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Affinity modification of human immunodeficiency virus reverse transcriptase and DNA template by photoreactive dCTP analogs

S.V. Doronin[a], M.I. Dobrikov[a], M. Buckle[b], P. Roux[b], H. Buc[b], O.I. Lavrik[a,*]

[a]*Institute of Bioorganic Chemistry, Siberian Division of the Russian Academy of Sciences, Prospekt Lavrentiev 8, 630090 Novosibirsk, Russian Federation*
[b]*Institut Pasteur, 25 Rue du Docteur Roux, F-75724 Paris cedex 15, France*

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Abstract New base-substituted analogs of dCTP containing an azido group have been synthesized and applied to a selective photoaffinity modification of HIV-RT (p66/p51 heterodimer). The labeling of only the 66 kDa subunit of HIV-RT was detected when the enzyme was first irradiated with the analogs and then template (5′-(d)GGTTAAATAAAATAGTAAGAATGTATAGCCCCTACCA-3′) and 5′ ^{32}P end-labeled 3′-(d)TTACATATCGGGGATGGT-5′ primer were added. The 5′ ^{32}P end-labeled primer elongated by dCTP analogs in the presence of both HIV-RT and DNA template is able to modify both subunits of HIV-RT and DNA template. This way of specific cross-linking to both DNA (RNA) template and HIV-RT opens up new possibilities to study the HIV-RT active site.

Key words: HIV reverse transcriptase; dCTP analog; Photoaffinity labeling

1. Introduction

A better understanding of the structure and function of HIV-RT is important for the search for specific inhibitors of AIDS. The method of affinity labeling is a very promising technique for both structure–function analysis and for selective inhibition of HIV-RT activity. dNTP analogs can be used as an effective tool for affinity labeling of HIV-RT.

In the present study two base-substituted analogs of dCTP were used for affinity labeling of HIV-RT and for the complementary addressed modification of DNA template in the ternary complex: enzyme–template–primer.

2. Materials and methods

Recombinant HIV-RT (heterodimer p66/p51) was purified from *E. coli* strain DH5 carrying pUC12N plasmid as described in [1] with some modifications. Non-radioactive dNTPs were purchased from Pharmacia. [γ-^{32}P]ATP (3000 Ci/mmol), [α-^{32}P]dTTP (3000 Ci/mmol) and protein molecular weight markers were purchased from Amersham. T_4 polynucleotidekinase was purchased from BRL. Other reagents were of analytical grade.

AlFABdCTP and FABdCTP were synthesized as described in [2,3].

Single-stranded synthetic DNA template and primer were prepared using LKB Gene Assembler and purified according to [4]. ^{32}P was introduced into the 5′-terminal position of the template and primer according to [4]. The primer was annealed to the template before being added to the reaction mixtures as described in [4,5]. A template/primer ratio of 2:1 was used in all experiments.

The standard mixture (50 μl) for measuring HIV-RT activity contained the following components: 50 mM Tris-HCl buffer, pH 7.8, 6 mM $MgCl_2$, 50 mM KCl, 1 mM DTT, 0.2 μM HIV-RT, 0.04 μM template–primer hybrid, dCTP and its analogs (0.01–100 μM). The reaction mixtures were incubated at 37°C. DNA synthesis and K_m and V_{max} values were determined as described in [4,5]. Quantitative measurements were conducted with PhosphorImager.

The composition of the reaction mixtures for photoaffinity labeling of HIV-RT was the same as for measuring HIV-RT activity. The reaction mixtures (10 μl) contained 0.2 μM HIV-RT, 50 μM of each of the dCTP analogs (FABdCTP or AlFABdCTP), 0.04 μM template–primer hybrid or 0.08 μM template: controls experiments were without DNA. 5′ ^{32}P end-labeled primer (3000 Ci/mmol) and [α-^{32}P]dTTP (3000 Ci/mmol) (5×10^{-5} M) were used. Photolysis was conducted by UV-light at a wavelength of > 300 nm.

The point of the DNA template modification was detected by gel-electrophoresis of the reaction mixture after piperidine treatment. DNA cleavage with piperidine was as described in [6]. Base-specific (G + A) cleavage of DNA was performed according to Maxam-Gilbert procedure described in [6].

3. Results and discussion

The structures of FABdCTP and AlFABdCTP are given in Fig. 1.

Fig. 2 shows the kinetics of the 5′-^{32}P-labeled primer extension with FABdCTP and AlFABdCTP by the catalytic activity of HIV-RT. As seen from Fig. 2, HIV-RT can use these analogs instead of dCTP in spite of the substitutions introduced into the dCTP base.

K_m and V_{max} values have been estimated for dCTP and both analogs. The K_m value for AlFABdCTP (1.7 μM) is similar to that of dCTP (2.6 μM) but FABdCTP exhibits an increase in K_m by one order of magnitude in comparison with dCTP (24 μM). V_{max} values for both analogs ($2.5 \cdot 10^{-2}$ s^{-1}) are nearly the same as that of dCTP. Therefore the analogs are useful substrates of this enzyme.

The incubation of 5′ ^{32}P end-labeled template in the presence of primer, FABdCTP and HIV-RT leads to the cleavage of template, probably due to some endonuclease impurities in the HIV-RT preparation (Fig. 3, lane 2). When the reaction mixture was UV-irradiated after primer elongation by FABdCTP, covalent attachment of 60% of the primer to the 5′ ^{32}P end-labeled template was detected (Fig. 3, lane 3). The primer covalent attachment to position G^{19} of the DNA template was shown by piperidine treatment of the modified DNA template (Fig. 3, lanes 6 and 7). Therefore 'complementary addressed modification' of the DNA template can perform by primer elongated with photoreactive dCTP analog in the presence of HIV-RT.

*Corresponding author. Fax: (7) (383) 235 1665.

Abbreviations: HIV-1, human immunodeficiency virus-1; HIV-RT, reverse transcriptase of HIV-1 (EC 2.7.7.49); FABdCTP, exo-*N*-[β-(*p*-azidotetrafluorobenzamido)-ethyl]-deoxycytidine 5′-triphosphate; AlFABdCTP, 5-[γ-*N*-(*p*-azidotetrafluorobenzamido)-aminoallyl]-deoxycytidine 5′-triphosphate; DTT, dithiothreitol.

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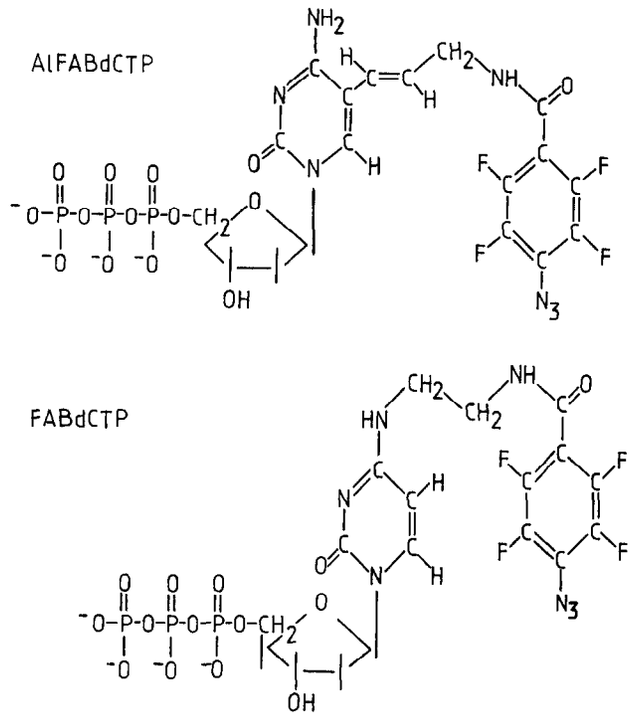

Fig. 1. Structural formula of AlFABdCTP and FABdCTP.

Fig. 4 shows the results of photoaffinity labeling of HIV-RT with FABdCTP. The primer elongation by FABdCTP or AlFABdCTP followed by UV-irradiation results in 5′ ^{32}P end-labeled primer attachment to both 66 and 51 kDa subunits of HIV-RT (Fig. 4, lane 4; Fig. 5, lane 5). In both experiments a high-molecular weight product (90 kDa) was detected. The nature of this product is under investigation.

The labeling of only the 66 kDa subunit of HIV-RT was detected when the enzyme was first irradiated with FABdCTP or AlFABdCTP and then the template and ^{32}P-labeled primer was added (Fig. 4, lanes 2 and 3; Fig. 5, lanes 3 and 4). The same pattern of subunit labeling was detected when the template–primer hybrid derivatized by FABdCTP was covalently linked to the enzyme and then elongated with [α-^{32}P]dTTP (Fig. 4, lane 5). Therefore, 'catalytically competent labeling' of HIV-RT takes place. This kind of affinity labeling was demonstrated earlier for several DNA and RNA polymerases [7].

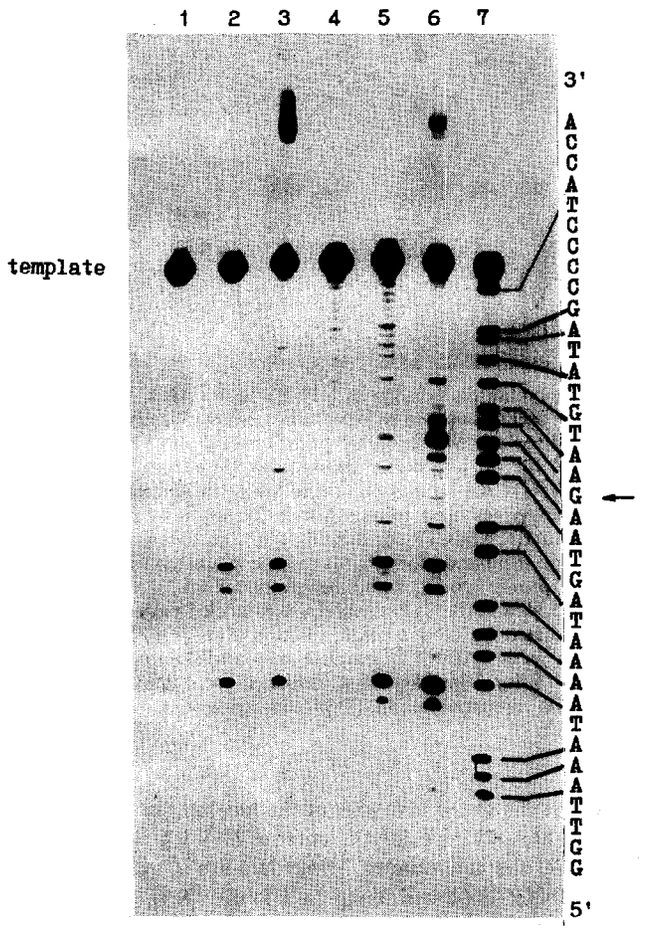

Fig. 3. 5′ ^{32}P end-labeled DNA template photoaffinity labeling with FABdCTP: (1) template; (2) template incubated for 5 min with enzyme, primer and FABdCTP; (3) template incubated for 2.5 min with enzyme, primer and FABdCTP and then UV-irradiated for 2.5 min; (4) template treated with piperidine; (5) template incubated for 5 min with enzyme, primer and FABdCTP and then treated with piperidine; (6) template incubated for 2.5 min with enzyme, primer and FABdCTP and then UV-irradiated for 2.5 min with the following treatment with piperidine; (7) G+A base-specific cleavage of the DNA template performed according to Maxam-Gilbert procedure.

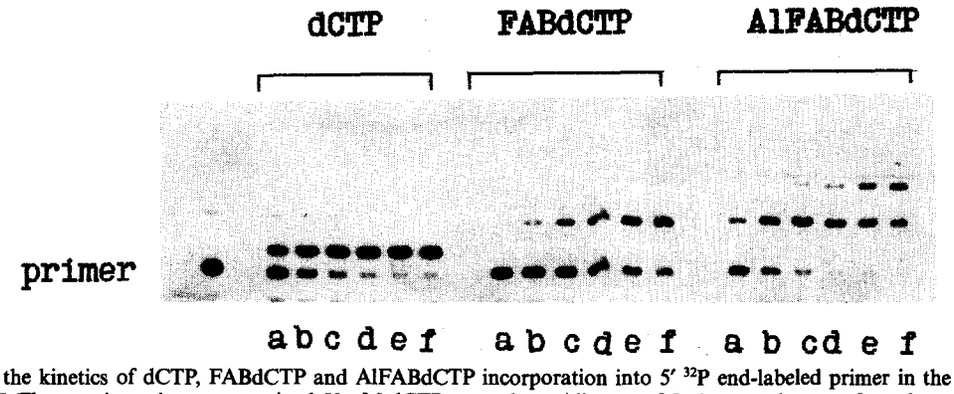

Fig. 2. Gel assay of the kinetics of dCTP, FABdCTP and AlFABdCTP incorporation into 5′ ^{32}P end-labeled primer in the presence of template catalyzed by HIV-RT. The reaction mixtures contained 50 μM dCTP or analogs. Aliquots of 5 μl were taken out for gel assay after 10 (a), 20 (b), 30 (c), 40 (d), 50 (e), 60 (f) s, respectively.

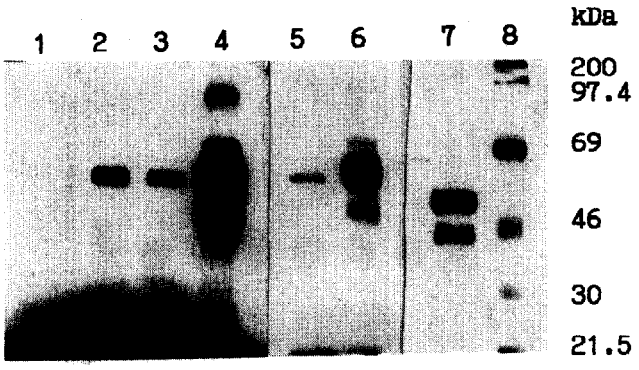

Fig. 4. Photoaffinity labeling of HIV-RT with FABdCTP. 5′ ^{32}P end-labeled primer was used in all experiments except (5) and (6): (1) enzyme incubated for 1 h with template–primer hybrid and FABdCTP (UV-irradiated for 2.5 min before incubation); (2) enzyme UV-irradiated with FABdCTP for 2.5 min with the following incubation for 1 h in the presence of the template–primer hybrid; (3) enzyme irradiated with the template and FABdCTP for 2.5 min with the following addition of primer and incubation for 1 h; (4) enzyme incubated with FABdCTP, template–primer hybrid 1 h and then irradiated for 2.5 min; (5) enzyme incubated with FABdCTP, template, non-labeled primer for 2.5 min, then irradiated for 2.5 min before addition of [α-^{32}P]dTTP; (6) enzyme incubated with FABdCTP, template–non-labeled primer hybrid and [α-^{32}P]dTTP for 3 min and then irradiated for 2.5 min; (7) HIV-RT (66/51 kDa) preparation; and (8) protein markers stained with G-250 Coomassie.

The results speak in favour of the localisation of the dNTP binding site and the catalytic center of HIV-RT on the 66 kDa subunit of the heterodimer. These data are in agreement with direct dNTP UV-crosslinking to HIV-RT [8,9], photoaffinity labeling of the enzyme with 4-thio UTP [10], and X-ray analysis data obtained for HIV-RT at 3 Å resolution [11].

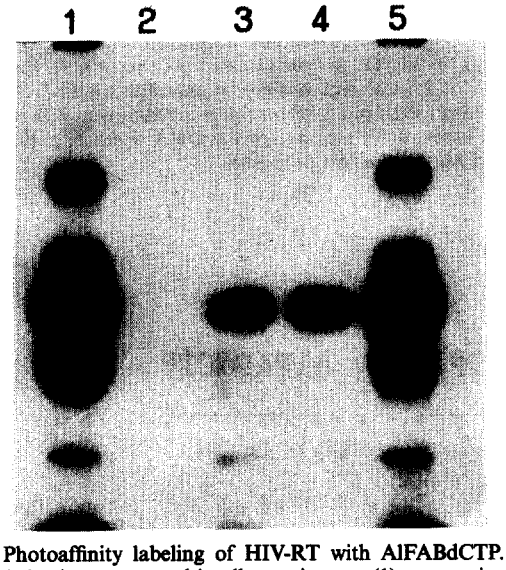

Fig. 5. Photoaffinity labeling of HIV-RT with AlFABdCTP. 5′ ^{32}P end-labeled primer was used in all experiments: (1) enzyme incubated with template–primer hybrid, AlFABdCTP for 2.5 min and UV-irradiated for 2.5 min; (2) enzyme incubated for 1 h with template–primer hybrid and AlFABdCTP UV-irradiated before incubation; (3) enzyme irradiated with AlFABdCTP for 2.5 min followed by incubation for 1 h in the presence of template and primer; (4) enzyme irradiated with AlFABdCTP and template followed by addition of primer and incubation for 1 h; (5) enzyme incubated with template, primer and AlFABdCTP, for 2.5 min followed by UV-irradiation for 2.5 min.

The labeling of both subunits after primer elongation by dCTP analogs followed by UV-irradiation of the complete reaction mixture (Fig. 4, lanes 4 and 6; Fig. 5, lanes 1 and 5) suggests the localisation of the template primer hybrid in the subunit contact region. This hypothesis is supported by our data obtained using photoreactive analogs of oligonucleotide primers containing azido groups at the 5′ end of the molecule [7].

Therefore both photoreactive analogs of dCTP are effective substrates of HIV-RT, and UV-irradiation of the reaction mixture induced the modification of both protein and DNA template in the ternary complex with the enzyme. Most of the 'reagent', namely photoreactive primer, synthesized in situ is used in the DNA modification. These base-substituted dNTP analogs can be envisioned as inhibitors of HIV replication mostly by the way of DNA (RNA) modification induced by the activity of HIV-RT.

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