A simple NMR analysis of the protonation equilibrium that accompanies aminoglycoside recognition: Dramatic alterations in the neomycin-B protonation state upon binding to a 23-mer RNA aptamer†

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Received (in Cambridge, UK) 10th August 2006, Accepted 27th September 2006
First published as an Advance Article on the web 23rd October 2006
DOI: 10.1039/b611597g

A complete characterisation of the protonation equilibrium that accompanies the molecular recognition of neomycin-B by a specific RNA receptor has been achieved by employing simple NMR measurements.

Aminoglycosides are highly potent, wide-spectrum bactericidals that target different RNA fragments.[1,2] Interestingly, the binding of these antibiotics to ribosomal RNA has been shown to be linked to the protonation of several amino groups of the drug in a pH dependent manner,[3] leading to the conclusion that drug protonation reactions are important thermodynamic participants in the formation of specific aminoglycoside–RNA complexes.[3] The link between binding and protonation has a profound influence on the thermodynamic parameters of the association process, which have been analysed in the past in various different ways.[3] It should be pointed out that although this phenomenon seems to be an essential feature of aminoglycoside recognition by ribosomal RNA, it has not yet been proved for different specific RNA receptors. From a general molecular recognition perspective, a proper description of the interaction of aminoglycosides with RNA requires a full characterization of the protonation equilibrium that accompanies the binding, implying knowledge of the drug protonation state in both its free and bound forms. On this basis, we herein propose a simple method to address this issue that may be also extended to other interacting molecular systems, for which protonation is involved in the recognition phenomenon. The proposed protocol is based on standard NMR experiments, whose analysis permits not only the determination of the net number of protons transferred to or from the aminoglycoside upon complex formation, but also the pointing out of the specific positions involved in the protonation process. As a key example, and in order to gain insights into the thermodynamic features of aminoglycoside binding to specific RNA receptors, we have analyzed the changes in the protonation state of neomycin-B, promoted by its interaction with a 23-mer RNA aptamer. Firstly, pK_a values of the antibiotic in D_2O were derived by pH titration experiments employing ^1H–^{13}C 2D-HSQC data. To avoid any pK_a alteration due to drug-counterion interactions,[4] a low concentration of antibiotics (10 mM) in their free base form were employed in all cases. The obtained results are summarised in Fig. 1 and Table 1. Thus, the most basic amino groups (pK_a > 8.5) are those located at position 6 of pyranose rings I and IV. The lowest pK_a value corresponds to the amino group at position 3 of ring II. In fact, this function presents a remarkably low basicity (pK_a = 5.4). This unusual behaviour can be attributed to the particular location of this amino group within the aminoglycoside scaffold and its electrostatic interactions with the other amino groups.

For paromomycin, it has been shown that the acid–base properties of aminoglycosides are sensitive to the presence of sulfate counterion.[4] Therefore, we decided to check the sensitivity of neomycin-B pK_a values to various ions in solution. Thus, pH titrations employing 2D-HSQC experiments in the presence of either 500 mM sodium sulfate, sodium chloride, sodium trifluoroacetate (TFA) or sodium phosphate were also performed. For sulfate, lower concentrations were also tested.

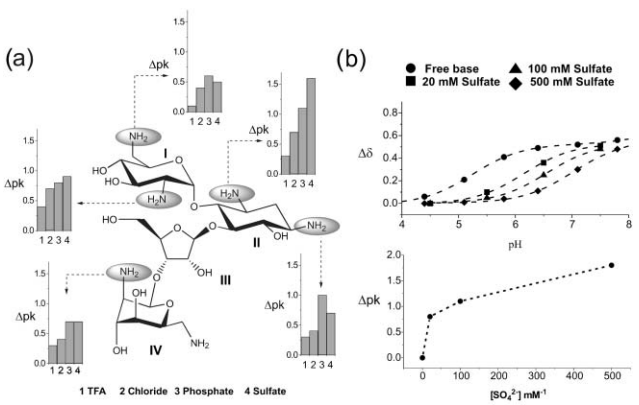

Fig. 1 (a) Increase of pK_a observed for some of the neomycin-B amino groups in the presence of 500 mM sodium trifluoroacetate, sodium chloride, sodium phosphate and sodium sulfate. The numbering employed for the different sugar units is shown. (b) pH titration profiles corresponding to the most affected antibiotic position, H3-II, at different sulfate concentrations. The ΔpK_a is represented as a function of the sulfate concentration in the lower panel.

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† Electronic supplementary information (ESI) available: Experimental details, and additional spectral and structural information. DOI: 10.1039/b611597g

Table 1 pK_a values derived for neomycin-B in their free and RNA bound states

	Free					Bound
	Free base	SO_4^{2-}	PO_4^{3-}	Cl^-	$CF_3CO_2^-$	Free base
2-I	7.6	8.5	8.4	8.3	8.0	>9.0
6-I	8.7	9.2	9.3	9.1	8.8	>9.0
1-II	8.1	8.8	9.1	8.5	8.4	>9.0
3-II	5.4	7.0	6.5	6.1	5.7	>9.0
2-IV	7.5	8.2	8.2	7.9	7.8	>9.0
6-IV	8.8	9.4	9.5	9.2	9.1	>9.0

It was observed that, in general, both sulfate and phosphate groups showed a larger influence on the aminoglycoside basicity than did the monoanionic species chloride and trifluoroacetate. In addition, trifluoroacetate had a lower impact on the pK_a values than chloride, probably due to the electron withdrawing nature of the fluorine atoms. These two observations are in agreement with the electrostatic nature of the antibiotic/counterion interaction.

As a next step, the protonation state of the drug in complex with a 23-mer RNA aptamer was analyzed by NMR. The employed methodology was based on the measurement of the $^1J_{C-H}$ couplings for the different H–C–NH_2/NH_3^+ fragments present in the drug. Given the different electron withdrawing characters of the amino function in its charged and uncharged forms, this J value was expected to be sensitive to its protonation state. This hypothesis has been confirmed both theoretically and experimentally (see ESI†). Thus, $^1J_{C-H}$ coupling constants for the HC–NH_2/NH_3^+ fragments present in aminoglycosides might be employed as sensors for the protonation state of each individual amino group within the drug in its complexed state. This methodology presents several advantages in respect of other alternatives. Firstly, it provides information, not only about the net number of protons transferred from or to the aminoglycoside upon complex formation, but also allows a straightforward identification of the particular amino group involved in this process. Secondly, the degree of protonation of each individual amino group within the drug at a given pH value can be established by a single measurement (instead of a full titration experiment). Finally, aminoglycoside CH–NH_2/NH_3^+ fragments exhibit distinct ^{13}C chemical shifts in the 45–57 ppm range. This spectral region is totally free of signals in the HSQC spectrum corresponding to DNA or RNA receptors (see Fig. 2).

By employing these simple measurements, the changes in the protonation state of neomycin-B that accompany its specific recognition by a 23-mer RNA aptamer were analyzed. The 3D structure of the complex has recently been described by NMR methods.[5] According to this data, the antibiotic is totally encapsulated within the binding pocket in the major groove of the receptor, and establishes several polar interactions that involve both charged and neutral groups. A spectral region of the coupled HSQC experiment corresponding to the neomycin-B/RNA complex is shown in Fig. 2. It can be observed that, despite the high molecular weight of the complex, the drug HC–NH_2/NH_3^+ atom pairs can be easily identified by their distinct carbon chemical shifts. The assignment of aminoglycoside HC–NH_2/NH_3^+ groups was based on the previous report of Patel *et al.*[5] To determine the degree of protonation of the drug within the complex, the

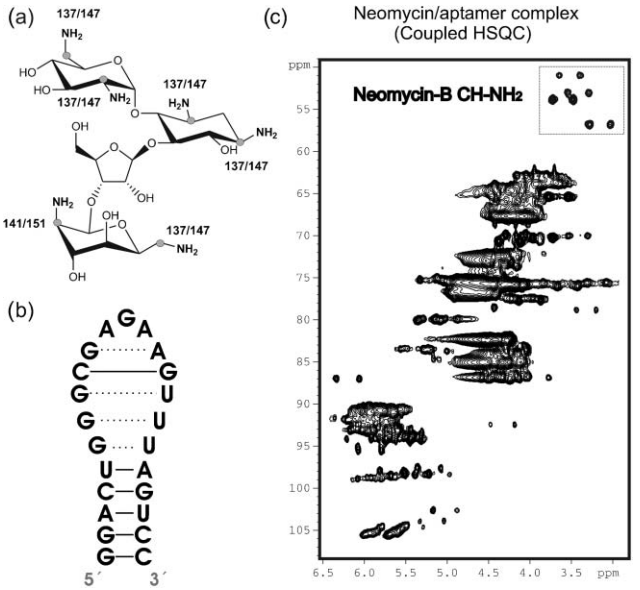

Fig. 2 (a) Values corresponding to the heteronuclear $^1J_{C-H}$ couplings for the HC–NH_2/NH_3^+ fragments present in neomycin-B in their protonated (right) and non-protonated (left) forms are shown on the structure of neomycin-B. (b) Schematic representation of the 23-mer RNA aptamer employed in our study. (c) Coupled $^1H/^{13}C$ HSQC experiment corresponding to the aminoglycoside/RNA complex.

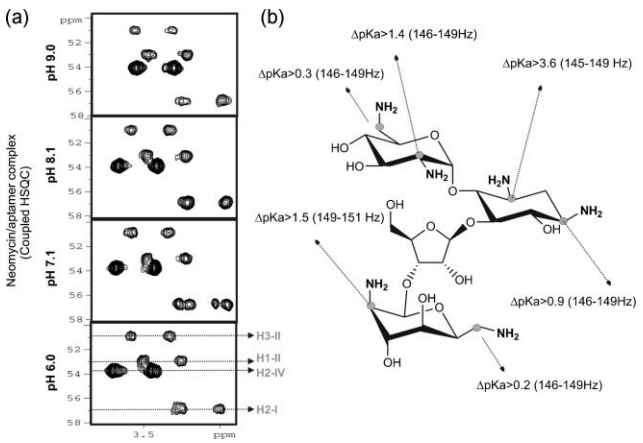

Fig. 3 (a) The HC–NH_2/NH_3^+ region in the coupled HSQC experiment corresponding to the aminoglycoside/RNA complex at different pH values. (b) pK_a alterations observed for the different amino groups upon complex formation. The $^1J_{C-H}$ values in the bound state are shown in brackets.

heteronuclear J couplings were measured at different pH values. The obtained results are summarized in Fig. 3.

From the NH_2/NH_3^+ chemical shifts, it has been proposed that the amino group at position 3 of ring II was deprotonated within the complex at neutral pH.[5] Strikingly, our data conclusively show that the antibiotic remains fully protonated up to pH 9.0 in the complexed state (higher pH values could not be tested due to degradation of the RNA receptor). Under these particular conditions, the binding process must be accompanied by a transfer of more than 4 protons from the buffer to the drug. For the NH_2-II group, this implies an increment in the pK_a of more

than 3.6 units, which suggests a huge contribution of this charge to the stability of the complex. Interestingly, this amino moiety is not involved in a direct interaction with a charged group within the binding pocket, according to the structural information available.[5] Instead, it forms two hydrogen bonds with a guanine base. This fact, together with the strongly negative electrostatic potential of the binding pocket, is sufficient to cause the large change in basicity.

Our results are in agreement with those reported for aminoglycoside recognition by ribosomal RNA and indicate that RNA binding-linked protonation of these antibiotics might be a general phenomenon. These subtle features of the binding reaction have been revealed by employing simple 2D NMR experiments that permit dissection of the energy and atomic features of the interaction process. The employed protocol can be extended to other systems of biological/biomedical interest, being especially suitable for the analysis of drug/DNA/RNA complexes, given the particular ^{13}C chemical shift dispersion of these receptors.

We thank the Ministerio de Educación y Ciencia (project CTQ2004-04494, Ramón y Cajal contract of F. C.) and Juan de la Cierva contract of J. R.). We also thank CESGA for computational support.

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