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Modified Pyrimidines Specifically Bind the Purine Riboswitch

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Riboswitches are highly structured elements within the 5′-untranslated regions of messenger RNAs (mRNAs) that specifically bind small molecule metabolites to regulate gene expression in a *cis*-fashion.[2−4] In recent years, over ten distinct riboswitches have been characterized, each recognizing a specific small molecule, including purine nucleobases,[5,6] amino acids,[7−9] and vitamin cofactors.[10,11] These regulatory elements are the focus of efforts to develop novel antibiotic therapeutics, as they are central to controlling essential metabolic pathways, particularly in Gram-positive bacteria, such as *Bacillus subtilis* and *Staphylococcus aureus*.[12] The adenine- and guanine-specific riboswitches, members of the purine riboswitch family, regulate expression of a diverse set of genes, including those involved in purine biosynthesis and salvage as well as purine and pyrimidine transport.[5,6]

The crystallographic structures of the 67-nucleotide guanine riboswitch aptamer domain complexed to hypoxanthine[13] as well as the structurally related adenine riboswitch bound to adenine[14] were recently solved. Despite binding different metabolites, the two RNAs adopt an almost identical three-dimensional fold (rmsd of 1.7 Å via superposition of the phosphates). These structures revealed that the purine nucleobase is completely engulfed within a three-way junction motif, resulting in direct recognition of every atom within the purine by either hydrogen bonding or van der Waals interactions. Furthermore, the specificity of the purine riboswitch for guanine versus that for adenine is effected by a single pyrimidine residue that forms a Watson−Crick interaction with the nucleobase ligand.[1,5] This was directly demonstrated by a structure of a natural guanine riboswitch from *B. subtilis* that regulates the *xpt-pbuX* operon containing a single point mutation (C74U), converting it into an RNA that binds adenine and related compounds with both high affinity and specificity, while completely abrogating guanine binding.[1]

To investigate whether ligands other than purines can efficiently bind the purine riboswitch, we tested the binding of a number of compounds using isothermal titration calorimetry to this mutant form of the guanine riboswitch (referred to as GR(C74U)). These included several pyrimidines bearing amino groups at the 5- and 6-positions that may mimic the N7 and N9 of purines (Tables 1 and S1 and Figure 1). Surprisingly, aminopyrimidines bound the purine riboswitch, albeit with weakened affinity as compared to that of adenine, underscoring the importance of the Watson−Crick interaction for productive ligand binding. Conversely, imidazole derivatives, including histamine and L-histidinol, displayed no detectable binding, further establishing that the six-membered ring of the purine is the primary recognition determinant.

To validate that these compounds would also bind natural adenine-responsive riboswitches, we tested one of the pyrimidine compounds for binding to the element that regulates the adenosine deaminase (*add*) gene in the human pathogen *Vibrio vulnificus*. As expected, 2,4,6-triaminopyrimidine (3APy) is capable of interacting with this RNA (Table 1). While 3APy binds the *add* riboswitch with ∼8-fold higher affinity than to GR(C74U), the two RNAs show similar $\Delta\Delta G$ values between adenine and 2,4,6-triaminopyrimidine. For both RNAs tested, the loss of the N9/C8 atoms of the purine ring (comparing 2,6-diaminopurine to 2,4,6-triaminopyrimidine, Table 1) results in ∼4 kcal/mol loss in affinity, likely reflecting not only the loss of hydrogen bonding and van der Waals interactions but also the potential energetic cost of having an internal cavity (Figure 2) and greater flexibility in the RNA backbone. These data reinforce previous studies that demonstrate that the factor that determines guanine versus adenine specificity in the purine riboswitch is the identity of the pyrimidine residue at position 74[1,5,6,13,14] and supports the GR(C74U) mutant as a model

Table 1. ITC Results for Binding of Nucleobases to Purine Riboswitch[a]

ligand	$K_{d,app}$ (μM)	ΔG (kcal mol^{-1})	$\Delta\Delta G^b$ (kcal mol^{-1})
2,6-diaminopurine[c]	0.02 ± 0.006	−11	−1.9
adenine[c]	0.4 ± 0.04	−8.8	
2,4,5,6-tetraaminopyrimidine	20 ± 0.1	−6.5	2.4
2,4,6-triaminopyrimidine	20 ± 3	−6.6	2.2
2,4-diaminopyrimidine	>1000	*d*	*d*
2,4,6-triamino-1,3,5-triazine	20 ± 1.0	−6.5	2.3
2,6-diaminopurine (*add*)	0.004 ± 0.001	−12	−2.5
adenine (*add*)	0.2 ± 0.03	−9.2	
2,4,6-triaminopyrimidine (*add*)	2 ± 0.4	−7.8	1.4

[a] Binding was measured in a solution containing 10 mM Na-HEPES, pH 7.5, 100 mM KCl, 10 mM $MgCl_2$ at 30 °C. Data obtained using GR(C74U) adenine-responsive riboswitch unless otherwise indicated. [b] $\Delta\Delta G = \Delta G$-(ligand) − ΔG(adenine). [c] Previously reported.[1] [d] Not determined.

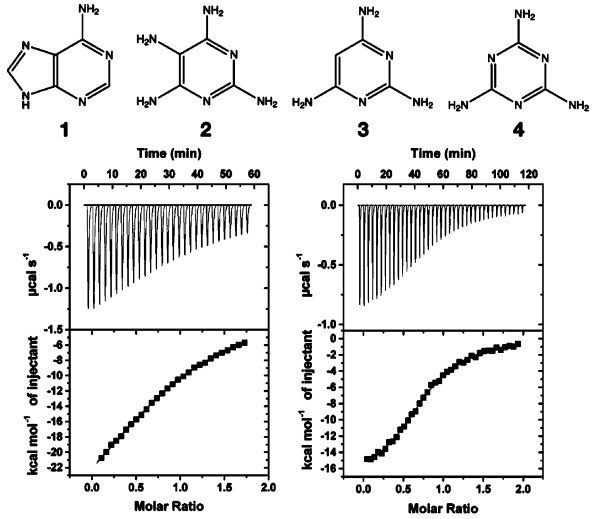

Figure 1. (Top) Structures of select compounds used in this study: **1**, adenine; **2**, 2,4,5,6-tetraaminopyrimidine; **3**, 2,4,6-triaminopyrimidine; **4**, 2,4,6-triamino-1,3,5-triazine. (Bottom) ITC data analysis of *xpt-pbuX* GR(C74U) (left) and *add* AR (right) binding 2,4,6-triaminopyrimidine. The mutant and native riboswitches bind ligand with similar affinity.

10.1021/ja063645t CCC: $33.50

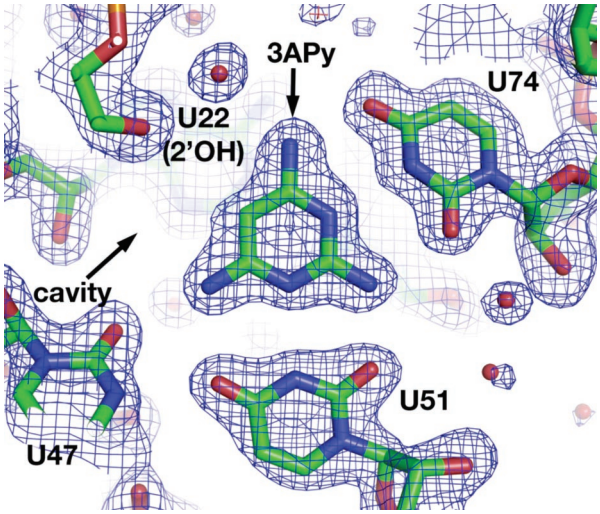

Figure 2. Crystal structure of the binding pocket of the GR(C74U) adenine-responsive riboswitch bound to 2,4,6-triaminopyrimidine (3APy). The mesh represents the electron density of a $2F_o-F_c$ simulated annealing omit map in which residues U51, U74, and 3APy were omitted from the model; map is contoured at 1.0σ.

system for investigating binding of adenine and related compounds to the purine riboswitch.

To determine if 3APy is capable of specifically binding to the purine riboswitch RNA in a fashion similar to purines, we solved the structure of this compound in complex with GR(C74U) using X-ray crystallography (Figure 2). This complex readily crystallized under conditions similar to that of previously reported complexes and yielded clear diffraction to ~1.6 Å resolution. The structure was solved using the molecular replacement method and refined to a final working crystallographic *R* factor of 19.5% and a free-*R* factor of 24.2% with excellent geometry (Supporting Information, Table S2). 2,4,6-Triaminopyrimidine occupies exactly the same position as the six-membered ring of hypoxanthine, forming hydrogen bonding contacts with U51 and U74 consistent with those observed in both the guanine and adenine riboswitches (Supporting Information, Figure S2). A previously observed base quadruple involving the nucleobase, U47, U51, U74, and the 2′OH of U22, does not require the participation of the latter, indicating its minor direct contribution to ligand recognition and further emphasizing the importance of the Watson−Crick interaction between pyrimidine 74 and the ligand.

Superposition of the GR−hypoxanthine and GR(C74U)−3APy structures about the binding pocket reveals that the two structures are nearly identical (rmsd = 0.65 Å, Supporting Information). However, there are several things to note about the pyrimidine-bound structure. The cavity created by the deletion of N7 and C8 of the purine is unoccupied by solvent, as evidenced by the lack of electron density even at 0.5σ. Modeling a single water into the cavity followed by one round of energy minimization revealed that a water molecule would not fit into the pocket without moderate steric clashes with functional groups in either the ligand or the RNA. Second, it is clear that the back side of the ligand binding pocket is rigidly locked into place despite the loss of hydrogen bonding between the purine N7 and U22 2′-hydroxyl group and van der Waals packing between atoms in U22 and U47 bases and the C8 of the purine. Thus, despite the flexibility of the binding pocket in the unbound state,[5] the binding pocket of the bound state, at least in this region, cannot adopt alternative conformations in response to smaller ligands. This is in contrast to the other side of the binding pocket, where U74 can shift its position in the presence of certain purine analogs (S.D.G. and R.T.B., manuscript in preparation).

In summary, we report that pyrimidines with modifications at their 5- and/or 6-positions are able to specifically bind to the aptamer domain of the purine riboswitch. The crystal structure directly demonstrates that the atoms of the 7- and 8-position of the purine are not required to organize the binding pocket around the ligand and that the U51−ligand−Py74 hydrogen bonding is sufficient for a stable interaction. As riboswitches are potentially excellent candidates for antibiotic therapeutics, it is important to understand the range of compounds that these genetic regulatory elements can specifically recognize. Our findings show that the five-membered ring of the purine can be readily modified or eliminated, suggesting pyrimidine compounds with a variety of one and two atom substitutions could efficiently target the purine riboswitch.

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Supporting Information Available: Experimental methods for the synthesis of RNA, isothermal titration calorimetry, crystallization of the RNA:3APy complex, and refinement of the structure. This material is available free of charge via the Internet at http://pubs.acs.org.

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