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Metabolic transformation of $AZTp_4A$ by Ap_4A hydrolase regenerates AZT triphosphate

Wayne H. Pitcher III, Thomas W. Kirby, Eugene F. DeRose, Robert E. London*

Department of Health and Human Services, Laboratory of Structural Biology, MR-01, National Institute of Environmental Health Sciences, P.O. Box 12233, Research Triangle Park, NC 27709, USA

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Abstract

The reverse transcriptase (RT) of HIV which has been inhibited by the incorporation of AZT into the primer strand is subject to a deblocking reaction by cellular ATP. This reaction yields unblocked primer plus the dinucleoside tetraphosphate, $AZTp_4A$. In the present study, we report that $AZTp_4A$ is an excellent substrate for the enzyme Ap_4A hydrolase (asymmetrical dinucleoside tetraphosphatase, EC 3.6.1.17), an enzyme that is widely distributed in many cell types. Progress of the reaction has been monitored by ^{31}P NMR, and it was found that hydrolysis results in the production of AZTTP:ATP in a 7:1 ratio. The $AZTp_4A$ was also hydrolyzed at a rate 1.8-fold more rapidly than Ap_4A. Spectrophotometric assays yielded Michaelis constants of 2.35 and 0.71 μM for Ap_4A and $AZTp_4A$, respectively. It, therefore, appears that Ap_4A hydrolase can play a useful role in the regeneration of the AZTTP, the active form of AZT, for the inhibition of HIV RT.
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Keywords: Ap_4A hydrolase; HIV; Reverse transcriptase; $AZTp_4A$; AZT; ^{31}P NMR

1. Introduction

Nucleoside analogs that inhibit HIV reverse transcriptase by acting as chain terminators represent a mainstay of clinically approved HIV therapy (Furman et al., 1986; Sandberg and Slikker, 1995). Unblocking of the terminated primer strand can theoretically result from reversal of the polymerase reaction to yield the nucleoside analog triphosphate. However, this reaction requires pyrophosphate, which is typically present at low concentrations due to its rapid intracellular degradation by alkaline phosphatase (Yoza et al., 1997). It has recently been noted that ATP, acting as AMP-tagged pyrophosphate, can support this reverse reaction, yielding a dinucleoside tetraphosphate (Meyer et al., 1998, 1999). Further, strains of HIV expressing mutant reverse transcriptase that facilitate the reverse ATP-dependent unblocking reaction have been identified (Arion et al., 1998; Meyer et al., 1999). In order to understand the possible significance of this unblocking reaction, it becomes necessary to consider the further metabolism of the dinucleoside tetraphosphate. The enzyme Ap_4A hydrolase (asymmetrical dinucleoside tetraphosphatase, EC 3.6.1.17), widely distributed in many cell types, acts as an asymmetric phosphatase to produce a mixture of NMP and NTP from dinucleoside tetraphosphate substrates. It has been demonstrated that di-2′,3′-dideoxynucleoside tetraphosphates ($ddNp_4ddN$) are substrates for this enzyme, although the hydrolytic reaction proceeds approximately five-fold more slowly than the corresponding hydrolysis of Ap_4A (Gunther Sillero et al., 1997). Thus, it appears that this enzyme could play a role in the regeneration of charged nucleoside analogs for further reaction with the primer strand. In the present study, we have performed ^{31}P NMR analyses of the metabolism of $AZTp_4A$ by the human hydrolase. The primary product of this reaction is AZTTP, indicating that this conversion could contribute to the

Abbreviations: AMP, adenosine 5′-monophosphate; Ap_4A, diadenosine 5′,5‴-P^1,P^4-tetraphosphate or adenosine(5′)tetraphospho(5′)adenosine; ATP, adenosine 5′-triphosphate; AZT, 3′-azido-3′-deoxythymidine; AZT-MP, 3′-azido-3′-deoxythymidine 5′-monophosphate; $AZTp_4A$, 3′-azido-3′-deoxythymidine(5′)tetraphospho(5′)-adenosine; AZTTP, 3′-azido-3′-deoxythymidine 5′-triphosphate; ddAMP, 2′,3′-dideoxyadenosine 5′-monophosphate; $ddAp_4A$, 2′,3′-dideoxyadenosine(5′)tetraphospho(5′)-adenosine; ddATP, 2′,3′-dideoxyadenosine 5′-triphosphate; HIV, human immunodeficiency virus; NMP, nucleoside 5′-monophosphate; NMR, nuclear magnetic resonance; NOE, nuclear OVERHAUSER enhancement; Np_4N', various dinucleoside tetraphosphates; NTP, nucleoside 5′-triphosphate; RT, reverse transcriptase; Up_4U, diuridine 5′,5‴-P^1,P^4-tetraphosphate

* Corresponding author. Tel.: +1-919-541-4879; fax: +1-919-541-5707.
E-mail address: london@niehs.nih.gov (R.E. London).

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regeneration of AZTTP and hence to the reblocking of the primer strand.

2. Materials and methods

2.1. Preparation of human Ap_4A hydrolase

The cDNA for the human Ap_4A hydrolase gene was subcloned from IMAGE clone 104884 (Research Genetics, Inc.). The following PCR primers were used to generate a DNA fragment with an NdeI restriction site at the start of the coding sequence and an EcoRI restriction site after the stop codon: 5′-GGGCATATGGCCTTGAGAGCATGTGG-3′; 5′-GGGAATTCAGGCCTCTATGGAGCAAAG-3′. The PCR product was digested with EcoRI and NdeI and ligated into similarly digested vector DNA, pET30a(+) (Novagen). The resulting plasmid, dubbed pET30AP4AH, allows for expression of Ap_4A hydrolase under the control of the T7 promoter. Thus, the construct was transformed into *Escherichia coli* strain BLR (DE32) (Novagen) and cells containing the plasmid were grown to mid-log phase ($A_{600} =\sim 0.6$) at 37 °C in LB medium containing 50 μg/ml kanamycin. Ap_4A hydrolase expression was induced by addition of isopropyl thio-β-D-galactoside (IPTG) to 1 mM, and the growth was continued for 3 h.

The harvested cells were resuspended in 50 mM Tris–HCl pH 8.0 with protease inhibitors added (10 μl Calbiochem Protease Inhibitor Cocktail Set III per ml of suspended cells), and were lysed by sonication in a Branson Sonifier 200 using a microtip probe at output level of six for 10× 30 s with 30 s cooling in an ice bath. The lysate was centrifuged at 30,000 × *g* for 15 min followed by addition of solid ammonium sulfate to the supernatant to 25% saturation. This was kept on ice 30 min and then centrifuged at 30,000 × *g* for 10 min. The supernatant was brought to 60% saturation with addition of solid ammonium sulfate and chilled on ice for 30 min and centrifuged as before. The 60% ammonium sulfate precipitate, containing Ap_4A hydrolase, was resuspended in a minimal amount of 50 mM Tris–HCl, pH 8.0, and the protein solution was applied to a 2.6 cm × 63 cm column of Sephacryl S-100. Enzyme was eluted with 50 mM Tris–HCl, pH 8.0. Fractions containing Ap_4A hydrolase were identified by SDS–polyacrylamide gel electrophoresis, pooled, and loaded onto an FPLC column (2.6 cm × 15 cm) containing Q-Sepharose (Amersham). The column was washed with 50 mM Tris–HCl, pH 8.0 and Ap_4A hydrolase was eluted with a linear gradient of 0–1000 mM NaCl in 50 mM Tris–HCl, pH 8.0. Fractions containing Ap_4A hydrolase were identified by SDS–polyacrylamide gel electrophoresis, pooled, and concentrated to a volume of 2 ml using a Centricon YM-3 filter unit (Millipore). The protein was chromatographed once again on a 2.6 cm × 63 cm column of Sephacryl S-100 and eluted with 50 mM Tris–HCl, pH 8.0. Fractions containing Ap_4A hydrolase were judged pure by SDS–polyacrylamide gel electrophoresis.

2.2. Preparation of other reagents

Ap_4A, AMP, and ATP were purchased from Sigma and were used without further purification. $AZTp_4A$ (free acid form) was obtained from TriLink Bio Technologies, Inc. (San Diego, CA) and was used without further purification after confirmation of purity by ^1H and ^{31}P NMR spectroscopy. HPLC analysis by TriLink indicated >97% purity. Concentrations of nucleotides and dinucleotides for NMR experiments were typically 5 mM (see Section 3).

2.3. NMR methods

^{31}P NMR spectra were acquired on a Varian Inova 500 NMR spectrometer at 25 °C using a Nalorac Z-SPEC triple resonance (^1H, ^{13}C, ^{31}P) probe operating at a ^{31}P frequency of 202.4 MHz. A one-pulse experiment was used to obtain ^{31}P spectra. Spectra were processed and analyzed with Varian VNMR software. Peaks for hydrolysis products of $AZTp_4A$ were identified by comparison of chemical shifts to those of Ap_4A hydrolysis products, ATP, AMP, and AZTTP. Identity of the AMP peak was confirmed by spiking the sample with authentic AMP and noting the increased intensity of the AMP resonance. Rates of hydrolysis were determined by fitting peak intensities for representative peaks (AMP/AZTMP) to an exponential equation using Kaleidagraph software. Phosphorus-31 NMR spectra were simulated using either an in house Mathematica program, or using the program NMR Simulator, "NMRSM", Revision 1.0 (http://www.chem.vt.edu/chem-dept/hbell/simulation/VTNMR.html) written by H.M. Bell, Virginia Tech, Blacksburg, VA, hbell@chemserver.chem.vt.edu.

2.4. Enzyme kinetics methods

Saturation kinetics of Ap_4A hydrolase were determined by computer fitting of substrate concentration–initial velocity data to the Michaelis–Menten equation. Initial velocities were measured by following the hyperchromicity at 259 nm caused by the hydrolysis of Ap_4A or $AZTp_4A$ (Pinto et al., 1991; Randerath et al., 1966). Hyperchromicity was estimated at 17% for Ap_4A and 5% for $AZTp_4A$. The reaction mixture consisted of 2 nM Ap_4A hydrolase, 10 μg alkaline phosphatase (to insure reaction irreversibility), 10 mM $MgCl_2$, and 50 mM imidazole, pH 7.0. Reaction volume was 1.0 ml. Reactions were initiated by the addition of Ap_4A or $AZTp_4A$ (final concentrations varied from 1 to 20 μM).

3. Results

3.1. Phosphorus-31 NMR characterization

The ^{31}P NMR spectrum of Ap_4A approximates a doublet of doublets, but is more accurately characterized as

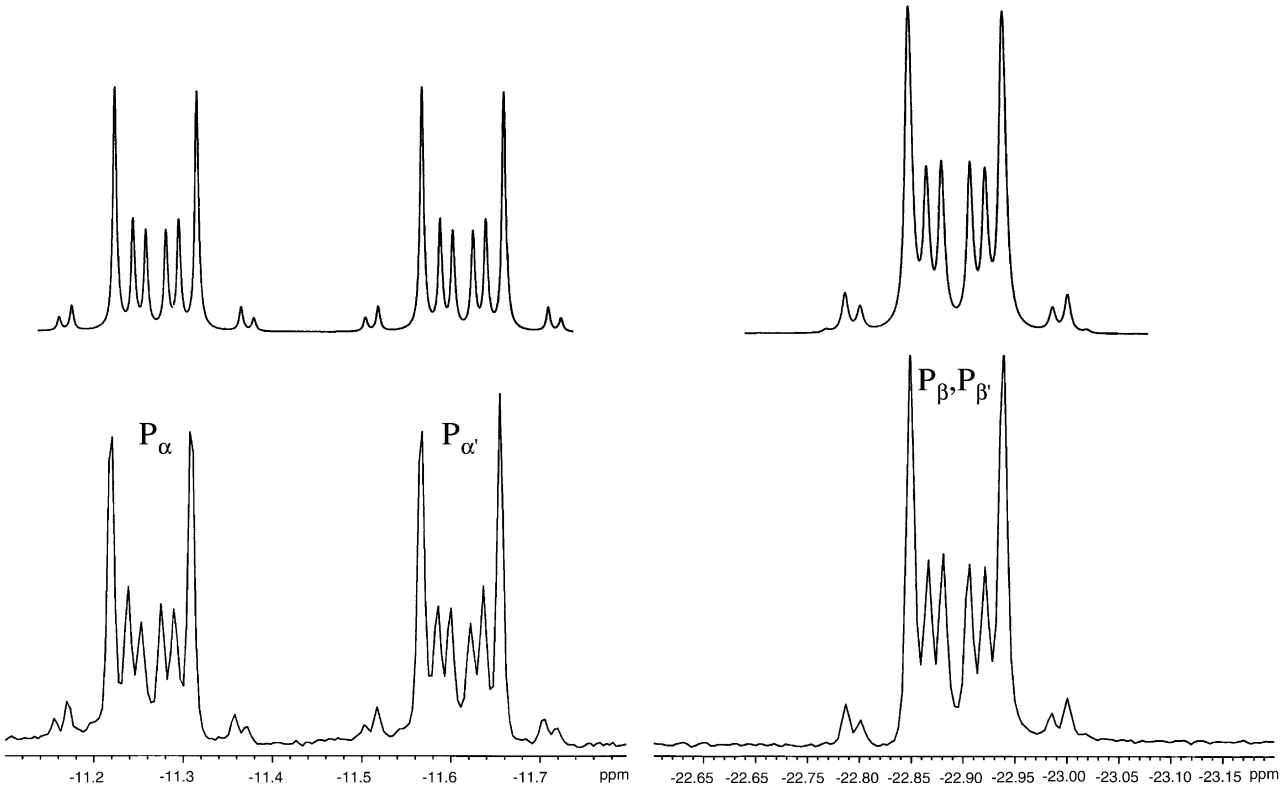


Fig. 1. Lower trace: ^{31}P spectrum of AZTp$_4$A; upper trace: simulated spectrum using the coupling constants given in the text. Sample contained 5 mM AZTp$_4$A in 50 mM imidazole buffer with 10 mM EDTA, pH = 7.0. Spectral parameters: 6000.6 Hz spectral width, 11,986 complex points, 0.999 s acquisition time, 256 scans.

an AA′XX′ spectrum. The α phosphates are centered at −11.3 ppm and the β phosphates at −23.0 ppm (referenced to external phosphoric acid standard). There are several reported NMR studies of Ap$_4$A (Plateau et al., 1981; Kolodny and Collins, 1986; Westkaemper, 1987; Tanner et al., 2002). Plateau et al. reported coupling constants of $^2J_{\alpha\beta} = 16.3$ and $^2J_{\beta\beta} = 13.7$ Hz, measured at pH 7.8 with 5 mM $MgCl_2$ present, while Kolodny and Collins, working at pH 7.0, reported $^2J_{\alpha\beta} = 15.36$ Hz, $^4J_{\alpha\beta'} = 3.02$ Hz, as well as an unexpectedly small coupling $^2J_{\beta\beta} = 10.77$ Hz between the two central β phosphates, and a surprising six bond coupling $^6J_{\alpha\alpha} = 5.73$ Hz between the two α phosphorus nuclei. We are aware of no precedent for such a long range phosphorus coupling, so we have also analyzed the spectrum using the formalism for an AA′XX′ spin system in a Mathematica calculation. The simulation predicts a maximum of 10 resolved resonances for both the AA′ and XX′ nuclei. The spectra for the αα′ and ββ′ nuclei appear identical and were readily fit using coupling constants: $^2J_{\alpha\beta} = 18.2$ Hz, $^2J_{\beta\beta} = 16.4$ Hz, $^4J_{\alpha\beta'} = 0.2$ Hz, and $^6J_{\alpha\alpha} = 0$. The values for these coupling constants are typical of structurally related molecules such as ATP (Gorenstein, 1984; Son et al., 1975). This discrepancy with the previous analysis most likely arises due to the limited signal/noise obtained in the earlier studies.

For AZTp$_4$A all of the ^{31}P resonances are theoretically inequivalent. The spectra were fit using the NMRSM program, with a shift difference of 4.0 Hz for the two β phosphorus nuclei. The observed and simulated ^{31}P spectra are shown in Fig. 1, and correspond to coupling constants: $^2J_{\alpha\beta} = {}^2J_{\alpha'\beta'} = 18.3$ Hz, $^2J_{\beta\beta'} = 16.3$ Hz, $^4J_{\alpha\beta'} = {}^4J_{\alpha\beta'} = 0.4$ Hz, and $^6J_{\alpha\alpha'} = 0$. The error in determination of the peak positions makes the values for the ^4J coupling interactions fairly uncertain.

3.2. Kinetics

After determining a concentration of Ap$_4$A hydrolase that would yield a rate sufficient to allow NMR observations over a period of several hours, ^{31}P studies were performed on both Ap$_4$A and AZTp$_4$A. NMR samples consisted of 5 mM Ap$_4$A or AZTp$_4$A, 100 nM human Ap$_4$A hydrolase, and 10 mM $MgCl_2$ in 50 mM imidazole buffer at pH 7.0 (600 ml total volume). Portions of sample spectra showing the course of AZTp$_4$A hydrolysis by Ap$_4$A hydrolase are shown in Fig. 2. Note the disappearance of the AZTp$_4$A α and β phosphate peaks and the appearance of the AMP and AZTTP phosphate peaks.

Phorphorus-31 NMR spectra of the monophosphate and γ phosphate peaks for the hydrolysis products of both Ap$_4$A and AZTp$_4$A are shown in Fig. 3. The ratio of products formed from AZTp$_4$A is 7:1 AZTTP:ATP (also equal to AMP:AZTMP), obtained by integration of the

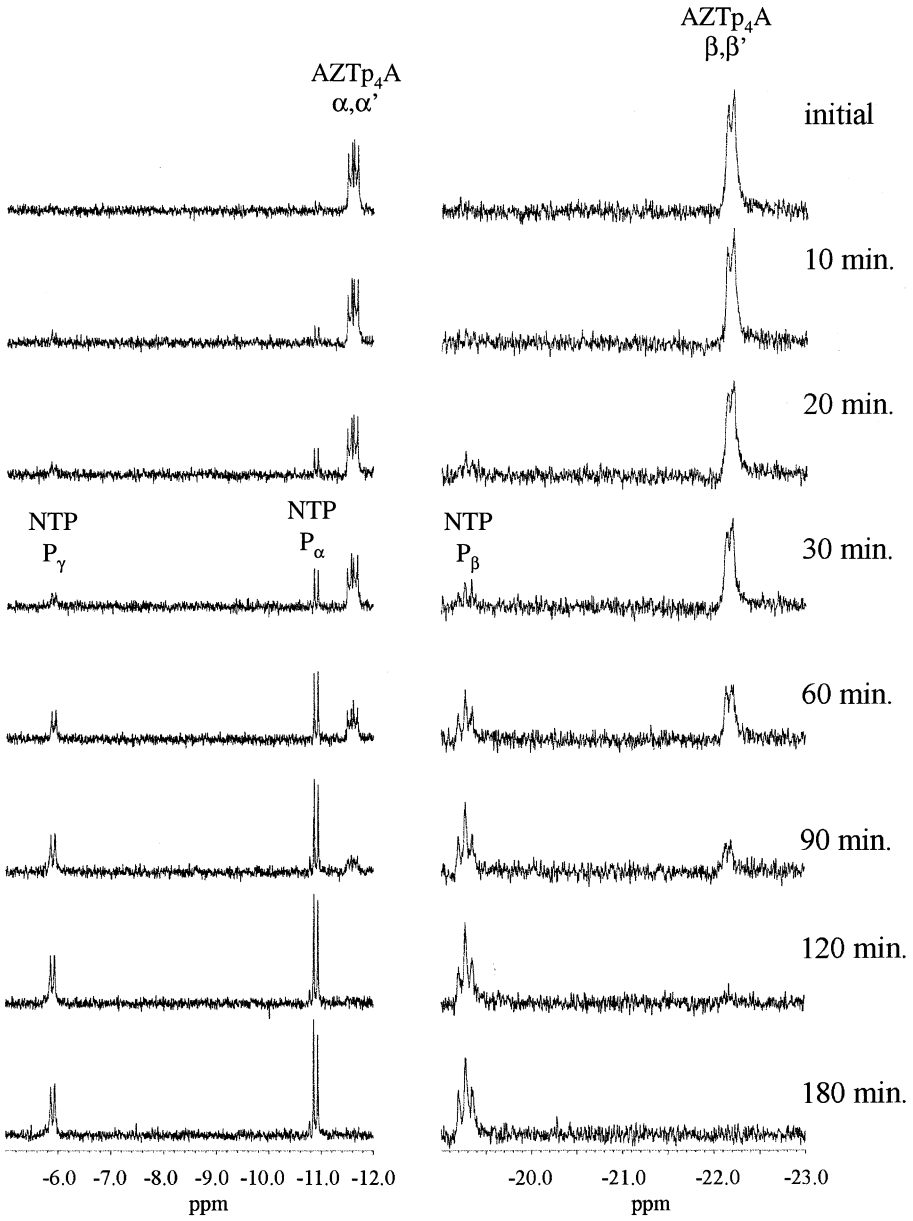
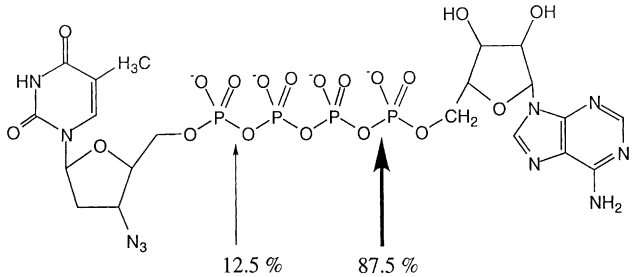


Fig. 2. ^{31}P NMR spectra of a sample initially containing 5 mM AZTp$_4$A, 100 nM Ap$_4$A hydrolase, 10 mM $MgCl_2$ in 50 mM imidazole buffer, pH = 7.0. Time points are as follows, from top to bottom: initial, 10, 20, 30, 60, 90, 120, and 180 min. Left hand panel, P_γ and P_α; right hand panel, P_β. NTP indicates nucleoside triphosphate resonances, which include both ATP and AZTTP peaks.

corresponding resonances:



We note that although the $^{31}P\{^1H\}$ NOEs for the α, β and γ phosphate groups may differ, the comparisons in Fig. 3 involve the α or γ resonances of the two nucleotides, which are expected to exhibit similar relaxation characteristics. A comparison of the rates of hydrolysis of Ap$_4$A and AZTp$_4$A by Ap$_4$A hydrolase under the conditions of the study is shown in Fig. 4. AZTp$_4$A is hydrolyzed 1.57 ± 0.19 times faster than Ap$_4$A. These data are derived from the resonance intensities of the AMP phosphate. Such a comparison neglects the slower rate of hydrolysis yielding ATP and AZTMP. Since under the conditions of the assay, the hydrolysis

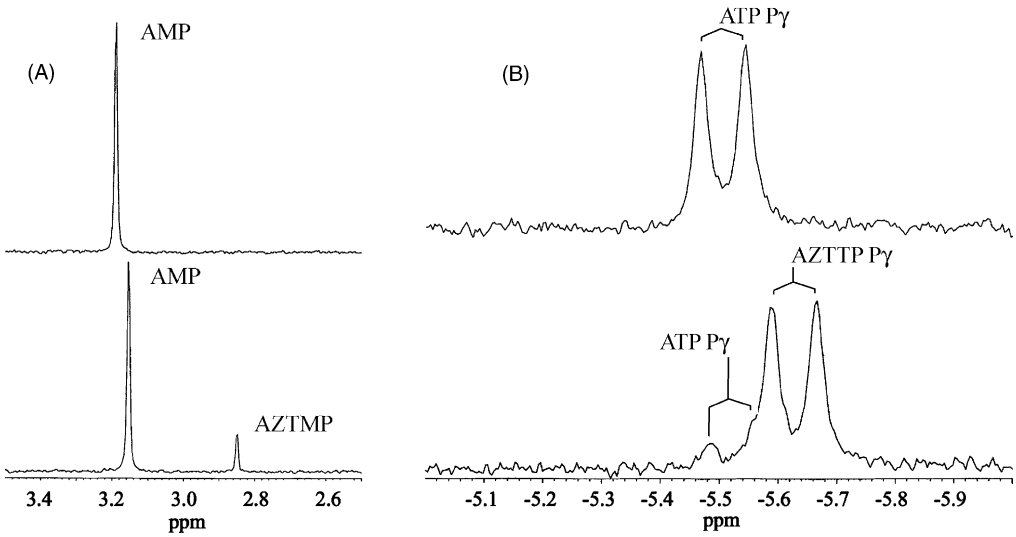


Fig. 3. ^{3}P NMR spectra showing the final hydrolysis products of AZTp$_4$A (lower trace) or Ap$_4$A (upper trace). The regions of the spectrum shown correspond to the nucleoside monophosphate (A) and to the nucleoside triphosphate P_γ (B). The small shift difference between AMP between the two samples arises due to a small pH difference.

reaction yielding AZTMP + ATP is proceeding at 1/7 the rate of the hydrolysis reaction yielding AMP + AZTTP, the total hydrolysis rate including both sets of products proceeds at a rate (8/7)1.57 = 1.79 relative to the hydrolysis of Ap$_4$A.

Assuming simple Michaelis–Menten kinetics, the initial rate of hydrolysis will be given by:

$$v_o = \frac{k_{cat}[E][S]}{K_M + [S]}$$

where E is the enzyme concentration, S the substrate concentration, and K_M the Michaelis constant. The K_M values determined spectrophotometrically were: 2.35 μM for Ap$_4$A and 0.71 μM for AZTp$_4$A. The value for AZTp$_4$A must be considered as a qualitative measure since it is measuring a composite of the two separate hydrolysis reactions of the AZTp$_4$A yielding either AMP and AZTTP or AZTMP and ATP. Further, the maximum absorbance change for complete hydrolysis of AZTp4A was only about 5% compared with a 20% change for hydrolysis of Ap$_4$A, so the accuracy of the measurement is limited. However, these K_M values are qualitatively similar to the values reported for Ap$_4$A hydrolysis by the Ap$_4$A hydrolases from other systems (Guranowski, 2000). For the substrate concentration used in the NMR studies, [S] ≫ K_M, so that $v_o \approx k_{cat}$[E]. Hence, the observed rate variation must arise from a difference in k_{cat} rather than K_M.

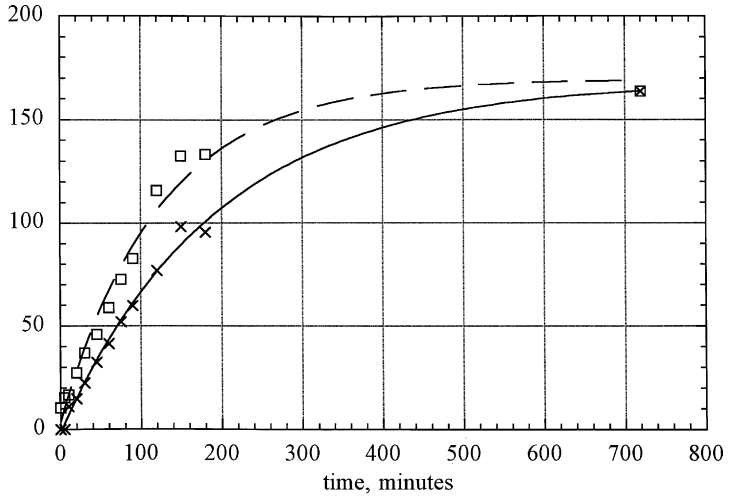


Fig. 4. Time course of Ap$_4$A and AZTp$_4$A hydrolysis by Ap$_4$A hydrolase. Hydrolysis of AZTp$_4$A and Ap$_4$A is measured by the intensity of the AMP peaks (□ and ×, respectively). The small shift difference observed for AMP between the two samples arises due to a small pH difference.

4. Discussion

RT-dependent removal of an AZT blocked primer terminus provides an important mechanism for the evasion of AZT by HIV RT. In support of this conclusion, mutant forms of RT have been identified that exhibit enhanced unblocking activity (Arion et al., 1998; Meyer et al., 1999; Boyer et al., 2001). Since $AZTp_4A$, the product of the unblocking reaction with ATP, is expected to be highly charged at neutral pH, it is unlikely that this compound will exit the cell. Hence, its subsequent fate is relevant to the effectiveness of AZT therapy. Since it has been demonstrated that the Ap_4A hydrolase has limited specificity for the base and for whether the 2′-oxy or deoxy sugar is present, it was anticipated that it might be able to hydrolyze $AZTp_4A$. Further, Ap_4A hydrolase is widely distributed in mammalian tissues, having been identified in Ehrlich ascites tumor cells (Moreno et al., 1982), human leukemia cells (Ogilvie and Antl, 1983), human placenta (Lazewska et al., 1993), human erythrocytes, leukocytes, and platelets (Hankin et al., 1995). Hence, the metabolism of $AZTp_4A$ by Ap_4A hydrolase could influence the effectiveness of AZT therapy. As observed here, $AZTp_4A$ is actually a somewhat better substrate for Ap_4A hydrolase than Ap_4A. Under the conditions of our study, the hydrolysis reaction of $AZTp_4A$ proceeded 1.8-fold faster than the hydrolysis of Ap_4A, and yielded a 7:1 ratio of AZTTP:ATP. This reaction is thus available for the regeneration of AZTTP, which can then serve as a substrate for the reverse transcriptase. Thus, almost 90% of the AZTTP precursor is regenerated at a rate dependent on the enzyme concentration in the cell. The AZTMP also is expected to be subsequently phosphorylated by thymidylate kinase (EC 2.7.4.9) to regenerate AZTTP (Furman et al., 1986). The success of AZT therapy may thus depend in part on the kinetic competition between the unblocking reaction and AZTTP regeneration by Ap_4A hydrolase. The concentrations of AZTTP in SIV-infected or uninfected peripheral blood mononuclear cells were reported to be 74 and 140 nM, respectively, about an order of magnitude lower than the levels of intracellular AZTMP (Qian et al., 1994). Thus, the ability of the enzyme to regenerate AZTTP rather than AZTMP represents a significant kinetic advantage for the reblocking reaction.

Although the structure of the human Ap_4A hydrolase has not yet been determined, the structures of the Ap_4A hydrolase derived from *Lupinus angustifolius* L., both free (Swarbrick et al., 2000) and as an ATP complex (Fletcher et al., 2002) have been determined by NMR spectroscopy. Studies utilizing a broad range of substrates and ^{18}O labeled water have shown that cleavage occurs between the P_γ and P_σ phosphate groups (Guranowski, 2000). Recognition of the Ap_4A substrate by the enzyme might be considered to involve two nucleoside subsites, N1 and N2, and a site that interacts with the bridging polyphosphate chain. Following such a model, binding of a ATP to the N1 subsite would be expected to result in an inert complex, while binding to the N2 subsite would place the α, β and γ phosphate groups in positions corresponding to the σ, γ, and β phosphate groups of the Ap_4A substrate, respectively. Such binding would presumably result in cleavage between P_α and P_β. The absence of any hydrolysis of ATP or other nucleoside triphosphates appears to indicate that the putative site N2 must have negligible affinity for the second nucleoside. This conclusion has been confirmed in the NMR analysis of the Ap_4A hydrolase complexed with ATP–MgF_x, which identified only a single mode of binding. It was thus concluded that only one adenosine interacts significantly with the enzyme, even in the case of diadenosine substrates, and therefore the second adenosine has no significant interactions with the protein (Fletcher et al., 2002). Based on these conclusions, the preferential formation of AZTTP versus ATP and the higher rate of hydrolysis of $AZTp_4A$ relative to Ap_4A indicate that the AZT moiety interacts more strongly with the N1 nucleoside binding subsite of the enzyme than adenosine. This conclusion is also supported by the lower Michaelis constant obtained for $AZTp_4A$ compared with Ap_4A. In the solution structure of the Ap_4A hydrolase–ATP–MgF_x complex, the ribose 2′ and 3′ hydroxyl groups are oriented outward from the binding cleft and have significant solvent exposure. This orientation explains the ability of the enzyme to accommodate bulky groups at the 2′ position, e.g. 2-deoxyadenylated Ap_4A (Guranowski et al., 2000). It appears from the structure of the enzyme–ATP–MgF_x complex noted above that the 3′-azide group should be easily accommodated in the active site. Further, there might be a stabilizing interaction with Trp-91, a residue that is conserved in the human enzyme, following the alignment given by Fletcher et al. (2002).

Hydrolysis of various Np_4N' substrates by most Ap_4A hydrolases indicate that there is relatively little preference for particular bases; for example, Up_4U was almost as good a substrate as Ap_4A (Vallejo et al., 1976). The dideoxy analogs $ddAp_4A$ and $ddAp_4ddA$ were found to undergo hydrolysis with an initial rate of about 20% that of Ap_4A (Gunther Sillero et al., 1997). Surprisingly, although the loss of the 2′ and 3′ hydroxyl groups reduces the hydrolytic rate, hydrolysis of $ddAp_4A$ yielded a product ratio [ddATP + AMP]/[ATP + ddAMP] of ∼3. This result appears to indicate that while the N1 binding site prefers the dideoxy nucleoside, the hydrolysis rate is slower. This combination of factors might indicate that the N2 binding site, while having very low affinity for the nucleoside generally, has still lower affinity for the dideoxynucleoside. Thus, the dideoxy substitution reduces affinity for both nucleoside-binding subsites, but the affinity for the N2 site is reduced to a greater degree than the affinity for N1. This contrasts with the analysis given above for $AZTp_4P$, in which the azido group is proposed to enhance binding to the N1 site.

In conclusion, the regeneration of AZTTP from $AZTp_4A$ may constitute an important mechanism in limiting the unblocking pathway and thus RT evasion of AZTTP. Future therapeutic approaches could account for and take advantage of this reblocking mechanism. The concentrations of

AZTTP derived through this pathway may be small compared with peak levels of AZTTP achieved after uptake and phosphorylation of the nucleoside, but would become more significant over longer time periods as intracellular AZT levels decline. Further, the ability of the Ap_4A hydrolase to regenerate primarily AZTTP, the active form of the intracellular drug, would facilitate the reblocking reaction. Although the exact physiological relevance of these findings is uncertain, previous studies have indicated that intracellular AZT nucleotide levels vary greatly among individuals (Tornevik et al., 1991), and thus, it is possible that there may be cases where the reblocking mechanism is important in cell-based or human systems. Further studies to elucidate the structural basis for the preferential generation of AZTTP from $AZTp_4A$ by the hydrolase are currently in progress.

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