Volume 312, number 2,3, 249–251 FEBS 11739 November 1992

Human immunodeficiency virus type 1 reverse transcriptase

Affinity labeling of the primer binding site

R.L. Mitina, S.V. Doronin, M.I. Dobrikov, D.R. Tabatadze, A.S. Levina and O.I. Lavrik

Novosibirsk Institute of Bioorganic Chemistry, Siberian Division of the Russian Academy of Sciences, Novosibirsk 630090, Russia

Received 3 September 1992

Affinity modification of the primer site of HIV1-RT was performed with an oligonucleotide derivative containing a photoreactive azido group at the 5′ end of $d(pT)_{10}$. The affinity of HIV1-RT for $d(pT)_{10}$ and for its derivative was first estimated by measuring the Michaelis constants of these two oligonucleotides acting as primers in the retrotranscription of poly(rA). The enzyme was then inactivated under UV-irradiation at 303–365 nm in the presence of ArN_3-$d(U^*T_9)$; the dependence of the rate of inactivation on primer concentration was found to be consistent with the K_m value. Last, selectivity of affinity modification was demonstrated through elongation of the covalently bound primer and selective protection of inactivation by $d(pT)_{10}$ or $tRNA^{Lys}$.

HIV1-RT; Photoaffinity modification; Primer-binding site

1. INTRODUCTION

HIV-RT catalyzes both RNA-directed and DNA-directed DNA synthesis in the cytoplasm shortly after retroviral infection of a cell. During the first step, the enzyme recognizes its natural primer $tRNA^{Lys}$ (subspecies 3). It subsequently performs retrotranscription, RNA degradation (via RNase H) template switching and DNA replication to yield a complete double-stranded DNA viral genome [1].

Several steps of this pathway are still open to debate. In particular, the initial recognition step could proceed via the recognition by the free enzyme of the preformed hybrid between primer and template [2–4]. Other studies favour a mechanism by which the free enzyme recognizes first the tRNA primer [5].

In both cases, the 3′ end of an oligonucleotide acting as primer must be crucial in the recognition process. Introduction of various modifying groups is therefore more easily done at other specific sites of the primer. In particular, 5′-end modified oligonucleotides are interesting tools to study the DNA polymerisation process, as previously shown for AMV-RT and DNA polymerase I from *E. coli* [6,7].

Correspondence address: R.L. Mitina, Institute of Bioorganic Chemistry, Lavrentieva st. 8, Novosibirsk 630090, Russia. Fax: (7) (383) 235 3459.

Abbreviations: HIV1-RT, reverse transcriptase of human immunodeficiency virus type-1; AMV-RT, reverse transcriptase from avian myeloblastosis virus; ArN_3-$d(U^*T_9)$, 5-*N*-(2′-nitro-5′-azidobenzoylglycyl)-aminoethyluridine nonathymidylate; NP-40, Nonidet-P40; BSA, bovine serum albumin.

2. MATERIALS AND METHODS

Oligo$(dT)_{10}$, poly(rA), dTTP were from NIKTI BAV (Russia). [^{3}H]dTTP (600 TBq/mol) from Izotop (Russia) and [α-^{32}P]dTTP (spec. act. >111 PBq/mol) from Izotop (Uzbekistan); DNase I from Fluka; $tRNA^{Lys}$ from yeast was purified according to method described for $tRNA^{Lys}$ from beef pancreas [8]. Other reagents were of analytical grade.

2.1. *Enzyme preparation*

The *E. coli* strain DH5 carrying pUC12N plasmid was described in [9] and kindly donated by Prof. H. Buc. The HIV1-RT homodimer (66/66) was obtained from overproducing strains of *E. coli* as described in [10]. The last agarose step was replaced by a chromatography on DNA-sepharose. Spontaneous cleavage of the homodimer was negligible.

2.2. *Synthesis of a photoreactive derivative of $d(pT)_{10}$: ArN_3-$d(U^*T_9)$*

The structure of this reagent is given in Fig. 1. Its synthesis as well as those of a succinimide ester of 2-nitro-5-azidobenzoic acid are described in [11,12].

2.3. *Enzyme modification under UV-irradiation: determination of HIV1-RT activity*

The reaction mixture for UV-irradiation contained (in 100 μl) the following standard components: 0.1 M Tris-HCl buffer (pH 8.2), 0.01 M $MgCl_2$, 0.15 M KCl, 0.1% NP-40, 0.1 mg/ml BSA, 10^{-8} M HIV1-RT, 10^{-6} to 10^{-8} M ArN_3-$d(U^*T_9)$. The reaction was started by UV-irradiation of the samples by a high pressure lamp (DPK-120) equipped with glass filters (BS-12 and UFS-1) providing irradiation in the range 303–365 nm. The power delivered was 5×10^{-4} J · s^{-1}. After 10, 20, 40, 80, and 160 s, aliquots (10 μl) were removed and added to the mixture (60 μl) for determination of HIV1-RT activity: 0.1 M Tris-HCl buffer (pH 8.2), 0.01 M $MgCl_2$, 0.15 M KCl, 10^{-3} M DTT, 0.1% NP-40, 0.1 mg/ml BSA, 10^{-6} M $d(pT)_{10}$, 10^{-5} M dTTP; 1 A_{260}/ml poly(rA), 1.5×10^{-3} Ci [^{3}H]dTTP.

The mixture used to monitor the HIV1-RT protection from inactivation contained the above standard components and 10^{-6} M oligo$(dT)_{10}$.

K_m values for $d(pT)_{10}$ and its analogue were estimated using the

program 'Enzfitter' (Control File Production Utility For Enzfitter Version 1.0 (C) Robin J. Leatherbarrow 1987). Errors were within 10%.

2.4. *Covalent labeling of HIV1-RT by ArN_3-d(U^*T_9)*

The reaction mixture (40 μl) for UV-irradiation contained: 0.1 M Tris-HCl buffer (pH 8.2), 0.01 M $MgCl_2$, 0.15 M KCl, 0.1% NP-40, 0.1 mg/ml BSA, 1 A_{260}/ml poly(rA), 1.8×10^{-6} M ArN_3-d(U^*T_9) and 10^{-7} M HIV1-RT. The mixture for the HIV1-RT protection from inactivation was supplemented with 10^{-5} M oligo(dT)$_{10}$ or 10^{-5} M yeast $tRNA^{Lys}$. After 3 min UV-irradiation 10^{-7} M [α-^{32}P]dTTP was added and the mixture was stored at 37°C for 1 h. The reaction mixture was then treated by DNase I (20 μg/ml) for 30 min at 37°C. The reaction was stopped by the addition of 10 μl of the following denaturing mixture: 5% SDS, 5% β-mercaptoethanol, 50% glycerol and 0.1% Bromophenol blue. The mixture was heated at 56°C for 10 min and electrophoresed according to Laemmli [13] in 10% acrylamide gel. The proteins were stained with G-250 Coomassie brilliant blue. The position of HIV1-RT subunits (66 kDa) was determined by using a calibration kit of proteins for SDS-electrophoresis from Pharmacia. The dried gels were then subject to radioautography.

3. RESULTS AND DISCUSSION

ArN_3-d(U^*T_9) appears to be a good substrate in the reaction of DNA polymerization catalyzed by HIV1-RT on a poly(rA) template. Using the methodology previously developed (cf. [14]), the K_m value for ArN_3-d(U^*T_9) is found to be equal to 1.6×10^{-7} M, while for the initial d(pT)$_{10}$ it is 8.5×10^{-8} M. It was previously found that the difference between the K_m and K_d values for the primers of DNA polymerases is generally negligible [14,15], so K_m may be used as a measure of their affinity. The K_d value for the photoreactive derivative is obtained according to Kitz and Wilson [16] using initial rates of enzyme inactivation under UV-irradiation, in the presence of increasing concentrations of ArN_3-d(U^*T_9) and in the absence of a complementary template. A K_d value around 4×10^{-7} M is found while at saturation, HIV1-RT activity is reduced by nearly 40% (Fig. 2). Qualitatively similar results are obtained when the hybrid between the template and the primer is preformed before irradiation. They will be presented elsewhere. Without the reagent, HIV1-RT loses only 10% of its initial activity under the same conditions; the oligonucleotide without the photoreactive group protects the enzyme from inactivation (Fig. 2).

This last observation, as well as the fact that the Michaelis constant is within experimental error equivalent to the dissociation constant, suggests that the photoaffinity modification of HIV1-RT by ArN_3-d(U^*T_9) should be directed to or near the site of enzyme interaction with oligo(dT)$_{10}$, at the primer or template binding site, though interactions with other nucleotide binding domains are also possible.

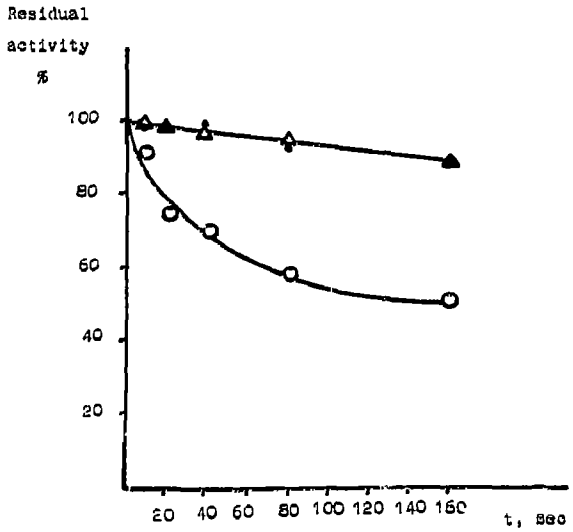

Fig. 2. Kinetic curves of HIV1-RT inactivation with ArN_3-d(U^*T_9) under UV-irradiation. Inactivation of HIV1-RT with 10^{-7} M ArN_3-(dU^*T_9) (○); without ArN_3-d(U^*T_9) (●); with 10^{-7} M ArN_3-d(U^*T_9) and 10^{-6} M oligo(dT)$_{10}$ (△). Other reaction conditions are specified in section 2.

To find out which nucleotide-binding domains are indeed involved in the reaction, we applied a special approach. The most convincing argument in favour of selectivity of affinity modification is the ability of a covalently bound residue of an affinity reagent to be transformed by the active center into a covalently bound residue of a product [17]. The method of selective labeling based upon 'catalytic competence' was intensively developed for analysis of DNA-dependent RNA polymerases [18]. In our case, the derivative of oligo(dT)$_{10}$ covalently bound with the active center of

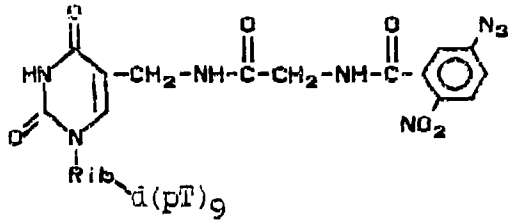

Fig. 1. Structure formula of the oligonucleotide derivative containing a photoreactive azido group on the 5′ end of d(pT)$_{10}$: ArN_3-d(U^*T_9).

1 2 3 4

Fig. 3. Radioautograph of electrophoretic patterns obtained for the HIV1-RT affinity, labeled with ArN_3-d(U^*T_9) and transformed into product by [^{32}P]dTMP incorporation. Labeling of HIV1-RT (66/66) with ArN_3-d(U^*T_9) in the presence of poly(rA) (1); poly(rA) was absent during the irradiation and present during the conversion of the label into product (2); affinity labeling performed in the presence of poly(rA) and oligo(dT)$_{10}$ (3); or $tRNA^{Lys}$ (4). Other reaction conditions are as stated in section 2.

the enzyme can be elongated by attachment of [α-^{32}P]dTMP from [α-^{32}P]dTTP in the presence of a complementary poly(rA) template. In this way a ^{32}P label is covalently bound to the enzyme. The derivatives bound outside the center do not catch radioactivity and remain invisible during the subsequent analysis.

The data on catalytic competent labeling of HIV1-RT are presented in Fig. 3. It has been shown that the p66 subunit of a homodimeric HIV1-RT (66/66) is labeled under these conditions.

No covalent labeling of HIV1-RT is observed during preliminary incubation of the enzyme with the reagent in the presence of oligo(dT)$_{10}$ or native primer tRNA^{Lys} (see Fig. 3, lanes 3 and 4).

Maximum labeling of HIV1-RT (66/66) is observed when the template, poly(rA), is added before UV-irradiation of the enzyme and the affinity reagent. If the template is added after UV-irradiation, together with the [α-^{32}P]dTTP substrate, the labeling is less extensive (compare lanes 1 and 2, Fig. 3).

Therefore the catalytic competence of the ArN_3-d(U*T$_9$) residue covalently bound with HIV1-RT favours an affinity modification of HIV1-RT primer-binding site. Because the most effective labeling of primer site takes place in the presence of a template, the efficiency of primer binding is template-dependent. However, the modification partially occurs in the absence of a complementary template showing that the primer can also interact with the free HIV-RT.

This efficient modification of the primer-binding site of HIV1-RT by photoaffinity analogue of d(pT)$_{10}$, will allow a detailed analysis of the mechanism of DNA polymerization catalyzed by HIV1-RT.

Acknowledgements: The authors thank Prof. H. Buc for careful reading and correcting the manuscript.

REFERENCES

[1] Gilboa, E., Mitra, S.W., Goff, S. and Baltimore, D. (1979) Cell 18, 93–100.
[2] Majumdar, C., Abbotts, J., Broder, S. and Wilson, S.H. (1988) J. Biol. Chem. 263, 15657–15665.
[3] Majumdar, C., Stein, C.A., Cohen, J.S., Broder, S. and Wilson, S.H. (1989) Biochemistry 28, 1340–1346.
[4] Kedar, P.S., Abbotts, J., Kovacs, T., Lesiak, K., Torrence, P. and Wilson, S.H. (1990) Biochemistry 29, 3603–3611.
[5] Sobol, R.W., Suhadolnik, R.J., Kumar, A., Lee, B.J., Hatfield, D.L. and Wilson, S.H. (1991) Biochemistry 30, 10623–10631.
[6] Lokhova, I.A., Nevinsky, G.A., Godovikova, T.S., Ivanova, E.M., Koshkin, A.A., Sergeev, D.S., Frolova, E.I., Rudenko, N.K., Khomov, V.V., Kavsan, V.M., Zarytova, V.F. and Lavrik, O.I. (1991) FEBS Lett. 281, 111–113.
[7] Mitina, R.L., Mustaev, A.A., Zaychikov, E.F., Khomov, V.V. and Lavrik, O.I. (1990) FEBS Lett. 272, 181–183.
[8] Fournier, M., Dorizzi, M., Serjer, C. and Labouesse, G. (1976) Biochimie 58, 1159–1165.
[9] Hizi, A., McGill, C. and Hughes, H. (1988) Proc. Natl. Acad. Sci. USA 85, 1218–1222.
[10] Hansen, S., Shulze, T. and Moelling, K. (1987) J. Biol. Chem. 262, 12393–12396.
[11] Dobrikov, M.I., Prichodjko, T.A., Safronov, I.V. and Shishkin, G.V. (1991) Siberian Chem. J. (in press).
[12] Dobrikov, M.I., Zarytova, V.F., Komarova, N.I., Levina, A.S., Lokha, A.S., Prikhodjko, T.A., Shishkin, G.V., Tabatadze, D.R. and Zaalishvili, M.M. (1991) Bioorg. Khim. (in press).
[13] Laemmli, U.K. (1970) Nature 227, 680–685.
[14] Nevinsky, G.A., Levina, A.S., Podust, V.N., Lavrik, O.I. and Holler, E. (1990) Biochemistry 29, 1200–1207.
[15] Nevinsky, G.A., Podust, V.N., Levina, A.S., Khalabuda, O.V. and Lavrik, O.I. (1985) Bioorg. Khim. 13, 357–369.
[16] Kitz, R. and Wilson, B.J. (1962) J. Biol. Chem. 273, 3245–3249.
[17] Groman, E.V., Schultz, R.M. and Engel, L.L. (1977) Methods Enzymol. 46, 54–59.
[18] Grachev, M.A., Kolocheva, T.I., Lukhtanov, E.A. and Mustaev, A.A. (1987) Eur. J. Biochem. 163, 113–121.