# Demethylation of 6-*O*-Methylinosine by an RNA-Editing Adenosine Deaminase

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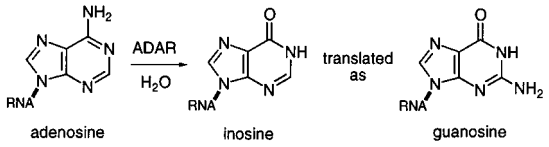
ADARs are *a*denosine *dea*minases that act on *R*NA and are responsible for RNA-editing reactions that occur in eukaryotic mRNAs, including the mRNAs of glutamate and serotonin receptors.[1] The deamination of adenosine in the mRNA results in inosine at that position. Since inosine is translated as guanosine, the ADAR reaction can lead to codon changes in the mRNA, resulting in the synthesis of variant protein structures (Scheme 1).

**Scheme 1**




In several cases, an ADAR capable of a particular editing reaction has been identified.[2−4] However, our understanding of the mechanism of the ADAR-catalyzed reaction is limited. Here we report the synthesis and analysis of substrate analogues designed to provide insight into the mechanistic relationship between the RNA-editing ADARs and the well-understood nucleoside-modifying enzyme adenosine deaminase (ADA).[5−8] These experiments illustrate significant mechanistic similarities and differences between the two classes of adenosine deaminase.

ADAR2 efficiently deaminates specific adenosines in the glutamate receptor B subunit pre-mRNA, including one that converts an arginine codon to a glycine codon (the R/G site).[9] Adenosine deaminase (ADA) is a nucleoside-modifying enzyme that has been extensively characterized structurally and mechanistically.[5−8] ADA uses a zinc-activated water molecule to affect hydrolytic deamination of its nucleoside substrate. The oxygen in the product of the ADAR reaction has also been shown to be derived from water.[10] However, ADARs share little sequence homology with ADAs.[11] Furthermore, the potent ADA inhibitor, coformycin, does not inhibit ADARs, even at high concentrations.[10] Therefore, the possibility exists that ADARs use an alternative mechanism for adenosine deamination.

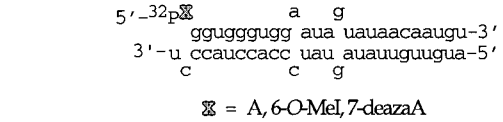
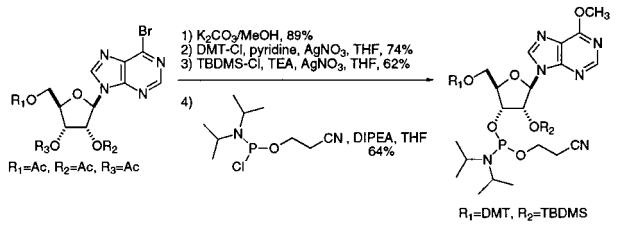



**Figure 1.** Synthetic oligoribonucleotide duplex substrate for ADAR2 where X indicates nucleotide analogues with base modifications at the R/G editing site as indicated.

We recently reported the discovery of ADAR2 substrates accessible by phosphoramidite chemical synthesis.[12] This has allowed us to alter the RNA-editing substrate at the atomic level via the appropriate phosphoramidite. Using this approach, we endeavored to define further the mechanistic relationship between ADAs and ADARs. We incorporated nucleoside analogues of known ADA reactivity into an editing substrate and analyzed the reaction of these analogues with ADAR2.

ADA can accommodate a wide variety of leaving groups at C6 of the purine and efficiently convert these analogues to inosine.[13,14] To determine if ADAR2 could similarly displace a substituent other than an amine from C6, we synthesized a phosphoramidite of 6-*O*-methylinosine (6-*O*-MeI) and used it to incorporate this analogue into RNA, generating a substrate bearing a 6-methoxy substituent on the purine at the editing site (Scheme 2). ADA had previously been shown to convert 6-*O*-MeI to inosine at approximately 1% the reaction rate with adenosine.[14]

**Scheme 2**




The necessary phosphoramidite was generated in four steps from 2′,3′,5′-tri-*O*-acetyl-6-bromopurine ribonucleoside, itself available in two steps from inosine via our recently reported procedure (Scheme 2).[15] This compound was converted to 6-*O*-MeI in excellent yield under mild conditions (5 equiv $K_2CO_3$ in methanol for 1 h at room temperature). For incorporation into RNA, 6-*O*-MeI was protected at the 5′-hydroxyl as the dimethoxytrityl ether and at the 2′-hydroxyl as the *tert*-butyldimethylsilyl ether in good yield. The resulting compound was then converted to the 3′-di-*iso*-propylaminocyanoethyl phosphoramidite under standard conditions. This phosphoramidite coupled quantitatively during automated RNA synthesis and 6-*O*-MeI survived conditions for RNA deprotection unaltered.[16] The RNA substrate chosen for these experiments is a mimic of the R/G editing site of the glutamate receptor B subunit pre-mRNA (Figure 1).

We compared the single turnover reaction of recombinant human ADAR2 with RNA substrates bearing various nucleosides at the R/G editing site using a thin-layer chromatography assay

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(16) Survival of the 6-*O*-MeI nucleoside to the conditions of RNA deprotection (0.05 M $K_2CO_3$/MeOH, rt, 4 h followed by 0.1 M TBAF/THF, rt, 36 h) was confirmed by MALDI-TOF mass spectral analysis of the 12 mer oligoribonucleotide 5′-CCUACCXUGAUG-3′, where X = 6-*O*-MeI.

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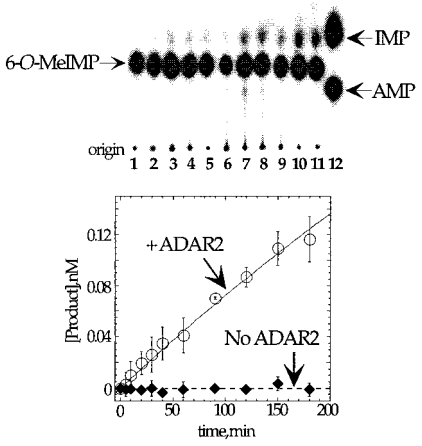


**Figure 2.** (Top) Storage phosphor autoradiogram of TLC plate used to separate products arising from the reaction of 54 nM ADAR2 with 1 nM substrate containing 6-*O*-MeI at the R/G editing site. Lanes 1−11, reaction times of 0, 5, 10, 20, 30, 40, 60, 90, 120, 150, and 180 min, respectively; lane 12, adenosine monophosphate (AMP) and inosine monophosphate (IMP) standards. (Bottom) Plot of product formation as a function of time. (○): Reaction in the presence of ADAR2. (◆): Reaction with no ADAR2 added.

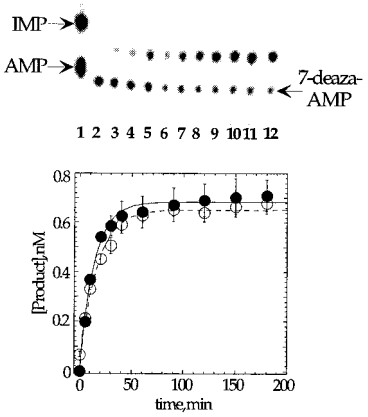


**Figure 3.** (Top) Storage phosphor autoradiogram of TLC plate used to separate products arising from the reaction of 1 nM substrate with 7-deazaadenosine occupying the R/G editing site and 54 nM ADAR2. Lane 1, AMP and IMP standards; lanes 2−12, reaction times of 0, 5, 10, 20, 30, 40, 60, 90, 120, 150, and 180 min, respectively. (Bottom) Plot of product formation as a function of time. (●): Adenosine at R/G site. (○): 7-Deazaadenosine at the R/G editing site.

previously described.[12] When 6-*O*-MeI was located at the editing site in the RNA substrate, ADAR2 converted it to inosine with a $k_{obs} = 0.001 \pm 0.0001$ min.$^{-1}$ (Figure 2). The reaction rate was slower than that observed for adenosine ($k_{obs} = 0.06 \pm 0.001$ min.$^{-1}$) (Figure 3) but faster than other analogues tested previously (e.g., 2′-*O*-methyladenosine).[12] Therefore, like ADA, ADAR2 does not require the 6-amino group of adenosine at the editing site. The overall reaction of ADAR2 with 6-*O*-MeI-containing RNA is a demethylation. We favor an $S_NAr$-like mechanism with attack by an activated water at C6 of the purine, leading to the loss of methanol in analogy to the ADA reaction. However, at this time, we cannot rule out an $S_N2$ attack at the methyl carbon where inosine functions as the leaving group. Nevertheless, the ADAR2-catalyzed conversion of 6-*O*-MeI to inosine argues against amine-specific deamination mechanisms, such as those involving Schiff base formation, for this enzyme.

ADA derives its specificity for purines via the formation of specific hydrogen bonds to the base. Each of the purine ring nitrogens available for hydrogen bonding (N1, N3, and N7) are contacted directly by amino acids that make up the ADA active site.[5] A direct contact to N7 is essential to nucleoside binding to ADA as 7-deazaadenosine (7-deazaA) is neither a substrate nor an inhibitor of this enzyme.[17] To determine if this interaction is also important in the ADAR2 reaction, we incorporated 7-deazaA into an RNA substrate using the known phosphoramidite.[18] In contrast to ADA, ADAR2 efficiently deaminated 7-deazaadenosine in this RNA substrate ($k_{obs} = 0.06 \pm 0.003$ min.$^{-1}$) (Figure 3). This result suggests that, if a hydrogen bonding contact is made by ADAR2 to N7 of the adenosine, this interaction does not significantly contribute to the rate-limiting step of the reaction.

ADARs and a related family of tRNA-specific adenosine deaminases have conserved sequences similar to the consensus sequence that makes up the active site of cytidine deaminases (CDAs).[7,19] CDA uses a zinc-activated water molecule to carry out deamination of its nucleoside substrate via attack at C4 of the pyrimidine and loss of ammonia in a reaction similar to that catalyzed by ADA. Using the comparison to CDAs, amino acids identified as possible active site residues for the ADARs, including putative metal-binding ligands, have been altered by site-directed mutagenesis with a corresponding loss of editing activity.[20] Although this suggests a similarity between CDAs and ADARs, we find no cytidine deamination in ADAR2 reactions, even if cytidine is placed at the editing site (data not shown). Given these observations, we believe ADAR2 uses a CDA-like active site, altered for adenosine recognition, for hydrolytic deamination of RNA substrates. This is consistent with ADAR2's ability to process 6-*O*-MeI in RNA at an editing site. However, the basis for ADAR2's adenosine selectivity is unknown at this time. The minimal sequence similarity between ADARs and ADA and the ability of ADAR2 to efficiently deaminate 7-deazaadenosine suggest that purine recognition is distinct for these two classes of adenosine deaminase. This may, in part, explain the lack of inhibition by the ADA inhibitor coformycin.

In summary, by comparing the reactivity of substrate analogues, we have shown that, although ADAR2 and ADA share mechanistic similarities, these enzymes have different functional group requirements in the reactive adenosine. Additional studies with RNA-containing nucleoside analogues will continue to define the role of substrate structure in the RNA-editing reaction and aid in the design of tight binding, active site-directed inhibitors. These compounds will be useful for future structural characterization of the ADAR2·RNA complex and for extending our understanding of the RNA-editing adenosine deamination reaction.

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**Supporting Information Available:** Synthetic procedures and NMR and HRMS data for all new compounds and procedures for the preparation and deamination of RNA substrates of ADAR2 (PDF). This material is available free of charge via the Internet at http://pubs.acs.org.

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