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Probing riboswitch–ligand interactions using thiamine pyrophosphate analogues†‡

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The *Escherichia coli* *thiM* riboswitch forms specific contacts with its natural ligand, thiamine pyrophosphate (TPP or thiamine diphosphate), allowing it to generate not only nanomolar binding affinity, but also a high degree of discrimination against similar small molecules. A range of synthetic TPP analogues have been used to probe each of the riboswitch–ligand interactions. The results show that the pyrimidine-sensing helix of *thiM* is exquisitely tuned to select for TPP by recognising the H-bonding donor and acceptors around its aminopyrimidine ring and also by forming π -stacking interactions that may be sensitive to the electronics of the ring. The central thiazolium ring of TPP appears to be more important for ligand recognition than previously thought. It may contribute to binding *via* long-range electrostatic interactions and/or by exerting an electron withdrawing effect on the pyrimidine ring, allowing its presence to be sensed indirectly and thereby allowing discrimination between thiamine (and its phosphate esters) and other aminopyrimidines found *in vivo*. The pyrophosphate moiety is essential for submicromolar binding affinity, but unexpectedly, it does not appear to be strictly necessary for modulation of gene expression.

Introduction

Riboswitches are folded regions of messenger RNA that are capable of directly binding essential metabolites including several coenzymes. Their function is to regulate gene expression in response to the cellular concentration of their cognate metabolite.¹ The aptamer of a riboswitch (the part of the sequence involved in binding its ligand) undergoes a conformational change upon ligand binding which, in turn, affects the structure of the downstream portion of the riboswitch (the expression platform) and this determines whether or not the mRNA is fully transcribed and/or translated into functional protein.

Escherichia coli has three thiamine pyrophosphate (TPP, coenzyme B₁) responsive riboswitches in operons associated with

TPP biosynthesis and transport: *thiMD*, *thiCEFGH* and *thiBPQ*.^{2,3} When complexed with TPP, the *thiM* riboswitch, found in the 5'-untranslated region of the *thiMD* mRNA, adopts a secondary structure that causes the expression platform to fold such that the ribosome binding site of the mRNA is sequestered in a base-paired region (see Fig. S1†).² TPP binding therefore prevents translation of the mRNA and triggers down-regulation of its own biosynthesis when it is available at sufficient concentrations within the cell.

The X-ray crystal structure of the *E. coli* *thiM* aptamer bound to TPP has been solved independently by two groups;^{4,5} the general architecture of the RNA can be described as Y-shaped⁴ with TPP bound in an extended conformation between the two arms of the “Y” (Fig. 1A and B). The crystal structure of another TPP binding riboswitch, from the *thiC* gene of *Arabidopsis thaliana*, reveals a very similar fold.⁶ TPP is comprised of an aminopyrimidine ring linked to a thiazolium ring (henceforth referred to as the A and B rings respectively, see Fig. 1B) with a pyrophosphate tail. As each arm of the *thiM* aptamer seems to interact only with one particular portion of the TPP molecule, they are referred to as the pyrimidine-sensing (P4–P5) and the pyrophosphate-sensing (P2–P3) helices. The A ring of TPP stacks between conserved purines in an interior loop within the pyrimidine-sensing helix. In addition, a network of hydrogen bonds (H-bonds) with two other conserved nucleobases holds the pyrimidine in place (Fig. 1C).

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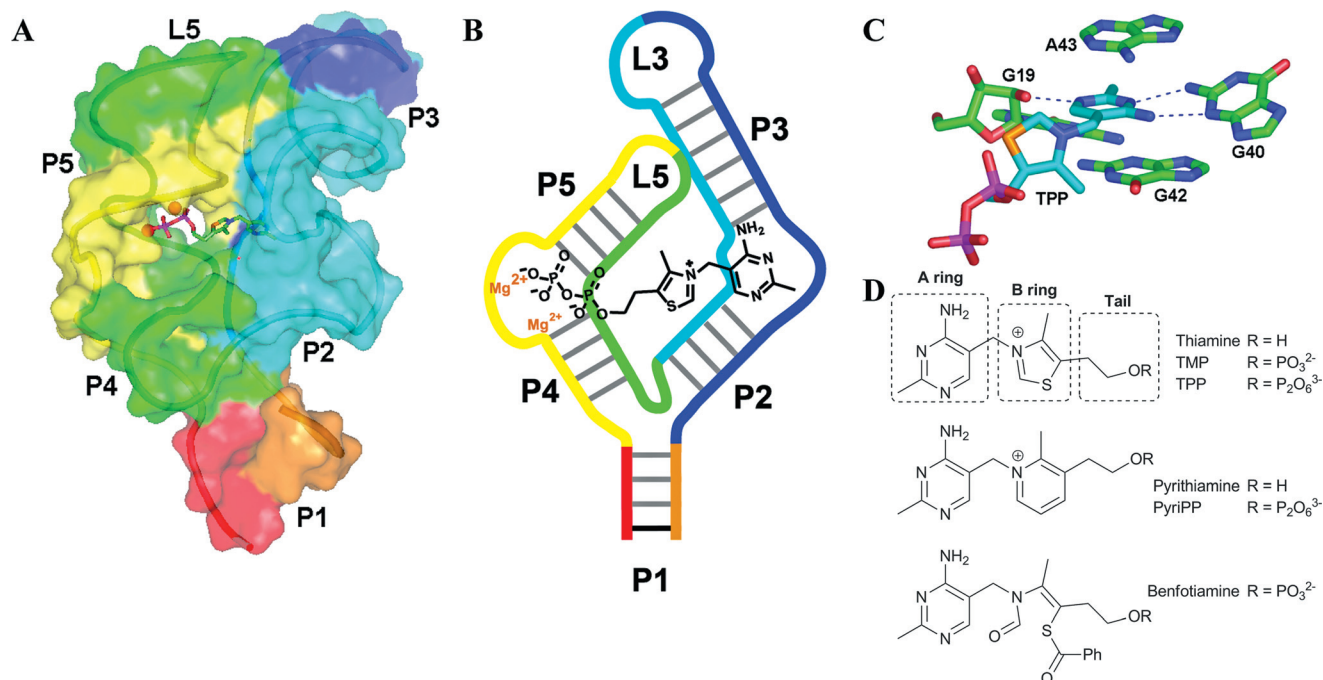


Fig. 1 (A) Structure of the *thiM* riboswitch from *E. coli* showing the pyrimidine-binding arm (P2/P3) and the pyrophosphate binding arm (P4/P5). TPP is in stick representation and the Mg²⁺ ions are orange spheres. (B) Schematic of the structure in (A) with the various regions of RNA coloured the same. (C) Detail of the 4-aminopyrimidine binding site; the 4-aminopyrimidine ring of TPP is π -stacked between G42 and A43 and hydrogen bonds to the guanine of G40 and to the 2'-OH of G19. The crystal structures are from PDB entry 2GDI⁵ and were drawn using PyMol (<http://pymol.org/>). (D) Thiamine pyrophosphate (TPP), its metabolic precursors (thiamine and TMP) and other synthetic *thiM* riboswitch ligands.

The pyrophosphate in contrast is not directly recognised by the RNA. Instead it is bound as a complex with two magnesium ions and the RNA mainly interacts with the metal ions and their inner-sphere co-ordination water molecules. The central B ring of TPP does not appear to engage in any close-range bonding to the RNA in the crystal structures.^{4,5}

In addition to TPP, the *thiM* aptamer is also known to bind to TPP's biosynthetic precursors – thiamine monophosphate (TMP) and thiamine, albeit with lower affinity.² Other ligands include the thiamine analogues pyrithiamine (pyri), pyrithiamine pyrophosphate (pyriPP) and benfotiamine (Fig. 1D), which have been co-crystallised with the *thiM* aptamer.⁴ These compounds all retain the aminopyrimidine ring of thiamine and only vary from thiamine and its phosphate ester derivatives at the central thiazolium ring. However, the *thiM* aptamer is capable of binding a much greater diversity of chemical structures, as we have demonstrated by finding a range of low molecular weight “fragment” ligands.⁷

In this paper we present a detailed structure–activity study on the binding of TPP analogues to the *E. coli thiM* aptamer. We systematically examine the consequences on binding of changing each part of the TPP molecule in turn (the A and B rings and the pyrophosphate tail). Using biophysical techniques developed for our fragment-based approach and a selection of synthetic thiamine analogues, we explore the specificity of the pyrimidine pocket, how pK_a and charge affect pyrophosphate binding, and also the extent to which the RNA recognises the central thiazolium ring of its ligand. In addition to *in vitro* binding studies, we investigate the analogues' ability to modulate *thiM* riboswitch-controlled gene expression, using an *in vitro* transcription/translation assay.

Results

Changing the A ring – interactions with the pyrimidine-sensing helix

We previously developed a competition equilibrium dialysis technique⁸ that allowed identification of ligands for a TPP riboswitch by their ability to displace radiolabelled thiamine from the riboswitch. A library of thiamine and pyrithiamine analogues in which the aminopyrimidine ring (A ring) was replaced by various aromatic heterocycles (**1–11**) was synthesised and tested for binding to the *thiM* aptamer using this method (Fig. 2). The compounds were grouped into three different series – **T**, **M** and **P**, depending on whether they possessed a 4-methylthiazolium, 2-methylpyridinium or pyridinium ring as their B ring.

Of the **T** series of analogues, **8T** (2,4-diaminopyrimidine) was the most potent, completely displacing the reporter ligand (99% displacement). Two other ligands, **1T** and **7T** (with a uracil and a dinitrophenyl ring respectively) also showed some activity (27% and 17% respectively). From the **M** and **P** series, **11P** (diamino-triazine) showed the greatest displacement of thiamine (51%). The next most potent compounds were **1M** and **1P** (both having the same uracil ring as **1T**; 27% and 32% displacement respectively) followed by **10P** (4-amino-6-methylpyrimidine) which showed modest displacing ability (20%).

Analogue **10P**, like TPP, has a methyl-4-aminopyrimidine ring. However, it is attached *via* the C-2 position to its B ring with the methyl group at C-6, whereas in TPP the pyrimidine is connected *via* C-5 and the methyl group is at C-2. The changed orientation of the A ring means that if it were to bind in the same way as TPP, one potential H-bond to the RNA would be lost

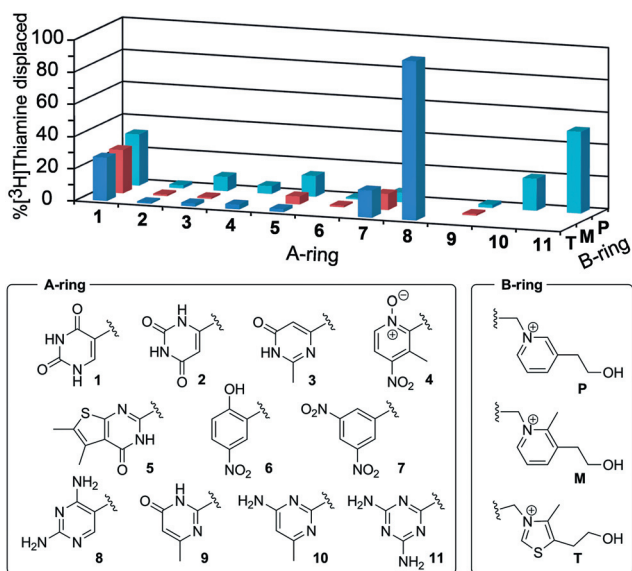


Fig. 2 Equilibrium dialysis results for the A-ring analogues. The compounds' ability to displace radiolabelled thiamine from *thiM* RNA is given as a percentage normalised against a positive control (500 μM TPP, 100% displacement) and a negative control (no added ligand, 0% displacement) on the vertical axis. Where there is no column, the compound was not synthesised/tested. Final concentrations: test compound = 500 μM , [^3H]thiamine = 100 nM and RNA = 10 μM . Approximate error (scintillation counting) for all equilibrium dialysis data is $\pm 9\%$.

compared to TPP and also that the B ring would be orientated differently and may have to adopt a twisted conformation to fit within the binding pocket. These factors cause **10P** to have only a moderate affinity for the aptamer despite having a similar type of A ring to TPP.

The dissociation constant for the most potent analogue, **8T**, was measured by isothermal titration calorimetry (ITC) and found to be 49 μM (Fig. S2 \ddagger).

Changing the B ring

In the X-ray crystal structure of thiamine bound to the *thiM* aptamer, the central B ring does not make any close contacts with the RNA, and so minor changes in ring B were not expected to have much effect on the binding. In agreement with this the dissociation constants for commercially available amprolium (8.8 μM) and pyriothiamine (1.8 μM , see Fig. S2 \ddagger), in which the thiazolium of thiamine is replaced by a pyridinium ring, are similar to that for thiamine (1.5 μM).⁸ Likewise compounds **1T**, **1M** and **1P** showed very similar levels of [^3H]thiamine displacement to each other, between 27 and 32% (Fig. 2). Compounds **3**, **5** and **7** showed small differences between the **T**, **M** and **P** series, but these differences are not likely to be significant given that the radioactivity counting error on each measurement is $\pm 9\%$.

To assess the tolerance of the *thiM* aptamer to other modifications at this position, we tested analogues where the thiazolium B ring of TPP was changed to other 5-membered aromatic heterocycles (**12–14**, triazole, 4-methyltriazole and thiophene respectively) or open-chain functionalities (**15–17**). Using

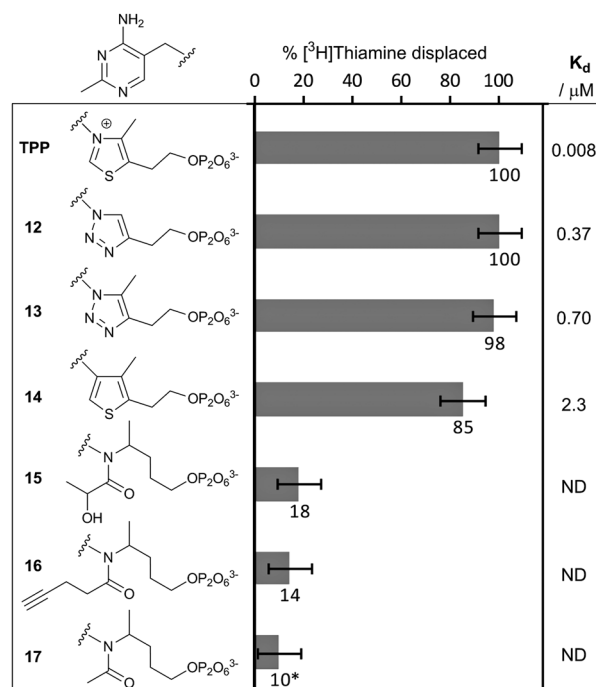


Fig. 3 Equilibrium dialysis and ITC results for the B-ring analogues. The percentage of [^3H]thiamine displaced was measured using 500 μM analogue (*except 720 μM for **17**). ND indicates that binding affinity was not determined by ITC.

competition equilibrium dialysis, as before, all the cyclic analogues were found to strongly displace [^3H]thiamine ($\geq 85\%$), but the open-chain analogues showed only marginal displacement (Fig. 3). The dissociation constants of **12–14** were measured by ITC to be in the range 0.37–2.3 μM .

Changing the tail – interactions with the pyrophosphate-sensing helix

The pyrophosphate group is required for tight binding and stabilization of the overall RNA fold – affinity drops from TPP (8 nM) to TMP (800 nM, Fig. S2 \ddagger) to thiamine (1.5 μM) as successive phosphates are removed. To explore whether other functionalities could be substituted for the pyrophosphate moiety, a series of triazole compounds bearing pyrophosphate analogues⁹ (**12**, **18–24**) were tested for binding by equilibrium dialysis (Fig. 4). The triazole pyrophosphate (**12**), difluoromethylene-diphosphonate (**19**), methylenediphosphonate (**20**) and *N*-phosphosulfamate (**21**) all strongly displaced thiamine (98–100%), whereas the remaining analogues with more varied functionality gave smaller displacements. The addition of an *N*-sulfocarbamate group (**22**, 59%) did not produce any additional binding over the alcohol (**18**, 61%) and the addition of the iminodiacetate (**23**) and malonate (**24**) groups impaired rather than improved binding.

The dissociation constants for the better ligands were measured by ITC (Fig. 4 and S3 \ddagger) and the most potent ligand by far was **20** (K_d = 9 nM) followed by **19**, then **12** and **21** which all had K_d values of between 0.1 and 1 μM . They in turn bound

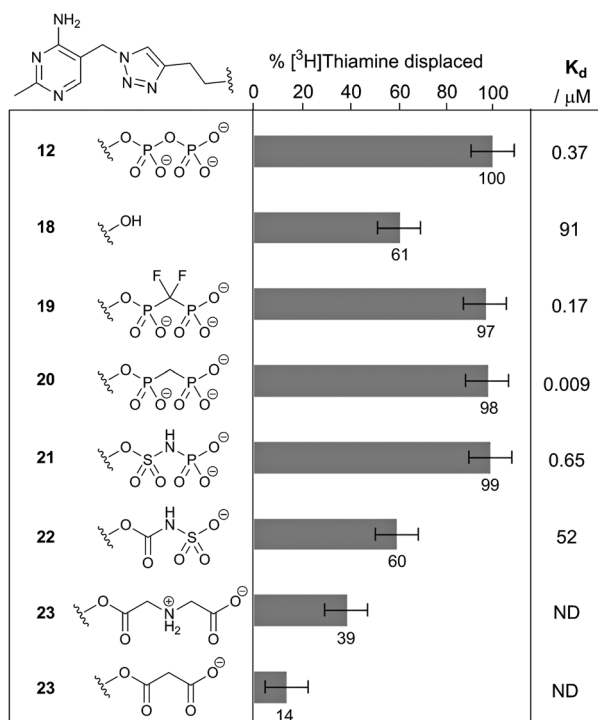


Fig. 4 Equilibrium dialysis results for the pyrophosphate analogues (tested at 500 μM). Dissociation constants for the more potent pyrophosphate analogues were obtained by ITC.

2–3 orders of magnitude better than **22** and **18** ($K_d = 52 \mu\text{M}$ and $91 \mu\text{M}$ respectively).

Effect of thiamine analogues on gene expression

The biophysical binding experiments indicated that many of the analogues tested have good binding affinity for the *thiM* aptamer. If in addition, these compounds were also able to affect the expression platform (when the whole riboswitch is present), they might alter gene expression. To test whether any of the analogues had such an effect, we assayed them using an *in vitro* transcription/translation (IVTT) method.⁷

The *thiM* riboswitch sequence (aptamer and expression platform) was cloned upstream of the *Renilla reniformis* luciferase gene and both were cloned into a plasmid bearing a T7 promoter. Luciferase was transcribed and translated using T7 RNA polymerase and *E. coli* S30 extract during a 2 h incubation in the presence of 100 μM ligand. The level of gene expression was assessed by measuring the luminescence of the sample upon addition of a luciferase substrate at the end of the incubation period and normalising it against a control reaction where no ligand was added.

The ability of the analogues to repress luciferase expression varied (Fig. 5). Methylenediphosphonate ester **20** was able to repress protein expression to the same extent as TPP (relative luminescence of 0.40 *versus* 0.39 for TPP). Difluoromethylenediphosphonate ester **19** and the pyrophosphorylated B-ring analogues **12–14** also had an effect, though less pronounced (relative luminescence of 0.68, 0.65, 0.62 and 0.66 respectively). The compounds **21**, **22** and **8T** (relative luminescence of 0.72,

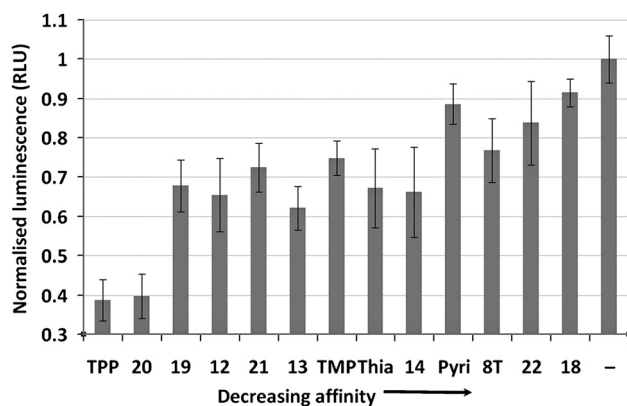


Fig. 5 The effect of various analogues on *thiM* riboswitch-controlled expression of luciferase. The level of luminescence is shown as a percentage of the luminescence observed with no added ligand. The analogues are arranged in order of binding affinity with the strongest binder (lowest K_d value) on the left and the weakest binder (highest K_d) on the right. Pyri = pyrithiamine, Thia = thiamine.

0.84, 0.77 respectively) affected gene expression to approximately the same extent as TMP (0.75) and thiamine (0.72), while pyrithiamine (0.82), amprolium (0.89), **22** (0.84) and **18** (0.92) had little or no significant activity. To exclude the possibility that the analogues were reducing the measured luminescence by interfering with the IVTT expression system or by inhibiting the luciferase enzyme, the experiments were repeated using a modified expression construct that had most of the riboswitch sequence removed (Fig. S4†). In those experiments, addition of TPP or any of its analogues had a negligible effect on the luminescence *versus* the control.

Discussion

Screening a range of TPP analogues offers insight into the importance of each part of the TPP molecule for molecular recognition and its influence on the conformational state of the riboswitch. Previously the only TPP analogues bearing modified pyrimidines that had been tested against the *thiM* aptamer were amprolium and oxythiamine (Fig. 6, insert). These showed only weak binding by in-line probing experiments.² Our ITC experiments showed that, while amprolium ($K_d = 7.5 \mu\text{M}$) is only slightly weaker a ligand than thiamine, oxythiamine did not show any heat of binding (Fig. S3†). Oxythiamine must form the less thermodynamically favoured enol tautomer in order to present the same pattern of H-bond donor/acceptors to *thiM* as thiamine. Amprolium, having the same arrangement of H-bond donors and acceptors as the aminopyrimidine ring of thiamine, binds better, but it is a weaker ligand, possibly due to steric effects from the propyl side-chain at C-2 of its aminopyrimidine ring (compared to the smaller methyl group at this position in thiamine).

From our studies with the **T**, **M** and **P** series of compounds (**1–11**), it is clear that it is the A ring that dictates binding, as varying the B ring has little effect. The most potent ligand in this series, **8T**, possesses a diaminopyrimidine capable of replicating the H-bonding pattern of TPP (Fig. 6). However, the

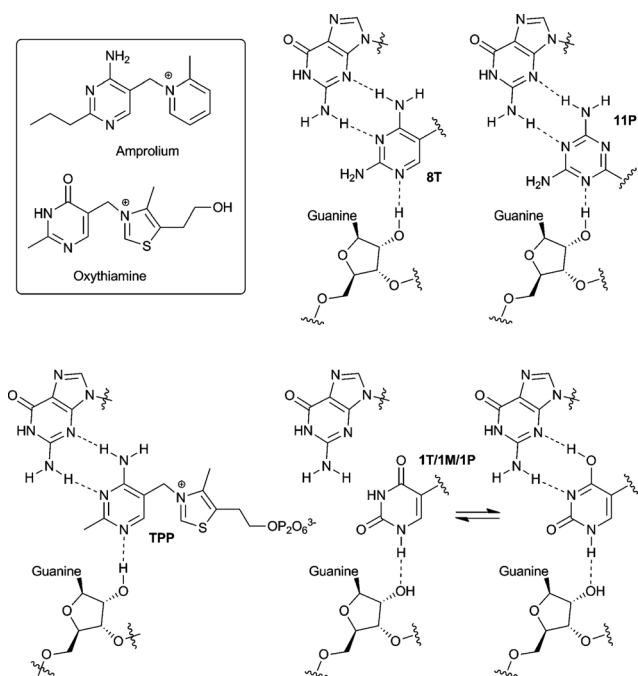


Fig. 6 The H-bonds that TPP forms to the pyrimidine-binding helix of the *thiM* aptamer and how some of the analogues might replicate these interactions.

replacement of the methyl group at C-2 in thiamine by a second amino group reduces its affinity (K_d increases from 1.5 μM to 49 μM). As the two groups are comparable in size, the loss of affinity is unlikely to be due to steric effects. The electron donating mesomeric effect of the amino group may affect both π -stacking and the $\text{p}K_a$ of the other nitrogen atoms around the ring, which would in turn affect the strength of H-bonding. In addition the loss of solvation of the additional amino group when it binds to the riboswitch may be detrimental.

Compound **11P** has the same H-bonding potential in ring A as **8T** and thiamine; however, it binds less tightly than either. This could be due to the fact that if it was to form the same H-bonds as thiamine and **8T**, its B ring would be positioned differently within the aptamer compared to the other two ligands and there may be steric interactions arising from the changed positioning of the second ring.

Pyrimidinediones **1T/1M/1P** all bind to the riboswitch (27–32% displacement of [^3H]thiamine) but with much less affinity than the mono- and di-aminopyrimidines, thiamine and **8T**. This can be explained by the fact that the preferred tautomer of **1** can only form one of the three hydrogen bonds observed in the pyrimidine binding site for thiamine. Tautomerisation to a less favourable enol tautomer (Fig. 6) would be required to allow the other two hydrogen bonds to the neighbouring guanine (alternatively, tautomerisation of the guanine to the less favourable imino tautomer would have the same effect). The negative effect of changing the relative position of attachment of ring B, which was observed with **11P** vs. **8T**, can also be seen with compound sets **1** and **2**, which both have uracil as the A ring but are attached to the B-ring *via* different positions. As a result of the less optimal substitution position, compounds **2** fail to displace any [^3H]thiamine at all.

In the crystal structures of the *thiM* aptamer–TPP complex, the central part of the TPP molecule (ring B) is solvent-exposed and forms no direct bonding interactions. As a consequence the RNA might be expected to accommodate ligands with significantly different B rings. This is supported by the fact that the apparent K_d for pyriPP binding to the *Bacillus subtilis tenA* riboswitch has been estimated at 160 nM, compared to 50 nM for TPP,¹⁰ and the *Aspergillus oryzae thiA* aptamer binds pyriPP with essentially the same affinity as TPP (K_d values of 50 nM and 56 nM respectively).¹⁰ Our data are consistent with these observations. ITC experiments show that thiamine and pyrithiamine bind with essentially the same affinity to the *thiM* aptamer. Our equilibrium dialysis experiments with the **T**, **M** and **P** series alcohols confirm that the aptamer does not distinguish to any great extent between the methylthiazolium, pyridinium and methylpyridinium rings, which are all positively charged.

It is notable, however, that analogues with a positively charged B ring bind much better than ones with a neutral B ring. For example, TPP binds *ca.* 300 times more tightly than dezaTPP **14**. One reason for this is probably electrostatic attraction of the positive charge towards the negatively charged RNA, although none of the backbone phosphates are particularly close. However, we believe there may be a second reason. In our previous paper,⁷ we reported the binding to the *thiM* riboswitch of a series of “fragments” including some 4-aminopyrimidines with various groups attached to C-5. Among these 4-aminopyrimidines there was a correlation between the electron withdrawing ability of the C-5 substituent and the binding affinity of the compounds. The same trend is observed for compounds **12–14**, which suggests that the electronic nature of the A ring is also important for strong π -stacking/H-bonding and the electron withdrawing effect of a positively charged B ring enhances the binding of the A ring. This is also supported by the observation that **8T**, with the electron-donating amino group at C-2 binds more weakly than thiamine. If this hypothesis is correct, it would explain how the riboswitch is able to differentiate thiamine and its phosphate esters from other 4-aminopyrimidines that it may encounter *in vivo*, for example hydroxymethylpyrimidine (HMP, a biosynthetic precursor to TPP), which has an identical A ring to TPP but only a CH_2OH in place of the B ring and which shows no apparent binding to the aptamer.⁷

Further support for the importance of the positively charged B ring comes from the fact that our neutral open chain analogues bind only very weakly to the *thiM* aptamer, although a contributing factor might be that their flexible nature makes it entropically less favourable to adopt a conformation that would allow the pyrimidine and the pyrophosphate to dock simultaneously to the RNA. The *E. coli thiM* aptamer can accommodate benfotiamine,⁴ which also has a non-cyclic functionality in place of the B ring and has a terminal phosphate (so it is a TMP mimic). In line with our results with open-chain analogues, benfotiamine is known to bind much less tightly than TMP.²

The absence of a terminal pyrophosphate does not preclude binding, but it does have a drastic effect on the affinity of the interaction with the riboswitch. TPP binds nearly 200 times better than thiamine, and a similar boost in affinity is observed when **18** is pyrophosphorylated to give **12** (250-fold reduction in K_d). When the pyrophosphate is replaced with other functionalities, there is a general trend in binding affinity: trianionic

compounds (**12**, **19**, **20**) > dianionic (**21**) > monoanionic (**22**, **23**, **24**). The same trend was seen when these compounds were tested against TPP-dependent enzymes.⁹ For the trianionic compounds, replacing the bridging oxygen of the pyrophosphate with a methylene group significantly improves the binding (40-fold lower K_d), whereas replacing it with a difluoromethylene group has a much smaller effect (2-fold lower K_d). The oxygen is not directly involved in forming any interactions to the RNA and the effect of the bridging group on the electronics of the other phosphate oxygens seems more important. There is a clear correlation between the pK_a of the phosphates/phosphonates and the binding affinity, with the best ligand being the methylene phosphonate ($K_d = 9$ nM), which has the least acidic protons (approximate pK_{a3} value for the parent acid is 7.45¹¹), followed by the difluoromethylene phosphonate ($K_d = 170$ nM, $pK_{a3} \approx 5.80$) and then the pyrophosphate ($K_d = 370$ nM, $pK_{a3} \approx 5.77$). The ability of the phosphate/phosphonate to stabilise electronic charge will obviously have an effect on its interaction with the magnesium ions and the RNA. Due to the presence of the two divalent cations, it is likely that all three of these compounds would be bound in the trianionic form. In this situation, the least acidic phosphonate has the least stabilised negative charge and would presumably form the strongest interaction to the oppositely charged magnesium ions.

The analogues that showed binding to the riboswitch aptamer were also tested for their ability to reduce gene expression, using an IVTT reporter gene assay. The assay monitored the riboswitch activity indirectly by measuring the luminescence produced by a catalytic reporter protein (*Renilla* luciferase) produced by IVTT. This system differs from previously published *thiM* reporter gene assays¹⁰ because it is performed *in vitro* using cell-free *E. coli* extracts, thus avoiding any doubts about cell-permeability, and it employs *Renilla* luciferase as the reporter gene, which gives high sensitivity. It is notable that with this system even TPP at 100 μ M only decreases expression of the reporter gene by *ca.* 60%. This is possibly because the concentrations of the components in the IVTT mixture mean that the TPP only has a limited amount of time to bind to the riboswitch before a ribosome binds to the ribosome binding site (RBS) and it is too late to prevent translation from starting.

The conformational changes that occur during switching have been studied in detail.^{12–15} The present model proposes that, in the absence of TPP, the pyrimidine-sensing helix (P2 and P3) is mostly preformed while the pyrophosphate-sensing helix (P4 and P5) is thought to have more conformational freedom allowing the riboswitch to exist in a fine equilibrium between two alternately folded states. In one, the two strands that make up the P4–P5 helix base-pair, forming the pyrophosphate binding pocket, leaving the RBS and start codon to be sequestered in another stem–loop within the expression platform. In the other, a strand of P4–P5 helix base-pairs with a portion of the expression platform, leaving the RBS and start codon single-stranded, thereby allowing initiation of translation (see Fig. S1†). The binding of TPP stabilises the pyrophosphate sensing helix and other tertiary interactions within the aptamer (especially a distal contact between P3 and loop L5 at the end of the P4–P5 arm), thereby favouring the former of the two states. This model suggests that, while the pyrimidine ring of TPP is important for molecular recognition, the pyrophosphate moiety of TPP is

required for the stabilisation of the OFF conformation of the riboswitch and therefore gene regulation.

Several factors must be borne in mind when comparing the thermodynamic K_d value for a compound binding to the aptamer with its effect on expression of a reporter enzyme: there could be significant differences in affinity between full length mRNA and the aptamer alone; switching could be kinetically rather than thermodynamically driven;¹⁶ the Mg^{2+} ion concentrations in the systems are likely to be different, *etc.* Nevertheless, our results suggest that there is a trend between the ligand binding affinity of the TPP analogues and the degree of gene repression. The most potent analogue, **20**, binds to the *thiM* aptamer with a very similar affinity to that of TPP and represses the expression of the *Renilla* luciferase to the same extent (approximately 60% reduction). Whereas, compounds **12–14**, **19** and **21**, which have K_d values approximately 20–250-fold higher than TPP, exert a lesser effect on gene expression, only reducing it by 21–38%. Interestingly, TMP shows the same behaviour as these analogues. The concentrations of all these compounds in the IVTT mixture is much higher than their measured K_d values, so at equilibrium the riboswitch should be almost fully bound to the compound. The fact that translation is not turned off as fully with these compounds as with TPP, therefore, suggests either that the system does not have time to reach equilibrium (as already suggested for TPP) or that the pyrophosphate-sensing helix is not so tightly held in place in the bound state and a small proportion of it exists in other conformations that allow binding of a ribosome (or a combination of these two effects). Further experiments to follow the conformational changes in the pyrophosphate-sensing helix would be needed to distinguish between these two possibilities.

The results obtained with the alcohol analogues (thiamine, pyrithiamine and **8T**) show that some regulation of expression does still occur, even in the complete absence of a pyrophosphate group. This is not what would be predicted from the model described above for the switching mechanism. This may hint at a more subtle model for switching whereby the pyrimidine-sensing helix, while preformed, is still flexible and docking of a ligand helps to rigidify it and thereby enhances the formation of the L5–P3 tertiary contact. This in turn shifts the equilibrium towards the OFF conformation. Although further studies will be necessary to clarify this point, our *in vitro* transcription–translation assay allowed for investigation of TPP analogues that would have not been possible with an *in vivo* system. Therefore, it should prove a valuable tool to complement crystallographic and *in vivo* expression techniques.

Conclusions

In conclusion, the studies reported here show that the *thiM* riboswitch of *E. coli* is highly selective for the 4-aminopyrimidine ring (A ring) of TPP, although other heterocycles can bind if they can offer the same pattern of hydrogen bond donors and acceptors. The riboswitch is not very selective for the thiazolium ring (B ring) of TPP, but positively charged rings bind much better than neutral ones and open-chain compounds are poor ligands. The pyrophosphate group of TPP is essential for tight binding but can be replaced by other trianionic groups of similar

shape. In particular the methylenediphosphonate group shows even better binding than pyrophosphate.

When tested in an *in vitro* transcription–translation (IVTT) system, the ability of the analogues to repress gene expression correlated with their binding affinity irrespective of whether they possessed a group capable of binding to the pyrophosphate-sensing helix. This suggests that binding of a ligand in the aminopyrimidine binding pocket alone can stabilise the whole aptamer structure, including the pyrophosphate-sensing helix (P4/P5, Fig. 1).

These observations should be borne in mind when designing effector ligands for TPP riboswitches, and again emphasise that RNA is capable of creating highly specific and selective interactions with small molecules.

Experimental

Materials

G-[³H] Thiamine hydrochloride was obtained from American Radiolabelled Chemicals Inc. Coelenterazine was from NanoLight Technologies. All other reagents and buffers were purchased from Sigma-Aldrich, Fisher or Melford. His₆-tagged T7 RNA polymerase (RNAP) was expressed and purified according to published procedures.⁷ All aqueous solutions were treated with 0.1% diethyl pyrocarbonate and autoclaved prior to use. All non-sterile vessels used for RNA handling were treated with RNase-AWAY (Molecular BioProducts) prior to use.

RNA preparation

The DNA encoding the *thiM*-RS was PCR-amplified from *E. coli* and cloned into pGEM-T easy vector (Promega), using standard molecular biology techniques. Its identity was confirmed by sequencing (GeneService). The fragment containing the T7 promoter and *thiM*-RS aptamer was amplified by PCR to obtain the 203 bp template for *in vitro* transcription (IVT). The 161 nt RNA containing the 78 nt *thiM* aptamer used in all the experiments was synthesised by IVT reactions containing 4 × 4 mM NTPs, 50–70 nM DNA template, 0.05–0.01 U μL⁻¹ inorganic pyrophosphatase (from *E. coli*, Sigma Aldrich), 10 μg mL⁻¹ T7 RNAP in 40 mM Tris pH 8.0, 20 mM MgCl₂, 10 mM DTT, 2 mM spermidine, and 0.01% Triton X-100. IVTs were incubated at 37 °C for 3 h, then 0.05 U μL⁻¹ RQ1 DNase (RNase-free, Promega) was added and the mixture was incubated for a further 15 min. All enzymatic reactions were stopped by the addition of EDTA (50 mM), extracted with 1× volume of citrate buffered phenol pH 4.3 and 1× volume of chloroform–isoamyl alcohol 24 : 1 and precipitated by addition of 1× volume of isopropanol and 0.1× volume of Na acetate pH 5.0 and cooled at –20 °C. The crude RNA was purified by 6% denaturing urea-PAGE, recovered by the crush/soak method in 10 mM phosphate buffer pH 7.0, 1 mM EDTA, concentrated by centrifugation (Vivaspin), precipitated in ethanol and redissolved in water. RNA was quantified by measuring the absorbance at 260 nm (Nanodrop, Thermo Scientific), and stored at –20 °C.

Competition equilibrium dialysis

Competition equilibrium dialysis experiments were carried out in DispoEquilibrium Dialyzers, 10 kDa MWCO (Harvard

Apparatus). Chamber A contained 200 nM [³H]thiamine (500 cpm pmol⁻¹), 500 μM test compound, 50 mM Tris pH 7.6, 50 mM NaCl, 5 mM MgCl₂, 30 μL total volume. Chamber B contained 10 μM RNA in 50 mM Tris pH 7.6, 50 mM NaCl, 5 mM MgCl₂, 30 μL total volume. The no ligand control contained no compound and the TPP control contained 500 μM TPP. Dialyzers were equilibrated overnight at 4 °C, then 20 μL aliquots were taken from each chamber, added to 5 mL of Optiphase HiSafe III scintillation cocktail (Perkin Elmer) and counted (Packard Tri-Carb 2100TR). The percentage of thiamine displacement was obtained by first calculating CpmB/(CpmA + CpmB), then by normalising these values onto the scale of no ligand control (0%) and TPP control (100%). The error on the percentages, estimated from the counting error, is approximately 9%.

Isothermal titration calorimetry

The RNA was dialysed exhaustively against binding buffer (50 mM Tris pH 7.6, 50 mM NaCl, 5 mM MgCl₂) for 18 h at 4 °C. The dialysis buffer was then used to make all subsequent dilutions and to prepare the solutions of the analogues. All experiments were carried out using a MicroCal ITC200 microcalorimeter at 25 °C, with reference power set to 5 μCal s⁻¹, initial delay 60 s and a stirring speed of 1000 rpm. An RNA concentration of 20–50 μM was used. Ligand concentrations were 1–0.2 mM. Each titration consisted of 25 injections (1 × 0.4 μL followed by 24 × 1.5 μL), spaced 80 s apart. For each ligand, a control titration of ligand into buffer was carried out and the resulting background curve was used to correct the RNA titrations. The data collected from each experiment were fitted with a single-site binding model using Origin ITC software (Microcal Software Inc.). The dissociation constant K_d was obtained by taking the inverse of K_a , and the error ΔK_d was estimated from the software calculated error ΔK_a , using the relationship $\Delta K_d = K_d \times (\Delta K_a / K_a)$.

In vitro transcription–translation (IVTT) assays

The plasmids used as DNA templates for *in vitro* transcription–translation (IVTT) were prepared according to standard molecular biology techniques and their identity was confirmed by sequencing (GeneService). IVTT assays were performed using a commercially available kit (Promega, *E. coli* T7/S30 Extracts for Circular DNA). A typical incubation (30 μL), prepared according to the manufacturer instructions, contained 7 ng μL⁻¹ of DNA template and 100 μM of compound (excluding negative controls). The IVTT reactions were incubated at 37 °C for 2 h, cooled on ice, then diluted 4-fold with luciferase buffer (50 mM Tris pH 7.6, 5 mM MgCl₂, 2 mM DTT, 0.4 mg mL⁻¹ of BSA). The luminescence was followed using a BMG microtitre plate reader (FLUOstar OPTIMA) equipped with a reagent injecting system. 55 μL aliquots of the diluted IVTT mix were placed in a black 96-well microtitre plate and 10 μL of 20 μM coelenterazine solution (in 200 mM HEPES pH 7.0, 1 M NaCl, 2 mM EDTA, 0.4 mg mL⁻¹ BSA, 1% ethanol) were added to each solution after a 20 s read delay and the luminescence increase was monitored every 2 s for 80 s. To obtain the luminescence counts, the

light signal was integrated over 80 s after injection. The assays were performed in duplicate.

Synthesis

See ESI.†

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