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1-Methylguanosine Substitutions of the Conserved Guanosine Residues Inactivate the Hammerhead Ribozyme

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Abstract: *A study is described in which a methyl group is introduced to the N^1-nitrogen of specific guanosine residues in the hammerhead ribozyme as a steric block to prevent hydrogen bonding interactions to the Watson-Crick face of each guanine base residue. The results of this study suggest the importance of hydrogen-bonding or other types of interactions to the Watson-Crick face of the three conserved G residues.*

The hammerhead ribozymes,[1] derived from structures present in the genomes of several plant pathogens,[2] are composed of three helices and include eleven consensus nucleotides that are responsible for the catalytic core of the complex. Cleavage of the RNA occurs as a transesterification reaction in which the 2'-hydroxyl at the cleavage site functions as an internal nucleophile and two products are generated; one contains a terminal 5'-hydroxyl and a second terminates as a 2',3'-cyclic phosphodiester.[1a,b] Divalent metal ions such as Mg^{2+} or Mn^{2+} are required for the cleavage reaction,[1b,3] and one or more of these cofactors may be necessary for cleavage activity.[4] Studies with phosphorothioate diesters suggest that an Mg^{2+} (Mn^{2+}) cofactor is bound to the pro-R oxygen in the unmodified complex and that transesterification occurs by an in-line mechanism.[5] Three other specific phosphodiesters within the conserved central core sequence appear to be necessary for efficient folding of the complex.[6]

Sequence mutations of the nucleotide residues present in the catalytic core result in dramatic decreases in cleavage activity[7] indicating a requirement for a conserved core of nucleotides. The incorporation of nucleoside analogues has permitted functional group mutagenesis at the atomic level within the catalytic complex. A series of analogue complexes lacking specific 2'-hydroxyls[3a,8] have been prepared and analyzed for cleavage efficiency. The importance of specific amino groups or N^7 nitrogens of the conserved purines in the hammerhead domain has been examined by replacement of single residues by nebularine, inosine 7-deazaadenosine, 7deazaguanosine, hypoxanthene or isoguanosine.[8f,9]

The present work focuses on possible interactions to the Watson-Crick face of the three conserved guanosine residues present in the catalytic core. In the present design, a methyl group added to the N^1-position of guanosine introduces a steric block to the central position of the three Watson Crick functional groups. This modification removes any possibility for hydrogen bonding at this site, but does not further alter the tautomeric form of the heterocycle. The introduction of the m^1G analogue for a conserved G residue within the catalytic core will effectively inhibit the formation of Watson-Crick G-C base pairs at each site (see Fig. 1a). Additionally, formation of base pairing interactions involving two of the three Watson-Crick functional groups[10,11] would likewise be inhibited (Fig. 1b and 1c). By comparison, the methyl group at position 1 should not interfere with Hoogsteen[10] base pairing interactions (Fig. 1d), or those involving the N^3-nitrogen and the N^2-amino group of guanosine, suggested to occur in some G-A base pairs[12] (Fig. 1e).

Five analogue ribozyme sequences were prepared using m^1G protected as the N^2-isobutyryl-5'-O-dimethyoxytrityl-2'-O-tbutyldimethylsilyl-3'-O-(β-cyanoethyl)-N,N-diisopropylphosphoramidite derivative.[13] Three complexes, G5m^1G, G8m^1G and G12m^1G (see Fig. 2) all involve the introduction of a methyl group to one of the three conserved guanosine residues present in the catalytic core. Two additional m^1G-containing sequences, GL2.1m^1G and

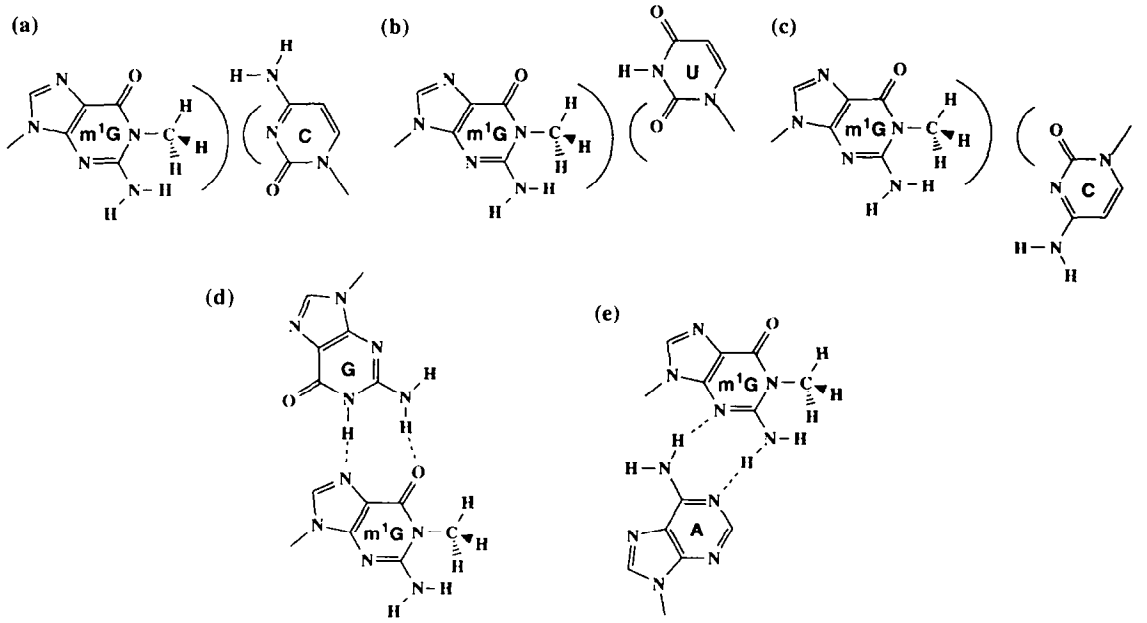

Figure 1. Selected base-pairing interactions that are inhibited (a, b, c) or permitted (d, e) by 1-methylguanosine.

G10.1m¹G (see Fig. 2) were prepared as potential positive and negative control complexes. The former complex places the methyl group on the G residue present in Loop II (L2.1), in a position of the complex that is not conserved. In fact the entire loop structure can be eliminated in favor of simple non-nucleoside linkers without any significant loss in cleavage activity.[14] Thus, we expected that the presence of the methyl group in Loop II would have relatively little effect upon the cleavage reaction (positive control). Conversely, there is increasing evidence that the base pair $G_{10.1}$-$C_{11.1}$ is required for efficient cleavage activity.[15] Introduction of the analogue at position $G_{10.1}$ should disrupt this base pair with consequent deleterious effects upon cleavage activity (negative control).

After synthesis and purification (PAGE) of each of the 33-nucleotide ribozyme sequences,[13-15] a small portion of material was hydrolyzed with S1 nuclease and calf intestinal alkaline phosphatase. Resolution of this hydrolysate by HPLC confirmed the presence of the m¹G residue in each complex. The absence of any unidentified peaks confirmed that no significant side reactions, or migration of the phosphodiester linkage had occurred during the assembly, deprotection and isolation of the sequences.

To analyze relative cleavage activity under single turnover conditions, we prepared solutions that contained the ribozyme sequence (0.6 μM) in excess of the substrate sequence (0.1 μM). The cleavage reactions were performed at 25 °C and pH 7.5, and under these conditions native complex exhibited a half-life of 0.6 min with a first order rate constant of 1.1 min^{-1}. This value is in agreement with that obtained for the identical complex prepared by enzymatic methods.[1b,7c] Although the precision of the data for the cleavage rates was quite good for identical batches of ribozyme and substrate, we have observed that the cleavage rates varied with different batches of purified sequences (but by no more than two-fold). Similar variations have been noted for other ribozyme sequences prepared by both chemical and enzymatic syntheses.[16] The values reported in this study were each obtained from single batches of ribozyme and substrate. The three conserved guanosine residues, G_5, G_8 and G_{12} in the ribozyme-substrate complex were each replaced by 1-methylguanosine. All three complexes in which the conserved guanosines of the conserved core were methylated

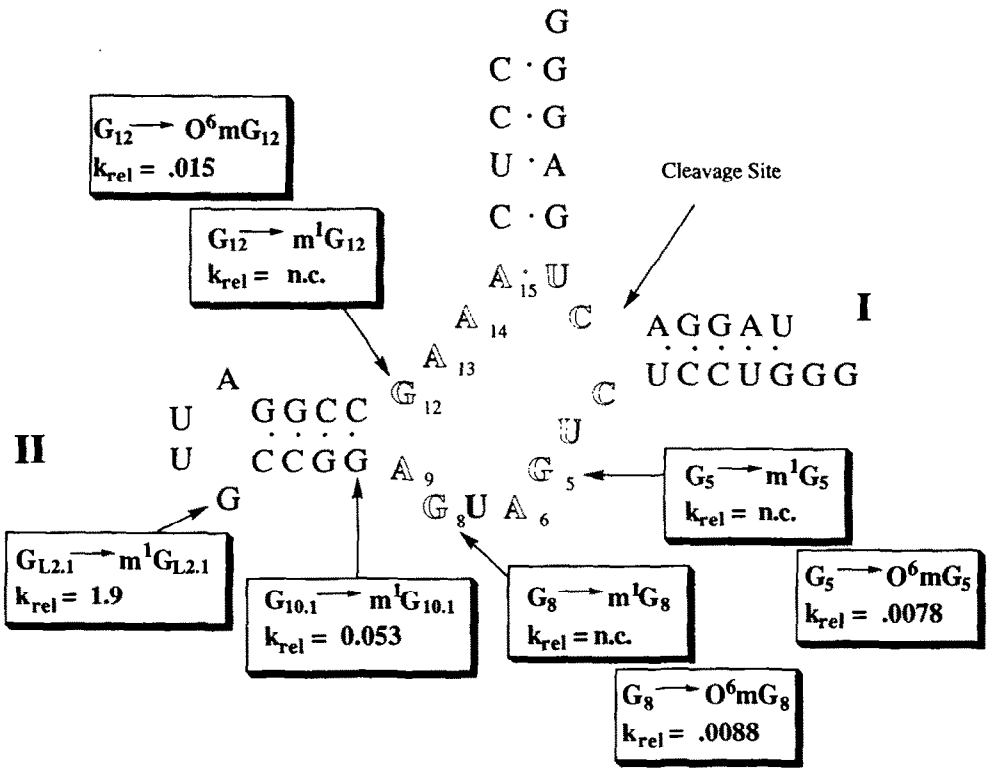

Figure 2. The location and relative cleavage activity of the m^{1}G-containing ribozymes. The data for the O^6-methylguanosine analogues reported by Grasby et al.[16] is illustrated for comparison purposes only (nc = no cleavage).

(G5m^{1}G, G8m^{1}G and G12m^{1}G) were inactive as cleavage catalysts; after a 24h incubation, less than 1% substrate cleavage could be detected above background (Fig. 2).

By comparison, replacement of $G_{L2.1}$ in Loop II by m^{1}G did not result in any loss in cleavage efficiency. This result was expected since the bases of Loop II are not conserved, and as noted above, the loop can be completely eliminated in some cases. On the other hand, it appears that the presence of the $G_{10.1}$-$C_{10.1}$ base pair is necessary for efficient cleavage activity.[15] Complexes in which this base pair has been eliminated in favor of a simple loop of nucleosides[15] or replaced by a non-nucleoside linker[14] exhibit very poor rates of cleavage. Replacement of $G_{10.1}$ by an m^{1}G residue results in cleavage activity reduced some 20-fold from that of the native complex (Fig. 2). This observation is consistent for a complex in which the $G_{10.1}$-$C_{10.1}$ base pair has been disrupted by the introduction of a methyl group, but the remaining base pairs of the stem maintain some structural integrity for the complex such that cleavage activity is retained, albeit at a relatively low level.

The effects on cleavage activity for the m^{1}G complexes can be compared with the results obtained previously for the related O^6-methylG complexes[17] in which the methyl group has been introduced at the O^6-carbonyl. In each case where the O^6-methyl analogue was introduced for one of the three core G residues a significant loss of cleavage efficiency was reported (see Fig. 2), but these complexes still retained some activity. By comparison, the three corresponding m^{1}G-containing complexes did not exhibit any significant cleavage activity.

Previously reported work, in which specific functional groups at selected sites within the hammerhead RNA complex have been deleted or otherwise altered, suggests a critical role for the functional groups on the Watson-Crick face

of the three conserved G residues present in the catalytic core. Replacement of G5 or G12 by inosine (deletion of the exocyclic amino group) results in complexes with poor cleavage activity.[8f,9a,d] Similarly, replacement of G5, G8 or G12 by 2-aminopurine[9d] (deletion of the O6-carbonyl and a change in the tautomeric nature of the N1 nitrogen), xanthene (replacement of the N2-nitrogen by a carbonyl), isoguanosine[9d] (reversing the C2 and C6 exocyclic functional groups), or as noted above, O6-methylguanosine,[17] all result in complexes with poor cleavage activity. By comparison, replacement of each of the conserved G residues by 7-deazaguanosine[9b] has relatively little effect upon cleavage activity. The present results involving m^1G substitutions are consistent with these previous studies, and suggest the presence of important transition state stabilizing interactions involving functional groups along the Watson-Crick face, rather than those of the Hoogsteen face or those involving of the minor-groove face, for these three G residues.

Acknowledgments

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