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Published on Web 02/28/2007

8-Azaguanine Reporter of Purine Ionization States in Structured RNAs

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Abstract: The fluorescent nucleotide analogue 8-azaguanosine-5′-triphosphate (8azaGTP) is prepared easily by in vitro enzymatic synthesis methods. 8azaGTP is an efficient substrate for T7 RNA polymerase and is incorporated specifically opposite cytosine in the transcription template, as expected for a nucleobase analogue with the same Watson−Crick hydrogen bonding face as guanine. 8-Azaguanine (8azaG) in oligonucleotides also is recognized as guanine during ribonuclease T1 digestion. Moreover, replacement of guanine by 8azaG does not alter the melting temperature of base-paired RNAs significantly, evidence that 8azaG does not disrupt stacking and hydrogen bonding interactions. 8azaGTP displays a high fluorescent quantum yield when the N1 position is deprotonated at high pH, but fluorescence intensity decreases significantly when N1 is protonated at neutral pH. Fluorescence is quenched 10-fold to 100-fold when 8azaG is incorporated into base-paired RNA and remains pH-dependent, although apparent pK_a values determined from the pH dependence of fluorescence intensity shift in the basic direction. Thus, 8azaG is a guanine analogue that does not perturb RNA structure and displays pH-dependent fluorescence that can be used to probe the ionization states of nucleobases in structured RNAs. A key application will be in determining the ionization state of active site nucleobases that have been implicated in the catalytic mechanisms of RNA enzymes.

Introduction

Fluorescence spectroscopy is a powerful technique commonly used to study nucleic acid structure and dynamics and nucleic acid interactions with ligands.[1,2] Since natural nucleobases and nucleosides have little or no fluorescence under common experimental conditions, studies often involve the attachment of a fluorophore to nucleic acid termini or incorporation of fluorescent nucleobase analogues. Nucleobase analogues, such as 2-aminopurine,[3−8] 2,6-diaminopurine,[3] formycin,[3] pyrrolocytosine,[9] 1,3-diaza-2-oxaphenothiazine,[10] and 2-amino-6-(2-thienyl)purine,[11] have been used as probes in fluorescence spectroscopy.[12,13] Analogues that resemble natural nucleobases in structure and biochemical properties and have high fluorescence quantum yields offer the most useful probes. 2-Aminopurine, a nucleobase analogue widely used for studies of nucleic acid structure and dynamics, displays a high quantum yield in solution ($\Phi = 0.68$) but undergoes significant fluorescence quenching when incorporated into oligonucleotides (>100-fold) and base-paired nucleic acids (>200-fold).[3,14] Furthermore, 2-aminopurine is a poor mimic of guanine in the context of a canonical base pair since it lacks the C6 carbonyl group and the N1 hydrogen (in the uncharged species that predominates at neutral pH, 2-aminopurine riboside, N1 p$K_a = 3.4$)[3] that are characteristic of the hydrogen bonding face of guanine (guanosine N1 p$K_a = 9.2$).[15]

8-Azaguanosine (8azaGuo) is a guanosine analogue with N8 in place of C8 that has a high quantum yield ($\Phi = 0.55$) when N1 is unprotonated at high pH but has the same Watson−Crick hydrogen bonding face as guanosine at neutral pH (8-azaguanosine N1 p$K_a = 8.05$)[16] (Figure 1). The first syntheses of 8-azaguanine (8azaG) and 8azaGuo were reported in 1945.[17] 8azaG was shown to be incorporated into RNA in *Bacillus*

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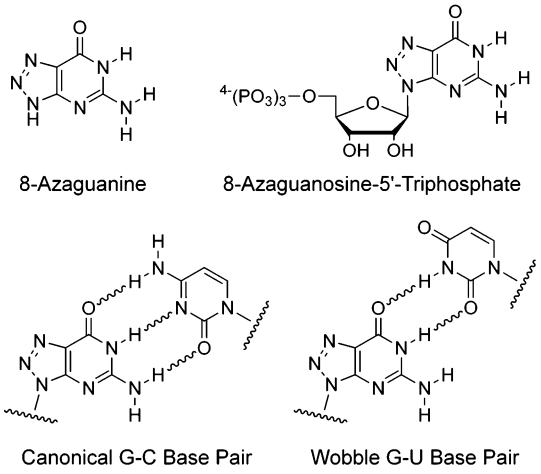

Figure 1. Chemical structures of 8-azaguanine, 8-azaguanosine-5′-triphosphate, and its base pairs with cytosine or uridine.

cereus grown in media with added 8azaG.[18] 8azaG also has been incorporated into *Escherichia coli* (*E. coli*) RNA polymerase transcripts in vitro and into mammalian RNA polymerase I transcripts in cultured cells.[19,20] 8azaGuo has been used to investigate the mechanism of purine nucleoside phosphorylase,[16,21,22] but 8azaG fluorescence has not yet been exploited as a reporter of nucleic acid structure or dynamics.

We have developed an efficient synthesis of 8azaGTP and incorporated the analogue into RNA by in vitro transcription using bacteriophage T7 RNA polymerase. As expected from the similarity between 8azaG and guanine, comparison of thermal melting profiles of fully or partially duplex RNAs in which one strand contained either a single 8azaG residue or a guanine residue revealed virtually no effect of 8azaG on RNA secondary structure stability. We also examined how fluorescence excitation and emission properties of 8azaG vary with RNA secondary structure and pH. The results demonstrate the potential of 8azaG as a nonperturbing fluorescent probe of nucleic acid structure and dynamics. 8azaG fluorescence depends on N1 deprotonation.[16]

The ionization state of active site nucleobases is a key feature of models of RNA catalytic mechanisms.[8,23−28] The crystal structure of the hairpin ribozyme complex with a vanadate transition-state mimic shows that N1 of an active site guanine, G8, is located within hydrogen bonding distance of the 2′ hydroxyl of the reactive phosphate.[29] On the basis of this structure, G8 has been proposed to act as a general acid−base catalyst, accepting a proton at the N1 position to activate the nucleophilic 2′ oxygen during cleavage.[29,30] To accept a proton as a general base catalyst, N1 of G8 must be in the deprotonated state. In an alternative model, G8 has been proposed to donate a hydrogen bond to the nucleophilic 2′ oxygen, providing electrostatic stabilization of negative charge that accumulates in the transition state as five electronegative oxygen atoms form transient bonds with phosphorus.[31,32] In this electrostatic stabilization model, N1 must be in a protonated state in order to donate a hydrogen bond.

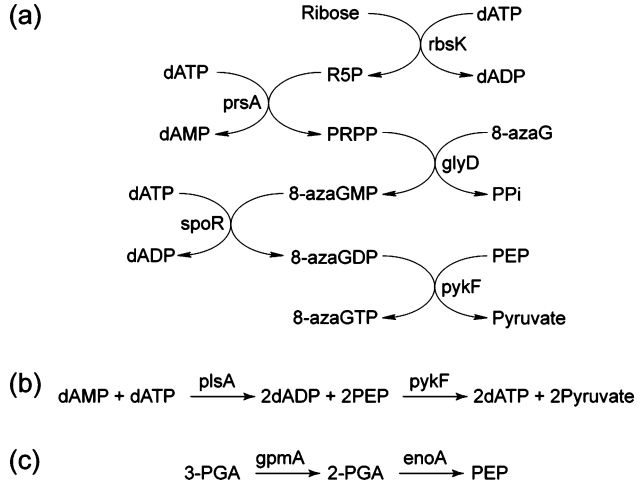

Figure 2. (a) Enzymatic synthesis of 8azaGTP from glucose and 8-azaguanine: ribokinase (*rbs*K), 5-phospho-D-ribosyl-α-1-pyrophosphate synthase (*prs*A), xanthine−guanine phosphoribosyltransferase (*gly*D), guanylate kinase (*spo*R), pyruvate kinase (*pyk*F). (b) In situ enzymatic regeneration of catalytic dATP: adenylate kinase (*pls*A). (c) In situ enzymatic generation of the phosphate donor phosphoenolpyruvate: phosphoglycerate mutase (*gpm*A); enolase (*eno*A).

Due to the problem of kinetic ambiguity, the protonation state of an active site nucleobase cannot be determined exclusively from the pH dependence of reaction rates.[33] Fluorescence spectroscopy has been used to investigate the protonation state of 2-aminopurine in a ribozyme active site,[8] but most investigations of protonation equilibria of nucleotide bases in RNA enzymes have relied on NMR spectroscopy.[34−38] Since N1 is the site in G8 that undergoes protonation and its protonated state is monitored by 8-azaguanine fluorescence, the pH dependence of 8-azaguanine fluorescence will make this analogue a useful reporter of the ionization state of active site guanines in investigations of catalytic RNAs and other structured RNAs.

Results and Discussion

(A) Enzymatic Synthesis. Our strategy for preparing 8-azaguanosine-5′-triphosphate (8azaGTP) was to use enzymes derived from the pentose phosphate and nucleotide salvaging pathways (Figure 2a). The efficient in vitro enzymatic synthesis

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of 8azaGTP began with the C5-phosphorylation of ribose by ribokinase (*rbs*K), to give ribose-5-phosphate (R5P) that was further phosphorylated at the C1 position by 5-phospho-D-ribosyl-α-1-pyrophosphate synthase (*prs*A) forming 5-phospho-D-ribosyl-α-1-pyrophosphate (PRPP).[39] Next, xanthine−guanine phosphoribosyltransferase (*gly*D) coupled 8-azaguanine (8azaG) to PRPP, forming 8-azaguanosine-5′-monophosphate (8aza-GMP). The monophosphate was subsequently converted to 8azaGTP by sequential action of guanylate kinase (*spo*R) and pyruvate kinase (*pyk*F). A key feature of the synthesis is the use of 5 equiv of dATP as phosphate donor that was regenerated using adenylate kinase (*pls*A) and *pyk*F (Figure 2b). The phosphoenolpyruvate (PEP) was in turn generated in situ (Figure 2c) from excess sodium 3-phosphoglycerate (3-PGA) with the coupled enzymes system of phosphoglycerate mutase (*gpm*A) and enolase (*eno*A).[40,41] The nucleotide analogue 8azaGTP was easily separated[42] in 60% isolated yield from the dATP and incorporated into RNA through transcription without further processing. It should be noted that five of the eight enzymes required to convert ribose into 8azaGTP are commercially available. The enzymes of the pentose phosphate and nucleotide salvaging pathways required to convert ribose into 8azaGMP are no longer commercially available in highly active forms. However, the purification procedures for these enzymes (*rbs*K, *prs*A, and *gly*D) have been described previously.[42,43] Alternatively, chemical syntheses of 8azaGTP are possible in significantly lower yields via Vorbrüggen glycosylation method[44] beginning from 8azaG (since the 8azaGuo is not commercially available), followed by phosphorylation of the resulting nucleoside.[45] The enzymatic synthesis route generates exclusively the correct diastereomer at the C1′ position of the ribose moiety, thus generating the NTP in a more efficient and cost effective manner and in higher yield.

(B) Incorporation of 8azaG into RNA. The RNA sequence that was chosen for these studies was designed to be useful for future investigations of the hairpin ribozyme catalytic mechanism (Figure 3a). The sequence of GR allows incorporation of a single guanine analogue during in vitro transcription in a location that corresponds to position 8 in the active site of a fully assembled ribozyme. The crystal structure of a hairpin ribozyme complex with a vanadate transition-state mimic places N1 of G8 within hydrogen bonding distance of the 2′-hydroxyl group that carries out nucleophilic attack on an adjacent phosphorus during self-cleavage.[29] A guanine analogue that reports directly on the protonation state of G8 through changes in fluorescence offers an approach for distinguishing possible catalytic roles for G8 in proton transfer and electrostatic stabilization that differ in the protonation state assigned to N1.[8,31,32]

A 20-nt oligonucleotide with a single 8azaG nucleobase, 8azaGR, was prepared though bacteriophage T7 RNA polymerase transcription of a partially duplex synthetic DNA template, as described previously,[42,46] except that reactions were primed with a GpG dimer and included 8azaGTP in place of GTP (Figure 3a). Transcription reactions with 8azaGTP gave isolated yields of approximately 4 nmol of 8azaGR/(mL of reaction).

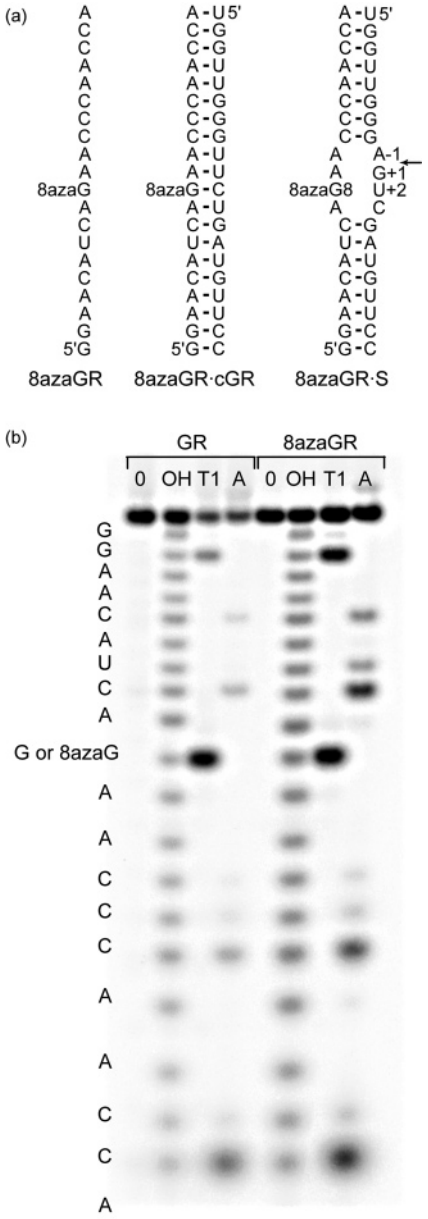

Figure 3. (a) Sequence of GR or 8azaGR and predicted base-pairing interactions in complexes with a perfectly complementary oligonucleotide, cGR, and an oligonucleotide, S, with the sequence of a hairpin ribozyme substrate. The GR·S complex is analogous to domain A of a hairpin ribozyme and is numbered accordingly, with an arrow indicating the reactive phosphodiester. (b) Sequencing of GR and 8azaGR through partial digestion of 3′-^{32}P-labeled oligonucleotides with ribonucleases T1 and A: lane 0, no treatment; OH, partial alkaline degradation; T1, partial ribonuclease T1 digestion; A, partial ribonuclease A digestion.

RNAs transcribed in reactions with 8azaGTP or GTP were subjected to enzymatic sequencing through partial digestion with ribonuclease T1, which cleaves specifically after G residues, or ribonuclease A, which cleaves after pyrimidine residues, and compared to products of partial alkaline hydrolysis (Figure 3b).

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Table 1. Thermal Melting Behavior of Complexes with 8azaG Modified and Unmodified RNAs[a]

	pH 7			pH 8			pH 9		
RNA complex	T_m	$\Delta H_m°$	$\Delta G°_{calc,25°C}$	T_m	$\Delta H_m°$	$\Delta G°_{calc,25°C}$	T_m	$\Delta H_m°$	$\Delta G°_{calc,25°C}$
8azaGR·cGR	339.8 ± 0.6	−94.1 ± 6.0	−11.5	338.6 ± 0.8	−84.1 ± 10.9	−10.0	337.9 ± 0.7	−78.9 ± 7.12	−9.3
GR·cGR	344.1 ± 2.0	−96.5 ± 39.2	−12.9	341.4 ± 0.5	−96.7 ± 4.4	−12.2	339.3 ± 0.8	−97.5 ± 12.9	−11.8
8azaGR·S	326.4 ± 0.5	−99.7 ± 9.5	−8.6	326.1 ± 0.7	−101.1 ± 9.9	−8.7	325.4 ± 2.5	−84.3 ± 33.2	−7.1
GR·S	330.0 ± 5.5	−86.5 ± 27.0	−8.4	326.8 ± 0.6	−83.7 ± 6.7	−7.3	326.8 ± 0.8	−119.1 ± 59.2	−10.4

[a] Thermal melt transition points (T_m) are in kelvin measured at a concentration of 1 μM for each RNA strand. Enthalpy ($\Delta H_m°$) values are reported in kcal^{-1}·mol^{-1}. Gibbs energy ($\Delta G°_{calc,25°C}$) values are reported in kcal·mol^{-1}.

Partial RNase T1 digestion of 3′-^{32}P-labeled transcripts that were isolated from transcription reactions containing GTP or 8azaGTP produced a major product with the size expected from cleavage of the phosphodiester bond 3′ of guanosine or 8-azaguanosine. Thus, 8azaG was specifically incorporated opposite the unique cytosine in the template during T7 RNA polymerase transcription in vitro and is recognized as guanine by RNase T1.

(C) Thermal Melting Profiles of 8azaG-Containing RNA. Although 8-azaguanosine retains the Watson−Crick hydrogen bonding face of guanosine, the change in charge distribution from substitution of C8 of guanine with N8 of 8azaG might alter the thermodynamic stability of an 8azaG:C base pair relative to a G:C pair. To evaluate the effect of an 8azaG substitution on RNA secondary structure stability, we compared melting profiles for the 8azaG-containing and unmodified RNAs annealed with a complementary oligonucleotide (cGR) that forms a perfectly paired duplex (GR·cGR or 8azaGR·cGR) or with a ribozyme substrate sequence (S) that forms a bulged duplex similar to domain A of the hairpin ribozyme[47] (GR·S or 8azaGR·S) (Figure 3a).

The T_m values obtained from plots of the percentage of hypochromicity versus temperature are given in Table 1. Perfectly paired 8azaGR·cGR duplexes displayed lower T_m values than their unmodified GR·cGR counterparts, with the difference ranging from 4.3°K at pH 7 to 1.4°K at pH 9. Perfectly base-paired RNA duplexes displayed significantly greater thermodynamic stability than RNA complexes with a 4-nt internal bulge, corresponding to loop A of the hairpin ribozyme, for RNAs with or without an 8azaG substitution. Thermal melting profiles showed smaller differences in stability for bulged duplexes that mimic the loop A domain of the hairpin ribozyme than for perfectly complementary duplexes, with T_m values varying less than 4°K between bulged duplexes with and without the 8azaG modification. T_m values decreased slightly with increasing pH, likely reflecting deprotonation of N1 and N3 hydrogen bond donors in G:C and U:A base pairs.

Comparison of $\Delta G°$ values calculated from the T_m and $\Delta H_m°$ values for 8azaGR·cGR and GR·cGR duplexes gave $\Delta\Delta G°$ values ranging from 1.4 kcal/mol at pH 7 to 2.5 kcal/mol at pH 9, with more favorable free energies characteristic of the unmodified complex. The nearest-neighbor sequence dependence of the free energy of RNA duplex formation is well-known for RNA helices with canonical matched and mismatched base pairs, allowing $\Delta G°$ values to be calculated for the same RNA duplex but with other matched and mismatched base pairs in place of the 8azaG:C pair.[48] $\Delta\Delta G°$ calculations show that RNA duplexes with C:G, A:U, or U:A pairs in the same position in a GR·cGR duplex as the 8azaG:C pair are less stable than the GR·cGR duplex that has a G:C pair. Free energy differences range from 0.6 kcal/mol for a duplex with a C:G pair in place of the G:C pair to as much as 2.9 kcal/mol for a duplex with a U:A base pair substitution. The duplex is less stable by 3.3 kcal/mol if the G:C pair is replaced by a G:U wobble pair. Substitution of C:C, U:C, or A:C mismatched base pairs for the G:C pair destabilize the duplex by 4.3 to 4.7 kcal/mol. Thus, $\Delta\Delta G°$ values calculated from differences in thermal melting behavior due to 8azaG:C and G:C pairs in 8azaGR·cGR and GR·cGR duplexes fall within the range expected for canonical base pairs in the same sequence context and are smaller than $\Delta\Delta G°$ values calculated for mismatched pairs. These results are consistent with the results of a computational study of 8azaG:C base-pairs[49] and with a previous study of the melting behavior of RNAs with low levels of 8-azaguanine acquired during growth of cultured cells in media with 8azaG.[50] Together with our evidence that 8azG is incorporated specifically opposite cytosine in the template strand during T7 RNA polymerase transcription in vitro, these results suggest that 8azaG forms a stable base pair with cytosine and does not significantly perturb RNA secondary structure stability.

(D) pH-Dependent Fluorescence of 8azaG-Modified RNA. The pH dependencies of 8-azaguanine and 8-azaguanosine fluorescence have been well-characterized.[16] Ionization of 8-azaguanosine occurs with a pK_a of 8.05.[16] Only the anionic form that is deprotonated at the N1 position displays significant fluorescence ($\Phi = 0.55$) with maximum absorption and emission observed at 278 and 362 nm, respectively. Fluorescence emission spectra for 8azaGTP and 8azaGR and for complexes formed between 8azaGR and cGR, in a perfectly base-paired duplex, or with S, in a bulged duplex analogous to the ribozyme loop A (Figure 3a), are shown in Figure 4. As is commonly observed with fluorescent nucleobase analogues, fluorescence emission was quenched when the analogue was incorporated into RNA. The fluorescence intensity measured for 8azaGR at 380 nm was about 10-fold less than that of 8azaGTP at pH 9 and 20-fold less at pH 7 (Figure 4a,b). For comparison, the quantum yield of 2-aminopurine nucleoside decreases more than 100-fold when it is incorporated into an oligonucleotide.[3] Fluorescence emission intensity for 8azaG in the bulged 8azaGR·S duplex was similar to emission intensity in single-stranded RNA (Figure 4b,c). Fluorescence intensity was lower still in the perfectly paired 8azaGR·cGR duplex with a decrease in emission intensity of about 100-fold relative to 8azaGTP (Figure 4a,d).

Despite the decrease in fluorescence observed when 8azaG is incorporated into RNA, sufficient intensity remained to allow measurements of the pH dependence of fluorescence emission

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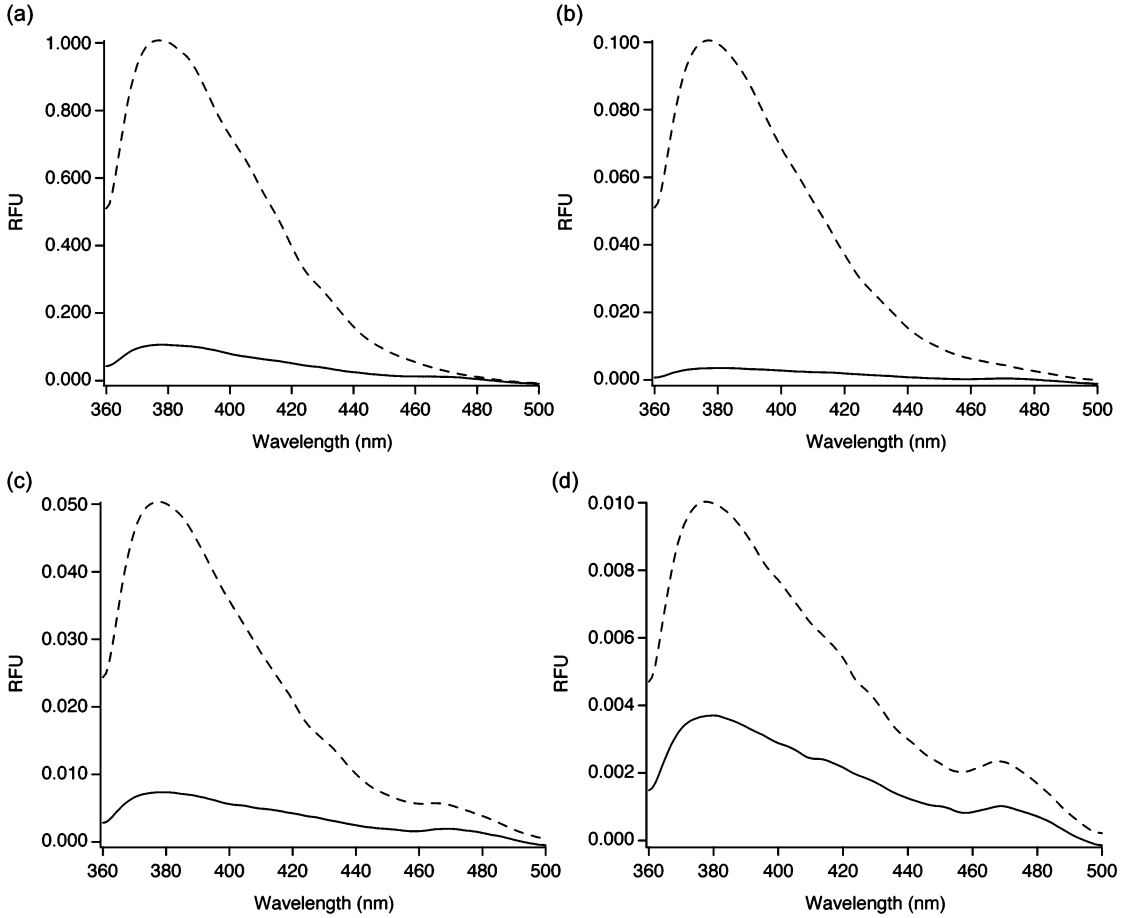

Figure 4. Representative normalized fluorescence emission spectra at pH 7.0 (solid lines) and 9.0 (dashed lines) for (a) 8-azaguanosine-5'-triphosphate, (b) 8-azaguanine in single-stranded RNA, (c) 8-azaguanine in a bulged duplex analogous to the loop A domain of the hairpin ribozyme, and (d) 8-azaguanine in perfectly paired duplex RNA.

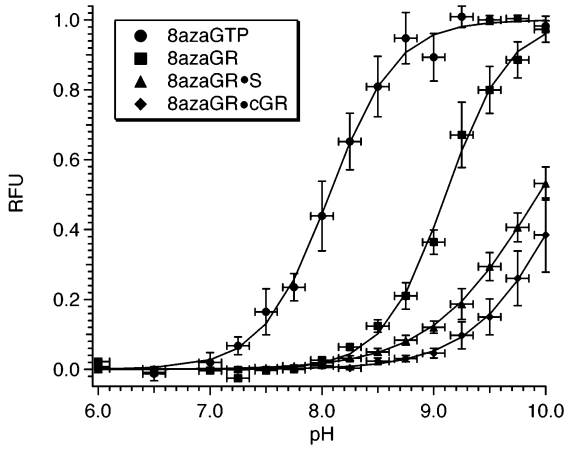

Figure 5. Relative fluorescence emission intensity at 380 nm of 8-azaguanosine-5′-triphosphate (●), 8-azaguanine in single-stranded RNA (■), 8-azaguanine in perfectly paired duplex RNA (◆), and 8-azaguanine in a bulged duplex analogous to the loop A domain of the hairpin ribozyme (▲). The excitation wavelength was 290 nm. Lines represent fits to eq 1. Apparent pK_a values obtained from the fit are given in Table 2.

(Figure 5). The pH dependence of the fluorescence integral between 370 and 390 nm was used to calculate apparent pK_a values for 8azaGTP and for 8azaG in single-stranded and duplex RNAs (Table 2). A value of 8.09 was obtained for the apparent pK_a of 8azaGTP, a value that is very close to the pK_a of 8.05 reported for 8-azaguanosine.[16] This difference is similar to the increase in the pK_a of guanosine triphosphate relative to guanosine and likely reflects the influence of the negatively charged triphosphate on the acidity of N1.[15,51,52]

Table 2. Spectral Properties of 8azaGTP and 8azaG in Single-Stranded and Duplex RNAs

compound	pK_a	max λ_{ex} (nm)	max λ_{em} (nm)
8azaGTP	8.1 ± 0.1	277.7 ± 1.6	378.0 ± 1.5
8azaGR	9.1 ± 0.1	285.3 ± 1.2	377.4 ± 1.6
8azaGR•S	9.9 ± 0.1	290.3 ± 1.0	378.8 ± 1.0
8azaGR•cGR	10.2 ± 0.4	289.3 ± 1.0	379.5 ± 1.2

8azaG clearly becomes more like guanine in the context of RNA since its pK_a shifts in the alkaline direction from 8.09 in 8azaGTP to 9.10 in the single-stranded 8azaGR oligonucleotide (Table 2). NMR studies have shown that pK_a values for guanine in RNA vary between 10.0 and 10.6, depending on the identity of the neighboring nucleobases.[53] Similar values were obtained by potentiometric pH titrations of guanosine dinucleotides, which gave pK_a values ranging from 9.4 to 10.4.[54,55] A more acidic pK_a of 9.3 was observed for guanine in 3′,5′-bisethylguanosine, which mimics a nucleic acid polymer but lacks contributions from stacking interactions.[53]

Although higher pK_a measurements were associated with

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larger errors, 8azaG clearly became more basic in a base-paired RNA duplex relative to single-stranded RNA, with an apparent pK_a of 10.2 observed for the perfectly paired 8azaGR·cGR complex. Interestingly, the pH dependence of fluorescence of 8azaG in the bulged 8azaGR·S duplex, which is analogous to loop A of the hairpin ribozyme, gave an intermediate pK_a value, suggesting that interactions within the loop have a modest influence on N1 acidity. In an NMR solution structure of an isolated loop A domain, G8 forms non-A-form stacking interactions and has an unusual *C*2′-endo sugar pucker. Rather than forming a canonical base pair, G8 functional groups form cross-strand hydrogen bonds with ribose oxygens; O6 of G8 forms a cross-strand hydrogen bond with the 2′-hydroxyl group of G+1, while its imino and amino protons interact with O4′ of U+2.[35] G8 forms quite different interactions in the context of a fully assembled ribozyme.[29,56,57] In the ribozyme active site, N1 and N2 of G8 are within hydrogen bonding distance of the 2′-oxygen nucleophile and the pro-R_P oxygen of the reactive phosphate, respectively. Future studies comparing the pH dependence of 8azaG8 fluorescence in an intact ribozyme with the pH dependence of catalytic activity might help to distinguish models in which unprotonated N1 of G8 mediates general base catalysis during cleavage or protonated N1 donates a hydrogen bond to provide electrostatic stabilization in the transition state.[8,28−32,56]

Conclusions

We report a highly efficient enzymatic synthesis of the nucleotide analogue 8-azaguanosine-5′-triphosphate, its site-specific incorporation into RNA, and physical properties of RNA with a single 8-azaguanine substitution. Synthesis of 8-azaguanosine-5′-triphosphate requires a single reaction to convert the nucleoside into the triphosphate for RNA transcription, with an isolated yield of 60%. Certain features of 8-azaguanine make this guanine analogue particularly useful for studies of RNA structure and function. First, the nucleotide is an efficient substrate for bacteriophage T7 RNA polymerase, allowing for site-specific incorporation into RNA through in vitro transcription. The nucleobase analogue could certainly be prepared as a phosphoramidite for chemical synthesis as well. In contrast to other commonly used guanine analogues, 8-azaguanine has the same Watson−Crick hydrogen bonding face and virtually the same pK_a for N1 protonation as guanine in the context of an RNA oligonucleotide, minimizing potential complications from RNA secondary structure destabilization. Furthermore, the 10- to 100-fold decrease in fluorescence intensity observed when 8-azaguanine is incorporated into an oligonucleotide is much less than the 100- to 1000-fold quenching observed for other nucleobase analogues when they are incorporated into nucleic acids. Finally, the changes in fluorescence properties that accompany N1 protonation make 8-azaguanine an excellent site-specific probe of the effects of a structured RNA environment on purine nucleobase ionization equilibria. These results establish the feasibility of exploiting the pH dependence of 8-azaguanine fluorescence to distinguish among alternative models of the hairpin ribozyme catalytic mechanism that differ in the protonation state assigned to an active site guanine.

Experimental Section

Synthesis of 8-Azaguanosine-5′-Triphosphate (8azaGTP). D-Ribose (375 mg, 2.5 mmol) and 8-azaguanine (8azaG, 380 mg, 2.5 mmol) were dissolved in 500 mL of synthesis buffer (0.0025 mM dATP, 0.1 mM kanamycin, 0.25 mM dAMP, 5 mM ampicillin, 10 mM $MgCl_2$, 20 mM dithiothreitol, 50 mM potassium phosphate, and 100 mM sodium 3-phosphoglycerate, pH 7.5) at 37 °C. The synthesis was started with the addition of 5 units of adenylate kinase (*pls*A, EC 2.7.4.3), 250 units of phosphoglycerate mutase (*gpm*A, EC 5.4.2.4), 375 units of enolase (*eno*A, EC 4.2.1.11), and 500 units of pyruvate kinase (*pyk*F, EC 2.7.1.40). The reaction was monitored by HPLC methods described elsewhere.[42] After 24 h, the conversion of dAMP to dATP appeared complete, and the synthesis of 8azaGTP was started with the addition of 5 units of ribokinase (*rbs*K, EC 2.7.1.15),[58] 5 units of 5-phospho-D-ribosyl-α-1-pyrophosphate synthetase (*prs*A, EC 2.7.6.1),[59] 5 units of guanine phosphoribosyltransferase (*gly*D, EC 2.4.2.7),[43] and 5 units of guanylate kinase (*spo*R, EC 2.7.4.8). After 240 h when the synthesis of 8azaGTP appeared complete, the reaction was brought to 0.5 M ammonium bicarbonate and pH 9.5 with addition of ammonium hydroxide and purified by boronate-affinity chromatography as previously described.[42] The 8azaGTP (1.5 mmol, 60% isolated yield) was dissolved in 15 mL of H_2O, adjusted to pH 7.6 with 1 M HCl, and then used in transcription reactions without further processing. Data: ^{1}H NMR (600 MHz, D_2O) δ (mult) 6.19 (d, H-1′), 5.14 (dd, H-2′), 4.73 (dd, H-3′), 4.42 (ddd, H-4′), 4.24 (dd, H-5′), 4.21 (dd, H-5″); ^{13}C NMR (151 MHz, D_2O) δ (mult) 161.4 (d, C-4), 158.8 (s, C-6), 154.8 (s, C-2), 127.8 (s, C-5), 91.5 (d, C-1′), 86.7 (d, C-4′), 75.5 (d, C-2′), 73.2 (d, C-3′), 68.2 (t, C-5′); HRMS (ESI-TOF$^+$) *m*/*z* calcd (M + 1$^+$) 524.9932, found 524.9932.

Preparation of RNAs. 8azaGR and GR RNAs (Figure 3a) were prepared by bacteriophage T7 RNA polymerase transcription of partially duplex templates according to published methods.[42,46] Templates were prepared by annealing synthetic deoxyoligonucleotides with the sequences 5′ CTA ATA CGA CTC ACT ATA (CTOP) and 5′ TGG TTG GGT TCT GAT GTT CCT ATA GTG AGT CGT ATT AG (BSGR). Reactions for transcription of 8azaGR contained 40 mM Tris-HCl (pH 8.1), 1 mM spermidine, 10 mM dithiothreitol, 0.01% Triton X-100, 80 mg/mL PEG-8000, 0.3 μM CTOP DNA, 0.3 μM BSGR DNA, 200 μM GpG, 24 mM total NTP (ATP = 12.00 mM, UTP = 1.33 mM, 8azaGTP = 1.33 mM, CTP = 9.33 mM), 38 mM $MgCl_2$, and 3000 units/(mL of T7 RNA polymerase). After 4 h at 37 °C, reactions were quenched with 0.1 volume of 0.5 M EDTA (pH 8.0), and extracted with an equal volume of phenol:chloroform:isoamyl alcohol (25:24:1) equilibrated with TE buffer (100 mM TrisCl, 1 mM EDTA, pH 8.0) and then with chloroform:isoamyl alcohol (24:1), equilibrated in the same buffer. The aqueous layer was passed through Sephadex G25 prepacked columns (NAP-25, Amersham Biosciences), and the eluate was dried to a volume of about 50 μL. Full length transcription products were fractionated by polyacrylamide gel electrophoresis and converted to sodium salts by DEAE chromatography as described.[60] After ethanol precipitation, RNA was dissolved in about 50 μL of water and desalted by gel filtration with P6 Micro Bio-Spin columns (Bio-Rad). The concentration was determined by UV absorbance at 260 nm assuming an extinction coefficient of 203 500 M^{-1}·cm^{-1} calculated using the nearest-neighbor method[61,62] and assuming that 8azaG is similar to guanine. On average, 3.6 nmol of 8azaGR was obtained per milliliter of transcription. The same procedure was used to prepare the unmodi-

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fied GR oligonucleotide except that transcription reactions included GTP instead of 8azaGTP and the GpG dinucleotide primer was omitted. cGR and S RNAs (Figure 3a) were prepared through chemical synthesis (Dharmacon).

Enzymatic Sequencing. 8azaGR or GR oligonucleotides were 3′-end labeled by reaction with [5′-^{32}P]pCp and RNA ligase, as described.[63] Alkaline hydrolysis was done in 0.5 M carbonate/bicarbonate buffer, pH 9.0, 100 mM EDTA with 25 μg/(μL of carrier RNA) at 90 °C for 10 min. Partial ribonuclease digestion was carried out with 0.4 units/(μL of ribonuclease T1) in 100 mM Tris-HCl, pH 8.0, for 4 min at room temperature or with 7.5 units/(μL of ribonuclease A) in 50 mM sodium acetate, pH 5.4 at room temperature for 2 min.

UV Thermal Melting Profiles. Changes in absorbance at 260 nm were measured at temperatures ranging from 0 to 90 °C in both directions, with increments of 1 °C/min, using a Cary 1 Bio UV−visible spectrophotometer (Varian). 8azaGR·cGR, 8azaGR·S, GR·cGR, and GR·S complexes were prepared with each strand at a concentration of 1 μM in 50 mM HEPES and 100 mM NaCl at pH 7.0, or in 10 mM phosphate buffer and 50 mM NaCl at pH 7.0, 8.0, or 9.0. Melting temperature (T_m) and the enthalpy of unfolding at the melting temperature ($\Delta H_m°$) were calculated by nonlinear least-squares fits to the van't Hoff equation,[62] using Igor software (Wavemetrics). The change in the heat capacity (ΔC_p) upon unfolding was assumed to be zero.

Fluorescence Measurements. A 2 μM amount of GTP or 8azaGTP, 5−20 μM 8azaGR, and 10−40 μM cGR or S were prepared in 50 mM NaCl and 50 mM MES (pH 6−6.50), HEPES (pH 7−7.75), TAPS (pH 8−8.75), or CHES (pH 9−10) and heated to 85 °C for 1 min and slowly cooled to 25 °C before data collection. Buffer pH was adjusted at 25 °C with NaOH. Fluorescence excitation and emission spectra were measured at 25 °C with a Molecular Devices Spectramax M2 plate reader that allowed simultaneous measurements of multiple samples with baseline correction for the buffers. Fluorescence excitation scans between 250 and 320 nm were recorded at 380 nm, and fluorescence emission scans between 360 and 500 nm were recorded with excitation wavelengths of 280, 290, and 300 nm. An apparent peak of emission intensity that appears at 470 nm is only detectable in the low emission intensity samples and likely reflects inaccuracies in the baseline correction by the plate reader fluorimeter that was used to collect these data. Apparent pK_a values were determined by nonlinear, least-squares fit analysis of the integrated fluorescence emission between 370 and 390 nm to an acid−base equilibrium (eq 1), using Igor software (Wavemetrics).

$$F = F_{B^-} + (F_{BH} - F_{B^-}) \frac{[H^+]}{K_a[H^+]} \quad (1)$$

Acknowledgment. This work was supported by the National Institutes of Health (Grant RO1 GM046422 to M.J.F. and Grant R42 GM074330 to Cassia LLC). C.P.D.C. is a Skaggs Postdoctoral Fellow. We are grateful to Sean Ryder for insightful suggestions and thank Gopi Kumar Mittapalli and Albert Eschenmoser's group for help with thermal melting experiments.

JA067699E

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