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Investigation of the dNTP-Binding Site of HIV-1 Reverse Transcriptase Using Photoreactive Analogs of dNTP

A. L. Zakharenko[1]*, D. M. Kolpashchikov[1], S. N. Khodyreva[1], O. I. Lavrik[1], and L. Menéndez-Arias[2]

[1]Novosibirsk Institute of Bioorganic Chemistry, pr. Lavrent'eva 8, Novosibirsk-90, 630090 Russia; fax: (3832) 33-3677; E-mail: sashaz@niboch.nsc.ru
[2]Centro de Biología Molecular "Severo Ochoa", CSIC-Universidad Autónoma de Madrid, 28049 Cantoblanco (Madrid), España; fax: (3491) 397-4799; E-mail: lmenendez@cbm.uam.es

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Abstract—The interaction of dNTPs with the active site of HIV-1 reverse transcriptase (HIV RT) has been investigated. The kinetic parameters of primer elongation catalyzed by wild-type HIV-1 RT and two of its mutants with substitutions for Tyr115 using dTTP and two of its photoreactive analogs were determined. The substitution for Tyr115 with alanine or tryptophan resulted in an increase in K_m values of dTTP and its analogs. Wild-type RT and its mutants were photoaffinity modified using photoreactive primer synthesized *in situ*. The modification was made in two variants: direct photocross-linking under UV irradiation and photosensitized modification using Pyr-dUTP as a sensitizer. The use of the sensitizer decreased the number of modification products and increased selective labeling of the catalytic subunit of both the mutant and wild-type RT.

Key words: HIV-1 reverse transcriptase, dNTP binding, photosensitizing modification

Human immunodeficiency virus type 1 reverse transcriptase (HIV-1 RT) is of great interest as a target in the fight against AIDS. The enzyme has been intensively studied with a broad spectrum of methods, e.g., X-ray structure analysis [1-4], site-directed mutagenesis [5-9], and affinity modification [10-15].

HIV-1 RT is composed of two subunits having molecular masses of 66 and 51 kD; it is a DNA- and RNA-dependent DNA polymerase, and it has RNase H activity. All three activities are associated with the large subunit. The two HIV-1 RT subunits are the products of a single gene, the small subunit resulting from a post-translational modification of the large one that eliminates the RNase H domain [16]. X-Ray structural analysis has shown that each subunit contains four common but differently folded subdomains [1-4]. The polymerase domain of the p66 subunit has an open, stretched conformation, whereas the polymerase domain of p51 is compacted, and its DNA-binding cleft is closed [1-4]. However, the small subunit may be able to bind substrates and catalyze DNA synthesis because the p51/p51 homodimer demonstrates a low level of DNA polymerase activity [17].

The results of X-ray structure analysis of HIV-1 RT in a ternary complex with DNA and dNTP were described in [4]. They revealed the amino acid residues, including Tyr115, involved in the dNTP-binding site. Using site-directed mutagenesis, some HIV-1 RT mutant forms with various amino acid residues in position 115 have been prepared [5-7]. The substitution of Tyr115 with

Abbreviations: HIV-1 RT) human immunodeficiency virus type 1 reverse transcriptase; Y115W and Y115A) its mutant forms with substitution for Tyr115 with tryptophan and alanine, respectively; FAB-4-ddUTP) 5-[*trans*-N-(2,3,5,6-tetrafluoro-4-azidobenzoyl)-3-aminopropenyl-1]-2′,3′-dideoxyuridine-5′-triphosphate (lithium salt); FAB-4-dUTP) 5-[*trans*-N-(2,3,5,6-tetrafluoro-4-azidobenzoyl)-3-aminopropenyl-1]-2′-deoxyuridine-5′-triphosphate (lithium salt); FAB-9-dUTP) 5-[*trans*-N-(N′-(2,3,5,6-tetrafluoro-4-azidobenzoyl)-4-aminobutyryl)-3-aminopropenyl-1]-2′-deoxyuridine-5′-triphosphate (lithium salt); FAB-11-dUTP) 5-[*trans*-N-(N′-(2,3,5,6-tetrafluoro-4-azidobenzoyl)-4-aminohexanoyl)-3-aminopropenyl-1]-2′-deoxyuridine-5′-triphosphate (lithium salt); FAB-13-dUTP) 5-[N-(N′-(2,3,5,6-tetrafluoro-4-azidobenzoyl)-4-aminooctanoyl)-*trans*-3-aminopropenyl-1]-2′-deoxyuridine-5′-triphosphate (lithium salt); FAB-*n*-dUTP) common name for perfluoroarylazide derivatives of dUTP; Pyr-dUTP) 5-[N-(4-(1-pyrenyl)-methylcarbonyl)-amino-*trans*-propenyl-1]-2′-deoxyuridine-5′-triphosphate (lithium salt); DTT) dithiothreitol; PAGE) polyacrylamide gel electrophoresis; SDS) sodium dodecyl sulfate.

* To whom correspondence should be addressed.

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other amino acids, as judged from the kinetic parameters of DNA synthesis, leads to decreased activity due to an increase of the K_m for dNTP [5-7]. Moreover, kinetic data indicate that the substitution of Tyr115 decreases the fidelity of DNA synthesis in comparison with the wild-type RT.

In addition to X-ray structure analysis and site-directed mutagenesis, affinity modification is also widely used to study the structure and function of DNA polymerases [10-15, 18]. Recently, a novel approach using a binary system of photoreagent and sensitizer was developed. This method allows for selective modification of the catalytic subunit of DNA polymerases in the presence of other DNA-binding proteins [19, 20]. Both components of the system are dNTP analogs, with a photoreactive group on the first and a photosensitizing group on the second component. The photoreactive dNTP analog is incorporated into the nascent primer strand by the enzyme, and the sensitizer binds to the dNTP-binding site of the DNA polymerase. When the resulting complex is irradiated with UV light of the appropriate wavelength, the sensitizer absorbs the energy and transfers it to the photoreactive group. Under the irradiation conditions used, the photoreagent is not activated in the absence of the sensitizer. The activation of the photoreagent leads to modification of the enzyme. The energy transfer efficiency is inversely related to the sixth power of the distance between the photoactivated and sensitizing groups [21], so activation is possible only when the photoactivated group is juxtaposed to the sensitizer, as occurs in the specific enzymatic complex.

We used two HIV-1 RT mutant forms, with Tyr115 substituted by alanine or tryptophan, to assess the role of Tyr115 in the interaction of dNTP with the enzyme. One of the goals of the study was to determine kinetic parameters of DNA synthesis catalyzed by the wild-type HIV-1 RT and its mutant forms.

Another goal was to optimize conditions for sensitized modification of HIV-1 RT. Because the enzyme is composed of two subunits containing dNTP-binding centers of diverse conformation and substrate-binding ability, the sensitized modification of HIV-1 RT may provide selective labeling of the catalytic subunit.

MATERIALS AND METHODS

Oligonucleotides. 36-mer template 5′-GGT TAA ATA AAA TAG TAA GAA TGT ATA GCC CCT ACC-3′ and 16-mer primer 5′-GGT AGG GGC TAT ACA T-3′ were synthesized at the Novosibirsk Institute of Bioorganic Chemistry. The following chemicals and materials were used: dNTP (Pharmacia, Sweden); [γ-^{32}P]ATP (Amersham, UK); X-ray film (Fuji, Japan); 1-pyrenebutyric acid (Fluka, Switzerland); other chemicals were of extra purity grade and manufactured in Russia. Pyr-dUTP was synthesized as described in [19], FAB-4-ddUTP as in [22], FAB-4-dUTP as in [23], and FAB-9-dUTP, FAB-11-dUTP, and FAB-13-dUTP as in [24].

Expression and purification of HIV-1 RT and its mutant forms. The large subunit of HIV-1 RT was expressed using the strain *Escherichia coli* DH5α carrying the plasmid p66(RT). The small p51 subunit of HIV-1 RT was expressed using the same strain carrying the plasmid pT51H. Cell cultures containing p66 and p51 were combined, and the enzymes were purified according to the protocol described by Martín-Hernández et al. [5]. The small subunit has 14 N-terminal residues among which are 6 histidine residues allowing metal-chelating affinity chromatography to be applied for the purification. Substitutions for the amino-acid residue in position 115 were introduced in both subunits. The purity of the enzyme preparations was estimated by SDS-PAGE [25]. It was not less than 95%. Protein concentrations were determined by the Bradford method [26] as quoted by [27]. The quantity of active enzyme was assayed by titration with the primer-template duplex as described previously [28].

Determination of kinetic parameters of DNA synthesis catalyzed by HIV-1 RT or its mutant forms. Reaction mixtures, 10 μl each, contained the following standard buffer components: 50 mM HEPES, pH 7.0, 150 mM potassium acetate, 15 mM $MgCl_2$, 5% PEG-6000, 1 mM DTT, 20 nM template-[^{32}P]primer, and 10 nM enzyme. Radioactive label was inserted into the oligonucleotide 5′-end by the method described in [29].

The reaction was initiated by the addition of dNTP and conducted at 37°C. Aliquots (5 μl) were taken at 10 sec intervals, and the reaction was terminated by addition of 5 μl of sample buffer (10 mM EDTA in 90% formamide). Reaction products were analyzed by electrophoresis in 20% polyacrylamide gel with 8 M urea.

The extended primer was determined quantitatively using Bio-Imaging Analyzer BAS-1500 (Fujifilm, USA) and Tina 2.09 software (Raytest Isotopenmessgerate GmbH, Staubenhardt, Germany). K_m and V_{max} were calculated using UltraFit 1.03 software for Macintosh (Biosoft) according to the Michaelis–Menten equation. K_m and V_{max} were established as the mean values of several experiments.

Determination of optimum concentrations for Mg^{2+} and Mn^{2+} in DNA synthesis catalyzed by HIV-1 RT or its mutant forms. Different concentrations of Mg^{2+} and Mn^{2+} were used to determine the optimal buffer conditions for enzymatic activity. Along with the standard components described above, the reaction mixtures contained 50 μM dTTP.

Study of the substrate properties of dUTP photoreactive derivatives in the presence of $MgCl_2$ and $MnCl_2$. Substrate properties of dUTP analogs were determined as described above. dTTP and its analogs were used at 50 μM for the

wild-type enzyme in the presence of $MgCl_2$ or $MnCl_2$. The dNTP concentration of 500 μM was used for the Y115W form in the presence of $MgCl_2$ or $MnCl_2$ and the Y115A form in the presence of $MnCl_2$. We used 1 mM dNTP for the mutant form Y115A in the presence of $MgCl_2$.

Photoaffinity modification of HIV-1 RT and its mutant forms using perfluoroarylazide derivatives of dUTP. The reaction mixtures for photoaffinity modification (total volume 25 μl) contained the following standard components: 50 mM Tris-HCl, pH 8.0, 10 mM $MgCl_2$, 50 mM NaCl, 10 μM FAB-*n*-dUTP (or 50 μM FAB-4-ddUTP), 100 nM template-[^{32}P]primer, 300 nM HIV-1 RT or its mutant forms. The concentration of FAB-ddUTP was chosen to be 5-fold greater than that of FAB-*n*-dUTP because of the difference in K_m values for deoxy- and dideoxynucleotides. The reaction mixtures were incubated for 30 min at 37°C and then irradiated for direct photomodification by UV light from a DRK-120 VIO-1 lamp (LOMO, St. Petersburg, Russia) at wavelength >280 nm (filter WG-280) for 50 sec. For the photosensitized modification, Pyr-dUTP or 1-pyrenebutyric acid was added after the incubation to final concentration 10 μM, and the mixture was irradiated for 10 min through filters FS-1 and BS-7 giving the wavelength range 365-450 nm. The reaction was terminated by the addition of the sample buffer [25].

Reaction products were analyzed by SDS-PAGE [25]. Gels were dried and autoradiographed.

Determination of the efficiency of HIV-1 RT photomodification using FAB-4-dUTP. Reaction mixtures (total volume 80 μl) contained the above-mentioned standard components and also 10 μM FAB-4-dUTP, 100 nM template-[^{32}P]primer, and 300 nM HIV-1 RT. To compare the modification efficiencies in the presence and in absence of photosensitizer, we prepared reaction mixtures with no photosensitizer and the reaction mixtures containing 10 μM 1-pyrenebutyric acid or 10 μM Pyr-dUTP as sensitizer. The reaction mixtures were incubated for 10 min at 37°C and irradiated as described above. After 5, 10, 15, 20, and 25 min of the irradiation, 2-μl aliquots were taken for analysis of the efficiency of primer elongation with dTTP analogs by polyacrylamide gel electrophoresis in the presence of 8 M urea, and 10-μl aliquots were taken for analysis of HIV-1 RT modification products by SDS-PAGE and subsequent analysis by autoradiography. The gel bands corresponding to the modification products were cut out, and their radioactivities were measured by the Cherenkov method using a Rackbeta 1211 scintillation counter (LKB, Sweden).

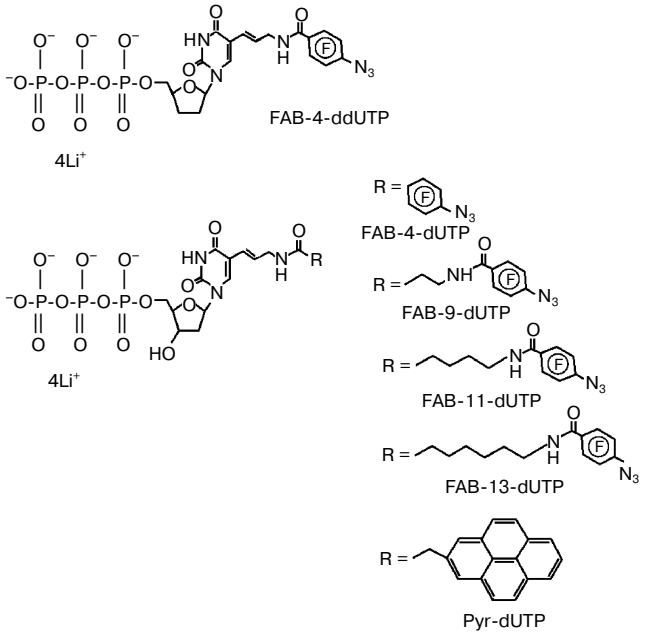

Fig. 1. Structural formulas of the photoreactive dUTP and ddUTP analogs.

RESULTS AND DISCUSSION

The structural formulae of the dUTP and ddUTP analogs are shown in Fig. 1. The perfluoroarylazide group is spaced 4 to 13 atoms (i.e., 15-22 Å) from the pyrimidine base. As we have shown previously, the modification efficiency for DNA polymerase β or replicative protein A (RPA) subunits depends on the linker length. The longer the linker is, the more effective DNA polymerase β labeling occurs. As for RPA, the modification efficiency function of the linker length is bell-shaped [30]. The difference in modification efficiency may result from different acceptors in the protein molecule that are available for the modification with nucleotide analogs of different linker length. So, it was interesting to estimate the efficiency of HIV-1 RT modification with some dTTP analogs of varying linker length.

Optimizing the bivalent metal ion concentrations. As reported previously, manganese ions cause mismatches of DNA replication catalyzed by various DNA polymerases [31-40]. Moreover, T7 DNA polymerase, *Taq* DNA polymerase, and *E. coli* DNA polymerase preferably incorporate dideoxynucleotides in the presence of manganese ions [41, 42]. So, we were interested in studying the effect of manganese ions on dNTP-binding by wild-type HIV-1 RT and its mutant forms.

Because optimum concentrations of both Mg^{2+} and Mn^{2+} in DNA synthesis vary significantly for various DNA polymerases, such as human placenta DNA polymerases α and β [37], avian myeloblastosis virus DNA polymerase [39], or *Thermus thermophilus* DNA polymerase [43], it was necessary to determine these parameters for HIV-1 RT and its mutant forms.

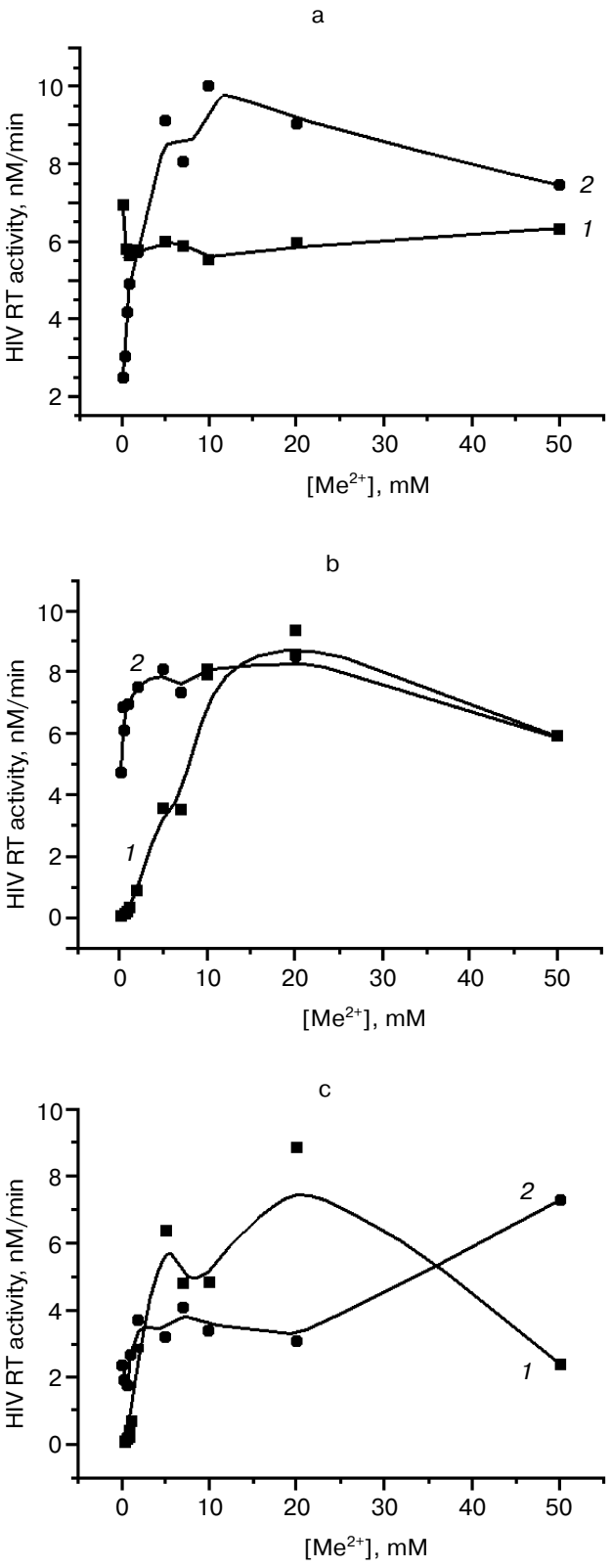

Fig. 2. Effect of Mg^{2+} (*1*) and Mn^{2+} (*2*) on the rate of DNA synthesis catalyzed by HIV-1 RT and its mutant forms: a) wild-type RT; b) Y115W; c) Y115A.

Wild-type HIV-1 RT demonstrates 2-fold increased reaction rate in the presence of $MnCl_2$ in comparison with $MgCl_2$ at concentrations higher than 5 mM. As for the mutant forms of HIV-1 RT, their reaction rates were the same at the same concentrations of Mg^{2+} or Mn^{2+}. Since the rate of DNA synthesis catalyzed by either HIV-1 RT or its mutant forms was maximal at ion concentrations less than 15 mM (both for Mg^{2+} and Mn^{2+}) and remained constant up to 50 mM (Fig. 2), 15 mM concentration for each ion was chosen for further experiments.

As compared with other DNA polymerases, such as human placenta DNA polymerases α and β [37], avian myeloblastosis virus DNA polymerase [39], or *Thermus thermophilus* DNA polymerase [43], HIV-1 RT has unusually broad concentration optima for both Mg^{2+} and Mn^{2+}. Also, it should be noted that the optimum concentrations of Mg^{2+} and Mn^{2+} are the same for HIV-1 RT, whereas other DNA polymerases show maximum activity at Mn^{2+} concentrations one order of magnitude lower than for Mg^{2+} concentrations [37, 39, 43].

Substrate properties of dUTP analogs in DNA synthesis catalyzed by HIV-1 RT or its mutant forms. We studied the substrate properties and kinetic parameters of photoreactive dUTP analogs to optimize conditions for the affinity modification of HIV-1 RT and its mutant forms. All of the dUTP derivatives are substrates and do not terminate the DNA synthesis catalyzed by either wild-type HIV-1 RT or its mutant forms (Fig. 3). For all of three enzymes, the primer is extended by a single deoxynucleoside-5′-monophosphate residue in the presence of Mg^{2+} and dTTP (Fig. 3, lanes *1* on the panels a, c, and e). When $MnCl_2$ substitutes for $MgCl_2$, the wild-type enzyme also catalyzes the incorporation of a single dTMP residue (panel b, lane *1*), whereas Y115W incorporates two (panel d, lane *1*) and Y115A incorporates at least seven dTMP residues (panel f, lane *1*).

When the other three of four native dNTPs were added, all of the enzymes catalyzed the synthesis of full-size DNA products in the presence of magnesium ions (Fig. 3, lanes *2* on the panels a, c, and e). Both wild-type HIV-1 RT and its mutant forms synthesize products with an extra base in comparison with template when manganese ions are presented (lanes *2* on the panels b, d, and f). All three enzymes have pausing sites in the complementary positions of template adenosines. When dTTP is replaced by its analog, bands corresponding to pausing sites become more intense in the mutant forms of the enzyme as well as with the wild-type HIV-1 RT.

Wild-type HIV-1 RT, as well as both of its mutant forms, also catalyze the incorporation of a single nucleotide in the presence of dUTP analog and magnesium ions (lanes *3*, *5*, *7*, and *9* on the panels a, c, and e).

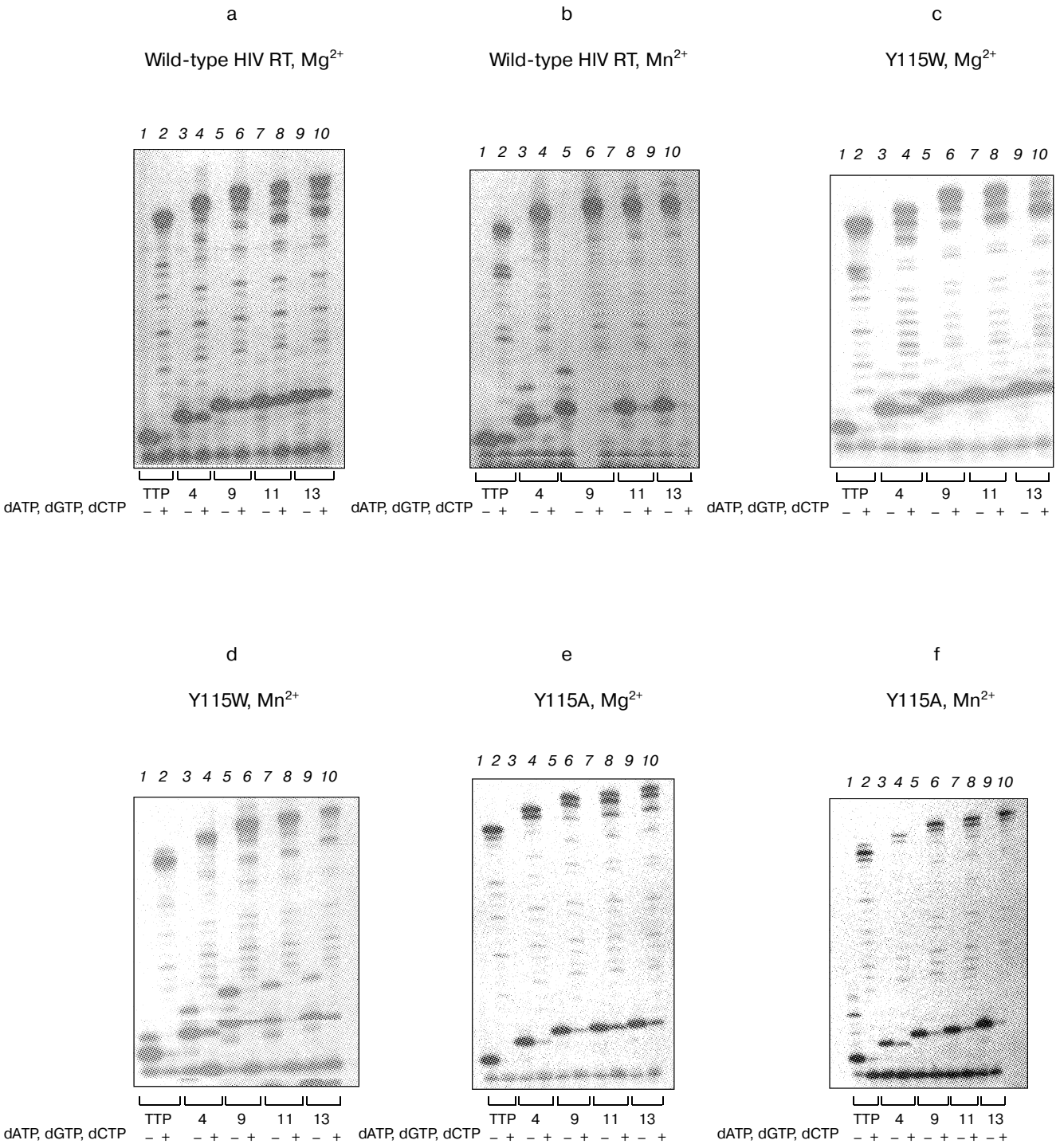

Fig. 3. Substrate properties of perfluoroarylazide dUTP-analogs in DNA synthesis catalyzed by wild-type HIV-1 RT in the presence of $MgCl_2$ (a) or $MnCl_2$ (b); Y115W in the presence of $MgCl_2$ (c) or $MnCl_2$ (d); Y115A in the presence of $MgCl_2$ (e) or $MnCl_2$ (f). The odd lanes show the results of electrophoretic separation of DNA-synthesis reaction products in the presence of TTP or its analog: the reaction mixtures contained HIV-1 RT or one of its mutant forms and TTP or corresponding FAB-*n*-dUTP along with the standard components (see "Materials and Methods"). The even lanes show the results of electrophoretic separation of DNA-synthesis reaction products in the presence of all four dNTPs: the reaction mixtures contained HIV-1 RT or one of its mutant forms, template-[^{32}P]primer, TTP or corresponding FAB-*n*-dUTP, dATP, dCTP, and dGTP, along with the standard components.

Replacing $MgCl_2$ with $MnCl_2$ causes the wrong incorporation of one or two FAB-*n*-dUTP residues by both the wild-type enzyme and the mutant form Y115W (lanes *3*, *5*, *7*, and *9* on the panels b and d), whereas Y115A incorporates a single residue of the reagent (lanes *3*, *5*, *7*, and *9* on panel f).

When dTTP analogs substitute for the native dTTP in the presence of the other three dNTPs and magnesium ions, the bands corresponding to full-size products decrease in density (lanes *4*, *6*, *8*, and *10* on the panels a, c, and e). This is apparently due to the modification of the DNA geometry with bulky substituents in the bases of the incorporated dTMP analogs.

Effect of amino acid substitution on dNTP binding. Site-directed mutagenesis results earlier showed that Tyr115 plays an important role in dNTP binding [5-7]. The enzyme activity was found to depend on the hydrophobicity of the side chain of the amino acid in position 115.

Steady-state kinetic parameters for the incorporation of the first nucleotide in the primer 3′-end were determined in the presence of $MgCl_2$ or $MnCl_2$ (table). As shown in the table, substitution of Tyr115 decreased the affinity of HIV-1 RT for dNTP, in agreement with previously reported data [5, 6]. Substitutions of Tyr115 had a relatively small effect on the k_{cat} values. On the other hand, the K_m values for dTTP varied from 0.29 μM for the wild-type enzyme to 98.5 μM for Y115A in the presence of $MgCl_2$, but varied moderately in the presence of $MnCl_2$ (from 1.3 μM for both wild-type HIV-1 RT and Y115W to 4.6 μM for Y115A). Thus, substitution of Tyr115 has virtually no effect on the K_m value in the presence of manganese ions, whereas a 300-fold difference in affinity exists between wild-type HIV-1 RT and Y115A in the presence of magnesium ions. Perhaps the nearby environment of the nucleotide varies significantly depending on the presence of magnesium or manganese ions. Indirect evidence for this conclusion lies in the fact that the K_m value for dNTP is nearly equal to that for ddNTP in the presence of manganese, but it is 100 times lower than that for ddNTP in the presence of magnesium ions [7].

The K_m values of dTTP analogs is 5-20 times greater than that of dTTP for wild-type HIV-1 RT as well as for both mutant forms in the presence of either $MgCl_2$ or $MnCl_2$. We were not able to determine the exact K_m values for dUTP derivatives in DNA synthesis catalyzed by Y115A in the presence of magnesium ions because of extremely high concentrations of dTTP analogs would be required.

Effect of substituent in the base on the nucleotide binding. The efficiencies of the incorporation of dTTP or its analogs (*f*) were determined as V_{max} to K_m ratios. The selectivities for the series of FAB-*n*-dUTP analogs are

Kinetic parameters for the reactions of dTTP, FAB-4-dUTP, and FAB-13-dUTP incorporation into the primer 3′-end catalyzed by HIV-1 RT and its mutant forms in the presence of either magnesium or manganese ions

Enzyme	dTTP			FAB-4-dUTP				FAB-13-dUTP			
	V_{max}, nM/min	K_m, μM	*f* *	V_{max}, nM/min	K_m, μM	*f*	$f_{dTTP}/f_{FAB\text{-}4\text{-}dUTP}$**	V_{max}, nM/min	K_m, μM	*f*	$f_{dTTP}/f_{FAB\text{-}13\text{-}dUTP}$
	In the presence of Mg^{2+}										
HIV RT***	30.4 ± 3.4	0.29 ± 0.08	105	29.5 ± 8.0	3.8 ± 0.9	7.76	13.7	29.7 ± 5.1	3.9 ± 1.8	7.61	13.6
Y115W	8.5 ± 3.4	10.9 ± 1.7	0.78	9.2 ± 3.2	50.1 ± 24.4	0.18	4.2	4.6 ± 1.4	72.2 ± 24.1	0.064	12.1
Y115A	12.7 ± 3.1	98.5 ± 34.2	0.13	n.d.	>200	n.d.	n.d.	n.d.	>200	n.d.	n.d.
	In the presence of Mn^{2+}										
HIV RT***	13.7 ± 1.0	1.3 ± 0.3	10.5	11.3 ± 1.7	27.3 ± 9.2	0.41	26.3	12.1 ± 2.3	33.4 ± 10.0	0.36	30.0
Y115W	7.9 ± 0.6	1.3 ± 0.4	6.1	8.7 ± 2.7	21.9 ± 10.7	0.40	15.2	9.7 ± 2.5	37.8 ± 11.5	0.26	23.7
Y115A	14.0 ± 0.6	4.6 ± 1.5	3.0	9.1 ± 0.9	26.6 ± 9.5	0.34	8.8	5.9 ± 0.9	27.3 ± 7.2	0.22	13.9

Note: Kinetic parameters are given in terms of mean value ± standard deviation; n.d., not determined.

* Incorporation efficiency values for dTTP or its analogs were calculated as V_{max} to K_m ratios for corresponding dNTPs.

** Selectivity values for dTTP in relation to perfluoroarylazide derivatives are given in terms of the ratios of *f* values for dTTP and corresponding FAB-*n*-dUTP.

*** Wild-type enzyme.

given as f_{dTTP} to $f_{FAB\text{-}n\text{-}dUTP}$ ratios. Selectivity values do not vary for both perfluoroarylazide dUTP derivatives in the case of both the wild-type enzyme and Y115W in the presence of $MgCl_2$. The efficiency of native dTTP incorporation is about 10 times higher that that of both analogs. The selectivity values in the case of Y115A in the presence of $MgCl_2$ were not calculated because the exact K_m values were not determined. Although the selectivity did not differ significantly between the wild-type HIV-1 RT and the mutant Y115A in the presence of $MnCl_2$, it tended to decrease.

Mutations in position 115 appear to be crucial for dTTP binding in the presence of $MgCl_2$, especially in the case of dTTP analogs. HIV-1 RT with an aromatic amino acid residue in position 115 possesses higher affinity to dTTP than Y115A, which contains the small hydrophobic amino acid residue. These observations are consistent with previously reported data from site-directed mutagenesis [5-7]. Michaelis constant values for dTTP and its analogs in the presence of $MnCl_2$ were within the same order of magnitude for all three enzymes. Hence, the linker length has virtually no effect on the binding of dTTP analogs.

Photoaffinity modification of HIV-1 RT and its mutant forms. Direct photomodification. When the enzyme is incubated with photoactive dTTP-analog in the presence of template-primer, the photoreactive nucleotide incorporates into the 3′-end of the primer, which binds to the enzyme under UV irradiation. On excitation of the photoreactive group of perfluoroarylazide analog with UV radiation at wavelength >280 nm, both HIV-1 RT subunits undergo photomodification resulting in at least four modification products (Fig. 4, lanes *1-4*). According to the data of X-ray structure analysis, the active site of the small subunit is in a closed state and unable to bind substrates [2]. Small subunits may form some fraction of homodimers possessing low DNA polymerase activity [17]. This homodimer has an active site that can bind substrates. We think that the modification of the small subunit may occur after the dissociation of the photoreactive primer from the active site located on the large subunit.

Some additional products have also been observed on modification of various DNA polymerases with various photoreactive dNTP-derivatives [10-12, 24, 30]. It should be noted that the electrophoretic mobility of the products of protein modification at various amino acids may vary significantly [44], so we suggest that the multiplicity of HIV-1 RT modification products may result from several acceptors disposed near the active site and accessible for modification with these derivatives.

Notice that the second from bottom product of HIV-1 RT modification decreases in density as the linker joining the photoreactive group and the reagent base is extended (Fig. 4, lanes *1-4*). To explain this fact, identification of the modification target is required. However, we propose that as the linker length changes, some changes occur in the group of amino acids accessible to modification by the perfluoroarylazide group of dTTP analogs. The multiple products of HIV-1 RT modification with dTTP derivatives cannot result from improper incorporation of two or more FAB-*n*-dUMP residues into the primer because the quantity of primer extended by more than one FAB-*n*-dUMP residue is negligible (data not shown).

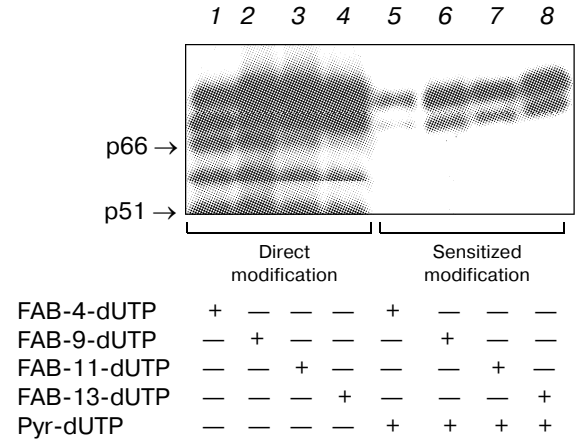

Fig. 4. Direct and photosensitized modifications of wild-type HIV-1 RT with perfluoroarylazide dUTP-derivatives of various linker lengths. The reaction mixtures contained HIV-1 RT, template-[^{32}P]primer, FAB-*n*-dUTP, Pyr-dUTP, and standard components (see "Materials and Methods"). Lanes *1-4* display results of electrophoretic separation of direct modification products: HIV-1 RT was incubated with template-[^{32}P]primer and corresponding FAB-*n*-dUTP and then irradiated as described in "Materials and Methods" for the direct modification. Lanes *5-8* display results of sensitized modification of HIV-1 RT with photoreactive dUTP-derivatives: HIV-1 RT was incubated with template-[^{32}P]primer and corresponding FAB-*n*-dUTP, then Pyr-dUTP was added, and the mixture was irradiated as described in "Materials and Methods" for photosensitized modification. On the left: positions of the large (p66) and small (p51) HIV-1 RT subunits.

Modification of HIV-1 RT and its mutants with FAB-4-ddUTP. As shown in Fig. 3, photoreactive dNTP-derivatives do not terminate the DNA synthesis. The dNTPs are also present in cell or nuclear extracts and can elongate primers after the photoreactive dNMP-analog incorporation. As this takes place, multiple primers are produced that contain the photoreactive group in various positions about the dNTP-binding site, thus complicating the interpretation of the data.

FAB-4-ddUTP was used for the synthesis of photoreactive primers that are not able to extend due to the absence of the 3′-hydroxyl group. The efficiency of modification of HIV-1 RT and its mutant form with the aid of primers carrying FAB-4-ddUMP on their 3′-ends and the

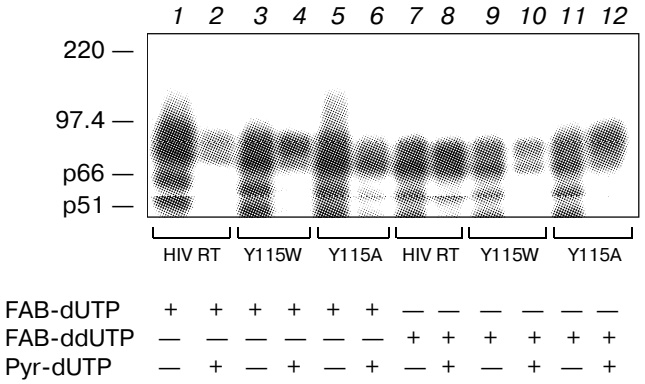

Fig. 5. Directed and photosensitized modifications of wild-type HIV-1 RT and its mutant forms with FAB-4-dUTP or FAB-4-ddUTP. The reaction mixtures contained HIV-1 RT, template-[^{32}P]primer, either FAB-4-dUTP or FAB-4-ddUTP, Pyr-dUTP, and the standard components listed in "Materials and Methods". The odd lanes display the results of electrophoretic separation of direct modification products: HIV-1 RT or its mutant forms were incubated with template-[^{32}P]primer and either deoxy- or dideoxy-UTP derivative and then irradiated as described in "Materials and Methods" for the direct modification. The even lanes display the results of sensitized modification of HIV-1 RT and its mutant forms with photoreactive dUTP-derivatives: the enzymes were incubated with template-[^{32}P]primer and corresponding dUTP analog, then Pyr-dUTP was added, and the mixtures were irradiated as described in "Materials and Methods" for the sensitized modification. On the left: positions of molecular weight standards and the large (p66) and small (p51) HIV-1 RT subunits (molecular mass values (kD) are given).

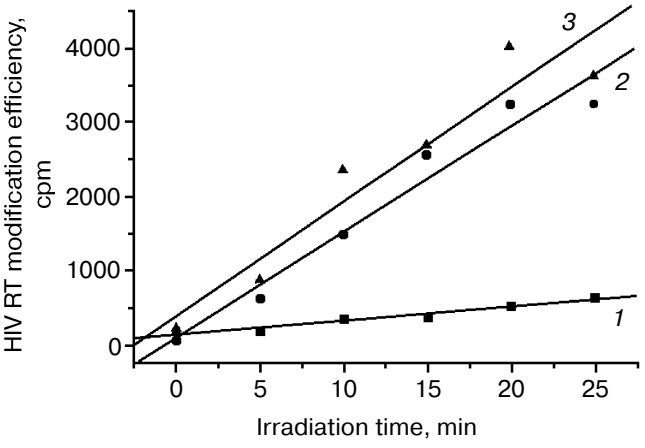

Fig. 6. HIV-1 RT modification efficiency in the absence (*1*) and in presence of various sensitizers—1-pyrenebutyric acid (*2*) and Pyr-dUTP (*3*).

product spectrum are virtually the same as those obtained using FAB-4-dUTP (Fig. 5). The number of modification products is reduced from four to two in the presence of a sensitizer (Fig. 5, even lanes). Thus, the same acceptor set is accessible for both the modification with FAB-4-ddUTP and modification with FAB-4-dUTP.

Photosensitized modification. We used photosensitized modification to increase the specificity of the labeling. When a photosensitizer is applied, only the catalytic subunit of HIV-1 RT is modified, and the number of modification products is reduced to two (Fig. 4, lanes *5-8*). Both HIV-1 RT mutants are also susceptible to both direct and sensitized modification producing products like those of the wild-type enzyme (Fig. 5, lanes *1-6*). It is necessary, however, to measure the kinetic parameters for the DNA-synthesis reactions catalyzed by the wild-type HIV-1 RT and its mutant forms in the presence of Pyr-dUTP to correctly estimate the contribution of the Tyr115 residue to the binding of the sensitizer.

Efficiency of HIV-1 RT photomodification in the presence of various sensitizers. The dependences of covalent addition of a photoreactive primer to the protein in the presence of Pyr-dUTP or 1-pyrenebutyric acid on the irradiation time are shown in Fig. 6. The initial rate of modification in the presence of Pyr-dUTP is 10 times higher than the rate of direct modification (Fig. 6, curves *3* and *1*, respectively). In the presence of 1-pyrenebutyric acid, the modification rate is less than twice lower than in the presence of a sensitizer containing a nucleotide residue (Fig. 6, curve *2*). Similar experiments were performed earlier on eucaryotic DNA polymerase β [19] and thermostable DNA polymerase from *Thermus thermophilus* [20]. The rate of photosensitized modification of these enzymes increased tenfold in the presence of Pyr-dUTP, but in the presence of 1-pyrenebutyric acid it was like that of direct modification. The sensitizing action of 1-pyrenebutyric acid on the HIV-1 RT photomodification process may be associated with a hydrophobic cavity near the enzyme active site in which the pyrenyl residue may bind. X-Ray structure analysis [45] suggests that this cavity could be the binding site of non-nucleoside HIV-1 RT inhibitors. The X-ray structure data also revealed such a cavity in the ternary HIV-1 RT–DNA–dNTP complex [4]. Thus, in the given system the sensitizer binding results not only from the specific interaction of the nucleotide part of the molecule with the enzyme dNTP-binding site, but also from the hydrophobic interactions of the pyrenyl residue with the protein.

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