Demonstration of the difference in binding affinity between the two binding sites of the ristocetin A asymmetric dimer

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The titration of ^{13}C labelled *N*-acetyl-D-ala-D-ala with the glycopeptide antibiotics ristocetin A and eremomycin, monitored by ^{13}C NMR spectroscopy, demonstrates that the two distinct binding sites of the ristocetin A asymmetric dimer have different affinities, whereas those of eremomycin show no detectable difference

In recent work, we have shown that glycopeptide antibiotics of the vancomycin group form dimers, where the dimerisation constants vary in the approximate range 10^1 to 10^6 dm^3 mol^{-1} (at *ca.* 298 K in aqueous solution) among the various members of the group examined.[1] We have also shown that the antibiotic ristocetin A,[2] and the related glycopeptide eremomycin,[3] form asymmetric dimers (K_{dim} 500 and 3 × 10^6 dm^3 mol^{-1} respectively). Thus, in both of these dimers, the peptide portions of the antibiotic are arranged in an antiparallel 'head-to-tail' fashion, but the saccharide portions which are attached to residue 4 are parallel or 'head-to-head'. Fig. 1 shows the structure of ristocetin A complexed with the cell wall analogue *N*-acetyl-D-Ala-D-Ala (*N*-Ac-DADA). Fig. 2 is a schematic representation of the molecular structure of the asymmetric dimer of ristocetin A, derived from intermolecular NOE enhancements at the dimer interface.[2] It is clear from this diagram that the two binding pockets are structurally different. On one side of the dimer, the binding pocket is capped by the rhamnose portion of the tetrasaccharide, whereas on the other side it is the arabinose which fulfils this role. It might therefore be expected that the binding constants for cell wall peptide analogues to these sites would also be different.[2] Here we show by a simple and unambiguous experiment that this is indeed the case.

In order to demonstrate that the two binding sites of ristocetin A had different affinities, a good spectroscopic probe providing clear, unambiguous signals with good resolution was required. Thus ^{13}C NMR was used to follow the titration of ^{13}C labelled *N*-Ac-DADA† into a solution of ristocetin A. The titration was easily monitored quantitatively by the integration of ^{13}C signals, which were essentially the only signals present due to the specific labelling of the terminal alanine carboxylate. During the titration two carbon signals were observed, a sharp resonance at δ 174.39 and a broader one at δ 174.59 (Fig. 3). Because of the high binding constant between *N*-Ac-DADA and

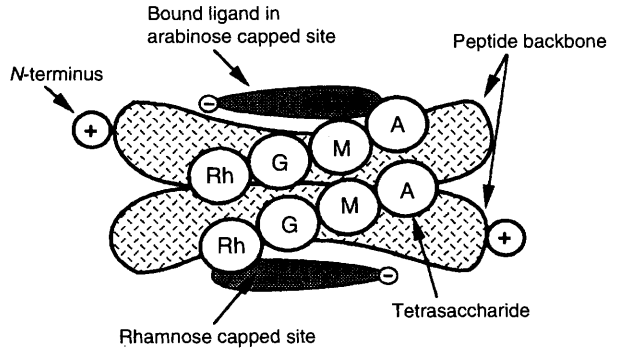

Fig. 2 Schematic showing the structure of the ristocetin A asymmetric dimer. The peptide backbone runs head to tail, but the ring 4 tetrasaccharide, represented by the circles, is head to head. As a consequence of the asymmetric structure, the two binding sites are structurally different. Rh = rhamnose, G = glucose, M = mannose, A = arabinose.

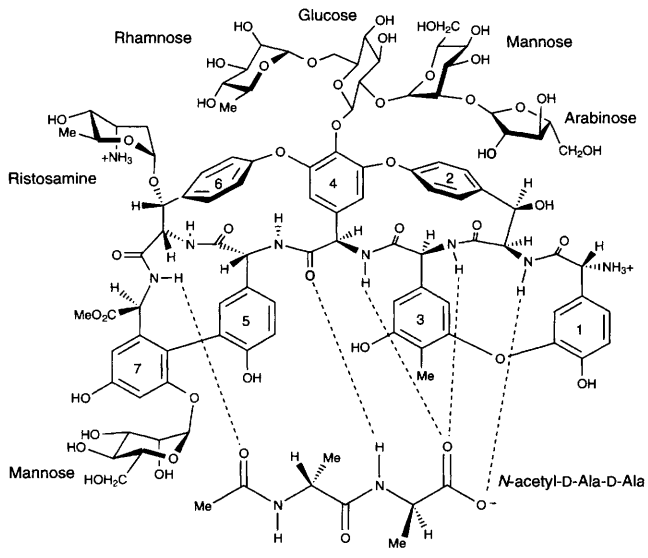

Fig. 1 Structure of the complex between ristocetin A and *N*-acetyl-D- Ala-D-Ala. Note the ring 4 tetrasaccharide, the parallel arrangement of which gives rise to asymmetry in the dimer (see Fig. 2).

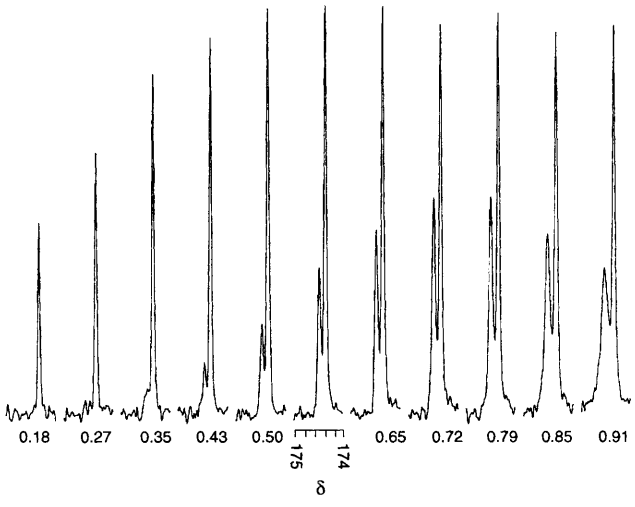

Fig. 3 ^{13}C NMR signals observed upon titration of *ca.* 1 equivalent of [1-^{13}C]-*N*-acetyl-D-Ala-D-Ala into a solution of ristocetin A. The titration show additions 2 to 12 of 13 additions (each of 10 μl) of ligand solution. The number of equivalents added is shown beneath each spectrum. Two signals are apparent; clearly the D_1 resonance appears first, the D_2 signal appearing later in the titration.

ristocetin (K = *ca.* 10^5 dm^3 mol^{-1}), both of these signals had to correspond to bound ligand. Furthermore, a high antibiotic concentration (50 mmol dm^{-3}) was used to ensure a very high proportion of dimer was present, and thus the two peaks could be assigned to the two different binding sites of the ristocetin dimer. Fig. 4 shows that as aliquots of ligand are added, the site designated D_1 is populated first, becoming saturated at just over 0.5 equiv. of ligand. The second site, designated D_2, starts to become populated at 0.3 equiv. of added ligand, most changes taking place after the D_1 site is already filled.‡ Thus, the two binding sites of the asymmetric ristocetin A dimer do have different affinities for cell wall analogues.

The experiment was repeated for the antibiotic eremomycin, which has an asymmetric dimer structure like ristocetin A (Fig. 5). The very high dimerisation constant of eremomycin allowed the use of a lower concentration (10 mmol dm^{-3}) of the antibiotic while still maintaining a very high dimer population. Eremomycin binds ligands much more weakly than ristocetin A,[4,5] leading to the observation of a signal for free ligand throughout the titration. Fig. 6 shows this free ligand signal as the one of largest amplitude (a singlet at δ 176.57). The bound signals are split into doublets (δ 175.81 and 175.64, 175.53 and 175.44), possibly due to a slow conformational exchange.§ Most importantly, it is clear from Fig. 6 that as the titration proceeds, the two sites are populated equally. Thus for eremomycin, although the binding sites are structurally different, there is no detectable difference between their binding affinities.

These experimental facts are in accord with modelling studies carried out on the two antibiotic–ligand complexes. Using the Macromodel[6] molecular modelling package, the structures were examined for hydrogen bonds between ligand and antibiotic. Changes in hydrophobic surface areas were also measured by separating ligand from antibiotic, one site at a time. The results show that for eremomycin, the number of hydrogen bonds made and the hydrocarbon surface buried are essentially the same, despite the differences in structure of the two sites. However, for ristocetin A, the rhamnose capped site makes two extra hydrogen bonds and buries an extra 20 Å^2 of hydrocarbon compared to the arabinose capped site. Previous consideration of the ristocetin dimer structure and evidence from monomeric complexes in Me_2SO had also suggested that the rhamnose capped site might have higher affinity.[2] Thus it would appear that the high affinity D_1 site is the one capped by rhamnose, whereas the lower affinity D_2 site is that capped by arabinose.

Glycopeptide antibiotics generally show cooperativity between ligand binding and dimerisation.[7] Thus the binding of ligand increases the dimerisation constant and *vice versa*. However, the antibiotic ristocetin A is anomalous in this respect. When it binds ligands such as *N*-Ac-DADA or di-*N*,*N*-acetyl-Lys-D-Ala-D-Ala, the extent of dimerisation is decreased. The difference in affinities of the two sites for ristocetin A may account for this apparent anticooperativity. It is possible that ristocetin A is in fact like all other glycopeptides, in that the D_1 dimer site binds ligand more strongly than does monomer, and that interactions at the dimer interface are also cooperatively enhanced. However, ristocetin A has a 'bad' D_2 site, constrained by the asymmetric dimer structure to be of lower affinity than D_1. The monomer is not so constrained and can adopt whatever conformation gives the best binding. If the binding energy of monomer is larger than that of D_2, and this difference more than outweighs the greater binding of D_1 over monomer and any other contributions from dimerisation, then the population of monomer will be increased when the antibiotic is fully bound. Eremomycin, with two sites of equal affinity, is not so affected, and thus exhibits strong cooperativity. We believe this work may have important consequences for control in biological signalling, which often involves asymmetric dimer receptors containing binding sites with different affinities.

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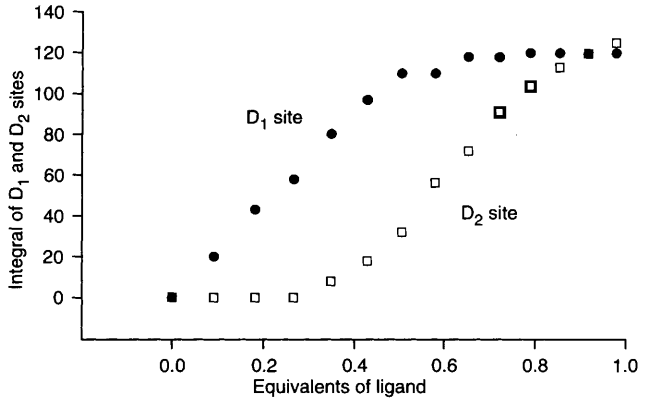

Fig. 4 Integrals of D_1 and D_2 peaks plotted against number of equivalents added. The D_1 site is clearly filled before the D_2 site.

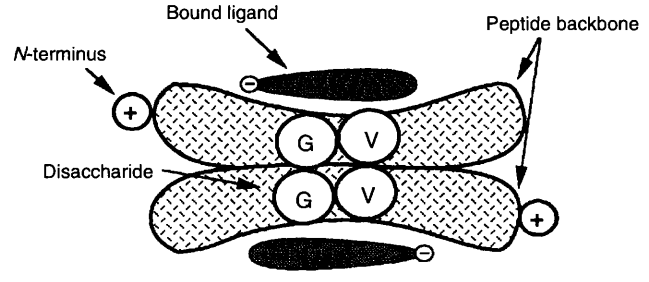

Fig. 5 Schematic representation of the eremomycin asymmetric dimer structure. Again, the peptide backbone is head to tail, but the ring 4 disaccharide is head to head. G = glucose, V = 4-*epi*-vancosamine.

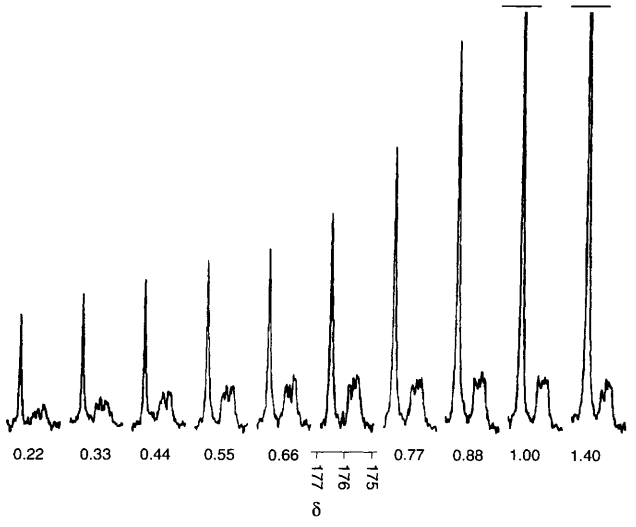

Fig. 6 ^{13}C NMR signals observed upon titration of an excess of [1-^{13}C]-*N*-acetyl-D-Ala-D-Ala into a solution of eremomycin. The number of equivalents of ligand added is shown beneath each spectrum. Due to the lower binding constant, free ligand (the largest peak) is always observed in this titration, but bound signals are equally populated, contrasting with Fig. 3.

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Footnotes

† [1-^{13}C]-D-alanine was prepared by resolving the diastereoisomeric menthyl esters of racemic labelled D-alanine. This was then coupled to *N*-acetyl-D-alanine by standard methods. NMR spectra were recorded using a Bruker AM400 spectrometer.

‡ The integrals of the signals from ligand bound to the D_1 and D_2 sites were determined by cutting and weighing of peaks. The integral of D_2 in the final point appears slightly larger than D_1. The error in the integrals (estimated at ±3%) could account for this, but it could also be real: a small amount of residual monomer is present, which dilution experiments showed to be overlapped with the D_2 peak.

§ Such splitting has so far been observed only for eremomycin among the glycopeptide antibiotics studied. This further splitting of the pair of lines associated with the formation of the asymmetric dimer may be due to a slow oscillation of ring 4, with energy minima corresponding to the residence of the 4b (4f) proton either at the front (back) face or back (front) face of ring 2 (ring 6). This hypothesis, incidental to the present work, is consistent with the finding that two populations of some proton resonances of ristocetin ψ-aglycone are observed (J. Mackay, unpublished work), despite the fact that the cause of asymmetric dimer formation (the saccharide attached to ring 4) is absent in ristocetin ψ-aglycone.

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