Interactions between Vancomycin and Cell-Wall Precursor Analogs Studied by Electrospray Mass Spectrometry[†]

Thomas STAROSKE,^{a)} Albert J. R. HECK,^{b)} Peter J. DERRICK^{b)}
and Dudley H. WILLIAMS*,^{a)}

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Non-covalent complexes between the glycopeptide antibiotic vancomycin and the cell-wall precursor analog *N*-acetyl-D-Ala-D-Ala, as well as its deuterated enantiomer *N*-acetyl-L-Ala-L-Ala-*d*₃ were probed using electrospray ionization mass spectrometry. In solution, the D,D-peptide interacts strongly with the antibiotic, whereas the L,L-peptide shows no observable complexation. Using a competition strategy, mass spectra were obtained from solutions containing vancomycin, *N*-acetyl-D-Ala-D-Ala, and *N*-acetyl-L-Ala-L-Ala-*d*₃. With acetonitrile-water as the solvent, the ratio of free vancomycin to vancomycin complex was much higher than expected from solution interactions and the specific D,D-peptide complex and the non-specific L,L-peptide complex were detected in a 1.7 : 1 ratio. In contrast, sprayed from an ammonium acetate buffer solution both the amount and specificity of observed complex reflected solution interactions (only D,D-peptide complex was found). Thus, our results demonstrate that in electrospray mass spectrometry the conditions must carefully be chosen and control experiments must be carried out in order to exclude non-specific aggregation as a possible cause of complex formation.

Introduction

During the last few years, mass spectrometry has been used in the investigation of non-covalent interactions. In particular, the development of electrospray ionization techniques has led to a variety of studies of molecular recognition processes, including estimations of binding constants. In such studies, solutions which closely mimic physiological conditions should be maintained.¹⁾ Despite the wide application of this method, there remains a need to establish that the complexes observed by mass spectrometry reflect structurally-specific solution interactions rather than non-specific aggregation during the ionization process. Recent studies report the detection of non-specific complexes (false positives) in control experiments with compounds which show no interactions in solution (e.g., with an enantiomer of a substrate of an enzyme) and ion abundances which do not reflect differences in binding constants in solution.²⁻⁵⁾

Vancomycin belongs to the glycopeptide group of antibiotics which act by binding to bacterial cell-wall precursors terminating in -Lys-D-Ala-D-Ala, thereby inhibiting cross-linking of the growing cell wall, resulting in cell death.^{6, 7)} These drugs are currently used to treat infections with multiply-resistant bacteria such as methicillin-resistant *Staphylococcus aureus* (MRSA). In addition to their clinical importance, extensive binding studies between vancomycin group antibiotics and cell-wall precursor analogs such as di-*N*-acetyl-Lys-

D-Ala-D-Ala (Ac₂KDADA) and *N*-acetyl-D-Ala-D-Ala (AcDADA) have shown that these systems serve as excellent models to probe non-covalent interactions in molecular recognition processes because of their relatively low molecular masses (ca. 1500 Da) and rigid structures.⁸⁾

In recent studies, complexes between glycopeptide antibiotics and cell-wall precursor analogs have been observed by electrospray mass spectrometry (ESI-MS)⁹⁾ and binding constants have been determined by correlations of relative ion abundance with ligand concentrations in solution.¹⁰⁾ Although it has been demonstrated that ligands with lower binding constants lead to less abundant ions of the non-covalent complexes, we believed that a different control experiment could help to test whether the binding is closely analogous to that occurring in solution, or may be of a less specific nature.

In solution, glycopeptide antibiotics bind strongly to the cell-wall precursor analog AcDADA. However, because of the stereospecificity of the binding, no significant complexation with -LALA terminating peptides is observed.^{11), 12)} Nevertheless, in fast atom bombardment mass spectrometry (FAB-MS) experiments with vancomycin aglycone and the ϕ -aglycone of ristocetin A, we have previously observed aggregates of the antibiotics not only with AcDADA but also with AcLALA.¹³⁾ In competition experiments using equimolar mixtures of AcDADA and AcLALA-*d*₃, both vancomycin aglycone and ristocetin A ϕ -aglycone showed adduct ions of similar abundances with both the D,D- and L,L-peptides.¹³⁾ As the formation of non-specific association products in FAB-MS is well known,¹⁴⁾ we wished to investigate whether ESI-MS reflects the molecular recognition of vancomycin and a cell-wall precursor analog in solution, and now report the results of such an experiment.

[†] This paper is dedicated to Dr. Makoto SUZUKI, in memory of both his contributions to science and his much valued friendship.

^{a)} Cambridge Centre for Molecular Recognition, Department of Chemistry (Lensfield Road, Cambridge, CB2 1EW, UK)

^{b)} Institute of Mass Spectrometry and Department of Chemistry, University of Warwick (Coventry, CV4 7AL, UK)

Experimental

Materials

Vancomycin hydrochloride was obtained from ICN Biomedicals. AcDAdA, LALA, acetic anhydride and acetic anhydride- d_6 were purchased from Sigma. AcLALA and AcLALA- d_3 were obtained upon acetylation of LALA with acetic acid and acetic anhydride- d_6 , respectively, in aqueous solution followed by solvent evaporation and lyophilization.

Sample preparation

Aqueous stock solutions of vancomycin (1 mM), AcDAdA (5 mM), and AcLALA- d_3 (5 mM) were used to prepare the samples. Dilution was carried out with acetonitrile–water (50 : 50, v/v) or ammonium acetate buffer (10 mM). To the buffer solution, 10% acetonitrile was added to facilitate ion evaporation. The following samples were obtained (no pH adjustments were made):

- vancomycin (30 μ M) in acetonitrile–water (pH 6.7).
- vancomycin (30 μ M), AcDAdA (50 μ M) and AcLALA- d_3 (50 μ M) in acetonitrile–water (pH 4.6).
- vancomycin (27 μ M) in buffer (pH 6.8).
- vancomycin (27 μ M), AcDAdA (50 μ M) and AcLALA- d_3 (50 μ M) in buffer (pH 6.6).

Mass spectrometry

A Fourier transform ion cyclotron resonance mass spectrometer (Bruker Daltronics, Billerica, MA) equipped with a shielded 9.4 T superconduction magnet (MagneX Scientific Ltd., Abingdon, UK), a cylindrical “infinity” ICR cell with diameter $d=0.06$ m, and an external electrospray ionization source (Analytica of Branford, Branford, MA), which has been described previously,¹⁵ was used. During the experiments, the

flow rate of the electrospray solutions was 72 μ l/h and the electrospray cone voltage was kept relatively low (ca. 20 V).

Results and Discussion

The basis of the molecular recognition event between glycopeptide antibiotics and cell-wall precursor analogs was revealed from investigations using ^1H NMR.¹⁶ The strongest interaction in this binding is that of the ligand carboxylate to the binding pocket of the antibiotic which possesses three adjacent amide NHs. Additional hydrogen bonds are formed between the amide groups of the antibiotic and the ligand (two additional hydrogen bonds in the case of AcDAdA). Binding in aqueous solution is also promoted by the hydrophobic effect, since the methyl groups of the Ala residues are partially buried against benzene rings of the antibiotics in the complexes.¹⁷ Thus, in solution, both electrostatic interactions (*e.g.*, ion-dipole, hydrogen bonds) and hydrophobic effects can contribute to the formation of a non-covalent complex. As the hydrophobic effect depends on the presence of a solvent, it does not contribute to the stability of aggregates in the gas phase in a mass spectrometer. Robinson *et al.* have concluded that differences in solution binding constants which are due to hydrophobic effects are not reflected in mass spectra.⁵

The antibiotic–ligand complexes are stable under various conditions, including a pH range of 4.5–7 and mixed aqueous–organic solvent systems.^{18,19} Therefore, although the samples used in our experiments differ somewhat in their composition of solvent and pH (see Experimental), the concentration of vancomycin–AcDAdA complex should be of a similar order of mag-

Fig. 1. FT-ICR electrospray ionization mass spectrum of vancomycin (30 μ M) in acetonitrile–water (50 : 50, v/v). The inset shows the isotopic distribution of doubly-protonated vancomycin.

Fig. 2. Mass spectrum of an acetonitrile-water solution containing vancomycin ($30\ \mu\text{M}$), AcDADA ($50\ \mu\text{M}$), and AcLALA- d_3 ($50\ \mu\text{M}$). The inset shows the isotopic distribution of the doubly-protonated vancomycin-AcDADA and vancomycin-AcLALA- d_3 complexes.

Fig. 3. Mass spectrum of vancomycin ($27\ \mu\text{M}$), AcDADA ($50\ \mu\text{M}$), and AcLALA- d_3 ($50\ \mu\text{M}$) in ammonium acetate buffer (10 mM) containing 10% acetonitrile. The inset shows the isotopic distribution of the doubly-protonated vancomycin-AcDADA complex.

nitude in all the solutions. On the basis¹¹⁾ of a binding constant of $2.0 \times 10^4\ \text{M}^{-1}$, we calculate free vancomycin and complex to be in a ratio of *ca.* 1.5:1. By UV

difference spectrophotometry the binding constant of AcLALA was found to be below the detection limit ($K < 300\ \text{M}^{-1}$).

Fig. 4. (a) Isotopic distribution of doubly-protonated vancomycin complexes in acetonitrile-water (see Fig. 2). (b) Calculated isotopic distribution of the vancomycin-AcdAdA complex. (c) Isotopic distribution of doubly-protonated vancomycin complex in buffer solution (see Fig. 3). Although the relative heights of the peaks in (c) are different from the calculated intensities in (b), integration of the signal shows that the intensities in (c) are very similar to the calculated.

Figure 1 shows the electrospray mass spectrum of vancomycin in acetonitrile-water with the doubly-protonated antibiotic as the most intense signal ($m/z \approx 725$). The spectrum of vancomycin in ammonium acetate buffer looks similar (spectrum not shown). After addition of equal amounts of AcdAdA (202 Da) and AclALA- d_3 (205 Da) to the vancomycin solution in acetonitrile-water, a signal due to the doubly protonated non-covalent complex between vancomycin and ligand was observed at $m/z \approx 826$ (Fig. 2). Isotopic resolution of this signal and comparison with the calculated isotopic distribution of the vancomycin-AcdAdA complex (Fig. 4) revealed that not only was the vancomycin-AcdAdA complex detected, but also the complex of the antibiotic with AclALA- d_3 in a ratio of *ca.* 1.7 : 1 (calculated after integration of the peaks). The spectrum also shows singly-protonated vancomycin at $m/z \approx 1449$. From the integrals of both singly and doubly-charged antibiotic and the complex with AcdAdA a ratio of *ca.* 7 : 1 was calculated for free to bound vancomycin (compared to a ratio of *ca.* 1.5 : 1 in solution). Like the actual observation of a complex with AclALA- d_3 , this ratio of 7 : 1 does not reflect the behavior of structurally-specific interactions in solution. In contrast, when the same compounds were sprayed from a solution of ammonium acetate buffer and 10% acetonitrile, no aggregation with AclALA- d_3 could be detected (Fig. 3). The spectrum shows only free vancomycin (singly and doubly-protonated, respectively) and the complex with AcdAdA in a ratio of *ca.* 1.4 : 1 (calculated as described above). This result is very close to the ratio of *ca.* 1.5 : 1 expected in a solu-

tion interaction. A comparison of the signals of the observed complexes and the calculated isotopic distribution of the expected vancomycin-AcdAdA complex is given in Fig. 4.

Conclusions

Our results demonstrate that the outcome of investigations of non-covalent interactions in solution by electrospray mass spectrometry can be dependent on the conditions chosen. An appropriate solvent system can exclude non-specific aggregation and can permit reproduction of solution abundance ratios. Control experiments with similar compounds which do not bind in solution are necessary in order to check for non-specific binding.

Given a careful choice of solution and interface conditions, for the cases reported here it appears that mass spectra can be obtained which reflect the structure-specific interactions which occur in solution.

Additional investigations, in which structurally-specific solution-phase non-covalent interactions between a range of glycopeptide antibiotics of the vancomycin group and a variety of bacterial cell-wall precursor analogs are probed, underline this conclusion.²⁰⁾

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References

- 1) M. Przybylski and M. O. Glocker, *Angew. Chem. Int. Ed. Engl.*, **35**, 806 (1996).
- 2) R. T. Aplin, C. V. Robinson, C. J. Schofield, and N. J. Westwood, *J. Chem. Soc., Chem. Commun.*, 2415 (1994).
- 3) J. B. Cunniff and P. Vouros, *J. Am. Soc. Mass Spectrom.*, **6**, 437 (1995).
- 4) J. Ding and R. J. Anderegg, *J. Am. Soc. Mass Spectrom.*, **6**, 159 (1995).
- 5) C. V. Robinson, E. W. Chung, B. B. Kragelund, J. Knudsen, R. T. Aplin, F. M. Poulsen, and C. M. Dobson, *J. Am. Chem. Soc.*, **118**, 8646 (1996).
- 6) H. R. Perkins, *Biochem. J.*, **111**, 195 (1969).
- 7) D. H. Williams, *Acc. Chem. Res.*, **17**, 364 (1984).
- 8) D. H. Williams, M. S. Searle, M. S. Westwell, J. P. Mackay, P. Groves, and D. A. Beauregard, *Chemtracts-Org. Chem.*, **7**, 133 (1994).
- 9) M. Hamdan, O. Curcuruto, and E. DiModugno, *Rapid Commun., Mass Spectrom.*, **9**, 883 (1995).
- 10) H.-K. Lim, Y. L. Hsieh, B. Ganem, and J. Henion, *J. Mass Spectrom.*, **30**, 708 (1995).
- 11) M. Nieto and H. R. Perkins, *Biochem. J.*, **123**, 789 (1971).
- 12) P. W. Smith, G. Chang, and W. C. Still, *J. Org. Chem.*, **53**, 1587 (1988).
- 13) D. H. Williams, C. Bradley, G. Bojesen, S. Santikarn, and L. C. E. Taylor, *J. Am. Chem. Soc.*, **103**, 5700 (1981).
- 14) M. Karas, U. Bahr, and U. Giessmann, *Mass Spectrom. Rev.*, **19**, 335 (1991).
- 15) A. J. R. Heck and P. J. Derrick, *Anal. Chem.*, **69**, 3603 (1997).
- 16) J. R. Kalman and D. H. Williams, *J. Am. Chem. Soc.*, **102**, 906 (1980).
- 17) D. H. Williams, J. P. L. Cox, A. J. Doig, M. Gardner, U. Gerhard, P. T. Kaye, A. R. Lal, I. A. Nicholls, C. J. Salter, and R. C. Mitchell, *J. Am. Chem. Soc.*, **113**, 7020 (1991).
- 18) M. Nieto and H. R. Perkins, *Biochem. J.*, **123**, 773 (1971).
- 19) R. Kannan, C. M. Harris, T. M. Harris, J. P. Waltho, N. J. Skelton, and D. H. Williams, *J. Am. Chem. Soc.*, **111**, 2946 (1988).
- 20) T. D. J. Joergensen, P. Roepstorff, and A. J. R. Heck, in preparation.

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