Materials and Methods

Chemically synthesized RNA oligonucleotides were purchased from Dharmacon and a DNA oligonucleotide was purchased from Integrated DNA Technologies. Cy3 and Cy5 N-succinimidyl esters were purchased from Amersham Biosciences. MgCl$_2$, PEG 400, and dextran were from Sigma and glycerol from Mallinckrodt. All other chemicals were from Fisher Scientific.

Oligonucleotide Sequences:

Two RNA constructs were used for this study, the GAAA tetraloop-receptor construct shown in Figure 1a and a non-docking control construct with a UUCG tetraloop substituted for the GAAA. The nucleotide sequences of the RNA oligos were:

1) 5’GGCGAAAGCCAAAAAAACGUGUCGUCCUAAGUCGGC
2) 5’GGCUUCGGCCAAAAAAACGUGUCGUCCUAAGUCGGC
3) 5’GCCGAUAUGGACGACACGCCCUCAGACGAGUGCG

Each RNA oligonucleotide included an amino group attached via a 3-carbon linker to the 5’ phosphate to facilitate site-specific fluorescence labeling. RNAs 1) and 3) were base-paired to form the GAAA tetraloop-receptor construct and 2) and 3) to form the UUCG construct. The constructs used in this study were also base-paired to a 5’-biotin modified DNA oligonucleotide with the sequence 5’CGCAGACTCGTCTGAG to form the bottom helix in Figure 1a. This biotin modification was used in previous studies and was retained here for direct comparison to the previous results.$^{1,2}$

Preparation of RNA:

The RNA oligonucleotide strands were 2’-deprotected following manufacturer protocols. All oligonucleotides were desalted by passing over NAP-5 G-25 size exclusion columns.
(Amersham Biosciences) prior to use. The RNA strands were labeled by incubating with Cy3 (FRET donor) or Cy5 (FRET acceptor) N-succinimidyl esters (Amersham Biosciences) according to manufacturer protocols. The labeled RNAs were purified by C-18 reverse-phase HPLC. Stock solutions of the assembled RNA constructs were made by mixing 1 µM of the appropriate Cy3-RNA strand, 1.5 µM of the complementary Cy5-RNA strand, and 2.0 µM of the biotin-DNA strand, heating to 70 °C for 3 min and slow cooling to room temperature. The stock solutions were then diluted to achieve the final RNA concentrations described below. All experiments were performed at 25 °C with solution conditions of 50 mM HEPES pH 7.5, 100 mM NaCl, and 0.10 mM EDTA. These conditions are referred to as 1X buffer.

**Procedure for Hydrostatic Pressure Experiments:**

For each experiment, approximately 1.0 mL of 20 nM RNA sample with a given Mg$^{2+}$ concentration was placed in a custom-made quartz cuvette that was sealed with movable pistons. All visible air bubbles were removed prior to sealing and pressurizing. The cuvette was placed in a custom-built high-pressure cell with sapphire windows to allow fluorescence data collection. Temperature in the cell was maintained at 25 °C by a circulating water bath. The cell was pressurized with a manually driven high-pressure generator (High Pressure Equipment, Erie, PA) using ethanol as the pressure transmitting fluid. Using a fluid to transmit pressure is much safer than using air, since the compressibility of the fluid (and hence the potential for $P\Delta V$ work in the event of a leak or break) is <100 times less than for air. This system has been rated to operate at pressures exceeding 3,000 bar. Pressures ranging from 1 to 2,500 bar were sampled in this study. For safety and to minimize temperature perturbations to the RNA, the hydrostatic pressure was slowly adjusted to each target value over a period of 1.5-2 min. and equilibrated for 6 min.
Fluorescence experiments were conducted using a spectrofluorometer modified to house the pressure cell assembly.

Fluorescence spectra were measured at each pressure with excitation at 500 nm (8 nm bandwidth) and with emission measured from 530 to 725 nm (8 nm bandwidth, 1 nm step-size, 1.0 s integration time per step). For each sample, two spectra were recorded at each pressure, one as pressure was incrementally increased and the other as it was incrementally decreased. Experiments were performed on three or more samples for each Mg\(^{2+}\) condition, and errors for the FRET data were calculated as the standard deviation between these experiments.

FRET efficiency was approximated as

\[
E_{\text{FRET}} = \frac{I_A}{I_D + I_A} \tag{1}
\]

where \(I_A\) is the total acceptor intensity and \(I_D\) is the total donor intensity. \(I_D\) was calculated by summing the intensities from 549-620 nm and \(I_A\) by summing from 655-725 nm. The \(I_D\) and \(I_A\) values were corrected to account for the effect of pressure on donor- or acceptor-only labeled RNA samples (data not shown). This correction reduced the magnitudes of the maximum pressure-induced changes in \(E_{\text{FRET}}\) by \(~0.01\).

**Determination of Thermodynamic Parameters from Hydrostatic Pressure Data:**

The equilibrium constant for GAAA tetraloop-receptor docking, \(K_{\text{dock}}\), was calculated at each pressure from the FRET data by assuming that the observed \(E_{\text{FRET}}\) is the population-weighted average of the \(E_{\text{FRET}}\) values for the docked and undocked states:

\[
E_{\text{FRET}} = e_d(f_{\text{docked}}) + e_u(1 - f_{\text{docked}}) \tag{2}
\]

where \(f_{\text{docked}}\) is the fraction docked and \(e_d\) and \(e_u\) are the \(E_{\text{FRET}}\) values for the docked and undocked states, respectively. By definition:

\[
K_{\text{dock}} = \frac{f_{\text{docked}}}{1 - f_{\text{docked}}} \tag{3}
\]
Substituting and solving for $K_{\text{dock}}$ yields:

$$K_{\text{dock}} = \frac{E_{\text{FRET}} - e_u}{e_d - E_{\text{FRET}}}$$

(4)

The $e_d$ value was estimated from the $E_{\text{FRET}}$ at 20 mM Mg$^{2+}$, which showed a maximum $E_{\text{FRET}}$ of 0.367 at ambient pressure (Figure S1). Since previously reported data from single-molecule experiments have shown that the docked population is $>95\%$ at 20 mM Mg$^{2+}$, the $E_{\text{FRET}}$ of 0.367 must be close to the maximum value. Thus, a value of 0.38 is a good estimate for $e_d$. An $e_u$ value of 0.13 was calculated from Equation 4 using the measured $E_{\text{FRET}}$ of 0.21 at zero Mg$^{2+}$ and 1 bar, the $K_{\text{dock}}$ of 0.49 at this [Mg$^{2+}$] from the single-molecule studies,$^1$ and the $e_d$ of 0.38. Varying the $e_d = 0.38$ and $e_u = 0.13$ values by $\pm 0.03$ changes the calculated $\Delta V$s by less than 50% for the 0, 0.20, and 1.0 mM Mg$^{2+}$ data, and this uncertainty was included in the errors reported in Table 1.

The $e_u$ value could not be estimated from the UUCG $E_{\text{FRET}}$ data because the previous single-molecule studies showed that the GAAA and UUCG constructs have significant populations of molecules that either do not have acceptor fluorophores or where the acceptor has photobleached.$^1$ These donor-only molecules alter the average $E_{\text{FRET}}$ observed for each construct in ensemble measurements. Thus, the measured $E_{\text{FRET}}$ for the undocked states will not necessarily be the same for the two constructs.

The thermodynamic relationship between pressure and chemical equilibrium is described by the equation$^3$:

$$\frac{\partial \ln(K_{\text{eq}})}{\partial P} = -\frac{\Delta V}{RT}$$

(5)

where $K_{\text{eq}}$ is the equilibrium constant for the reaction, $P$ is the hydrostatic pressure, $\Delta V$ is the partial molar volume change for the reaction, $R$ is the gas constant, and $T$ is absolute
temperature. Thus, the $\Delta V$ for GAAA tetraloop-receptor docking was determined at each $[\text{Mg}^{2+}]$ by plotting $\ln(K_{\text{dock}})$ versus $P$ fitting to the equation (where $b$ is the y-intercept):

$$\ln(K_{\text{dock}}) = -P\left(\frac{\Delta V}{RT}\right) + b$$  \hspace{1cm} (6)

*Effects of hydrostatic pressure on solution conditions:*

A 2,500 bar pressure increase will cause an 11% decrease in the volume of a 0.100 molal solution of NaCl at 25 ºC, which has a solution compressibility of $4.47 \times 10^{-5}$ bar$^{-1}$. The resulting 11% increase in $[\text{Mg}^{2+}]$ would cause a small $E_{\text{FRET}}$ increase of $\leq 0.004$, as determined from the previously reported $K_{1/2}$ and the maximum change in $E_{\text{FRET}}$ for $\text{Mg}^{2+}$-induced GAAA tetraloop receptor docking.\(^2\) The effect of pressure on the $pK_a$ of the HEPES buffer used here is also small, with a change of only $\sim 0.03$ up to 1000 bar.\(^5\) Thus, the observed decrease in GAAA tetraloop-receptor docking does not result from changes in $[\text{Mg}^{2+}]$ or pH induced by hydrostatic pressure. The data for the non-docking UUCG tetraloop construct confirms that the observed pressure-dependent FRET change results from disruption of GAAA tetraloop-receptor docking (Figure S2).

*Procedure for Cosolute Experiments:*

All experiments were performed at 25 ºC in 1X buffer. RNA samples (0.500 mL, 20 nM RNA) with a given Mg$^{2+}$ concentration were placed in 1.5 mL quartz fluorescence cuvettes in a spectrofluorometer (Photon Technology International). Fluorescence spectra were recorded using the same wavelengths described above, with 4 and 8 nm bandwidths for excitation and emission, respectively, and integration times ranging from 0.3-0.5 s per 1 nm step.

To achieve the desired final concentrations of cosolute, concentrated solutions of PEG 400 (MW = 400 g/mol), sucrose (MW = 342.3 g/mol), or glycerol (MW = 92.1 g/mol) were
serially pipetted into the RNA sample in the fluorometer. For the experiments with dextran (MW = 12,000 g/mol), the highest weight percent dextran data point was collected first, with the lower percents achieved by diluting the RNA/dextran sample with a 1X buffer/Mg\(^{2+}\) solution. The 0% dextran data were collected in separate experiments. Addition of concentrated dextran to 0% samples to reach 14.7% dextran yielded similar \(E_{\text{FRET}}\) values for this concentration to those observed after serial dilution of RNA samples initially containing 34.4% dextran. The concentrated cosolute solutions were prepared by dissolving the pure substance in a solution of 1X buffer with the same MgCl\(_2\) concentration as the RNA sample. Thus, all solutions had the same molality of 1X buffer and MgCl\(_2\). These experiments were performed at constant buffer/Mg\(^{2+}\) molality rather than molarity to minimize changes in Mg\(^{2+}\) activity resulting from the excluded volume of the cosolute. Experiments at constant buffer/Mg\(^{2+}\) molarity yielded similar results (data not shown). The concentrated cosolute solutions were filtered prior to use using either a Nalgene CN filter unit (0.2 \(\mu\)m pore size) or a MFS cellulose acetate syringe-tip membrane (0.22 \(\mu\)m pore size) to remove any light scattering particles.

\(E_{\text{FRET}}\) values were calculated from the fluorescence data by the same procedure described above for the hydrostatic pressure data, including correcting the \(I_D\) and \(I_A\) values for effects measured on Cy3- or Cy5-only RNA samples. The effect of PEG 400 on the non-docking UUCG tetraloop construct was more pronounced than the other cosolutes but still much smaller than its effect on the GAAA construct (Figure S3a). To better represent the cosolute effects specific to GAAA tetraloop-receptor docking, the reported \(E_{\text{FRET}}\) values for the GAAA construct were corrected for effects on the UUCG construct:

\[
E_{\text{FRET, corrected}} = E_{\text{GAAA}} - (E_{\text{UUCG}} - E^*_{\text{UUCG}}) \quad (7)
\]
where $E_{GAAA}$ is the $E_{\text{FRET}}$ for the GAAA construct at a given cosolute weight percent, $E_{UUCG}$ is the $E_{\text{FRET}}$ for the UUCG construct at the same weight percent, and $E_{UUCG}^{\text{U}}$ is the $E_{\text{FRET}}$ for the UUCG construct in the absence of cosolute. This correction has little effect on the sucrose, glycerol, or dextran data but does somewhat reduce the apparent FRET increase with PEG 400 concentration (Figure S3). The data for PEG 400 and for sucrose at 1.0 mM MgCl$_2$ shown in Figure 1d were corrected by Equation 7.

**References:**


Figure S1. Effect of hydrostatic pressure on GAAA tetraloop-receptor docking at 20 mM Mg$^{2+}$.

$E_{\text{FRET}}$ is plotted vs. pressure for the GAAA tetraloop construct at 20 mM MgCl$_2$ (●). The data measured at 0 (□), 0.20 (■), and 1.0 (○) mM MgCl$_2$ that were presented in Figure 1b are shown for comparison.
Figure S2. Hydrostatic pressure dependence of the non-docking UUCG tetraloop construct. 

$E_{FRET}$ is plotted vs. pressure for the UUCG tetraloop construct (▲) at 1.0 mM MgCl$_2$. The

$\Delta E_{FRET}$ for the UUCG construct is < 0.01 between 1 and 2,500 bar. The data from Figure 1b for

the GAAA construct under the same conditions is shown for comparison (○).
Figure S3. Effects of various cosolutes on the GAAA tetraloop and UUCG tetraloop constructs. 
$E_{\text{FRET}}$ is plotted as a function of the weight percent of each cosolute for the GAAA tetraloop-
receptor construct at 1.0 mM (●) and 0.20 mM (□) MgCl$_2$ and for the UUCG construct at 1.0 
mM (○) and 0.20 mM (■) MgCl$_2$. (a) Effect of PEG 400 on the GAAA and UUCG tetraloop 
constructs. (b) Same data as (a) with the $\Delta E_{\text{FRET}}$ from 0% PEG 400 for the UUCG construct 
subtracted from the $E_{\text{FRET}}$ for the GAAA construct (Equation 7). (c) Effect of sucrose on the 
GAAA and UUCG tetraloop constructs. (d) Sucrose data corrected for $\Delta E_{\text{FRET}}$ for the UUCG 
construct. (e) Effect of glycerol on the GAAA and UUCG tetraloop constructs. (f) Glycerol data 
corrected for $\Delta E_{\text{FRET}}$ for the UUCG construct. (g) Effect of dextran 12,000 on the GAAA and 
UUCG tetraloop constructs. (h) Dextran 12,000 data corrected for $\Delta E_{\text{FRET}}$ for the UUCG 
construct. The 1.0 mM Mg$^{2+}$ data in (b) and (d) are also shown in Figure 1d.