

2'-Fluoro Substituents Can Mimic Native 2'-Hydroxyls within Structured RNA

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DOI 10.1016/j.chembiol.2011.07.014

SUMMARY

The ability of fluorine in a C-F bond to act as a hydrogen bond acceptor is controversial. To test such ability in complex RNA macromolecules, we have replaced native 2'-OH groups with 2'-F and 2'-H groups in two related systems, the *Tetrahymena* group I ribozyme and the Δ C209 P4-P6 RNA domain. In three cases the introduced 2'-F mimics the native 2'-OH group, suggesting that the fluorine atom can accept a hydrogen bond. In each of these cases the native hydroxyl group interacts with a purine exocyclic amine. Our results give insight about the properties of organofluorine and suggest a possible general biochemical signature for tertiary interactions between 2'-hydroxyl groups and exocyclic amino groups within RNA.

INTRODUCTION

The use of chemical modifications has become a powerful tool to explore structure-function relationships in biologically important macromolecules (Chatterjee and Muir, 2010; Das et al., 2005; Hahn and Muir, 2005). Hydroxyl groups are ubiquitous in Nature and can act as hydrogen bond donors, hydrogen bond acceptors, or both. Replacement of a hydroxyl group attached to a carbon with a fluorine atom removes the hydrogen-bond donor capability in the newly introduced group and, because of the high electronegativity of fluorine relative to carbon (Pauling, 1932), fluorine would seem suited to act as a hydrogen-bond acceptor. However, the ability of fluorine in a C-F bond to act as a hydrogen bond acceptor is controversial.

Results from several computational studies and crystallographic surveys (Carosati et al., 2004; Dunitz, 2004; Dunitz and Taylor, 1997; Howard et al., 1996; Mehta and Sen, 2010; Müller et al., 2007; Murray-Rust et al., 1983; O'Hagan and Rzepa, 1997; Offen et al., 2009) suggest that hydrogen bonds to fluorine atoms are possible. However, it is often proposed that these hydrogen

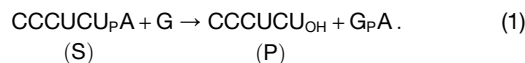
bonds are not as strong as hydrogen bonds to hydroxyls and that constraints within the system prevent the hydrogen bond donor from gaining access to better hydrogen bond acceptors than fluorine (Dunitz, 2004; Dunitz and Taylor, 1997). Much of the functional data pertains to fluorinated sugars in the context of sugar-modifying enzymes, but complexities associated with these systems frequently confound the analysis, including uncertainty about the influence of stereoelectronic effects on binding energies and about the rate-limiting step in the reaction (Martin et al., 1990; McCarter et al., 1992; Namchuk and Withers, 1995; Percival and Withers, 1992; Persson et al., 2001; Street et al., 1986; Street et al., 1989). To our knowledge, rigorous integration of structural and functional data has been possible for only one case, xylanase, which allowed Wicki et al. (2007) to conclude that organofluorine can accept a hydrogen bond.

Here, we have extended this rigorous analysis to the behavior of organofluorine in RNA systems. Using two well-studied RNA systems, the *Tetrahymena* group I ribozyme and the Δ C209 P4-P6 RNA domain derived from this ribozyme, we have determined the effects arising from replacement of a native 2'-OH group by a 2'-F versus a 2'-H atom.

RESULTS AND DISCUSSION

A 2'-F Atom Acts as Hydrogen-Bond Acceptor in the *Tetrahymena* Group I Ribozyme

The *Tetrahymena* group I ribozyme catalyzes the site-specific attack of an exogenous guanosine molecule (G) on the phosphoryl group of an oligonucleotide substrate (S) (Equation 1) (Houglund et al., 2006).



The ribozyme exists in at least two different conformations along the reaction coordinate, referred to as the "open complex" and the "closed complex" (Bevilacqua and Turner, 1991; Herschlag, 1992). Functional (Forconi et al., 2009, 2010) and structural (Lipchock and Strobel, 2008) data strongly suggest that the exocyclic amino group of the guanosine nucleophile donates a hydrogen bond to the 2'-OH group of residue A261 in the

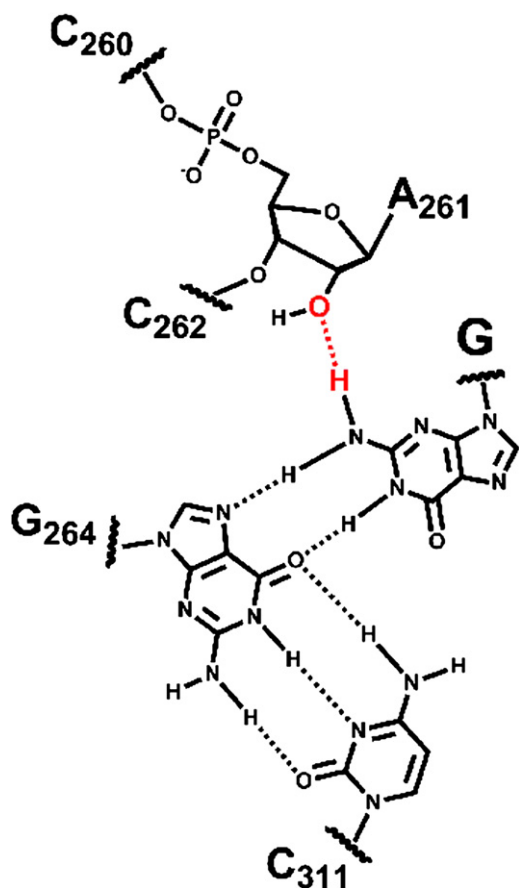


Figure 1. Schematic Representation of the Interactions Made by the Base of the Guanosine Nucleophile in the Closed Complex of the *Tetrahymena* Group I Ribozyme

The hydrogen bond between G and residue A261 is shown in red.

closed complex (Figure 1) but that this hydrogen bond is not formed in the open complex.

If fluorine cannot replace oxygen as a hydrogen-bond acceptor partner for the exocyclic amino group of G, then G would be expected to bind to the closed complex of a ribozyme containing a 2'-F atom at position 261 with reduced affinity relative to the wild-type ribozyme. Conversely, if fluorine flawlessly replaces oxygen as a hydrogen-bond acceptor, then the affinity of G for the closed complex would be unaffected by the introduction of a 2'-F atom at position 261.

To distinguish these possibilities, we used a semisynthetic protocol (Moore and Sharp, 1992) to generate a ribozyme containing a single 2'-F or 2'-H substitution at position A261 (herein referred to as the A261F and the A261H ribozymes, respectively), and we measured their reactivity using presteady state kinetics. To ensure that the introduction of a fluorine atom does not unexpectedly alter the properties of the ribozyme, we measured the affinity of a guanosine analog, AUCG, for the open complexes of the wild-type (A261OH) and the modified ribozymes; as noted above the hydrogen bond between the exocyclic amino group of G and the 2'-OH of A261 is not formed in the open complex (Forconi et al., 2010). AUCG was used instead of G because it binds tighter without altering the reaction mechanism (Moran et al., 1993; Russell and Herschlag, 1999), thereby allowing precise determination of the nucleophile affinity for the open complex without incurring problems related to the poor solubility of G. The affinity of AUCG for the open complexes of the three ribozymes is the same within 2-fold (Table 1), suggesting that there is no general effect on the guanosine-binding site from these single-atom substitutions.

We then measured AUCG affinity for the closed complexes of the three ribozymes. As noted above, the exocyclic amino group of the nucleophilic guanosine donates a hydrogen bond to the 2'-OH of residue A261 in this complex (Figure 1) (Forconi et al., 2009, 2010). Consistent with formation of this hydrogen bond, the affinity of AUCG for a ribozyme containing a 2'-H at position A261 is reduced by ~6-fold compared to the A261OH ribozyme (Table 1) (Forconi et al., 2010). In contrast, we found identical AUCG affinity for the A261OH and the A261F ribozymes (Table 1; see Figure S1 and Table S1 available online).

The simplest explanation for this result is that a hydrogen bond is formed between the exocyclic amino group of AUCG and the 2'-F of the A261F ribozyme. A strong prediction from this model is that an oligonucleotide lacking the exocyclic amino group would bind the closed complexes of the A261OH, A261F, and A261H ribozymes with the same affinity. In agreement with this prediction, the affinities for AUCI, which lacks the exocyclic amino group on the nucleophilic base, are within 2-fold for these three ribozymes (Table 2).

An alternative explanation is that the 2'-F substitution introduces a binding pocket for a localized water molecule and that this water molecule accepts a hydrogen bond from the guanosine nucleophile. However, this model is less likely as a rearrangement would be required that is not needed in the case of a direct interaction. Regardless, the fluorine atom would contribute to localization of the water molecule near the exocyclic

Table 1. Binding and Reactivity of AUCG, Measured at pH 6.9 and 50 mM Mg²⁺

Ribozyme	$(K_g^{\text{AUCG}})(\mu\text{M})$		k_c (min ⁻¹)	$(k_{\text{cat}}/K_M)_{\text{closed}}^{\text{AUCG}}$ (M ⁻¹ min ⁻¹)
	Open	Closed		
A261OH	6.5 ± 0.9 (1.0)	0.58 ± 0.10 (1.0)	0.076 ± 0.003 (1.0)	1.2 × 10 ⁵ (1.0)
A261F	10 ± 2 (1.5)	0.64 ± 0.06 (1.1)	0.017 ± 0.001 (0.22)	2.3 × 10 ⁴ (0.20)
A261H	5.0 ± 2.0 (0.77)	3.2 ± 0.4 (5.5)	0.0040 ± 0.0001 (0.053)	1.1 × 10 ³ (0.0092)

Values in parentheses represent values relative to the A261OH ribozyme. See also Figure S1 and Table S1.

Table 2. Binding Affinities of AUCI for the Closed Complexes of Different Ribozymes, Measured at pH 8.1 and 50 mM Mg²⁺

Ribozyme	$(K_d^{AUCI})_{\text{closed}}$ (μM)
A261OH	360 \pm 40
A261F	290 \pm 10
A261H	390 \pm 100

amino group of AUCG in this model, again suggesting that the fluorine atom can accept a hydrogen bond.

Another possibility is that the preferred nucleoside conformation of the analogs could contribute to the observed effects. The 2'-F, 2'-OH, and 2'-H nucleotides have been reported to have fractional occupancies of the 3'-endo conformation of 0.81, 0.49, and 0.35, respectively (Guschlbauer and Jankowski, 1980). Indeed, at residues populating the 2'-endo conformation, substitution of 2'-F nucleotides is known to inhibit RNA activity (Ortoleva-Donnelly et al., 1998; Ryder and Strobel, 1999). In the group I structural models most relevant to the catalytic structure of the ribozyme (Lipchock and Strobel, 2008) the sugar ring of residue A261 adopts a 2'-endo conformation. On the basis of conformational preferences alone, the A261F substitution would be expected to impart 2.3-fold weaker rather than the observed 5-fold stronger binding of AUCG relative to A261H (Table 1). Thus, the simplest explanation to account for our observations posits that the 2'-F atom mimics the hydrogen-bond acceptor properties of the hydroxyl group.

We also measured the reactivity of the closed complexes of the three ribozymes noted above with bound AUCG (k_c , Table 1). The A261H ribozyme reacts \sim 20-fold slower than wild-type with saturating AUCG, whereas the A261F ribozyme reacts only \sim 4-fold slower (Table 1 and Figure 2). As the 2'-fluorine at position 261 in the A261F ribozyme cannot donate a hydrogen bond, a possible explanation is that a hydrogen bond donation from the 2'-OH group of A261 to the ribozyme is also important for the chemical step. Inspection of published group I ribozymes crystal structures (Golden et al., 2005; Lipchock and Strobel, 2008; Stahley and Strobel, 2005) suggests the *pro-R_P* phosphoryl oxygen and the 5'-bridging oxygen of residue C262 as putative partners in such a hydrogen bond. A role of the *pro-R_P* phosphoryl oxygen of residue C262 in the chemical step is supported by the \sim 10-fold reduced rate constant for the chemical step when this phosphoryl oxygen is substituted by a sulfur atom (Houglund et al., 2005). Alternatively, subtle conformational rearrangements upon fluorine substitution might affect the chemical step, presumably by altering the relative positioning within the active site (Forconi et al., 2010, 2011).

The overall effect of the 2'-H substitution at position A261 is 110-fold decrease in reactivity (Table 1) ($k_{\text{cat}}/K_M^{AUCG}$)_{closed}, whereas the 2'-F substitution gives only a 5-fold decrease. The difference in reactivity between the A261F and A261H ribozymes is larger than expected for a differential sugar puckering preference (see also above) and suggests that there can be significant energetic consequences from hydrogen bonding to this fluorine.

Additional Support for the Ability of 2'-F Atoms to Act as Hydrogen-Bond Acceptors

To more broadly evaluate whether 2'-F can supplant important 2'-OH groups within structured RNA, we used 2'-deoxy- and

2'-deoxy-2'-fluoronucleotide α -thiotriphosphates to perform nucleotide analog interference mapping experiments (Ryder et al., 2000) in the context of Δ C209 P4-P6 RNA. This RNA, derived from the *Tetrahymena* ribozyme, consists of two sets of coaxially-stacked helices that pack against one another through tertiary interactions to form a compact architecture even in the absence of the remainder of the ribozyme. Crystallographic analyses have defined the P4-P6 structure at atomic resolution (Cate et al., 1996; Juneau et al., 2001; Ye et al., 2008), and biochemical analyses have defined its folding thermodynamics and kinetics (Battle and Doudna, 2002; Matsumura et al., 2003; Sattin et al., 2008; Schlatterer et al., 2008; Takamoto et al., 2004; Young and Silverman, 2002). Changes in global compaction of the domain upon folding allow separation of folded and unfolded molecules by nondenaturing gel electrophoresis (Murphy et al., 1994), which provides a convenient assay for folding interference (Basu and Strobel, 1999; Schwans et al., 2003).

Previous work has demonstrated that 15 sites of 2'-deoxynucleotide interference within this domain identify residues bearing important 2'-OH groups, and these 15 sites coincide with all of the crystallographically inferred 2'-OH interactions (Schwans et al., 2003, 2004). An additional site, A151, shows weak 2'-H interference, although structural models provide no evidence for a hydrogen bond.

To examine the effect of 2'-fluoro substitution at residues involved in hydrogen bonds, we compared the 2'-fluoro and 2'-deoxynucleotide interference maps to generate a 2'-fluoro/2'-deoxynucleotide interference profile for every residue (Figure 2). We determined the sites of 2'-fluoro/2'-deoxynucleotide interference using nondenaturing gel electrophoresis to separate folded from unfolded molecules as previously described (Basu and Strobel, 1999; Schwans et al., 2003). To achieve maximal sensitivity to folding interference without compromising signal we conducted the folding reactions and nondenaturing gel electrophoresis at the $[\text{Mg}^{2+}]_{1/2}$ for folding of Δ C209 P4-P6 (0.45 mM MgCl_2). At this Mg^{2+} concentration, the 2'-fluoronucleotides interfered with folding at 13 of the 15 2'-deoxynucleotide interference sites, indicating that the 2'-F atom cannot replace the 2'-OH at these sites without energetic penalty. These sites of 2'-fluoro/2'-deoxynucleotide interference coincide with residues that bear hydroxyl groups implicated as hydrogen bond donors (ribose zipper motifs and residues where the 2'-hydroxyl lies within hydrogen bond distance of N1 or N3 of purines; see Table S2), consistent with the loss of hydrogen bond donor ability expected for both 2'-fluoro and 2'-deoxynucleotides. However, 2 of the 15 sites can accommodate the 2'-F atom without inducing folding interference, indicating that a fluorine atom can supplant the role of the hydroxyl group at those sites. The same interference pattern was obtained at 2.0 mM MgCl_2 (data not shown).

In the crystal structure the hydroxyl groups at the two sites showing 2'-H but not 2'-F interference have two features in common: (1), both reside within range to accept a hydrogen bond from an exocyclic amine; and (2), neither appears to donate a hydrogen bond, as no heteroatoms bearing nonbonding electron pairs reside nearby (Figure S2). The latter feature may allow fluorine to accept a hydrogen bond from the exocyclic amine without incurring unfavorable interactions with local atoms.

In the RNA folding reaction, hydrogen bond formation at an individual residue is invariably coupled to the formation of other

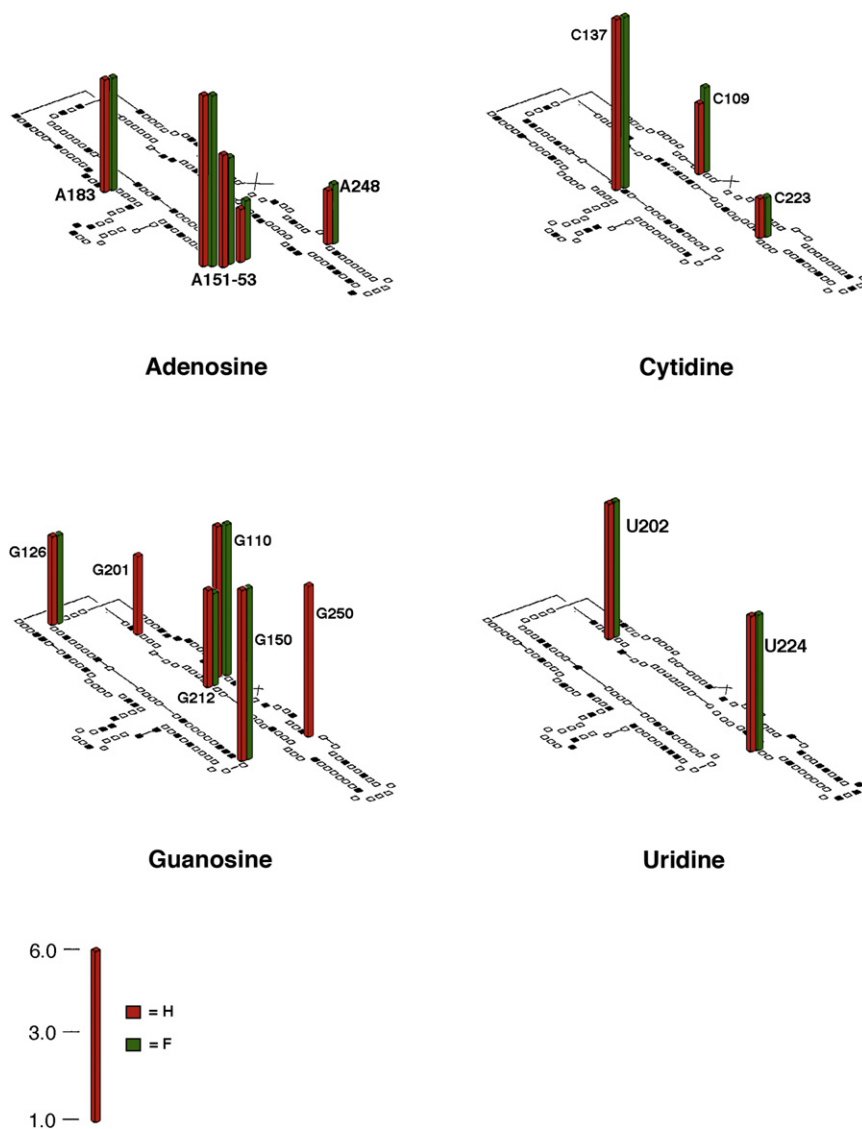


Figure 2. Δ C209 P4-P6 Secondary Structure with Histogram Indicating the 2'-F/2'-H Interference Profile at 0.45 mM $MgCl_2$

Sites of 2'-F and 2'-H interference are represented by the green and red bars, respectively. Sites of unique 2'-F interference correlate with residues that adopt a 2'-endo conformation in the structural models, as previously observed for the hairpin ribozyme (Ryder and Strobel, 1999), and are omitted for clarity. Interference values were calculated as described by Ryder et al. (Ryder et al., 2000). Only sites with interference values >1.5 are shown to take into account the experimental errors. See also Table S2 and Figure S2.

fluorine acting as a hydrogen bond acceptor. In each case the hydroxyl group interacts with a purine exocyclic amine. These results are consistent with the suggestion from analysis of crystal structures that the ability of a 2'-F atom to substitute for a 2'-OH group may be limited to interactions with protons attached to a nitrogen atom (Murray-Rust et al., 1983). If so, deleterious effects caused by 2'-deoxynucleotides but not 2'-fluoronucleotides may represent a general biochemical signature for tertiary interactions between 2'-hydroxyl groups and exocyclic amino groups within RNA. Additionally, the ability of fluorine to substitute at these positions appears to depend upon the absence of hydrogen bond acceptors to the native hydroxyl group. Additional analyses, akin to the study herein, in which structural and functional data are integrated, will be required to probe potential hydrogen

interactions. Nevertheless, the ability of 2'-analogs to mimic an interaction mediated by a specific 2'-OH group can be assessed by the differential effect of 2'-H and 2'-F substitution at that residue. In the simplest model, if a hydrogen bond is made in the folded structure, a more stable folded structure is expected compared to a molecule lacking this interaction. Thus, if the 2'-substitution disrupts this interaction, a less stable folded structure is expected, and if the 2'-substitution does not disrupt this interaction the stability of the folded structure is expected to be similar to the 2'-OH containing molecule. The observation that two sites with 2'-F substitution in P4-P6 RNA do not affect folding provides additional evidence that a 2'-F atom can mimic the hydrogen-bond acceptor properties of the native hydroxyl group.

SIGNIFICANCE

In this work we identified three different contexts where a 2'-F, but not a 2'-H, can effectively substitute for the native 2'-OH, suggesting that a hydrogen bond is maintained with

bonds to fluorine from other groups and thereby reveal whether our findings can be generalized to other hydrogen bond partners and other RNA systems.

EXPERIMENTAL PROCEDURES

Ribozyme preparations and kinetic assays were performed essentially as previously described (Forconi et al., 2009, 2010). Full details are given in the Supplemental Experimental Procedures. 2'-Fluorocytidine and 2'-fluorouridine were purchased from ChemGenes and 2'-fluoroguanosine was synthesized as described by Kawasaki et al. (1993). The nucleosides were converted to the α -thiotriphosphates and purified as previously described for 2'-mercaptonucleoside- α -thiotriphosphates (Schwans et al., 2003). Interference mapping experiments were conducted as previously described by Schwans et al. (2003) and references therein.

SUPPLEMENTAL INFORMATION

Supplemental Information includes two figures, two tables, and Supplemental Experimental Procedures and can be found with this article online at doi:10.1016/j.chembiol.2011.07.014.

ACKNOWLEDGMENTS

This work was supported by grants from the NIH to D.H. (GM 49243) and J.A.P. (AI081987), by NIH grant P20 RR-016461 from the National Center for Research Resources (to M.F.), and by a grant to the College of Charleston from the Howard Hughes Medical Institute through their Undergraduate Education program.

Received: May 3, 2011

Revised: June 20, 2011

Accepted: July 6, 2011

Published: August 25, 2011

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