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Designed Alteration of Binding Affinity in Structure-Switching Aptamers through the Use of Dangling Nucleotides

Sladjana Slavkovic, Sophie R. Eisen, and Philip E. Johnson*

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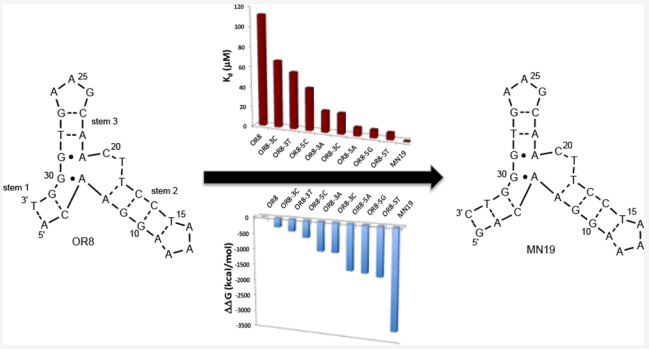
ABSTRACT: The ability to change binding affinity in a controlled fashion is a key step in the rational design of biomolecules in general and functional nucleic acids in particular. Here, we use dangling nucleotides to alter the binding affinity of structure-switching aptamers. Dangling nucleotides can stabilize or destabilize a nucleic acid structure with a known ΔG°_{37}. When the dangling nucleotide stabilizes the structure, less free energy from ligand binding is needed to fold the molecule and hence the ligand is observed to bind tighter than in the absence of the unpaired nucleotide. For a destabilizing dangling nucleotide, the opposite occurs, and the observed binding is weaker. We demonstrate this concept using both the cocaine-binding aptamer and the ATP-binding aptamer systems. We find that for both aptamers there is a direct, but different, relationship between the predicted stabilization and the change in the observed binding free energy.



The ability to alter the function of biomolecules in a designed manner is an important goal in biotechnology. For functional nucleic acids, altering nucleic acid structure and thereby its function is greatly aided by the known thermodynamic contributions to stability of adding or changing base pair identity.[2] An important class of functional nucleic acids consists of structure-switching aptamers. For these aptamers, ligand binding is coupled with a structural transition from an unfolded or loosely folded ligand-free structure to a folded ligand-bound structure.[3−5] Structure-switching aptamers are important in biosensor development where the structural change is converted into a measurable signal corresponding to the binding event. Previous methods of improving aptamer binding affinity have varied from changing the selection methods to altering the sequence of selected aptamers in an essentially random manner to changing the positioning of the aptamer in a molecular beacon.[6−8] Other studies, also looking at structure-switching aptamers, introduced a population-shift mechanism in which stabilizing the nonbinding state is reflected in a tighter observed affinity.[9−11]

The cocaine-binding aptamer is a useful model system for aptamer design.[12−17] This aptamer is structured as a three-way junction centered near a tandem AG mismatch (Figure 1).[18] Two facets of this aptamer that make it particularly interesting are that it undergoes a transition between having a ligand-induced folding mechanism when stem 1 is ≤3 bp long and being prestructured with a ≥4 bp stem 1.[18,19] Additionally, the cocaine-binding aptamer has substantial binding promiscuity in that it binds quinine-based ligands much tighter than cocaine, the ligand for which it was originally selected.[20−25] Previously, we introduced binding selectivity for tighter binding ligands into this aptamer by altering the length of stem 1. When stem 1 is shortened to 2 bp [OR8 (Figure 1)] instead of 3 bp [MN19 (Figure 1)], more free energy from ligand binding is needed to fold the aptamer. Only the tighter binding ligand, quinine, can both fold and bind the OR8 aptamer, while cocaine is not capable of binding OR8.[19]

Here, we fine-tune the binding affinity of the structure-switching cocaine-binding aptamer in a designed manner by introducing unpaired or dangling 3′ or 5′ nucleotides. Unpaired nucleotides can stack against the terminal base pair[26−28] and stabilize, or even destabilize, a nucleic acid structure by a known ΔG°_{37} value.[1] This trend of stacking by dangling nucleotides leads to purines generally being more stabilizing than pyrimidine nucleotides. Previously, we used dangling nucleotides to improve the NMR spectra in an RNA stem−loop structure.[29] For structure-switching aptamers, or aptamers that undergo a ligand-induced folding mechanism, adding a stabilizing dangling nucleotide should reduce the free energy needed to fold the molecule. This free energy needed

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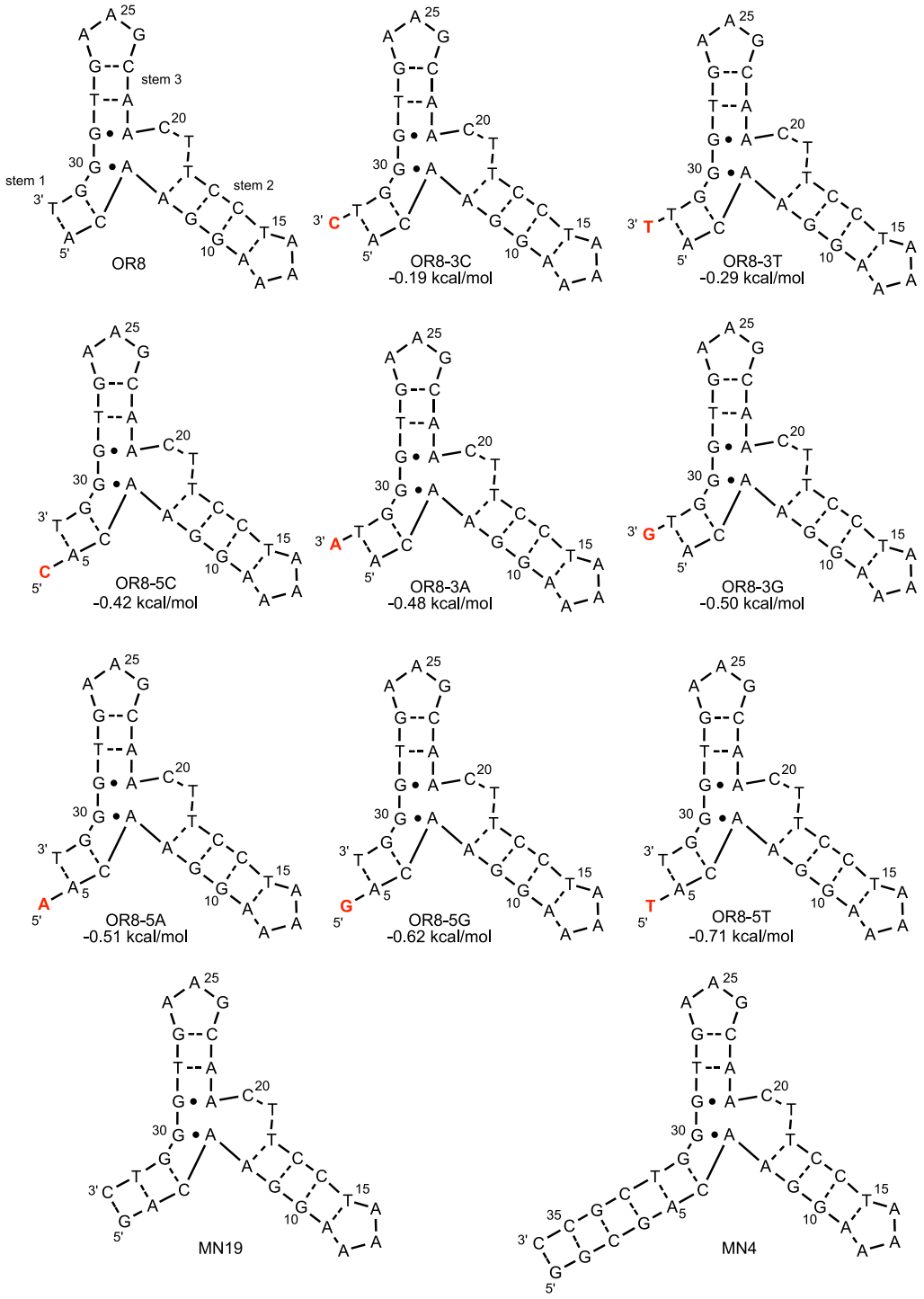

Figure 1. Secondary structures of the cocaine-binding aptamers containing dangling nucleotides as well as the MN4, OR8, and MN19 aptamers. The dangling nucleotides are colored red. The numbering of the nucleotides matches what we have used previously and is derived from the MN4 construct. ΔG°_{37} values for the dangling nucleotide constructs are indicated below the aptamer names and are from ref 1. Dashes between nucleotides indicate Watson−Crick base pairs, while dots indicate non-Watson−Crick base pairs.

for folding is obtained from the ligand binding free energy, and more free energy would be left over for the apparent ΔG of binding and observed in the measured K_d value.

MATERIALS AND METHODS

Materials. Aptamer samples were obtained from Integrated DNA Technologies (IDT, Coralville, IA) and used without further purification. DNA samples were dissolved in distilled deionized water, exchanged three times in a 3 kDa molecular weight cutoff concentrator with 1 M NaCl, and washed at least three times with distilled deionized water. All cocaine-binding aptamer samples were exchanged with a buffer of 20 mM Tris (pH 7.4), 140 mM NaCl, and 5 mM KCl three times before use. ATP-binding aptamer samples were exchanged with a buffer of 10 mM HEPES (pH 7.6), 100 mM NaCl, and 2 mM $MgCl_2$ three times before use.[30] Aptamer concentrations were

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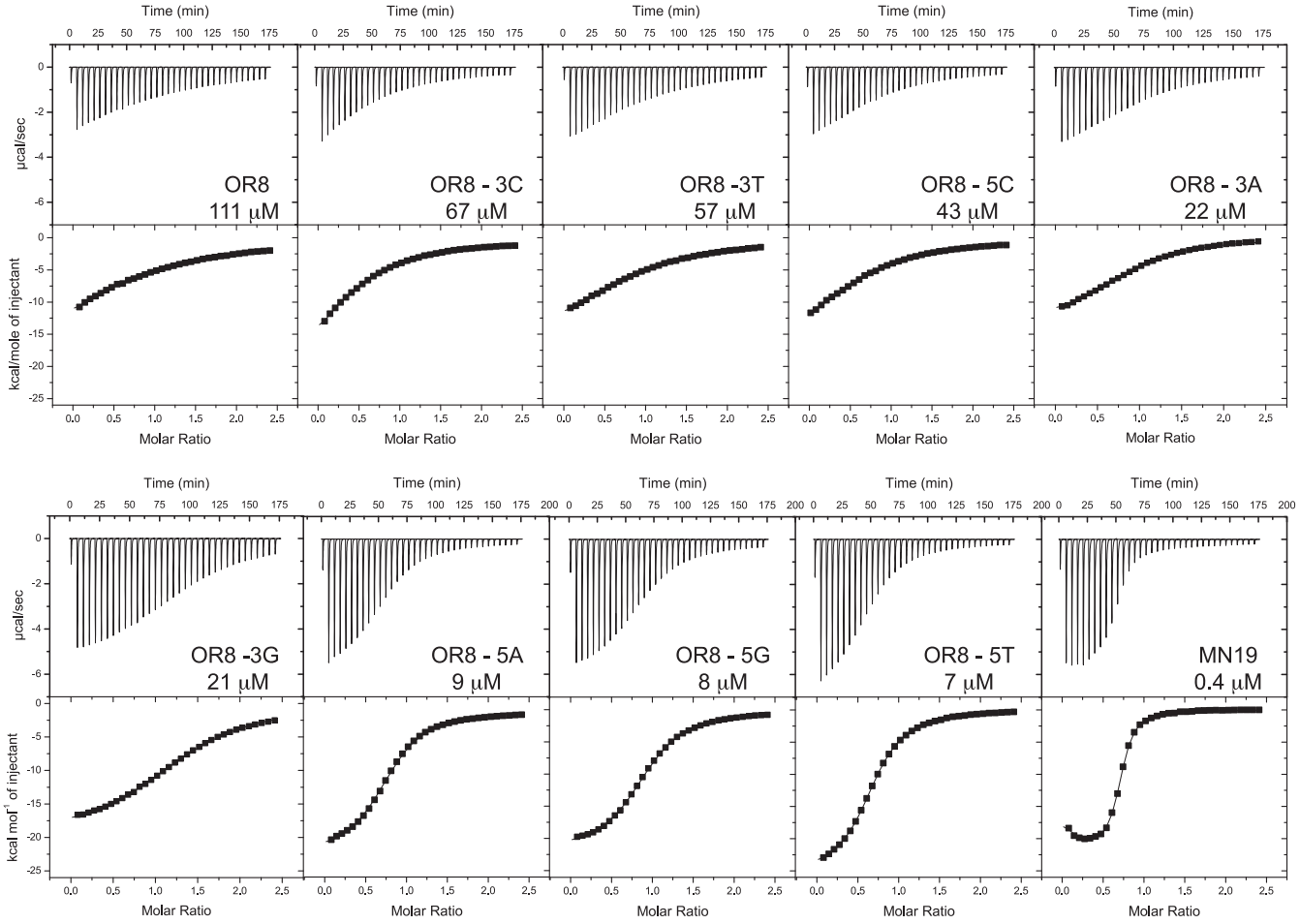

Figure 2. ITC data showing the binding interaction of different cocaine-binding aptamers with quinine. On top are the raw titration data showing the heat resulting from each injection of quinine into an aptamer solution. The bottom shows the integrated heat plot after correcting for the heat of dilution. Data were acquired at 15 °C in 20 mM Tris (pH 7.4), 140 mM NaCl, and 5 mM KCl.

determined by absorbance spectroscopy using their calculated extinction coefficients. Quinine and adenosine were obtained from Sigma-Aldrich. Stock solutions of the ligands for binding experiments were prepared by dissolving the calculated weight into the appropriate buffer.

Isothermal Titration Calorimetry. Isothermal titration calorimetry (ITC) was performed using a MicroCal VP-ITC instrument in a manner similar to that previously described.[31] Samples were degassed before analysis with a MicroCal ThermoVac unit. All experiments were corrected for the heat of dilution of the titrant. Titrations were performed with aptamer samples in the cell and with the ligand as the titrant, in the needle. All aptamer samples were heated in a boiling water bath (100 °C) for 3 min and cooled in an ice−water bath for 5−20 min prior to use in a binding experiment to allow the DNA aptamer to anneal in an intramolecular fashion.

All ITC binding experiments were performed at 15 °C. ITC experiments involving the cocaine-binding aptamer were performed with an aptamer concentration of 98 μM and using a quinine concentration of 1.12 mM. ITC experiments involving the ATP-binding aptamer were performed with an aptamer concentration of 100 μM and using an adenosine concentration of 3.12 mM. All binding experiments consisted of an initial delay of 60 s, a first injection of 2 μL, and a 300 s delay. The 34 subsequent injections were 8 μL in volume, spaced every 300 s. The first point was removed from all of the data sets due to the different injection volume and delay parameters. One-site ITC data were fit to a one-site binding model using manufacturer-provided Origin 7 software. Two-site binding data were fit to a two-independent site binding model developed by Freiburger et al.[32] using Matlab 14 software.

RESULTS

Cocaine-Binding Aptamer Dangling Nucleotide Constructs. The quinine binding thermodynamics of the eight different cocaine-binding aptamer constructs (Figure 1) with dangling nucleotides were determined using ITC methods. For comparison, both OR8 and MN19 constructs, comprised of stem 1 lengths of 2 and 3 bp, respectively, were also measured. These dangling nucleotide constructs preserve the identity of the terminal AT base pair of OR8. We wished to maintain the terminal AT base pair as in a previous study we switched the AT base pair to be a TA base pair, in the context of a 6 bp stem 1 (MN14), and the affinity for cocaine was decreased to 31 from 7 μM.[33] We chose to perform ITC experiments at 15 °C as the OR8 quinine complex unfolds at 27−28 °C[19] and using a higher temperature would weaken the observed binding affinity.

The thermograms for all 10 constructs are shown in Figure 2. With the exception of MN19, all binding curves were fit to a 1:1 binding model. For MN19, the thermogram shows a

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distinct nonsigmoidal shape indicative of two-site binding as observed previously at lower aptamer and NaCl concentrations.[34] The data for MN19 were therefore fit to a two-independent site binding model. The thermodynamic binding parameters for these cocaine-binding aptamer constructs are listed in Tables 1 and 2.

Table 1. Binding Affinities and Free Energies of Quinine Binding by Aptamers Used in This Study[a]

	K_d (μM)	ΔG_{bind} (kcal mol^{-1})	$\Delta\Delta G_{bind}$ (kcal mol^{-1})	ΔG°_{37} (cal mol^{-1})[c]
OR8	111 ± 4	−5.2 ± 0.2	–	–
OR8-3C	66 ± 4	−5.5 ± 0.4	−0.29 ± 0.43	−190
OR8-3T	57 ± 1	−5.6 ± 0.1	−0.38 ± 0.23	−290
OR8-5C	43 ± 1	−5.8 ± 0.1	−0.55 ± 0.23	−420
OR8-3A	22 ± 1	−6.2 ± 0.1	−0.94 ± 0.23	−480
OR8-3G	21 ± 1	−6.2 ± 0.2	−0.94 ± 0.27	−500
OR8-5A	8.7 ± 0.3	−6.7 ± 0.2	−1.46 ± 0.38	−510
OR8-5G	8.3 ± 0.1	−6.7 ± 0.1	−1.49 ± 0.22	−620
OR8-5T	7.2 ± 0.1	−6.8 ± 0.1	−1.57 ± 0.21	−710
MN19[b]	0.4 ± 0.1	−8 ± 1	−3.22 ± 1.25	−1150

[a]Data acquired at 15 °C in 20 mM Tris (pH 7.4), 140 mM NaCl, and 5 mM KCl. The values reported are averages of two to five individual experiments. [b]Data were fit to a two-independent site binding model with the fits for the high-affinity site reported here. [c]Values from ref 1.

Table 2. Thermodynamic Parameters of Aptamers Used in This Study upon Binding to Quinine[a]

	ΔH (kcal mol^{-1})	$-T\Delta S$ (kcal mol^{-1})
OR8	−10.1 ± 0.1	4.9 ± 0.3
OR8-3C	−15 ± 2	10 ± 2
OR8-3T	−16.9 ± 0.2	11.3 ± 0.2
OR8-5C	−16 ± 3	11 ± 3
OR8-3A	−16.6 ± 0.1	10.5 ± 0.1
OR8-3G	−19.8 ± 0.1	13.6 ± 0.2
OR8-5A	−20 ± 3	14 ± 3
OR8-5G	−21 ± 2	14 ± 2
OR8-5T	−25.7 ± 0.1	19.0 ± 0.1
MN19[b]	−17 ± 1	9 ± 2

[a]Data acquired at 15 °C in 20 mM Tris (pH 7.4), 140 mM NaCl, and 5 mM KCl. The values reported are averages of two to five individual experiments. [b]Data were fit to a two-independent site binding model with the fits for the high-affinity site reported here.

ATP-Binding Aptamer Dangling Nucleotide Constructs. To test the generality of introducing dangling nucleotides into structure-switching aptamers to alter binding affinity, we also introduced dangling nucleotides into a single-site version of the ATP-binding aptamer (Figure 3).[30] The adenosine binding thermodynamics of two different ATP-binding aptamer constructs with dangling nucleotides were determined using ITC methods. To provide a comparison with the dangling nucleotide constructs, both the ATP1d and ATP1e aptamers, comprised of stem lengths that differed by 1 bp, were also analyzed (Figure 3). The two dangling nucleotide constructs were chosen as their unpaired nucleotides are the most stabilizing and destabilizing possible combinations for a terminal TA base pair.[1] These two dangling nucleotide constructs have a stability difference of 1213 cal mol^{-1}, which is similar to the 1150 cal mol^{-1} range of stability in the cocaine-binding aptamer dangling nucleotide constructs analyzed. Additionally, the dangling 5′-G in ATP1e-5G against

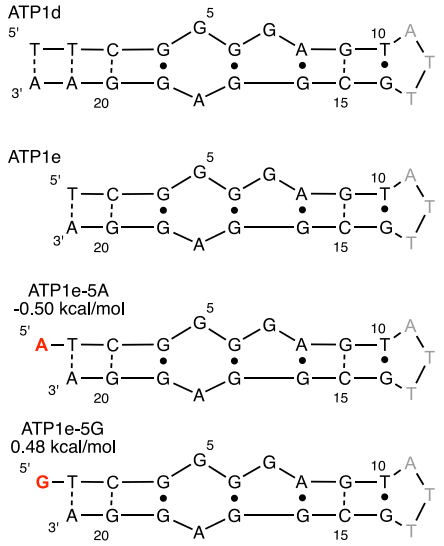

Figure 3. Secondary structures of the ATP-binding aptamers containing dangling nucleotides as well as the ATP1d and ATP1e aptamers. The dangling nucleotides are colored red. Dashes between nucleotides indicate Watson−Crick base pairs, while dots indicate non-Watson−Crick base pairs. ΔG°_{37} values for the dangling nucleotide constructs are indicated below the aptamer names and are from ref 1.

a TA base pair is the most destabilizing possibility reported of all potential dangling nucleotide combinations. The thermograms for all four ATP-binding aptamer constructs are shown in Figure 4. All binding curves were fit to a 1:1 binding model. The thermodynamic binding parameters for the ATP-binding aptamer constructs for adenosine are listed in Tables 3 and 4.

DISCUSSION

We have shown that the addition of dangling nucleotides to structure-switching aptamers will increase or decrease the affinity of the aptamer depending on whether the dangling nucleotide stabilizes or destabilizes the DNA structure (Tables 1 and 3). We think this concept will apply to other structure-switching aptamers in which dangling nucleotides can be introduced. To explain what is occurring, we propose that all of the different constructs of the same aptamer type bind the ligand with the same amount of free energy. For the cocaine-binding aptamer binding quinine (QN), this amount is $\Delta G_{QN,bind}$ (Figure 5). The nucleotide composition at the ligand-binding site is the same in all aptamers, while the changes to the aptamer that affect stability are located at the end of stem 1, located away from the binding site; therefore, the binding free energy is highly likely to be the same for the different constructs. Interaction of quinine with an aptamer results in some of the $\Delta G_{QN,bind}$ going into or "paying" for folding the aptamer, with the remaining binding free energy manifesting itself as an apparent binding affinity, $\Delta G^{app}_{QN,bind}$ (Figure 5). This apparent ΔG value is what is measured by the ITC and manifested in the observed affinity.

We previously demonstrated a similar concept with the cocaine-binding aptamer by altering the length of stem 1 to obtain an aptamer (OR8) that bound only the tighter-binding ligand quinine, and not the weaker-binding ligand cocaine.[19] This demonstrated that quinine binding is tight enough to both fold and bind OR8; however, the binding of cocaine does not have enough free energy to fold OR8, so no binding is

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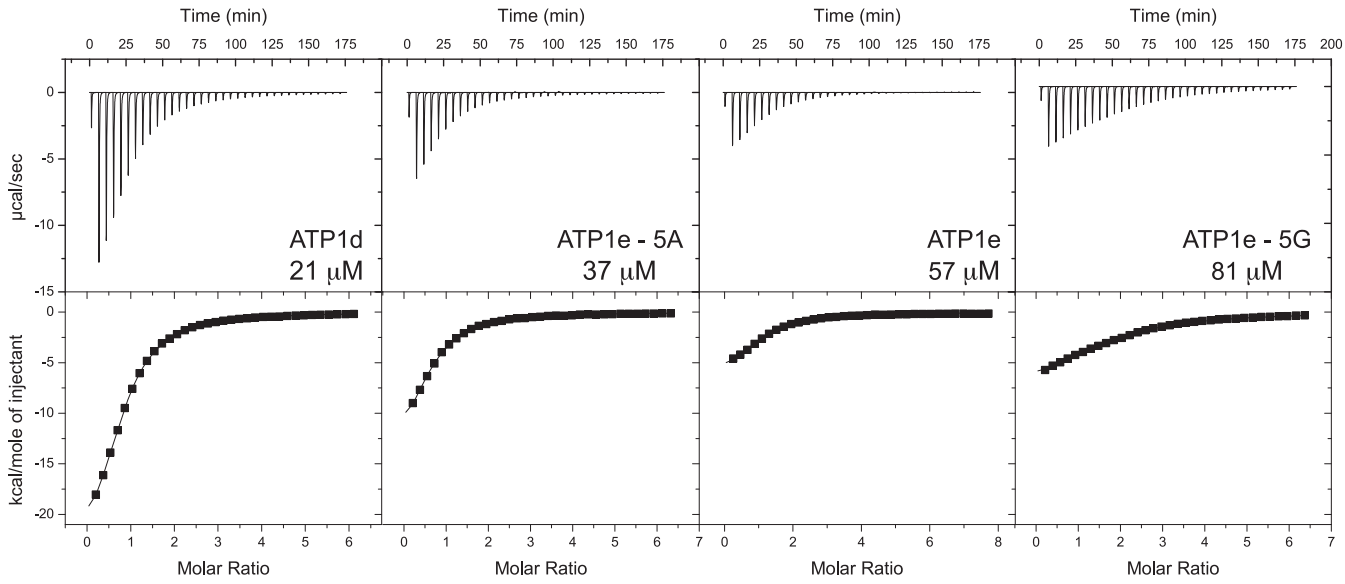

Figure 4. ITC data showing the interaction of the different ATP-binding aptamers with adenosine. On top are the raw titration data showing the heat resulting from each injection of ligand into the aptamer solution. The bottom shows the integrated heat plot after correcting for the heat of dilution. Data were acquired at 15 °C in 10 mM HEPES (pH 7.6), 100 mM NaCl, and 2 mM $MgCl_2$.

Table 3. Binding Affinities and Free Energies of Adenosine Binding by the ATP-Binding Aptamers Used in This Study[a]

	K_d (μM)	ΔG_{bind} (kcal mol^{-1})	$\Delta\Delta G_{bind}$ (kcal mol^{-1})	ΔG°_{37} (cal mol^{-1})[b]
ATP1e	57 ± 5	−5.6 ± 0.5	–	–
ATP1e-5A	37 ± 3	−5.9 ± 0.5	−0.3 ± 0.8	−500
ATP1e-5G	81 ± 7	−5.5 ± 0.2	0.1 ± 0.6	480
ATP1d	21 ± 2	−6.2 ± 0.6	−0.6 ± 0.8	−733

[a]Data acquired at 15 °C in 10 mM HEPES (pH 7.6), 100 mM NaCl, and 2 mM $MgCl_2$. The values reported are averages of two or three individual experiments. [b]Values from ref 1.

Table 4. Thermodynamic Parameters of Adenosine Binding by the ATP-Binding Aptamers Used in This Study[a]

	ΔH (kcal mol^{-1})	$-T\Delta S$ (kcal mol^{-1})
ATP1e	−8.1 ± 0.8	3 ± 1
ATP1e-5A	−17.3 ± 0.2	11.5 ± 0.2
ATP1e-5G	−8 ± 2	3 ± 2
ATP1d	−23.6 ± 0.4	17.4 ± 0.4

[a]Data acquired at 15 °C in 10 mM HEPES (pH 7.6), 100 mM NaCl, and 2 mM $MgCl_2$. The values reported are averages of two or three individual experiments.

observed. We extended this analysis for the quinine derivative amodiaquine, the tightest binding ligand for the cocaine-binding aptamer reported to date.[25] Amodiaquine can fold and bind a cocaine-binding aptamer (OR7) comprised of only 1 bp in stem 1. Therefore, OR7 requires even more free energy from ligand binding to fold than does OR8, which has 2 bp in stem 1. Quinine, a weaker binding ligand than amodiaquine, does not bind OR7.

In this study, we thought that the incorporation of dangling nucleotides into a structure-switching aptamer should alter the binding affinity in a more fine-grained manner than by adding or removing base pairs (Figure 5). Dangling nucleotides can stabilize or destabilize a nucleic acid structure in a known manner depending on the identity of the 5′ or 3′ added nucleotide and the identity of the terminal base pair.[1,35] If the dangling nucleotide is stabilizing, as almost all combinations are, less free energy from the binding affinity is needed to fold the aptamer and consequently the observed binding affinity increases (Figure 5). This is what we observe with all of the cocaine-binding aptamer dangling nucleotide constructs (Table 1). We note that the binding thermodynamics for the OR8 and MN19 aptamers match what we observed previously when corrected for temperature. Additionally, we had not previously observed two-site binding for the MN19 construct at this NaCl concentration (140 mM). However, at the high aptamer concentration used here, 98 μM, we do observe quinine binding at the weaker second site.

As a confirmation of the trend seen with the cocaine-binding aptamer, and to extend results to include a destabilizing dangling nucleotide construct, we incorporated dangling nucleotides into the single-site version of the ATP-binding aptamer developed by Zhang and Liu.[30] The ATP aptamer is also recognized as undergoing a structure-switching transition from an unstructured or loosely structured free state to a structured bound state.[36] We thought that the large change in affinity between the 2 and 3 bp aptamers ATP1d and ATP1e represented a limitation of the amount of free energy available from adenosine binding to fold the destabilized aptamer ATP1e being reached. Consequently, we chose to add dangling nucleotides to this construct. Our adenosine binding results for ATP1d (Tables 3 and 4) match what was previously reported.[30] For ATP1e, our affinity reported here is significantly higher than previously reported for the same conditions. We attribute this to us working at a much higher aptamer concentration of 100 μM rather than 10 μM and therefore being able to better define the binding curve as we are working at a higher Wiseman "c" parameter (c value).[37,38]

The binding affinities of the two dangling nucleotide ATP-binding aptamers were measured using ITC methods and match the trends observed with the cocaine-binding aptamer. ATP1e-5A, which is more stable than ATP1e, has a higher affinity for adenosine than ATP1e (Table 3). ATP1e-5G, which has the destabilizing dangling 5′-G, has a lower affinity for adenosine than ATP1e (Table 3).

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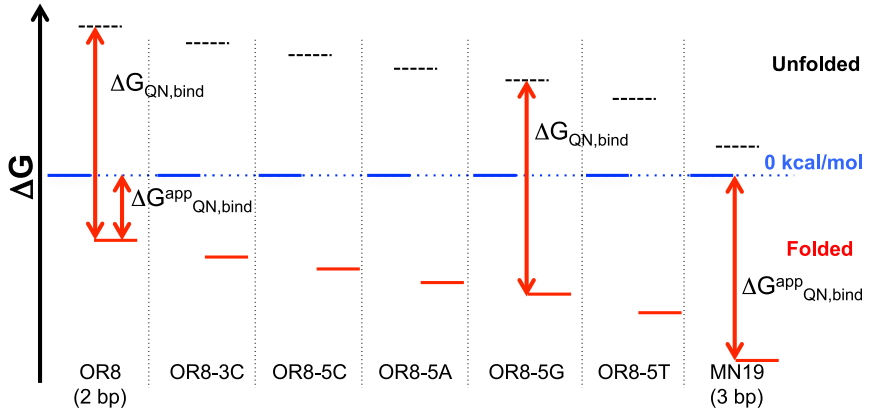

Figure 5. Free energy diagram of ligand binding by some of the dangling nucleotide cocaine-binding aptamer constructs. Each aptamer has solid blue, dotted black, and solid red lines representing the unfolded free state, the hypothetical folded free state, and the folded bound state, respectively.

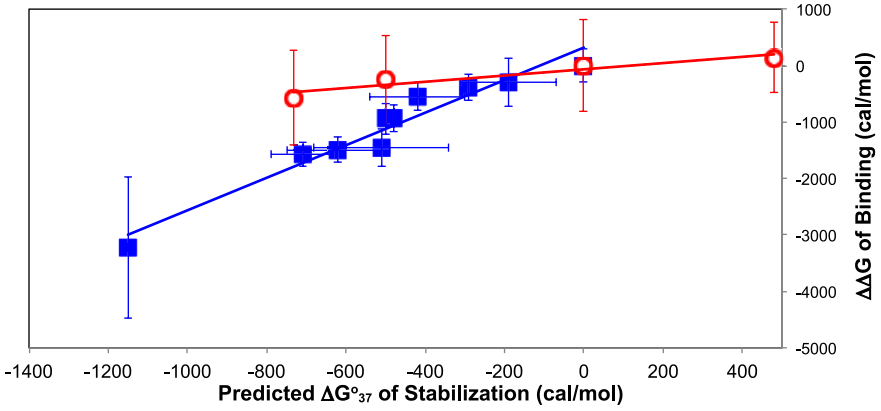

Figure 6. Plot of the observed change in ΔG of binding ($\Delta\Delta G_{bind}$) vs predicted ΔG°_{37} values upon adding a dangling nucleotide. The blue filled squares are the data for the cocaine-binding aptamer series, while the empty red circles are for the ATP-binding aptamers.

For the cocaine-binding and ATP-binding aptamers, we observe that the dangling nucleotides with the largest contributions to stabilization display the largest increase in observed binding affinity (Tables 1 and 3). We plotted the difference in the free energy of binding ($\Delta\Delta G_{bind}$), obtained from the K_d values, against the predicted ΔG°_{37} values for the added dangling nucleotide (Figure 6). Also plotted are the data for aptamer constructs extended by 1 bp. The data for the cocaine-binding aptamer and ATP-binding aptamer follow separate straight-line relationships. Neither data set has a slope of 1. This, at least partially, arises from the fact that the predicted stabilization data are tabulated for 37 °C while both binding data sets were acquired at 15 °C. We will note that for the ATP-binding aptamer dangling nucleotide constructs there are significant differences in K_d values, but they do not have a significant difference in $\Delta\Delta G_{bind}$ values (Table 3). This demonstrates that adding dangling nucleotides has different effects in different systems and may not always result in significant differences in $\Delta\Delta G_{bind}$ values.

We propose that the two data sets do not fall on the same straight line as the addition of a dangling nucleotide in these two separate aptamer systems results in different contributions to decreasing the required free energy of folding for each system. One difference between these two aptamers is that the ligand-binding sites in the aptamers are at a different distance from the site of stabilization by the dangling nucleotide. For the cocaine-binding aptamer, the high-affinity ligand-binding site is at the three-way junction, close to nucleotides T19, C20, G30, and G31.[18] This is 2−3 bp from the site of stabilization by the addition of dangling nucleotides at the terminal base pair in OR8 (Figure 1). For the ATP-binding aptamer, the binding site is at the sole unpaired G nucleotide [G7 (Figure 3)]. The binding site is 5 bp from where the addition of dangling nucleotides stabilizes the free aptamer structure. Another factor that could contribute to the different effect of adding dangling nucleotides is the different three-dimensional structure formed by the two aptamers. Other reasons could involve the difference in the nature of how the two ligands interact with the aptamers due to factors such as the size and shape of the ligand and the role played by the different noncovalent forces involved in ligand binding. Finally, different types of forces could drive the folding of the two different aptamers, with the added dangling nucleotides affecting the different folding events in different ways. It is likely that a combination of these factors results in the different effect on ligand affinity that adding dangling nucleotides has in the cocaine-binding and ATP-binding aptamer systems.

AUTHOR INFORMATION

Corresponding Author

Philip E. Johnson − *York University, Toronto, Canada;* orcid.org/0000-0002-5573-4891; Phone: 416-736-2100, ext. 3319; Email: pjohnson@yorku.ca

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Other Authors

Sladjana Slavkovic − *York University, Toronto, Canada*
Sophie R. Eisen − *York University, Toronto, Canada*

Complete contact information is available at:
https://pubs.acs.org/10.1021/acs.biochem.9b00630

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Notes

The authors declare no competing financial interest.

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