Measurement of the different affinities of the two halves of glycopeptide dimers for acetate

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The titration of sodium acetate into solutions of the vancomycin group antibiotics eremomycin and chloroeremomycin has been studied by observing the downfield movement of the ^{1}H NMR signals due to amide protons in the antibiotic binding pocket; this change in chemical shift has been used to determine the different affinities of the two halves of the dimers for acetate ligand.

The vancomycin antibiotics are clinically important in the treatment of infections by Gram-positive bacteria and are currently the last line of defence against methicillin-resistant *Staphylococcus aureus* (MRSA).[1,2] They act by binding to the terminal -Lys-D-Ala-D-Ala sequence of bacterial cell wall precursors, inhibiting transglycosylation and ultimately causing cell death.[3,4] In recent work, we have shown that these glycopeptide antibiotics form asymmetric homodimers in solution and that dimerisation is, in all but one case, cooperative with binding to bacterial cell wall precursor analogues.[5] The asymmetry arises from a head-to-head orientation of the saccharide portions attached to residue 4 of the antibiotics, disrupting the C_2-symmetry of the head-to-tail peptide backbones.[6–9] A consequence of this asymmetry for the antibiotic ristocetin A is that the two halves of its dimer bind ligands with different affinities.[10,11] However, eremomycin, which also forms an asymmetric dimer, was shown to bind this ligand with approximately equal affinities into each side of such a dimer. Thus, although the asymmetric eremomycin dimer might be expected in principle to bind ligands in each half of the dimer with different affinities, the observation of similar or identical affinities suggested a smaller interaction of the ligand with the disaccharide of eremomycin compared to the tetrasaccharide of ristocetin A.

Previously, it has not been possible to directly measure binding affinities of ligands to antibiotic dimers. This is because binding affinities have been measured at low antibiotic concentrations (typically $\leqslant$50 μm) by UV difference spectrophotometry,[4] capillary zone electrophoresis[12] or isothermal calorimetry.[13] At such concentrations, weakly dimerising antibiotics, such as vancomycin and ristocetin A, are present at $>$90% monomer, and strongly dimerising antibiotics, such as eremomycin and chloroeremomycin, are present at $>$50% monomer.

We now describe a method, analogous to the UV difference spectrophotometric method, for the determination of binding constants of acetate to antibiotic at a concentration of 5 mM ($>$95% dimer). The binding of bacterial cell-wall precursors is crucially dependent on the recognition of their terminal carboxy groups and acetate thus provides the simplest model for the study of this interaction.

UV difference spectrophotometry plots the change in absorbance of the antibiotic as a function of ligand concentration. The method described here plots the change in ^{1}H NMR chemical shift of the amide protons w_2, w_3 and w_4 (Fig. 1) as a function of ligand concentration, at pH 4.5 and 300 K. These three protons, and in particular w_2, have been extensively studied previously and a strong correlation has been observed between their limiting downfield chemical shift (shift when $>$95% antibiotic is bound) and the free energy of association for a variety of ligands.[14,15] Table 1 lists the chemical shifts of w_2, w_3 and w_4 of eremomycin and chloroeremomycin when free and when fully bound to acetate. For both antibiotics, two signals are observed for each of these protons: one signal for each half of the asymmetric dimer. Each of these signals is time-averaged between the bound and free state, as acetate binding is in fast exchange on the NMR time scale. As a result, they move gradually downfield during titration of acetate into the antibiotic, reflecting the proportion of the antibiotic bound by acetate at a particular ligand concentration. Thus, the chemical shift of each of these protons is analogous to the change in absorbance measured by UV difference spectrophotometry, and a binding

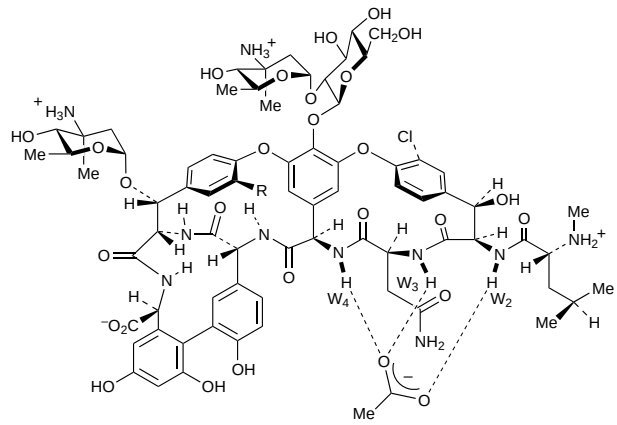

Fig. 1 Structures of the antibiotics eremomycin (R = H) and chloroeremomycin (R = Cl) binding to acetate. The three amide protons mentioned in the text, w_2, w_3 and w_4, are labelled. Hydrogen bonds to acetate are shown by dashed lines.

Table 1 Chemical shifts of amide protons in the antibiotic binding pocket when free and when fully bound to acetate. The protons from each half of the dimer are distinguished by the presence or absence of an asterisk (*). All values are in ppm relative to internal trimethylsilyl propionate (TSP, δ = 0)

		δ					
Antibiotic	State	w_2*	w_2	w_3*	w_3	w_4*	w_4
Eremomycin	Free	8.50	8.45	8.23	8.23	7.91	7.84
Eremomycin	Bound	10.46	10.16	9.29	9.19	8.61	8.37
Chloroeremomycin	Free	8.84	8.69	8.39	8.29	7.78	7.78
Chloroeremomycin	Bound	10.52	10.37	9.27	9.25	8.75	8.70

Table 2 Binding constants of acetate to eremomycin and chloroeremomycin measured by following the chemical shifts of individual amide protons at varying concentrations of acetate. The presence or absence of an asterisk (*) distinguishes protons in each half of the dimer. w_2* of chloroeremomycin was not visible above the baseline at enough concentrations of acetate for a meaningful binding constant to be determined

	K_b/dm^3 mol^{-1}					
Antibiotic	w_2*	w_2	w_3*	w_3	w_4*	w_4
Eremomycin	19.5 ± 0.4	8.8 ± 0.6	14.8 ± 1.0	8.6 ± 0.5	17.4 ± 0.8	6.3 ± 0.6
Chloroeremomycin		13.5 ± 5.2	24.4 ± 0.7	12.9 ± 0.6	21.0 ± 1.6	17.5 ± 1.5

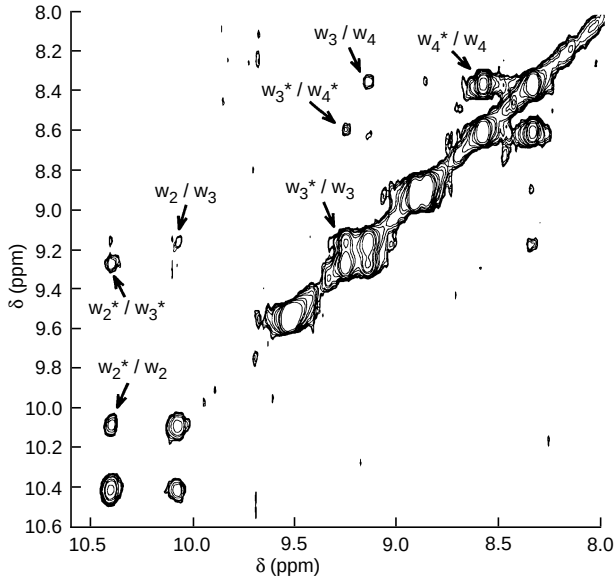

Fig. 2 Portion of the 50 ms NOESY spectrum of eremomycin fully bound to acetate. Labelled are NOE cross peaks between adjacent protons in the same binding pocket and chemical exchange cross peaks between equivalent protons in each half of the dimer. These confirm the assignments of the separate peaks to a particular half of the dimer.

constant can be calculated from the titration curves using a least-squares curve fitting procedure.[16]

Table 2 shows the binding constants measured from these curves. A two-dimensional NOESY spectrum with a mixing time of 50 ms was used to confirm the assignments of the protons in each of the binding pockets. Due to the short mixing time, the extent of chemical exchange was low and thus strong NOE cross peaks were only observed between signals from protons within the same binding pocket (Fig. 2).

There are two points of note. Firstly, the binding affinities of acetate to chloroeremomycin are greater than those to eremomycin. This is similar to previously measured binding constants which also show ligand affinities for chloroeremomycin which are greater than those for eremomycin.[5] Secondly, for both antibiotics the binding constant for one half of the dimer is greater than that to the other half; by a factor in the region of 1.7–2.8 in the case of eremomycin, and by a factor in the region of 1.2–1.9 in the case of chloroeremomycin.

The resonance of w_2 can also be used to demonstrate the very useful correlation between limiting chemical shift and free energy of binding mentioned previously.[14,15] If points for w_2 and w_2* are added to such a curve for eremomycin, using the binding free energies measured in this study, it can be seen that both lie almost exactly on this curve (Fig. 3), *i.e.* the protons from the half of the dimer with the greater affinity for acetate are also further downfield. This emphasises the sensitivity of the correlation between binding affinity and limiting chemical shift of w_2 and how it may potentially be used to extract binding affinities from chemical shift data.

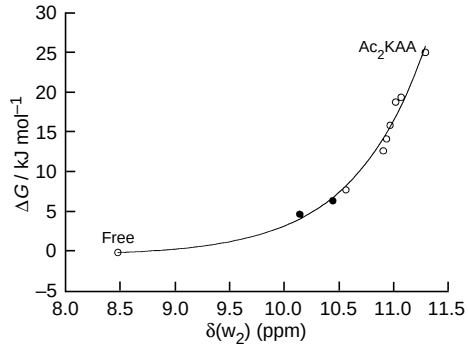

Fig. 3 Plot of free energy of association against limiting chemical shift of w_2 for a variety of bacterial cell-wall precursor analogues binding to eremomycin. The two solid black points are those for w_2 and w_2* in the eremomycin/acetate complex, as determined in the present study.

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Footnote

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