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Boronate affinity adsorption of RNA: possible role of conformational changes ☆

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Abstract

Batch equilibrium adsorption isotherm determination is used to characterize the adsorption of mixed yeast RNA on agarose-immobilized *m*-aminophenylboronic acid. It is shown that the affinity-enhancing influence of divalent cations depends strongly on the precise nature of the cation used, with barium being far more effective than the conventionally-used magnesium. This adsorption-promoting influence of barium is suggested to arise primarily from ionic influences on the structure and rigidity of the RNA molecule, as the adsorption of ribose-based small molecules is not similarly affected. The substitution of barium for the standard magnesium counterion does not greatly promote the adsorption of DNA, implying that the effect is specific to RNA and may be useful in boronate-based RNA separations. RNA adsorption isotherms exhibit sharp transitions as functions of temperature, and these transitions occur at different temperatures with Mg^{2+} and with Ba^{2+}. Adsorption affinity and capacity were found to increase markedly at lower temperatures, suggestive of an enthalpically favored interaction process. The stoichiometric displacement parameter, Z, in Ba^{2+} buffer is three times the value in Mg^{2+} buffer, and is close to unity.

Keywords: Adsorption isotherms; *m*-Aminophenylboronic acid; RNA; Inorganic cations

1. Introduction

Interest in nucleic acid separations has recently been driven by remarkable advances in genetic technology and nucleic acid biochemistry. The separation of RNA has become important both in applications in which RNA is the desired product, such as ribozyme science and rRNA probe methods, and in applications requiring the removal of contaminating RNA from other, desired products such as plasmid DNA.

One of the few chemical differences between RNA and DNA which can serve as a basis of separation is the presence of the vicinal 2′,3′ *cis*-diol at the 3′ end of the RNA molecule. This feature is absent from the deoxyribose backbone of DNA, and is exploited as the basis of recognition by boronate affinity methods. Boronic acid chromatography has been used for tRNA and (ribo)nucleotide isolation since the 1970s [1–4]. Several workers have sub-

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sequently demonstrated the application of boronic acid chromatography to separation of nucleic acids and their derivatives, and of sugars and glycosylated proteins [5–18]. In addition, it has been demonstrated that modification of the *m*-aminophenylboronic acid group to confer a lower pK_a on the boronic acid moiety can enhance the range of pH over which these adsorbents are useful [19–21].

In the present work we have used batch equilibrium adsorption isotherm measurement and several supporting techniques to characterize the adsorption of mixed RNAs on *m*-aminophenylboronic agarose under a variety of conditions.

2. Experimental

m-Aminophenylboronic acid agarose PBA-60 (60–100 μmol boron/ml gel), PBA-30 (30–50 μmol boron/ml gel) and PBA-10 (10–15 μmol boron/ml gel) were obtained from Amicon. Total yeast RNA (R-6750), salmon sperm DNA (D-1626), adenosine and AMP were obtained from Sigma as were most other chemicals used. Water was purified using a Millipore Milli-Q water purification system. Glycine, 50 m*M* at pH 9.0 was used as the common buffering agent. Divalent cations were added as their chloride salts, and the pH was adjusted to 9.0 by adding concentrated NaOH or HCl. Buffers were prepared fresh for every set of experiments and were stored at 4°C when not in use.

Nucleic acid concentrations were estimated from absorbance at 260 nm measured over a 1-cm light path on a diode-array UV spectrophotometer (Beckman), with appropriate dilution to bring the absorbance to between 0.10 and 1.0. Using gravimetrically-prepared solutions, the extinction coefficient of the mixed RNA used was experimentally found to be 16.25 ($\pm$1.36, n=3) $(mg/ml)^{-1}\ cm^{-1}$. This value was found to be independent of pH and ionic strength over the ranges used in this work. Molar extinction coefficients used for AMP and adenosine were obtained from Dawson et al. [22].

The isotherm measurement procedure was derived from those of Roush et al. [23] and Gill et al. [24,25]. Adsorption isotherm experiments were carried out in microcentrifuge tubes (Baxter) of 1900 μl nominal volume (2150 μl actual working volume). For each isotherm, 100 μl of the adsorbent stock suspension at 10^7 beads/ml (determined using a hemocytometer after freshly mixing) was added to tubes already containing increasing volumes of RNA stock solution (in experimental binding buffer), and a sufficient volume of the experimental binding buffer to fill the remainder. A small hole in the cap of each tube allowed nearly complete filling with liquid to minimize air volume while avoiding leakage. Tubes were equilibrated in an end-over-end rotator (Cole-Parmer) at 5 rpm for the equilibration time (usually overnight; results were time-invariant after 5 h), then centrifuged at 16 000 *g* for 2 min in an Eppendorf microcentrifuge (Brinkman) to settle the agarose beads. The unbound (free) RNA in the supernatant liquid was assayed by carefully removing 2 ml of the supernatant, and determining RNA concentration spectrophotometrically. Adsorption of RNA on PBA was weak enough under some experimental conditions that no wash was performed before adding elution buffer. The RNA present in 150 μl of liquid carried over from the first supernatant was accounted for in calculating the quantity of adsorbed RNA from the absorbance of the second (post-elution) supernatant. The bound RNA was then eluted from the beads by adding 2 ml of 500 m*M* Tris–HCl, pH 8.5 as a competitive eluent to each tube, and equilibrating all the tubes in the rotator for at least 10 h (results were time-invariant after 5 h). The tubes were then centrifuged and the previously-bound RNA determined by absorbance. Explicit mass balances calculated for each point from the amounts of RNA added and recovered closed with an average of less than 10–20% of the RNA unaccounted for. It was verified that RNA did not adsorb nonspecifically to an appreciable extent to the microcentrifuge tubes or the pipette tips used, based on the insignificant decrease in absorbance at 260 nm of dilute solutions after extended exposure to large surface areas. All experiments were performed in triplicate, and were conducted within an enclosure maintained at the experimental temperature, with all buffers pre-equilibrated within the enclosure for at least 12 h.

3. Results

To allow mass recoveries to be calculated for each

adsorption experiment, complete elution of the adsorbed RNA was required. A variety of compounds were tested as potential eluents based on observations in the literature. Acetate buffer at pH 5.0 (50 m*M*) eluted approximately half the adsorbed RNA. EDTA (100 m*M*) was effective at either pH 6.0 or pH 9.0, but produced high background absorbance at 260 nm. The competitive eluents sorbitol, mannitol (each at 100 m*M* in 50 m*M* glycine with 50 m*M* $MgCl_2$, pH 9.0) and Tris (at 100 or 500 m*M*, and at pH 6.0 or 8.5) were also effective, giving recoveries indistinguishable from 100%. We chose 500 m*M* Tris–HCl, pH 8.5 for routine use in recovering adsorbed RNA since pH is not well controlled at pH 6.0 with Tris.

It is a standard practice to include Mg^{2+} in mobile phases used for boronate affinity separation of RNA, putatively for screening of electrostatic repulsion between the negatively charged phenylboronic acid ligand and the RNA backbone phosphates. Batch adsorption studies of RNA on PBA-60 were used to identify the optimum concentration of Mg^{2+} (as $MgCl_2$). RNA adsorption on PBA-60 was found to increase with increasing $MgCl_2$ concentration; the enhancement saturates above 100 m*M* (results not shown), and this concentration was chosen for further studies.

Adsorption isotherms were measured for RNA on PBA-60 in the presence of a variety of divalent cations at 100 m*M* (Fig. 1). Adsorption affinity and capacity are markedly increased by use of divalent cations other than magnesium, notably barium. NaCl is ineffective in promoting adsorption even at high ionic strength (1000 m*M*), suggesting that cation promotion of RNA adsorption does not depend solely on the screening of electrostatic repulsion.

To test whether adsorption enhancement by divalent cations is mediated by the boronate–diol interaction alone, we measured adsorption isotherms for adenosine and AMP, which possesses a potentially interactive vicinal diol. In contrast to RNA, AMP and adenosine adsorb better in the presence of Mg^{2+} than of Ba^{2+} (Fig. 2). Taken with the results for RNA, this reversal is inconsistent with a simple electrostatic screening mechanism for enhancement by divalent cations. To test the role of *cis*-diols in divalent cation-promoted adsorption of RNA on PBA-60, as well as to examine the practical utility of Ba^{2+}-promoted adsorption in RNA/DNA separations, the adsorption of double-stranded DNA frag-

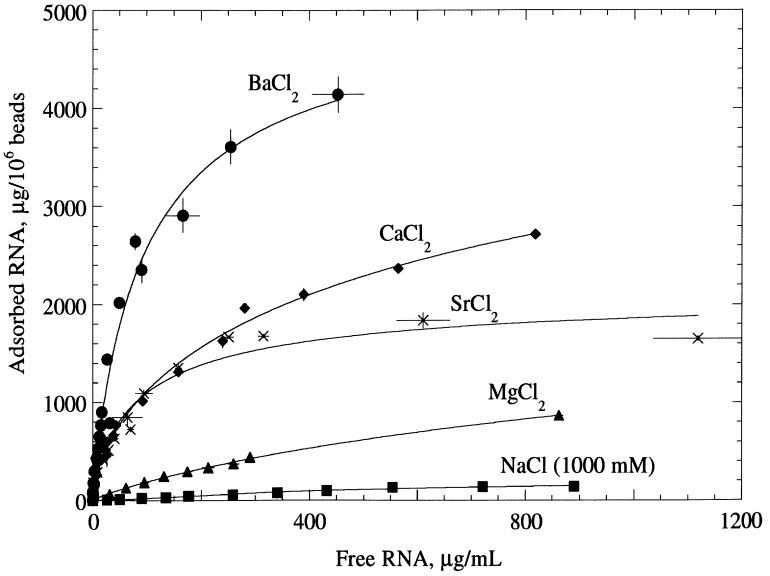

Fig. 1. Batch adsorption isotherms of RNA on PBA-60 at 4°C. Effect on RNA adsorption of 1000 m*M* NaCl or 100 m*M* divalent cation (chloride salt) in 50 m*M* glycine, pH 9.0.

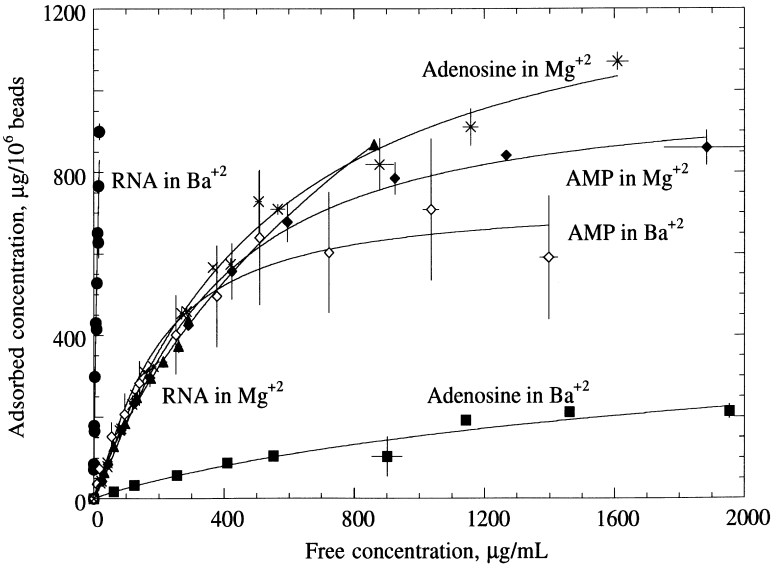

Fig. 2. Batch adsorption isotherms of RNA, AMP and Adenosine on PBA-60 at 4°C. Effect of Ba^{2+} and Mg^{2+} on RNA, adenosine and AMP adsorption. Binding buffer: 50 m*M* glycine, pH 9.0 and 100 m*M* $BaCl_2$ or $MgCl_2$.

ments resulting from *Rsa*I digestion of Salmon sperm genomic DNA (mean length ~400 base pairs) was examined. While RNA is favored in media containing either Ba^{2+} and Mg^{2+}, the adsorption of DNA (which lacks the *cis*-diol important for boronate interaction) is also enhanced in the presence of Ba^{2+} (Fig. 3).

The present work employed mixed RNA species from yeast, in part because of the unavailability of homogenous RNA in sufficient quantities, and in part

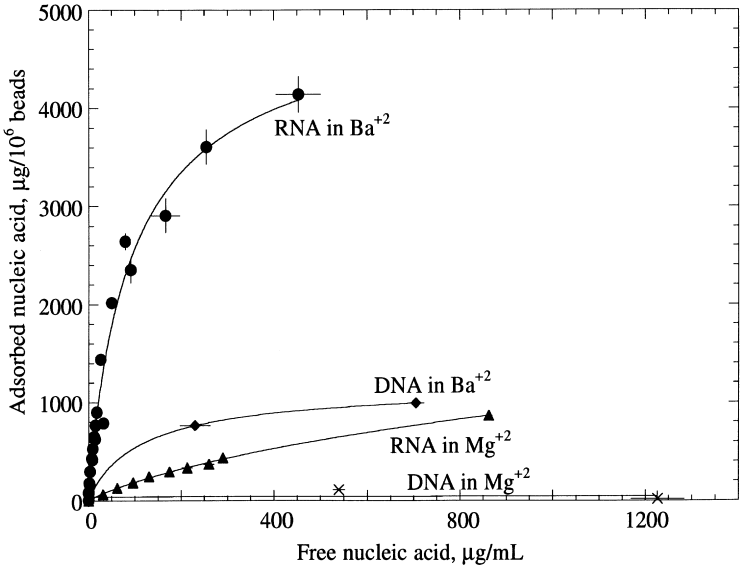

Fig. 3. Batch adsorption isotherms of RNA and DNA on PBA-60 at 4°C. Effect of Ba^{2+} and Mg^{2+} on RNA and DNA adsorption. Binding buffer: 50 m*M* glycine, pH 9.0 and 100 m*M* $BaCl_2$ or $MgCl_2$.

because boronate media often are used to separate mixed RNA from cell extracts. To determine whether specific components of the RNA mixture were selectively adsorbed, agarose-gel electrophoresis was used to analyze RNA adsorbed and eluted from the beads. No systematic fractionation by the adsorbent was observed (results not shown). In the presence of both Ba^{2+} and Mg^{2+} buffers, both the eluted and the unbound RNA gave electrophoretic band patterns generally similar to that of the original sample. While the degree of resolution of the complex samples was poor, there was a slight tendency for the highest molecular mass species not to be adsorbed (results not shown). As each RNA molecule has only a single free 2′,3′ diol, independent of its length, the ''specific binding energy'' (on a mass basis) may be higher for shorter molecules, which may also experience less steric hindrance and easier mass transport.

To test the importance of the boronate ligand in mediating adsorption, experiments were performed with phenylboronate agarose (PBA) matrices of varying boronate ligand density. RNA adsorption increases with increase in boronate loading (Fig. 4), in a similar way in both Ba^{2+} and Mg^{2+} buffers, as boron loading varies from 10–15 μ*M* boron/ml gel (PBA-10), through 30–50 μ*M* boron/ml gel (PBA-30), to 60–100 μ*M* boron/ml gel (PBA-60). This suggests that the boronate ligand mediates adsorption, i.e., that RNA adsorption in Ba^{2+} or Mg^{2+} is due primarily to specific interactions.

RNA adsorption isotherms were measured in the presence of Mg^{2+} and Ba^{2+} at a variety of temperatures (Figs. 5 and 6). The affinity of adsorption (judged by initial slopes, and by Hill equation correlation of these data; [26]) is higher at lower temperatures, implying an exothermic adsorption process. The isotherms for a given ion exhibit a rather abrupt transition, which occurs over a relatively narrow range of temperature. For Ba^{2+} buffer this transition is at about 4°C; for Mg^{2+} buffer it is at about 25°C. The ion-dependence of this transition temperature further supports a structural mechanism for the adsorption-promoting effect of divalent cations, and its occurrence might also be exploited for temperature-swing elution of RNA from boronate adsorbents.

Isotherms were measured for adsorption of RNA to PBA-60 in both Ba^{2+} and Mg^{2+} buffers, in the presence of varying concentrations of the competitive eluent ribose (Figs. 7 and 8). These competitive adsorption data were analyzed according to a stoi-

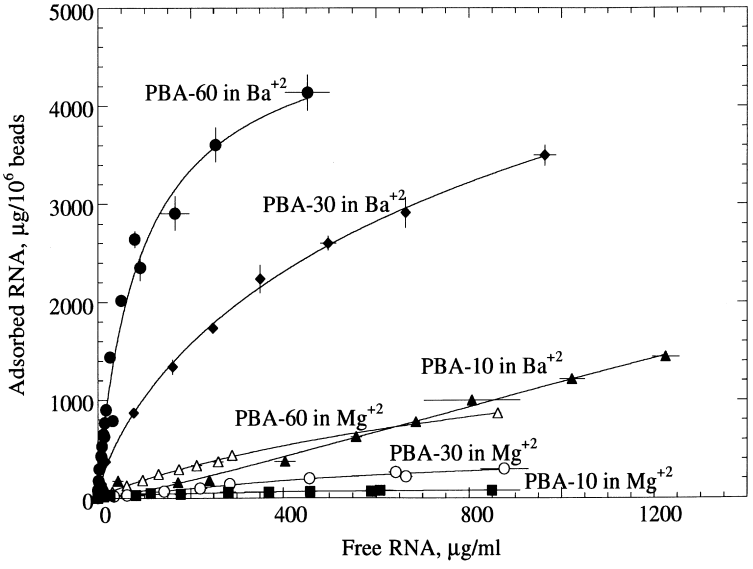

Fig. 4. Batch adsorption isotherms of RNA on PBA-60, PBA-30 and PBA-10 at 4°C. Effect of phenyl boronate loading on RNA adsorption in Ba^{2+} and Mg^{2+} buffers. Binding buffer: 50 m*M* glycine, pH 9.0 and 100 m*M* $BaCl_2$ or $MgCl_2$.

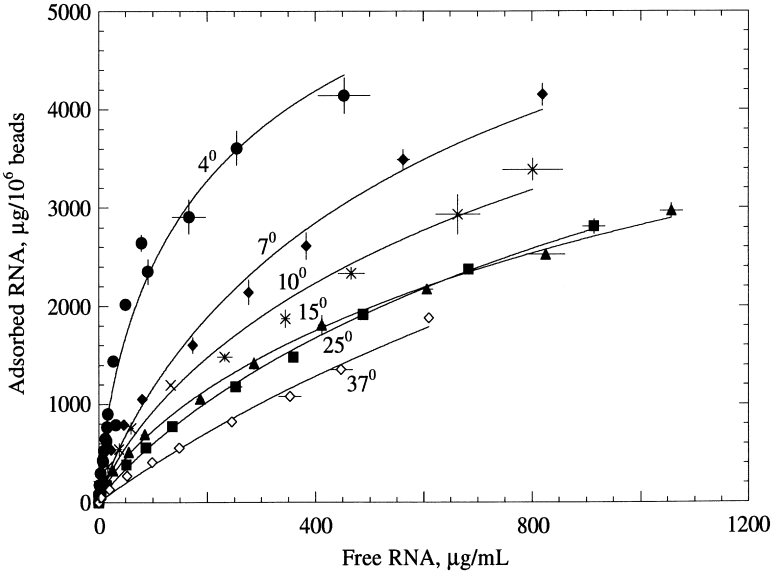

Fig. 5. Batch adsorption isotherms of RNA on PBA-60. Effect of temperature (°C) on RNA adsorption in Ba^{2+} buffer. Binding buffer: 50 m*M* glycine, 100 m*M* $BaCl_2$, pH 9.0.

chiometric displacement-type model as developed by Boardman and Partridge [27] and Kopaciewicz et al. [28]. Making the assumption that each RNA adsorbed displaces a definite number, *Z*, of ribose molecules from the boronate adsorbent surface, and that ribose does not interact with RNA in solution (this eliminates the factor of two which applies in ion-exchange interactions in which the 1:1 displacing

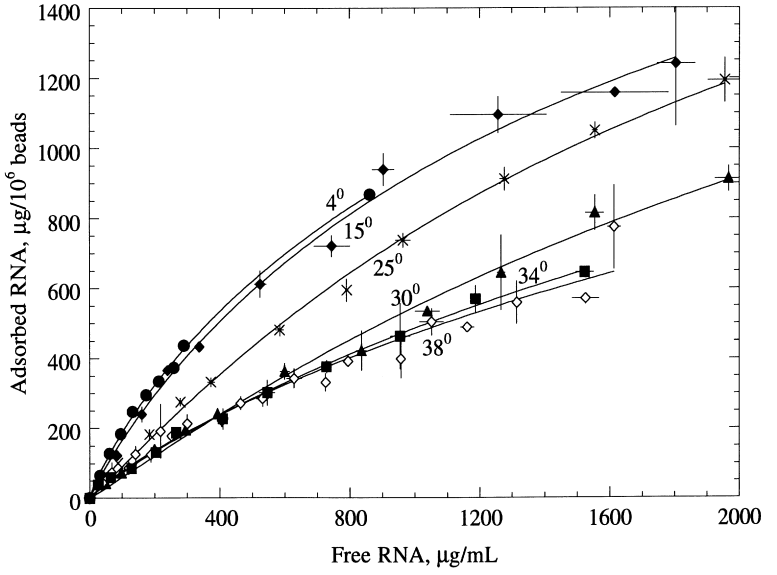

Fig. 6. Batch adsorption isotherms of RNA on PBA-60. Effect of temperature (°C) on RNA adsorption in Mg^{2+} buffer. Binding buffer: 50 m*M* glycine, 100 m*M* $MgCl_2$, pH 9.0.

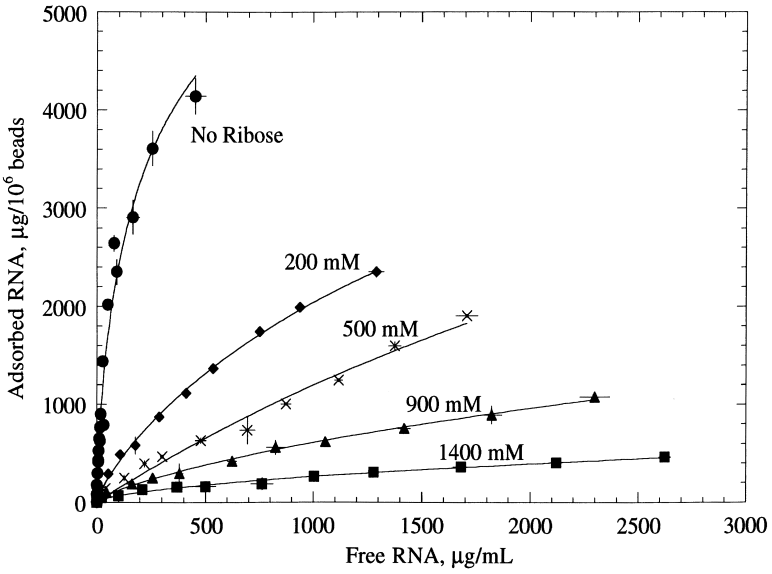

Fig. 7. Batch adsorption isotherms of RNA on PBA-60 at 4°C. Effect of ribose concentration on RNA adsorption in Ba^{2+} buffer. Binding buffers: varying concentrations of ribose in 50 m*M* glycine, 100 m*M* $BaCl_2$, pH 9.0.

ion is assumed also to interact with the protein in solution; [23,25,28]), we can formulate a mass-action equilibrium analysis. The relative affinity of adsorption at a given ribose concentration is taken as the initial slope of the isotherm, measured over the first 3–5 points for Ba^{2+}, and the first 8–10 for Mg^{2+} isotherms, which were less curved (calculated values of *Z* are relatively insensitive to the number

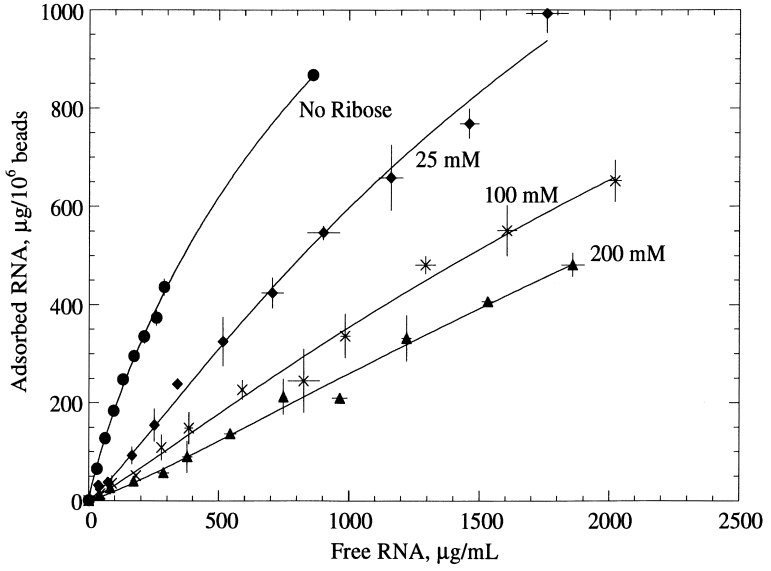

Fig. 8. Batch adsorption isotherms of RNA on PBA-60 at 4°C. Effect of ribose concentration on RNA adsorption in Mg^{2+} buffer. Binding buffers: varying concentrations of ribose in 50 m*M* glycine, 100 m*M* $MgCl_2$, pH 9.0.

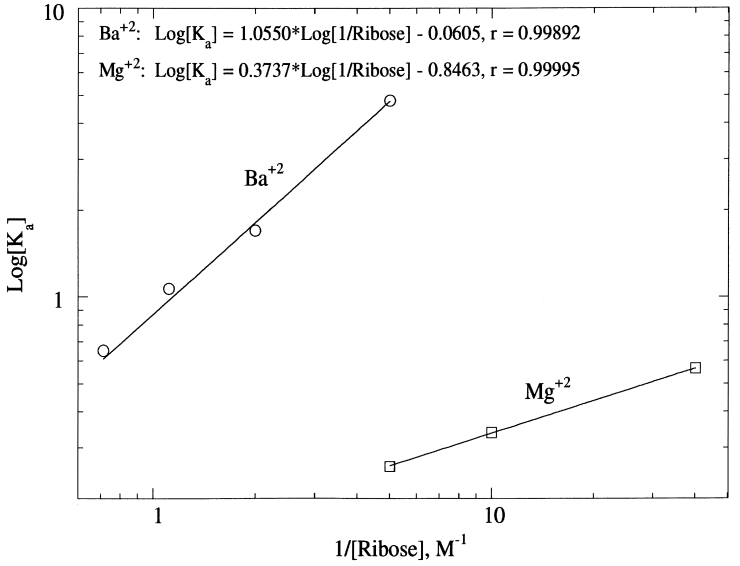

Fig. 9. Calculation of stoichiometric displacement parameter, Z, from log–log plot of adsorption affinity (K_a) vs. reciprocal of ribose concentration. K_a values were obtained from the initial slopes of the isotherms in Figs. 7 and 8.

of points used). Values of the displacement parameter Z are derived from a linear least-squares fit of the affinity of adsorption K_a as a function of ribose concentration, as shown in Fig. 9. While each set of data is well-fit by the equation, the values of Z are 1.08 for Ba^{2+} and only 0.374 for Mg^{2+}, i.e., about one-third the value for Ba^{2+}.

4. Discussion

We have presented several lines of evidence which suggest that enhancement by divalent cations of RNA adsorption on boronate matrices involves mechanisms other than simple electrostatic screening. First, NaCl is ineffective in promoting adsorption even at higher ionic strengths than those at which $BaCl_2$ and $MgCl_2$ are effective. Second, the adsorption-enhancing effect of Ba^{2+} is not observed with AMP, which possesses a negatively charged phosphate as well as a potentially interactive vicinal diol, but lacks the complex structure of a polymeric nucleic acid molecule. Third, adsorption of diol-free DNA is also enhanced in the presence of Ba^{2+} (though RNA is still favored).

In addition, RNA adsorption isotherms exhibit an abrupt transition with increasing temperature, and this transition occurs at different temperature ranges in the presence of Mg^{2+} (about 4°C) and Ba^{2+} (about 25°C). The existence of this transition, and its dependence on ionic environment, is consistent with structural modulation of adsorption. The difference in apparent number of ribose molecules displaced upon RNA adsorption in Ba^{2+} and in Mg^{2+} buffers is also consistent with an RNA-structural explanation.

An alternative explanation, potential cation-induced changes in the structure of the adsorbent matrix, deserves consideration. While such a change could occur through shielding of electrostatic repulsion among boronate ligands on the matrix, this mechanism would apply equally well to NaCl, which was found ineffective. A specific boronate–cation interaction is also possible, but this would be expected to alter the adsorption of the small diol-bearing molecules adenosine and AMP, and this effect was not observed. In other work, a slight difference in RNA melting behavior in the presence of Mg^{2+} and of Ba^{2+} was observed by temperature-programmed heating spectrophotometry, though these results must be regarded as preliminary because of the complex nature of the samples and

thermal transitions involved [26]. Changes in the conformation of tRNA molecules have been observed in the presence of cations such as NH_4^+ [29], Eu^{+3} [30], and Mg^{2+} [31–35], as reviewed by Draper [35]. Thus, a cation influence on the conformation of RNA molecules is not unexpected, and we believe that conformational changes in RNA mediate the influence of divalent cations on boronate affinity adsorption of RNA.

Acknowledgements

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