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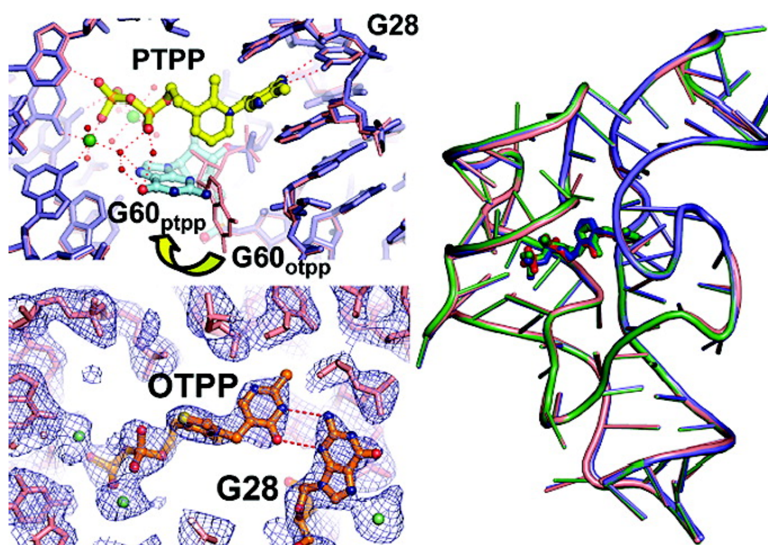

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Structural Basis of Thiamine Pyrophosphate Analogues Binding to the Eukaryotic Riboswitch

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Several metabolites regulate their own production by directly interacting with highly conserved regions of mRNA capable of forming a discrete tertiary structure.[1] These regions of mRNA are called riboswitches,[2] and more than 10 essential compounds have been shown to regulate their cellular levels in this manner. Several riboswitches are targeted by antibiotics,[3,4] making them promising drug targets.[5,6]

Structural and biochemical studies on riboswitches revealed that the small-molecule ligands bind the RNA with high specificity and affinity.[7] For example, riboswitches efficiently discern the biologically relevant form of these metabolites, e.g., the phosphorylated versus the non-phosphorylated form of riboflavin[8] or the enzymatically active sulfonium configuration of *S*-adenosyl methionine (SAM).[9] Crystal structures of several riboswitches, including the bacterial and the eukaryotic thiamine pyrophosphate (TPP)-specific riboswitches,[10–15] uncovered that the bound ligands are deeply buried, far more so than typically observed for *in vitro* selected oligonucleotides or aptamers.[16] Furthermore, the binding pockets of riboswitches form via a ligand-mediated induced-fit mechanism that involves the reorganization of RNA elements.[17]

The TPP-sensing riboswitch is found in most bacteria and several archaea and is, so far, the only riboswitch identified in eukaryotes.[18,3] TPP, commonly referred to as vitamin B1, is composed of a pyrimidine ring, a central thiazole ring, and a pyrophosphate group (Figure 1A). It is an essential metabolite in all organisms through its role as a cofactor in a broad range of enzymatic reactions.[19] Two thiamine analogues, oxythiamine and pyrithiamine, have been used extensively to produce thiamine deficiency in model organisms (Figure 1A).[20] Oxythiamine has a hydroxyl group replacing the exocyclic amino group in the pyrimidine ring, while pyrithiamine possesses a central pyridine ring rather than a thiazole ring. In the cell, these analogues are metabolized into their pyrophosphate derivatives,[21] which inhibit many TPP-dependent enzymes. Although it has been shown that oxythiamine can reduce the growth rate of *Saccharomyces cerevisiae*, presumably by binding to enzymes,[22] there is no biochemical or structural information regarding the binding of oxythiamine pyrophosphate (OTPP) to the TPP riboswitch and its potential as an antibiotic. Finally, while pyrithiamine pyrophosphate (PTPP) has been shown to exert an antibiotic effect via interaction with TPP-specific riboswitches in bacteria and plants,[4] no structural information is available for this complex, either.

To better understand the molecular basis of these interactions, we solved the structures of the eukaryotic TPP-sensing riboswitch from *Arabidopsis thaliana* in complex with OTPP and PTPP at 2.65 and 2.0 Å resolution, respectively. We were also able to improve the diffraction properties of the TPP-bound riboswitch crystals to 2.25 Å resolution.

The refined model of the TPP-bound riboswitch contains 163 water molecules and 10 Mg^{2+} ions (Figure 1B). Overall, it is very similar to the lower resolution structure,[14] except for the conformation of the TPP pyrophosphate moiety, where the higher resolution map reveals an extended conformation bound to two Mg^{2+} ions and a closely coordinated network of water molecules (Figure 1C and Supporting Information). The observed conformation of the pyrophosphate and its interaction with the two Mg^{2+} ions are similar to those observed in the bacterial TPP-specific riboswitch.[23,13]

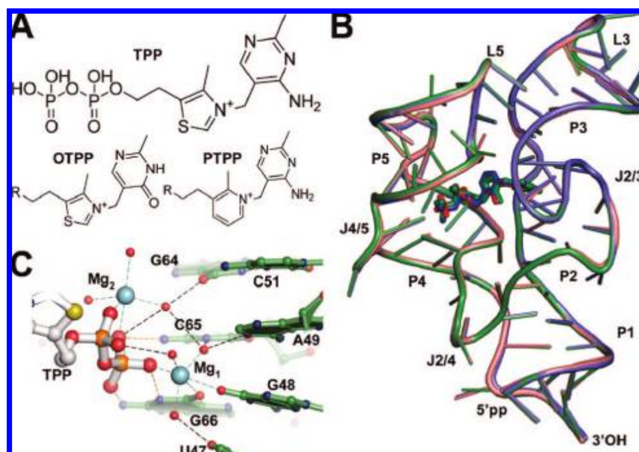

Figure 1. (A) Chemical structure of thiamine pyrophosphate (TPP), oxythiamine pyrophosphate (OTPP), and pyrithiamine pyrophosphate (PTPP), R = $P_2O_6H_3$. (B) Superposition of the *A. thaliana* riboswitch structures in complex with TPP (green), OTPP (red), and PTPP (blue). (C) Close view of the pyrophosphate moiety of TPP, with its bound magnesium ions and water molecules in cyan and red, respectively.

OTPP and PTPP were chemically synthesized as previously described for PTPP[4] (see Supporting Information for details). We crystallized the analogue/riboswitch complexes and solved the structures by molecular replacement. Refined models were obtained with final crystallographic working *R* factors and free-*R* factors of 20.9/25.7, 23.3/26.9, and 20.7/25.1, for the TPP-, OTPP-, and PTPP-bound *A. thaliana* riboswitch, respectively (Table S1).

While the overall structure of the riboswitch in complex with OTPP or PTPP is similar to its structure in complex with the natural ligand (Figure 1B),[14] important differences emerge in the binding interactions of these analogues.

The structure of the OTPP complex reveals that the interactions between the pyrophosphate moiety and the riboswitch are, as expected, nearly identical to those observed for TPP. In contrast, one might have predicted that the substitution of the exocyclic amino group of TPP by a keto group at the C4 position of the pyrimidine ring of OTPP would lead to a different mode of interaction with the ligand-binding pocket. Instead, the OTPP molecule interacts with the purine ring of G28 with the same geometry as observed for the TPP pyrimidine ring (Figure 2B; see also Supporting Information). Since the most favored tautomeric form of the OTPP in solution has the N3 position protonated (Figure 2A),[24] the actual mode of binding implies that the pyrimidine ring

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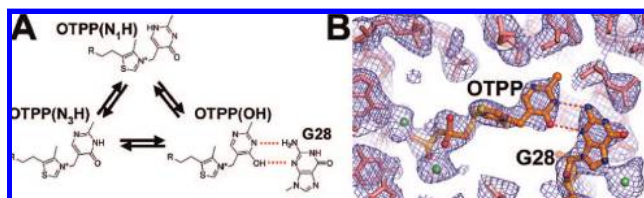

Figure 2. (A) Schematic representation of the tautomeric equilibrium of OTPP and its influence on riboswitch binding. (B) Close view of the OTPP interaction with G28. The blue mesh is a $2F_o - F_c$ simulated annealing omit map where OTPP has been omitted from the calculations.

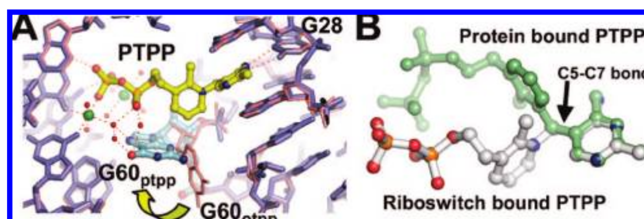

Figure 3. (A) Close view of the PTPP interaction site. The yellow arrow indicates the movement of nucleotide G60 toward the pyridine ring when PTPP is bound instead of OTPP (or TPP). OTPP- and PTPP-bound riboswitch structure are superimposed and shown in salmon and blue, respectively. (B) Superposition of the PTPP conformations observed when bound to protein (PDB code 2f17) and our structure.

of the OTPP is stabilized in its enol form (Figure 2B). Therefore, the structure supports the biochemical data indicating that the oxythiamine, the non-phosphorylated form of the thiamine analogue, displayed a strongly reduced affinity to the *Escherichia coli* 165 thiM RNA riboswitch, as the binding of the enol tautomer is energetically disfavored.[25]

The specific contacts between the RNA and the pyrophosphate and pyrimidine moieties of the antimicrobial PTPP observed in the structure of its complex with the riboswitch are the same as in the case of TPP-riboswitch complex. Nevertheless, the absence of the sulfur atom in the pyridine ring of PTPP reduces the acidic character of the central ring and permits closer interactions with the base of guanosine 60 (G60). This nucleotide base is not well ordered in the TPP- or OTPP-containing structures but becomes clearly visible in the PTPP-bound riboswitch structure, where it stacks against the edge of the PTPP central ring (Figure 3A). This conformation of G60 is further stabilized by water-mediated hydrogen bonds with the PTPP α-phosphate (Figure 3A).

There are some substantial differences in the way TPP analogues are recognized by the riboswitch when compared to their enzyme binding mode. TPP derivatives bind the riboswitch with a stretched conformation of their respective rings, i.e., the thiazole and the pyrimidine ring for OTPP or the pyridine and the pyrimidine ring for PTPP. This arrangement differs significantly from their V-shaped conformations when bound to proteins.[26] The difference in the relative orientation of the two rings is 90° around the C5−C7 bond (Figure 3B). This property could be explored to synthesize riboswitch-specific TPP analogues that would efficiently target the riboswitch but not affect the activity of enzymes that require TPP as a cofactor.

The crystal structures of OTPP and PTPP in complex with the *A. thaliana* TPP-specific riboswitch, described here, provide detailed chemical information on these complexes with important implications for drug design. The OTPP-riboswitch complex reveals that the pyrimidine ring of OTPP is stabilized in its enol form when bound to the riboswitch. The structure of PTPP, a well-characterized antimicrobial, in complex with the riboswitch highlights new interactions possibly leading to a tighter binding pocket, as exemplified by the rotation of the base of G60.

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Supporting Information Available: Experimental details and additional figures. This material is available free of charge via the Internet at http://pubs.acs.org.

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