Structure–activity relationships in HIV-1 reverse transcriptase revealed by radiation target analysis

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Abstract

Radiation target analysis is a powerful technique that can be used to determine both the structural and functional sizes of macromolecules. We have used this technique to probe the structure–function relationships of the recombinant forms of HIV-1 reverse transcriptase (RT). For the p66/p51 and p66/p66 dimeric forms of HIV-1 RT, both the structural and functional target sizes corresponded to that of the dimeric protein, indicating that a primary ionization in one subunit of the HIV-1 RT enzyme results in the concomitant polymer scission of both subunits. In contrast to p66/p51 and p66/p66 RT, the individually isolated p51 subunit of HIV-1 RT inactivated as a monomer. However, in the presence of a DNA template/primer substrate, the radiation inactivation analyses of p51 yielded a structural target size corresponding to that of a dimeric protein. This would indicate that the DNA substrate acted as a scaffold or template for p51 RT homodimer formation. In light of this observation, radiation inactivation studies can readily be applied to other DNA polymerase enzymes, such as the murine leukemia virus RT, for which the functional form of the enzyme has yet to be determined.

Keywords: HIV-1; reverse transcriptase; radiation inactivation; dimer; monomer

HIV-1 reverse transcriptase (RT) is a multifunctional enzyme responsible for conversion of the viral single-stranded RNA genome into double-stranded DNA. RT exhibits two enzymatically distinct activities: a DNA polymerase activity that synthesizes DNA by using either RNA or DNA templates (termed RNA-dependent [RDDP] or DNA-dependent DNA polymerase [DDDP] activity, respectively) and a ribonuclease H (RNase H) activity that degrades the RNA strand of RNA/DNA hybrids (Telesnitsky and Goff 1997). HIV-1 RT is an asymmetric heterodimer composed of a 560-amino-acid 66-kD subunit (p66) and a 440-amino-acid

51-kD (p51) subunit. The p51 polypeptide is derived from the p66 by proteolytic cleavage of its C-terminal RNase H domain (di Marzo Veronese et al. 1986). The p66/p51 HIV-1 RT heterodimer contains one DNA polymerization active site and one RNase H active site, which both reside in the p66 subunit at spatially distinct regions (Kohlstaedt et al. 1992). Although the p51 subunit contains the same amino acid sequence that comprises the DNA polymerase domain of the p66 subunit, the polymerase active site in p51 is not functional (Wang et al. 1994). Numerous structural studies have indicated that the p66 subunit shows an overall architectural similarity to the Klenow fragment of *Escherichia coli* DNA polymerase I, and consists of the “fingers,” “palm,” “thumb,” and “connection” subdomains (Kohlstaedt et al. 1992; Jacobo-Molina et al. 1993; Ren et al. 1995; Rodgers et al. 1995; Das et al. 1996; Hsiou et al. 1996; Huang et al. 1998; Sarafianos et al. 2001). The p51 subunit contains these same polymerase subdomains; however, their spatial arrangement differs markedly to those of the p66 subunit. Although the p66 subunit adopts an “open” cata-

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lytically-competent conformation that can accommodate a nucleic acid template strand, the p51 subunit is in a "closed" conformation and is considered to play a largely structural role (Wang et al. 1994).

The development of prokaryotic expression vectors encoding the amino acid sequences for RT p66 and p51 has enabled the preparation of large quantities of the p66/p51 form of RT, as well as the pure p51 and p66 polypeptides (Le Grice et al. 1995; Fletcher et al. 1996). The isolated p66 polypeptides dimerize to form p66/p66 RT homodimers (Restle et al. 1990). Furthermore, p66/p66 HIV-1 RT exhibits levels of DNA polymerase and RNase H activities similar to those of the p66/p51 RT heterodimer (Fletcher et al. 1996). However, differences in the affinity of the inter-subunit interactions between the p66/p51 and p66/p66 RT enzymes have been noted (Divita et al. 1995; Sluis-Cremer et al. 2000). The dissociation constant (K_d) for the p66/p51 RT heterodimer has been calculated to be 150 nM, whereas the K_d for the p66/p66 RT homodimer is only 2.7 μ M (Divita et al. 1995; Sluis-Cremer et al. 2000). In contrast to the RT p66 polypeptide, the p51 subunit exhibits a low propensity to self-dimerize—the K_d for p51/p51 RT homodimer formation has been estimated to be 670 μ M (Restle et al. 1990). Despite this inability to form stable dimers, a significant level of DNA polymerase activity has been associated with the purified RT p51 subunit (Bavand et al. 1993; Fletcher et al. 1996; Dufour et al. 1998). This latter observation is particularly surprising when one considers that the DNA polymerase activities of RT are confined exclusively to dimeric forms of the enzyme (Restle et al. 1990).

In this study, we have evaluated the importance of the inter-subunit interactions on the enzymatic functioning of the recombinant forms of HIV-1 RT by using radiation inactivation, a technique that has proven to be very useful in defining the structure–function relationships of a large number of other proteins (Kempner 1999). Radiation target analysis is based on the direct action of ionizing radiation on macromolecules (Harmon et al. 1985), which can be achieved by irradiation at very low temperatures. Under these conditions, radiation energy from a single electron impact anywhere in a polypeptide chain is transmitted throughout the polypeptide, cleaving the polymer backbone and destroying all function. Based on the fact that the probability of a single electron impact on a protein molecule is directly proportional to the mass of the polypeptide, this technique can be used to determine the target size of both the structure of the polypeptide and the enzymatic activity associated with it (Saccomani et al. 1981). The radiation-sensitive mass of a variety of enzymes has been determined by radiation inactivation (Kempner 1988). Our results show some unexpected and interesting relationships between the subunits of HIV-1 RT and the effects that the nucleic acid template/primer (T/P) substrate has on them.

Results

Target size of the recombinant forms of HIV-1 RT determined by SDS–gel electrophoresis

Radiation damage in proteins includes the destruction of covalent bonds in the backbone of polypeptide chains, which results in the formation of fragmented polymers. These fragments separate from the intact polypeptide on SDS-PAGE, and the analysis of the remaining intact polypeptide yields the target size of the radiation-sensitive structure (Saccomani et al. 1981). In the irradiated HIV-1 RT samples, the Coomassie dye stain intensity of the 66,000 and 51,000 Mr bands, resolved by SDS–gel electrophoresis, permitted the estimation of the residual amount of each RT subunit as a function of radiation dose. The inactivation profiles for the p66 subunit in both the p66/p51 and p66/p66 forms of RT, as well as the p51 subunit in the p66/p51 form of RT, could mathematically be described as simple exponential decays (Fig. 1A). However, the average target sizes for the physical structures of the individual p66 and p51 RT subunits were not calculated to be 66 or 51 kD as expected, but ranged from 115 to 123 kD (Table 1). These results imply that a single radiation interaction in one polypeptide resulted in gross structural damage in the other subunit, presumably due to efficient transfer of some of the radiation-deposited energy between the subunits. In contrast to the p66/p51 and p66/p66 RT dimeric proteins, the purified RT p51 subunit yielded a target size of 44 kD (Table 1), indicating that the individually isolated subunit inactivated structurally as a monomer. This result can be interpreted in one of two ways: Either the p51 subunit exists as a stable

Figure 1. Radiation inactivation profiles for p66/p51, p66/p66, and p51 HIV-1 RT determined by SDS-PAGE (A) and enzyme activity (B). (A) The p66 and p51 subunits of p66/p51 RT are designated by solid circles and open circles, respectively; the p66 subunits in p66/p66 RT, by solid triangles; and p51 RT, by open triangles. (B) p66/p51 RT is designated by solid circles; p66/p66 RT, by open circles; and p51 RT, by triangles. The data shown are from three independent experiments and are reported as mean $\pm$ 1 SD.

Table 1. Target sizes (in kD) for HIV-1 reverse transcriptase

Enzyme	Structural target size		Functional target size		
	p66	p51	RDDP	DDDP	RNase H
p66/p51 RT	121.5 ± 17.7	123 ± 15.8	132.4 ± 25.1	112.2 ± 10.5	104.0 ^b
+T/P (126.3 kD)	132.8 ^a	123.9 ^a	131.7 ^a	133.0 ^a	120.6 ^a
+T/P (15.5 kD)	93.9 ^a	95.0 ^a	91.5 ^a	87.9 ^a	87.6 ^a
p66/p66 RT	114.9 ± 9.2	—	117.0 ± 6.1	117.3 ± 4.4	111.6 ^b
p51 RT	—	43.7 ± 5.0	—	52.9 ± 4.7	—
+T/P (126.3 kD)	—	107.9 ^a	—	— ^c	—
+T/P (15.5 kD)	—	95.4 ^b	—	— ^c	—

Unless otherwise stated all experiments were carried out in triplicate, and the data are reported as an average value ± SD.

^a Target size derived from a single experiment.

^b Average target size derived from two independent experiments.

^c Radiation inactivation profiles were complex and composed of two exponents. See Fig. 2.

monomer in solution at the protein concentration used in this study (2 µg/µL = 40 µM), or that the inter-subunit energy transfer, observed in p66/p51 RT and p66/p66 RT, does not occur in a putative p51/p51 RT homodimer. Size-exclusion high-performance liquid chromatography (SEC-HPLC) analyses favor the first alternative (Fig. 2); however, we can not rule out the possibility of weakly interacting p51 subunits forming p51/p51 homodimers that are unstable during the SEC-HPLC chromatographic run.

Figure 2. SEC-HPLC elution profiles of HIV-1 p66/p51 (A) and p51 (B) RT. SEC-HPLC was carried out as described in Materials and Methods. The SEC column was equilibrated by using molecular mass markers for gel filtration chromatography ranging in size from 12,000 to 200,000 D. The void volume of the column is indicated (V_0). (A) The p66/p51 RT eluted as a dimeric protein with an apparent molecular weight of 117,000. The p66/p66 RT homodimer also eluted as a single peak yielding an apparent molecular weight consistent with its dimeric structure (data not shown). (B) The individually isolated p51 subunit eluted as a monomer; the apparent molecular weight was determined to be 55,000.

Target size of the recombinant forms of HIV-1 RT determined by enzymatic activity

The enzymatic activity of the p66/p51 and p66/p66 recombinant forms of HIV-1 RT decreased as an exponential function of radiation exposure (Fig. 1B), yielding a functional target size of ~104 to 132 kD, depending on the nature of the enzymatic assay (Table 1). Thus, a primary ionization anywhere in the RT dimer resulted in a complete loss of all biological activity. The DNA polymerase activity (DDDP activity) of the p51 subunit also decreased as an exponential function of radiation dose (Fig. 1B); however, it yielded a functional target size of 53 kD (Table 1). Thus, the target sizes for the p51 subunit determined by SDS-PAGE and DNA polymerase activity are consistent with the individually isolated p51 subunits of HIV-1 RT existing as monomers in solution.

Target analyses of HIV-1 RT in the presence of T/P

To evaluate the effect of the DNA T/P substrate on the quaternary structure of the p66/p51 and p51 recombinant forms RT, radiation inactivation experiments were carried out in which the RT (p66/p51 or p51 alone) was incubated with the homopolymeric T/P poly(dC)-oligo(dG). Two forms of poly(dC)-oligo(dG) were used that differed in molecular mass: a “large” form in which the length of the poly(dC) template was ~370 nucleotides (1.2×10^5 D) and a “small” form in which the length of the poly(dC) template was 30 nucleotides (9.2×10^3 D). In both instances, an 18-nucleotide poly(dG) primer (6.3×10^3 D) was annealed to the template.

Analysis of the calculated target sizes for enzymatic activity and polypeptide structure indicates that the presence of the T/P does not significantly impact on the radiation inactivation profile of p66/p51 HIV-1 RT (Table 1). However, significant differences in the radiation inactivation

profiles of RT p51 in the presence of T/P, assessed by both DDDP activity and SDS-PAGE analyses, were noted (Fig. 3A,B). For the radiation inactivation profiles evaluated by SDS-PAGE (Fig. 3A), the p51 subunit complexed with T/P was inactivated with an apparent molecular mass of 95 to 108 kD. This result indicates that the T/P substrates induce the formation of a p51/p51 RT homodimer in which radiation energy deposited in one of the subunits destroys both subunits. Interestingly, the size of the template in the poly(dC)-oligo(dG) had no impact on the RT p51 radiation inactivation profile: The structural target sizes calculated for RT p51 complexed with either the large or small form of poly(dC)-oligo(dG) yielded similar results (Table 1). The inactivation profile of RT p51-T/P complex monitored by DDDP activity yielded a biphasic inactivation profile (Fig. 3B) that could be best fit by the sum of two exponential decays. Evaluation of this more complex inactivation profile indicated that 15% of the DDDP activity was due to a structure of ~42 kD, whereas 85% was associated with a 184-kD structure.

Discussion

Radiation inactivation of the recombinant forms of HIV-1 RT

Radiation target sizes can be determined for any surviving parameter that can be measured in irradiated samples. The

Figure 3. Radiation inactivation profiles for the RT p66/p51-T/P and p51-T/P complexes determined by SDS-PAGE (A) and enzyme activity (B). The profiles represent averaged data for irradiation experiments carried out with both the "long" and "short" T/P substrates. (A) The p66 and p51 subunits of p66/p51 RT are designated by solid circles and open circles, respectively, and the RT p51 subunit, by triangles. The radiation inactivation profile determined for the unliganded RT p51 is illustrated by a dashed line. (B) The inactivation profile for the RT p51-T/P complex is designated by open circles, RT p51 alone, by a dashed line, and RT p66/p51 alone, by a solid line. The inactivation profile could be best fit to a double exponential decay in which 85% of the enzyme yielded a functional target size of 184 kD and 15% a target size of 42 kD.

measured property is lost whenever a radiation hit occurs in a molecular structure that controls that property.

For the p66/p51 and p66/p66 forms of HIV-1 RT, both the structural target sizes for the individual subunits (as determined from the stain intensity of the monomer band on SDS-gel electrophoresis) and the functional target sizes (obtained from the residual RDDP, DDDP, and RNase H activities) correspond to the molecular masses of the dimeric proteins (i.e., 110 to 130 kD). From these data, it must be concluded that a primary ionization in either the p66 or p51 subunit destroys both subunits, resulting in the concomitant loss of enzymatic activity. That these dimeric forms of HIV-1 RT inactivate functionally as dimers is not surprising when one considers that the enzymatic activities of RT are tightly coupled to the quaternary structure of the enzyme (Restle et al. 1990, 1992). In contrast, our observation that the individual subunits in the dimer inactivated with molecular masses corresponding to the dimeric form of the enzyme was unexpected. The phenomenon of radiation energy transfer along a single polypeptide chain is most typically seen (Kempner 1999). However, the phenomenon of radiation energy transfer across noncovalent interactions is not frequently observed in multimeric proteins, and the mechanism by which this process occurs remains unclear (Kempner 1999). Previous studies using the avidin-biotin system gave no evidence for radiation damage appearing across the strong noncovalent interactions (Kempner and Miller 1990). Accordingly, other properties of the inter-subunit interaction such as size, shape, and complementarity might be considered. The extensive interactions and high affinity between the two subunits of p66/p51 may be related to the mechanism(s) of this radiation damage.

In contrast to the p66/p51 and p66/p66 forms of HIV-1 RT, the individually isolated p51 subunit is inactivated as a monomeric protein for both structure and function. This observation is consistent with data indicating that this subunit does not readily dimerize (Restle et al. 1990; Becerra et al. 1991). In this regard, the concentration of protein used in our study (~40 μ M) is much less than the K_d estimated for p51/p51 homodimer formation (670 μ M). Even though p51 polypeptides have a low affinity for one another, significant DNA polymerase activity has been associated with the isolated subunit, even in assays in which the effective concentration of the subunit ranges from 20 to 200 nM (Bavand et al. 1993; Fletcher et al. 1996; Dufour et al. 1998). This would indicate that either the p51 subunit is functionally active as a monomer, or the homodimeric form of the enzyme is induced by the presence of the T/P substrate.

Radiation inactivation analyses of HIV-1 RT-T/P complexes

To address the possibility that the T/P substrate induced homodimer formation in the p51 RT subunits, we carried

out radiation inactivation experiments in which both the p66/p51 and p51 forms of RT were complexed with the homopolymeric substrate poly(dC)-oligo(dG). For p66/p51 RT, the presence of T/P had no effect on the radiation inactivation profiles and calculated target sizes evaluated by either RT activity or SDS-PAGE. Furthermore, preliminary experiments have indicated that the T/P was inactivated independently of RT and yielded a target size consistent with its molecular mass. In contrast to p66/p51 RT, the p51 subunit appeared to be inactivated structurally as a homodimer in the presence of the T/P, thereby indicating that the nucleic acid substrate provided a scaffold or template for RT p51/p51 homodimer formation. As described in the Introduction, the p51 subunit in the p66/p51 RT heterodimer adopts a closed conformation that is enzymatically inert and unable to accommodate T/P binding. Therefore, one must assume that the p51 must undergo significant structural rearrangements to bind T/P and to form a p51/p51 homodimeric protein. Molecular modeling studies have indicated that the monomeric form of the p51 subunit would maintain the same thermodynamically favored "closed" conformation that is observed for the subunit in the p66/p51 RT heterodimer (Wang et al. 1994). In light of this prediction, it may be possible that the T/P substrate induces an "open" conformation in the p51 subunit that then permits it to form a stable homodimer.

As opposed to the radiation inactivation profile determined by SDS-PAGE, the inactivation profile for the RT p51-T/P complex evaluated by DNA polymerase activity was more complex (Fig. 3B). Complex inactivation curves (such as that seen in Fig. 3B) have been ascribed to the presence of active structures of different masses (Kempner 1995). In this regard, each species is independently inactivated, resulting in an inactivation profile that is the sum of exponentials. Resolution of the components introduces considerable error in the radiation target sizes, especially in the larger ones. In the present case, it is estimated that 15% of the DDDP activity in these samples is due to a structure similar in size to the p51 subunit. The bulk of the activity (~85%) is due to a much larger structure equivalent to two or more p51 subunits. A simple explanation for these results is that 15% of the p51 subunits in these samples were not complexed to T/P.

Conclusions

In this work, we have shown by radiation inactivation analyses that the nucleic acid T/P substrate provides a scaffold or template that allows the monomeric p51 subunit of HIV-1 RT to form homodimeric forms of the enzyme. Although such forms of the enzyme might not be present during the HIV-1 viral life-cycle, our results highlight the fact that significant conformational changes in the RT subunits can be induced by cofactors such as nucleic acids. Furthermore,

we have established that radiation inactivation analyses provide a useful technique for evaluating quaternary structural changes in RT, and therefore, it can readily be applied to other DNA polymerase enzymes (such as the MLV RT) for which the functional form of the enzyme has not yet been established.

Materials and methods

The homopolymeric T/Ps poly(rA)-oligo(dT)₁₂₋₁₈ and poly(dC)-oligo(dG)₁₂₋₁₈, as well as [³H]-TTP and [³H]-dGTP, were purchased from Amersham Pharmacia Biotech. Invitrogen carried out the synthesis of the 30 nucleotide poly(dC) and 18 nucleotide oligo(dG) primers. All other reagents were of the highest quality available and were used without further purification.

The recombinant forms of HIV-1 RT were expressed and purified as described previously (Fletcher et al. 1996). Samples for irradiation were then diluted to 2 mg/mL in 20 mM Tris-HCl (pH 7.9, 20°C) and 100 mM NaCl. This concentration of protein has previously been shown to be high enough to yield meaningful radiation target sizes and avert the need for free radical scavengers (Eichler et al. 1987; Kempner and Miller 1994). Aliquots of 0.25 mL were placed in 2-mL glass ampoules and frozen rapidly on dry ice. Samples were held at -80°C except during irradiation, which was carried out at -135°C. Radiation exposures of 0 to 100 Mrads were obtained from 10-MeV electrons produced by a linear electron accelerator (Armed Forces Radiobiology Research Institute) as described (Harmon et al. 1985). After irradiation, samples were stored at -80°C until required for analysis, at which time they were rapidly thawed and aliquots were removed for assay. The remaining material was then refrozen in aliquots on dry ice and stored at -80°C for further use. These samples were subjected to only a single additional thaw cycle before being discarded.

HIV-1 RT RDDP and DDDP activities were determined by using a fixed time assay. Reaction mixtures (50 µL total volume) contained 50 mM Tris-HCl (pH 7.8, 37°C), 60 mM KCl, 10 mM MgCl₂, and 5 µg/mL of either poly(rA)-oligo(dT)₁₂₋₁₈ (for RDDP activity) or poly(dC)-oligo(dG)₁₂₋₁₈ (for DDDP activity), and either 20 µM [³H]TTP or 10 µM [³H]dGTP. Reactions were initiated by the addition of 50 to 80 ng of RT (9 to 12 nM final concentration). Reaction mixtures were incubated for 20 min at 37°C and then quenched with 250 µL of ice-cold 10% trichloroacetic acid (TCA) containing 20 mM sodium pyrophosphate. Quenched samples were left on ice for 20 min, then filtered by using 1.2-µm glass fiber type C filter multiwell plates (Millipore), and washed sequentially with 10% TCA containing 20 mM sodium pyrophosphate and 100% ethanol. The extent of radionucleotide incorporation was determined by liquid scintillation spectrometry.

RT RNase H activity was assayed as described previously (Starres and Cheng 1989). Briefly, reaction mixtures (50 µL total volume) contained 50 mM Tris-HCl (pH 8.0, 37°C), 60 mM KCl, 10 mM MgCl₂, and 2 µg/mL of poly([³H]rG)-poly(dC). Generally, 50 ng of RT were used. Reactions were carried out for 20 min at 37°C and then quenched by placing the tubes on ice, followed by the addition of 100 µL of cold 7% perchloric acid. After 30 min on ice, the reaction mixtures were centrifuged at 12,000g for 15 min. One hundred microliters of the supernatants was carefully removed, and the radioactivity was determined by liquid scintillation analysis.

SDS-PAGE was performed according to the method of Laemmli (1970). Aliquots of the irradiated proteins were electrophoresed,

and gels were stained with Coomassie blue G250 stain (Biorad). Densitometric scans of the gels permitted quantitative determinations of the amount of monomers surviving in each irradiated sample.

SEC-HPLC was performed on a Gilson HPLC system using a 7.8- × 600-mm Biosep SEC-3000 column (Phenomenex) and a mobile phase of 20 mM Tris-HCl (pH 7.9, 20°C) and 100 mM NaCl at a flow rate of 1 mL/min. The sample injection volume was 20 µL, and elution profiles were recorded simultaneously at 260 and 280 nm. The column was equilibrated by using molecular mass markers for gel filtration chromatography (Sigma) ranging in size from 12,000 to 200,000 D.

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