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# Activation and Deactivation of a Broad-Spectrum Antiviral Drug by a Single Enzyme: Adenosine Deaminase Catalyzes Two Consecutive Deamination Reactions

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**Ribavirin is an approved broad-spectrum antiviral drug. A liver-targeting prodrug of ribavirin, viramidine, is in clinical trial in an attempt to provide a better therapeutic index. The conversion of viramidine to ribavirin, and of ribavirin to an inactive metabolite through adenosine deaminase, is reported. Kinetic analysis indicates that adenosine deaminase is likely involved in activation of viramidine in vivo, and the process is highly pH sensitive. The differential activities of two consecutive deamination reactions are kinetically studied and interpreted based on adenosine deaminase structural information. A comprehensive understanding of the viramidine and ribavirin deamination mechanism should help in designing better nucleoside therapeutics in the future.**

Ribavirin (1-β-D-ribofuranosyl-1,2,4-triazole-3-carboxamide) is a broad-spectrum antiviral drug (18, 19). Its use in aerosol therapy is approved for the treatment of severe respiratory syncytial virus infection in children (28), and its use in combination therapy with alpha interferon (native or pegylated form) is the standard treatment for chronic hepatitis C virus infection (4, 11). Since its discovery in 1970, significant efforts have been made to elucidate ribavirin's mechanism of action. Current data suggest that it has pleiotropic modes of action. Ribavirin itself can be considered a prodrug that is converted to ribavirin 5′-monophosphate (RMP), ribavirin 5′-diphosphate, and ribavirin 5′-triphosphate (RTP) in vivo through sequential action of three cellular kinases (25). RMP has been shown to inhibit the host inosine monophosphate dehydrogenase, thereby resulting in a decreased intracellular pool of GTP available for viral replication (21). RTP is the major form of metabolites that accumulate in vivo (14). It has been shown to be an effective inhibitor of viral guanylyltransferase and mRNA (guanine-$^{7}$N-)-methyltransferase, causing the synthesis of nascent RNA with abnormal 5′-cap structures, and thus inhibits viral transcription (9). All three phosphate metabolites, RMP, ribavirin 5′-diphosphate, and RTP, have been implicated as direct inhibitors of viral polymerases (15). Recently it was reported that RTP can be incorporated into the viral genome as a mutagen and induces virus error catastrophe (1, 2). On the indirect-mechanism-of-action side, ribavirin has been shown to elicit immune-based antiviral activities through shifting of the host immune response from type 2 to type 1, a pattern of T-cell-mediated immunity that favors elimination of intracellular viral pathogens (22).

For the treatment of chronic hepatitis C virus infection, the dose of ribavirin is limited by the side effect, hemolytic anemia, through the accumulation of RTP in the red blood cells. In an attempt to deliver ribavirin more specifically to the liver and less to the red blood cells to improve the therapeutic ratio, a number of ribavirin derivatives were explored to develop the next generation of broad-spectrum antiviral drugs with higher potency and fewer side effects. One of the interesting compounds is the 3-carboxamidine derivative of ribavirin, viramidine (previously known as ribamidine) (Fig. 1) (27). The in vitro activity of viramidine against RNA viruses is similar to that of ribavirin. Pharmacokinetic studies indicated that viramidine is converted to ribavirin in vivo (C.-C. Lin et al., unpublished data). Since the major site of conversion is believed to be in the liver, viramidine may represent a liver-targeting prodrug of ribavirin with a projected improved toxicity profile. These hypotheses were confirmed in additional animal pharmacokinetic and toxicology studies (Lin et al., unpublished data). Clinical studies on viramidine are ongoing to explore its clinical utility as a next-generation ribavirin. To further characterize the metabolic conversion of viramidine to ribavirin, the enzymatic mechanism of conversion of viramidine to ribavirin in vitro has been studied.

Crystal structural analysis of ribavirin suggested that ribavirin adopts a conformation similar to that of a purine ribonucleoside, with the carbonyl oxygen and amino nitrogen of the 3-carboxamide group arranged spatially similarly to the O-6 and N-1 atoms of inosine and guanosine (16). Conceivably, viramidine may closely resemble the conformation of adenosine, with two nitrogen atoms of the 3-carboxamidine occupying stereochemically the same positions as the 6-amino nitrogen and N-1 atom of adenosine (Fig. 1). For the purine metabolic pathway, adenosine undergoes a hydrolytic deamination to convert to inosine after releasing an ammonia molecule (13). This deamination is catalyzed by an efficient zinc metalloenzyme, adenosine deaminase (ADA) (26). ADA is ubiquitous in virtually all mammalian tissues (23). Conformational eclipsing of 3-carboxamidine of viramidine with the 6-amino of adenosine prompted us to speculate that ADA could catalyze the hydrolytic deamination of viramidine as it does to adenosine. The aim of our study was to test the hy-

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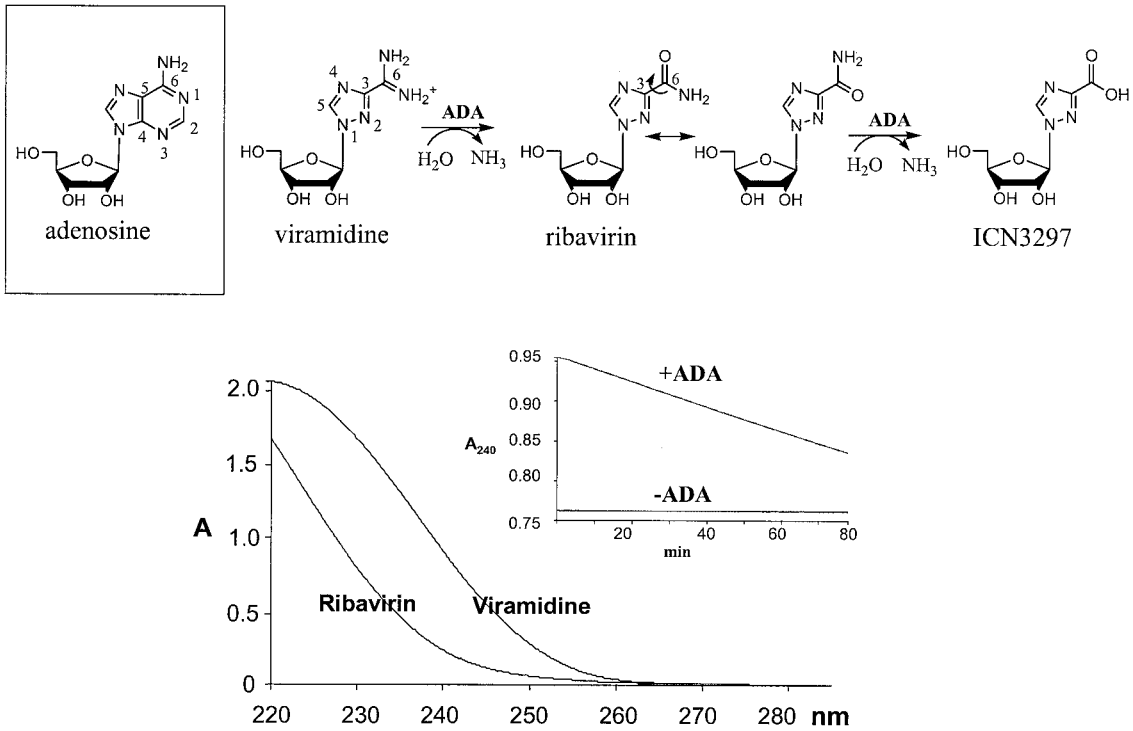


FIG. 1. Adenosine deaminase catalyzes hydrolytic deamination of viramidine to ribavirin, and ribavirin to ICN3297, determined by UV spectral analysis. The difference of absorption at 240 nm from UV spectra of viramidine and ribavirin (0.2 mM) was utilized to monitor the first conversion. The insert shows the time course of conversion of viramidine (0.2 mM) to ribavirin by adenosine deaminase (0.2 μM) and the negative control (no enzyme).

pothesis of ADA-catalyzed conversion of viramidine to ribavirin.

Mammalian ADA is evolutionarily conserved. For example, human (3) and bovine ADA (GenBank accession no. AF280603, submitted by S. J. Kelly and M. S. Hershfield, 2000.) share 88% amino acid sequence identity. Based on the published crystal structure, all the key catalytic residues in the active site are 100% conserved among various mammalian ADA species (26). Human ADA and the enzyme isolated from calf intestine exhibit almost identical $K_m$ and $k_{cat}$ values for adenosine and other nucleoside substrates (20). Using a highly purified mammalian ADA from calf intestine (Roche Bioscience, Indianapolis, Ind.), its steady-state kinetics with adenosine as a substrate was determined. A standard assay was performed at 23°C in Dulbecco's phosphate-buffered saline (PBS) buffer (pH 7.3) with the ADA concentration fixed at 2 nM. Initial velocities at various concentrations of adenosine were determined by following the conversion to inosine at 265 nm on a Perkin-Elmer $\lambda_{40}$ UV/VIS (visible) spectrophotometer. By plotting the initial velocities against adenosine concentrations and fitting the data into the Michaelis-Menten equation, $k_{cat}$ (180 $s^{-1}$) and $K_m$ (28 μM) values were determined. The enzyme catalytic efficiency ($k_{cat}/K_m$) was 6,400 $mM^{-1}$ $s^{-1}$ (Table 1). The kinetic parameters are in good agreement with those reported in the literature (6).

To test ADA's ability to convert viramidine to ribavirin, a similar spectroscopic method was developed. Analysis of UV spectra of viramidine and ribavirin indicated that there is a significant absorption difference at 240 nm between the two compounds (Fig. 1), suggesting that the change in UV absorption can be used to follow the deamination of viramidine to ribavirin. Mixing viramidine with ADA resulted in a linear

TABLE 1. Steady-state kinetic parameters of deamination of three nucleoside substrates by adenosine deaminase[a]

| Substrate | Wavelength[b] (nm) | Extinction coefficient ($mM^{-1}$ $cm^{-1}$) | $K_m$ (mM) | $k_{cat}$ ($s^{-1}$) | Catalytic efficiency ($mM^{-1}$ $s^{-1}$) | Relative activity |
|---|---|---|---|---|---|---|
| Adenosine | 265 | 14 | 0.028 ± 0.001 | 180 ± 10 | 6,400 | $1.7 \times 10^5$ |
| Viramidine | 240 | 3.9 | NA | 1.9 ± 0.5 | 0.19 | 5 |
| Viramidine (pH = 9) | 240 | 3.9 | 0.46 ± 0.08 | 28 ± 1 | 61 | 1,600 |
| Ribavirin | 230 | 3.6 | NA | 0.26 ± 0.02 | 0.037 | 1 |

[a] The kinetic parameters were determined in PBS buffer (pH 7.3) except for the second set of data for viramidine, which was determined in phosphate buffer (pH 9.0). The resultant parameters and standard deviations are based on at least two sets of data. $K_m$ values for viramidine and ribavirin at pH 7.3 cannot be accurately determined due to the limit of substrate concentration in the assay to keep the absorption in the linear range. Catalytic efficiency is estimated based on at least two sets of data.

[b] Wavelength to monitor the depletion of substrate and formation of product in the assay.

decrease of absorption at 240 nm, indicating that ADA catalyzed the hydrolytic deamination of viramidine (Fig. 1). No deamination was observed in the absence of ADA or in the presence of 1 nM deoxycoformycin (Calbiochem, La Jolla, Calif.), a potent and highly specific inhibitor for ADA (data not shown). Further analysis of the reaction products by mass spectroscopy confirmed ribavirin as the major reaction product (data not shown). Steady-state kinetic analysis was conducted to determine the reaction parameters. However, an accurate $K_m$ value was elusive due to the highest concentration of viramidine that we could use in the assay to keep the compound's UV absorption in the linear range. Available data points gave an estimated $k_{cat}$ of 1.9 $s^{-1}$ and the estimated catalytic efficiency of 0.19 $mM^{-1}s^{-1}$ (Table 1). In spite of the high $K_m$ value, reasonable turnover was observed at lower concentration of viramidine due to the high catalytic constant. For example, at a 100 μM concentration of viramidine and a 1 μM concentration of ADA, the observed rate constant was 1.3 $min^{-1}$, suggesting that the high $K_m$ value should not be a hurdle for ADA-catalyzed viramidine deamination. Considering that the kinetic analysis was conducted at 23°C, the enzyme catalytic efficiency would be increased by three- to fourfold at the physiological temperature (37°C). It would be in the same magnitude as that for ADA-catalyzed conversion of an antiherpetic prodrug, 2,6-diamino-9-(2-hydroxyethoxymethyl)purine, to acyclovir (20).

The spontaneous hydrolytic deamination of viramidine to ribavirin was investigated. To quantitatively assess this tendency, viramidine (0.3 mM) was dissolved in Dulbecco's PBS buffer and sealed in a quartz cuvette. The nonenzymatic deamination reaction was monitored by a UV/VIS spectrophotometer following the disappearance of viramidine at 240 nm in a period of 34 days (data not shown). The second-order rate constant ($k$) was calculated with the water concentration fixed at 55 M. It was $1.2 \times 10^{-9}$ $s^{-1} \cdot M^{-1}$ from the average of two sets of data. From the relationship $t_{1/2} = 0.693/k$, the half-life ($t_{1/2}$) of viramidine in PBS was determined to be about 140 days. This result indicates that spontaneous hydrolysis of viramidine in solution is minimal. The half-life of viramidine is of the same magnitude as those for adenosine and cytidine (7). ADA enhances the rate of viramidine deamination by a factor of $10^7$-fold.

Ribavirin has been reported to undergo hydrolytic deamination in vivo to convert to a carboxylic derivative, ICN3297, a metabolite that does not possess any antiviral activity (Fig. 1) (12). Analysis of ribavirin's crystal structure showed that there are two ribavirin molecules in a crystal unit, and in both molecules the 3-carboxamide oxygen is *trans* to the glycosidic bond (16). A 180° rotation of the C-3–C-6 carboxamide bond will move the amino group to the *trans* position and pose it at the same position as the 6-amino of adenosine (Fig. 1). Potentially, ADA could catalyze another round of deamination to convert ribavirin to ICN3297. To investigate this possibility, a UV spectroscopic method was developed to monitor the conversion at 230 nm where there is a significant difference in absorption between the two compounds (data not shown). By mixing a 1 μM concentration of ADA with ribavirin (0.2 mM), a slow conversion was observed. Further steady-state kinetic analysis by varying the ribavirin concentration resulted in an estimated $k_{cat}$ of 0.26 $s^{-1}$. An accurate determination of $K_m$ was elusive due to the limit of ribavirin concentration in the assay in order to keep the absorption in the linear range. The estimated catalytic efficiency was 0.037 $mM^{-1}$ $s^{-1}$, which is about fivefold lower than that with viramidine and is $1.7 \times 10^5$-fold less than that with the native substrate adenosine (Table 1). Nevertheless, the results indicate that ADA is able to slowly convert ribavirin to ICN3297.

ADA is believed to catalyze adenosine deamination with an addition-elimination or $S_N2$ mechanism. In this reaction, active site residue Asp295 acts as a general base for deprotonation of a water molecule (Fig. 2). This water molecule is further activated by the active site-bound zinc ion to undergo a nucleophilic attack of C-6 of adenosine to form a tetrahedral intermediate with N-1 of adenosine, simultaneously accepting a proton from Glu217 (26). Collapse of the tetrahedral intermediate eliminates the ammonium molecule and results in the formation of inosine. Similar deamination mechanisms for viramidine and ribavirin are proposed in Fig. 2. It has been reported that the hydrogen bond between N-3 of adenosine and the backbone amide of Gly184 in ADA is critical for substrate recognition (10, 26). Both viramidine and ribavirin have a triazole base and lack the pyrimidine ring of adenosine. The absence of the N-3–Gly184 interaction likely causes weak substrate binding, resulting in high $K_m$ values for viramidine and ribavirin. Moreover, free rotation of the C-3–C-6 bond in viramidine and ribavirin, a single bond with some double-bond features, may contribute to the weak substrate binding and lower catalytic efficiency. Evidently the estimated $k_{cat}$ values for viramidine and ribavirin are about 100-fold lower than that for adenosine.

Steady-state kinetic analysis indicates that viramidine is a better substrate than ribavirin, with an approximately sevenfold-higher $k_{cat}$. Structurally, viramidine and ribavirin are almost identical except that viramidine has an imino group in the place of a carbonyl group of ribavirin (Fig. 1). It has been reported that 1-deazaadenosine is a potent inhibitor but cannot be deaminated by ADA (10), implying that N-1 of adenosine is critical for catalysis. While viramidine has equivalent nitrogen at the N-1 position, ribavirin has carbonyl oxygen there. In the cocrystal structure of ADA with purine riboside (26), N-1 of adenosine forms a hydrogen bond with the side chain of Glu217. A similar O-H···N hydrogen bond could be formed between viramidine and Glu217 (Fig. 2A). However, for ribavirin, an O-H···O hydrogen bond between the carbonyl oxygen and Glu217 replaces the O-H···N hydrogen bond as shown in Fig. 2B. During the deamination of ribavirin, formation of the tetrahedral intermediate could result in a negative charge on the carbonyl oxygen. This additional negative charge may interact with the vicinal zinc ion and His238 and alter the local conformation of the active site. This subtle change during the catalysis may be responsible for a lower $k_{cat}$ value for ribavirin compared to viramidine.

Viramidine was titrated with 0.1 N HCl or NaOH using a Mettler DL21 titrator (Mettler-Toledo-AG, Im Langacher, Switzerland) to determine the $pK_a$ of the functional groups. Two $pK_a$s, of 5.4 and 10.5, were observed and assigned to the triazole ring and carboxamidine, respectively. To explore the effect of protonated carboxamidine on deamination activity, a pH-rate profile of ADA using adenosine, viramidine, or ribavirin as a substrate was needed. The studies were conducted in

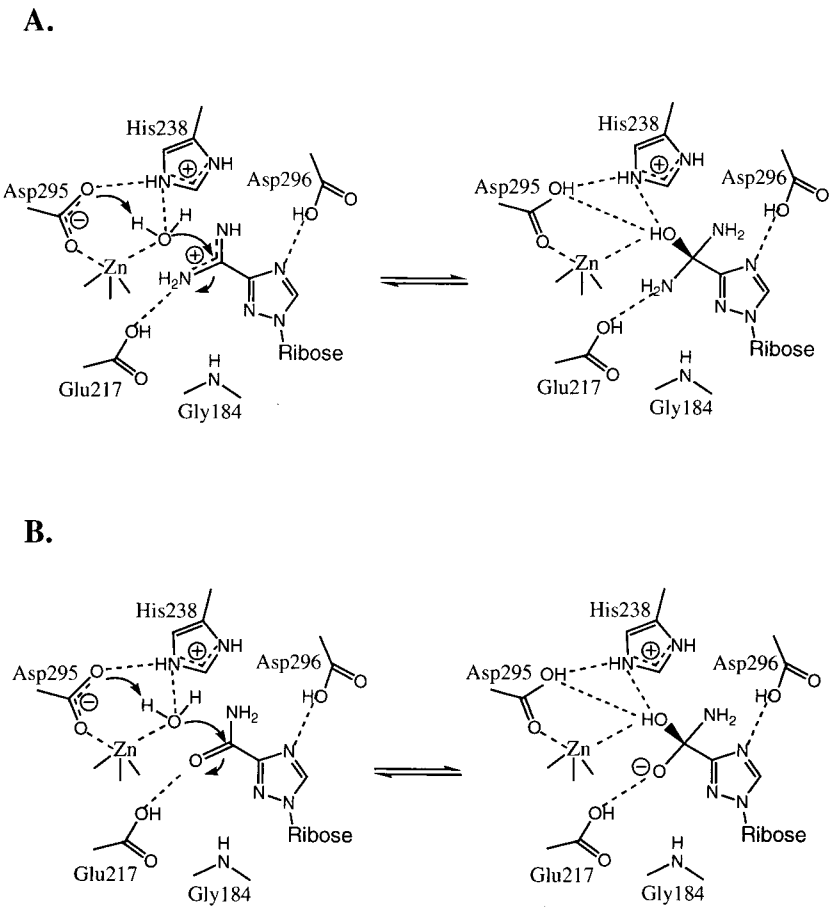


FIG. 2. Proposed deamination reaction mechanism of viramidine and ribavirin by adenosine deaminase. The interaction map between the ADA active site and viramidine (A) or ribavirin (B) is based on the cocrystal structure of adenosine deaminase with purine ribonucleoside (26).

a phosphate buffer (50 mM) and 100 mM NaCl. The pHs of the respective buffers were adjusted to 4.0, 5.0, 6.0, 7.0, 8.0, 9.0, 9.5, 10.0, 10.5 and 11.0. The high Michaelis constant for viramidine and ribavirin prevented accurate determination of the $k_{cat}$ and $K_m$ values at each pH value. Therefore, we simply measured the initial velocity at each pH, with the substrate concentration fixed at half of the estimated $K_m$. The bell-shaped pH-rate curves were fitted to the equation shown below to determine the apparent $pK_a$ (acidic side) and $pK_b$ (basic side) values.

$$v' = v/[1 + 10^{(pH-pKa)} + 10^{(pKb-pH)}]$$

The stability of ADA activity over the pH range was investigated. In the assay, ADA was found to be stable for most of the pH values with which the studies were conducted. However, protein denaturation was observed after 5 min for pH values above 10. Thus, the linear activity-time course in the first 2 min was utilized to determine initial velocity ($v_i$).

As shown in Fig. 3, the pH profiles for both adenosine and ribavirin were bell shaped, with the apparent $pK_a$ values of 5.6 and 5.9 and $pK_b$ values of 8.3 and 8.4, respectively. The results are consistent with those reported for adenosine (17, 24), implying that changing the substrate from adenosine to ribavirin does not alter the catalytic reaction mechanism, nor does it change the pH profile. Interestingly, the pH-$v_i$ profile for viramidine, another triazole nucleoside, is different. The initial velocity went higher with the increase of pH, and it reached maximum around pH 10 (Fig. 3). A further increase of pH from 10.0 resulted in a lower level of enzyme activity, which is likely due to the rapid denaturation of the protein. The optimal pH for deamination of viramidine coincides with the $pK_a$ value

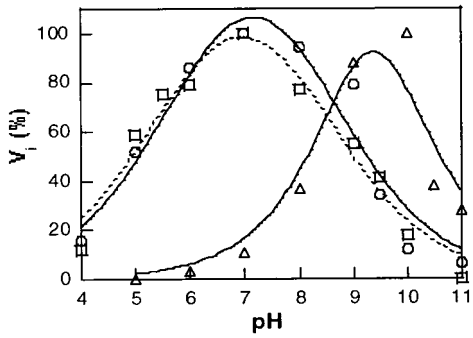


FIG. 3. The pH-rate profiles of adenosine deaminase with adenosine (□), viramidine (△), or ribavirin (○) as a substrate. The experiments were conducted in a phosphate buffer. The initial velocities at various pH values were fitted to the equation given in the text. One representative set of experimental data is shown here.

of the carboxamidine, which is 10.5. This result suggests that deprotonation of viramidine is responsible for the unusually high optimal pH of 10.0. Apparently ADA prefers the deprotonated form of viramidine as a substrate. Further steady-state kinetic analysis of the viramidine deamination reaction at pH 9.0 showed that the $K_m$ value was 0.46 mM and the enzyme turnover number increased to 26 $s^{-1}$ (Table 1). This result represents a 320-fold enhancement of catalytic efficiency ($k_{cat}/K_m$) compared to the reaction at pH 7.3, which is only 100-fold less active than that with native substrate adenosine. It further illustrates that the positive charge on the carboxamidine interferes with both catalysis ($k_{cat}$) and substrate binding ($K_m$). From the proposed model on Fig. 2A, the charge-charge repulsion among the protonated carboxamidine, the active site zinc ion, and the His238 side chain may cause a subtle change of the active site at the ground state and make it suboptimal for substrate binding and catalysis. These pH-rate studies suggest that viramidine would not be converted to ribavirin efficiently by ADA in an acidic environment such as the stomach, where the level of ADA specific activity is one of the highest in human tissues (23). But it would be more readily converted in the lower intestines, where the environment is more basic, with a pH value around 8.0.

It is rare for an enzyme to be demonstrated to catalyze two consecutive reactions on a single substrate. Notably, for carbohydrate metabolism, several hexose dehydrogenases, including dTDP galactose 6-dehydrogenase, GDP-mannose 6-dehydrogenase, and UDP-D-glucose dehydrogenase, convert sugars to the carboxylic acid in two consecutive oxidations. In the metabolism of amino acids, anthranilate 1,2-dioxygenase catalyzes the sequential deamination and decarboxylation to convert anthranilate to catechol. But to our knowledge, ADA may be the only known enzyme that has been shown to catalyze two consecutive deamination reactions on a single substrate. The capability to perform consecutive deamination reactions lies partially in the catalytic power and loose control of substrate specificity of adenosine deaminase. It also depends on the availability of two identically reactive amino/imino groups on viramidine. The consecutive deamination is likely to follow a dissociative mechanism, since the accumulation of ribavirin was observed in vitro as determined by mass spectral analysis.

The enzyme-activated prodrug approach is a strategy that has been successfully employed to improve physicochemical and pharmacokinetic properties of potential therapeutic agents. The ubiquity and abundance of ADA in human tissues makes it ideal for activation of nucleoside prodrugs (23). Indeed, several ADA-activated 6-amino purine nucleoside prodrugs have been reported. 2,6-Diamino-9-(2-hydroxyethoxymethyl)-purine was reported as a prodrug for better absorption through the gut and higher plasma levels of antiherpetic acyclovir (20). A 6-amino derivative of F-dideoxyinosine (F-ddI) was prepared as a more acid-stable prodrug for central nervous system delivery (5). 2,6-Diaminopurine dioxolane is currently in phase I and II clinical trials as a potentially more aqueous soluble and bioavailable prodrug of guanine dioxolane nucleoside for treatment of HIV infection (8). 6-Aminocarbovir was designed for enhanced systemic and central nervous system delivery (29). Viramidine is the first example of an ADA-activated nucleoside prodrug that lacks the canonical purine base.

In summary, our data demonstrate that adenosine deaminase is able to catalyze the conversion of viramidine to ribavirin and ribavirin to an inactive metabolite, ICN3297. The first deamination reaction is highly pH sensitive and relatively efficient, whereas the second one is slower. From the kinetic analysis, it is predicted that ribavirin, the active metabolite of viramidine, will accumulate before being metabolized to the inactive metabolite. It is remarkable that a single enzyme can activate an antiviral prodrug and then slowly deactivate it. Based on the in vitro catalytic efficiency data and the existence of abundant ADA in virtually all mammalian tissues, adenosine deaminase likely contributes to the conversion of viramidine to ribavirin in vivo. However, we cannot rule out the possibility of additional enzymes, such as AMP deaminase, being involved in the deamination. Further in vivo experiments are required to ultimately determine the exact role that adenosine deaminase plays in metabolizing viramidine. From the pH profile studies, we discovered that the deamination of viramidine has an unusually high optimal pH that is related to the deprotonation of viramidine. The viramidine deamination is highly pH sensitive, suggesting that the deamination rates may vary in different human tissues and compartments. The striking differences in catalytic efficiency between adenosine and viramidine or ribavirin are likely due to the lack of a pyrimidine ring of the latter nucleosides. Unfavorable electrostatic interaction between the enzyme active site and the substrate in the ground state (viramidine) as well as in the tetrahedral intermediate (ribavirin) may contribute to the lower catalytic efficiency. A comprehensive understanding of the viramidine metabolism and enzymatic mechanism should help in designing better prodrugs of nucleoside therapeutics in the future.

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