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Studying aminoglycoside antibiotic binding to HIV-1 TAR RNA by electrospray ionization mass spectrometry

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Abstract

The recognition of the aminoglycosides neomycin and streptomycin by HIV-1 TAR RNA was studied by electrospray ionization mass spectrometry (ESI-MS). Members of the aminoglycoside family of antibiotics are known to target a wide variety of RNA molecules. Neomycin and streptomycin inhibit the formation of the Tat protein–TAR RNA complex, an assembly that is believed to be necessary for HIV replication. The noncovalent complexes formed by the binding of aminoglycosides to TAR RNA and the Tat–TAR complex were detected by ESI-MS. Neomycin has a maximum binding stoichiometry of three and two to TAR RNA and to the Tat–TAR complex, respectively. Data from the ESI-MS experiments suggest that a high affinity binding site of neomycin is located near the three-nucleotide bulge region of TAR RNA. This is consistent with previous solution phase footprinting measurements [H.-Y. Mei et al., Biochemistry 37 (1998) 14204]. Neomycin has a higher affinity toward TAR RNA than streptomycin, as measured by ESI-MS competition binding experiments. A noncovalent complex formed between a small molecule inhibitor of TAR RNA, which has a similar solution binding affinity as the aminoglycosides, and TAR RNA is much less stable than the RNA–aminoglycoside complexes to collisional dissociation in the gas phase. It is believed that the small molecule inhibitor interacts with TAR RNA via hydrophobic interactions, whereas the aminoglycosides bind to RNAs through electrostatic forces. This difference in gas phase stabilities may prove useful for discerning the types of noncovalent forces holding complexes together. (Int J Mass Spectrom 193 (1999) 115–122) © 1999 Elsevier Science B.V.

1. Introduction

Protein–RNA interactions are involved in many cellular processes including transcription, translation, RNA degradation, and nucleocytoplasmic transport [1]. An emerging technique for studying the noncovalent interactions between proteins and oligonucleotides is electrospray ionization mass spectrometry (ESI-MS), as the measurement of molecular weight provides a direct means for determining the stoichiometry of the binding partners [2–9]. MS with electrospray ionization has demonstrated to be an extremely soft ionization method. Even the results from weak interactions, such as noncovalently bound complexes, can survive the transition from the condensed phase to the vapor phase for their ultimate detection by mass spectrometry. The observation of small molecule binding to larger oligonucleotide targets is difficult for many other biophysical techniques, such

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as gel electrophoresis. Thus, because of its superior mass resolution and accuracy, ESI-MS may prove to be an extremely valuable tool also for studying the effects of small molecule inhibitors of targeted protein–RNA complexes. It provides an ideal method to directly determine drug binding stoichiometry [10] and potentially, relative and absolute binding affinities [2,11–14].

Replication of the human immunodeficiency virus (HIV) requires the complexation of the viral transactivator protein, Tat, to the transactivation responsive element (TAR), which is located at the 5′-end of mRNA [15,16]. TAR is a small RNA hairpin consisting of a stem-loop structure with an essential three-nucleotide pyrimidine bulge. Tat protein contains an arginine-rich region near its C-terminus that interacts specifically with this pyrimidine bulge. A smaller length peptide containing the basic C-terminal sequence (Tat peptide) has nearly equal binding specificity and affinity to TAR RNA as full length Tat protein [17–20]. Because the Tat–TAR complexation is critical for HIV replication, drugs that interfere with the interaction of Tat protein/peptide and TAR RNA may be promising inhibitors of HIV replication [21–23].

Previously, we demonstrated the utility of ESI-MS for studying the Tat–TAR noncovalent complex [6]. We have extended this study by applying mass spectrometry toward the examination of the action of neomycin and streptomycin on the Tat peptide–TAR RNA system. Neomycin and streptomycin are aminoglycoside antibiotics known to specifically bind RNA molecules [24,25] and to interfere with the complexation between Tat protein and TAR RNA [17]. Neomycin and streptomycin inhibit binding of Tat peptide to TAR RNA with IC_{50} values (concentration necessary for 50% inhibition) of 0.92 and 9.5 μM, respectively [22]. A stem-loop structure like that found in TAR RNA is necessary for binding of the antibiotics [23,26]. By using ESI-MS, we demonstrate that the binding stoichiometry of aminoglycosides as well as their relative affinities to TAR RNA and the Tat–TAR complex can be determined.

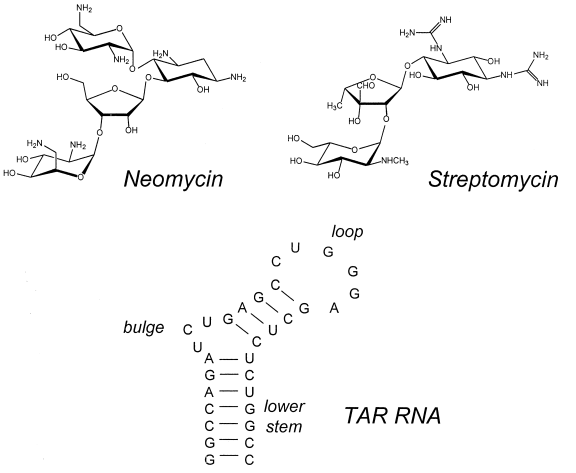

Fig. 1. Structures of TAR RNA (31-mer), neomycin, and streptomycin.

2. Experimental

2.1. TAR RNA, Tat peptide and aminoglycosides

TAR RNA (the shortened 31-mer form of the full-length 59-mer, M_r 9941; Fig. 1) was chemically synthesized using phosphoramidite chemistry, purified by polyacrylamide gel electrophoresis, and characterized by enzymatic sequencing and ESI-MS [6,17]. Gel-purified RNA samples for ESI-MS binding studies were further de-salted by cold ethanol precipitation as ammonium acetate salts [27,28]. The 40-amino acid Tat peptide (M_r 4644) was synthesized by solid phase synthesis using standard Boc chemistry protocols and purified by reversed phase high-performance liquid chromatography (HPLC). The aminoglycoside antibiotics, neomycin (as neomycin B) and streptomycin, were purchased from Sigma Chemical Company (St. Louis, MO) and were used without further purification.

2.2. Sample preparation

Lyophilized TAR RNA was diluted in 10 mM ammonium acetate, pH 6.9, to give 1–10 μM concentration solutions. To ensure proper folding of the TAR RNA structure, the RNA solutions were annealed by

heating for 4 min at 95 °C and cooled slowly. Prior to ESI-MS experiments, 1,2-cyclohexanediamine tetraacetic acid (CDTA, 0.3 mM) [28] was added to the TAR RNA solution to further reduce formation of cation adducts. The addition of methanol (to 10% *v/v*) enhanced the stability of the ESI-MS signal without altering the resulting mass spectra. Tat peptide was dissolved in 10 mM ammonium acetate, pH 6.9 and 0.01% Nonidet-P40 to give 0.5 mM concentration stock solutions. Nonidet-P40, a nonionic surfactant, was added to prevent the aggregation of peptides. Neomycin and streptomycin were dissolved in 10 mM ammonium acetate, pH 6.9, to give 1 mM concentration stock solutions. For the aminoglycoside binding studies with TAR RNA or Tat–TAR complex, an appropriate amount of neomycin or streptomycin was added to give aminoglycoside/TAR RNA ratios in the range of 1:1 to greater than 10:1. The solutions were equilibrated for 10–20 min prior to mass spectral acquisition.

2.3. Mass spectrometry

ESI-MS was performed with a double focusing hybrid mass spectrometer (EBqQ geometry, Finnigan MAT 900Q, Bremen, Germany) with a mass-to-charge (*m/z*) range of 10 000 at 5 kV full acceleration potential [29]. A position-and-time-resolved-ion-counting (PATRIC) scanning focal plane detector with an 8% *m/z* range of the *m/z* centered on the array detector was used [30]. For most of the experiments, an ESI interface based on a heated glass capillary inlet was used. Warm nitrogen gas (~60 °C) countercurrent to the electrospray aided droplet and ion desolvation [31]. The nitrogen flow rate (3 L min^{-1}) can influence the amount of residual solvation observed for the multiply charged ions as well as the sensitivity of ESI, especially for ESI from aqueous solutions. Low energy gas phase collisions, controlled by adjustment of the voltage difference between the tube lens at the metallized exit of the glass capillary and the first skimmer element (ΔV_{TS}), were also used to augment the desolvation of the ESI-produced droplets and ions. Additionally, electrospraying the aqueous solutions slightly "off-axis" relative to the capillary inlet (approximately 5°–10° angle) was necessary to acquire spectra with minimal ion solvation and to increase sensitivity. Spraying "on-axis" relative to the capillary inlet produced broad peaks because of poorly desolvated ions. Especially important for ESI of aqueous solutions, a stream of sulfur hexafluoride coaxial to the spray suppressed corona discharges in both positive and negative ion modes. Solution flow rates delivered to the ESI source were typically in the 0.5–1.0 μL min^{-1} range.

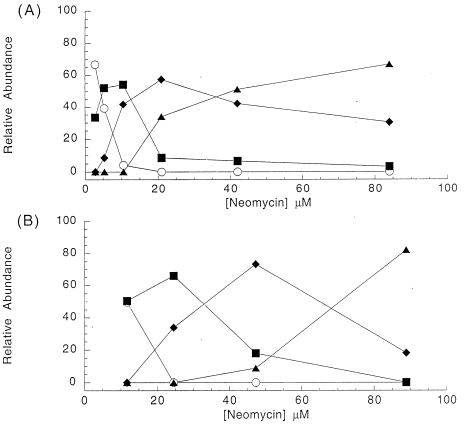

Fig. 2. Titration plot of TAR RNA (10 μM concentration) with neomycin in the (A) positive ion and (B) negative ion modes of ESI-MS. Open circle: free TAR RNA, closed square: TAR/neomycin complex, closed diamond: TAR/$neomycin_2$ complex, and closed triangle: TAR/$neomycin_3$ complex.

Some of the ESI-MS experiments utilized a heated metal capillary interface and a low flow micro-ESI source [6]. The temperature of the metal capillary inlet was maintained at 150 °C. The micro-ESI source allowed for solution flowrates to 150 nL min^{-1}.

3. Results and discussion

3.1. Aminoglycoside binding to TAR RNA

Positive ion and negative ion ESI-MS of the titration of TAR RNA (9941 MW) with neomycin (614.6 MW) are qualitatively similar (Fig. 2). In both cases, the maximum number of neomycin molecules bound to TAR RNA is three and the neomycin binding appears to occur sequentially. Multiple bind-

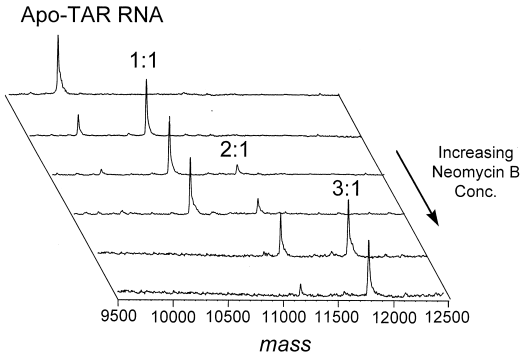

Fig. 3. Negative ion ESI mass spectra (deconvoluted spectra) of the titration of neomycin to TAR RNA (1.5 μM concentration). The concentrations of neomycin from the top spectrum to the bottom spectrum are 0, 1.4, 1.9, 2.4, 4.0, and 6.5 μM. The maximum binding stoichiometry of neomycin binding to TAR is three.

ing of neomycin occurs only at the higher concentration levels of neomycin (Fig. 3). Increasing the neomycin concentration to levels higher than depicted in Fig. 2 and 3 did not change the maximum stoichiometry of neomycin binding. Also, streptomycin binds to TAR RNA with a maximum stoichiometry of three. From solution phase measurements, an initial neomycin molecule is believed to specifically recognize the stem region immediately below the three-nucleotide bulge element of TAR RNA [22,26], with other weaker affinity or nonspecific interactions contributing to the binding of additional neomycin molecules (*vide infra*). In separate experiments, neomycin does not bind to the Tat peptide, indicating that neomycin interacts specifically with TAR RNA.

The mass spectrometry results agree qualitatively with data obtained by nondenaturing polyacrylamide gel electrophoresis (i.e. gel mobility shift assays), which also show sequential and multiple bindings of neomycin to TAR RNA [22,26]. However, whereas the determination of stoichiometry from gel mobilities is subject to interpretation, the stoichiometry of neomycin complexation can easily be measured from the ESI mass spectrum (Fig. 3).

Although several publications have reported the ESI-MS measurement of solution binding affinities for micromolar binding affinity systems [2,11–14], it can be difficult to measure the absolute binding constants of small molecules to specific targets with higher affinities (i.e. submicromolar). However, *relative* binding affinities can be more easily determined from competitive binding experiments monitored by ESI-MS [12,32]. Under competitive binding conditions where the TAR RNA to neomycin to streptomycin concentrations are equimolar, only neomycin is observed to bind to TAR RNA in both positive and negative ion ESI-MS (Fig. 4). Streptomycin binding to TAR RNA is not observed in the presence of neomycin, even though streptomycin binds to TAR RNA in separate direct binding studies. Thus, TAR

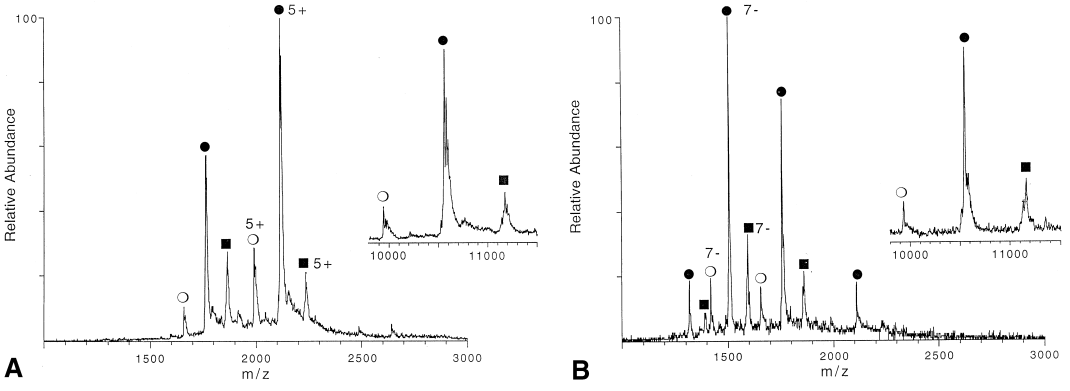

Fig. 4. Competitive binding studies of TAR RNA with neomycin and streptomycin at a 1:1:1 ratio (10 μM concentration each). (A) Positive ion ESI and (B) negative ion ESI mass spectra are shown, with the deconvoluted (mass domain) spectra depicted in the insets. Only complexes between neomycin and TAR RNA are observed; there is no evidence for streptomycin binding to TAR RNA. Open circle: free TAR RNA, closed circle: TAR/neomycin complex, and closed square: TAR/neomycin$_2$.

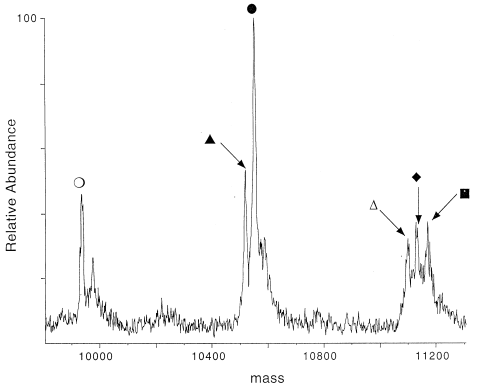

Fig. 5. The negative ion ESI deconvoluted mass spectrum of the noncompetitive binding study of TAR RNA (10 μM concentration) with neomycin and streptomycin at a 1:1:10 concentration ratio. Even though there is ten times as much streptomycin as neomycin, the TAR/neomycin complex still dominates the spectrum. Open circle: free TAR RNA, closed circle: TAR/neomycin complex, closed square: TAR/neomycin$_2$, closed triangle: TAR/streptomycin, open triangle: TAR/streptomycin$_2$, and closed diamond: TAR/neomycin/streptomycin.

RNA binds more strongly to neomycin than streptomycin, in agreement with gel mobility shift assays, as the IC_{50} value for streptomycin is an order of magnitude greater than for neomycin [22]. Under noncompetitive conditions where the TAR RNA to neomycin to streptomycin ratio is 1:1:10, both neomycin and streptomycin are observed to bind to TAR RNA and ternary complexes are formed as well (Fig. 5). The neomycin–TAR RNA complex dominates the spectrum even though streptomycin is present at ten times the amount of neomycin. From the relative abundances of the neomycin–TAR and streptomycin–TAR complexes and the concentration difference between neomycin and streptomycin [4,5], TAR RNA is estimated to have an affinity twenty times greater to neomycin than to streptomycin.

3.2. Aminoglycoside binding to the Tat peptide–TAR RNA complex

Positive ion and negative ion ESI-MS of the titration of the 1:1 stoichiometry Tat peptide–TAR RNA complex with neomycin show a decreasing relative abundance of the Tat–TAR complex and an increasing relative abundance of the ternary complex between Tat peptide, TAR RNA, and neomycin with increasing neomycin concentration. A maximum of two neomycin molecules bind to the Tat–TAR complex (data not shown [33,34]). With higher neomycin concentrations (~10:1 neomycin:Tat–TAR), after the formation of the Tat–TAR–neomycin$_2$ complex, the complex dissociates to form TAR–neomycin and TAR–neomycin$_2$ complexes and free Tat peptide. We believe that dissociation of the complex occurs in solution and is not an artifact of the electrospray process, as all experimental conditions were kept constant over the full range of the titration experiment. Conditions used to observe the Tat–TAR–neomycin$_2$ complex were used to observe the dissociation of the complex at higher neomycin concentrations. In addition, the ESI-MS results are qualitatively consistent with the gel mobility shift assays [22,23], which show a decrease in the amount of the Tat–TAR complex and the formation of TAR–neomycin complexes with increasing amounts of neomycin added. Formation of the ternary complex between Tat, TAR RNA, and neomycin is not observed in the gel mobility shift assays; however, the gel mobility assay may not be able to resolve the ternary complex from the Tat–TAR complex.

Competition experiments were used to determine the relative binding affinities of neomycin and streptomycin to the 1:1 Tat–TAR complex. Under competitive binding conditions (i.e. equimolar concentrations), both positive and negative ion ESI-MS show only neomycin binding to the Tat–TAR complex (Fig. 6). Again, although streptomycin binds to the Tat–TAR complex in separate experiments, streptomycin does not bind to the complex in the presence of neomycin, as the Tat–TAR complex has a greater affinity to neomycin than to streptomycin.

3.3. Binding sites of neomycin to TAR RNA

The neomycin binding sites on the TAR RNA structure can be deduced from the available data. Both the mass spectrometry results and the gel mobility shift assays indicate that there is multiple binding of neomycin to TAR RNA. A maximum binding stoi-

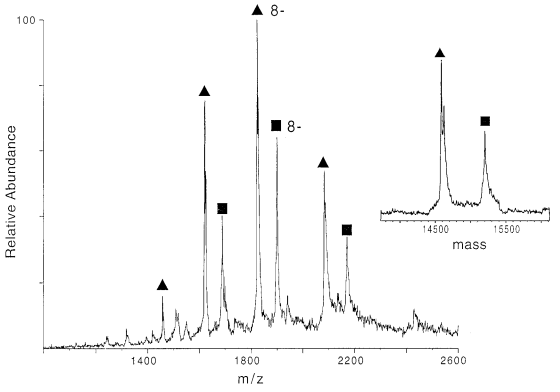

Fig. 6. The negative ion ESI mass spectrum (and deconvoluted spectrum in the inset) of the competitive binding experiment of 1:1 Tat peptide–TAR RNA complex (10 μM concentration) with neomycin and streptomycin at a ratio of 1:1:1. Triangle: Tat/TAR, and square: Tat/TAR/neomycin.

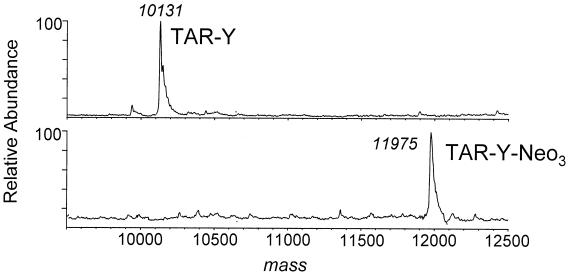

Fig. 7. The negative ion deconvoluted ESI spectra of TAR RNA (1.5 μM concentration) binding to 2,4,5,6-tetraaminoquinazoline (referred to as Y) and neomycin. The top spectrum shows the mass spectrum from a solution containing compound Y and TAR RNA at a 4:1 molar ratio. The bottom spectrum shows the mass spectrum from a solution containing compound Y, neomycin, and TAR RNA at a 4:6:1 molar ratio.

chiometry of three and two neomycin molecules to TAR RNA and Tat–TAR, respectively, was measured by ESI-MS [33,34]. Previous studies, including our ESI-MS measurements [6], have established the bulge region of TAR RNA as the recognition site for Tat protein/peptide [16,20,35–37]. Therefore, a region near the bulge is a likely site for neomycin binding. In fact, the stem region immediately below the bulge was suggested to be the primary binding site from ribonuclease protection experiments [23,26]. Using a mutant form of TAR RNA, further assays showed that the loop region of TAR RNA is also a binding site for neomycin. ESI-MS data for a modified TAR RNA, in which the loop region of TAR RNA was replaced with a polyethyleneglycol linker [17], showed binding of only two molecules of neomycin, compared to three for the unmodified TAR (data not shown).

3.4. Solution phase and gas phase interactions

The types of interactions that govern noncovalent binding in solution can be distinguished by the ESI-MS gas phase measurements. Electrostatic forces are greatly strengthened in a solventless environment, and thus complexes held together by electrostatic interactions are extremely stable in the gas phase. Electrostatic interactions in solution are decreased by its dielectric constant [38,39]. Previously, we had observed that ions for the Tat peptide–TAR RNA complex were very stable, as the complex was not observed to dissociate at very high ESI interface energies [i.e. in-source collisionally activated dissociation (CAD) induced by increasing ΔV_{TS}] [6]. Another noncovalent complex between a highly positively charged molecule and a negatively charged macromolecule is the zinc finger HIV nucleocapsid protein and ψ-RNA; again the protein–RNA gas phase complex is stable against dissociation [9].

Aminoglycosides are known to bind to RNAs through charge–charge interactions [40,41]. Many of the amino groups of neomycin are positively charged at neutral pH. The neomycin–TAR RNA complexes (e.g. TAR–neomycin, TAR–neomycin$_2$, TAR–neomycin$_3$) were not observed to dissociate at high ΔV_{TS}. Similarly, the complexes between neomycin and the Tat–TAR complex observed in the gas phase are stable to dissociation attempts.

Interactions that are largely governed by hydrophobic interactions in solution appear to be weakened in vacuum. Robinson's group had noted that the apparent relative affinities measured by ESI-MS for acyl CoA derivatives hydrophobic binding to acyl CoA-binding protein did not correlate with their solution affinities [42]. The labile dissociation of the gas phase complex could account for this discrepancy. A small molecule, identified from screening a Parke-

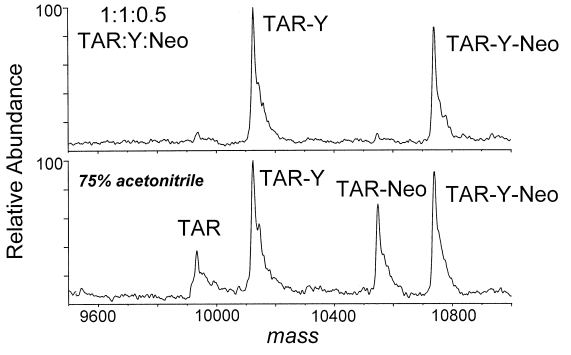

Fig. 8. The negative ion deconvoluted ESI spectra of a 1:1:0.5 concentration ratio of TAR RNA (5 μM concentration), 2,4,5,6-tetraaminoquinazoline (referred to as Y), and neomycin. The top spectrum shows only formations of the TAR–Y and TAR–Y–neomycin complexes. Compound Y is selectively dissociated from the complexes upon addition of acetonitrile to 75% *v/v* (bottom).

Davis chemical compound library as a TAR RNA inhibitor, 2,4,5,6-tetraaminoquinazoline (referred to as Y; MW 190.2) [23], does not share the same binding site(s) with neomycin, as the TAR–Y–neomycin$_3$ complex can be formed (Fig. 7). A maximum stoichiometry of only one molecule of Y binds to TAR. The TAR–Y complex is relatively unstable in the gas phase compared to the electrostatically bound TAR–neomycin. In-source CAD dissociates only Y from the TAR–Y–neomycin and TAR–Y–neomycin$_3$ complexes (data not shown). Similarly, increasing the organic solvent (acetonitrile) content to 75% *v/v* reduces the relative proportion of TAR–Y complexation, whereas the abundance of the TAR–neomycin complex remains unchanged (Fig. 8). These observations are consistent for compound Y binding to TAR by hydrophobic-type interactions.

The different relative stabilities of gas phase interactions have implications for using ESI-MS to determine solution phase absolute and relative binding affinities. For compounds that bind to a target molecule with similar-type binding mechanisms, and thus may have similar gas phase stabilities, determining their relative binding affinities by ESI-MS should not be problematic. However, if hydrophobic interactions are in play, the lability of the gas phase complex may conspire to reduce the confidence of the MS data.

4. Conclusions

Using ESI-MS, a method for determining the stoichiometry of small molecule inhibitor binding to noncovalent protein–RNA complexes was developed. For the aminoglycoside example presented, neomycin and streptomycin are likely to target the structure of TAR RNA, as no direct binding was observed with Tat peptide. The maximum binding stoichiometry of neomycin to TAR RNA is three, and a maximum of two molecules of neomycin bind to the Tat peptide–TAR RNA complex prior to dissociation of the peptide–RNA complex. The gas phase ESI-MS data are qualitatively consistent with solution data gathered from nondenaturing gel mobility assays. Relative binding affinities are easily measured by competitive binding studies using ESI-MS. Previous to these measurements, the exact neomycin binding stoichiometries to TAR RNA and to the Tat–TAR complex were not known. This information is important for understanding the structural aspects of small molecule recognition by RNA.

The charge–charge interactions between the aminoglycosides and TAR RNA are extremely stable in the gas phase, whereas hydrophobic binding is weakened in a solventless environment. This phenomenon may cast doubt for applying ESI-MS as a *general* technique to study noncovalently bound complexes. However, at the present time, it may be premature to make this judgement without additional knowledge. ESI-MS has already made contributions to numerous structural studies involving noncovalent complexes [3]. The method will continue to be useful to biochemists and medicinal chemists, as it provides a largely unambiguous means to measure the size of complexes and the stoichiometry of binding. Ultimately, ESI-MS may provide a tool for rapidly screening compounds to support drug discovery efforts.

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