Published in final edited form as:

J Mol Biol. 2007 July 27; 370(5): 993–1005.

Effects of osmolytes on RNA secondary and tertiary structure stabilities and RNA-Mg²⁺ ion interactions

Dominic Lambert and David E. Draper*

Department of Chemistry, Johns Hopkins University, Baltimore, MD 21218

Summary

Osmolytes are small organic molecules accumulated by cells in response to osmotic stress. Although their effects on protein stability have been studied, there has been no systematic documentation of their influence on RNA. In this work, the effects of nine osmolytes on the secondary and tertiary structure stabilities of six RNA structures of differing complexity and stability have been surveyed. Using thermal melting analysis, *m*-values (change in ΔG° of RNA folding per molal concentration of osmolyte) have been measured. All the osmolytes destabilize RNA secondary structure, although to different extents, probably because they favor solubilization of base surfaces. Osmolyte effects on tertiary structure, however, can be either stabilizing or destabilizing. We hypothesize that the stabilizing osmolytes have unfavorable interactions with the RNA backbone, which becomes less accessible to solvent in most tertiary structures. Finally, it was found that as a larger fraction of the negative charge of an RNA tertiary structure is neutralized by hydrated Mg²⁺, the RNA becomes less responsive to stabilizing osmolytes and may even be destabilized. The natural selection of osmolytes as protective agents must have been influenced by their effects on the stabilities of functional RNA structures, though in general, the effects of osmolytes on RNA and protein stabilities do not parallel each other. Our results also suggest that some osmolytes can be useful tools for studying intrinsically unstable RNA folds and assessing the mechanisms of Mg²⁺ - induced RNA stabilization.

Keywords

proline; urea; glycerol; trimethylamine oxide; betaine

Introduction

To respond to changes in osmotic pressure, some cells synthesize or take up small organic solutes known as osmolytes. These osmoregulatory compounds have also been shown to help cells cope with other environmental stresses such as high temperature, pressure or desiccation, and to affect the stabilities of proteins and DNA ^{1; 2}. Osmolytes belong to diverse chemical families including methylamines, amino acids, sugars and polyols. Most osmolytes, those referred to as compatible or protective osmolytes, tend to stabilize protein structure, while urea, the principal non-compatible osmolyte, is a denaturant. The concentrations of these molecules are apparently regulated so as to maintain the osmolarity of the cell without perturbing macromolecular functions, for instance by adjusting the ratio of protective and denaturing

*Address correspondence to this author at draper@jhu.edu..

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

osmolytes to maintain protein stability³ or by adjusting the intracellular concentrations of osmolytes and ions to maintain protein – DNA interactions⁴.

Studies of osmolytes have primarily focused on their effects on protein stability and enzyme function^{1; 5}. Structured RNA molecules are also important components of the cell, and any changes to the stabilities of tRNAs, hairpins controlling transcription termination, or riboswitches (to name a few possibilities) could alter gene expression in dramatic ways. In one study, the ratio of denaturing and stabilizing osmolytes (urea and TMAO, respectively) needed to maintain the tertiary structure of a tRNA was found to be about the same as the ratio needed to maintain protein stability⁶. But in general, there is no reason to suppose that RNA and proteins, which are stabilized by very different balances of forces, should respond to osmolytes in the same ways, and there are few data that bear on this question. In this work, we provide a first systematic look at the degree to which RNA structures are sensitive to osmolytes by surveying the effects of nine different osmolytes on five RNAs with increasing structural complexity (Figure 1). A sixth RNA with the same overall structure but different stability as the RNA in Figure 1D has also been included.

Protein – osmolyte interactions have been quantitatively described in thermodynamic terms. The critical factor is the partitioning between water and osmolyte at solvent-exposed surfaces of a protein. While stabilizing osmolytes tend to be excluded from the protein surface, forcing the polypeptide to adopt a compactly folded structure with a minimum of exposed surface area, denaturing osmolytes accumulate at the surface and promote unfolding^{7; 8; 9; 10}. Accumulation or exclusion occurs primarily at polar surfaces, particularly the backbone amides^{9; 11; 12}. Thus, the sensitivity of a protein to osmolytes depends primarily on the degree to which its polar peptide backbone becomes buried upon folding into the native structure and the relative strength of osmolyte *vs.* water interactions with peptide groups¹².

Structured RNAs differ from proteins in two respects that are relevant to the way these molecules interact with osmolytes. First, functional RNAs *in vivo* may be entirely secondary structure or also include tertiary interactions; in contrast, extensive protein secondary structure is usually not observed in the absence of the complete tertiary fold. The extents to which base *vs.* backbone surfaces are buried upon folding RNA secondary or tertiary structures differ considerably. It is thus possible that an osmolyte could have qualitatively different effects on RNAs that rely to different degrees on sets of tertiary interactions (in addition to secondary structure) for function. Second, all RNAs have a high negative charge density and consequently strong interactions with ions¹³, a factor that is usually not as important to protein folding. Osmolytes could therefore affect RNA stability indirectly, by modulating the thermodynamic activity of the ions, the strength of the Coulombic interactions between ions and RNA, and the energetics of ion dehydration.

With these considerations in mind, the survey of osmolyte effects on RNA reported here examines both RNA secondary structure stability and the formation of several different RNA tertiary structures which differ in their solvent accessible surface areas and in their interactions with ions. We find that all osmolytes destabilize RNA secondary structure to some degree, but many osmolytes stabilize tertiary structure. The latter stabilizing influence is, in most cases, strongly attenuated or even reversed by high Mg^{2+} ion concentrations. Besides the relevance of these results to the *in vivo* role of osmolytes, our findings suggest that some osmolytes could be useful tools for RNA folding studies.

Results

Choice of RNAs and osmolytes for study

The five RNA structures chosen for our survey of osmolyte effects on folding transitions are shown in Figure 1. The simple hairpin (Figure 1A) was designed to be a representative secondary structure with both A-U and G-C base pairs. The other RNAs are all naturally-occurring RNAs with tertiary structure, and were selected on the basis of several criteria. First, in order to measure the effect of an osmolyte on the RNA stability, the tertiary folding transition had to be resolvable in the UV melting profile of each RNA. An example profile of each RNA is shown in supplementary Figure S1. Second, by analogy with proteins, we expected that accumulation or exclusion of osmolytes at RNA surfaces would lead to stabilization or denaturation of the molecules. Thus, RNAs showing different proportions of solvent accessible surface area (SASA) in the folded state were chosen (see below). Lastly, RNA tertiary structures tend to be strongly stabilized by Mg^{2+} ions, but different mechanisms may dominate between RNAs¹³. At one extreme, “diffuse” ions, which remain fully hydrated and do not directly contact the RNA, contribute to the stability of all RNA structures^{14; 15; 16}. At the other extreme, “chelated” ions are substantially dehydrated and bind to a specific set of RNA ligands^{13; 17}. Because osmolytes may affect the free energy of ions in various RNA environments differently, RNAs stabilized to different degrees by diffuse and chelated Mg^{2+} ions were included.

We calculated the SASA of each RNA in Figures 1B–E from its atomic coordinates, and divided the exposed surface into contributions from base, sugar, or phosphate. The results (Figure 2) are expressed as SASA per nucleotide; smaller values correspond to an overall more compact structure. For reference, the average exposure of an internal nucleotide in an A-form helix or an A-form single strand are shown. A-form geometry maintains a high degree of stacking between bases and undoubtedly underestimates the solvent exposure of single-stranded nucleotides in unfolded RNAs. To obtain a more realistic estimate of nucleotide exposure in a single strand, we extracted single strand, non-A-form segments of RNA from several crystal structures and averaged the SASA of these nucleotides (see Materials and Methods). Though the component surface areas obtained in this way are much larger than those calculated for nucleotides in A-form conformations, they are still 55–70% of the calculated maximal surface areas of a fully extended polynucleotide¹⁸. Comparing the SASA of single- and double-stranded RNA nucleotides in Figure 2, it is apparent that the major change in SASA upon RNA secondary structure formation is burial of the bases; changes in backbone exposure are relatively minor.

There are distinct differences in SASA among the selected RNA tertiary structures. The 58mer rRNA fragment (Figure 1D) is the most compact structure (smallest total SASA per nucleotide) and has a significantly higher degree of phosphate burial than the other RNAs. Both Mg^{2+} and K^+ ions are found chelated to buried backbone oxygens in this RNA and are energetically important for folding^{17; 19}. Two sequence variants of this RNA were studied, U1061A and GACG; U1061A RNA is more stable²⁰. The Beet Western Yellow Virus (BWYV) pseudoknot (Figure 1C) is the least compact RNA. Mg^{2+} stabilizes the structure, but only as a diffuse ion¹⁶. The tertiary unfolding transition reported here involves only the disruption of loop L2 interactions in the minor groove of helix H1 (Figure 1C)²¹; changes in SASA per nucleotide are probably small compared to the other RNAs described here. Formation of the tar-tar* complex (Figure 1B), by the interaction of two hairpin loops, is accompanied by large change in the SASA of bases and the creation of an unusual tunnel in the major groove²². Finally, the A-riboswitch (Figure 1E) shows the smallest base SASA among the RNAs. Formation of its tertiary structure requires binding of an adenine base derivative. Because the ligand interactions with the RNA depend on base stacking and hydrogen bonding in the same way as

any intramolecular interactions of a base with the rest of an RNA, osmolytes should perturb both ligand binding and overall RNA stability by similar mechanisms.

The osmolytes used in this survey were selected to be representative of different chemical classes of naturally occurring osmolytes: amino acids (proline and betaine), methylamines (betaine and TMAO), and polyols or alcohols (sorbitol, sucrose, glycerol, ethylene glycol, and methanol). Methanol is not a natural osmolyte but was included in this survey because it has been previously observed to stabilize RNA tertiary structure²³.

Approach: UV-thermal melting analysis and *m*-value determination

By monitoring the UV melting profiles of an RNA in the presence of increasing osmolyte concentration, as exemplified by the GACG RNA melting profiles in Figure 3A, we obtain the T_m and ΔH° of each RNA unfolding transition as a function of osmolyte molality (see Materials and Methods for data analysis). Figure 3B shows the behavior of all four unfolding transitions that were fit to the GACG RNA melting profiles, plotted as $1/T_m$ vs. osmolyte molality. In this RNA, the first unfolding transition is disruption of tertiary structure and the last corresponds to the melting of the small helix H2b. Only the osmolyte dependence of the first transition has been compiled for this RNA, because it is not well understood what secondary structures are unfolding in the first and second transitions²⁴, and the last transition is poorly determined in the presence of certain osmolytes. The slopes of these plots are multiplied by an appropriate factor to obtain the free energy change as a function of osmolyte concentration (see Materials and Methods), known as the *m*-value²⁵. In the case of proteins, the folding free energy tends to vary linearly with osmolyte concentration²⁶, a phenomenon that has been attributed to the small magnitude of the equilibrium constant for exchanging osmolyte and water at the protein surface²⁷. A linear free energy dependence is observed for almost all of the osmolyte-RNA combinations reported in this study, though in some cases (marked by an asterisk in figures) the free energy of folding reaches a minimum value by 1 – 2 *m* osmolyte. Such behavior might be expected if an osmolyte binds specific site(s) on an RNA surface much more strongly than does water.

Osmolytes destabilize RNA secondary structures

Besides melting experiments with the designed hairpin, three other hairpin unfolding reactions were resolved as transitions in the melting profiles of the RNAs with tertiary structure; these are the tar and tar* hairpins and the hairpin containing helix H1 of BWYV RNA (Figure 1B and 1C, respectively). With one unusual exception, all of the tested osmolytes destabilized all of these hairpins, though to widely varying degrees (Figure 4A). Surprisingly, proline is the most efficient denaturant of RNA secondary structure, not urea (Figure 4A). The BWYV H1 hairpin consists entirely of G-C base pairs, which could be a factor in its more effective denaturation by proline and betaine relative to the other hairpins. Intriguingly, glycerol greatly stabilizes the BWYV H1 hairpin, while it acts as a mild denaturant on the other RNAs. The stabilizing effect of glycerol is also unusual in reaching a plateau at about 1 *m* (Figure 4B). This “saturation” behavior suggests that glycerol forms unusually strong interactions with the H1 hairpin (see Discussion).

m-values for urea and duplex RNAs have been reported²⁸. The value obtained for a six base pair duplex is about two-thirds larger than we obtain for the six base pair hairpin, after making an estimated correction for the difference between molar and molal units used in the two studies. The discrepancy may arise from the different salt conditions (100 *mM* KCl in our study, vs. 0.5 M NaCl) and experimental methods, which derive *m*-values at much higher urea concentrations than those used here.

Osmolyte effects on RNA tertiary structure can be either stabilizing or destabilizing

We next examined the effects of osmolytes on the unfolding of RNA tertiary structure (Figure 5). These measurements were made in the presence of K^+ as the only stabilizing cation, except for the two 58mer rRNA fragments which require some Mg^{2+} to fold. (The influence of Mg^{2+} on osmolytes is addressed in the following section.) Urea and proline are uniformly destabilizing. The effects of sorbitol, betaine, and sucrose are RNA-dependent, while the remaining alcohols and TMAO tend to be stabilizing, in some cases strongly so. There is considerable variation from RNA to RNA in the effectiveness of an osmolyte. For example, the U1061A and GACG variants of the rRNA fragment, which differ by only four nucleotides in sequence (Figure 1D), differ by nearly a factor of two in sensitivity to methanol and ethylene glycol. Because the tested RNAs differ in solvent exposure of bases and backbone (Figure 2), it is perhaps not surprising that their responses to an osmolyte are variable (see Discussion).

Osmolyte effects on RNA-ion interactions

As pointed out above, RNA tertiary structures tend to be strongly stabilized by Mg^{2+} , even in the presence of a large excess of monovalent cations. Osmolytes could indirectly affect RNA stability by altering the activity or solvation free energy of ions and thus the free energy of either diffuse or chelated ion – RNA interactions. If such indirect effects are negligible, then Mg^{2+} -induced stabilization and osmolyte effects should be additive. To see if this is the case, we compared RNA-osmolyte m -values measured with K^+ as the only cation to measurements made in K^+/Mg^{2+} mixtures (Figure 6). The K^+/Mg^{2+} ratio was adjusted in each case to insure that Mg^{2+} was responsible for substantial stabilization of the RNA. These comparisons could only be done with the tatar*, BWYV, and A-riboswitch RNAs, which have stable structures in the absence of Mg^{2+} .

Urea, sorbitol, and methanol are the least sensitive to the presence of Mg^{2+} with all three RNAs. Among some of the other osmolytes, instances of large, Mg^{2+} -induced increases in m -values are seen (proline, betaine, TMAO). There are fewer cases of a significant Mg^{2+} -induced decrease in m -value (BWYV RNA with ethylene glycol, sucrose). Two of the most stabilizing conditions for BWYV RNA, glycerol in the absence of Mg^{2+} and ethylene glycol in its presence, again show saturation behavior suggesting relatively strong binding of the osmolyte to the folded RNA.

It thus appears that Mg^{2+} and many osmolytes are capable of either synergistic or competitive effects on RNA stability, depending on the RNA and specific conditions. To characterize the interplay between Mg^{2+} and osmolytes further, we measured the stability of several RNAs over wide ranges of Mg^{2+} concentrations, with or without an osmolyte present (Figure 7). Ethylene glycol and TMAO were chosen for study, to see if their opposite effects on BWYV RNA stability extend to other RNAs. The principal finding is that Mg^{2+} strongly attenuates the stabilizing effects of the two osmolytes (compare Figures 7A–C). At high enough concentrations, Mg^{2+} either renders the osmolyte ineffective (Figures 7A and C) or converts it to a somewhat destabilizing osmolyte (Figure 7B). Similar experiments with the A-riboswitch and proline, betaine, or methanol showed similar trends as seen in Figure 7B: the stabilizing effect of Mg^{2+} was attenuated by the osmolyte, and the stabilizing osmolytes (betaine and methanol) became destabilizing above 1 mM Mg^{2+} (data not shown). Some synergism between Mg^{2+} and ethylene glycol with BWYV RNA is observed at very low Mg^{2+} concentrations (Figure 7A). Presumably the diverse results of the measurements in Figure 7 reflect the strong Mg^{2+} -dependence of osmolyte – RNA interactions, which may vary from synergistic with Mg^{2+} to competitive as the Mg^{2+} concentration increases.

Mg^{2+} ions in the presence of BWYV RNA remain hydrated¹⁶, and it is likely that the same is true for the A-riboswitch RNA: Mg^{2+} ions observed in the riboswitch crystal structure have

at least one shell of hydrating water²⁹. To ask whether Mg^{2+} has the same attenuating effect on osmolytes when the RNA is stabilized by both diffuse and chelated ions, we performed similar experiments on the GACG rRNA fragment. As Mg^{2+} concentration is increased, the stabilizing effect of ethylene glycol becomes smaller but is not eliminated (Figure 7D). Of most interest is the fact that the stabilizing effect of TMAO is nearly constant over the entire range of Mg^{2+} concentrations (Figure 7E), the only instance we observed of an osmolyte and Mg^{2+} acting in an additive way to stabilize RNA tertiary structure.

Finally, we note that *m*-values for the secondary structure transitions observed in the melting of the tar-tar* complex and BWYV RNA were unaffected by the exchange of K^+ for the K^+/Mg^{2+} mixture in the experiments reported in Figure 6 (data not shown). This result is consistent with Mg^{2+} having a much more dramatic effect on RNA tertiary structure stability than on secondary structure.

Discussion

Osmolyte effects on RNA stability: general considerations

A framework for considering the effects of osmolytes on the stabilities of RNA structure is proposed in Figure 8. Unfolding of native (N state) RNA structures has been described as occurring in a hierarchical fashion²⁸. Tertiary interactions are disrupted first, leading to the formation of an intermediate (I state) characterized by the presence of only secondary structure. When further destabilized, RNAs in this intermediate state melt into a completely unfolded, single-stranded RNA (U state). This unraveling of a complex RNA tertiary structure is accompanied by a decrease in the negative charge density and a concomitant reduction in the number of excess monovalent and divalent cations needed to neutralize the phosphate negative charges¹³. When RNA unfolding is allowed to proceed in the presence of osmolytes, the small organic solutes have the potential to shift the equilibria between the different folding states (N vs. I and I vs. U) through favorable or unfavorable interactions with one conformation over another. Although the survey of osmolyte effects on RNA folding transitions presented in this work reveal a wide range of effects, we suggest that the majority of these observations can be rationalized in terms of a few basic considerations as to (i) the preferential interactions of nucleic acid bases with an osmolyte compared to water; (ii) the unfavorable interactions of an osmolyte with the ribose-phosphate backbone (i.e., a preference of the backbone for solvation by water), and (iii) the attenuation of osmolyte discrimination between I and N state RNAs by high concentrations of Mg^{2+} . In a few cases, relatively strong interactions of an osmolyte at specific RNA sites may also be an important factor. These themes are considered in more detail in the following sections.

Osmolytes destabilize RNA secondary structure

The I → U unfolding RNA unfolding transition is accompanied by a large increase in the SASA of bases, and relatively little change in ribose or phosphate exposure to solvent (Figure 2). All osmolytes tested destabilize RNA helices (Figure 4), which is consistent with preferential accumulation of osmolyte at the surfaces of bases. To the extent that literature data are available, they tend to confirm the favorable interaction of the osmolytes studied here with bases or nucleosides. Urea, methanol, and ethylene glycol increase the solubility of bases^{30; 31}. Urea and betaine preferentially accumulate around single stranded DNA, and are either excluded from (betaine) or are unaffected by (urea) double strand DNA surfaces^{32; 33}.

Favorable and unfavorable effects of osmolytes on RNA tertiary stability

In contrast to the I → U transition, the I → N RNA folding transition is not dominated by changes in the exposure of one type of surface area (Figure 8): some I state singled stranded regions may become more stacked, helix grooves may be filled by bases, and the development

of a more compact structure tends to bury ribose-phosphate backbone. The I $\rightarrow$ N transition also tends to be more sensitive to the ionic composition of the solvent, especially the presence of Mg^{2+} , than secondary structure unfolding. Thus, the balance between an osmolyte's affinity for bases (destabilizing N), exclusion from backbone (stabilizing N), and synergism or competition with Mg^{2+} , will dictate how an osmolyte affects the folding of a certain RNA. RNAs in which these three factors are weighted differently may respond to the presence of the same osmolyte in disparate ways.

Two of the osmolytes we studied, urea and proline, consistently denature tertiary structure (Figure 5). These osmolytes must either favor backbone exposure as well as base exposure, or the osmolyte preference for exposed base surfaces overwhelms any favorable free energy associated with the burial of backbone.

With the exception of sorbitol, the remaining osmolytes we studied (betaine, polyols, methanol, and TMAO) are effective stabilizers of at least some RNA tertiary structures (Figure 5). Because these same osmolytes tend to destabilize secondary structure, we argue that these compounds favor burial of RNA backbone- that is, favorable osmolyte interactions with base surfaces must be outweighed by unfavorable interactions with backbone. There are few literature data from which the preferential interaction of osmolytes with nucleic acid backbone can be deduced, but we note that most osmolytes are excluded from the protein peptide backbone, and it is this unfavorable interaction that largely accounts for the stabilization of proteins by osmolytes^{12; 34}. (The denaturants urea and guanidine hydrochloride are exceptions, favorably interacting with the peptide unit.) The hypothesis that RNA-stabilizing osmolytes are excluded from the polar RNA backbone is consistent with their exclusion from polar protein backbone.

Osmolyte – ion opposition in RNA stabilization

Monovalent salts strongly stabilize both RNA and DNA secondary structure. Changes in water activity or solvent dielectric constant caused by osmolyte addition could, in principle, modulate the activities of ions and their effects on RNA stability. However, the monovalent salt dependence of DNA helix denaturation is affected by osmolytes (ethylene glycol, betaine, and urea) to an extent that is at the edge of statistical significance^{33; 35}. Similarly small effects of Na^+ have been seen with ethylene glycol and the denaturation of a DNA triplex, a system which has a higher charge density approaching that of an RNA pseudoknot³⁵. Mg^{2+} ions interact much more strongly than monovalent ions with RNA, especially with compact tertiary structures, but their possible modulation of osmolytes – nucleic acid interactions have not been explored. We have therefore focused on the possible interplay between Mg^{2+} and osmolytes in this survey of osmolyte effects.

Initial experiments comparing the effects of osmolytes on RNA stability in the presence of either K^+ as the only cation or a $\text{Mg}^{2+}/\text{K}^+$ mixture showed that the ionic composition of the buffer increases or decreases the *m*-values of many osmolyte-RNA systems, suggesting that osmolytes and ions may strongly influence each others' interaction with RNAs, but no clear pattern of effects was apparent (Figure 6). A consistent theme emerged when RNA stability over a wide range of Mg^{2+} concentrations was measured in the presence or absence of an osmolyte: the stabilizing influence of the osmolyte is strongly attenuated with increasing Mg^{2+} concentration or, viewed in a reciprocal way, the stabilizing effect of Mg^{2+} is attenuated in the presence of osmolyte. In individual cases, low concentrations of Mg^{2+} may add to the osmolyte-induced stabilization (Figure 7A), or high Mg^{2+} concentrations may cause the osmolyte to become destabilizing (Figure 7B). We conclude that the stabilizing effects of Mg^{2+} and most of the osmolytes studied here are not additive. Because Mg^{2+} ions tend to be held close to the RNA surface³⁶, it is perhaps to be expected that strong interactions would arise between the ions and the water/osmolyte layers solvating the RNA. Relatively strong

interactions between Mg^{2+} and osmolytes are also likely; for instance, Mg^{2+} forms ion pairs with the carboxylate anions found in proline and betaine³⁷. But detailed speculations as to why Mg^{2+} and osmolyte act in opposition cannot be made without more information about the accumulation (or exclusion) of osmolyte, water, and ions near the surfaces of I and N state forms of the RNA.

As mentioned in Results, BWYV and A-riboswitch RNAs are probably stabilized predominantly by diffuse Mg^{2+} ions, while with GACG RNA a partially dehydrated Mg^{2+} ion at a buried chelation site contributes most of the Mg^{2+} -dependent folding free energy at lower monovalent ion concentrations¹⁵. It is therefore interesting that Mg^{2+} and TMAO are additive in their stabilizing effects with this RNA (Figure 7E). This result implies that the opposing free energies of Mg^{2+} dehydration and burial at the electronegative binding site are either unaffected by TMAO, or affected in parallel fashion to give the same net free energy advantage.

Sequence- and structure specific effects

Some of the osmolytes studied here are known to vary in their preferences for G,C- or A,U-rich sequences. For example, urea has a stronger affinity for A and T³³ and betaine is more effective in destabilizing G-C base pairs³⁸. Such preferences probably account for some of the variation we see in osmolyte effects between RNAs. For instance, betaine and proline have larger *m*-values for denaturation of the BWYV RNA hairpin H1, which is entirely G-C pairs, than with the other hairpins tested.

A potential sequence-specific effect of an osmolyte is the unusual stabilization of the BWYV helix 1 by glycerol, which shows “saturation” behavior suggestive of an unusually strong interaction with the helix (Figure 4B). Glycerol has been observed as part of an RNA minor groove network of hydrogen bonds involving waters, 2'-hydroxyls, and the 2- and 3-amino groups of G³⁹. An accumulation of hydrogen-bonded glycerol molecules in the helix H1 minor groove, which is entirely G-C, might account for its unusual stabilizing effect. When the 2'OH is missing from a helix (i.e., in DNA), glycerol is destabilizing and has no base composition preferences^{33; 40}.

The only other instance of saturation behavior we observed was with ethylene glycol stabilizing the tertiary fold of BWYV RNA, though in this case it is the hydrogen bonding of loop 2 bases in the helix H1 minor groove that is stabilized. We know of no precedent for specific binding of ethylene glycol to RNA surfaces. The cases of these two osmolytes having unusually strong effects with specific RNAs suggests that RNA control elements (such as riboswitches) could have been devised by natural selection to sense the concentrations of specific osmolytes.

Comparison of osmolyte effects on proteins and RNA and *in vivo* implications

m-values for the folding of small proteins have been measured for most of the osmolytes used in the present study. The values obtained for a carboxyamidated form of ribonuclease lacking disulfide bonds (104 residues, ~5600 Å² folded surface area) are typical¹²: urea is destabilizing, *m* ≈ 2 kcal/mol/M; proline and betaine are moderately stabilizing, *m* ≈ -0.6 kcal/mol/M; sucrose and sorbitol are more effective (*m* ≈ -1.6 kcal/mol/M) and TMAO is the strongest stabilizer (*m* ≈ -2 kcal/mol/M). The range of these protein *m*-values is comparable to the range of RNA *m*-values, but the specific effects are much different. With regard to RNA tertiary structure, both betaine and proline can be destabilizing, the latter strongly so, in contrast to their consistent stabilization of protein structure. Sorbitol is weakly destabilizing with RNA tertiary structure, but moderately stabilizing with proteins. Only sucrose and TMAO have roughly similar effects on protein and RNA structure, though the magnitude of the effects of these osmolytes on RNAs in cells will depend on the degree to which RNA structures are neutralized by Mg^{2+} *in vivo*.

It has been assumed that a selective advantage for cellular accumulation of protecting osmolytes in response to water stress is their compatibility with *protein* structure. But some of these protecting osmolytes are clearly not compatible with RNA tertiary structure. The function of numerous crucial cellular components depends on the folding of RNAs into specific tertiary structures and, in many cases, their ability to switch between alternative conformations. Translational repression^{41; 42}, riboswitches⁴³, and the control of plasmid copy number⁴⁴ are examples of regulatory systems dependent on the proper folding of RNA tertiary structures similar to those used in the present study. The spliceosome, ribosome and other RNA-containing complexes also rely on RNA tertiary interactions for their function. Although the effects of osmolytes on such complexes has not been systematically explored, it is known that betaine stimulates translation *in vitro*⁴⁵, and both betaine and TMAO stimulate 50S subunit reconstitution⁴⁶.

The selective pressures on cellular accumulation of osmolytes are probably also acting at the level of RNA secondary structure. Hairpin formation regulates transcription termination⁴⁷ and modulates translational efficiency⁴⁸; stable base pairing is required for the function a host of short RNA sequences that regulate transcription, translation, and RNA modification⁴⁹. All osmolytes appear to destabilize such structures, at least weakly. It appears that cells must regulate the concentrations of various ions and small organic molecules in ways that integrate their sometimes opposing contributions to the stability of proteins, duplex RNA, and RNA tertiary structures.

Potential uses for osmolytes in RNA folding studies

In protein studies, osmolytes have been used to measure folding free energies²⁵, to force marginally stable structures to fold⁵⁰, and to probe the area and chemical nature of surfaces exposed in folding or binding reactions⁵¹. Several similar applications of osmolytes to RNA folding studies are possible. First, urea has been used to measure the RNA surface area exposed in folding intermediates^{28; 52}; its consistent destabilization of RNA secondary structure (Figure 4) and relative insensitivity to the presence of divalent ions (Figure 6;²⁸) are useful features for this application. Betaine has been used as a probe of phosphate burial in protein – DNA complexes^{51; 53}, and could potentially be used in a similar way to quantitate phosphate burial in the formation of RNA structure. These applications of osmolytes to RNA folding are currently limited by the lack of models for base and backbone exposure in U and I state RNAs, which are needed for calculations of the changes in SASA taking place in a folding reaction. Second, the apparent differential effect of TMAO on the energetics of Mg²⁺ ion interactions in different RNA environments (Figure 7) may be a useful tool for elucidating the mechanisms by which Mg²⁺ stabilizes RNA interactions in complex RNAs. Finally, the stabilizing effects of TMAO and other osmolytes could also be useful for studying RNAs with inherently unstable structures or for extending the range of solution conditions under which an RNA folds. Methanol has been used in this way to maintain folding of GACG RNA with a wider range of ion types and concentrations than otherwise possible²³.

Material and Methods

Chemicals and solutions

All solutions were prepared using distilled deionized water at 18.3MΩ resistivity. Betaine ((carboxymethyl)trimethylammonium)) monohydrate (>99% pure), D-sorbitol (>99.5% pure), L-proline (>99.5% pure), urea (99.5% pure), and potassium chloride (>99.5% pure) were purchased from Fluka. Trimethylamine oxide (TMAO) (>98% pure) and glycerol (>99% pure) and MOPS (99.5% pure) were obtained from Sigma. Methanol (>99.8% pure), ethylene glycol (100%) and sucrose (99.9% pure) were purchased from E.M. Science, J.T.Baker and Roche, respectively. Magnesium chloride (>99.8% pure) was purchased from Aldrich. Because it is

difficult to keep the latter salt dry during storage, solution concentrations were determined by titration with EDTA according to described procedures⁵⁴. All chemicals were used without further purification.

The short hairpin RNA (Figure 1A), tar and tar* (Figure 1B) were purchased from Dharmacon. BWYV RNA (Figure 1C), the two 58mer rRNA fragment variants (Figure 1D) and the A-riboswitch (Figure 1E) were prepared by *in vitro* transcription with T7 RNA polymerase from double-stranded synthetic DNA template (BWYV RNA) or linear plasmid DNA (58-mer and A-riboswitch RNAs) by described methods^{16; 55}. RNAs were purified either by chromatography under denaturing conditions on ion exchange columns or by denaturing polyacrylamide gel electrophoresis followed by electroelution, as described^{16; 54}.

Before use, RNAs were extensively equilibrated with the appropriate buffers, using Centricon filter units (Millipore, Billerica, MA). Osmolyte studies have variously used molar or molal concentration scales. Molal units (*m*) are more convenient for our purposes, in which we wish to keep both water and ion concentrations constant as osmolyte concentration increases, and have been used throughout. MOPS buffer was adjusted to pH 7.0 with KOH (KMOPS). For each RNA studied, buffers of 10 mM KMOPS pH7.0 and 2 μ M EDTA with various KCl or KCl/MgCl₂ concentrations were used unless otherwise indicated. Hairpin: 0.1 mM EDTA and 100 mM KCl. Tar-tar*: 400 mM KCl or 100 mM KCl and 250 μ M MgCl₂. BWYV RNA: 0.1 mM EDTA and 40 mM KCl or 14 mM KCl and 10 μ M MgCl₂, except buffers for use with TMAO that had higher buffer concentrations but the same K⁺ concentrations: 36 mM KMOPS pH 7.0 and 30 mM KCl or 10 μ M MgCl₂. U1061A RNA: 0.1 mM EDTA, 100 mM KCl and 1.2 mM MgCl₂. GACG RNA: 0.1 mM EDTA, 100 mM KCl and 3.2 mM MgCl₂. A-Riboswitch RNA: 5 μ M 2,6-diaminopurine (DAP), 250 mM KCl or 100 mM KCl and 1.2 mM MgCl₂. Experiments on osmolyte effect on Mg²⁺-induced stability were done using same KMOPS/EDTA buffer with the following KCl concentrations: BWYV RNA: 14mM KCl; GACG RNA: 100mM KCl; A-Riboswitch RNA: 5 μ M 2,6-diaminopurine and 100mM KCl. MgCl₂ was added to these buffers as needed.

All osmolyte solutions were prepared gravimetrically. A common buffer was made to which was added precise weights of EDTA, KCl and MgCl₂ as needed. Each osmolyte was then weighed and added to this buffer to make a stock solution. Precisely one mL of each solution was weighed to determine its density. Densities were used to calculate the amount of stock needed for the volumetric preparation of each thermal melt analysis sample.

UV thermal analysis

Melting experiments were performed in a Cary 400 spectrophotometer with 1 cm path length cuvettes. For each RNA, absorbance data were collected at both 260 and 280 nm from 5° to 95° C (except for the tar-tar* complex which was collected from 2° to 95°C) and plotted as the first derivative of absorbance with respect to temperature (a melting profile). To simplify data analysis, sequential two-state transitions, defined by T_m , ΔH° (assumed to be independent from temperature), and absorbance changes, were used and fit globally to both the 260 and 280 nm data as described⁵⁶. Low temperature baselines for RNAs with tertiary structure are frequently small or zero, and only in a few cases were manually adjusted to optimize the fitted curve. Three unfolding transitions were fitted for the BWYV RNA melting profiles, as previously reported^{16; 21}. Three and four transitions were fitted to the U1061A and GACG 58mer rRNA fragments, respectively^{20; 23}. Characteristics of the A-riboswitch in melting experiments have not previously been reported. We find that the T_m of the first unfolding transition depends on the concentration of ligand (in this case, 2,6-diamino-purine, DAP) as expected for unfolding of tertiary structure (D. Leppley & D.E.D., unpublished observations), and, depending on the salt and osmolyte concentrations, have fit the melting profiles with either two or three transitions to extract the T_m and ΔH° of the first unfolding transition. Absorbance

data for the small hairpin, which melts in a single transition, were analyzed by standard methods incorporating low and high temperature baselines as fitted variables⁵⁷. See supplementary Figure S1 for examples of deconvoluted melting profiles for each RNA in the absence of osmolytes.

For each RNA, the averages and standard deviations of T_m and ΔH° were calculated from multiple melting experiments in the absence of osmolyte; errors averaged about ± 0.5 deg for T_m and $\pm 7\%$ for ΔH° (complete lists are in Supplementary Table S1). To determine the fitted parameter errors for individual melts and to confirm that a deconvolution of the data into unique values of T_m and ΔH° had been obtained, a bootstrap method was used⁵⁸. In this analysis, parameters are fit to a large number of artificial data sets generated by a Monte Carlo method. Statistics compiled on the fitted parameters provide confidence intervals for each variable and detect correlations between variables (see ref. ⁵⁶ for further details).

The effect of an osmolyte on the stability of an RNA was calculated from $\Delta\Delta G^\circ = (\Delta H^\circ)(T_0) (1/T_m - 1/T_0)$, where ΔH° and T_0 were the enthalpy and T_m of an RNA transition in the absence of osmolytes (Supplementary Table S1). Alternatively, the slope of a plot of $(1/T_m)$ vs. osmolyte molality is multiplied by $(\Delta H^\circ)(T_0)$ to obtain the so-called m -value, $(\partial(\Delta G^\circ)/\partial m_{\text{osmolyte}})$. This equation assumes two-state behavior of the secondary or tertiary folding transition. There is evidence that ΔH° is affected by some osmolytes⁴⁰, but the effects are small and we did not see significant trends in ΔH° with osmolyte molality, within the error of the experiments. In the case of the hairpin, which was analyzed as a single unfolding transition, we also recast the melting curves as the fraction RNA folded as a function of temperature, from which ΔG° for folding could be obtained for a temperature range bracketing the hairpin T_m . Thus it was possible to find ΔG° vs. m_{osmolyte} at a common temperature. This approach gave results in reasonable agreement with the one described above, as long as the transition regions from melting curves for each osmolyte concentration overlap sufficiently.

Solvent Accessible Surface Area calculations and molecular modeling

All solvent accessible surface area (SASA) calculations were performed using the program Surface Racer⁵⁹ with the Richards parameters and a 1.4 Å probe radius. As a rough estimate of the average surface accessibility of residues in single-stranded RNA, we summed and averaged the results from five segments of RNA that clearly do not adopt A-form helical structure (residues from the following PDB files: 1ATO:9–12; 1CX0:149–158; 1NBS:183–190; 1U9S:219–222 and 437D:18–21). Likewise, to evaluate the SASA of an internal residue in a single- or double-stranded RNA in A-form geometry, we used models generated using MC-Sym (3.3.2) for the helix of hairpin RNA (Figure 1A)⁶⁰. SASA calculated for the other RNAs shown in Figure 1 included all residues except for residue at position 1 (GTP) in the BWYV RNA, using the following PDB coordinate files: 1KIS, 437D, 1HC8 and 1Y26. SASAs of the tar and tar* hairpins were calculated for the individual chains extracted from the coordinate file, without attempting to re-model the hairpin loop regions. The solvent exposure of bases in these loops is therefore underestimated.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

We thank Desirae Leippely for the plasmid encoding the A-riboswitch RNA and for samples of purified A-riboswitch RNA, Dr. Ross Shiman for helpful advice on experimental protocols, and Dr. François Major for access to version 3.3.2 of MC-Sym.

This work was supported by NIH grant 1RO1 GM58545.

References

1. Yancey PH, Clark ME, Hand SC, Bowlus RD, Somero GN. Living with water stress: evolution of osmolyte systems. *Science* 1982;217:1214–22. [PubMed: 7112124]
2. Record MT Jr, Courtenay ES, Cayley DS, Guttman HJ. Responses of *E. coli* to osmotic stress: large changes in amounts of cytoplasmic solutes and water. *Trends Biochem Sci* 1998;23:143–8. [PubMed: 9584618]
3. Yancey PH, Somero GN. Counteraction of urea destabilization of protein structure by methylamine osmoregulatory compounds of elasmobranch fishes. *Biochem J* 1979;183:317–23. [PubMed: 534499]
4. Record MT Jr, Courtenay ES, Cayley S, Guttman HJ. Biophysical compensation mechanisms buffering *E. coli* protein-nucleic acid interactions against changing environments. *Trends Biochem Sci* 1998;23:190–4. [PubMed: 9612084]
5. Somero GN. Protein adaptations to temperature and pressure: complementary roles of adaptive changes in amino acid sequence and internal milieu. *Comp Biochem Physiol B Biochem Mol Biol* 2003;136:577–91. [PubMed: 14662287]
6. Gluick TC, Yadav S. Trimethylamine N-oxide stabilizes RNA tertiary structure and attenuates the denaturing effects of urea. *J Am Chem Soc* 2003;125:4418–9. [PubMed: 12683801]
7. Courtenay ES, Capp MW, Anderson CF, Record MT Jr. Vapor pressure osmometry studies of osmolyte-protein interactions: implications for the action of osmoprotectants in vivo and for the interpretation of “osmotic stress” experiments in vitro. *Biochemistry* 2000;39:4455–71. [PubMed: 10757995]
8. Felitsky DJ, Cannon JG, Capp MW, Hong J, Van Wynsberghe AW, Anderson CF, Record MT Jr. The exclusion of glycine betaine from anionic biopolymer surface: why glycine betaine is an effective osmoprotectant but also a compatible solute. *Biochemistry* 2004;43:14732–43. [PubMed: 15544344]
9. Courtenay ES, Capp MW, Record MT Jr. Thermodynamics of interactions of urea and guanidinium salts with protein surface: relationship between solute effects on protein processes and changes in water-accessible surface area. *Protein Sci* 2001;10:2485–97. [PubMed: 11714916]
10. Timasheff SN. Water as ligand: preferential binding and exclusion of denaturants in protein unfolding. *Biochemistry* 1992;31:9857–64. [PubMed: 1390769]
11. Auton M, Bolen DW. Additive transfer free energies of the peptide backbone unit that are independent of the model compound and the choice of concentration scale. *Biochemistry* 2004;43:1329–42. [PubMed: 14756570]
12. Auton M, Bolen DW. Predicting the energetics of osmolyte-induced protein folding/unfolding. *Proc Natl Acad Sci U S A* 2005;102:15065–8. [PubMed: 16214887]
13. Draper DE, Grilley D, Soto AM. Ions and RNA folding. *Annu Rev Biophys Biomol Struct* 2005;34:221–43. [PubMed: 15869389]
14. Misra VK, Draper DE. Mg(2+) binding to tRNA revisited: the nonlinear Poisson-Boltzmann model. *J Mol Biol* 2000;299:813–25. [PubMed: 10835286]
15. Misra VK, Draper DE. The linkage between magnesium binding and RNA folding. *J Mol Biol* 2002;317:507–21. [PubMed: 11955006]
16. Soto AM, Misra V, Draper DE. Tertiary structure of an RNA pseudoknot is stabilized by “diffuse” Mg(2+) ions. *Biochemistry* 2007;46:2973–83. [PubMed: 17315982]
17. Misra VK, Draper DE. A thermodynamic framework for Mg²⁺ binding to RNA. *Proc Natl Acad Sci U S A* 2001;98:12456–61. [PubMed: 11675490]
18. Alden CJ, Kim S-H. Solvent-accessible Surfaces of Nucleic Acids. *J Mol Biol* 1979;132:411–434. [PubMed: 533898]
19. Conn GL, Gittis AG, Lattman EE, Misra VK, Draper DE. A compact RNA tertiary structure contains a buried backbone-K⁺ complex. *J Mol Biol* 2002;318:963–73. [PubMed: 12054794]
20. Lu M, Draper DE. Bases Defining an Ammonium and Magnesium Ion-Dependent Tertiary Structure Within the Large Subunit Ribosomal RNA. *J Mol Biol* 1994;244:572–585. [PubMed: 7527467]
21. Nixon PL, Giedroc DP. Energetics of a strongly pH dependent RNA tertiary structure in a frameshifting pseudoknot. *J Mol Biol* 2000;296:659–71. [PubMed: 10669615]
22. Chang K-Y, Tinoco I. The Structure of an RNA “Kissing” Hairpin Complex of the HIV TAR Hairpin Loop and its Complement. *J Mol Biol* 1997;269:52–66. [PubMed: 9193000]

23. Shiman R, Draper DE. Stabilization of RNA tertiary structure by monovalent cations. *J Mol Biol* 2000;302:79–91. [PubMed: 10964562]
24. Laing LG, Draper DE. Thermodynamics of RNA Folding in a Highly Conserved Ribosomal RNA Domain. *J Mol Biol* 1994;237:560–576. [PubMed: 7512652]
25. Pace CN. The stability of globular proteins. *CRC Crit Rev Biochem* 1975;3:1–43. [PubMed: 238787]
26. Santoro MM, Bolen DW. A test of the linear extrapolation of unfolding free energy changes over an extended denaturant concentration range. *Biochemistry* 1992;31:4901–7. [PubMed: 1591250]
27. Schellman JA. Selective binding and solvent denaturation. *Biopolymers* 1987;26:549–59. [PubMed: 3567326]
28. Shelton VM, Sosnick TR, Pan T. Applicability of urea in the thermodynamic analysis of secondary and tertiary RNA folding. *Biochemistry* 1999;38:16831–9. [PubMed: 10606516]
29. Serganov A, Yuan YR, Pikovskaya O, Polonskaia A, Malinina L, Phan AT, Hobartner C, Micura R, Breaker RR, Patel DJ. Structural basis for discriminative regulation of gene expression by adenine- and guanine-sensing mRNAs. *Chem Biol* 2004;11:1729–41. [PubMed: 15610857]
30. Herskovits TT, Harrington JP. Solution studies of the nucleic acid bases and related model compounds. Solubility in aqueous alcohol and glycol solutions. *Biochemistry* 1972;11:4800–11. [PubMed: 4540188]
31. Herskovits TT, Bowen JJ. Solution studies of the nucleic acid bases and related model compounds. Solubility in aqueous urea and amide solutions. *Biochemistry* 1974;13:5474–83. [PubMed: 4549016]
32. Hong J, Capp MW, Anderson CF, Saecker RM, Felitsky DJ, Anderson MW, Record MT Jr. Preferential interactions of glycine betaine and of urea with DNA: implications for DNA hydration and for effects of these solutes on DNA stability. *Biochemistry* 2004;43:14744–58. [PubMed: 15544345]
33. Nordstrom LJ, Clark CA, Andersen B, Champlin SM, Schweinfus JJ. Effect of Ethylene Glycol, Urea, and N-Methylated Glycines on DNA Thermal Stability: The Role of DNA Base Pair Composition and Hydration. *Biochemistry* 2006;45:9604–9614. [PubMed: 16878995]
34. Liu Y, Bolen DW. The peptide backbone plays a dominant role in protein stabilization by naturally occurring osmolytes. *Biochemistry* 1995;34:12884–91. [PubMed: 7548045]
35. Spink CH, Chaires JB. Effects of hydration, ion release, and excluded volume on the melting of triplex and duplex DNA. *Biochemistry* 1999;38:496–508. [PubMed: 9890933]
36. Misra VK, Draper DE. The interpretation of Mg^{2+} binding isotherms for nucleic acids using Poisson-Boltzmann theory. *J Mol Biol* 1999;294:1135–47. [PubMed: 10600372]
37. Archer DW, Monk CB. Ion association constants of some acetates by pH (glass electrode) measurements. *J Chem Soc (London)* 1964:3117–22.
38. Rees WA, Yager TD, Korte J, Hippel PHv. Betaine Can Eliminate the Base Pair Composition Dependence of DNA Melting. *Biochemistry* 1993;32:137–144. [PubMed: 8418834]
39. Rypniewski W, Vallazza M, Perbandt M, Klussmann S, Delucas LJ, Betzel C, Erdmann VA. The first crystal structure of an RNA racemate. *Acta Crystallogr D Biol Crystallogr* 2006;62:659–64. [PubMed: 16699193]
40. Spink CH, Garbett N, Chaires JB. Enthalpies of DNA melting in the presence of osmolytes. *Biophys Chem*. 2006
41. Draper DE. How do proteins recognize specific RNA sites? New clues from autogenously regulated ribosomal proteins. *Trends Biochem Sci* 1989;14:335–338. [PubMed: 2678632]
42. Schlax PJ, Xavier KA, Gluick TC, Draper DE. Translational repression of the *E. coli* α operon mRNA: Importance of an mRNA conformational switch and a ternary entrapment complex. *J Biol Chem* 2001;276:38494–501. [PubMed: 11504736]
43. Mandal M, Boese B, Barrick JE, Winkler WC, Breaker RR. Riboswitches control fundamental biochemical pathways in *Bacillus subtilis* and other bacteria. *Cell* 2003;113:577–86. [PubMed: 12787499]
44. Eguchi Y, Tomizawa J. Complexes formed by complementary RNA stem-loops. Their formations, structures and interaction with ColE1 Rom protein. *J Mol Biol* 1991;220:831–42. [PubMed: 1715406]

45. Brigotti M, Petronini PG, Carnicelli D, Alfieri RR, Bonelli MA, Borghetti AF, Wheeler KP. Effects of osmolarity, ions and compatible osmolytes on cell-free protein synthesis. *Biochem J* 2003;369:369–74. [PubMed: 12374569]
46. Semrad K, Green R. Osmolytes stimulate the reconstitution of functional 50S ribosomes from in vitro transcripts of *Escherichia coli* 23S rRNA. *RNA* 2002;8:401–11. [PubMed: 11991636]
47. von Hippel PH. An integrated model of the transcription complex in elongation, termination, and editing. *Science* 1998;281:660–5. [PubMed: 9685251]
48. de Smit MH, van Duin J. Control of Prokaryotic Translational Initiation by mRNA Secondary Structure. *Prog in Nucleic Acid Res and Molec Biol* 1990;38:1–164. [PubMed: 2183291]
49. Storz G, Altuvia S, Wassarman KM. An abundance of RNA regulators. *Annu Rev Biochem* 2005;74:199–217. [PubMed: 15952886]
50. Baskakov I, Bolen DW. Forcing thermodynamically unfolded proteins to fold. *J Biol Chem* 1998;273:4831–4. [PubMed: 9478922]
51. Hong J, Capp MW, Saecker RM, Record MT Jr. Use of urea and glycine betaine to quantify coupled folding and probe the burial of DNA phosphates in lac repressor-lac operator binding. *Biochemistry* 2005;44:16896–911. [PubMed: 16363803]
52. Fang X, Littrell K, Yang XJ, Henderson SJ, Siefert S, Thiyagarajan P, Pan T, Sosnick TR. Mg²⁺-dependent compaction and folding of yeast tRNA^{Phe} and the catalytic domain of the *B. subtilis* RNase P RNA determined by small-angle X-ray scattering. *Biochemistry* 2000;39:11107–13. [PubMed: 10998249]
53. Kontur WS, Saecker RM, Davis CA, Capp MW, Record MT Jr. Solute probes of conformational changes in open complex (R_{Po}) formation by *Escherichia coli* RNA polymerase at the lambdaPR promoter: evidence for unmasking of the active site in the isomerization step and for large-scale coupled folding in the subsequent conversion to R_{Po}. *Biochemistry* 2006;45:2161–77. [PubMed: 16475805]
54. Grilley D, Soto AM, Draper DE. Direct quantitation of Mg²⁺-RNA interactions by use of a fluorescent dye. *Methods in Enzymology*. 2006in press
55. Grilley D, Soto AM, Draper DE. Mg²⁺-RNA interaction free energies and their relationship to the folding of RNA tertiary structures. *Proc Natl Acad Sci U S A* 2006;103:14003–14008. [PubMed: 16966612]
56. Draper, DE.; Bukhman, YV.; Gluck, TC. Thermal Methods for the Analysis of RNA Folding Pathways. In: Beaucage, SL.; Bergstrom, DE.; Glick, GD.; Jones, RA., editors. *Current Protocols in Nucleic Acid Chemistry*. section 11.3. John Wiley & Sons; New York: 2000.
57. Petersheim M, Turner DH. Base-stacking and Base-Pairing Contributions to Helix Stability: Thermodynamics of Double-Helix Formation with CCGG, CCGGp,CCGGAp, CCGGUp, and ACCGGUp. *Biochemistry* 1983;22:256–263. [PubMed: 6824629]
58. Press, WH.; Teukolsky, SA.; Vetterling, WT.; Flannery, BP. *Numerical Recipes in C*. Cambridge University Press; Cambridge: 1992.
59. Tsodikov OV, Record MT Jr, Sergeev YV. Novel computer program for fast exact calculation of accessible and molecular surface areas and average surface curvature. *J Comput Chem* 2002;23:600–9. [PubMed: 11939594]
60. Gendron P, Lemieux S, Major F. Quantitative analysis of nucleic acid three-dimensional structures. *J Mol Biol* 2001;308:919–36. [PubMed: 11352582]
61. Egli M, Minasov G, Su L, Rich A. Metal ions and flexibility in a viral RNA pseudoknot at atomic resolution. *Proc Natl Acad Sci U S A* 2002;99:4302–7. [PubMed: 11904368]
62. Conn GL, Draper DE, Lattman EE, Gittis AG. Crystal structure of a conserved ribosomal protein-RNA complex. *Science* 1999;284:1171–4. [PubMed: 10325228]

Abbreviations used

TMAO

trimethylamine oxide

BWYV

beet western yellow virus

DAP

2,6-diamino-purine

SASA

solvent accessible surface area

Figure 1.

Schematics of the secondary and tertiary structures of the RNAs used in this study. Horizontal black bars and black dots represent Watson-Crick and non-canonical base pairs, respectively. Gray bars represent base-base tertiary interactions, and thin lines with arrowheads denote 5'–3' backbone connectivities. (A) A designed short hairpin. (B) Tar-tar* kissing-loop complex (1KIS)²². (C) Beet Western Yellow Virus (BWYV) pseudoknot (1L2X)⁶¹. (D) 58mer rRNA fragment binding L11 protein. The sequence is the U1061A variant of the *E. coli* rRNA (1HC8)⁶². Dashed arrows indicate four point mutations in the GACG RNA construct²⁰. (E) A-riboswitch adapter region (1Y26)²⁹. The adenine ligand is shown in outline typeface.

Figure 2.

Solvent-accessible surface area calculations. Average exposures ($\text{\AA}^2$) were calculated per nucleotide for (A) bases, (B) sugars and (C) phosphates in different RNA conformations and constructs. From left to right in each panel the calculations represent an average of 30 single-stranded residues observed in RNA crystal structures; an internal residue within an A-form single-strand (ss) RNA or double-strand (ds) RNA; the tar and tar* hairpins; the tar-tar* complex; BWYV RNA, 58mer rRNA fragment (U1061A RNA); and A-riboswitch RNA.

Figure 3.

Extraction of transition T_m s and m -values from UV melting profiles. (A) Example melting profiles of GACG in various molalities of TMAO. The temperature dependence of the absorbance is plotted as the first derivative (dA_{260}/dT) for the following TMAO concentrations: 0 (red), 0.46 (blue), 0.92 (cyan), 1.38 (light green) and 1.84 m (dark green). The least squares best fit profile for dA_{260}/dT data in the absence of TMAO is shown (red line) along with its deconvoluted transitions 1 (black line), 2 (large dashed line), 3 (small dashed line) and 4 (dotted line). (B) Plots of reciprocal T_m s of each unfolding transition (line-coded as in A) vs. TMAO molality; slopes of these lines were used in calculating m -values. Error

bars, derived from bootstrap analysis of the melting profiles, are shown for all points; in some cases, the bars are smaller than the data points.

Figure 4.

Osmolyte effects on RNA secondary structure stability in the absence of Mg^{2+} (K^+ concentrations are different for each RNA; see Materials and Methods.) (A) m -values obtained for the indicated RNA hairpins in the presence of 0 – 2 m of various osmolytes. The m -value relates to the folding reaction; hence a positive value indicates osmolyte-induced destabilization of the RNA. The effect of glycerol on BWVY RNA (indicated by an asterisk) is the maximum stabilization free energy induced by the osmolyte (see panel B). (B) Effects of glycerol on secondary and tertiary structure stabilities of BWVY RNA in the absence of Mg^{2+} (44 mM K^+). Reciprocal T_m s from secondary (transition 3, square) and tertiary (transition 1, circle) structure melting transitions at different glycerol molalities are shown with error bars

(sometimes smaller than the points) derived from the bootstrap procedure. The reported stabilization energy (panel A) is calculated from the T_m at the intersection of the two least squares lines.

Figure 5.

Osmolyte effects on RNA tertiary structure stability in the presence of K⁺ as the only cation (tar-tar*, BWYV, and A-riboswitch RNAs) or Mg²⁺ and K⁺ (U1061A and GACG RNAs) (see Materials and Methods for all salt concentrations). *m*-values are plotted for each RNA in the presence of the indicated osmolytes, except for the effect of glycerol on BWYV RNA (indicated by an asterisk) which is the maximum stabilization free energy observed at a saturating level of ~1 *m* osmolyte and is -2.0 kcal/mole/*m* (Figure 4B).

Figure 6.

Osmolyte effects on RNA tertiary structure stability in buffers containing either K⁺ as the only cation or mixtures of K⁺ and Mg²⁺. Details of the salt concentrations used with each RNA are given in Materials and Methods. *m*-values for the tar-tar* complex, BWYV RNA, and A-riboswitch RNA are shown. Asterisks indicate that ΔG° vs. osmolyte molality did not remain linear to 2 *m* osmolyte; the maximum free energy of stabilization is reported instead of an *m*-value. The stabilization by glycerol of BWYV RNA in the absence of Mg²⁺ was -2.0 kcal/mole/*m*. For ethylene glycol and BWYV RNA in buffer with K⁺ and Mg²⁺, the value is -0.86 kcal/mole/*m*.

Figure 7.

Effects of osmolytes on Mg^{2+} -dependence of RNA tertiary stability. The reciprocal T_m s for tertiary structure unfolding in the absence (open) or presence (closed) of osmolytes are plotted for BWYV RNA with 2 *m* ethylene glycol (A), A-riboswitch RNA with 2 *m* ethylene glycol (B) or 1.84 *m* TMAO (C), and a 58mer rRNA fragment (GACG RNA) with 2 *m* ethylene glycol (D) or 1.84 *m* TMAO (E) are depicted with corresponding bootstrap errors (some are smaller than data points). K^+ concentrations were 14 *mM* for BWYV RNA, and 104 *mM* for the other two RNAs. Reciprocal T_m s at 0 *mM* Mg^{2+} are boxed.

Figure 8.

Representation of the potential effects of osmolytes on RNA folding equilibria. Unfolded (U), Intermediate (I) and Native (N) RNA states are depicted. The predominant solvent-accessible surfaces of each state are indicated in boxes.