BINDING SITES FOR ZINC(II) IN BACITRACIN

Neal W. Cornell and Donald G. Guiney, Jr.
Department of Chemistry, Pomona College, Claremont, California 91711

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Summary: Formation of the bacitracin-Zn^{+2} complex has been studied by spectrophotometric titration, optical rotatory dispersion and nuclear magnetic resonance. ORD spectra indicate that the bacitracin thiazoline provides one coordination site for metal ions. A second site is suggested by titration data which give a pK of 6.2 for complex formation, thus implicating the histidine residue. NMR studies provide additional evidence in agreement with titration data. In the presence of Zn^{+2} the imidazole C-4 and C-2 hydrogen peaks are shifted downfield by 0.18-0.20 ppm, suggesting that the metal ion coordinates with the nitrogen atom at position 3 in the histidine residue.

Bacitracin is a dodecapeptide antibiotic produced by *Bacillus licheniformis*, and it has several interesting chemical features (1,2). Among these are a thiazoline ring structure which is essential for antibiotic activity (2), and the stabilizing and activating effects of divalent metal ions (2, 3,4). The most extensive chemical studies of metal ion activation have been performed by Craig and his co-workers (2) who have developed techniques for large scale purification of bacitracin A, the major active component in commercial preparations. Because the bacitracin system provides a model for metal ion activation of enzymes, we have conducted studies aimed at defining the sites in bacitracin which participate in metal binding. Results presented here are interpreted to indicate that the thiazoline ring and the histidine residue provide two coordination sites for Zn^{+2}. A role for histidine in forming bacitracin-metal ion complexes was suggested previously (2,5). In addition, the ease with which unambiguous NMR spectra can be obtained with the bacitracin-Zn^{+2} complex may be useful in extending this spectroscopic technique to the study of protein-metal ion complexes.

Materials and Methods

Bacitracin, purchased from Calbiochem, had antibiotic activity of 50-55 units/mg (5), was a gray-yellow powder and, when chromatographed on car-

boxymethyl-Sephadex G-25, was indicated to be a mixture of several UV-absorbing materials. Fractionation of commercial bacitracin was achieved by a modification of the chromatographic procedure of Konigsberg and Craig (6), and details of the purification process will be published elsewhere (4). The purified, lyophilized material used in our experiments was a white powder which dissolved in H_2O to give a colorless solution and antibiotic activity of 81-87 units/mg. All reagents were prepared and glassware rinsed with distilled H_2O which had been passed through a demineralizing column and had a measured conductance of 1 μmho/cm or less. Optical rotatory dispersion (ORD) spectra of aqueous solutions were obtained with a Cary 50 spectropolarimeter. NMR studies were performed with a Varian 60 instrument. Because of lowered H_2O solubility of the bacitracin-metal ion complex, NMR spectra were run on solutions made with CD_3OD using tetra-methyl silane (TMS) as an internal standard.

Results and Discussion

It was previously found that addition of Zn^{+2} to solutions of bacitracin A resulted in an enhancement of the UV-absorption maximum at 253 mμ. At pH 6.34 the enhancement was progressive with increasing Zn^{+2}, reaching a maximum at a bacitracin:Zn^{+2} ratio of 1:12. From such measurements it was possible to determine an association constant for the metal ion complex of 2.5×10^3 M^{-1} (2). These results have been reproduced with the Zn^{+2} complex of chromatographically purified bacitracin which has an association constant of 3.0×10^3 M^{-1} at pH 6.40 (4).

Craig, *et al.* (2) obtained ORD spectra for bacitracin A which are also reproduced with the purified bacitracin used in the present studies. As can be seen in Fig. 1, there are two UV Cotton effects, one near 250 mμ and the other at approximately 220 mμ. The former has been tentatively attributed to the thiazoline ring and the latter to the cyclic hexapeptide of bacitracin (2). Two types of experiments performed by us have reinforced the suggested source of the long wavelength Cotton effect. First, the circular di-

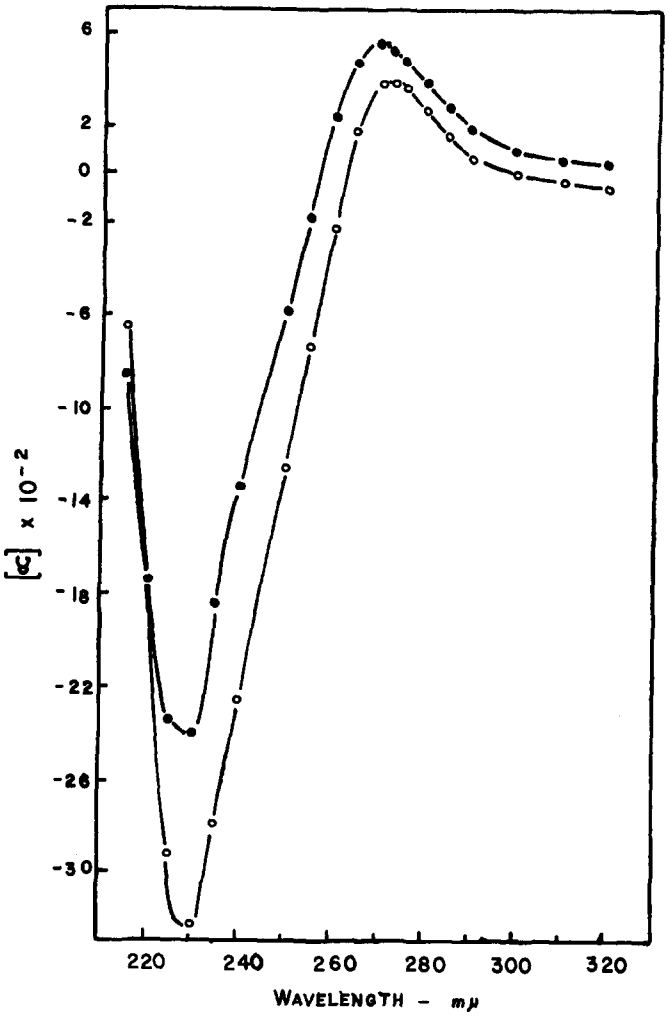

Fig. 1. ORD spectra of bacitracin and bacitracin:Zn^{+2} complex. •—• Bacitracin in H_2O, pH 6.8. Between 320–250 mμ, concentration of bacitracin was 0.1% (w/v). That solution was diluted fivefold for wavelengths below 250 mμ. o—o As before but with tenfold excess of $ZnCl_2$ added.

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chroism (CD) spectrum of bacitracin has a maximum at 253 mμ, the absorption maximum of the thiazoline ring. Second, the thiazoline derivative of N-acetylglutathione (7) in aqueous solution at pH 4 also has a CD maximum at 253 mμ. (In addition, CD spectra more precisely position the short wavelength Cotton effect at 218 mμ.)

Also shown in Fig. 1 is the ORD spectrum for the bacitracin:Zn^{+2} complex. Both Cotton effects are perturbed by the presence of Zn^{+2}, and these data suggest that the thiazoline ring provides one coordination site for Zn^{+2}. This interpretation is consistent with the report that bacitracin F, in which the thiazoline has been oxidized to the thiazole, does not form a complex with Zn^{+2}.

We have also attempted to identify other coordination sites for metal ions. One possibility is the imidazole ring of the histidine residue which, in the conformation proposed for bacitracin, is in the vicinity of the thiazoline (1,2,6). Spectrophotometric titrations and NMR spectra provide strong evidence that, in fact, the histidine residue is involved in metal binding to bacitracin. The titration studies are illustrated by Fig. 2. Aqueous solutions of 0.15 mM bacitracin and 0.15 mM $ZnCl_2$ were adjusted to the indicated pH's with either HCl or KOH and the spectra from 310-220 mμ were read. On plotting maximum absorption of the thiazoline peak against pH, a pK of 6.2 is indicated for the formation of Zn^{+2} complex.

Although the pK determined from Fig. 2 is presumptive evidence impli-

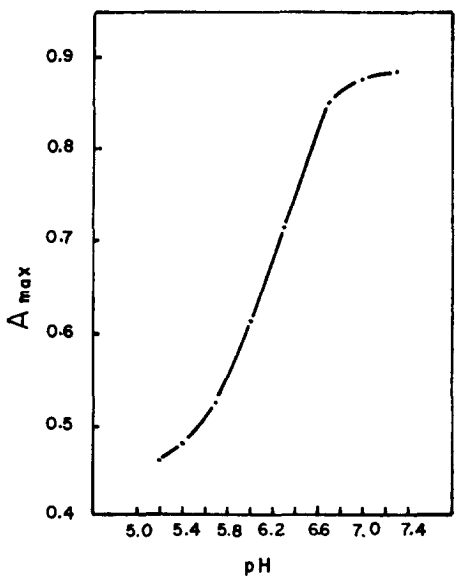
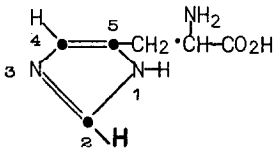


Fig. 2. Interaction of Zn^{+2} and bacitracin as a function of pH.

cating the histidine residue, it seemed desirable to evaluate Zn^{+2} binding by an additional, independent method. NMR spectroscopy is appropriate here since the aromatic character of the imidazole ring produces a ring current effect which shifts the C-4 hydrogen signal about 6.8-7.2 ppm and the C-2 hydrogen signal about 7.8-8.2 ppm downfield from TMS.



These two imidazole hydrogen peaks are clearly seen in the NMR spectrum of bacitracin (Fig. 3). The C-2 hydrogen peak appears at 7.85 and the C-4 peak at 6.88 ppm downfield. The only other peak in this region is the sharp signal from the five phenylalanine ring protons which is seen at 7.22 ppm. The upfield region of the spectrum is difficult to interpret in terms of specific bacitracin protons, but we have tentatively identified a large, broad peak at 0.95 ppm as arising from the eight side chain CH_3- groups. More work is needed before unequivocal assignments can be made to peaks in the upfield region.

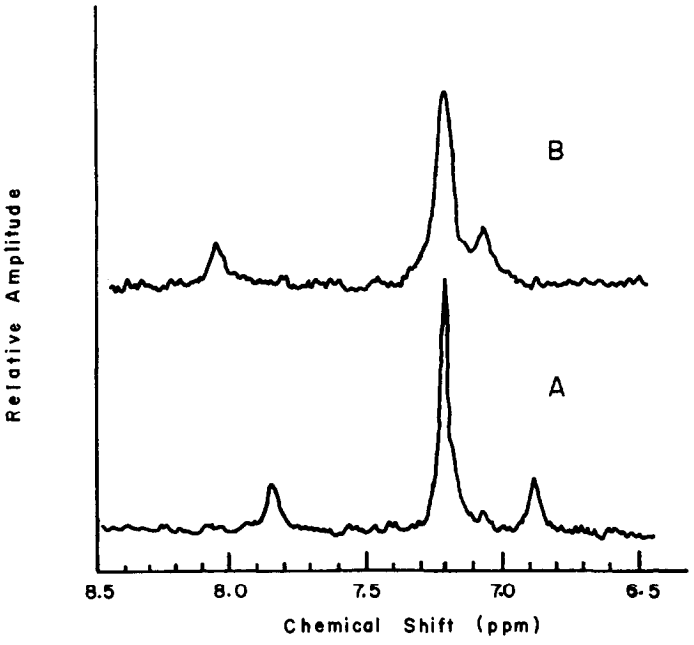


Fig. 3. NMR spectra of (A) bacitracin and (B) bacitracin:Zn^{+2} complex. Compositions of the two solutions are given in Table 1.

Table 1

CHEMICAL SHIFTS IN THE BACITRAIN NMR SPECTRUM

Source of Signal	Shift without Zn^{+2} [a] CPS	(ppm)	Shift with Zn^{+2} [b] CPS	(ppm)	Difference $\Delta\delta$ (ppm)
Saturated (CH_3-)	57	0.95	56	0.93	-0.02
Phenyl ring protons of phenylalanine	433	7.22	433	7.22	0.00
Histidine C-4 hydrogen	413	6.88	424	7.06	0.18
C-2 hydrogen	471	7.85	483	8.05	0.20

[a] Bacitracin, 0.05 M in CD_3OD with tetra-methyl silane as internal reference.

[b] As above but with 0.5 M $ZnCl_2$.

Spectra obtained in the presence and absence of Zn^{+2} (Fig. 3 and Table 1) support the conclusion drawn from the titration data; i.e., the histidine residue of bacitracin provides a coordination site for metal ion binding. It is interesting to note (Table 1) that the histidine C-4 and C-2 hydrogen peaks are shifted almost equally when bacitracin complexes Zn^{+2}. This is in contrast to our observations with free histidine in D_2O where addition of Zn^{+2} to the solution results in a downfield shift of 0.48 ppm for the C-2 hydrogen while the C-4 hydrogen is shifted by only 0.14 ppm. Such differences are understandable if, as proposed by others (8,9), the nitrogen at position 1 is the primary coordination site in the imidazole ring of the histidine molecule. The equal downfield shifts seen for the C-2 and C-4 imidazole hydrogen peaks when bacitracin complexes with Zn^{+2} indicates that the metal ion has equal effects on lowering the electron density at the two positions. Consequently, we conclude that nitrogen at position 3 is the coordinating atom of imidazole in bacitracin. Although neither the titration data nor the NMR data alone are sufficient evidence, considered together they provide strong support for the con-

clusion that the histidine residue is involved in binding of Zn^{+2} to bacitracin. In future experiments with purified bacitracin F and other chemical derivatives, efforts will be made to identify additional coordination sites including those in the thiazoline ring.

Finally, it is interesting to note in Fig. 3 and Table 1 that the phenyl ring hydrogens of the phenylalanine residues undergo no shift when Zn^{+2} binds to bacitracin. This observation might indicate that the phenyl ring is constrained by interaction with the amino-terminal isoleucine in the manner proposed by other studies (1,2,6).

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