Ligand-Induced Dissociation of the Asymmetric Homodimer of Ristocetin A Monitored by ^{19}F NMR Spectroscopy

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Abstract: In this paper we demonstrate the use of ^{19}F-labelled ligands in the study of the therapeutically important vancomycin group of antibiotics. The substantial simplification of spectra that occurs when such labelled ligands are employed is used to study the anticooperativity between ligand binding and dimerisation of ristocetin A. The general utility of ^{19}F-labelled ligands is demonstrated, with the caveat that the positioning of the label has to be carefully considered. With the growing therapeutic importance of the vancomycin group of glycopeptide antibiotics, such a tool may prove valuable in the detailed study of their mode of action.

Keywords: antibiotics · asymmetric homodimer · cooperative effects · NMR spectroscopy · glycopeptides

Introduction

The vancomycin group of antibiotics is therapeutically important in the treatment of Gram-positive bacterial infections.[1,2] They inhibit cell wall growth by binding to the Lys-D-Ala-D-Ala of the cell-wall precursors on the bacterium surface,[3–6] ultimately causing death of the bacterium.[7] Previously we have shown that most of these antibiotics dimerise, and that dimerisation is important in their mode of action.[8–12] In particular, the antibiotic binding cooperativity to cell-wall precursors that are attached to the bacterial cell membrane increases with increasing dimerisation constant.[9] This cooperativity is additional to that found in free solution, where ligand binds to dimer more strongly than to monomer, that is dimerisation is cooperative with ligand binding, even in free solution.[10]

Ristocetin A (Figure 1) is the exception to the rule. It has a dimerisation constant of $500\,M^{-1}$ when free, but this falls to $350\,M^{-1}$ when fully bound to ligand.[13] Most of the vancomycin group of antibiotics possess a saccharide (usually a disaccharide) attached to residue 4 and in the dimer; these sugars are oriented in a head-to-head fashion (Figure 2). This breaks the C_2 symmetry of antiparallel backbones of the dimer; thus, the two halves may have different affinities for ligand. Ristocetin A possesses a tetrasaccharide at residue 4, and therefore the asymmetry should be more pronounced than for other members of the

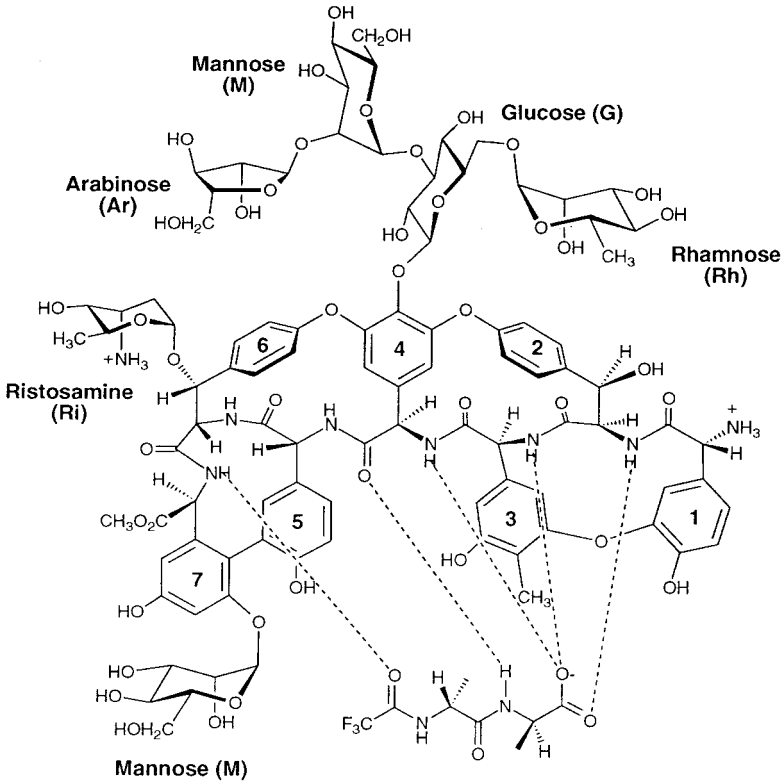

Figure 1. Exploded view of the complex formed between ristocetin A and TFAcDAdA.

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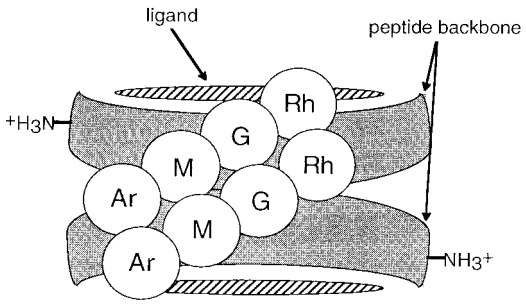

Figure 2. Schematic view of the ristocetin A dimer, illustrating how the parallel arrangement of the tetrasaccharides breaks the C_2 symmetry of the antiparallel antibiotic peptide backbones.

group. This was first confirmed by titrating 13C-labelled ligands against two different dimers: ristocetin A and eremomycin.[14] Further work with ristocetin A demonstrated that although it exhibits overall anticooperativity, the binding of the first equivalent of ligand actually promoted dimerisation, and it was the second binding event that led to the overall drop in dimerisation constant.[15]

Results and Discussion

The above-mentioned analysis[15] of the ristocetin A system was made possible by observing the 1H NMR signals of certain protons, both of the antibiotic itself and of the added ligand, *N*-α-*N*-ε-diacetyl-lysyl-D-alanyl-D-alanine (diAcKDADA). However, ligands with deuteration at multiple sites were required to simplify the spectra. In this work we use analogous ^{19}F-labelled ligands to monitor the course of the net anticooperativity of ligands binding to ristocetin A by ^{19}F NMR spectroscopy. This strategy has a number of distinct advantages. First, the relative sensitivity of ^{19}F in NMR spectroscopy is very high, second only to ^{1}H (^{19}F = 0.83, ^{1}H = 1). Second, owing to the absence of any other ^{19}F signals, the spectra are far simpler and easier to obtain; for example, there is no need for solvent suppression and there are no difficulties with receiver gain. Consequently the spectra are simpler to interpret and the integrals can be evaluated more accurately. Finally, the ^{19}F label can be readily introduced into the ligands by replacement of an acetyl group with a trifluoroacetyl group.

The ligand chosen for our study of antibiotic ristocetin A was *N*-α-trifluoroacetyl-D-alanyl-D-alanine (TFAcDADA). There were two main reasons for this choice. First, it binds relatively strongly to ristocetin A ($K_b = 1.3 \pm 0.1 \times 10^5\,\text{M}^{-1}$, as measured by UV difference spectrophotometry). Therefore at the concentrations of antibiotic used in this study, equimolar amounts of ligand and antibiotic result in almost complete binding, > 95% of antibiotic bound. Second, in the complex between ristocetin A and TFAcDADA, the trifluoroacetyl group should be located in the vicinity of the aromatic rings and of the tetrasaccharides. This should maximise the sensitivity of the change in chemical shift of the trifluoroacetyl group with respect to its position in the complex.

Spectra were acquired by using antibiotic concentrations of 10 mM and 50 mM, and a solvent mixture of 20% v/v CD_3CN/D_2O. This solvent mixture was used to minimise non-specific aggregation of the antibiotic and thereby to sharpen the observed resonances. In addition, this solvent mixture allowed us to perform NMR experiments at temperatures below 0 °C. A sample was prepared containing antibiotic (50 mM) and TFAcDADA (12.5 mM) in 20% v/v CD_3CN/D_2O with 0.1 M phosphate buffer (pH 6), and 1D spectra were obtained at various temperatures (Figure 3). The results indicate that at

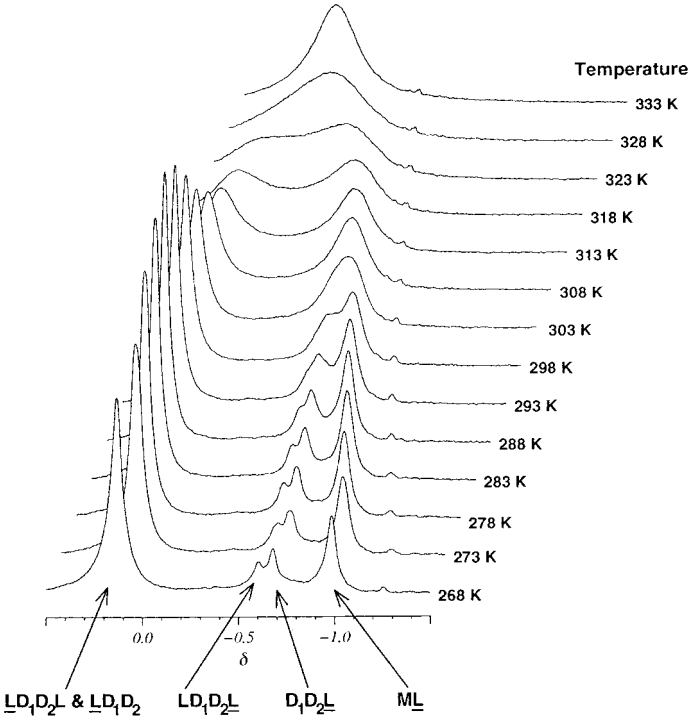

Figure 3. Plot illustrating the temperature variation in the ^{19}F spectrum of a 1:0.25 complex of ristocetin A (50 mM) and TFAcDADA (12.5 mM) in 20% v/v CD_3CN/D_2O.

room temperature and above, some of the species present are in intermediate or fast exchange. At 268 K, however, the previously broad peaks are resolved into four distinct peaks. These peaks (Figure 3) were identified by using results from further titration experiments (Figure 4). Reading from right to left in Figure 3 they are: ligand bound to monomer (ML), ligand bound to the less-favoured side of the singly bound dimer (D1D2L), ligand bound to the less-favoured side of the doubly bound dimer (LD1D2L), and a peak corresponding to ligand bound to the more-favoured side of the singly bound dimer (LD1D2), and ligand bound to the more-favoured side of the doubly bound dimer (LD1D2L). In the titration performed at an antibiotic concentration of 10 mM (Figure 4), the signals were sufficiently sharp for these last two species to be identified at a number of points during the titration (between 6 and 8 mM ligand added). Subsequently the signal for free ligand L was found to appear upfield of the ML signal, and a small signal due to a trace fluoride impurity in the antibiotic was observed further downfield still (Figure 4). ^{19}F-^{19}F NOESY spectra recorded with a number of short mixing times (10, 20, 50 and 100 ms) demonstrated that the signals due to ligand bound to each of the two halves of the dimer do

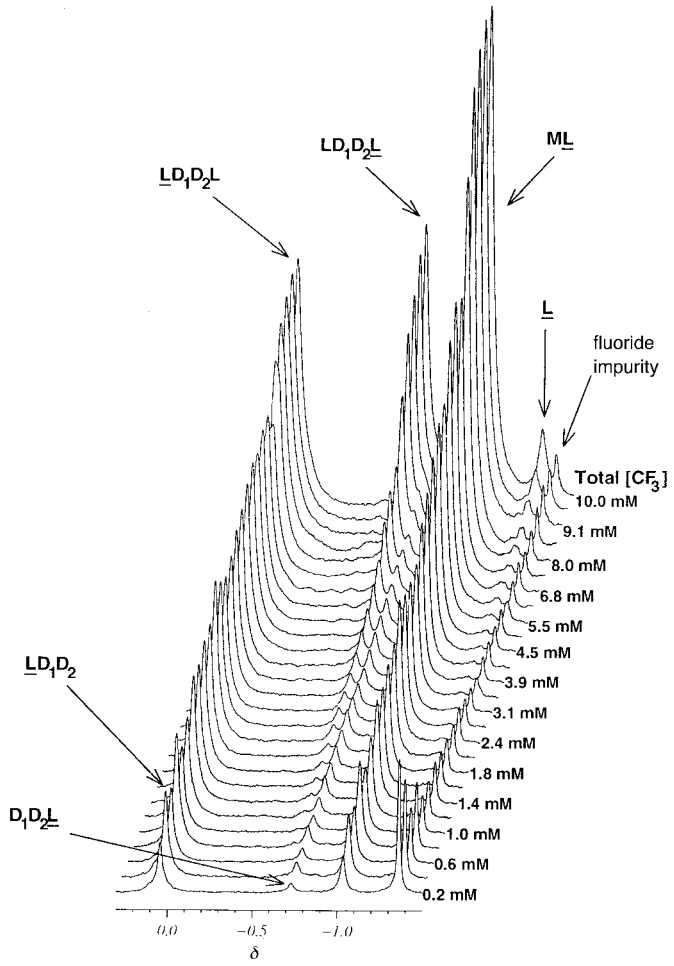

Figure 4. Plot illustrating the ^{19}F spectra from a titration of 1 equivalent of TFAcDADA into a solution of ristocetin A (10 mM) in 20% v/v CD_3CN/D_2O at 268 K. For clarity the intensity of the spectra at the lowest concentrations have been scaled up so that all spectra can be shown on the same plot.

The dimerisation constant of the unbound antibiotic was determined under the same conditions of solvent and temperature by monitoring the chemical shift of a proton on the dimer interface at different concentrations of antibiotic. By using the method described previously,[16] a value of $3.3 \pm 0.5 \times 104\,M^{-1}$ was obtained. Therefore, given the total concentration of all bound species and the dimerisation constant of the unbound antibiotic, concentrations of the free dimer (D1D2) and monomer (M) could be calculated, and hence the concentrations of all the relevant species in the solution for every titration point.

Figure 5 shows the variation of the concentration of the various species throughout the titration and clearly, the less-favoured side of the dimer (D2) is not appreciably filled until the more-favoured side (D1) is almost completely bound, consistent with previous observations.[14,15] However, by

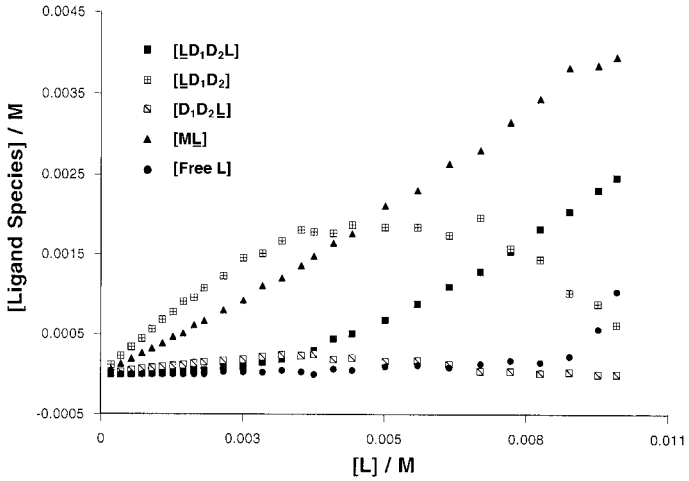

Figure 5. Plot of concentration of the different fluorine-containing species against total concentration of TFAcDADA added. The plot was obtained from the titration data illustrated in Figure 4.

not exchange directly. Rather, this exchange proceeds via the bound monomer species, or free ligand, the signal from which was too low to be observed.

Figure 4 illustrates the spectra from titration of TFAcDADA against a solution of ristocetin A (10 mM in 20% v/v CD_3CN/D_2O with 0.1M phosphate buffer, pH 6.0, at 268 K). Initially, the only signals observed were from LD1D2, D1D2L, ML and a small amount of a fluoride impurity present in the antibiotic solution. Direct integration of the signals for LD1D2 and D1D2L gave a value for the difference in binding energies between the two halves of the dimer of $4.4 \pm 0.3\,kJ\,mol^{-1}$, similar to that found previously.[15] As further ligand was added, the amount of doubly bound dimer and ligand-bound monomer increased. Similar results were obtained in a titration by using an antibiotic concentration of 50 mM.

The concentrations of the various species present can be determined by direct integration of the peaks, since the total concentration of the trifluoroacetyl group is known for every point on the titration. Although the peaks corresponding to LD1D2 and LD1D2L are overlapped severely, their concentrations can be determined since [LD1D2L] = [LD1D2L]. examining the final concentrations of dimer and monomer, it is clear that there has been a substantial overall drop in the dimerisation constant for the fully bound dimer ($K_d = 190 \pm 10\,M^{-1}$) using TFAcDADA as ligand, that is the anticooperativity is much more pronounced than with diAcKDADA. Further, examination of a plot of total dimer against ligand added (Figure 6) shows that, unlike the case of diAcKDADA where cooperativity was observed for the addition of the first equivalent of ligand, here anticooperativity was observed even for this first addition.

Previous work has demonstrated that for ristocetin A binding to diAcKDADA, there is a difference in binding energies (at 283 K) between the two halves of the dimer. The values are 27.9 and 23.0 $kJ\,mol^{-1}$, respectively, when the other side of the dimer is vacant, or 29.6 and 24.7 $kJ\,mol^{-1}$, respectively, when the other side of the dimer is filled.[15] The binding energy to the monomer is 26.7 $kJ\,mol^{-1}$, that is between the values for the two different sites of the dimer. Therefore, binding to the more-favoured dimer site is favoured over binding to monomer. However, the filling of the less-favoured site is anticooperative with respect to dimerisation, as this site has a smaller binding energy than

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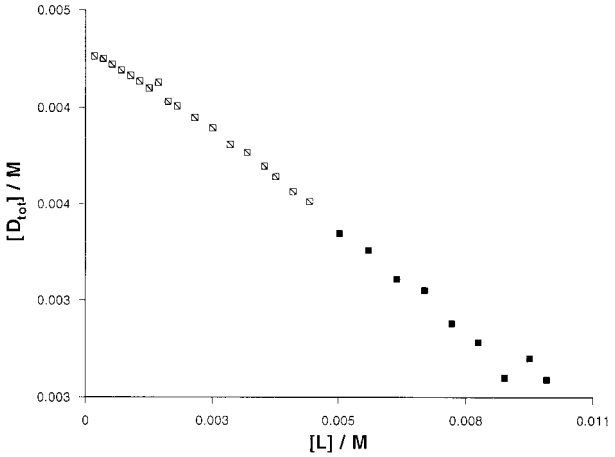

Figure 6. Plot of total concentration of dimer against total concentration of TFAcDAdA added. The plot was obtained from the titration data illustrated in Figure 4.

monomer, regardless of whether or not the other side of the dimer is filled.[15]

What is the physical cause of this difference in binding energies? As has been mentioned previously, the asymmetry is induced by the head-to-head orientation of the tetrasaccharides, breaking the C_2 symmetry of the head-to-tail arrangement of the antibiotic backbones. It is known that these sugars protrude over the binding sites, and given the length of the saccharide in the case of ristocetin A (four sugars as opposed to two for most of these antibiotics), the sugars will impinge upon both binding pockets more than for other antibiotics. Owing to the asymmetry, one pocket will be influenced by rhamnose and the other by arabinose (see Figure 2). The differences between the interactions of these different sugars with the ligand gives an insight into the observed differences in binding energies.[17]

In the present case, the trifluoroacetyl group is significantly more bulky than the corresponding acetyl group, and so relative to acetyl, these interactions may be less favourable owing to steric hindrance. The sugars are tightly packed together in the dimer with very little room for flexibility, and it is plausible that dissociation to monomer would allow sufficient flexibility for the unfavourable steric contacts in the ligand–bound dimer to be reduced.

Conclusions

It has been demonstrated that the use of ^{19}F NMR spectroscopy combined with labelling of ligands is a powerful tool for the study of the mode of action of the vancomycin group of antibiotics. Although in this case, the positioning of the trifluoroacetyl group appears to influence the degree of anticooperativity observed, it has produced useful information about binding to this antibiotic. It has also demonstrated the advantages that can be obtained (relative to 1H NMR spectroscopy) from the use of ^{19}F NMR spectroscopy in terms of simplification of spectra. For future studies, the trifluoroacetyl group could be readily placed in other parts of an extended ligand where its interaction with the antibiotic would not be so sterically demanding. Given the abundance of aromatic rings in these antibiotics, distinct chemical shift changes between bound and unbound ligand species would still be likely, thus preserving the usefulness of this technique for the study of the mode of action of these antibiotics.

Experimental Section

Materials: Ristocetin A, donated by Abbott Laboratories, Chicago, was used without further purification. The ligand used in these studies, TFAcDAdA, was produced by using standard Boc peptide chemistry, analogous to that published previously.[12]

NMR spectroscopy: All 1H NMR experiments were performed on a Varian Unity-500 spectrometer at 268 K. One-dimensional spectra were recorded by using 32000 complex data points.

All ^{19}F NMR experiments were performed on a Bruker AM-400 spectrometer equipped with a ^{19}F/^{1}H probe. One-dimensional spectra were recorded at typically 376.47 MHz by using 8000 complex data points over a spectral width of 6.9 ppm.

NMR titrations with ristocetin A: Ristocetin A concentrations used were 10 mM and 50 mM. Antibiotic solutions were prepared by dissolving a suitable quantity of antibiotic (15.5 mg and 77.6 mg, respectively) in phosphate buffer (750 μL, 0.1M, pH 6) and were adjusted to pH 6. These were lyophilised, and the residues were dissolved in D2O (750 μL) and lyophilised. Finally, the samples were dissolved in a 20% v/v CD_3CN/D_2O mixture (750 μL). 500 μL of these solutions were put directly into an NMR tube and 200 μL were added to the appropriate amount of TFAcDAdA (1.8 mg and 9.0 mg, respectively) so that, when mixed with the the other 500 μL, the final concentrations of ligand were 10 mM and 50 mM, respectively. In the titrations, ligand solution was added in aliquots of 2–10 μL.

Measurement of association constants by UV difference spectrophotometry: All experiments were performed with a dual beam Uvikon 940 spectrophotometer equipped with a thermocirculator to maintain a constant temperature. A ristocetin A concentration of 50 μM was used. Ristocetin A samples were dissolved in 20% v/v CH_3CN/H_2O (0.1M phosphate buffer, pH 6) and were adjusted to pH 6. Ligand samples were prepared by dissolving appropriate quantities of ligand in antibiotic solution and adjusting to pH 6 where necessary. The ligand concentration was calculated such that the addition of 500 μL ligand solution to 1900 μL stock antibiotic solution would give >95% bound antibiotic. Antibiotic solution (1900 μL) was added to both reference and sample cells, and a spectrum was recorded between 190–300 nm. Ligand solution (500 μL) was added to the sample cell and a further spectrum was recorded between the same wavelengths. The difference spectrum showed two distinct peaks, at 245 and 290 nm. The sample cell contents were replaced with fresh antibiotic solution (1900 μL) and aliquots of ligand solution (10–50 μL) were added. The absorbances at 244, 245, 290 and 291 nm were noted for each point, and the difference between the sums of the absorbances at 290,291 and 244, 245 nm was calculated. The association constant was determined by curve-fitting a plot of absorbance difference against ligand concentration, using the program Kaleidagraph (Abelbeck Software). Determinations were always carried out in triplicate; the average result is quoted, and the error is derived from the result with the largest deviation from the average.

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