Intracellular Substrates for the Primer-Unblocking Reaction by Human Immunodeficiency Virus Type 1 Reverse Transcriptase: Detection and Quantitation in Extracts from Quiescent- and Activated-Lymphocyte Subpopulations

Anthony J. Smith,¹ Peter R. Meyer,¹ Deshratn Asthana,² Margarita R. Ashman,²
and Walter A. Scott^{1*}

Departments of Biochemistry and Molecular Biology¹ and Psychiatry,² University of Miami School of Medicine, Miami, Florida

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Treatment of human immunodeficiency virus type 1 (HIV-1)-infected patients with 3'-azido-3'-deoxythymidine (AZT) selects for mutant forms of viral reverse transcriptase (RT) with increased ability to remove chain-terminating nucleotides from blocked DNA chains. We tested various cell extracts for the presence of endogenous acceptor substrates for this reaction. Cell extracts incubated with HIV-1 RT and [³²P]ddAMP-terminated DNA primer/template gave rise to ³²P-labeled adenosine 2',3'-dideoxyadenosine 5',5'''-P₁,P₄-tetrphosphate (Ap₄ddA), ddATP, Gp₄ddA, and Ap₃ddA, corresponding to the transfer of [³²P]ddAMP to ATP, PP_i, GTP, and ADP, respectively. Incubation with [³²P]AZT monophosphate (AZTMP)-terminated primer/template gave rise to the analogous ³²P-labeled AZT derivatives. Based on the rates of formation of the specific excision products, ATP and PP_i levels were determined: ATP was present at 1.3 to 2.2 mM in H9 cells, macrophages, and unstimulated CD4⁺ or CD8⁺ T cells, while PP_i was present at 7 to 15 μM. Under these conditions, the ATP-dependent reaction predominated, and excision by the AZT-resistant mutant RT was more efficient than wild type RT. Activated CD4⁺ or CD8⁺ T cells contained 1.4 to 2.7 mM ATP and 55 to 79 μM PP_i. These cellular PP_i concentrations are lower than previously reported; nonetheless, the PP_i-dependent reaction predominated in extracts from activated T cells, and excision by mutant and wild-type RT occurred with similar efficiency. While PP_i-dependent excision may contribute to AZT resistance in vivo, it is likely that selection of AZT-resistant mutants occurs primarily in an environment where the ATP-dependent reaction predominates.

Human immunodeficiency virus type 1 (HIV-1) replication is inhibited by the incorporation of chain-terminating nucleotide analogs at the 3' end of the nascent DNA chain during reverse transcription. Viral replication in the presence of these drugs results in selection of resistance mutations in the viral sequences encoding reverse transcriptase (RT), the replicative enzyme for HIV. Mutations at six codons in RT (M41L, D67N, K70R, L210W, T215Y or -F, and K219Q) are commonly selected by the nucleoside inhibitor 3'-azido-3'-deoxythymidine (AZT) and have been shown to confer AZT resistance through an increased ability to remove the chain terminator after it has been incorporated into DNA (1, 29, 46). These mutations are also correlated with significant resistance to other chain-terminating nucleosides (33, 34, 47).

Excision has been shown to occur by RT-catalyzed transfer of the chain-terminating residue to a variety of acceptor substrates in vitro by a reaction that is related to pyrophosphorolysis (1, 5, 16, 29, 31, 42, 43). The intracellular acceptor for this reaction is unknown, but likely candidates include nucleoside triphosphates and nucleoside diphosphates as well as inorganic pyrophosphate (PP_i).

In this report, we show that cell extracts contain a mixture of

acceptor substrates for the excision reaction. ATP or PP_i predominates depending on the cell type and its activation status. For these experiments, it was necessary to avoid errors in the measurement of PP_i levels in the extracts, which can be affected by a number of factors: the breakdown of unstable intracellular compounds to form PP_i during the extraction procedure, contaminating platelets, and cellular enzymes that alter PP_i levels during the extraction process. Results obtained with an optimized extraction procedure disagree with widely quoted values of 130 to 150 μM for intracellular PP_i concentration in unstimulated lymphocytes (3); our results are more in agreement with the data of De La Rosa et al. (8) that PP_i concentrations are less than 10 μM. We also show that PP_i concentrations increased to 55 to 79 μM in highly stimulated T cells. At this level of PP_i, pyrophosphorolysis predominated and the overall rate of excision was similar for wild-type (WT) and AZT-resistant enzymes. To explain selection of AZT resistance mutations in vivo, it is possible that PP_i-dependent excision is enhanced in the mutant virus but not detected in the in vitro assays. Alternatively, selection may occur in an intracellular environment that favors ATP-dependent excision over pyrophosphorolysis.

Preliminary reports of this work have been presented previously (A. J. Smith, P. R. Meyer, D. Asthana, M. R. Ashman, and W. A. Scott, Abstr. XII Int. HIV Drug Resist. Workshop, abstr. 38, 2003; A. J. Smith, P. R. Meyer, and W. A. Scott, Abstr. XIII Int. HIV Drug Resist. Workshop, abstr. 25, 2004).

* Corresponding author. Mailing address: Department of Biochemistry and Molecular Biology, University of Miami School of Medicine, P.O. Box 016129, Miami, FL 33101-6129. Phone: (305) 243-6359. Fax: (305) 243-3065. E-mail: wscott@med.miami.edu.

MATERIALS AND METHODS

Cells, cell subfractionation, and activation. The H9 T-lymphoid cell line was obtained from the AIDS Research and Reference Reagent Program, National Institutes of Health, and maintained in RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS) (heat-inactivated) and 45 $\mu\text{g}/\text{ml}$ gentamicin. Peripheral blood from healthy donors was obtained from Continental Blood Bank Service (Miami, FL), and the mononuclear cell fraction (PBMC) was isolated by centrifugation through Ficoll/Hypaque. Purified cell subpopulations were positively selected from the PBMC mixture by magnetic separation with anti-CD4-, anti-CD8-, or anti-CD14-conjugated microbeads (MidiMACS; Miltenyi Biotec) to >96% purity as determined by flow cytometry. CD4⁺ and CD8⁺ T cells were either extracted immediately (unstimulated T cell extracts) or suspended at 10^6 cells/ml in RPMI 1640 medium supplemented with 10% FBS, 10 units/ml interleukin 2 (Roche Diagnostics), 2 $\mu\text{g}/\text{ml}$ phytohemagglutinin (GIBCO BRL), and 45 $\mu\text{g}/\text{ml}$ gentamicin, and incubated for 48 h before extraction (activated T-cell extracts). CD14⁺ cells were either extracted immediately (monocyte extracts) or induced to differentiate into macrophages by incubation for 7 days at 10^6 cells/ml in Macrophage-SFM medium (GIBCO BRL) supplemented with 500 units/ml of recombinant granulocyte-macrophage colony-stimulating factor (Immunex) in the absence of FBS and gentamicin. On day 7, the medium was replaced and the cells were cultured for an additional 48 h in the presence of 100 units/ml gamma interferon (PeproTech) and 100 ng/ml *Escherichia coli* lipopolysaccharide (Sigma). All cell cultures were maintained at 37°C in a humidified atmosphere containing 5% CO₂.

The purity of cell subpopulations from each donor was determined by flow cytometry using monoclonal antibodies anti-CD45, anti-CD3, anti-CD4, anti-CD8, anti-CD14, or anti-CD69 conjugated to fluorochromes allophycocyanin, fluorescein isothiocyanate, phycoerythrin, or peridinin chlorophyll protein (Becton Dickinson) in four-color flow cytometry assays. Flow cytometric analysis was performed using a FACSCalibur flow cytometer and CellQuest software (Becton Dickinson) with gating for viable cells.

Preparation of cell extracts. Cell extraction was similar to the procedure described by De La Rosa et al. (8), modified as described below. Cells were harvested by centrifugation, and cell pellets were resuspended in boiling water (75 $\mu\text{l}/10^6$ cells), incubated at 100°C for 70 s, and cooled in ice. After brief centrifugation, each extract was passed through a 3K MICROSEP microconcentrator (Pall Gelman) to remove residual enzymatic activities, and the spin column was washed once with warm water. The filtrates were combined and lyophilized, reconstituted in 200 μl 20 mM Tris-HCl (pH 7.6), and stored at -20°C. Recovery of ATP increased during incubation at 100°C, reaching a plateau after 70 s; however, the PP_i level increased linearly with additional incubation at 100°C, indicating that PP_i was released/formed from cellular constituents during the extraction step. Formation of PP_i during boiling water or perchloric acid extractions has been described by De La Rosa et al. (8), who showed that the breakdown of cellular pools of 5'-phosphoribosyl-1-pyrophosphate was a likely source for this PP_i. Measurements of PP_i in extracts prepared after various times of incubation at 100°C showed that PP_i formed during extraction for 70 s ranged from 1.6 to 7.4 pmol/ 10^6 cells for different cell types, resulting in a mean background correction of 7.6 ± 1.5 μM intracellular PP_i (concentration averaged over various cell types after adjustment for differences in cell volume).

Recovery of ATP and PP_i was tested by adding [γ -³²P]ATP (Perkin-Elmer) or [³²P]PP_i (prepared from [γ -³²P]ATP by digestion with snake venom phosphodiesterase [Boehringer Mannheim] and purified by polyacrylamide gel electrophoresis) just after the addition of boiling water to the cell pellet to begin the extraction procedure. In the absence of the MICROSEP filtration step, [³²P]ATP was stable, but [³²P]PP_i was degraded to [³²P]phosphate and also incorporated into higher-molecular-weight compounds. When the filtration step was included in the procedure, loss of [³²P]PP_i was less than 3%. Recovery of [γ -³²P]ATP was also greater than 97%.

Removal of [³²P]ddAMP from blocked primers by WT and mutant HIV-1 RT. DNA primer L32 (5'-CTACTAGTTTTCTCCATCTAGACGATACCAGA-3') was annealed with DNA template WL50 (5'-GAGTGCTGAGGTCTTCATTC TGGTATCGTCTAGATGGAGAAAAGTAG-3') and chain terminated by extending with [α -³²P]ddATP (Amersham) as previously reported (31). Expression and purification of WT HIV-1 RT (RT^{WT}) and D67N/K70R/T215Y/K219Q mutant HIV-1 RT (RT^{AZT}) were described previously (29, 31). The [³²P]ddAMP-terminated L32 primer/WL50 template was reisolated and incubated at a concentration of 5 nM with 200 nM RT^{WT} or RT^{AZT} at 37°C in a 10- μl reaction volume containing RB buffer (40 mM HEPES, pH 7.5, 20 mM MgCl₂, 60 mM KCl, 1 mM dithiothreitol, 2.5% glycerol, and 80 $\mu\text{g}/\text{ml}$ bovine serum albumin). Cell extract (3 μl extract/10 μl reaction) or ATP and PP_i were added to provide acceptor substrates for excision, and 0.8 μM ddATP was included in

the reaction mixture to prevent reincorporation of the labeled excision products. The reaction was terminated by heating at 90°C for 3 min. The ³²P-labeled products were separated by electrophoresis on a 20% denaturing polyacrylamide gel and quantitated by phosphorimaging using ImageQuant version 3.3 (Molecular Dynamics). The percentage of total radioactivity appearing in each excision product was plotted versus time, and the initial rate of excision product formation was determined from the linear portion of the curve.

Removal of [³²P]AZT monophosphate (AZTMP) from blocked primers by WT and mutant HIV-1 RT. DNA primer L33 (5'-CTACTAGTTTTCTCCATCTAG ACGATACCAGAA-3') was annealed with template WL50 and chain terminated by extending with [α -³²P]AZT triphosphate (AZTTP) (synthesized as previously described [29]). The reaction was carried out as described in the previous section except that 0.8 μM ddTTP was included in place of ddATP to prevent reincorporation of excision products.

Estimation of PP_i and ATP concentrations in cell extracts. Initial rates of [³²P]ddATP and adenosine 2',3'-dideoxyadenosine 5',5''-P₁[³²P]P₄-tetraphosphate ([³²P]Ap₄ddA) synthesis were determined as described above, using reaction mixtures containing a constant amount of cell extract and increasing amounts of unlabeled PP_i or ATP. The added acceptor substrates enhanced the rate of formation of the respective excision products. The percentage of radioactivity in [³²P]ddATP or [³²P]Ap₄ddA was determined after a defined incubation period, and the rate of product formation was calculated from the concentration of labeled primer-template in the reaction mixture, the percentage converted to specific product, and the time of incubation. The rate of [³²P]ddATP or [³²P]Ap₄ddA synthesis was plotted versus the concentration of added PP_i or ATP, respectively, and the linear portion of the curve was fit to the equation $y = mx + b$, where x and y are the concentrations of acceptor substrate (PP_i or ATP) added and excision product (Ap₄ddA or ddATP) formed, respectively. The concentration of PP_i or ATP in the cell extract is equal to b/m .

RESULTS

Acceptor substrates for excision of ddAMP by HIV-1 RT are present in H9 cell extracts. Incubation of [³²P]ddAMP-terminated DNA primer/template with HIV-1 RT^{AZT} and H9 cell extract led to a time-dependent decrease in radioactive primer and increase in low-molecular-weight labeled products (Fig. 1A). These products were identified as Ap₄ddA, ddATP, Gp₄ddA, and Ap₃ddA by their comigration with authentic compounds (formed by incubation of HIV-1 RT with [³²P]ddAMP-terminated DNA primer/template and the appropriate acceptor substrate [data not shown]) and resistance to digestion by calf intestinal phosphatase (Fig. 1A, lane 12). The identification of dinucleoside tetra- and triphosphates on the basis of electrophoretic mobility and sensitivity to enzyme digestion has been described previously (31). Ap₄ddA, ddATP, Gp₄ddA, and Ap₃ddA correspond to products resulting from the transfer of [³²P]ddAMP from the primer terminus to ATP, PP_i, GTP and ADP, respectively (Fig. 1B). The rate of Ap₄ddA formation was approximately 3 times that of ddATP or Gp₄ddA and 10 times that of Ap₃ddA.

The boiling-water extraction procedure was chosen after comparison with established extraction methods for measurement of nucleotide pools (2, 19, 23, 37, 48, 50) by adding [³²P]ATP or [³²P]PP_i to the cells at the time of extraction and evaluating their recovery in the extract by gel electrophoresis (see Methods). After extraction for 16 h at -20°C with 70% methanol, the electrophoretic mobility of the [³²P]ATP was unaltered; however, about a third of the [³²P]PP_i was cleaved to free phosphate, while the rest was incorporated into higher-molecular-weight species (data not shown). By contrast, greater than 97% of both compounds was unchanged after the boiling-water extraction followed by centrifugation through a 3K MICROSEP microconcentrator as described in Methods.

Incubation of [³²P]ddAMP-terminated primer/template with

FIG. 1. Excision of $[^{32}\text{P}]\text{ddAMP}$ by HIV-1 RT^{AZT} , using acceptor substrates in H9 cell extract. (A) $[^{32}\text{P}]\text{ddAMP}$ -terminated L32 primer/WL50 template was incubated at 37°C without (–) or with (+) HIV-1 RT^{AZT} and H9 cell extract for the times indicated at the bottom of the figure. The products were separated by electrophoresis on a 20% sequencing gel. The labeled compounds formed during the incubation are indicated to the right and left of the figure. The 40-min samples are shown before (lanes 2, 5, and 11) and after (lanes 3, 6, and 12) digestion with 2 units of calf intestinal phosphatase (CIP) for 15 min at 37°C . (B) Products expected from excision of $[^{32}\text{P}]\text{ddAMP}$ and transfer to various acceptor substrates.

cell extract in the absence of HIV-1 RT^{AZT} (Fig. 1A, lanes 1 to 3) or incubation with HIV-1 RT^{AZT} in the absence of cell extract (lanes 4 to 6) resulted in the slow appearance of a phosphatase-sensitive compound identified as ddAMP, suggesting low levels of exonuclease contamination in the reaction mixture. All quantitative determinations were corrected for this background. A phosphatase-resistant compound (identified as $[^{32}\text{P}]\text{ddAp}_4\text{ddA}$) was observed during incubation of labeled primer/template and RT in the absence of cell extract (lanes 4 to 6). A small amount of ddATP was added to the reaction mixtures to prevent reincorporation of the labeled excision products; the presence of this compound led to the formation of ddAp₄ddA in the absence of other substrates. This product was not observed when H9 cell extract was added to the reaction mixture (lanes 7 to 12).

To estimate the content of ATP and PP_i in H9 cells, HIV-1

TABLE 1. Intracellular levels of ATP and PP_i^a

Cell type	Intracellular amt of:			
	ATP (pmol/10 ⁶ cells)	PP _i (pmol/10 ⁶ cells) ^b	ATP (mM) ^c	PP _i (μM) ^{b,c}
H9 cells	1,600 ± 300	18 ± 7	1.3 ± 0.2	15 ± 4
Unstimulated CD4 ⁺ T cells	470 ± 60	1.8 ± 1.3	2.2 ± 0.3	8 ± 6
Activated CD4 ⁺ T cells	1,230 ± 100	68 ± 11	1.4 ± 0.1	79 ± 15
Unstimulated CD8 ⁺ T cells	360 ± 50	2.6 ± 1.0	1.7 ± 0.2	12 ± 8
Activated CD8 ⁺ T cells	2,300 ± 540	47 ± 5	2.7 ± 0.6	55 ± 5
Monocytes	1,100 ± 230	13 ± 1	2.3 ± 0.5	27 ± 3
Macrophages	1,500 ± 390	7 ± 1	1.6 ± 0.4	7 ± 1

^a Reactions were performed as described in the legend for Fig. 2. Numbers are reported as ± SEM for two to five determinations. Data for primary cell subpopulations were obtained from five independent blood donors.
^b Corrected for PP_i formed during the extraction at 100°C as described in Materials and Methods. Corrections in pmol/10⁶ cells: H9 cells, 7; unstimulated CD4⁺ and CD8⁺ T cells, 1.6; activated CD4⁺ and CD8⁺ T cells, 6.4; monocytes, 4; macrophages, 7. Correction for intracellular PP_i concentration was 7.6 μM for all cell types.
^c Concentrations calculated using the following values for cell volume: H9 cells, 1.2 pL/cell (11); activated CD4⁺ and CD8⁺ T cells, 0.86 pL/cell; unstimulated CD4⁺ and CD8⁺ T cells, 0.21 pL/cell; monocytes, 0.48 pL/cell; macrophages, 0.96 pL/cell (51).

RT^{AZT} and $[^{32}\text{P}]\text{ddAMP}$ -terminated DNA primer/template were incubated with H9 cell extract and increasing amounts of unlabeled ATP or PP_i as shown in Fig. 2. As expected, the addition of ATP to the reaction mixture resulted in increased formation of $[^{32}\text{P}]\text{Ap}_4\text{ddA}$ (Fig. 2A and B), while the addition of PP_i increased the formation of $[^{32}\text{P}]\text{ddATP}$ (Fig. 2C and D). In each case, the formation of the other removal products was diminished. The amounts of ATP and PP_i present in the H9 cell extract were estimated from the linear portions of the plots of product versus added ATP or PP_i as described in Methods. For the experiments shown in Fig. 2, it was determined that 10⁶ H9 cells contained approximately 2,000 pmol of ATP and 15 pmol of PP_i. The results of multiple experiments are summarized in Table 1.

Acceptor substrates for excision of ddAMP by HIV-1 RT are present in primary immune cell extracts. Primary PBMCs were prepared from healthy donors, and purified cell subfractions were isolated by positive selection using magnetic beads conjugated with monoclonal antibodies to human CD4, CD8, and CD14 surface markers; the purity of each cell subfraction was greater than 96% (data not shown). Cell extracts were prepared from unstimulated or activated CD4⁺ and CD8⁺ subfractions as described in Methods. Activation of CD4⁺ and CD8⁺ T cells with interleukin 2 and phytohemagglutinin for 48 h resulted in upregulation of CD69 on 83% of the CD4⁺ cell subpopulation and 70% of the CD8⁺ subpopulation (data not shown). A portion of the CD14⁺ cell subfraction (monocyte fraction) was treated as described in Methods to induce differentiation into macrophages (greater than 90% by microscopic examination), which were extracted for subsequent analysis.

Removal of $[^{32}\text{P}]\text{ddAMP}$ from the DNA primer/template by transfer to acceptor substrates present in the primary cell extracts was carried out as described for H9 cell extracts (above). In all cases, $[^{32}\text{P}]\text{Ap}_4\text{ddA}$ and $[^{32}\text{P}]\text{ddATP}$ were formed in greater amounts than other products, indicating that ATP and PP_i were the predominant acceptor substrates in these extracts. Extracts from unfractionated PBMCs produced $[^{32}\text{P}]\text{ddATP}$

FIG. 2. Estimation of intracellular ATP and PP_i concentrations in H9 cells. (A) $[^{32}P]$ ddAMP-terminated L32 primer/WL50 template was incubated with 3 μ l H9 cell extract (3 μ l corresponds to 3.4×10^5 H9 cells) in the absence (–) or presence (+) of HIV-1 RT^{AZT} and additional ATP (concentrations given in μ M at the bottom of the panel) in a total volume of 10 μ l for 10 min at 37°C. The products were separated by electrophoresis as with Fig. 1. Each reaction condition is shown in duplicate except for the highest two ATP concentrations. (B) Rate of Ap_4ddA formation in the experiment in panel A as a function of added ATP concentration. Rates are given in nM Ap_4ddA formed after 10 min incubation. The inset shows the linear portion of the plot. Linear regression gave a slope (m) of 0.01 (nM increase in Ap_4ddA per μ M increase in ATP) and y intercept (b) of 0.69 nM. The ratio ($b/m = 69 \mu$ M) corresponds to the ATP concentration derived from the H9 cell extract (2,000 pmol ATP per 10^6 H9 cells). (C) Reactions were carried out as in (A) except that the 3- μ l H9 cell extract added to the reaction mixture corresponded to 2.0×10^5 H9 cells, and PP_i was added instead of ATP (added PP_i concentrations are indicated at the bottom of the panel). Phosphorimager exposure was longer than in (A) to increase sensitivity to the PP_i excision product. All reactions are shown in duplicate except for the 0.8 to 6.4 μ M PP_i concentrations. (D) Rate of ddATP formation in the experiment shown in panel C as a function of added PP_i concentration. Rates are given in nM ddATP formed after 10 min incubation. Slope ($m = 1.13$; nM increase in ddATP per μ M increase in PP_i) and y intercept ($b = 0.31$ nM) were determined by linear regression (inset). The ratio ($b/m = 0.3 \mu$ M) corresponds to the PP_i concentration derived from the H9 cell extract (15 pmol PP_i per 10^6 H9 cells).

almost exclusively (data not shown). This is most likely explained by the presence of a high concentration of PP_i due to incomplete removal of platelets, which contain high levels of this acceptor substrate (20, 45, 49). The low levels of PP_i detected in the extracts of unstimulated PBMC subpopulations suggest that platelets were effectively depleted during the purification of immune cell subfractions (Table 1).

When extracts from unstimulated $CD4^+$ and $CD8^+$ T cells provided the acceptor substrates for the removal reaction, the rate of formation of $[^{32}P]Ap_4ddA$ exceeded that of $[^{32}P]ddATP$ by threefold (Fig. 3A and C), indicating that ATP was the dominant substrate; however, when extracts prepared from activated cells were used in the removal reaction, the rate of formation of $[^{32}P]ddATP$ exceeded $[^{32}P]Ap_4ddA$ formation by eightfold for $CD4^+$ T cells (Fig. 3B) and fivefold for $CD8^+$ T cells (Fig. 3D), indicating that PP_i was the dominant substrate in these extracts. Extracts from $CD14^+$ monocytes formed $[^{32}P]Ap_4ddA$ and $[^{32}P]ddATP$ at similar rates (Fig. 3E), while extracts from differentiated macrophages supported the formation of $[^{32}P]Ap_4ddA$ at about two times the rate of $[^{32}P]ddATP$ formation (Fig. 3F).

Intracellular concentrations of ATP and PP_i were estimated for each primary immune cell subpopulation as described for H9 cells (Table 1). Intracellular ATP concentrations ranged from 1.4 to 2.7 mM among different cell subpopulations and activation conditions, in agreement with published values (14, 19, 38, 53). Intracellular concentrations of PP_i were estimated

FIG. 3. Excision of ddAMP by HIV-1 RT^{AZT} using ATP and PP_i in primary, human immune cell extracts. $[^{32}P]$ ddAMP-terminated L32 primer/WL50 template was incubated with HIV-1 RT^{AZT} and immune cell extract for various times at 37°C, and the products were identified by PAGE. Percentage of total radioactivity present in Ap_4ddA (closed circles) or ddATP (open circles) was determined by phosphorimaging. Reaction mixtures contained extracts from unstimulated $CD4^+$ T cells (A), activated $CD4^+$ T cells (B), unstimulated $CD8^+$ T cells (C), activated $CD8^+$ T cells (D), monocytes (E), or macrophages (F).

FIG. 4. Excision of ddAMP or AZTMP by HIV-1 RT^{WT} and RT^{AZT}, using stimulated or unstimulated CD4⁺ T-cell extracts. [³²P] ddAMP-terminated L32 primer/WL50 template or [³²P]AZTMP-terminated L33 primer/WL50 template was incubated with RT^{WT} (open circles) or RT^{AZT} (closed circles) and stimulated or unstimulated CD4⁺ T-cell extract for various times at 37°C, and the products were identified by PAGE. Percentage of total radioactivity present in each excision product was determined by phosphorimaging, and percentages were then added together to yield total removal (percentage of the input primer/template). Reaction mixtures contained extracts from unstimulated CD4⁺ T cells and ddAMP-terminated primer (A) or AZTMP-terminated primer (B) or extracts from stimulated CD4⁺ T cells and ddAMP-terminated primer (C) or AZTMP-terminated primer (D).

to be 8 to 12 μ M in unstimulated CD4⁺ and CD8⁺ T cells, increasing to 55 μ M in activated CD8⁺ cells and 79 μ M in activated CD4⁺ cells. The level of PP_i in CD14⁺ monocytes was 27 μ M, which decreased to 7 μ M upon differentiation into macrophages. Unstimulated CD4⁺ or CD8⁺ T cells and undifferentiated CD14⁺ monocytes were extracted immediately after the cell subpopulations were isolated, while stimulated T cells and differentiated macrophages were incubated in culture prior to extraction. It is possible that the concentrations of PP_i and ATP were perturbed during the fractionation procedure. If so, this may affect the values obtained for the unstimulated cells. It is unlikely that any perturbation that occurred during cell fractionation would affect PP_i or ATP concentrations after incubation in culture for at least 48 h. These results suggest that the levels of PP_i are influenced by the activation status of the cells while ATP levels are much less affected.

Comparison of excision by RT^{WT} and RT^{AZT} in the presence of CD4⁺ T-cell extracts. The excision activities of RT^{WT} and RT^{AZT} were compared using different CD4⁺ T-cell extracts to determine conditions that provide the mutant enzyme with a greater ability to remove chain terminators. Figure 4A shows that the rate of excision of [³²P]ddAMP from ddAMP-terminated primer/template by HIV-1 RT^{AZT} was three- to sixfold greater than that by RT^{WT} when the acceptor substrates were provided from an extract of unstimulated CD4⁺ T cells. This result is consistent with the demonstration that ATP is the dominant acceptor substrate in unstimulated CD4⁺ T cells (Fig. 3 and Table 1), since we and others have shown that primer unblocking by the mutant enzyme is greater than that of wild-type RT when the acceptor substrate is a nucleoside triphosphate (4, 5, 25, 28–30, 32, 42, 43). Similar results were

FIG. 5. Excision of ddAMP by HIV-1 RT^{AZT}, using ATP and PP_i when both are present at the estimated intracellular concentrations. [³²P]ddAMP-terminated L32 primer/WL50 template was incubated without (–) or with (+) HIV-1 RT^{AZT} for the times indicated at 37°C with a mixture of 2.2 mM ATP and 10 μ M PP_i (A, C) or 2.9 mM ATP and 80 μ M PP_i (B, D). The products were identified by PAGE. The positions of labeled products Ap₄ddA and ddATP are shown at the right of panels A and B. For panels C and D, radioactivity in excision products Ap₄ddA (closed circles) and ddATP (open circles) was determined by phosphorimaging and expressed as a percentage of total counts in the lane.

observed for excision of [³²P]AZTMP from AZTMP-terminated primer/template (Fig. 4B). When the acceptor substrates were provided by an extract of highly stimulated CD4⁺ T cells, no difference was observed in excision rates for RT^{WT} and RT^{AZT} (Fig. 4C and D). This is consistent with the demonstration that PP_i is the predominant acceptor substrate present in extracts of stimulated CD4⁺ T cells (Fig. 3 and Table 1) and that the rates of pyrophosphorolysis are similar for RT^{WT} and RT^{AZT} (4, 5, 25, 28–30, 32, 42, 43).

Excision by RT^{WT} or RT^{AZT} in the presence of mixtures of ATP and PP_i. Since the preparation of cell extracts and addition of the reagents in the excision assay result in an overall dilution of the contents of the cell ranging from 30- to 100-fold, we compared the excision activities of RT^{WT} and RT^{AZT} in the presence of the major acceptor substrates, ATP and PP_i, at concentrations that correspond to the estimated intracellular levels of these compounds. In a mixture containing 2.2 mM ATP and 10 μ M PP_i, the ATP-dependent removal reaction predominated (Fig. 5A and C). Comparison with the results for unstimulated CD4⁺ T-cell extract (Fig. 3A) indicates that the excision products are formed in approximately the same ratio. In a mixture containing 2.9 mM ATP and 80 μ M PP_i, the product ratio was reversed (Fig. 5B and D), in agreement with the results for activated CD4⁺ T cells (Fig. 3B). These results show that the relative contributions of ATP and PP_i to the excision reaction are maintained upon extensive dilution.

To more specifically define the ATP/PP_i conditions required for an excision advantage with the AZT-resistant enzyme, additional reactions were carried out with mixtures of 2.9 mM

FIG. 6. Excision of ddAMP or AZTMP by HIV-1 RT^{WT} and RT^{AZT}, using 2.9 mM ATP and different concentrations of PP_i. [³²P] ddAMP-terminated L32 primer/WL50 template (A, C, E) or [³²P]AZTMP-terminated L33 primer/WL50 template (B, D, F) was incubated with RT^{WT} (open circles) or RT^{AZT} (closed circles) for 1 min (ddAMP-terminated primer) or 3 min (AZTMP-terminated primer) at 37°C, and the products were identified by PAGE. Concentration of each excision product formed (panels C-F) was determined from the percentage of radioactivity recovered in each product, and the concentrations were added together to yield total removal (panels A, B).

ATP and concentrations of PP_i ranging from 0.01 to 100 μM (Fig. 6). At low concentrations of PP_i, total excision of ddAMP by RT^{AZT} occurred at about 10 times the rate of excision by RT^{WT} (Fig. 6A); however, this difference was smaller at PP_i concentrations between 10 and 30 μM; no difference was observed between the enzymes when the PP_i concentration was greater than 35 μM. PP_i-dependent excision was minimal with both enzymes except when PP_i concentration exceeded about 10 μM (Fig. 6C). ATP-dependent excision predominated at low PP_i concentrations but was suppressed when the PP_i concentration was greater than 10 μM (Fig. 6E).

Similar experiments were performed to measure the excision of AZTMP (Fig. 6B, D, and F). At low PP_i concentrations, total excision of AZTMP by RT^{AZT} occurred at about six times the rate of excision by RT^{WT}; however, no difference was observed between RT^{WT} and RT^{AZT} at PP_i concentrations greater than 50 μM (Fig. 6B). These results suggest that enhanced excision by RT^{AZT} is only manifested in an environment where the PP_i concentration is less than 35 μM (for ddAMP removal) or 50 μM (for AZTMP removal).

DISCUSSION

Cell extracts contain several compounds that can serve as acceptor substrates for HIV-1 RT-mediated excision. In most

extracts tested, the predominant product was a dinucleoside tetraphosphate produced by ATP-mediated excision. The exceptions were activated primary human CD4⁺ and CD8⁺ T cells, where the predominant product was the dideoxynucleoside triphosphate produced by PP_i-mediated excision. Our results suggest that concentrations of PP_i in T lymphocytes are much lower than previously suggested (8 to 12 μM in unstimulated T cells and 55 to 79 μM in activated T cells); nonetheless, the PP_i concentrations found in activated cells are sufficient to account for most of the excision, because the rate with PP_i is much faster than with ATP. We tested mixtures of 2.9 mM ATP and various concentrations of PP_i and showed that ATP-dependent excision of ddAMP or AZTMP predominated when the PP_i concentration was less than 35 μM or 50 μM, respectively. PP_i-dependent excision predominated when PP_i concentrations were greater than those values. These results present a paradox in terms of selection for AZT resistance mutations, since ATP-mediated excision, but not PP_i-mediated excision, is elevated in RT^{AZT} by comparison with RT^{WT} (4, 5, 25, 28–30, 32, 42, 43). Yet our results have shown that the overall rate of excision is the same for RT^{AZT} and RT^{WT} when the reaction is carried out at the concentrations of ATP and PP_i found in activated CD4⁺ T cells, where the bulk of viral replication and hence selection for AZT resistance mutations seems most likely to occur.

While there is evidence that cell-free virus particles are capable of reverse transcription, DNA synthesis occurs predominantly after entry of the viral core into the cytoplasm and appears to be coupled with disruption of the core structure (15). Biochemical analysis has identified a variety of nucleoprotein complexes that are active in DNA synthesis, although the significance of these complexes in the infection process has not been unambiguously demonstrated (10, 35). Structures active in reverse transcription have been identified cytologically by the ability to incorporate fluorescently labeled deoxynucleotides (26). The ability to label these cytoplasmic complexes with substrate indicates that they are permeable to deoxynucleotides and, presumably, also to ATP and PP_i. The majority of reverse transcription complexes are attached to microtubules and cytoplasmic dynein, which are proposed to facilitate their movement to the perinuclear region in preparation for entry into the nucleus and integration in the host chromosomes (26).

The major site of cellular ATP synthesis in lymphocytes is the mitochondrial matrix, and ATP utilization occurs predominantly through protein synthesis, membrane transport, RNA and DNA synthesis, and maintenance of the mitochondrial proton gradient (6). Concentration gradients created by ATP consumption are reequilibrated through the action of enzymes such as adenylate kinase, which provides a relay system for rapid energy transfer from the mitochondria to the sites of energy utilization (9). Subcellular distribution of ATP was studied in four human cell lines, using luciferase constructs targeted to specific cellular compartments (12). Levels of ATP were similar in the cytosol, the subplasma membrane region, and the nucleus. A twofold-higher level was detected in the mitochondrial matrix. Therefore, the intracellular ATP concentration is maintained in a relatively narrow range (1 to 5 mM) in most cell types, including lymphocytes and lymphocyte-derived cell lines (8, 17, 53; this report), and it is likely that

reverse transcription occurs in the presence of these concentrations.

Cellular PP_i is formed in numerous metabolic reactions (44, 52), with major contributions from the activation of amino acids for protein synthesis, activation of fatty acids for oxidation, and formation of phosphodiester bonds in nucleic acid synthesis. It has been frequently suggested (21) that PP_i is rapidly cleaved by inorganic pyrophosphatases located throughout the cell and that this lowers the local PP_i concentration and energetically drives these reactions in the forward direction. Russell (44), however, has pointed out that the pyrophosphatase reaction does not attain equilibrium *in vivo*, at least in rat liver, and that PP_i may have an additional role. It is possible that the PP_i concentration may be elevated in certain circumstances, resulting in the transient reversal of PP_i -generating reactions; however, in a general sense, biochemical or cellular mechanisms must exist that limit the extent of the reverse reactions to assure that biosynthetic reactions will go forward. The subcellular distribution of PP_i and pyrophosphatases has not been extensively described; yet a wide range of PP_i concentrations, ranging from 4 μ M to 400 μ M, has been reported in cell extracts (3, 8, 18, 24, 41, 44). This range is explained, at least in part, by events that occur during or after preparation of the cell extracts. We encountered several sources of variability. (i) Platelets, which contain very high levels of PP_i (8, 20, 45, 49), may be present at variable levels as a contaminant in unfractionated PBMC preparations. (ii) PP_i may be lost during extraction due to residual enzyme activities that degrade PP_i to inorganic phosphate or incorporate it into higher-molecular-weight compounds. (iii) PP_i may be formed by the breakdown of other cellular constituents, such as phosphoribosyl pyrophosphate, during extraction (8).

De La Rosa et al. (8) have shown that specific procedures must be followed to obtain PBMC preparations that are free of platelets. These authors measured PP_i at a level of 20 to 90 pmol/ 10^6 cells in unfractionated, unstimulated PBMC preparations ($\sim$ 50 to 210 μ M, assuming an average cell volume of 0.42 pL for unfractionated PBMCs [8]). This value decreased to $\sim$ 4 to 7 μ M PP_i when the platelet contamination was removed. The most commonly quoted value for PP_i level is 133 ± 20 pmol/ 10^6 cells in unstimulated PBMCs, reported by Barshop et al. (3) ($\sim$ 130 to 400 μ M, depending on assumptions about cell volume), but these authors did not address the possibility of platelet contamination or production of PP_i from cellular constituents during perchloric acid extraction. High levels of PP_i were also observed in unfractionated PBMCs in the present study (data not shown); however, PP_i levels were much lower in PBMC subfractions, emphasizing the importance of platelet removal during isolation of cell subpopulations.

Cold methanol extraction, which yields satisfactory recovery of nucleotides (13, 37, 48), failed to inactivate enzymes that degrade [32 P] PP_i or incorporate the label into higher-molecular-weight compounds during the extraction. Persistence of active enzymes after methanol extraction has been previously reported (36). We were able to minimize the loss of PP_i during extraction by using boiling water as the extraction agent combined with MICROSEP filtration of the extracts. We also demonstrated that PP_i is released by the breakdown of cellular constituents during the 100°C extraction procedure and that it was necessary to control the length of the heat treatment and

to apply a correction for this background. We observed a 4- to 10-fold increase in PP_i levels in CD8⁺ and CD4⁺ T cells after 48 h of mitogenic stimulation. This is larger than that previously reported after 8 h of stimulation (8); however, major metabolic changes that would lead to substantial PP_i formation probably occur later than 8 h (39, 40). The level of PP_i in primary CD14⁺ monocytes (27 μ M) decreased by about 75% after differentiation into macrophages, consistent with reduced proliferation in these terminally differentiated cells (27, 54).

There is general agreement that the rate of ATP-mediated excision is substantially greater for AZT-resistant RT than for WT RT (5, 16, 22, 25, 29, 32, 34, 42, 43). While agreeing that ATP-mediated excision is the largest biochemical difference between mutant and WT RT and that an increase in PP_i -mediated excision is not observed in kinetic assays, Ray et al. (42, 43) have suggested that pyrophosphorolysis may also play a role in AZT resistance. Mutant RT dissociates more slowly from AZTMP-terminated primer/template than WT RT (7), which could result in a net increase in formation of either PP_i - or ATP-mediated excision products. Ray et al. (42) have suggested that this difference may not be evident in kinetic assays. In addition, mutant RT is less sensitive than WT RT to inhibition by the next complementary deoxynucleoside triphosphate, which could lead to increased excision by the mutant enzyme using either PP_i or ATP in the presence of physiological concentrations of deoxynucleoside triphosphates (43). Whether these effects are of sufficient magnitude to support a role for PP_i -dependent excision in AZT resistance *in vivo* is yet to be determined.

A more general question is how selection of AZT-resistant mutants can occur when ATP-mediated excision may be overwhelmed by pyrophosphorolysis in actively replicating CD4⁺ T cells. Assuming that reactions carried out with purified recombinant WT and mutant RTs and with synthetic chain-terminated primer/template are representative of the reactive species in the intracellular reverse transcription complexes, several possible explanations for this phenomenon should be considered: (i) The affinity of RT for PP_i may be lower *in vivo* than that observed *in vitro* due to intracellular ionic conditions, the presence of cellular factors, etc., so that the ATP-dependent reaction may be sufficient to account for mutant selection *in vivo*. (ii) Alternative mechanisms for enhanced PP_i -dependent excision by mutant enzyme not detected *in vitro* may operate *in vivo* as discussed above (42, 43). (iii) Access of PP_i to the RT active site may be restricted by a mechanism that does not affect ATP-mediated excision. For example, a pyrophosphatase enzyme specifically bound to the reverse transcription complex, to cytoplasmic dynein, or to the microtubule network could prevent PP_i -dependent excision during normal viral DNA synthesis and thereby protect viral replication from inhibition by transient accumulation of PP_i . (iv) PP_i detected in whole-cell extracts may be primarily localized *in vivo* in a specific subcellular compartment where reverse transcription does not occur. (v) Selection for AZT resistance mutations may actually occur primarily in unstimulated or partially stimulated CD4⁺ T cells, monocytes, or macrophages where the metabolite levels support the predominance of the ATP-dependent excision reaction. Currently available data do not allow us to determine whether any of these mechanisms plays a role in mutant selection.

In summary, our studies demonstrate that intracellular pools of ATP are sufficient to catalyze the excision of AZTMP or ddAMP from nascent DNA chains. Except for PP_i in activated T cells, other substrates make only a minimal contribution to the total excision activity. A role for PP_i in excision-mediated resistance cannot be excluded, but it seems more likely that mechanisms exist to ensure that DNA synthesis in the reverse transcription complex is not affected by fluctuations in the PP_i concentration. The identification of factors that control excision in the intracellular environment will be important for our understanding of the *in vivo* selection of this class of drug resistance mutations.

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