

# The Mechanism of Adenosine to Inosine Conversion by the Double-Stranded RNA Unwinding/Modifying Activity: A High-Performance Liquid Chromatography–Mass Spectrometry Analysis<sup>†</sup>

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**ABSTRACT:** We have used directly combined high-performance liquid chromatography–mass spectrometry (LC/MS) to examine the mechanism of the reaction catalyzed by the double-stranded RNA unwinding/modifying activity [Bass & Weintraub (1988) *Cell* 55, 1089–1098]. A double-stranded RNA substrate in which all adenosines were uniformly labeled with <sup>13</sup>C was synthesized. An LC/MS analysis of the nucleoside products from the modified, labeled substrate confirmed that adenosine is modified to inosine during the unwinding/modifying reaction. Most importantly, we found that no carbons are exchanged during the reaction. By including H<sub>2</sub><sup>18</sup>O in the reaction, we showed that water serves efficiently as the oxygen donor in vitro. These results are consistent with a hydrolytic deamination mechanism and rule out a base replacement mechanism. Although the double-stranded RNA unwinding/modifying activity appears to utilize a catalytic mechanism similar to that of adenosine deaminase, coformycin, a transition-state analogue, will not inhibit the unwinding/modifying activity.

An activity that modifies adenosines to inosines within double-stranded RNA (dsRNA) has been characterized (Bass & Weintraub, 1988; Wagner et al., 1989). This activity was originally discovered in the South African clawed toad *Xenopus laevis* (Bass & Weintraub, 1987; Rebagliati & Melton, 1987) and was later found in *Drosophila melanogaster* (B. Bass, unpublished results), *Caenorhabditis elegans* (M. Krause, unpublished results), and a number of mammalian tissues as well as cultured cells (Wagner et al., 1990). Although the activity appears to be ubiquitous among the phyla of the animal kingdom, its function remains unknown.

The modification of an adenosine to an inosine within dsRNA results in an inosine–uridine mismatch and local melting of the helix. For this reason, the activity was originally characterized as a dsRNA unwinding activity (Bass & Weintraub, 1987; Rebagliati & Melton, 1987). More recently, the activity has been referred to as the dsRNA unwinding/modifying activity (Bass & Weintraub, 1988; Wagner et al., 1989). The dsRNA unwinding/modifying activity is very specific for dsRNA. Competition assays indicate that the activity will not bind to single-stranded RNA, single-stranded DNA, double-stranded DNA (Bass & Weintraub, 1987; Wagner & Nishikura, 1988), or adenine nucleosides or nucleotides (B. Bass, unpublished results).

There are two known mechanisms by which adenosine is converted to inosine in biological systems: hydrolytic deamination and hypoxanthine (the inosine base) insertion. Adenosine deaminase and AMP deaminase, enzymes involved in purine metabolism, remove the amino group from adenosine and replace it with an oxygen from water to produce inosine

and ammonia. The mechanism of adenosine deaminase and AMP deaminase is thought to be a hydrolytic deamination in which water directly attacks the base, the enzyme acting as a general base catalyst (Frick et al., 1986). The direct water attack on the substrate is thought to produce a tetrahedral intermediate. This hypothesis is based on the fact that compounds that mimic the tetrahedral intermediate, such as coformycin, are potent inhibitors of adenosine deaminase and AMP deaminase (Frick et al., 1986; Merkler et al., 1990). Further, the recently determined X-ray crystal structure of adenosine deaminase complexed with a transition-state analogue is consistent with the proposed tetrahedral intermediate (Wilson et al., 1991).

The second mechanism of converting adenosine to inosine is exemplified by the mammalian enzyme that converts adenosine to inosine in the first position of the anticodon of tRNA. This enzyme is postulated to cleave the glycosyl bond, remove adenine, and insert hypoxanthine (Elliott & Trewyn, 1984). This might be surprising in light of the chemical ease of hydrolytic deamination; however, this is not the only RNA modification enzyme that uses a base replacement mechanism. For example, tRNA–guanine transglycosylase removes guanine from the primary transcript of certain tRNAs and replaces it with the hypermodified base queuine [reviewed by Nishimura (1983)].

In order to examine the mechanism of the dsRNA unwinding/modifying activity, we used a dsRNA substrate that contained adenosine uniformly labeled with <sup>13</sup>C and examined the nucleoside products by directly combined high-performance liquid chromatography–mass spectrometry (LC/MS).<sup>1</sup> We observed that all of the carbons contained in the adenosine are retained during the reaction. Further, by running the modification reaction in H<sub>2</sub><sup>18</sup>O, we have shown that water can act

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<sup>1</sup> Abbreviations: M, neutral nucleoside molecule; B and S, base and sugar fragments of a nucleoside, respectively; accordingly, BH<sub>2</sub><sup>+</sup> corresponds to a protonated free base; LC/MS, high-performance liquid chromatography–mass spectrometry.

efficiently as an oxygen donor in vitro. These results are consistent with a hydrolytic deamination and rule out a base replacement mechanism. Finally, the mass spectrometric analysis clearly verifies that the modified base is inosine as suggested by previous chromatographic analyses (Bass & Weintraub, 1988; Wagner et al., 1989).

#### MATERIALS AND METHODS

**Synthesis of [ $^{13}\text{C}$ ]A-dsRNA.** Uniformly  $^{13}\text{C}$ -labeled adenosine 5'-monophosphate ([ $^{13}\text{C}$ ]5'-AMP) was isolated from the rRNA fraction of *Escherichia coli* B cells grown on [ $^{13}\text{C}$ ]acetate. The labeled cells were obtained from the National Stable Isotopes Resource, Los Alamos National Laboratory. Nucleic acids were extracted from the lyophilized cells by an adaptation of Zubay's method (Zubay, 1962). Cells were rehydrated in extraction buffer [10 mM  $\text{Mg}(\text{C}_2\text{H}_3\text{O}_2)_2$ /1 mM Tris-HCl (pH 7.4); 40 mL of buffer/g] and disrupted in a Dounce homogenizer. The disrupted cells were extracted twice with buffer-saturated phenol. The nucleic acid was precipitated by addition of 2.5 volumes of ethanol and storage overnight at  $-20^\circ\text{C}$ . The precipitate was recovered by centrifugation for 30 min at 10 000 rpm at  $4^\circ\text{C}$  and dried in vacuo. This crude nucleic acid precipitate was extracted twice with 1 M NaCl, and the insoluble (rRNA-rich) fraction was dissolved in a minimal volume of extraction buffer. The nucleic acid was then ethanol-precipitated as above.

Seventy-three milligrams of nucleic acid (estimated from 20  $A_{260}$  units = 1 mg) was dissolved in 22 mL of 50 mM Tris-HCl (pH 7.4)/5 mM  $\text{MgCl}_2$  and incubated with 13  $\mu\text{g}$  of RNase-free DNase (Bethesda Research Laboratories) for 1 h at  $37^\circ\text{C}$ . The RNA was then ethanol-precipitated as above; the pellet was washed with 70% ethanol and dried in vacuo.

The resultant RNA (72 mg) was solubilized with two 20-mL portions of 1 mM Tris-HCl (pH 7.4) and then mixed with 0.2 mL of 1 M  $\text{Na}_2\text{C}_2\text{H}_3\text{O}_2$  (pH 5.3). Seventy units of nuclease P1 was added, and the solution was incubated 48 h at  $45^\circ\text{C}$ . The digest was stored at  $-20^\circ\text{C}$  until used.

AG 1-X8 ( $\text{Cl}^-$ ) resin (100–200 mesh, Bio-Rad) was converted to the acetate form as directed by the supplier. A  $10 \times 2.5$  cm Econo column (Bio-Rad) was filled with resin to a height of 7 cm and equilibrated with 10 mM  $\text{NH}_4\text{C}_2\text{H}_3\text{O}_2$  (pH 4.3). Labeled nucleotide 5'-monophosphates were eluted using an  $\text{NH}_4\text{C}_2\text{H}_3\text{O}_2$  (pH 4.3) step gradient (Sinsheimer & Koerner, 1951). The 5'-AMP fraction was eluted with 0.2 M  $\text{NH}_4\text{C}_2\text{H}_3\text{O}_2$ , dried in a SpeedVac (Savant Instruments), and stored at  $-20^\circ\text{C}$ .

Isotopic enrichment of [ $^{13}\text{C}$ ]5'-AMP was determined from the 70-eV electron ionization mass spectrum of the trimethylsilyl derivative of adenosine. Uniformly labeled [ $^{13}\text{C}$ ]adenosine ([ $^{13}\text{C}$ ]A) was obtained by dephosphorylation of the [ $^{13}\text{C}$ ]5'-AMP by alkaline phosphatase. The nucleoside was further purified by HPLC using a Supelcosil LC-18-T column (Supelco) with a flow rate of 1.0 mL/min of water and a linear gradient solvent program of 0–60% methanol over 60 min. Two micrograms of HPLC-purified nucleoside was dried into a  $50 \times 0.4$  mm tube, fashioned from 0.4 mm i.d. Pyrex tubing, and incubated for 1 h at  $100^\circ\text{C}$  in 8  $\mu\text{L}$  of solution consisting of 10:1 (v/v) Sylon BFT [*N,O*-bis(trimethylsilyl)trifluoroacetamide/1% trimethylchlorosilane] and dry pyridine. One microliter of solution was used to acquire several mass spectra on a VG 70-SEQ instrument (VG Instruments). The normalized partial mass spectrum (average of six scans) of the ( $\text{M}-\text{CH}_3$ ) $^+$  ion cluster for the tetrakis-(trimethylsilyl) derivative of [ $^{13}\text{C}$ ]A consisted of the following ( $m/z$ )/% relative abundance values: 540/1.7; 541/1.4;

542/1.3; 543/1.2; 544/1.6; 545/2.8; 546/3.2; 547/9.2; 548/32.6; 549/79.6; 550/100.0; 551/41.2; 552/19.2; 553/5.4; 554/1.8. Deconvolution of the isotopic cluster (Yamamoto & McCloskey, 1977) indicated a  $^{13}\text{C}$  enrichment of 91.2% for the principal labeled species. The preparation contained 1.5% all- $^{12}\text{C}$  material.

Uniformly  $^{13}\text{C}$ -labeled ATP ([ $^{13}\text{C}$ ]ATP) for transcription reactions was created by mixing 0.1  $\mu\text{M}$  ATP, 1.0 mM [ $^{13}\text{C}$ ]5'-AMP, 400  $\mu\text{g}/\text{mL}$  myokinase (Boehringer Mannheim), 1200  $\mu\text{g}/\text{mL}$  pyruvate kinase (Boehringer Mannheim), and 120 mM phosphoenolpyruvate, in  $1\times$  reaction buffer ( $10\times = 1$  M triethanolamine, 25 mM  $\text{MgCl}_2$ , and 100 mM KCl titrated to pH 7.6) and incubating for 5 h at  $25^\circ\text{C}$ . The resultant ATP was purified by HPLC using a Supelcosil LC-18-T column with a flow rate of 1.0 mL/min of 0.1 M  $\text{KH}_2\text{PO}_4$ /8 mM tetrabutylammonium hydrogen sulfate (pH 6.0) and a linear gradient solvent program of 0–30% methanol over 30 min. Fractions containing ATP were pooled and concentrated by a SpeedVac concentrator before subsequent purification or storage. The purified ATP samples were desalted by HPLC using two passes over a Supelcosil LC-18-T column with a flow rate of 1.0 mL/min of water and a linear gradient solvent program of 0–30% methanol over 30 min.

The dsRNA containing adenosine that was uniformly labeled with  $^{13}\text{C}$  ([ $^{13}\text{C}$ ]A-dsRNA) was synthesized using previously published methods (Bass & Weintraub, 1987). As described, the dsRNA consisted of the sense and antisense transcripts of the chloramphenicol acetyltransferase (CAT) gene. For these experiments, the transcription reactions had 0.5 mM [ $^{13}\text{C}$ ]ATP instead of the unlabeled ATP and contained no radiolabeled ATP.

**Preparation of Partially Purified Extracts of the Unwinding/Modifying Activity.** Partially purified preparations of the unwinding/modifying activity were made by the following method. A high-speed supernatant fraction (S100) from *Xenopus laevis* eggs was prepared as described (Bass & Weintraub, 1987). The S100 material was dialyzed against buffer A [10 mM Tris-HCl (pH 7.9), 20% glycerol, 10 mM NaCl, 25 mM KCl, 1.1 mM  $\text{MgCl}_2$ , 0.5 mM dithiothreitol (DTT), and 0.1 mM EDTA] and further purified by ion-exchange chromatography at  $4^\circ\text{C}$ . Briefly, approximately 300 mL of S100 extract (2 g of protein) was adsorbed to a column ( $1.6 \times 35$  cm) of TSK-DEAE-650m (Toyopearl) previously equilibrated in buffer A, and subsequently washed with 10 column volumes of buffer A. Proteins bound to the column were eluted with a 25–500 mM KCl gradient in buffer A at 20 mL/h. Fractions of 6 mL each were collected and assayed for activity as previously described (Bass & Weintraub, 1988). Active fractions which eluted between 0.18 and 0.21 M KCl, and contained approximately 6% of the total starting protein, were pooled and dialyzed at  $4^\circ\text{C}$  against buffer A and then stored in small aliquots at  $-80^\circ\text{C}$ .

**Modification Reactions for LC/MS Analysis.** Modification reactions were carried out on  $1.5 \times 10^{-12}$  mol of [ $^{13}\text{C}$ ]A-dsRNA in a volume of 1 mL. Final in vitro reactions contained 1.6 mg/mL partially purified protein, 0.5 mM DTT, 25 mM Tris-HCl (pH 7.8), 1600 units/mL RNasin, 10% glycerol, 5 mM NaCl, 12.5 mM KCl, 0.55 mM  $\text{MgCl}_2$ , 0.05 mM EDTA, and 1.5 nM (0.8  $\mu\text{g}/\text{mL}$ ) [ $^{13}\text{C}$ ]A-dsRNA. Modification reactions were incubated for 4 h at  $25^\circ\text{C}$  and were stopped by the addition of 200  $\mu\text{L}$  of proteinase K mix [50 mM Tris-HCl (pH 7.5), 50 mM NaCl, 5 mM EDTA, 0.5% SDS, and 400  $\mu\text{g}/\text{mL}$  proteinase K] followed by incubation for 1 h at  $37^\circ\text{C}$ . The modification reaction conditions were designed to ensure a complete reaction, which results in

the conversion of 50% of the adenosines to inosine (Bass & Weintraub, 1988). For the negative control samples, the reaction mix was incubated with 200  $\mu$ L of proteinase K mix for 1 h at 37 °C prior to the addition of [U- $^{13}$ C]A-dsRNA and a mock incubation for 4 h at 25 °C. Subsequently, both the negative control reaction and the modification reaction were treated the same. Samples were phenol- and chloroform-extracted, ethanol-precipitated in 2.5 M  $\text{NH}_4\text{C}_2\text{H}_3\text{O}_2$ , and washed with 70% ethanol. Samples were resuspended in  $\text{H}_2\text{O}$ , heated to 97 °C, immediately cooled on ice, and then digested for 6 h in 20 mM  $\text{NH}_4\text{C}_2\text{H}_3\text{O}_2$  (pH 5.0) with 120 units of ribonuclease T2 at 37 °C, followed by digestion with 60 units of nuclease P1 in the same buffer for 2 h at 56 °C.

The  $\text{H}_2^{18}\text{O}$  reactions were carried out the same as above except 42% of the reaction volume was 97.17 atom %  $\text{H}_2^{18}\text{O}$  (Merck Isotopes).

**LC/MS Analysis.** The chromatographic system employed for analyses was a Beckman Instruments Model 342, a dual-pump system with high-pressure mixing. Chromatographic separations were accomplished on a 4.6  $\times$  250 mm LC-18S analytical column (Supelco) preceded by a 4.6  $\times$  30 mm RP-18 guard column (Applied Biosystems). The analytical and guard columns were maintained at 30 °C in a thermostatically controlled column heater (Model TCM, Waters Associates). Ultraviolet absorbance at 254 and 280 nm was monitored with a Model 440 detector (Waters Associates) serially connected between the column and the mass spectrometer. The aqueous buffer was 250 mM  $\text{NH}_4\text{C}_2\text{H}_3\text{O}_2$ , pH 6.0, and the organic modifier was 60:40 (v/v) water/acetonitrile. The buffer was vacuum-degassed and filtered through a 0.2- $\mu$ m Nylon-66 filter in a single step with a solvent filter/degasser. Organic modifier was degassed by stirring vigorously under vacuum for 20 min. The mobile phase flow rate was 2 mL/min, and the gradient profile used was a modification (Phillipson et al., 1987) of a gradient previously reported by Buck et al. (1983).

The total effluent from the liquid chromatograph was passed into a thermospray ion source of a noncommercial quadrupole mass spectrometer, previously described by Edmonds et al. (1985). The thermospray controller was a commercially available design from Vestec Corp. The relevant thermospray parameters were the vaporizer control temperature of  $\sim$ 130 °C and the vaporizer tip temperature of  $\sim$ 260 °C. The vapor temperature at the point of ion sampling was 295 °C. Mass spectra were obtained by scanning with the instrument from  $m/z$  100 to  $m/z$  360 every 1.7 s. The instrument was controlled by a Vector/One data system (Teknivent Corp.). A detailed treatment of the interpretation of thermospray mass spectra of nucleosides and analysis of RNA hydrolysates is given by Pomerantz and McCloskey (1990).

**Modification Reactions and Control Reactions for the Inhibitor Experiments.** Modification reactions and control reactions for the inhibitor experiments were carried out in a volume of 20  $\mu$ L under the same buffer conditions as the modification reaction described above. The cofomycin was isolated from *Streptomyces antibioticus* and obtained from Calbiochem. The dsRNA substrate was internally labeled with [ $^{32}$ P]ATP as described previously (Bass & Weintraub, 1987). Six femtomoles of the dsRNA and 32  $\mu$ g of the partially purified extract were used per reaction. Reactions were stopped with 5  $\mu$ L of proteinase K mix as described above.  $^{32}$ P-Labeled 5'-AMP, 5'-GMP, 5'-UMP, 5'-CMP, and 5'-IMP were prepared for use as markers and substrate by phosphorylation of the nucleoside 3'-monophosphate with [ $\gamma$ - $^{32}$ P]ATP followed by removal of the 3'-monophosphate with nuclease

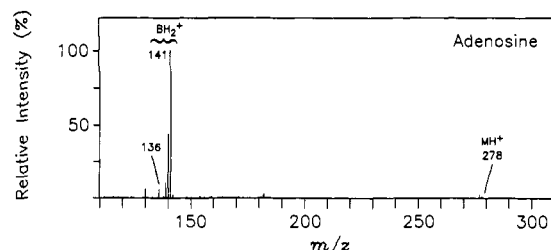


FIGURE 1: Thermospray mass spectrum from the LC/MS analysis of adenosine derived from the [U- $^{13}$ C]A-dsRNA. The protonated free base ( $\text{BH}_3^+$ ) isotope cluster and the completely  $^{13}\text{C}$  labeled molecular ion ( $\text{MH}^+$ ) are marked.

P1 as described previously (Bass & Weintraub, 1988). For the reactions spiked with AMP deaminase, we used 0.02 unit of rabbit muscle 5'-AMP deaminase (Sigma). Where indicated, heat inactivation of modification extract was carried out at 65 °C for 10 min. After proteinase K treatment, samples that did not contain dsRNA were phenol-, chloroform-, and ether-extracted, dried in a SpeedVac concentrator, and resuspended in 10  $\mu$ L of water. Samples that contained dsRNA were phenol-extracted, chloroform-extracted, and subsequently ethanol-precipitated. To digest the RNA to 5'-mononucleotides, samples were treated with P1 nuclease. Samples were resuspended in 20  $\mu$ L of TE [25 mM Tris-HCl (pH 7.8) and 1 mM EDTA], and 30 units of P1 nuclease and incubated at 45 °C for 30 min; 30 more units of P1 nuclease was added, and then the samples were incubated at 65 °C for an additional 30 min. Prior to analysis, samples were phenol-, chloroform-, and then ether-extracted, dried in a SpeedVac concentrator, and resuspended in 10  $\mu$ L of water. Thin-layer chromatography was carried out on a cellulose plate with saturated  $(\text{NH}_4)_2\text{SO}_4/0.1 \text{ M NaC}_2\text{H}_3\text{O}_2$  (pH 6.0)/2-propanol (79:19:2, by volume) as the chromatographic solvent.

## RESULTS

The dsRNA unwinding/modifying activity has not been purified. The work done previously (Bass & Weintraub, 1988) and the work described here were done with partially purified extracts (see Materials and Methods). These extracts contained many nucleic acid, protein, and possibly small-molecule contaminants. For this reason, the identity of all the reactants and products, and therefore the mechanism of the reaction, has been difficult to characterize. In order to circumvent the problems of a crude system, we have investigated the reaction mechanism using isotopically labeled reactants and directly combined LC/MS. This technique allows the separation of nucleosides and provides relative retention times, UV absorbance information, and complete mass spectra of the individual nucleosides. Therefore, the isotopic distribution for each nucleoside derived from a complex mixture of nucleic acids can be determined, providing a means of establishing the exact number of skeletal carbon atoms transferred in the adenosine to inosine conversion.

An 800 base pair RNA duplex (Bass & Weintraub, 1987) containing adenosine that was uniformly labeled with  $^{13}\text{C}$  was synthesized. This construct will be referred to as [U- $^{13}$ C]A-dsRNA. Theoretically, the method of synthesis of [U- $^{13}$ C]A-dsRNA should yield dsRNA that is 98.5% isotopically labeled adenosine (see Materials and Methods). Indeed, an LC/MS analysis of the adenosine in the [U- $^{13}$ C]A-dsRNA (Figure 1) showed that the dsRNA contained very little unlabeled adenosine (e.g., the  $\text{BH}_3^+$   $m/z$  136 ion<sup>1</sup> abundance is low). The labeled adenosine used to make the dsRNA contained an average of 91 mol %  $^{13}\text{C}$  (see Materials and Methods). The labeled adenosine gives an isotopic mass

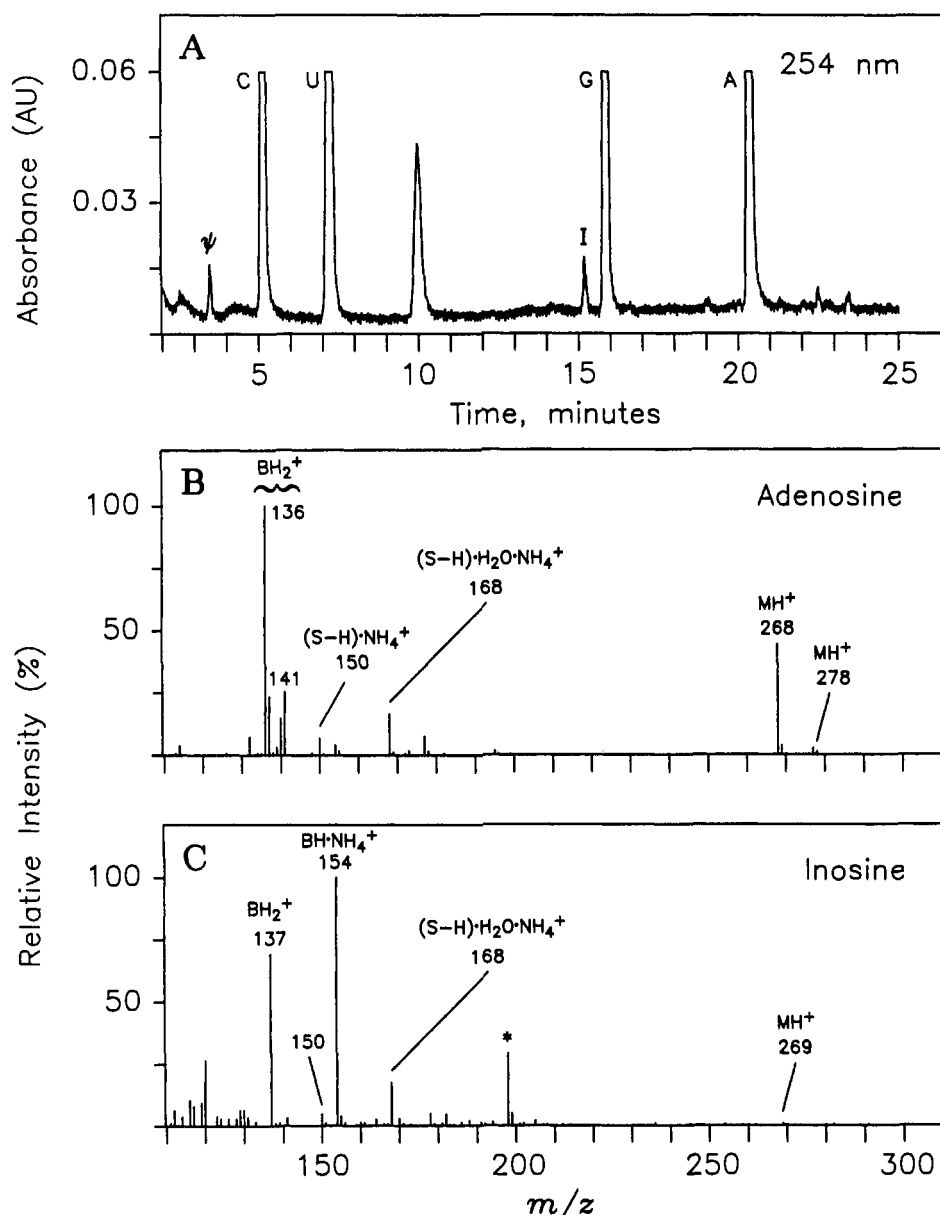


FIGURE 2: LC/MS analysis of nucleosides from the negative control modification reaction. (A) Chromatogram of the nucleosides with UV detection at 254 nm. The peak at 10 min is unidentified, but the mass spectrum indicated that it is not a nucleoside (data not shown). (B) Thermospray mass spectrum of adenosine showing the protonated free base ( $BH_2^+$ ) isotope cluster, the sugar ions corresponding to the loss of one hydroxyl hydrogen from the sugar fragment, and the formation of an adduct with  $NH_4^+$  or  $NH_4^+$  and  $H_2O$  [ $(S-H)\cdot H_2O\cdot NH_4^+$ ], and the unlabeled and completely labeled molecular ions ( $MH^+$ ). (C) Thermospray mass spectrum of inosine showing  $BH_2^+$ ,  $(S-H)\cdot NH_4^+$  ( $m/z$  150),  $(S-H)\cdot H_2O\cdot NH_4^+$ ,  $MH^+$ , and the ion corresponding to free base with an  $NH_4^+$  adduct ( $BH\cdot NH_4^+$ ). The peak marked with an asterisk is a background ion.

pattern for adenosine  $BH_2^+$  ions in which the ion with five  $^{13}C$  atoms ( $m/z$  141) is the most abundant and the ions with three and four  $^{13}C$  atoms ( $m/z$  139 and 140) are less abundant (Figure 1). Although thermospray LC/MS allows the definitive determination of the number of  $^{13}C$  atoms in a particular ion species, it should be noted that minor variations in observed abundance ratios within isotopic clusters (e.g., compare Figure 3C,  $BH_2^+$  and  $BH\cdot NH_4^+$  clusters) normally occur as a consequence of the thermospray process (Pomerantz & McCloskey, 1990).

In order to examine the modified nucleosides, the [ $U-^{13}C$ ]A-dsRNA was incubated with a partially purified preparation of the unwinding/modifying activity or with an inactivated preparation as a negative control. Prior to analysis by LC/MS, the RNA was deproteinized, ethanol-precipitated, and then digested with ribonucleases to produce the nucleosides.

**The Extracts Contain Nucleic Acid.** The LC/MS data showed that, as expected, the extract used to modify the [ $U-^{13}C$ ]A-dsRNA contained a large amount of nucleic acid. The chromatograms of Figures 2–4 show a large excess of ribonucleoside compared to what was added in the form of dsRNA substrate. Although the amount of nucleoside derived from the extract was greater than that derived from the substrate RNA, the labeled adenosine base ions were easily detectable in the mass spectra of all the samples ( $m/z$  139–141 in panels B of figures 2–4). The extract nucleic acid contained the modified nucleosides pseudouridine and inosine. The presence of pseudouridine was shown by the UV absorbing peak at 3.5 min in the chromatogram of the negative control (Figure 2A). The identity of pseudouridine was confirmed by mass spectrometry (data not shown). Unlabeled inosine was also found in the nucleoside samples of the negative control (Figure 2, panels A and C), and was identified by its HPLC elution time

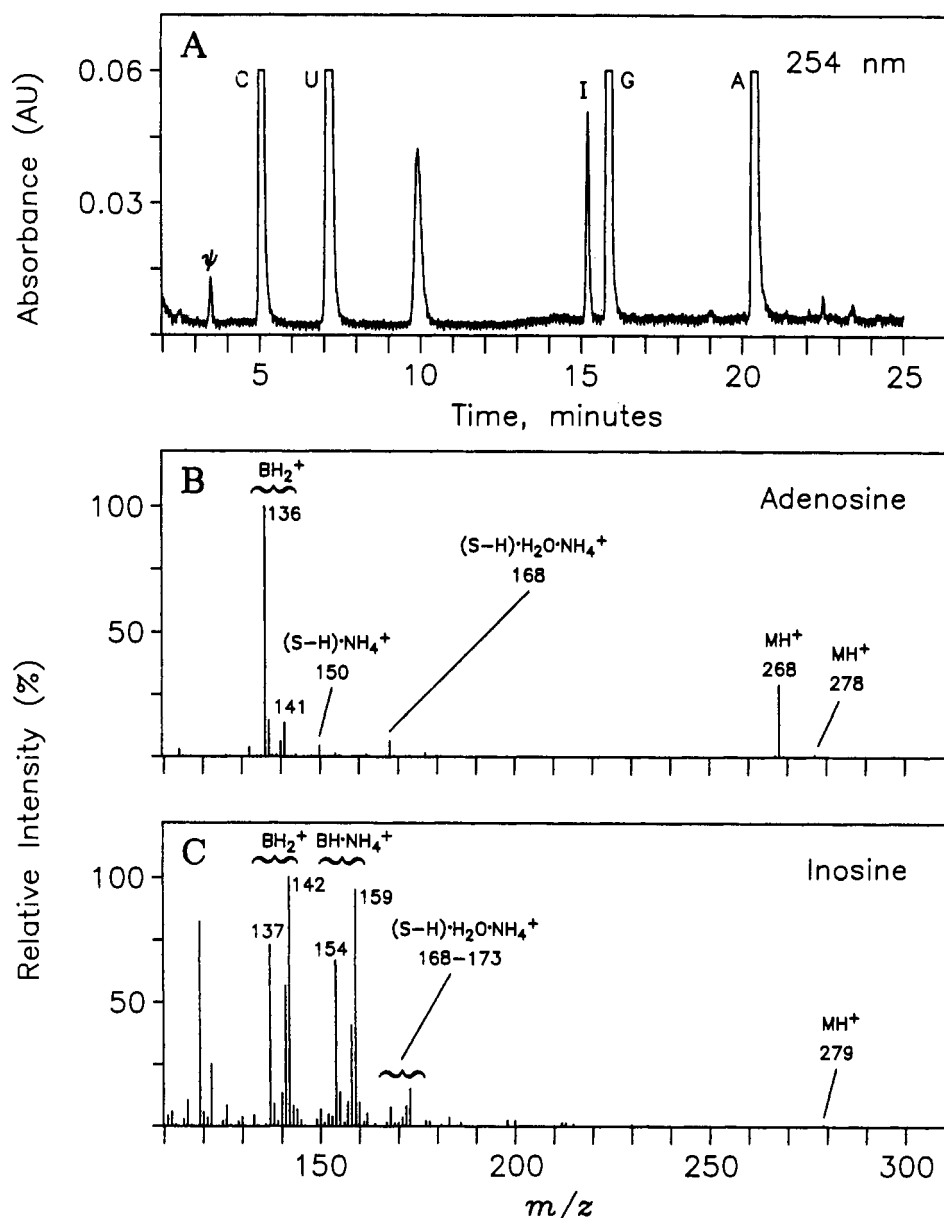


FIGURE 3: LC/MS analysis of the nucleosides from the modification reaction. (A) and (B) as in Figure 2. (C), same as in Figure 2 but the  $BH_2^+$ ,  $BH \cdot NH_4^+$ , and  $(S-H) \cdot H_2O \cdot NH_4^+$  ions are labeled as isotope clusters.

and mass spectrum (Pomerantz & McCloskey, 1990). Since adenosine can be chemically deaminated to inosine, and adenosine deaminase is sometimes found as a contaminant in ribonuclease preparations, we considered the possibility that the inosine in the negative control samples was created during the ribonuclease digestion. However, the mass spectrum of the inosine in the negative control (Figure 2C) showed no ions corresponding to inosine derived from the labeled adenosine (e.g.,  $m/z$  142, 159, and 173). This indicated that the inosine in the negative control samples was present in the extract since both labeled and unlabeled adenosine would be converted to inosine if deamination occurred during digestion of the RNA.

**All Labeled Carbon Atoms Are Retained during the Conversion of Adenosine to Inosine by the Unwinding/Modifying Activity.** The chromatogram of the nucleosides from the modified RNA (Figure 3A) showed an increase in the amount of UV-absorbing material at the retention time of inosine compared to the negative control. The mass spectrum of inosine from the modified RNA (Figure 3C) showed a new cluster of peaks at  $m/z$  140–142. These peaks were 3–5  $m/z$  units greater than the unlabeled inosine  $BH_2^+$  ion ( $m/z$  137), and the isotopic pattern of these peaks was very similar to the

pattern of peaks in the adenosine spectra (Figure 1 and panels B in Figures 2–4,  $m/z$  139–141) that represent the labeled adenosine  $BH_2^+$  ions. Therefore, these peaks were assigned as labeled inosine  $BH_2^+$  ions. The presence of labeled inosine  $BH_2^+$  ions is the expected result if the inosine were derived from the labeled adenosine without any loss of carbon from the purine nucleoside skeleton. The peaks at  $m/z$  157–159 were assigned as labeled inosine  $BH \cdot NH_4^+$  ions, and their presence supports the assignment of  $m/z$  140–142 as labeled inosine  $BH_2^+$  ions.

The peaks at  $m/z$  171–173 in the spectrum of inosine from the modified RNA (Figure 3C) were assigned as labeled sugar ions [ $(S-H) \cdot H_2O \cdot NH_4^+$ ; Pomerantz & McCloskey, 1990] and showed that, as expected, the sugar in the inosine came from the adenosine and all the original carbons were retained. These results confirmed that the unwinding/modifying activity converts adenosine to inosine as suggested by previous chromatographic experiments (Bass & Weintraub, 1988; Wagner et al., 1989) and established that no carbons are exchanged during the reaction.

**Water Can Efficiently Act as an Oxygen Donor in Vitro.** Modification and control reactions were carried out on the

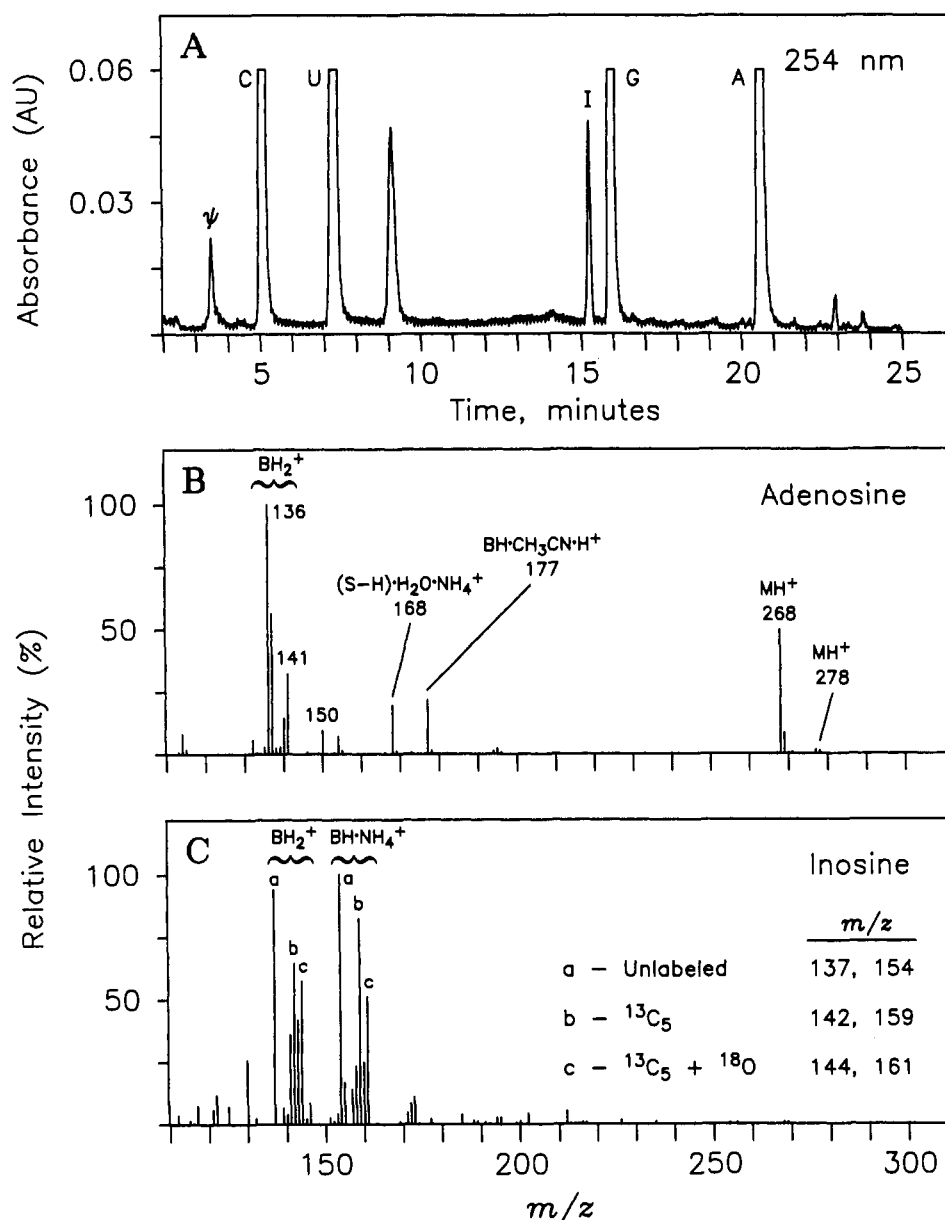


FIGURE 4: LC/MS analysis of nucleosides from  $H_2^{18}O$  modification reactions. (A) Chromatogram of the nucleosides with UV detection at 254 nm. (B) Thermospray mass spectrum of adenosine showing the  $BH_2^+$  isotope cluster,  $(S-H)\cdot NH_4^+$  ( $m/z$  150),  $(S-H)\cdot H_2O\cdot NH_4^+$ , the unlabeled and completely labeled  $MH^+$ , and the free base complexed with acetonitrile and a proton ( $BH\cdot CH_3CN\cdot H^+$ ). (C) Thermospray mass spectrum of inosine showing  $BH_2^+$  and  $BH\cdot NH_4^+$  isotope clusters. The peaks at  $m/z$  171–173 are labeled  $(S-H)\cdot H_2O\cdot NH_4^+$  ions.

[U- $^{13}C$ ]A-dsRNA in the presence of approximately 42%  $H_2^{18}O$  (by volume) and analyzed by LC/MS as before. The results of the negative control (data not shown) were similar to the negative control experiment shown in Figure 2. The results of the LC/MS analysis of the modification reaction in the presence of  $H_2^{18}O$  are shown in Figure 4. The chromatogram (Figure 4A) and the adenosine mass spectrum (Figure 4B) showed no significant differences from the modification experiment in Figure 3 (panels A and B). The inosine mass spectrum showed a new set of abundant ions at  $m/z$  144 and 161. These corresponded to inosine base ions,  $BH_2^+$  ( $m/z$  144) and  $BH\cdot NH_4^+$  ( $m/z$  161) with five  $^{13}C$  and one  $^{18}O$  atom incorporated. These results indicated that water acts as an efficient oxygen donor in vitro.

**Coformycin Does Not Inhibit the Unwinding/Modifying Activity.** The retention of all the  $^{13}C$  atoms and the incorporation of  $^{18}O$  into the inosine product rule out base replacement as the mechanism of the unwinding/modifying activity. The results are consistent with the hydrolytic deamination mechanism of adenosine deaminase and AMP

deaminase. Since coformycin and deoxycoformycin inhibit both adenosine deaminase and AMP deaminase, we thought that these transition-state analogues might also inhibit the unwinding/modifying activity. To test this hypothesis, we used [ $^{32}P$ ]A-labeled dsRNA ([ $^{32}P$ ]A-dsRNA) and followed the production of IMP products by thin-layer chromatography (TLC); modification was monitored in the presence and absence of coformycin. Coformycin did not inhibit the unwinding/modifying reaction at early or late time points in the reaction (Figure 5, compare lanes 6 and 7; data not shown). The concentration of coformycin used to try to inhibit the reaction was 12 mM, 6-fold more than the amount used to inhibit the conversion of AMP to IMP by AMP deaminase (Figure 5, compare lanes 2 and 3). Previous studies have indicated the stereochemistry at the hydroxyl-bearing C8 position of coformycin and deoxycoformycin is critical for their function as inhibitors; the 8R isomer binds about  $10^7$  times more tightly than the 8S isomer (Schramm & Baker, 1985). The coformycin used in the experiment shown in Figure 5 was the 8R isomer. However, reasoning that the dsRNA un-

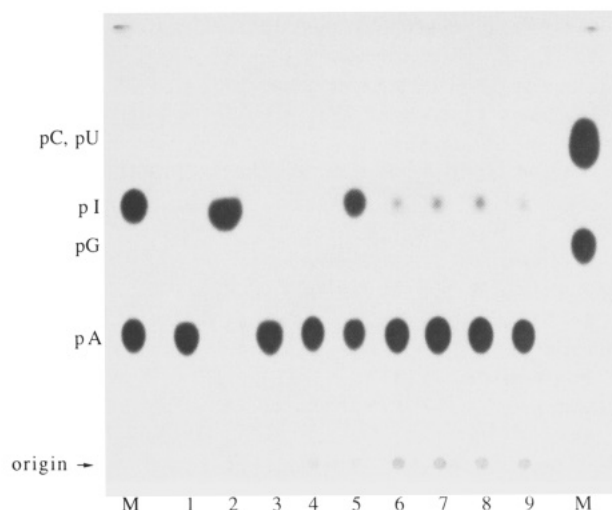


FIGURE 5: Test of possible inhibitors of the unwinding/modifying activity. Unwinding/modifying reactions were carried out on either [ $^{32}$ P]5'-AMP or double-stranded RNA internally labeled with [ $^{32}$ P]A ([ $^{32}$ P]A-dsRNA). Some of the reactions were spiked with AMP deaminase. The reaction times and inhibitor concentrations are given in the individual lane descriptions. Reactions were deproteinized and digested to the 5'-mononucleotides (if necessary) as described under Materials and Methods. Mononucleotide products were separated by TLC and visualized by autoradiography. Lane 1, 2-h modification reaction using 5'-AMP as substrate. Lane 2, 2-h modification reaction using 5'-AMP as substrate with the addition of AMP deaminase. Lane 3, same reaction as lane 2 but with 2 mM coformycin. Lane 4, 2-h incubation of AMP deaminase in a heat-inactivated extract using [ $^{32}$ P]A-dsRNA as substrate. Lane 5, 2-h modification reaction using [ $^{32}$ P]A-dsRNA as substrate. Lane 6, 12-min modification reaction using [ $^{32}$ P]A-dsRNA as substrate. Lane 7, same as lane 6 but with 12 mM coformycin. Lane 8, same as lane 6 but with 12 mM inosine. Lane 9, same as lane 6 but with 12 mM adenosine. Lanes marked with an M are marker lanes. The mobilities of all mononucleotides are indicated to the left of the autoradiogram.

winding/modifying activity may exhibit a different stereoselectivity, we also tested the ability of 8(*S*)-deoxycoformycin to inhibit the modification reaction (data not shown). Similar to the results with the R isomer, we found that 8(*S*)-deoxycoformycin (6 mM) did not inhibit the reaction.

Since inosine and adenosine in the context of dsRNA are the substrate and product of the modification reaction, we also tested the ability of these nucleosides to inhibit the reaction. We found that inosine and adenosine do not inhibit the unwinding/modifying activity even at high concentrations (Figure 5, lanes 8 and 9). Figure 5 also shows that AMP deaminase does not convert adenosine to inosine within dsRNA (lane 4) and that the unwinding/modifying extracts do not have any AMP deaminase activity (lane 1). We conclude that coformycin, adenosine, and inosine are not inhibitors of the unwinding/modifying activity.

## DISCUSSION

We have used directly combined high-performance liquid chromatography-mass spectrometry to examine the mechanism of the dsRNA unwinding/modifying activity. A dsRNA substrate that contained adenosines uniformly labeled with  $^{13}\text{C}$  was prepared, and the carbon atoms were monitored with LC/MS. The mass spectrometric analysis verified that the modified base was inosine, as suggested by previous chromatographic analyses (Bass & Weintraub, 1988; Wagner et al., 1989). Most importantly, we observed that all 10 carbon atoms are retained during the conversion of adenosine to inosine by the unwinding/modifying activity. By performing the modification reaction in the presence of  $\text{H}_2^{18}\text{O}$ , we further demonstrated that water is the oxygen donor in vitro. To-

gether, these results exclude a base replacement mechanism and suggest the reaction occurs by a hydrolytic deamination mechanism, similar to the reactions catalyzed by adenosine deaminase and AMP deaminase.

Despite the apparent similarity of the mechanism to that of adenosine deaminase and related enzymes, we find that the transition-state analogue coformycin, which inhibits these enzymes, does not inhibit the dsRNA unwinding/modifying activity. This result may simply reflect the different substrate specificities of the enzymes, emphasizing that more than just a nucleoside is required for binding to the active site of the dsRNA unwinding/modifying activity. Alternatively, it is possible that formation, or exposure, of the active site requires a conformational change that is dependent on the initial binding to dsRNA. According to this model, coformycin would not inhibit the dsRNA unwinding/modifying activity because the active site would not exist, or would be masked, until dsRNA was bound.

Of course, it is also possible that coformycin does not inhibit the reaction because the unwinding/modifying activity does not deaminate by the same mechanism as adenosine deaminase. In particular, it should be noted that, although we favor a hydrolytic deamination mechanism, we cannot yet exclude a transamination type mechanism. Enzymes that deaminate by a transaminase reaction mechanism have pyridoxal phosphate as a cofactor (Braunstein, 1973). The pyridoxal phosphate forms a Schiff base with the leaving group nitrogen and serves to activate the carbon-nitrogen bond for subsequent hydrolysis. Since C6 of adenosine has partial double bond character and is sufficiently electrophilic for a direct attack by water, we see no reason for further activation. Furthermore, as yet we see no evidence that catalysis by the unwinding/modifying activity requires a pyridoxal phosphate cofactor. Of course, to rule out a transamination mechanism, we must demonstrate that ammonia is released during the unwinding/modifying reaction. This experiment is not yet feasible and must await the purification of the unwinding/modifying protein(s).

An interesting sidelight to this work is the detection of inosine within the nucleic acid that contaminates our partially purified protein preparations. For several reasons, we believe that this inosine came from RNA rather than from free nucleoside or nucleotide in the extract. First, pseudouridine is created posttranscriptionally (Johnson & Söll, 1970), so the abundance of pseudouridine in the samples indicates that there was a large amount of RNA in the extracts. Second, the unwinding/modifying extracts were exhaustively dialyzed and partially purified by DEAE chromatography, and furthermore, after the modification reactions were performed, the samples were ethanol-precipitated prior to ribonuclease digestion. All of these steps would be expected to remove free nucleosides and nucleotides.

It is generally accepted that among cellular RNAs, only tRNA molecules contain inosine; however, careful analyses have not been performed. As discussed, our samples contain inosine and pseudouridine but none of the other modified nucleotides expected (Buck et al., 1983; Dirheimer, 1983) if tRNA were the source of these modified nucleotides. This raises the exciting possibility that inosine is more common within cellular RNAs than once thought. In fact, it seems possible that a subset of the cellular RNAs contaminating the extracts are substrates for the unwinding/modifying activity in vivo. Alternatively, it is possible that concentration of the unwinding/modifying activity by purification caused cellular RNAs that would not normally be substrates for the activity



to be modified. Regardless, these results provide the first hint that substrates for the unwinding/modifying activity exist in the *Xenopus* egg.

In summary, we believe the unwinding/modifying activity uses a catalytic mechanism that is similar to that of adenosine deaminase and AMP deaminase. Since a great deal is known about the adenosine and AMP deaminases, we should be able to test this hypothesis in future studies. For example, the recent X-ray crystallographic study of adenosine deaminase complexed with a transition-state analogue reveals the presence of a previously undetected zinc cofactor bound at the active site (Wilson et al., 1991; during catalysis by adenosine deaminase, the metal ion is proposed to serve as an electrophile, activating the water for attack at C6. In future studies, it should be straightforward to determine if the unwinding/modifying activity exhibits a similar requirement for a zinc cofactor. It has also been noted that the adenosine deaminases and AMP deaminases share a set of evolutionarily conserved amino acids (Chang et al., 1991). The crystal structure indicates that a subset of these conserved residues is present at the active site and contributes to catalysis (Wilson et al., 1991). Once we have purified the dsRNA unwinding/modifying activity, it will be interesting to see if the amino acid sequence of the protein(s) exhibits the previously noted homologies.

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