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Synthetic substrate analogs for the RNA-editing adenosine deaminase ADAR-2

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ABSTRACT

We have synthesized structural analogs of a natural RNA editing substrate and compared editing reactions of these substrates by recombinant ADAR-2, an RNA-editing adenosine deaminase. Deamination rates were shown to be sensitive to structural changes at the 2'-carbon of the edited adenosine. Methylation of the 2'-OH caused a large decrease in deamination rate, whereas 2'-deoxyadenosine and 2'-deoxy-2'-fluoroadenosine were deaminated at a rate similar to adenosine. In addition, a duplex containing as few as 19 bp of the stem structure adjacent to the R/G editing site of the GluR-B pre-mRNA supports deamination of the R/G adenosine by ADAR-2. This identification and initial characterization of synthetic RNA editing substrate analogs further defines structural elements in the RNA that are important for the deamination reaction and sets the stage for additional detailed structural, thermodynamic and kinetic studies of the ADAR-2 reaction.

INTRODUCTION

RNA editing is a term used to describe the structural alteration, insertion or deletion of nucleotides in RNA (1). If the modification occurs in messenger RNA (mRNA), it can result in the translation of a protein sequence different from that predicted by the DNA sequence of the gene. A number of mRNA sequences from a variety of organisms are now known to arise from RNA editing (2–4). Thus, this process can play an essential role in the transfer of information that takes place during protein expression.

Processing of the mRNA for mammalian GluR-B, a subunit of a glutamate-gated ion channel, involves editing reactions where genomically encoded sequences are altered in the pre-mRNA by deamination of adenosines (4). The deamination of adenosine (A) in the mRNA results in inosine (I) at that position. Because inosine is translated as guanosine (G), the editing reaction causes a functional G for A replacement (Fig. 1). At two of the edited sites in GluR-B mRNA, the deamination reaction causes a codon change. The Q/R site is so named because at that site a glutamine codon is altered by adenosine deamination such that the new sequence encodes for arginine. The R/G site is located in an arginine codon that is converted to a sequence that encodes for glycine. An additional site (+60 hot spot) is also deaminated efficiently, but occurs in an intronic sequence and causes no amino acid changes in the protein. Specific deamination sites in other pre-mRNA sequences have also been identified (3).

Two different proteins edit GluR-B pre-mRNA *in vitro*. ADAR-1 is purified from tissue sources as an ~120 kDa protein (5,6) and efficiently modifies the hot spot and R/G sites (7,8). ADAR-2 is an ~80 kDa protein that deaminates the Q/R and R/G sites and, less efficiently, the hot spot site (7,8). Both ADARs 1 and 2 have been cloned and overexpressed (9). These enzymes have multiple copies of a sequence motif (double-stranded RNA-binding motif; dsRBM) found in double-stranded RNA binding proteins such as PKR, the RNA-dependent protein kinase (10). This observation is consistent with the requirement for double-stranded-RNA secondary structure in the RNA editing substrates.

Amino acid sequences C-terminal to the RNA binding motifs have been identified that may comprise the deamination active site (11,12). Nucleoside deaminases, such as cytidine deaminase and adenosine deaminase, have been extensively characterized, both structurally and mechanistically (13). The active sites of these enzymes are composed of amino acid sequences that are highly conserved among species. ADARs 1, 2 and a recently reported yeast tRNA-specific adenosine deaminase have conserved amino acid sequences similar to some of these consensus sequences (14). However, certain nucleoside deaminase consensus sequences appear to be altered or missing in the ADARs, suggesting that the ADAR active site is different from that of cytidine or adenosine deaminase. This is not surprising when one considers that adenosine deaminase will not react with adenosines in oligoribonucleotides and ADARs do not deaminate free adenosine (15). Therefore, a protein architecture distinct from the nucleoside deaminases has evolved to carry out the deamination of adenosine within the context of structured RNA. Our current knowledge of the mechanism of this RNA-dependent adenosine deamination reaction is extremely limited. For instance, little is known of the elements in the RNA, beyond a duplex secondary structure, that are recognized during the enzyme reaction.

Here we compare the reactivities of several ADAR-2 substrates having subtle structural differences introduced by phosphoramidite chemical synthesis. We show that replacing the 2'-hydroxyl group with a 2'-methoxy group results in a large decrease in deamination rate at the methylated site. However, 2'-deoxyadenosine and 2'-deoxy-2'-fluoroadenosine are deaminated at a rate similar to that found for adenosine. We also show that a duplex containing as few as 19 bp of the stem

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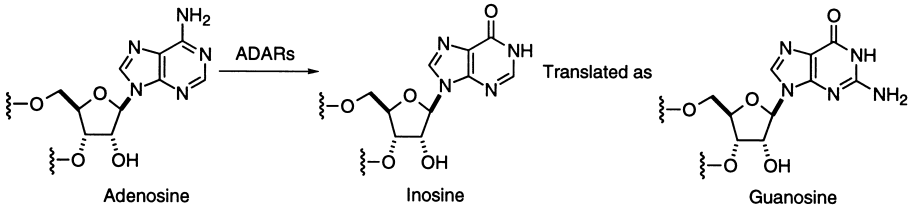

Figure 1. Deamination of adenosine by ADARs can change the coding properties of mRNA.

structure adjacent to the R/G editing site of the GluR-B pre-mRNA supports deamination of the R/G adenosine by ADAR-2.

MATERIALS AND METHODS

General

Distilled, deionized water was used for all aqueous reactions and dilutions. Biochemical reagents were obtained from Sigma/Aldrich unless otherwise noted. 5'-Dimethoxytrityl-2'-*tert*-butyldimethylsilyl protected adenosine, guanosine, cytidine and uridine phoshoramidites were purchased from ChemGenes. 2'-OMe-guanosine and 2'-OMe-adenosine phosphoramidites were purchased from Glen Research. 2'-Deoxyadenosine phosphoramidite was purchased from Perkin Elmer/ABI. 2'-Deoxy-2'-fluoroadenosine phosphoramidite was synthesized as previously described (16). [γ-^{32}P]ATP (6000 Ci/mmol) was obtained from DuPont NEN. Nuclease P1 and proteinase K were purchased from Boehringer Mannheim.

Synthesis and purification of RNAs

Oligoribonucleotides were prepared on a Perkin Elmer/ABI Model 392 DNA/RNA synthesizer with β-cyanoethyl phosphoramidites. Deprotection was carried out in NH_3-saturated methanol for 24 h at room temperature followed by 0.1 M tetrabutylammonium fluoride in tetrahydrofuran for 24 h at room temperature. Deprotected oligonucleotides were purified by polyacrylamide gel electrophoresis, visualized by UV shadowing and extracted from the gel by the crush and soak method with 0.5 M NH_4OAc, 0.1% SDS, 0.1 mM EDTA. Extinction coefficients for these RNAs were calculated as the sum of the extinction coefficients of the component nucleotides (17). Concentrations were determined by UV absorbance at 260 nm.

Protein expression and purification

$(His)_6$-tagged rat ADAR-2 was overexpressed in *Pichia pastoris* under the transcriptional control of a methanol-inducible promoter and purified as described (9,18). A standard curve for protein concentration was generated by resolving known amounts of bovine serum albumin on a 10% SDS–PAGE gel, visualizing the bands by Sypro-Orange (Molecular Dynamics) staining and quantitating the protein bands using a Molecular Dynamics STORM 840 PhosphorImager and ImageQuant software. This standard curve was then used to approximate the concentration of ADAR-2. This value was not corrected for the concentration of ADAR-2 active sites.

Deamination assay

For a particular experiment, the appropriate oligonucleotide was labeled at the 5' end using [γ-^{32}P]ATP (6000 Ci/mmol) and T4 polynucleotide kinase. The unincorporated [γ-^{32}P]ATP was removed using a Microspin G-25 column (Amersham Pharmacia). The amount of ^{32}P incorporation was determined using scintillation counting. The 5' end-labeled strand was hybridized with the unlabeled complement in 25 mM Tris–HCl, pH 8, 1 mM EDTA and 100 mM NaCl. The mixture was heated at 95°C for 5 min and allowed to slow-cool overnight to room temperature. The duplex was purified on a 12% non-denaturing polyacrylamide gel (29:1 acrylamide:bisacrylamide). The appropriate band was visualized by PhosphorImaging, excised and extracted into 500 µl of 200 mM NaCl, 0.1 mM EDTA overnight at room temperature. Polyacrylamide particles were removed using a Spin-X (Costar) centrifuge column. The RNA duplex was ethanol precipitated and redissolved in deionized water. The duplex concentration was determined by scintillation counting and the volume was adjusted to give a 50 nM stock solution which was stored at –80°C. Each deamination reaction contained ~130 nM ADAR-2, 1 nM labeled RNA duplex and assay buffer containing 15 mM Tris–HCl, pH 8, 6% glycerol, 0.5 mM dithiothreitol, 60 mM KCl, 1.5 mM EDTA, 0.003% NP-40, 160 U/ml RNasin, 1.0 µg/ml torula RNA. The concentration of ADAR-2 used in each assay was sufficient for quantitative binding of the substrate as evidenced by a gel mobility shift analysis unless otherwise noted. Reaction mixtures were incubated at 30°C for varying times. At each time point, an aliquot was removed and the reaction was quenched by proteinase K treatment (5), followed by digestion with nuclease P1 and resolution of the resulting 5'-mononucleotides by thin layer chromatography (TLC) as previously described (19). Storage phosphorimaging plates (Kodak) were pressed flat against TLC plates and exposed in the dark for 12–17 h. A Molecular Dynamics STORM 840 PhosphorImager was used to obtain all data from the storage screens. The data were analyzed by performing volume integrations of the regions corresponding to starting material, product and background sites using the ImageQuant software.

RESULTS

An oligonucleotide duplex substrate

In order to facilitate a structure/function analysis of ADAR-2 substrate analogs, we designed an RNA duplex substrate accessible by chemical synthesis and similar in length and sequence to a hairpin stem previously shown to be deaminated

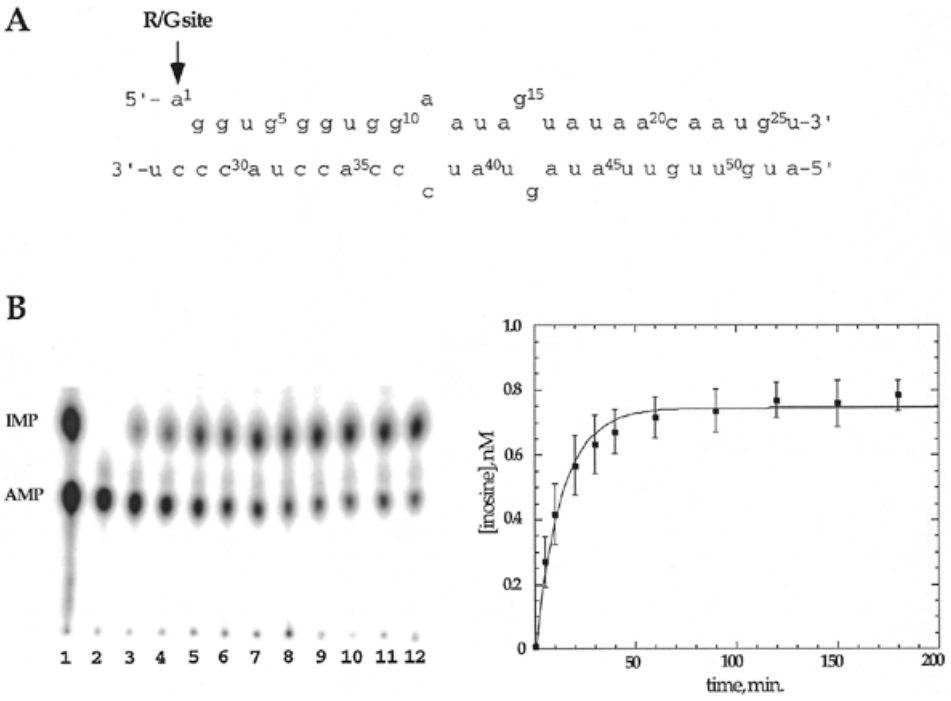

Figure 2. (**A**) Synthetic oligoribonucleotide duplex substrate for ADAR-2. (**B**) (Left) Storage phosphor autoradiogram of TLC plate used to separate deamination products arising from reaction of ADAR-2 with the synthetic duplex substrate. Lane 1, adenosine monophosphate (AMP) and inosine monophosphate (IMP) standards; lanes 2–12, reaction times of 0, 5, 10, 20, 30, 40, 60, 90, 120, 150 and 180 min, respectively. (Right) Plot of product formation as a function of time. The data were fit to the equation % inosine$_t$ = % inosine$_\infty$(1 − e$^{-kobs \cdot t}$) using the least squares method of KaleidaGraph. Data points reported are the average ±SD for three independent experiments.

by ADAR-2 *in vitro* (Fig. 2A) (20). The key difference between the structure of our substrate and the small substrate reported earlier is the removal of the loop connecting the two strands of the hairpin stem. This allows for the generation of a substrate via the synthesis of two relatively short oligoribonucleotides and their hybridization to form the necessary duplex. Using this duplex as a parent structure, we can readily make subtle structural changes at individual nucleotides using non-standard phosphoramidites during the synthesis of the two strands.

The deamination reaction catalyzed by ADAR-2 at the adenosine corresponding to the R/G editing site was monitored by following the conversion to inosine using a TLC assay previously described for a 5'-^{32}P-labeled substrate (Fig. 2B) (21). In the presence of an excess of ADAR-2 at 30°C, this substrate is deaminated to ~80% completion. Measuring the percent product formed as a function of time and fitting the data to a first order rate equation yields a $k_{obs} = 6.6 \pm 2.9 \times 10^{-2}$ min.$^{-1}$.

Substrate analogs with ribose modifications at the R/G editing site

Analogs of the substrate were prepared with different substituents at the 2'-carbon of the adenosine at the R/G editing site to probe structural requirements in the sugar of the edited nucleotide. The 2'-hydroxyl group at this position was replaced with a hydrogen (2'-deoxyadenosine), a fluorine (2'-deoxy-2'-fluoroadenosine) or a methoxy group (2'-*O*-methyladenosine) using the corresponding protected phosphoramidites during oligoribonucleotide synthesis (Fig. 3A). These substrates were compared by measuring the rates of product formation in the presence of an excess of ADAR-2 at 30°C (Fig. 3B). Replacement of the ribose hydroxyl with either hydrogen (H) or fluorine (F) leads to only a small decrease in rate (<4-fold difference in k_{obs}) relative to the adenosine-containing duplex (OH) (Table 1). However, methylation of this hydroxyl (OCH_3) has a more detrimental effect, leading to a >200-fold decrease in k_{obs} (Table 1). To determine if the large decrease in deamination rate observed with the substrate bearing a 2'-*O*-methyl substitution was dependent on the site of methylation, a new duplex substrate was prepared that had the guanosine 5 nt in the 3' direction from the deamination site (position 6) 2'-*O*-methylated. The large detrimental effect seen for methylation at the R/G site was no longer detected with this substrate as it was deaminated at a rate similar to that of the duplex bearing no 2'-*O*-methyl substitution (Table 1). We also observed only a minor effect on the reaction with the guanosine immediately adjacent to the deamination site 2'-*O*-methylated (data not shown).

The effect of duplex length on the deamination reaction

To define further the structural requirements for reaction at the R/G site adenosine, we prepared duplexes differing in length by removing base pairs from the end opposite the deamination site. Substrates predicted to have 19 and 17 bp were prepared and compared with the parent structure with 23 bp (Fig. 4A). Each of these duplexes retains the two base mismatches found naturally in the R/G hairpin stem.

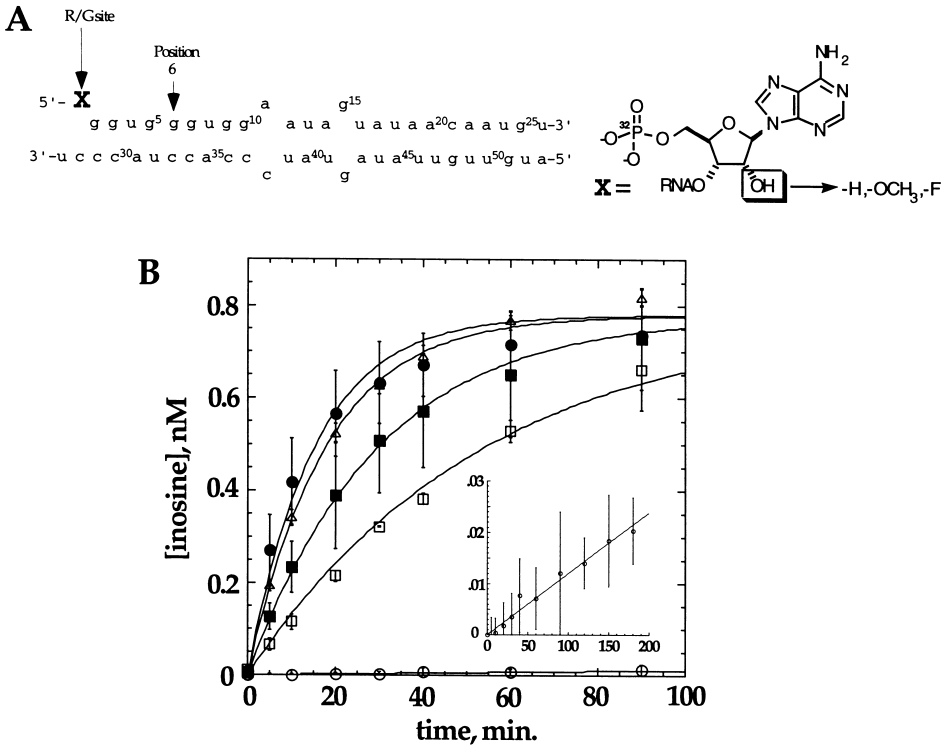

Figure 3. (**A**) Duplex substrates with ribose modifications. (**B**) Plot of product formation as a function of time for modified substrates with ribose modifications. Closed circle, adenosine at R/G site; open square, 2'-deoxyadenosine at R/G site; open circle, 2'-*O*-methyladenosine at R/G site; closed square, 2'-deoxy-2'-fluoroadenosine at the R/G editing site; triangle, adenosine at the R/G editing site, 2'-*O*-methylguanosine at position 6. The data were fit to the equation % inosine$_t$ = % inosine$_\infty$(1 – e$^{-kobs \cdot t}$) using the least squares method of KaleidaGraph. End points of ~80% were used for each fit, including that for the 2'-*O*-methyladenosine-containing substrate. Data points reported are the average ± SD for three independent experiments. Inset, plot of product formation as a function of time for substrate with a 2'-*O*-methyladenosine at the R/G editing site.

Table 1. Rate constants for R/G site adenosine deamination by ADAR-2

Substrate	k_{obs}, min^{-1}	k_{rel}
R/G site 2'-OH	$6.6 \pm 2.9 \times 10^{-2}$	1.0
R/G site 2'-F	$3.4 \pm 1.7 \times 10^{-2}$	0.5
R/G site 2'-H	$1.9 \pm 0.2 \times 10^{-2}$	0.3
R/G site 2'-OMe	$\sim 2.4 \pm 0.6 \times 10^{-4}$	~0.004[a]
Position 6 2'-OMe	$4.7 \pm 0.1 \times 10^{-2}$	0.7
19 bp duplex	$4.1 \pm 1.1 \times 10^{-2}$	0.6
17 bp duplex	NR	NR[b]

[a]Rate constant obtained assuming the same end point as other R/G site analogs.
[b]Rate constant not reported due to apparent lower end point under these reaction conditions.

When the reactivities of these substrates were compared as above, the duplexes with 23 and 19 bp were deaminated at the R/G site at similar rates to ~80% completion (Fig. 4B; Table 1). However, the 17 bp duplex deaminates more slowly and yields ~3-fold less product at the end point of the reaction than the longer duplexes (Fig. 4B). The 17 bp duplex also appears to bind less effectively to the protein in comparison to the longer duplexes when assayed by gel mobility shift (data not shown).

DISCUSSION

In this study, comparisons of ADAR-2 deamination reactions have been made for a series of substrate analogs that were obtained by chemical synthesis of the RNA. To our knowledge, this is the first example of substrates for ADAR-2 generated by phosphoramidite synthesis. The approach of mutating RNA substrates at the atomic level by removing or replacing individual functional groups has proven to be useful in mechanistic analyses of other RNA modifying enzymes and ribozymes (22–26). We have shown that a short oligonucleotide duplex generated by chemical synthesis is recognized and processed by ADAR-2. Also, methylation of the 2'-hydroxyl group at the editing site significantly decreases the rate of the deamination reaction, whereas other 2'-modified adenosine analogs placed at this position are deaminated more rapidly. This observation suggests that the ADAR-2 active site cannot accommodate the bulk of the additional methyl group in the substrate at this position. However, given ADAR-2's ability to process a 2'-deoxyadenosine and a 2'-deoxy-2'-fluoroadenosine at the editing site, the enzyme appears to be flexible with regard to features such as the presence of hydrogen bonding

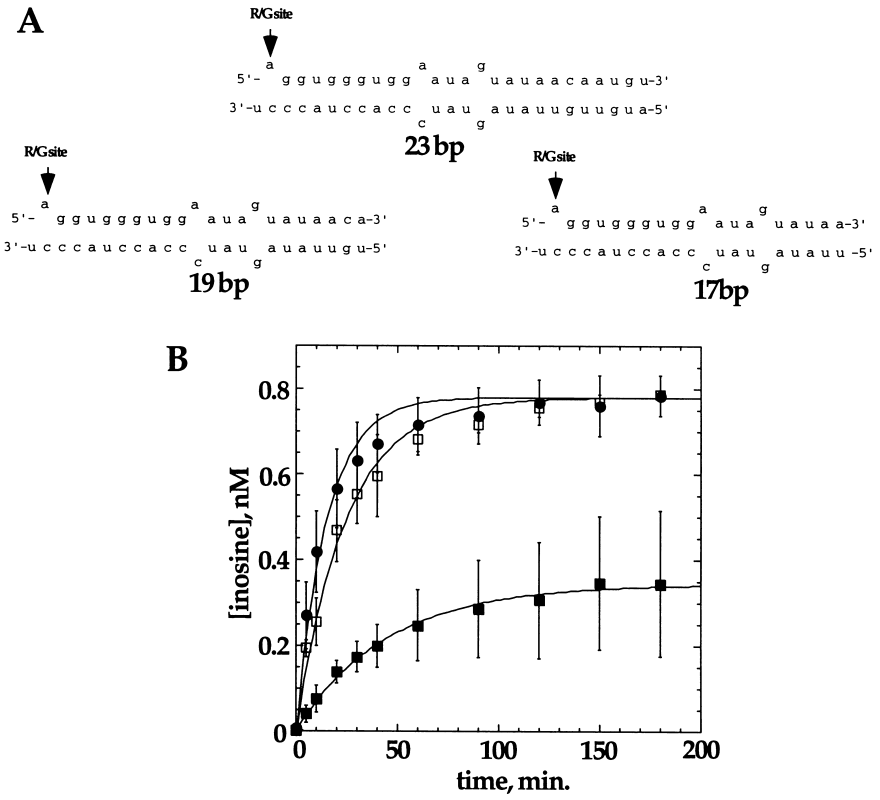

Figure 4. (**A**) Duplex substrates of different lengths. (**B**) Plot of product formation as a function of time for duplex substrates of different lengths. Circle, 23 bp; open square, 19 bp; closed square, 17 bp. The data were fit to the equation % inosine$_t$ = % inosine$_\infty$(1 – e$^{-kobs \cdot t}$) using the least squares method of KaleidaGraph. Data points reported are the average ± SD for three independent experiments.

functional groups at the 2' carbon and preferred sugar conformation at the edited nucleotide (2'-endo for 2'-deoxyadenosine versus 3'-endo for adenosine and 2'-deoxy-2'-fluoroadenosine).

2'-*O*-Methylation at a position other than the editing site did not necessarily inhibit the deamination reaction under our conditions. This result is interesting in light of a number of reports that indicate 2'-*O*-methylation can inhibit complexation by dsRBM-containing proteins. For instance, 2'-*O*-methylation at specific locations on an RNA duplex inhibited the binding of the dsRBM-containing RNA-binding domain of the RNA-dependent protein kinase (27). Also, a duplex formed between a 2'-*O*-methylated oligoribonucleotide and an mRNA molecule was not deaminated by a sample of nuclear extract containing ADARs, whereas a similar duplex that was not 2'-*O*-methylated was deaminated in high yield (28). In addition, the recently reported crystal structure of a dsRBM from *Xenopus laevis* RNA-binding protein A (Xlrbpa) bound to a short RNA duplex shows numerous contacts between the protein and RNA 2'-hydroxyl groups (29). One interpretation of our results is that the dsRBMs of ADAR-2 are able to accommodate the disruptive effect of a single 2'-*O*-methyl substitution and maintain high affinity binding with other contacts. Alternatively, there may be a subset of 2'-hydroxyl groups on an ADAR-2 substrate that are not contacted by the dsRBMs of the protein during catalysis and methylation at these sites has little effect on the deamination reaction at a nearby adenosine.

Mechanistic and structural studies of RNA-editing enzymes will be more easily executed if minimal substrate structures are defined. Here we have shown that a duplex lacking 4 bp of the R/G stem is sufficient to support the deamination reaction of ADAR-2 at the R/G adenosine under our assay conditions. Therefore, if contacts are made by ADAR-2 to these 4 bp, they are not essential for reaction at the R/G site. A smaller duplex with 17 bp is deaminated more slowly and to a lower overall extent by the enzyme. This lower reactivity is likely due to diminished binding affinity and may be a result of the decreased thermodynamic stability of the shorter duplex.

Conclusion

The RNA editing adenosine deaminase ADAR-2 will recognize and process substrate analogs prepared by phosphoramidite chemical synthesis. Using these analogs, we have shown that methylation of the 2'-hydroxyl group of the nucleotide at the editing site significantly decreases the rate of the deamination reaction. We have also shown that a duplex containing as few as 19 bp supports deamination of the GluR-B R/G adenosine by ADAR-2. This identification and initial characterization of synthetic RNA editing substrate analogs further defines elements in the RNA that are important for the deamination reaction, and sets the stage for the analysis of other substrate and transition state analogs of increased structural complexity as well as detailed structural, thermodynamic and kinetic studies of this enzyme.

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