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Covalent Stabilization of a Small Molecule-RNA Complex

Hayden Peacock, Radhika Bachu, and Peter A. Beal*

‡ Department of Chemistry, University of California, One Shields Ave, Davis, CA 95616

Abstract

We demonstrate covalent bond formation between an RNA aptamer containing a cysteamine-tethered nucleobase and helix-threading peptides (HTPs) containing α -bromoacetamide N-termini. The reaction is high yielding and inhibited by a DNA strand Watson-Crick complementary to the aptamer sequence indicating covalent reaction is dependent on the high affinity HTP-binding site present in the folded aptamer. These results are important for future structural studies of HTP-RNA complexes and methods for the discovery of new high affinity analogs via covalent tethering strategies.

Keywords

aptamer; helix-threading peptide; nucleoside analog; covalent tethering; templated reaction

Methods to introduce specific covalent bonds within biological macromolecules have seen use in a variety of applications. For instance, bio-orthogonal reactions, like the Staudinger-Bertozzi ligation, can be used to label and image specific cell surface molecules[1]. Also, bio-orthogonal triazole-forming cycloadditions of azides and alkynes have been used to functionalize specific sites in proteins to, for instance, introduce purification handles[2]. The selectivity of reaction in these and related examples comes from the inherently selective nature of the reactive functional groups (phosphine + azide, alkyne + azide, etc). Selectivity in the formation of covalent bonds with biological macromolecules can also arise via the formation of specific noncovalent complexes that bring reactive groups together. This approach, typified by Meares' recent use of covalent bonds to enhance antibody-antigen interactions[3], is particularly useful as a means of stabilizing complexes prior to structural studies[4,5] or as a strategy for the discovery of new high affinity ligands for biological receptors[6]. While this approach has been applied broadly to the study of small molecule-protein[7–9], small molecule-DNA[10,11] and DNA-protein interactions[4,5], there is a paucity of data on the use of covalent chemistry to stabilize complexes involving small molecules and RNA[12]. Here we describe an example of covalent stabilization of a small molecule-RNA complex.

Our laboratory has reported the development of helix-threading peptides (HTPs) for targeting binding sites within duplex RNAs[13–18]. These compounds have an aromatic heterocycle modified at opposite edges such that binding by intercalation places substituents in both the minor and major grooves at duplex binding sites (i.e. threading intercalation).

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*Corresponding author: (530) 752 4132 beal@chem.ucdavis.edu.

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Our previous studies demonstrated that compounds of this type bind preferentially to sites in duplex RNA bearing a 5'-PyPu-3' sequence flanked on each 3' side by bulges or loops. Selective binding sites for HTPs have been identified in a bacterial ribosomal RNA[13], a human miRNA precursor[17] and adjacent to an editing site in a human pre-mRNA[18]. However, optimization of the affinity of these ligands for specific target sites has been hampered by the lack of high-resolution structural data for an HTP-RNA complex. Stabilization of these complexes by introducing covalent linkages should prove helpful in these efforts.

We have analyzed the binding mode of HTPs to an RNA aptamer containing a high affinity site using directed hydroxyl radical cleavage[14] (Figure 1). The cleavage patterns observed suggested that the N-terminus of the bound HTP is proximal to the first two adenosines of the AAUU internal loop (nucleotides 21 and 22 of RNA used in this study, Figure 1), which is directly adjacent to the putative intercalation site (5'-CpG-3' step). Guided by these results, we incorporated complementary reactive functional groups into the RNA and HTP to allow for stabilization of the complex via a covalent link. Thus, we replaced nucleotide 21 or 22 with an adenosine analog containing an *S*-tritylcysteamine modification at the purine 2-position (RNAs **1** and **2**, respectively), which can be deprotected after RNA synthesis to reveal a nucleophilic thiol. We also synthesized three 2-phenylquinoline HTPs with an α -bromoacetamide electrophile at each N-terminus, and three different lengths of N-terminal peptide domain (Figure 1).

To install the thiol-containing nucleobase into RNA, we prepared the corresponding phosphoramidite (**1**, Scheme 1). First, tri-*O*-acetyl protected 2-fluoropurine ribonucleoside was treated with *S*-tritylcysteamine, yielding the *N*²-alkylated nucleoside **2** via nucleophilic aromatic substitution. This was followed directly by deprotection with ammonia/methanol. The 5'-DMTrO, 2'-OTBDMS, β -cyanoethyl phosphoramidite was prepared from **2**, and used to incorporate the new nucleoside analog into the RNA aptamer via solid phase synthesis. After cleavage from the solid support, the RNA (still containing the S-Tr protecting group) was purified via denaturing polyacrylamide gel electrophoresis, and treated with silver nitrate to reveal the free thiol[19,20]. Complete deprotection of the trityl group was confirmed via MALDI-MS. The RNA was stored frozen in reducing buffer (containing tris(2-carboxyethyl)phosphine (TCEP)) until use. The deprotected RNA was stable when stored in buffer at -80 °C for several months.

The electrophilic 2-phenylquinoline HTPs were prepared via solid-phase peptide synthesis incorporating the 2-phenylquinoline amino acid using procedures based on our previous report[16]. The α -bromoacetamide functional groups were installed by performing a final coupling with bromoacetic acid. Upon completion of the synthesis, the peptides were purified by HPLC and characterized by high-resolution ESI-MS.

Using MALDI-MS, we evaluated the effect of combining RNA1 (0.1 μ M) bearing the 2-cysteamine purine analog at position 21 with HTP1 (shortest N-terminal peptide domain of three HTPs prepared) (1 μ M) (Figure 2A, B). Importantly, we observed a new peak in the mass spectrum of the reaction mixture consistent with the predicted structure of the RNA-HTP covalent conjugate (calculated = 9937.4, observed = 9936.7). The progress of the reaction was evaluated by obtaining mass spectra at various time points after an iodoacetamide quench. The relative intensities of peaks corresponding to HTP-RNA conjugate and iodoacetamide-quenched RNA varied with reaction time. These data were used to plot a reaction progress curve and the data were fitted to a first order exponential yielding a rate constant $k = 5.5 \times 10^{-2} \text{ min}^{-1}$ for the covalent conjugation reaction (Figure 2C). The measured rate constant is approximately 6 times greater than the rate constant reported for a similar conjugation reaction occurring between an in vitro selected 5'-

phosphorothioate RNA and an α -bromoacetamide peptide[21]. Our work reported here is distinguished from this earlier example by the fact that we have engineered a covalent bond into the RNA-ligand complex *post*-selection, as opposed to using covalent bond formation as a criterion for RNA selection.

While our results clearly show the formation of a covalent conjugate, it was important to establish whether the reaction was dependent on selective binding of the HTP to the aptamer RNA. Previous studies in our laboratory have shown that HTPs of this type bind poorly to perfectly matched duplexes[13,15]. Thus, we annealed a Watson-Crick complementary single stranded DNA to the thiol-tethered RNA aptamer generating a hybrid duplex that lacks the high affinity HTP binding site but still bears the reactive thiol (Figure 3A). As expected for a reaction requiring the aptamer binding site, the hybrid duplex did not react with HTP1 under these conditions (Figure 3B). This inhibitory effect was shown to require the DNA sequence to be complementary to the aptamer as a non-complementary DNA did not inhibit conjugation (Figure 3C). In addition, we tested to see if an equivalent concentration of iodoacetamide (1 μ M) would react with the RNA under the same conditions. After 1h, we observed no alkylation product in response to iodoacetamide treatment (data not shown), whereas under identical conditions HTP1 gave approximately 90% conversion to the covalent conjugate.

The yields of covalent conjugates for reactions involving the two thiol-modified RNAs (0.1 μ M) and three bromoacetamido-HTPs (1 μ M) were monitored by MALDI-MS of the reaction mixtures quenched with iodoacetamide at 5 min time points (Figure 4). Reactions of HTP2 and HTP3 gave higher yields than HTP1, for both RNAs. However, these reactions were only partially inhibited by WC complementary DNA, suggesting that nonspecific reaction may be contributing to the yield in these cases. This may be due to increased reactivity of HTPs 2 and 3, in which the bromoacetamide is located adjacent to an alpha amino acid (Val or Ser, respectively) rather than to the (4-aminophenyl)methyl moiety.

RNA2 gave conjugate yields similar to those observed for RNA1 with each of the HTPs tested suggesting a similar placement of reactive thiol in the complexes involving RNAs 1 and 2 (Figure 4). As described above, no high-resolution structure of the HTP-binding aptamer is available to evaluate the positions of the modified adenosines. However, an RNA aptamer that binds the dye malachite green is similar in sequence to the HTP aptamer used in this study. Importantly, a crystal structure[22] of this aptamer is available in which a ligand (tetramethylrosamine, an analog of malachite green) is intercalated into a 5'-CpG-3' step adjacent to an AAU loop. In this structure, the C2 positions of the loop adenosines are within three angstroms of each other (Figure 5). The minimal difference in the reactions of RNAs 1 and 2 observed in this study is consistent with positioning of the loop adenosines similar to those found in the AAU loop of the malachite green aptamer.

In summary, we have shown that the complex between an RNA-binding helix threading peptide and its RNA target can be stabilized by a covalent bond formed via reaction of thiol-containing RNA and bromoacetamide-HTP. The reaction was inhibited by a DNA strand that is Watson-Crick complementary to the RNA target confirming its dependence on the high affinity HTP binding site found in the RNA alone. These results are important for future structural studies of HTP-RNA complexes and methods for the discovery of new high affinity HTP analogs via covalent tethering strategies.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

A) Predicted secondary structure of the HTP-binding RNA aptamer used here. Sites of incorporation of the *S*-tritylcysteamine modified nucleobase are indicated by X and the putative binding site for helix threading peptide (HTPs) is indicated by the blue box. Treatment of the *S*-trityl protected RNAs with AgNO₃ followed by dithiothreitol (DTT) revealed the free thiol modification (**2AP-SH**). **B)** Structures of the α -bromoacetamide HTPs used.

Figure 2.

(A) Reaction of thiol-bearing RNA and HTP1. (B) MALDI mass spectrum of the reaction between RNA1 (0.1 μ M) with HTP1 (1 μ M) at 1 h, showing approximately 90% yield of the RNA1-HTP1 conjugate. The reaction was quenched with an excess of iodoacetamide. (C) Plot of fraction product versus time for the reaction between RNA1 (0.1 μ M) and HTP1 (1 μ M). Fraction product was determined by quenching the reaction in an excess of iodoacetamide at various time points and comparing MALDI-MS peak areas of product and starting material. The data were fitted to the equation $\%P_t = \%P_{\infty}(1 - e^{-k_{\text{obs}}t})$ using the least squares method. Data points are the average $\pm$ SD for at least two independent experiments. N.B. Peaks marked § are consistent with ions resulting from the loss of guanine (-151 m/z) and peaks marked * are consistent with ions resulting from the addition of the MALDI matrix (6-aza-2-thiothymine, $+143$ m/z). Reaction conditions: 50 mM Bis-Tris•HCl, 100 mM NaCl, 10 mM MgCl_2 , pH 7.0 containing 0.1 mM TCEP.

Figure 3.

(A) Depiction of the inhibitory effect of Watson-Crick complementary (WC) DNA on the reaction between RNA1 and HTP1. (B) MALDI mass spectrum of the reaction between RNA1 (0.1 uM), after heating and cooling with WC DNA, and HTP1 (1 uM), showing inhibition of product formation. The reaction was quenched at 1 h with an excess of iodoacetamide. (C) MALDI mass spectrum of the reaction between RNA1 (0.1 uM), after heating and cooling with non-complementary (NC) DNA, and HTP1 (1 uM), showing presence of the HTP1-RNA1 conjugate. The reaction was quenched at 1 h with an excess of iodoacetamide. Reaction conditions: 50 mM Bis-Tris•HCl, 100 mM NaCl, 10 mM MgCl₂, pH 7.0 containing 0.1 mM TCEP.

Figure 4.

Fraction product at 5 min for reaction between RNAs (0.1 μ M) and HTPs (1 μ M). Reactions were performed either in the absence of DNA, after heating and cooling with non-complementary DNA (NC DNA) or after heating and cooling with Watson-Crick complementary DNA (WC DNA). Quenching was achieved by addition of an excess of iodoacetamide. Data points are the average $\pm$ SD for at least two independent experiments. ND = not detected. Reaction conditions: 50 mM Bis-Tris•HCl, 100 mM NaCl, 10 mM MgCl_2 , pH 7.0 containing 0.1 mM TCEP.

Figure 5.

A portion of the crystal structure (left) and sequence (right) of the malachite green aptamer bound to tetramethylrosamine (in red). Highlighted in yellow are the C2 positions of the two adenosines (A₁ and A₂) within the three-nucleotide bulge directly adjacent to the ligand intercalation site (blue box). PDB code: 1F1T.

Scheme 1.

Synthesis of *N*²-(2-(triphenylmethylthio)ethyl)-2-aminopurine ribonucleoside phosphoramidite. Reagents and conditions: (i) (a) *S*-trityl-cysteamine, DMF (b) $\text{NH}_3(\text{sat})$ in MeOH, 76%; (ii) DMTrCl, DMAP, pyridine, 66%; (iii) TBDMSCl, Et_3N , DMAP, THF, 38%; (iv) 2-cyanoethyl-(*N,N*-diisopropylamino)chlorophosphite, DIPEA, THF, 79%.