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Quantifying the ion atmosphere of unfolded, single-stranded nucleic acids using equilibrium dialysis and single-molecule methods

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ABSTRACT

To form secondary structure, nucleic acids (NAs) must overcome electrostatic strand–strand repulsion, which is moderated by the surrounding atmosphere of screening ions. The free energy of NA folding therefore depends on the interactions of this ion atmosphere with both the folded and unfolded states. We quantify such interactions using the preferential ion interaction coefficient or ion excess: the number of ions present near the NA in excess of the bulk concentration. The ion excess of the folded, double-helical state has been extensively studied; however, much less is known about the salt-dependent ion excess of the unfolded, single-stranded state. We measure this quantity using three complementary approaches: a direct approach of Donnan equilibrium dialysis read out by atomic emission spectroscopy and two indirect approaches involving either single-molecule force spectroscopy or existing thermal denaturation data. The results of these three approaches, each involving an independent experimental technique, are in good agreement. Even though the single-stranded NAs are flexible polymers that are expected to adopt random-coil configurations, we find that their ion atmosphere is quantitatively described by rod-like models that neglect large-scale conformational freedom, an effect that we explain in terms of the competition between the relevant structural and electrostatic length scales.

INTRODUCTION

The formation of secondary structure through Watson–Crick base pairing is essential to the structure and function of RNA and DNA in both biology (e.g. RNA ribozymes (1) and riboswitches (2)) and biotechnology (e.g. DNA origami (3)). Both species of nucleic acid (NA) are strongly negatively charged, and so in forming double-stranded helices they must overcome significant electrostatic (ES) repulsion. This is accomplished through screening of the ES potential by counterions specifically bound to the NA structure (as in the case of chelated divalent ions) or loosely associated in an ion atmosphere (4). To fully account for the ES free energy of secondary structure formation, we require a quantitative description of the association of ions with both the folded, helical state and the unfolded, single-stranded state.

A number of studies have probed the stoichiometry of ion association with double-stranded DNA (5–10). For salt species with high mean ion activity coefficients (e.g. NaCl), these results can be modelled by solutions to the non-linear Poisson–Boltzmann (NLPB) equation (10). Much less is known about the interaction of ions with unfolded, single-stranded NAs (ssNAs): some early papers (11–13) have reported changes in counterion activity as a DNA helix is thermally or chemically denatured and, more recently, anomalous small-angle X-ray scattering (ASAXS) results have quantified the ion atmosphere of a single-stranded DNA oligonucleotide at one particular concentration of RbCl salt (14).

Here, we rigorously measure the stoichiometry of ion association with single-stranded DNA and RNA using three independent, complementary techniques: the first a direct approach of Donnan equilibrium dialysis experiment, the other two indirect thermodynamic cycle analyses using the known double-stranded results in combination with either single-molecule magnetic tweezer experiments or existing thermal denaturation data. The salt-dependent results that we obtain by all three methods are in good agreement. We then identify the relevant physical parameters underlying the observed behaviour by comparing the results to models of the ion atmosphere incorporating varying amounts of structural and conformational detail.

Quantifying the ion atmosphere

The various NA conformational states are related through the thermodynamic cycle depicted in Figure 1, where the

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The Na\textsuperscript{+} ion excess per nucleotide, $\bar{\Gamma}_c$, is determined from the molar concentrations of sodium in the NA solution, $c_{\text{Na}}$, and in the bulk, $c_{\text{bulk}}$, and from the concentration of nucleotides, $c_p$:

$$\bar{\Gamma}_c^\text{molar} = \frac{c_{\text{Na}}}{c_p} - \frac{c_{\text{bulk}}}{c_p}. \quad (7)$$

The various concentrations were measured using inductively coupled plasma atomic emission spectroscopy (AES) (iCAP 6300, Thermo Scientific) calibrated using standard solutions of sodium and phosphorus (Fluka). The details

Figure 1. Thermodynamic cycle diagram for mechanical unfolding of an NA hairpin. The helix state refers to the intact hairpin, the stretch state to the hairpin after being mechanically unfolded by an applied force and the coil state to the unfolded hairpin in the absence of force. Each state is characterized by a salt-dependent preferential cation interaction coefficient (ion excess), $\Gamma_i$, and each transition is associated with a change in ion excess, $\Delta \Gamma_{i,j}$.

helix state ($\Gamma_C$)

coil state ($\Gamma_{C}$)

stretch state ($\Gamma_{S}$)

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of the calibration are given in the Supplementary Data. To begin with a thermodynamic identity connecting changes in the relevant free energy for magnetic tweezers experiments, the grand canonical (Landau) potential of mean extension, \( d\Omega \), and changes in the experimental parameters \( X \) (molecular extension), \( f \) (applied force), \( \Gamma \), (excess of a particular species of ion) and \( \mu \), (chemical potential of that ion in bulk solution): 

\[
d\Omega = -Xdf - \sum_{\text{species}} \Gamma_i d\mu_i. \tag{5}
\]

In an experiment, \( f \) and \( \mu \) are directly controlled and \( X \) is measured; therefore, Equation (5) can be used to derive several relations to extract \( \Gamma \), the change in ion excess between conformational states, from the experimental data (34). In this paper, we report results using one of these methods, a generalized Clausius–Clapeyron relation, although we show in Supplementary Figure S6 that the results of the other methods are in agreement. This Clausius–Clapeyron relation is given by

\[
\Delta\Gamma = \frac{\Delta X}{2k_B T}\left(\frac{\partial f_c}{\partial \ln c}\right) \tag{6}
\]

where \( \Delta X \) is the change in extension between the folded and unfolded states, \( f_c \) is the equilibrium unfolding force of the hairpin, \( c \) is the bulk molar salt concentration and \( \alpha \) is the concentration-dependent activity correction factor tabulated in the supporting information of Dittmore et al. (33).

The salt-dependent values of \( f_c \) are extracted from two-state folding/unfolding trajectories as shown in Figure 2B. For each trace, collected at a particular \( c \) and constant \( f \), a histogram is made and the equilibrium constant calculated as a ratio of occupancy times: \( K = T_{\text{folded}} / T_{\text{unfolded}} \). For each salt concentration, \( K \) is plotted versus \( f \) and fit with a two-state Boltzmann model to obtain \( f_c \). The derivative \( (\partial f_c / \partial \ln c) \) in Equation (6) is evaluated separately for the \( f_c \) data of each trial, yielding \( \Delta\Gamma_{H-S} \).

RESULTS

Coil ion excess measured by D-AES

We measured the ion excess of the unfolded coil state, \( \Gamma_c^{\text{molar}} \), directly using dialysis and atomic emission spectroscopy (D-AES). This method relies on the phenomenon of Gibbs–Donnan equilibrium (38), in which the NA solution is placed in grand canonical equilibrium, across a membrane, with a salt reservoir. Attractive interactions with cations, repulsive interactions with anions and EV effects all lead to perturbations in the molar ion concentrations in the sample compartment compared with the reservoir. These perturbations, which we measure by AES, yield \( \Gamma_c^{\text{molar}} \) through Equation (4). In these experiments we used 50 nucleotide NA homopolymers that do not exhibit appreciable secondary structure formation or base stacking (24, 25). D-AES results for \( \Gamma_c^{\text{molar}} \) of both dT\(_{50}\) DNA and rU\(_{50}\) RNA are shown in Figure 3. There is known to be a length dependence to the NA ion excess (23, 39); as such, the results we present can only be extrapolated to NAs of other lengths through appropriate correction.
Figure 2. Single-molecule magnetic tweezers experiment to measure the change in ion excess, \( \Delta \Gamma_{H-S} \), when an RNA hairpin is mechanically unfolded. (A) Diagram of the molecular construct. (B) Sample trace showing hopping between two conformational states, corresponding to two molecular extensions, \( X \), and extraction of the corresponding equilibrium constant, \( K \). The unfolding force, \( f_c \), is then obtained from a plot of \( \ln K \) versus applied force. (C) Plot of \( f_c \) as a function of bulk NaCl concentration for the RNA hairpin. (D) \( \Delta \Gamma_{H-S} \) for an RNA hairpin (open circles) and previously reported (33) values for a DNA hairpin (open circles) using the generalized Clausius–Clapeyron method. Error bars reflect standard errors of the mean.

Figure 3. Values of \( \bar{\Gamma}_{i,n}^{\text{molar}} \) (i.e. including both ES and EV effects) for (A) DNA and (B) RNA, determined directly by D-AES (solid points) and indirectly by thermodynamic cycle analysis using NLPB calculations and either single-molecule experiments (33,35,42) (open diamonds) or melting experiments (49) (line/shaded region). Also plotted is the DNA ASAXS result of Gebala et al. (10) in RbCl (star).

Figure 4. Structural model of dsDNA (A) and naive model of ssDNA (B) used in NLPB calculations, each having 50 nucleotides. Equivalent models for RNA were also used. (C) Ion excess of dsDNA (thick solid line) and dsRNA (thin solid line) in molar units, calculated by numerical evaluation of the NLPB equation as a function of bulk salt concentration. The use of NLPB to estimate \( \Gamma_H \) is validated by comparison with the experimental results of Gebala et al. (10) (squares). Similar NLPB results for models of the ssRNA structure neglecting any conformational freedom are plotted for DNA (thick dashed line) and RNA (thin dashed line).

NLPB calculations

We obtain \( \Gamma_H \), the ion excess of the folded, helical state, using numerical solutions to the NLPB equation on model A-form (RNA) and B-form (DNA, Figure 4A) double helices under various bulk salt concentrations. In using the
canonical double-helix geometries, we assume that the helices do not undergo significant conformational fluctuations over time or significant conformational changes between salt concentrations. Molecular dynamics simulations of dsDNA, which show no clear trend in various structural parameters with salt (40), support this assumption. In using the Poisson–Boltzmann equation, we assume that ion–ion effects are sufficiently weak that the ion density can be treated as a continuous mean field, which is well established in the case of monovalent ions (41). Agreement with experimental data further validates both assumptions. Figure 2C shows the results of our NLPB calculations, in molar units, for dsDNA and dsRNA as solid lines; co-plotted are the results of DNA buffer equilibration/AES studies that show reasonable agreement with the NLPB (10). That there is some disagreement, especially at high-salt, could arise from the non-ideal solution behaviour of NaCl; Gebala et al. have shown that NLPB calculations best reproduce experimental ion counting data for ion species with the highest mean ion activity coefficients (10). We use the average extent of this disagreement as an estimate of the NLPB error.

The dashed lines in Figure 4C show results of similar NLPB calculations for simple models of the ssNAS (i.e. the dsDNA models with one strand removed, Figure 4B). We do not expect such models to truly capture the physics of the single-stranded system, as the flexible ssNAS exist in a conformational ensemble that is unsampled by these models. Shkel and Record have successfully used such modelling to explain some ssDNA properties (31). These naive models give a handle on certain local aspects of the problem: the effects of close co-localization of strands and of the charge spacing along the backbone. We see the first effect by comparing the dsDNA and ssNA curves in Figure 4C: for a particular species and at a particular salt concentration, the ion excess of the ssNA is slightly lower than that of the dsDNA (3–6% for DNA, 3–10% for RNA). This reduction is expected based on the reduced charge density of the single-stranded state, which puts the system closer to the Debye–Hückel limit. We see the second effect, that of the charge spacing along the backbone, by comparing the DNA and RNA curves. Because DNA has a greater charge spacing along the sugar-phosphate backbone than RNA (0.70 nm versus 0.59 nm) (19), it has a smaller local charge density and is thus also closer to this limit.

Single-molecule measurement of \( \Delta \Gamma_{H-S} \)

We use single-molecule magnetic tweezers experiments (32) to measure the mechanical force needed to unfold a RNA helix as a function of bulk salt concentration. The experimental geometry is sketched in Figure 2A and the method used to determine the equilibrium unfolding force, \( f_e \), is illustrated in Figure 2B. We then apply a thermodynamic relation (34) to these \( f_e(c) \) data to extract the change in ion excess associated with this unfolding, \( \Delta \Gamma_{H-S} \).

Figure 2C plots the equilibrium unfolding force, \( f_e \), for the RNA helix. We see that \( f_e \) increases linearly with \( \ln c \) over low-to-moderate salt concentrations and flattens off as \( c \) approaches 1 M. Equation (6) is applied to these data to obtain \( \Delta \Gamma_{H-S} \), which is non-constant across most of the salt range studied (Figure 2D); similar results for DNA (33) are co-plotted. In both cases ions are released when the NA helix unfolds and, at sufficiently high salt, the number released decreases with increasing \( \ln c \). This is consistent with a screening length argument. At high salt the Debye length becomes short enough that all charges on the macromolecule are electrostatically isolated; thus, the differences in charge arrangement between the helix and stretch states become unimportant and the difference in ion excess between the states, \( \Delta \Gamma_{H-S} \), goes to zero.

Completing the thermodynamic cycle

Starting from the \( \Gamma_H \) results of Figure 4C, an indirect measurement of \( \Gamma_C \) can be reached using either of two routes through the thermodynamic scheme of Figure 1: via single-molecule stretching experiments, in which

\[
\Gamma_C = \Gamma_H + \Delta \Gamma_{H-S} + \Delta \Gamma_{S-C},
\]

or via oligonucleotide melting studies, in which

\[
\Gamma_C = \Gamma_H + \Delta \Gamma_{H-c}.
\]

Results can be obtained in either molar or molal units by using \( \Gamma_H \) values integrated in the appropriate way from the NLPB calculations.

Evaluation of the mechanical stretching method (Equation (7)) requires \( \Gamma_H \) and \( \Delta \Gamma_{H-S} \) obtained as discussed above, and also \( \Delta \Gamma_{S-C} \), the change in the ion excess as the stretched state is relaxed to a random coil without refolding. Prior studies have reported \( \Delta \Gamma_{S-C} \) data for homopolymers of DNA (35) and RNA (42) that do not form secondary structure. These homopolymers were on the order of 1000 nucleotides in length, and may exhibit length-dependent effects when compared with the 50-mers we use in the present studies. An exact correction for such effects is not known to us, but would have only a minor effect: \( \Delta \Gamma_{S-C} \) contributes only a few percent to the value of \( \Gamma_C \). Also, whereas the previously reported \( \Delta \Gamma_{S-C} \) values were obtained by fitting the data with a surface having a particular functional form, analyzing the data in a way that does not depend on the choice of model function leads us to conclude that, within uncertainty, \( \Delta \Gamma_{S-C} \) is salt-independent. As such, we arrive at per-nucleotide \( \bar{\Delta} \Gamma_{S-C} \) values of 0.027 ± 0.003 for RNA and 0.043 ± 0.005 for DNA. Using these results, we can then evaluate Equation (7) to obtain the first indirect evaluation of \( \Gamma_C \), which is plotted in molar units alongside the D-AES results in Figure 3.

Alternatively, we can complete the thermodynamic cycle using Equation (8), which relies on the NLPB results for \( \Gamma_H \) and also on \( \Delta \Gamma_{H-c} \) data from oligonucleotide melting experiments (43–48). We are unaware of suitable data for RNA melting, so we confine our analysis by this method to DNA. Oweczarzy et al. (49) have reported melting temperatures for DNA oligonucleotides as a function of length, base composition and salt concentration. We analysed all of the 25 base pair data in their dataset using

\[
\Delta \Gamma_{H-c} = \frac{1}{2a\beta} \frac{N - 2}{N} \frac{dT_m}{d\ln c},
\]

which follows from Olmsted et al. (23), where \( T_m \) is the duplex melting temperature, \( N \) is the number of base pairs.
and $\beta = \frac{RT^2_m}{\Delta H^o}$ is a compound constant measured by calorimetry of NA conformational transitions and having a value of $\beta = 55 \pm 10\%$ (50). Performing this analysis, the details of which are given in the Supplementary Data, gives $\Delta T_{\text{H-C}}$ over [NaCl] = 69-1025 mM. We extrapolate to lower salt concentrations by assuming constant $\Delta T_{\text{H-C}}$, an assumption that is supported by other melting experiments (50). Finally, we evaluate Equation (8) to obtain the second indirect measure of $\Gamma_C$, which is also plotted, in molal units, in Figure 3A.

**DISCUSSION**

Despite being based on different experimental techniques, the various methods discussed above agree on a consistent trend in the salt dependence of the ion excess of random-coil, single-stranded DNA and RNA (Figure 3). The linear decrease in molal ion excess at high salt (see plot with linear axes, Supplementary Figure S8) is due to EV effects; ES effects, which are of greatest interest to understanding NA–ion interactions, manifest as perturbations to this behaviour. We can isolate the ES effects by correcting the data into molal units. This is done, for the thermodynamic cycle measurements, by using the molal values of $\Gamma_H$ from the NLPB and, for the D-AES measurements, by adding an EV correction. This correction is obtained by multiplying the EV of the NA—estimated from the structural model of Figure 4B incorporating a Stern layer—by the bulk salt concentration. Such EV-corrected, molal data are plotted in Figure 5, where we now see that the data lie, roughly, between the expected low-salt Manning ($\bar{\Gamma}_C \rightarrow 0.75–0.79$, assuming thin-cylinder geometry) and high-salt Debye–Hückel ($\bar{\Gamma}_C \rightarrow 0.5$) limits. Below, we explore electrostatics models to quantitatively reproduce the $\bar{\Gamma}_C(c)$ curves between these limits.

**Uniform mean-field model**

Because the ssNAs are so flexible and exist in an ensemble of random-coil conformations, we hypothesized that the chain would act as a uniform charge distribution, with the charge density given by the average over all configurations. In a simple model of this picture, we treat the system as consisting of two compartments in Gibbs–Donnan equilibrium with each other: one a bulk reservoir and the other a region of uniform charge density, $\rho$. This charged region represents the NA coil, ignoring any effects that may arise from the shape of the NA or the discreteness of its charge. Following the derivation given in the Supplementary Data, we solve for the per-nucleotide cation excess of this model of the coil state:

$$\bar{\Gamma}_C = 1 \frac{1}{2} - \sqrt{1 + \left(\frac{ce}{\rho}\right)^2} - \frac{ce}{\rho},$$  \hspace{1cm} (10)

where $c$ is the salt concentration in the bulk, $e$ is the fundamental charge and $\rho$ is the absolute value of the uniform volume charge density.

It is possible to connect this model to physical NA coils via the charge density parameter. If we treat the NA coil as a sphere, with charge $Q$ and radius $R$, then $\rho = \frac{Q}{4 \pi R^2}$ or, using the definition of the radius of gyration, $R_g$,

$$\rho = \frac{Q}{4 \pi R_g^2 \pi R^3},$$ \hspace{1cm} (11)

Salt-dependent $R_g$ values for dT$_{50}$ have been measured using small-angle X-ray scattering by Sim et al. (51); we are not aware of similar measurements for RNA. We can insert these $R_g$ values into Equation (11) and, in turn, into Equation (10) to obtain semi-theoretical predictions of $\bar{\Gamma}_C$ based on the uniform mean-field (UMF) model and the experimental $R_g$ values. These predictions are co-plotted with the molal data in Figure 5A.

This simple, UMF model exhibits $\bar{\Gamma}_C \rightarrow 0.5$ high-salt limiting behaviour (Supplementary Figure S10); however, its quantitative agreement with the experimental data is poor. Our hypothesis of dominant conformational flexibility, leading the system to behave as if it is uniformly charged, is thus not borne out. We require an alternative model, including some non-uniformity in the charge distribution, to explain the data.
Non-uniform mean-field models

Because all of our experiments are carried out in the presence of added salt, ES interactions will be appreciable only on length scales shorter than the Debye screening length, \( \kappa^{-1} \). The conformational flexibility that is central to the UMF model occurs only on long length scales compared with \( \kappa^{-1} \). As such, we now hypothesize that it is the short-length-scale (i.e. sub-Debye-length) charge distribution of the system, which is rod-like, that dominates the interactions with the ion atmosphere.

One way to account for the local charge distribution in the ssNA coil is through the single-stranded NLPB calculations of Figure 4C, which are based on a naive structural model (Figure 4B) that does not sample the available conformations of the chain but does account for the discreteness of the source charge and its spacing along the backbone. Because this naive model is based on the canonical double-helical structure, it incorporates some degree of base-stacking, not present in the random-coil dT_{20} or Ru_{50}, that may introduce error into the modelling. Figure 5 compares these NLPB results with the molal DNA and RNA data, showing good agreement across the full range of salt concentration in both cases.

An alternative, structurally simpler model lacking discrete charges is that of Landy et al. (52), in which the NA is treated as a charged rod with radius \( a \). In the low ionic strength, excess salt limit, this model predicts that the cation excess of the negatively charged rod is given by

\[
\Gamma^c \approx \frac{\xi^2}{2\xi^2} \left[ 1 - \left( \frac{K_0(\kappa a)}{K_1(\kappa a)} \right)^2 \right] + \frac{1}{2}, \quad (12)
\]

where \( \xi \) is the Manning parameter (17) (\( \xi = 1 \) for ssDNA, \( \xi = 1.19 \) for ssRNA), \( \kappa^{-1} \) is the Debye length and the \( K_i \) are the modified Bessel functions of the second kind; this equation is valid for \( \xi \geq 1 \). For the ssNAs, the choice of \( a \) is non-obvious. Here, for both RNA and DNA, we set \( a = 0.24 \) nm: the distance of closest approach of Na\(^+\) ions to the backbone phosphates in molecular dynamics simulations of dsRNA (53). As shown in Figure 5, the charged rod model also agrees with the data except at the very lowest salt concentrations.

That both the single-stranded NLPB and charged rod models agree with the EV-corrected \( \Gamma^c_{\text{molal}} \) data indicates that the monovalent ion excess of ssNAs is insensitive to their large-length-scale conformation. Rather, it is the spacing of the charges along the strand that dominates. This effect is seen by comparing the \( \Gamma^c \) results for RNA (0.59 nm spacing along sugar-phosphate backbone) and DNA (0.70 nm spacing) in Figures 3 and 5. That the ion excess is insensitive to the conformation of the NA is consistent with the small magnitude of \( \Delta\Gamma_{S-C} \), the change in ion excess between two states that differ, primarily, in their conformation (i.e. extended versus coiled). It is also consistent with our hypothesis that, due to ES screening on length scales larger than the Debye length, the interactions between the ion atmosphere and the NA are dominated by the rod-like charge distribution on short length scales.

CONCLUSION

We have presented new D-AES measurements of the absolute number of ions associated with unfolded DNA and RNA oligonucleotides, single-molecule measurements of the change in number of ions associated with an RNA hairpin as it is mechanically unfolded and NLPB calculations of the absolute number of ions associated with double-helical and single-stranded DNA and RNA. Using these results, along with existing data on a number of NA systems, we have obtained three different measurements of the monovalent ion atmosphere, \( \Gamma^c \), of unfolded, single-stranded DNA and two different measurements of unfolded RNA. We have also reported measurements of the change in ion excess as an unfolded NA folds to form secondary structure. These \( \Delta\Gamma_{H-C} \) results, which show non-trivial salt dependence (Supplementary Figure S7), contribute to understanding the free energy change associated with secondary structure formation.

We interpret the observed salt-dependent \( \Gamma^c \) behaviour as an interpolation between two limiting regimes: a low-salt limit described by Manning counterion condensation theory and a high-salt, Debye–Hückel limit in which the charge is equally offset by cation association and anion depletion. The detailed behaviour at intermediate salt concentrations depends on the strandedness and charge spacing of the NA, but does not depend appreciably on its large-scale conformation, presumably due to the short-range nature of the screened electrostatics. This indicates that, when considering free energies of ion interactions in the context of NA structure formation, much of the molecular complexity on large length scales can likely be ignored.

SUPPLEMENTARY DATA

Supplementary Data are available at NAR online.

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