHz bacitracin A ^{1}H NMR spectrum at pH 5.8 with the decoupling field applied at (A) no decoupling, (B) 5.24 ppm, (C) 3.58 ppm, (D) 2.06 ppm, and (E) 4.65 ppm.

to coupling between these two protons. Thus, resonance c and also resonance a (not shown) are reduced to doublets by irradiating at 3.58 ppm. This further strengthens the thiazoline assignments.

Figure 5D shows the result of decoupling at 2.06 ppm (resonance g, Figure 4) in the methylene region. This methylene multiplet is unresolved but has an intensity consistent with its arising from a single pair of methylene protons. The resultant partial collapse of the multiplet at 4.26 ppm (resonance b, Figure 4) and the concomitant collapse of the methyl doublet at 1.06 ppm (resonance h, Figure 4) shown in Figure 6B support the assignment that these resonances arise from the same residue, the terminal isoleucine. It should be noted that the position of the methylene multiplet at 2.06 ppm; i.e. downfield of the remainder of the group of methylene protons, is also consistent with the assignment of the protonated (at pH 5.8) terminal isoleucine.

Figure 5E details the effect of decoupling at 4.65 ppm. This is within the region of the spectrum, partially obscured by the residual solvent peak, where the α-carbon protons are expected

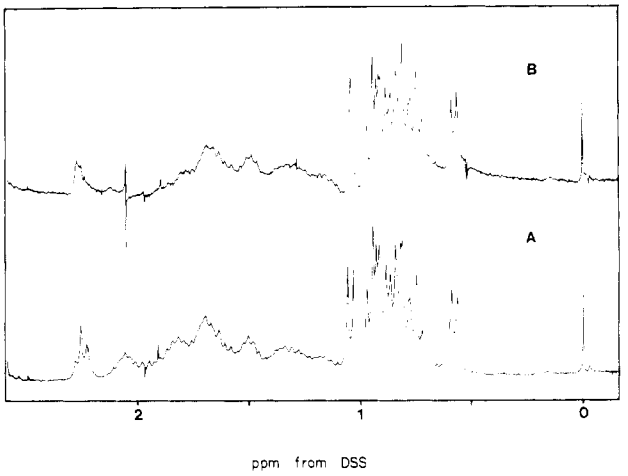

FIGURE 6: 0–2.5-ppm region of the 270-MHz bacitracin A ^{1}H NMR spectrum at pH 5.8: (A) with no decoupling; (B) with decoupling at 2.06 ppm.

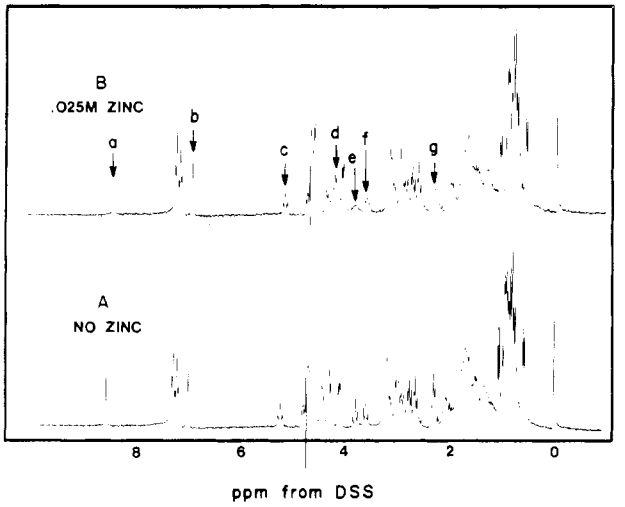

FIGURE 7: 220-MHz ^{1}H NMR spectrum of bacitracin A at pH 4.8 in the (A) absence and (B) presence of Zn^{2+}. The letters denote resonances affected by metal. See text for discussion.

to resonate. The resultant collapse of the multiplet at 2.60 ppm (resonance e, Figure 4), tentatively assigned above as the methylene adjacent to the aspartate carboxyl, allows positive identification. Since only the carboxyl-bound methylene of the aspartate, and not that of the glutamate, is also adjacent to an α carbon, the resonance at 2.60 ppm must arise from the aspartic acid residue. The resonance at 2.24 ppm (resonance f, Figure 4) then is due to the γ-methylene protons of the glutamic acid residue. This is in accord with the tentative assignment (vide supra) based on relative chemical shifts and pK values.

Metal Binding Studies. Figure 7 shows the effect of 0.025 M $ZnCl_2$ on the bacitracin A 220-MHz ^{1}H NMR spectrum at pH 4.8. Under these conditions, with a total bacitracin A concentration of 10 mM, the antibiotic is ~50% saturated with Zn^{2+}. The major resonances affected are labeled in Figure 7B. Resonances a and b, the histidine C-2 and C-4 protons, respectively, are shifted upfield and broadened, with the C-2 proton being more affected. Resonance c, the thiazoline methine proton, and resonance d, the terminal isoleucine α-carbon proton (partially obscured by a group of resonances), are both shifted upfield. Resonances e and f, due to the thiazoline methylene protons, and resonance g, corresponding to the glutamic acid γ-carbon protons, are all shifted downfield and broadened.

It is apparent from Figure 7 that both upfield and downfield shifts occur upon Zn^{2+} binding. This is to be expected since the direction of the shift is dictated by the ionization state of the group to which the metal is binding. Thus, for an uncharged or negatively charged group, binding of the metal ion results in a decrease in electron density in the vicinity of the functional group and a subsequent downfield shift of the resonances of proximal groups. Conversely, metal ion binding to a positively charged group involves displacement of a proton which, having a higher charge density, is more effective at lowering the electron density around this group. Thus, metal ion binding in this case results in an upfield shift of neighboring protons. In Figure 7, then, the histidine resonances shift upfield due to Zn^{2+} binding and the concomitant release of a proton from the protonated (at pH 4.8) imidazole. The large downfield shifts of the thiazoline methylene protons indicate Zn^{2+} binding at this neutral (at pH 4.8) group, while Zn^{2+} binding to the negatively charged glutamate carboxyl (~75% ionized) is indicated by the large downfield shift in the γ-methylene protons.

The α-carbon proton of the amino-terminal isoleucine shifts upfield upon addition of Zn^{2+}, consistent with the binding of the metal to the protonated amino group and consequent proton release. This, however, would require more protons to be released than are observed (Scogin et al., 1980). The model proposed in the accompanying paper (Scogin et al., 1980) can be used to explain this discrepancy. This model requires that the binding of a divalent metal ion results in a lowering of the pK of the terminal amino group, which is not directly involved in the metal binding. This pK is reduced from 7.8 to 5.7, a change which is reflected in the ^{1}H NMR spectrum by an upfield shift of the α-carbon proton due to a now significant fraction of unprotonated amino group (at pH 4.8).

It is interesting that while the thiazoline methylene protons shift downfield upon addition of Zn^{2+}, the methine resonance shifts slightly upfield. This upfield shift is most likely due to the deprotonation of (or possible metal binding to) the adjacent isoleucine amino group, but it could also be a reflection of a change in environment due to a conformational change accompanying Zn^{2+} binding. In either case, the large downfield shifts of the methylene resonances suggest that the Zn^{2+} binds to the sulfur rather than the nitrogen of the thiazoline ring.

The broadening observed in the shifted resonances of Figure 7B is a consequence of an exchange rate of the Zn^{2+} between bacitracin-bound and free environments that is intermediate on the ^{1}H NMR time scale; i.e., of the same order as the chemical shift difference of the affected resonance between the two environments. It is possible for resonances on the same molecule to exhibit different exchange rates. Exchange in the fast exchange region would be easily detected by the required chemical shift change. Slow exchange would result in two separate resonances, one for each environment. In the unlikely case that the Zn^{2+}-bound functional group were motionally restricted to a large enough extent that its resonance would be difficult to detect, binding would still be apparent in the reduced intensity of the unbound resonance. Since for the present case, in which an ~50% reduction in intensity would be expected, no resonances are unshifted and markedly reduced in intensity, we may conclude that no groups other than those already indicated are involved in Zn^{2+} binding.

Conclusions

The chemical shifts resulting from protonation and metal binding to the peptide may be due to the direct influence of changes in electron density or may result indirectly from an

altered conformation of the peptide. The results presented can be reasonably interpreted by assuming the simplest case, where changes in electron density alone are responsible for the observed effects. Glutamic acid, histidine, and the thiazoline moiety all appear to be involved in zinc binding, but there is no evidence for the involvement of the aspartic acid residue. The N-terminal amino group may be involved in binding or may experience a lowering in pK upon binding. When combined with the proton release data of the accompanying paper (Scogin et al., 1980), it appears likely that the latter possibility obtains. The proton release data also exonerate the ornithine amino of involvement. These results can be compared to the findings of others.

Cornell & Guiney (1970), using ^{1}H NMR and optical rotatory dispersion, found evidence of Zn^{2+} binding to the histidine and thiazoline moieties of bacitracin. No evidence for or against participation by other groups was reported. Further, the ^{1}H NMR data, obtained from a methanol solution, suggested preferential binding of Zn^{2+} to the N-3 nitrogen of the imidazole ring since equal shifts were observed for the C-2 and C-4 proton resonances. By contrast, our results, from aqueous solutions, reveal much larger shifts for the C-2 proton than for the C-4 proton. This would imply either preferential binding to the N-1 nitrogen or nonpreferential binding since the latter condition would also result in a greater effect on the C-2 proton (between the two nitrogens) than on the C-4 proton.

Wasylishen & Graham (1975) observed the effect of Cu^{2+} and Mn^{2+} on the ^{13}C NMR spectrum of bacitracin. They concluded that Cu^{2+} binds to the histidine ring and the aspartic and glutamic acid carboxyl groups over the pH range 4.5–7.4. At pH 6.6, Mn^{2+} was found to bind to the same groups, as well as to the thiazoline.

By contrast, Garbutt et al. (1961) found that Mn^{2+} did not bind to bacitracin at pH <7. Cu^{2+} was found to bind to the same groups implicated in the study by Wasylishen & Graham, while Zn^{2+}, Co^{2+}, and Ni^{2+} were found to bind to the histidine (and presumably the thiazoline) but not the carboxyl groups.

These two studies may have been hindered by their reliance on preparations containing a mixture of bacitracins. In addition, solutions of bacitracin A at 0.08–0.2 M, the concentrations reported by Wasylishen & Graham (1975), show signs of significant aggregation in the presence of divalent metal ions. The influence this might have on the observed metal binding is unclear. The ^{13}C NMR experiments also might be sensitive to weak binding of these paramagnetic metals to secondary sites of interaction on the peptide.

Craig et al. (1969) concluded that bacitracin A bound to zinc via the histidine imidazole, the peptide nitrogen of the histidine, the thiazoline nitrogen or sulfur, and the free amino group of the terminal isoleucine. Their deductions concerning the terminal amino group were based partly on the result of a proton release experiment which is not compatible with results from this laboratory (Scogin et al., 1980).

The conformational restrictions placed on the Zn^{2+}–bacitracin A complex by the groups implicated in binding should greatly aid the elucidation of the conformation. Determination of the backbone conformation by measurement of the $J_{NH,\alpha CH}$ coupling constants (Barfield & Karplus, 1969) can be facilitated by the structural limitations imposed by the required orientation of side-chain groups involved in Zn^{2+} binding. The resultant conformation should be very reliable.

Acknowledgments

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