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Apolar chemical environments compact unfolded RNAs and can promote folding

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Abstract

It is well documented that the structure, and thus function, of nucleic acids depends on the chemical environment surrounding them, which often includes potential proteinaceous binding partners. The nonpolar amino acid side chains of these proteins will invariably alter the polarity of the local chemical environment around the nucleic acid. However, we are only beginning to understand how environmental polarity generally influences the structural and energetic properties of RNA folding. Here, we use a series of aqueous-organic cosolvent mixtures to systematically modulate the solvent polarity around two different RNA folding constructs that can form either secondary or tertiary structural elements. Using single-molecule Förster resonance energy transfer spectroscopy to simultaneously monitor the structural and energetic properties of these RNAs, we show that the unfolded conformations of both model RNAs become more compact in apolar environments characterized by dielectric constants less than that of pure water. In the case of tertiary structure formation, this compaction also gives rise to more energetically favorable folding. We propose that these physical changes arise from an enhanced accumulation of counterions in the low dielectric environment surrounding the unfolded RNA.

INTRODUCTION

Over the years, it has become increasingly apparent that the functions of structured RNAs are intimately related to the many conformations that they adopt in aqueous solutions (1). To better understand the folding of these biological polymers, biophysical and biochemical studies are often conducted in physiologically inspired aqueous solutions that aim to mimic various components (e.g., inorganic or macromolecular) of intracellular environments (2). For example, the role of inorganic counterions (3–6) such as K⁺ (7,8) and Mg²⁺ (9,10) in RNA folding has been an active area of research for decades (11,12). Furthermore, the abundance of essential nucleoprotein complexes in biology has led to numerous studies of protein-assisted RNA folding (13), in which certain types of positively charged RNA-

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AUTHOR CONTRIBUTIONS

E.D.H. conceived project, designed experiments, provided samples, analyzed data, and wrote the manuscript. S.M.G. designed experiments, performed experiments, analyzed data, and wrote the manuscript.

SUPPORTING MATERIAL

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binding proteins have been proposed to function as macromolecular counterions that can facilitate conformational transitions in nucleic acids (14,15). From these studies, it is clear that electrostatic contributions can greatly alter the kinetics and energetics of RNA structure formation.

In addition to acting as a counterion, any protein bound to an RNA will also alter the local chemical environment around the RNA, resulting in something that is generally less polar than water (16), which we will refer to as an apolar environment. Because polarity arises from the separation of electrical charges, apolar environments will inevitably alter the electrostatic considerations associated with RNA folding. Unfortunately, a complete understanding of how they influence the structural and energetic aspects of RNA folding has not yet been fully established, with only a handful of ensemble biochemical studies suggesting that they can indeed alter the folding energetics and/or enzymatic activity of RNA (17–25). Not only are the effects of environmental polarity important for a more complete understanding of RNA folding and RNA-protein interactions, but they also have significant implications for biotechnology as well, for which several mainstream applications routinely use solvent systems that are less polar than water, e.g., polymerase chain reaction (26), RNA precipitation (27), phenol-chloroform extraction (28), and reverse-phase high-performance liquid chromatography (29). Therefore, we set out to further investigate the influence of apolar chemical environments on the fundamental principles governing RNA folding using a series of aqueous-organic mixtures (Fig. 1 A). Unfortunately, polarity can be a difficult property to quantify, particularly for systems containing multiple cosolvents (34). However, the dielectric constant, or relative permittivity (ϵ_r), is a bulk property that is closely related to polarity and has been extensively measured for nearly a century (31). Furthermore, many common organic solvents have dielectric constants that are lower than water, and therefore, by mixing water with a miscible cosolvent, we can produce a series of apolar chemical environments for RNA folding studies.

Past efforts to study the structural and energetic properties of RNAs in apolar environments have primarily been carried out using ensemble spectroscopic techniques (18,24,35). Although limited in number, these efforts have started to unveil new insights into the role of polarity in RNA folding. However, the data obtained from these ensemble approaches only report on the average properties of a large population of molecules, making it difficult to identify differential effects on distinct subpopulations of molecules (e.g., folded and unfolded). To avoid these shortcomings, we use single-molecule Förster resonance energy transfer (FRET) to systematically study how different apolar environments influence the structural and energetic aspects of RNA folding using two different model systems, each dissolved in various aqueous-organic cosolvent systems. FRET is a powerful photophysical phenomenon that can be monitored spectroscopically to measure nano-meter-scale distances between two neighboring fluorescent probes. When these probes are attached to an RNA of interest, one can use a sensitive fluorescence microscope to measure structural changes within individual molecules (36,37), which make it possible to obtain information about the conformational and energetics properties of distinct subpopulations associated with heterogeneous molecular ensembles.

The two fluorescently labeled model RNA-folding systems used in this study are 1) a hairpin RNA (HP-RNA) construct (Fig. 1 B, *left*) with a 5 basepair stem and a large poly-rA loop (14) and 2) a GAAA tetraloop-tetraloop receptor RNA (TL-RNA) construct (Fig. 1 B, *right*) derived from the *Tetrahymena* ribozyme (38). Previous biophysical investigations of these two model RNAs revealed that both display cooperative two-state folding and unfolding behavior during the formation of either secondary (HP-RNA) or tertiary (TL-RNA) structural interactions (14,38). To understand the influence of solvent polarity on the structural and energetic properties of RNA folding, the two FRET-labeled RNAs were each dissolved in different cosolvent systems prepared by mixing water with several different organic solvents: methanol (MeOH); ethanol (EtOH); isopropyl alcohol (IPA); acetone, which is also known as dimethyl ketone (DMK); and acetonitrile (ACN). The results of these experiments show that apolar chemical environments characterized by dielectric constants lower than that of water invariably compact the unfolded states of these two RNAs, which, in some cases, results in more energetically favorable folding.

MATERIALS AND METHODS

Chemicals and reagents

Doubly distilled water was used to prepare all solutions. KCl, NaOH, Na₂HPO₄, NaH₂PO₄, HEPES, EDTA, MeOH, and IPA were purchased from Sigma-Aldrich (St. Louis, MO). EtOH (190 proof) was purchased from Decon Labs (King of Prussia, PA). DMK and ACN were purchased from EMD Millipore (Burlington, MA) and Thermo Fisher Scientific (Fair Lawn, NJ), respectively. All the chemicals, solvents, and reagents used in this research were of the highest purity grade available at the time.

Preparation of the hairpin RNA construct

A commercially synthesized (Integrated DNA Technologies, Coralville, IA) custom-designed bireactive RNA construct was purchased with the following nucleotide sequence: 5'-/5ThioMC6-D/ GUC UUC CAA AAA AAA AAA AAA AAC CAA AAA AAA AAA AAA AA/iAmMC6T/ GAA GAG AAA AAA-3' (HP-RNA).

The length of the poly-rA loop and the number of complementary basepairs in the hairpin RNA (HP-RNA) construct were specifically designed to balance the rate constants describing formation and disruption of the 5 basepair duplex such that the folding of this secondary structural element is nearly isoenergetic in solution conditions with near-physiological salt concentrations while also maintaining a conformational change that could be easily resolved with FRET. This bireactive oligonucleotide was dissolved in a buffered aqueous solution to covalently and site-specifically couple the FRET donor and acceptor fluorophores to the RNA. Specifically, a maleimide-functionalized CF660R (acceptor) fluorophore was coupled to the RNA via a 6-carbon thiol-modified phosphate (/5ThioMC6-D/) at the 5' end of the oligonucleotide and an *N*-hydroxysuccinimide (NHS)-functionalized Cy3B (donor) fluorophore was coupled to the RNA via a 6-carbon amino-modified deoxythymidine (/iAmMC6T/) at nucleotide position 48 (Fig. 1 B). The doubly labeled and high-performance liquid chromatography (HPLC)-purified HP-RNA was flash

frozen and stored at -70°C until use. A more detailed description of labeling reactions and purification process can be found elsewhere (14).

Preparation of the GAAA tetraloop receptor construct

The three-piece construct was assembled following a previously established protocol (38). The two RNA oligos (ExonanoRNA, Columbus, OH) and one DNA oligo (Integrated DNA Technologies) were purchased as custom-synthesized material with the following sequences: 5'-/Am6/ GGC GAA AGC CAA AAA AAC GUG UCG UCC UAA GUC GGC-3' (TL-RNA1); 5'-/Am6/ GCC GAU AUG GAC GAC ACG CCC UCA GAC GAG UGCG-3' (TL-RNA2); 5'-/5Biosg/ CGC ACT CGT CTG AG-3' (TL-DNA3).

The nucleotide sequences for the two interacting secondary structural elements of the GAAA TL-RNA construct were taken from the *Tetrahymena* ribozyme and linked together with a short single-stranded RNA linker, which controls the effective local concentration of the two species. The length of the linker was chosen such that the resulting folding reaction is nearly isoenergetic in solution conditions with near-physiological salt concentrations while also maintaining a conformational change that could be resolved with FRET. The 6-carbon amino-modified phosphate (/Am6/) at the 5' ends of both RNA oligos was covalently labeled with either CF660R (TL-RNA1) or Cy3B (TL-RNA2) using NHS-coupling chemistry (Fig. 1 B). Briefly, the RNA samples were each dissolved in 0.1 M sodium phosphate buffer at pH 8.0. Approximately, 10 nmol of the RNA was repeatedly diluted to $\sim 500\ \mu\text{L}$ using additional sodium phosphate buffer and concentrated to $\sim 25\ \mu\text{L}$ using 3 kDa MWCO centrifuge filter tube (Millipore) to filter out any small reactive primary amino-groups present in the starting material. The concentrated RNA oligo solutions were each mixed with solutions containing ~ 10 -fold molar excess of either the NHS-functionalized donor or acceptor dyes (~ 100 nmol, in $\sim 20\ \mu\text{L}$ dimethyl sulfoxide) and then allowed to incubate for 30 min in dark. After incubation, the reaction mixtures were run through pre-equilibrated (0.1 M sodium phosphate (pH 7.0) with 1 mM EDTA) Zeba spin desalting columns (Thermo Fisher Scientific, Waltham, MA) three consecutive times according to the manufacturer's guidelines to remove excess free dye. The degree of labeling was determined by measuring the absorbance ratio of the purified sample at 260 nm as well as at either 560 nm (Cy3B) or 660 nm (CF660R) using a DS-11 FX + spectrophotometer (DeNovix, Wilmington, DE). The labeled TL-RNA1 and TL-RNA2 oligos were further purified using a reversed-phase C18 column (Dr. Maisch, Ammerbuch-Entringen, Germany) and an HPLC system equipped with a multiwavelength light detector (1260 Infinity II; Agilent Technologies, Santa Clara, CA). After labeling and purification, the TL-RNA construct was assembled by annealing the three oligos (i.e., TL-RNA1, TL-RNA2, and TL-DNA3). The annealing was achieved by incubating 6 nmol aliquots ($100\ \mu\text{L}$ each) of the three oligos in 0.1 M sodium phosphate buffer at pH 7.0 with 1 mM EDTA for 2 h at room temperature. Annealed TL-RNA construct was flash frozen in liquid nitrogen and stored at -70°C until use. A biotin linker (/5Biosg/) was included in the design of the TL-RNA to enable future single-molecule FRET experiments in which the RNA will be immobilized to the surface of a glass coverslip.

Sample preparation

Measurements were performed in 15-well microscope slides (Ibidi, Planegg, Germany) containing 40 μL of the RNA sample solution, which was prepared using an appropriate volume of concentrated RNA stock solution diluted into the measurement buffer to achieve a final RNA concentration of ~ 100 pM. The resulting RNA sample solution also contains the following components: 25 mM HEPES, 12.5 mM NaOH, 250 mM KCl, 0.01% v/v Tween 20, and a specified amount of organic solvent. HEPES and NaOH were used to buffer the solution pH. KCl was added to ensure that the folded and unfolded states of the two model RNA constructs were approximately equally populated in the absence of organic solvents and thus that the folding reactions were nearly isoenergetic. Tween 20 was added to prevent adsorption of the RNA to the hydrophobic surfaces of the 15-well microscope slides. Organic solvents were used to lower the dielectric constant of the cosolvent system. For each measurement, the final value of ϵ_r was interpolated from published data on binary aqueous-organic solvent mixtures (30–32).

Single-molecule FRET spectroscopy with alternating continuous-wave excitation

All single-molecule measurements were performed using a slightly modified MicroTime200 system (PicoQuant, Berlin, Germany) consisting of an inverted confocal fluorescence microscope equipped with two excitation sources and four detectors (Fig. 2 A). Two dual-mode diode lasers (QuixX 515-80PS and QuixX 642-140PS; Omicron, Rodgau, Germany) operating in the continuous-wave mode are used to produce ~ 100 μW of vertically polarized light at wavelengths of 515 and 642 nm to excite the donor and acceptor fluorophores, respectively. Alternating excitation (39) of the two fluorophores is carried out at 20 kHz using a signal generator (577-4C; Berkeley Nucleonics, San Rafael, CA) to modulate the output of the two lasers. After being reflected off a dual-band dichroic mirror (ZT532/640rpc; Chroma, Bellows Falls, VT), the laser light is then focused to a diffraction-limited spot within the sample using a 60 \times magnification 1.2 NA water immersion microscope objective (UPlanSAPO; Olympus, Tokyo, Japan). The fluorescence emitted from the sample is collected using the same objective in an epifluorescence configuration and directed toward the detection unit, where it first passes through a 100- μm -diameter confocal pinhole. Then, the emission is spatially separated via a polarizing beam splitter (BS Cube Polarizer; PicoQuant), resulting in two streams of linearly polarized fluorescence: one parallel to the polarization of the laser light, the other perpendicular. The two streams are then each split again using a long-pass dichroic mirror (635LPXR; Chroma) to spectrally resolve donor and acceptor emission, resulting in a total of four separate detection streams. Band-pass filters in the appropriate detection streams reject any fluorescence not associated with the donor (582/64 BrightLine; Semrock, New York, NY) and acceptor (690/70 BrightLine; Semrock) fluorophores. Each stream of fluorescence terminates at a single-photon avalanche photodiode-based detector (SPCM-AQRH-14-TR; Excelitas Technologies, Waltham, MA), where the arrival times of individual photons are recorded by a time-correlated single-photon counting module (HydraHarp 400; Pico-Quant) with the temporal resolution set to 16,384 (2^{14}) ps. The duration of each measurement was 900 s.

Data analysis

Under our experimental conditions, the confocal volume is most often devoid of fluorescent molecules, with an average occupancy of less than 0.01 molecules. Nevertheless, individual fluorescently labeled molecules will inevitably diffuse through the confocal volume, where they will be excited by the two alternating lasers, resulting in a transient burst of fluorescent photons (Fig. 2 B). The detection records of the photons emitted from these freely diffusing fluorescently labeled RNA molecules were analyzed using Mathematica 12.0 (Wolfram Alpha) in conjunction with Fretica, a C++ based MATH-LINK module for the analysis of time-correlated single-photon-counting single-molecule FRET data (40). The data analysis workflow employed during this research is as follows: first, time-gating was used to determine which laser (either 515 or 642 nm) was active for every detected photon in each of the four streams. Then, photons were assigned to 500 μ s time bins based on their absolute arrival time (Fig. 2 C). Time bins with a total photon count rate ($^{Tot}n = {}^1n_{515} + {}^1n_{642} + {}^2n_{515} + {}^2n_{642} + {}^3n_{515} + {}^3n_{642} + {}^4n_{515} + {}^4n_{642}$) of less than 20 photons per bin were used to calculate the average back-ground photon count rate for each of the four streams during either 515 or 642 nm excitation. Then, corrected photon count rates (N) were determined for all time bins by accounting for background, spectral cross talk, direct excitation of the acceptor, and nonidentical excitation and detection efficiencies of the donor and acceptor fluorophores. Those time bins with a corrected total photon count rate ($^{Tot}N = {}^1N_{515} + {}^1N_{642} + {}^2N_{515} + {}^2N_{642} + {}^3N_{515} + {}^3N_{642} + {}^4N_{515} + {}^4N_{642}$) of more than 20 photons per bin were considered bursts of fluorescence arising from single molecules diffusing through the confocal volume (41). Corrected photon count rates associated with the acceptor and donor fluorophores ($^{Acc}N = {}^1N + {}^3N$ and $^{Don}N = {}^2N + {}^4N$, where $^{Tot}N = ^{Acc}N + ^{Don}N$) during 515 and 642 nm excitation were used to calculate values for both the FRET efficiency (E) and fluorescence stoichiometry (S) using Eq. 1 and Eq. 2, respectively (Fig. 2 D).

$$E = ^{Acc}N_{515} / (^{Acc}N_{515} + ^{Don}N_{515}). \quad (1)$$

$$S = ^{Tot}N_{515} / (^{Tot}N_{642} + ^{Tot}N_{515}). \quad (2)$$

The fluorescence stoichiometry (S) of a burst arising from a molecule containing only donor or acceptor fluorophores will yield values near $S = 1$ and $S = 0$, respectively. Therefore, values of fluorescence stoichiometry are used to restrict our analysis and interpretation of FRET efficiencies to only those bursts arising from molecules containing active donor and acceptor fluorophores (i.e., $0.25 < S < 0.75$). This allows us to effectively filter out unwanted contributions from any potential donor-only or acceptor-only molecules that, for example, may not have been removed during the HPLC purification of the RNA constructs.

The FRET efficiency (E) values from bursts with $^{Tot}N > 50$ were then compiled into histograms (Fig. 2 D), in which the widths of the resulting distributions were largely limited by shot noise (Fig. S1). Histograms were then fitted using Gaussian distributions to determine the mean FRET efficiency, $\langle E \rangle$, and fractional abundance, θ , of the folded and unfolded subpopulations. Based on several repeated measurements under identical conditions, typical experimental uncertainties associated with $\langle E \rangle$ and θ are ± 0.02 and

± 0.03 , respectively. The fractional abundance of each subpopulation was used to calculate the equilibrium constant for folding ($K_{fold} = \theta_f / \theta_u$) and thus the standard state Gibbs free energy difference ($\Delta G_{fold}^\circ = -RT \ln K_{fold}$, where R is the gas constant) between the two subpopulations at $T = 294.2$ K. For the ease of data interpretation, all ΔG_{fold}° values in apolar solvent conditions were referenced to aqueous conditions, i.e., $\Delta G_{fold}^\circ(\text{mixture}) - \Delta G_{fold}^\circ(\text{H}_2\text{O})$.

RESULTS

Although the importance of electrostatics in RNA folding has been extensively studied over the past 50 years, we are only beginning to understand how the polarity of the local chemical environment around the RNA influences the structural and energetic aspects of folding. As such, the main objective of this study is to determine how local chemical environments that are less polar than water, and perhaps akin to those experienced by an RNA at the surface of a protein (16), influence the folding of structured nucleic acids. To accomplish this task, we chose to use binary mixtures of water and various organic solvents to systematically adjust the polarity of buffered solutions containing either secondary or tertiary structural elements capable of folding into well-defined structures. Our findings indicate that the apolar environments provided by these cosolvent systems profoundly impact the structural properties of RNAs as well as the energetics of folding.

Dielectric constant of binary aqueous-organic solvent mixtures

Several different scales have been introduced to characterize the polarity of pure solvents. Empirical scales for solvent polarity are often based on a variety of spectroscopic, equilibrium, or kinetic measurements of the solvent or solutes dissolved within it (34). Multiparameter approaches have also been developed that are based on other measurable physical properties of the solvent, such as the refractive index or dielectric constant (34). Despite these efforts, solvent polarity remains difficult to quantify, particularly for miscible aqueous-organic mixtures (34). Therefore, we simply chose to use literature-reported values of the relative dielectric constant (or permittivity), ϵ_r , to quantify the changing polarity of these cosolvent systems (Fig. 1 A). In this study, the structural and energetic aspects of RNA folding were studied in the presence of different binary mixtures of water and various amounts of MeOH, EtOH, IPA, ACN, and DMK. Because the dielectric constants of these pure organic solvents are lower than that of pure water, increasing amounts of organic solvent produce mixtures with decreasing values of ϵ_r (Fig. 1 A).

Two model RNA constructs

The donor and acceptor fluorophores of the HP-RNA construct are separated in primary structure by a long sequence of adenosines (see Materials and methods). Importantly, the five complementary nucleotides on either side of this sequence allow the two ends of the oligonucleotide to temporarily basepair with each other (14), resulting in the formation of a short, duplex-like, secondary structural element (Fig. 1 B). The HP-RNA molecules that adopt more expanded and unfolded conformations will have lower transfer efficiency values than the more compact folded conformations. Similarly, the fluorophores of the TL-RNA construct are located near the GAAA tetraloop and its cognate receptor. When

the tetraloop docks into its receptor, the resulting tertiary structure is temporarily held in place by multiple noncanonical hydrogen-bonding and base-stacking interactions (33). In this way, folding of the TL-RNA brings the two fluorophores closer together, giving rise to molecules with higher transfer efficiencies than their unfolded counterparts (38). For both RNAs, structure formation is governed by the molecular forces they experience in solution, including Coulombic repulsions between nucleotide phosphates, van der Waals interactions, hydrogen bonds, and steric clashes. Any alterations to the above factors, including those brought about by the polarity of the local chemical environments, can affect the structure and relative energetic stability of the folded and unfolded conformations.

RNA folding in an aqueous solvent

In single-molecule FRET measurements under standard aqueous conditions, both RNA constructs adopt two distinct conformations, as indicated by the bimodal distributions observed in transfer efficiency histograms (Fig. 3, A and B, *top*). The mean transfer efficiency and fractional abundance of each subpopulation were quantified by fitting the distributions to a sum of two normal distributions (see Materials and methods). For the HP-RNA (Fig. 3 A, *top*), the high-transfer-efficiency folded subpopulation has a mean value of $^{HP}\langle E \rangle_f \approx 0.95$ and the low-transfer-efficiency unfolded subpopulation has a mean value of $^{HP}\langle E \rangle_u \approx 0.25$. Under these conditions, the fractional abundance of the folded conformation is $^{HP}\theta_f = 47.5\%$. The folded and unfolded conformations of the TL-RNA construct (Fig. 3 B, *top*) are located at slightly more intermediate transfer efficiencies with $^{TL}\langle E \rangle_f \approx 0.8$ and $^{TL}\langle E \rangle_u \approx 0.4$, respectively. Here, the fractional abundance of the folded conformation of the TL-RNA is $^{TL}\theta_f = 29.2\%$.

Importantly, the fractional abundance of the folded and unfolded conformations can then be used to estimate the equilibrium constant and thus the standard state Gibbs free energy change associated with these two conformational equilibria. For the two RNA constructs under standard aqueous conditions, we get $^{HP} \mathcal{G}_{fold} = 0.056 \text{ kcal mol}^{-1}$ and $^{TL} \mathcal{G}_{fold} = 0.525 \text{ kcal mol}^{-1}$ for the HP-RNA and TL-RNA constructs, respectively. These free energy values under our standard aqueous conditions will serve as a reference point for additional measurements conducted in binary aqueous-organic solvent mixtures.

RNA folding in apolar environments

To study the structural and energetic properties of RNA folding in apolar chemical environments, we carried out similar measurements in binary aqueous-organic solvent mixtures consisting of increasing amounts of MeOH (Fig. 3). From these histograms, one can see how the mean transfer efficiency, $\langle E \rangle$, and fractional abundance, θ , of the two distinct subpopulations change as the RNAs are exposed to increasingly apolar conditions. To further elucidate the structural and energetic aspects of RNA folding in apolar chemical environments, additional single-molecule FRET experiments were conducted using EtOH and IPA as additional protic solvents, as well as ACN and DMK as aprotic solvents (Fig. S2).

Apolar chemical environments compact unfolded RNAs—Upon inspection of the MeOH data sets (Fig. 3), one can clearly see that increasingly apolar environments,

containing higher amounts of organic cosolvent, tend to increase the mean transfer efficiency of the unfolded subpopulations of both the HP-RNA and TL-RNA constructs. This trend can be quantified and visualized in plots of $\langle E \rangle$ vs. ϵ_r (Fig. 4, A and B), where ϵ_r systematically decreases as the amount of organic solvent increases. The same general trend is also apparent when using any of the other four organic cosolvents, i.e., more apolar environments, characterized by lower values of ϵ_r , consistently yield higher values of $^{HP}\langle E \rangle_u$ and $^{TL}\langle E \rangle_u$.

The overall magnitude of the increase is largely independent of cosolvent identity, suggesting that other, nondielectric properties of these aqueous-organic mixtures (e.g., index of refraction) are not responsible for the observed trends (Table S1). Additionally, minor solvent-dependent changes in the photophysical properties of the fluorophores (e.g., fluorescence lifetime) cannot account for the observed changes in mean transfer efficiency (Tables S2 and S3). Therefore, this systematic shift to higher mean transfer efficiencies suggests that the unfolded conformations associated with the HP-RNA (Fig. 4 A) and TL-RNA (Fig. 4 B) constructs become more compact in apolar environments.

Interestingly, the mean transfer efficiency of the folded conformation of the HP-RNA was largely insensitive to the polarity of the local chemical environment. This finding likely suggests that the geometry and orientation of the canonical basepairing interactions responsible for the formation of RNA secondary structure are not heavily influenced by the polarity of the local chemical environment. Presumably, this is because the bases are sequestered within the interior of the resulting duplex, where the polarity of the local chemical environment is dominated by the dielectric properties of the nucleic acid rather than the solvent. However, our quantitative analysis does reveal a subtle increase in the mean transfer efficiency associated with the folded conformation of the TL-RNA construct in aqueous-organic mixtures (Fig. 4 B). Although the change in transfer efficiency is quite small, it persists across all cosolvents, and therefore this finding may be indicative of a slight molecular compaction and/or conformational rearrangement that occurs when RNA tertiary structures are exposed to apolar conditions.

Apolar chemical environments can make folding more energetically favorable

—One of the many advantages of single-molecule FRET over conventional ensemble methods is its ability to study individual subpopulations (e.g., folded and unfolded) within a heterogeneous sample. The transfer efficiency histograms for both the HP-RNA and TL-RNA clearly exhibit at least two easily distinguishable subpopulations across a wide range of solution conditions (Fig. 3). Using our Gaussian fitting analysis, we were able to calculate the fractional abundance, θ , of the folded and unfolded species in each of the measurements, which reflects the relative concentrations of the two subpopulations in solution.

The direct relationship between the folding equilibrium constant ($K_{fold} = \theta_f / \theta_u$) and the Gibbs free energy change associated with folding ($\Delta G_{fold} = -RT \ln K_{fold}$) allows us to use our single-molecule FRET data to determine how the polarity of the local chemical environment alters the energetics of RNA folding. To accomplish this, we calculate the difference between ΔG_{fold} in the binary aqueous-organic solvent mixtures and pure water, where $\Delta G_{fold} = \Delta G_{fold}(\text{mixture}) - \Delta G_{fold}(\text{H}_2\text{O})$. As summarized in the plots

of G°_{fold} vs. ϵ_r (Fig. 4, C and D), folding free energies associated with the two RNA constructs responded quite differently to increasing amounts of the various organic cosolvents. Indeed, previous investigations of nucleic acid folding in aqueous-organic mixtures have also reported differential effects on the energetics of secondary and tertiary structure formation (19,21). Here, the values of G°_{fold} for TL-RNA systematically increase under apolar experimental conditions with lower values of ϵ_r (Fig. 4 D), suggesting that the energetic stability of this tertiary interaction is primarily influenced by the changing polarity. In contrast, the values of G°_{fold} for the HP-RNA vary nonmonotonically with increasing organic composition and are highly dependent on the identity of the cosolvent (Fig. 4 C), clearly indicating that cosolvent polarity is not the only physicochemical properties of these cosolvents that influences the folding energetics of this RNA.

Upon further inspection, our data provide a potential explanation for the highly variable folding energetics associated with the HP-RNA (Fig. 4 C). First, at low to intermediate concentrations, the protic cosolvents tend to be stabilizing, whereas the aprotic cosolvents tend to be destabilizing. Second, the changes in folding energetics for the HP-RNA correlate quite well with the cosolvents ability to act as a hydrogen-bond acceptor (HBA), as quantified via the β_1 scale (42). Specifically, folding becomes slightly more favorable in solutions where the cosolvents are relatively strong HBAs (i.e., IPA $\beta_1 = 0.68$; EtOH $\beta_1 = 0.62$; MeOH $\beta_1 = 0.54$), whereas folding becomes slightly more unfavorable in solutions where the cosolvents are less effective HBAs (i.e., DMK $\beta_1 = 0.49$; ACN $\beta_1 = 0.37$). Together, these observations seem to indicate that these aqueous-organic mixtures not only alter the polarity of the solution, but they also alter hydrogen-bonding interactions within or between the solvent and the solutes (e.g., RNA and ions) and that these alterations noticeably influence the energetics associated with HP-RNA folding.

Furthermore, the cosolvent identity and concentration-dependence of $-G^{\circ}_{fold}$ for the HP-RNA (Fig. 4 C) mirrors reported values (43) for the change in solvation entropy (H°_{solv}) associated with transferring KCl from pure water to a binary aqueous-organic solvent mixture (Fig. S4). The strong relationship between these two thermodynamic properties suggests that the folding energetics observed for the HP-RNA are not dominated by the changing polarity (as observed with the TL-RNA), but rather that they are dominated by the changing energetics of counterion solvation. More specifically, folding of the HP-RNA becomes more favorable when solvation of the dominant salt (e.g., KCl) is less enthalpically favorable relative to pure water, making it easier for the RNA to displace the solvent and engage in direct electrostatic interactions with the ion. Because this behavior was not apparent for both RNAs, it is likely that any potassium ions taken up during folding of the HP-RNA are partially desolvated and directly coordinated by the RNA, whereas the ions taken up during the folding of the TL-RNA (8) are likely to remain fully solvated.

RNA folding in salty environments

The above results show that apolar chemical environments compact the unfolded structural ensembles of both RNAs and, in the case of tertiary structure formation, shift the conformational equilibrium to favor folding. These observations are surprisingly similar to the effect that monovalent cations have on the structural and energetic properties of

RNA folding (14,44), perhaps because both can profoundly alter the electrostatics of RNA folding. To more robustly assess these similarities, measurements were conducted on the HP-RNA and TL-RNA in purely aqueous solutions over a wide range of KCl concentrations (Fig. 5). As expected, the unfolded conformations of the two model RNAs also became more compact upon addition of KCl. Furthermore, this compaction was also accompanied by a stabilization of the folded conformations associated with both constructs.

DISCUSSION

It is generally accepted that the molecular structures arising from RNA folding are held in place by hydrogen bonds, the stacking of the aromatic bases, and electrostatic interaction between the negatively charged phosphates and any counterions present in solution (45,46). Over the years, numerous research endeavors have been conducted with the goal of providing a fundamental understanding of these inter- and intramolecular interactions. However, we are only beginning to understand how the polarity of the local chemical environment influences the structural and energetic aspect of the folding process.

In a recent study, Nordén and co-workers observed local breathing and unstacking of double-stranded DNA in “semihydrophobic” solutions containing water and various amounts of ethylene glycol ethers such as diglyme and PEG-400 (47). Using ensemble spectroscopic techniques, Nakano and Sugimoto have also suggested that aqueous-organic solvent mixtures can alter the structural stability and catalytic activity of various nucleic acids (17–25) and generally attribute these effects to decreases in both the dielectric constant and thermodynamic activity of water. However, the insights gleaned from these studies are not strictly limited to a fundamental understanding of the physical principles governing RNA folding. For example, the cytosolic environment is also thought to be slightly apolar, and therefore, aqueous-organic solvent mixtures have also been used to mimic the cellular milieu (19). The effects of solvent polarity on RNA folding may also shed light on the molecular function of many important macromolecular machines assembled from both protein and nucleic acid subunits because the surfaces of these protein subunits are substantially less polar than the surrounding aqueous environment (16,20).

In this study, we explored the effect of solvent polarity on both the structural and energetic properties of RNA secondary and tertiary structure formation using single-molecule fluorescence techniques. Importantly, the use of single-molecule approaches allows us to simultaneously monitor the structural and energetic properties of RNAs using quantitative experimental observables (i.e., the mean transfer efficiency $\langle E \rangle$ and Θ , the fractional abundance) associated with distinct subpopulations (e.g., folded and unfolded) within heterogeneous molecular ensembles. We chose to systematically alter solvent polarity, quantified via its dielectric constant, by mixing increasing weight percentages of five different organic solvents with water (Fig. 3 A). Our results show a clear compaction of unfolded RNAs in increasingly apolar environments with dielectric constants less than that of pure water, regardless of which solvents are used. The overall extent of compaction, reflected by an increase in the $\langle E \rangle$, scales with the decreasing dielectric constant of the binary aqueous-organic solvent mixtures (Fig. 4, A and B). Importantly, a similar degree

of compaction can also be achieved by simply increasing the concentration of monovalent cations in a purely aqueous solution (Fig. 5). How can we explain these observations?

As described by Coulomb's law (Eq. 3), the magnitude of the electrostatic force, F , between two species separated by a distance, r , with net charges, q_1 and q_2 , is inversely proportional to the relative dielectric constant or permittivity, ϵ_r , of the surrounding medium.

$$F \propto \frac{1}{4\pi\epsilon_r} \frac{q_1 q_2}{r^2}. \quad (3)$$

Thus, the attractive force between an RNA molecule and any neighboring counterions in solution will increase in apolar environments where the value of ϵ_r is less than that of pure water. The enhanced Coulombic interaction between 1) the electrostatic potential generated by negatively charged phosphates and 2) nearby solvated cations will increase the total positive charge density in the ion atmosphere (48) surrounding the RNA. This uptake of counterions from the bulk will then lead to more effective screening of the intramolecular electrostatic repulsion between phosphate groups relative to a purely aqueous solvent, allowing the unfolded RNA to more readily sample compact structures. Indeed, our experimental observations in apolar conditions are remarkably consistent with such a prediction.

Furthermore, the compaction of these unfolded RNAs increases the effective local concentration of the nucleotides involved in the formation of higher-order structures. Compaction may also bias the unfolded ensemble to mimic the compact transition states commonly observed in RNA folding experiments (49–51). In both cases, the net result would be an RNA that is primed to fold, which would increase the folding rate constant and lead to more energetically stable folded structures. Notably, we also observed this stabilizing effect for the folding of the TL-RNA construct. Similar observations supporting the notion that apolar chemical environments stabilize the formation of RNA tertiary structures have also been reported previously (52,53). Based on these findings, we conclude that apolar environments, characterized by dielectric constants less than that of pure water, can promote folding via the following physical mechanism: the low dielectric environment facilitates recruitment of counterions, which leads to more compact unfolded structures that are primed to fold, thereby shifting the equilibrium to favor the folded state.

CONCLUSIONS

In this study, we altered the polarity of the local chemical environment surrounding two model RNA using binary mixtures of water and various organic cosolvents. Our results indicate that apolar environments, characterized by dielectric constants less than that of water, generally compact unfolded RNA molecules. We propose that these apolar environments give rise to enhanced condensation of counterions around the negatively charged phosphate groups of the RNA, which minimizes interphosphate repulsion and allows the unfolded RNA to sample more compact conformations. Indeed, our conclusion is bolstered by a recent study that showed nanoscale hydrophobic environments increase

the strength of salt bridges formed in an otherwise aqueous media (54), a phenomenon first discussed with regard to protein folding nearly 70 years ago (55).

Furthermore, we observed that this molecular compaction is also accompanied by an energetic stabilization of folded RNA tertiary structures, presumably reflecting a reduction of the conformation search associated with the folding process. Here, it is of course important to note that environments near the surfaces of proteins are quite apolar, where the value of ϵ_r is thought to be three to four times lower than that of pure water (16). From this simplified point of view, it is reasonable to expect the local chemical environment around an RNA within a ribonucleoprotein complex may in fact be quite apolar, which, as we have shown, can alter the structural and energetic properties of RNA folding. These dielectric effects associated with apolar environments at the surfaces of proteins may also help explain why the unfolded nucleic acid clients of RNA folding chaperones become significantly more compact after associating with their proteinaceous binding partners (14).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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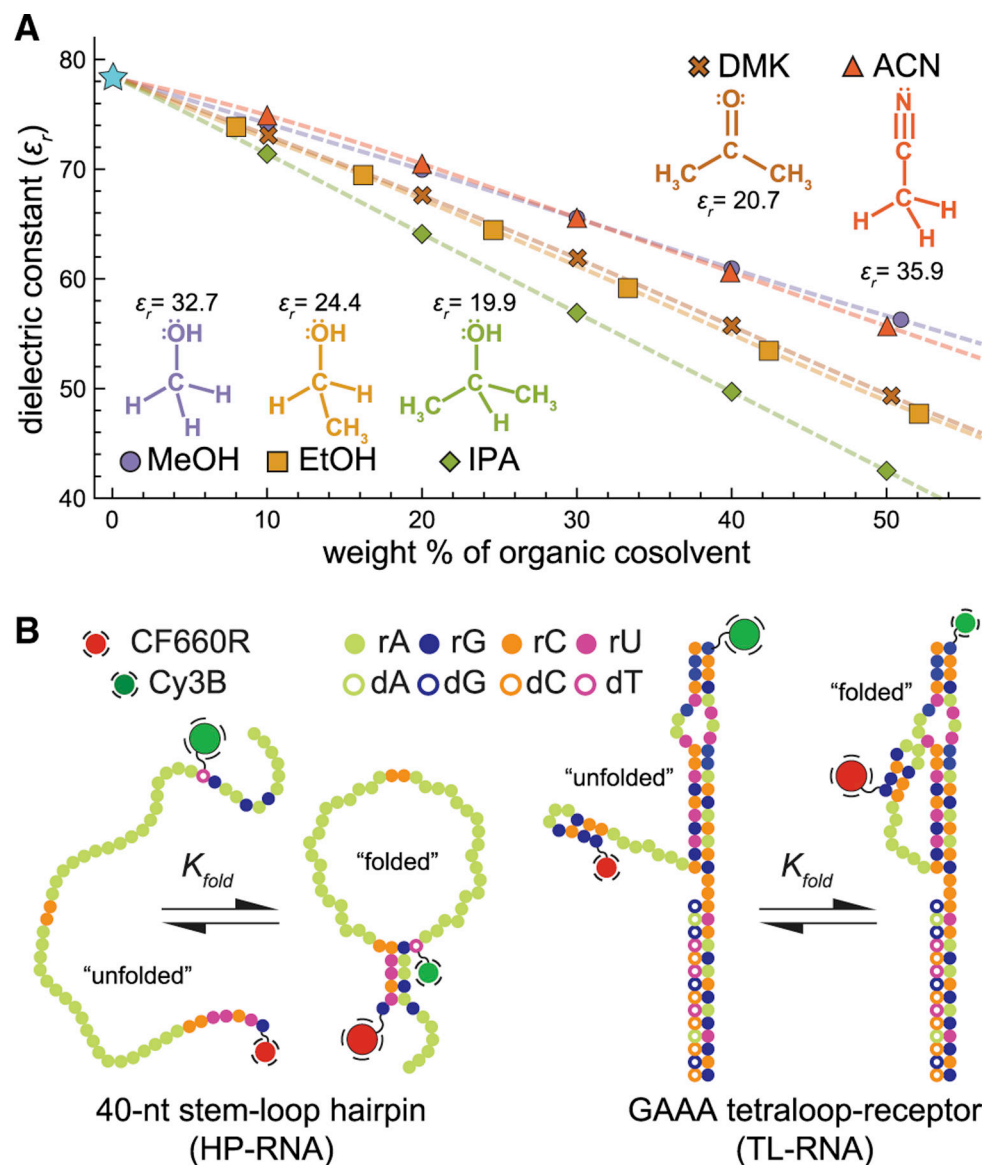
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WHY IT MATTERS

The biological functions of noncoding RNAs are often closely related to their ability to form intricate three-dimensional structures. These structures are stabilized by several weak interactions within the RNA and between the RNA, the solvent, and any other solutes in solution. Here, we demonstrate that chemical environments that are less polar than water (and akin to those that an RNA might experience when bound to a protein or in any number of biotechnological applications) generally compact unfolded RNA conformations and, in some cases, can shift conformational equilibria to favor the formation of folded structures. These findings shed light on the physical rules that govern how noncoding RNAs adopt intricate structures essential to all living entities.

**FIGURE 1.**

Formation of RNA secondary and tertiary structures in apolar environments. The relative dielectric constant (ϵ_r) is used to quantify solvent polarity. (A) Reported values of ϵ_r for several binary aqueous-organic solvent mixtures at 25°C. Solvent polarity monotonically decreases with increasing amounts of organic solvent. Values for water-MeOH, water-EtOH, and water-DMK were taken from (30). References (31) and (32) were used to derive the dielectric constants of water-IPA and water-ACN mixtures, respectively. (B) Color-coded sequence diagrams of the two model RNA constructs used in this study. Folding of the HP-RNA (*left*) results in the formation of five canonical basepairs, which hold together the hairpin secondary structure with a large poly-rA loop. Folding of the TL-RNA (*right*) involves docking the GAAA tetraloop into its cognate receptor. This common tertiary structural motif is held together by several noncanonical interactions (33). For the single-molecule fluorescence studies, both RNAs were modified with Cy3B and CF660R, which

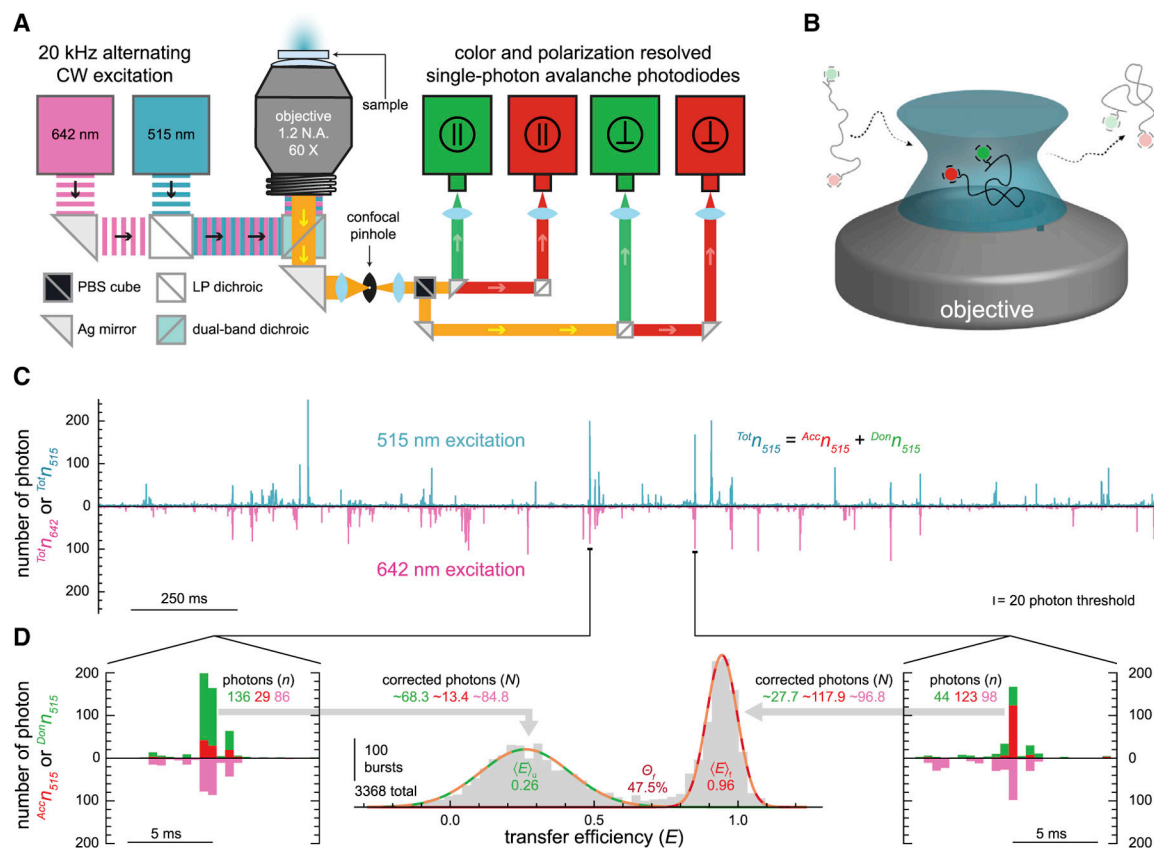
function as the FRET donor and acceptor fluorophores, respectively. For both RNAs, the efficiency of energy transfer from the donor to the acceptor is highest in the folded conformation.

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**FIGURE 2.**

Experimental design and analysis. (A) Schematic overview of a single-molecule confocal fluorescence microscope. The alternating (20 kHz) output from two diode lasers (515 and 642 nm) is directed into the back aperture of the water immersion objective using a dual-band dichroic mirror resulting in a diffraction-limited focal spot. The Stokes-shifted fluorescence emitted from the sample is collected by the same objective and directed through a 100 μm pinhole to remove out-of-plane light. A polarizing beam splitter (PBS) cube and a set of long-pass (LP) dichroic mirrors spatially separates the emission into four photon streams based on polarization and color. The emitted photons in each of the four streams are detected by four separate single-photon avalanche photodiodes. (B) Cartoon depicting the confocal volume of an inverted confocal microscope (not to scale). The donor and acceptor-labeled RNA molecules that stochastically diffuse through the confocal volume get excited by the focused laser beams producing a burst of photons. (C) Representative 2.5 s segment of a 900 s fluorescence time trace with 500 μs time bins depicting numerous bursts of photons resulting from alternating 515 and 642 nm excitation of the FRET-labeled RNAs (100 pM RNA, 25 mM HEPES, 12.5 mM NaOH, 250 mM KCl, 0.01% v/v Tween 20). (D) Insets on the left and right are representative 12.5 ms segments of the data in (C), each depicting a burst of photons. Transfer efficiency (E) and stoichiometry (S) values are calculated for each burst of photons recorded during the measurement using Eq. 1 and Eq. 2, respectively. The values of E arising from those bursts with more than 50 total corrected photons emitted by FRET-labeled molecules (i.e., $0.25 < S < 0.75$) are then compiled into a transfer efficiency histogram. Histograms are fitted to a sum of two Gaussian functions to

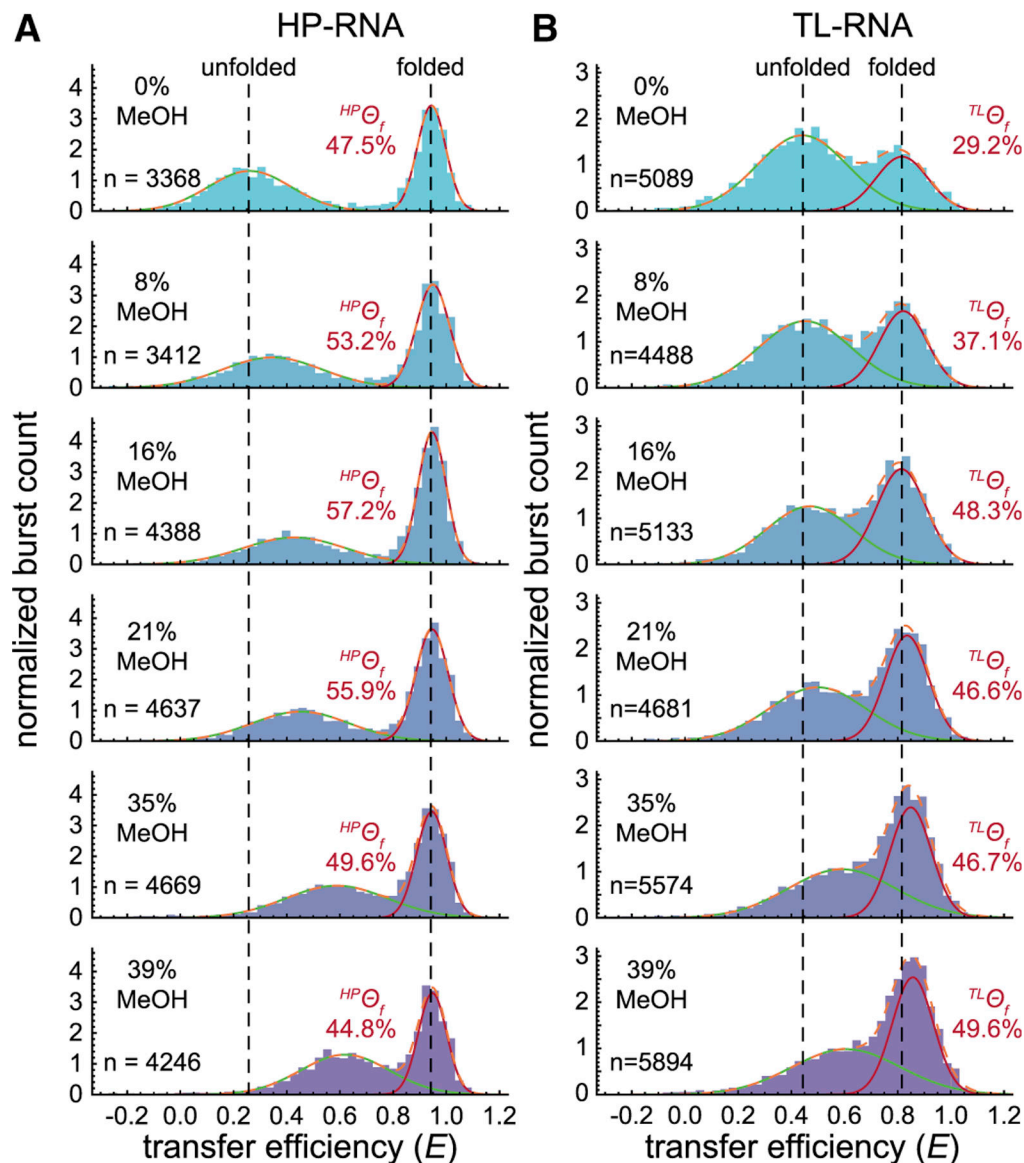
quantify the mean transfer efficiency, $\langle E \rangle$, and fractional abundance, θ , of the folded and unfolded subpopulations, with typical experimental uncertainties of $\langle E \rangle \pm 0.02$ and $\theta \pm 0.03$.

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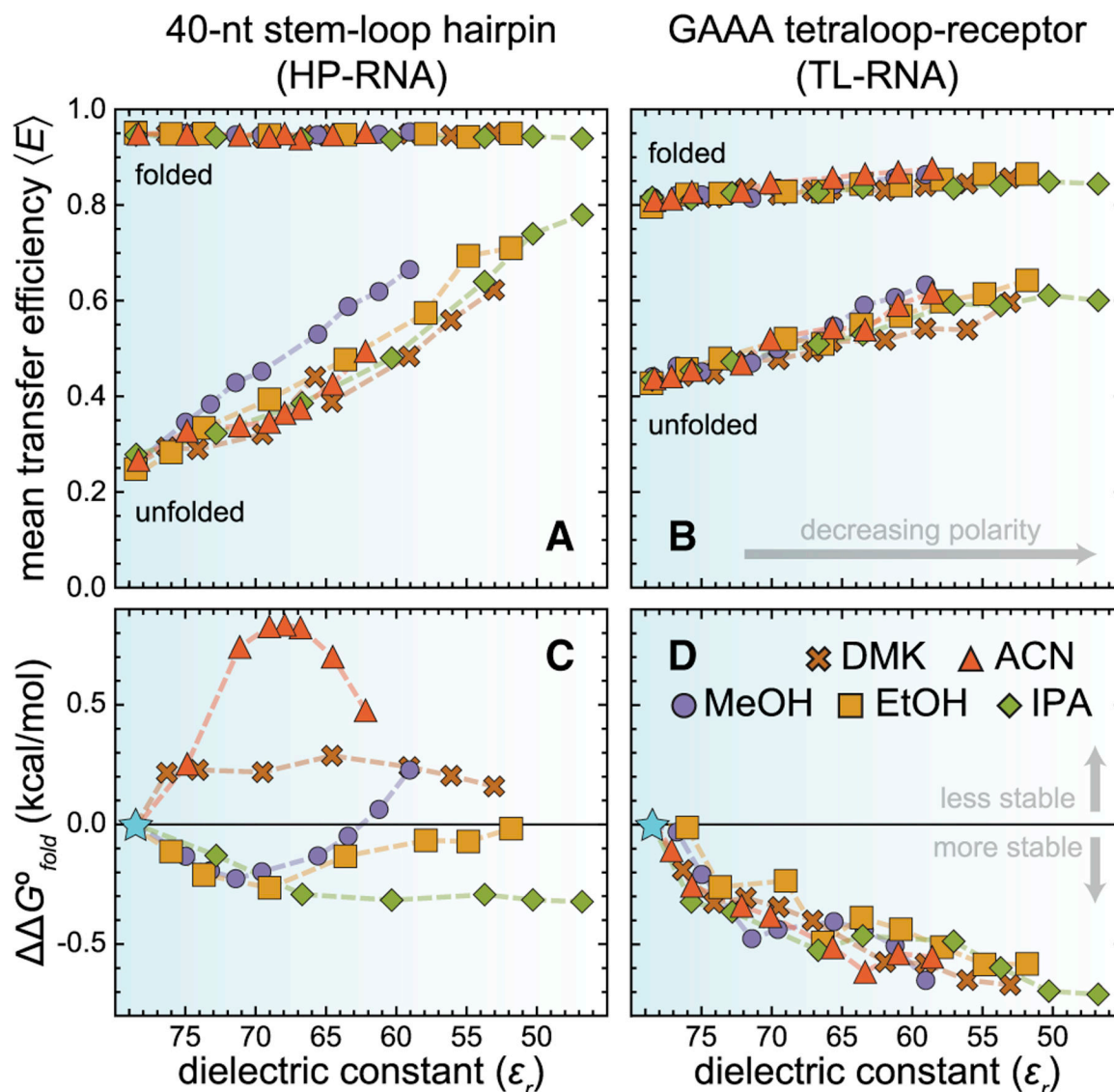
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**FIGURE 3.**

Effect of MeOH on RNA folding. Single-molecule transfer efficiency histograms for the HP-RNA (*A*) and TL-RNA (*B*) constructs in various mixtures of H₂O-MeOH are shown. Vertical dashed lines show how the high- and low-transfer-efficiency subpopulations change relative to the pure aqueous conditions (*top*). For the HP-RNA construct, the addition of MeOH as a cosolvent greatly increases the mean transfer efficiency of the unfolded subpopulation while slightly modulating the fractional abundance of the folded subpopulation. For the TL-RNA construct, MeOH increases the mean transfer efficiency of both subpopulations in addition to systematically increasing the fractional abundance of the folded subpopulation (100 pM RNA, 25 mM HEPES, 12.5 mM NaOH, 250 mM KCl, 0.01% v/v Tween 20, and the specified amount MeOH).

**FIGURE 4.**

Apolar chemical environments compact unfolded RNAs. The mean transfer efficiencies of the folded and unfolded subpopulations of the (A) HP-RNA and (B) TL-RNA constructs plotted against the relative dielectric constant, ϵ_r , of various binary aqueous-organic solvent mixtures. Apolar conditions, characterized by values of ϵ_r that are less than pure water (≈ 78.5), strongly affect the mean transfer efficiency of the unfolded conformations of both RNAs, with almost no dependence on cosolvent identity. Notably, the mean transfer efficiency of folded HP-RNA, $^{HP}\langle E \rangle_f$ and the folded TL-RNA, $^{TL}\langle E \rangle_f$ were much less sensitive to changes in ϵ_r . The differential free energy change for RNA folding ($\Delta\Delta G^\circ_{fold}$) in apolar environments is shown for two RNA constructs designed to probe the formation of (C) secondary structure (HP-RNA) and (D) tertiary structure (TL-RNA). Based on our definition of $\Delta\Delta G^\circ_{fold}$ (see Materials and methods), negative values represent conditions that stabilize the folded conformation (100 pM RNA, 25 mM HEPES, 12.5 mM NaOH, 250 mM

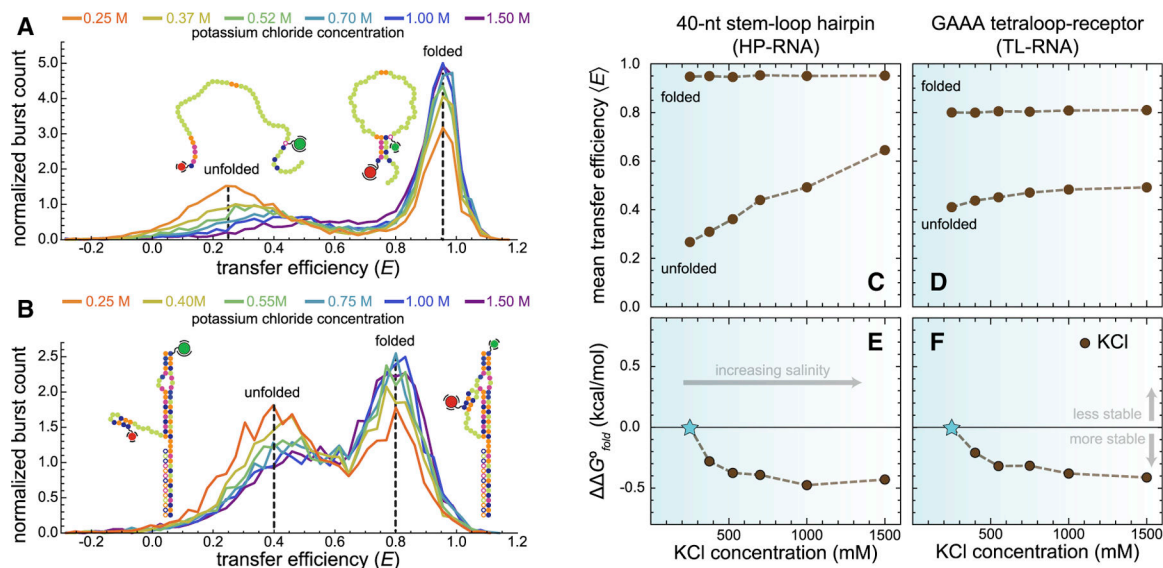
KCl, 0.01% v/v Tween 20, and an amount of organic solvent to achieve the specified value of ϵ_r . The corresponding weight percentages for each measurement are shown in Fig. S3).

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**FIGURE 5.**

Effect of KCl concentration on the conformational dimensions and folding equilibria of RNA secondary and tertiary structure formation. Transfer efficiency histograms show compaction of the unfolded conformations of the (A) HP-RNA and (B) TL-RNA at elevated KCl concentrations. Conversely, the concentration of KCl has almost no effect on the mean transfer efficiency associated with folded conformations of the HP-RNA and TL-RNA, as shown by the dashed line at $^{HP}\langle E \rangle_f \approx 0.95$ and $^{TL}\langle E \rangle_f \approx 0.8$, respectively. However, in both cases, the two RNAs tend to favor the folded conformation at high concentrations of KCl (100 pM RNA, 25 mM HEPES, 12.5 mM NaOH, 0.01% Tween 20, and, unlike the previous experiments, no organic solvent but instead variable concentrations of KCl). Mean transfer efficiencies of the folded and unfolded subpopulations of the (C) HP-RNA and (D) TL-RNA constructs are plotted against KCl concentration. The differential free energy change for folding ($\Delta\Delta G_{fold}^{\circ}$), relative to 250 mM KCl, associated with the HP-RNA and (E) TL-RNA (F) is plotted against KCl concentration.