Protein Science (1996), 5:278-286. Cambridge University Press. Printed in the USA.
Copyright © 1996 The Protein Society

Chemical crosslinking of the subunits of HIV-1 reverse transcriptase

ZEGER DEBYSER AND ERIK DE CLERCQ
Rega Institute for Medical Research, Minderbroedersstraat 10, B-3000 Leuven, Belgium

(RECEIVED June 20, 1995; ACCEPTED November 21, 1995)

Abstract

The reverse transcriptase (RT) of the human immunodeficiency virus type 1 (HIV-1) is composed of two subunits of 66 and 51 kDa in a 1 to 1 ratio. Because dimerization is a prerequisite for enzymatic activity, interference with the dimerization process could constitute an alternative antiviral strategy for RT inhibition. Here we describe an in vitro assay for the study of the dimerization state of HIV-1 reverse transcriptase based on chemical crosslinking of the subunits with dimethylsuberimidate. Crosslinking results in the formation of covalent bonds between the subunits, so that the crosslinked species can be resolved by denaturing gel electrophoresis. Crosslinked RT species with molecular weights greater than that of the dimeric form accumulate during a 1–15-min time course. Initial evidence suggests that those high molecular weight species represent trimers and tetramers and may be the result of intramolecular crosslinking of the subunits of a higher-order RT oligomer. A peptide that corresponds to part of the tryptophan repeat motif in the connection domain of HIV-1 RT inhibits crosslink formation as well as enzymatic activity. The crosslinking assay thus allows the investigation of the effect of inhibitors on the dimerization of HIV-1 RT.

Keywords: chemical crosslinking; human immunodeficiency virus type 1; oligomerization; reverse transcriptase; RT inhibitors

The attraction of the reverse transcriptase (RT) of the human immunodeficiency virus type 1 (HIV-1) as an antiviral target for AIDS therapy has made this enzyme the focus of intensive research during recent years. RT plays a central role in the viral replication cycle. Through its multiple enzymatic activities, the viral RNA genome is copied into a double-stranded DNA that is subsequently integrated as provirus in a host chromosome (for reviews see Goff, 1990; Jacobo-Molina & Arnold, 1991). It is now clearly established that inhibition of the HIV-1 reverse transcription process can lead to a significant, albeit temporary, reduction of the viral load in HIV-1-infected patients (Wei et al., 1995). Potent nucleoside and non-nucleoside RT inhibitors have been developed and extensively described (De Clercq, 1992). Nucleoside analogues generally have more toxic side effects than the HIV-1 specific non-nucleoside RT inhibitors. However, the clinical efficacy of both classes of drugs is compromised by the selection of drug-resistant virus strains (for a review see Larder, 1993). Both the detection of vast amounts of HIV in the lymph nodes during the early stages of infection (Pantaleo et al., 1993) and the rapid turnover of actively replicating virus (Ho et al., 1995; Wei et al., 1995) support intervention with potent antivirals at an early stage of infection. Combination therapy with antiviral drugs directed at different targets of the viral replication cycle is probably required to prevent selection of drug-resistant virus strains. Against this background, we want to explore alternative antiviral strategies in an effort to suppress HIV infection. One such strategy for RT inhibition, as originally proposed by Restle et al. (1990), is based on the requirement of dimerization for the enzymatic activity of HIV-1 RT. Inhibitors of RT dimerization may inhibit RT activity and thus viral replication.

The reverse transcriptase of HIV-1 is found in equimolar amounts of a 66-kDa (p66) and a 51-kDa (p51) form in infected cells and in virions (Di Marzo-Véronese et al., 1986; Lightfoote et al., 1986). The biologically active form of HIV-1 RT is believed to be a heterodimer composed of the 51-kDa and the 66-kDa subunit (for review see Le Grice, 1993). This conclusion is based on gel filtration (Chandra et al., 1986) and glycerol gradient centrifugation analysis (Hansen et al., 1988). The 51-kDa subunit originates from the 66-kDa form through a specific proteolytic cleavage by the HIV-1 protease. The polymerase domain of RT is located in the 51-kDa N-terminal region, whereas the remaining C-terminal 15 kDa of the p66 subunit corresponds to the RNase H domain. Both domains are separated by a relatively poorly conserved central region called the "tether" (Johnson et al., 1986). The three-dimensional structure of the heterodimeric HIV-1 RT complexed with the non-nucleoside inhibitor

Reprint requests to: Zeger Debyser, Rega Institute for Medical Research, Minderbroedersstraat 10, B-3000 Leuven, Belgium; e-mail: zeger.debyser@uz.kuleuven.ac.be.

nevirapine (Merluzzi et al., 1990), was determined by X-ray crystallography (Kohlstaedt et al., 1992). The structure of a ternary complex with a ds DNA template-primer substrate and the Fab fragment of a monoclonal antibody was subsequently reported (Jacobo-Molina et al., 1993). Although both subunits contain four subdomains, denoted the fingers, thumb, palm, and connection, the overall arrangement of the subdomains is strikingly different in p66 and p51. In both crystal structures, RT is thus present as an asymmetric heterodimeric enzyme.

Dimerization of HIV-1 RT appears to be required for enzymatic activity. The monomers are generally believed to be inactive (Restle et al., 1990). Analytical ultracentrifugation was used to study the interaction between both subunits (Becerra et al., 1991). The association constants measured by sedimentation analysis are $4.92 \times 10^5\ M^{-1}$ for the heterodimer and $5.1 \times 10^4\ M^{-1}$ for homodimeric p66. Prior to the elucidation of the three-dimensional structure of the enzyme, two amino acid repeat motifs located in the central tether domain (residues 252–428 of p66), were proposed to be involved in the hydrophobic protein–protein interactions within the heterodimer (Baillon et al., 1991). The first motif is a leucine zipper-like motif and is located between positions 282 and 310. Site-directed mutagenesis of leucine at position 289 into lysine resulted in a p66 mutant that is unable to dimerize with either p66 or p51, whereas the same mutation introduced into p51 did not affect its capacity to form heterodimers (Goel et al., 1993). The second repeat motif consists of a repeat of five tryptophan residues separated from each other by three amino acids and is located between positions 398 and 414 (Baillon et al., 1991). Supporting evidence for their role in the dimerization process stems from the dimerization inhibition observed with a synthetic peptide corresponding to the residues of HIV-1 RT at positions 389–407 (Divita et al., 1994). According to the resolved crystal structures, the important intersubunit interactions are located between the p66 palm and p51 fingers, between the p66 and p51 connection domains, and between p66 RNase H and the p51 thumb (Ding et al., 1994). Whereas the tryptophan repeat is located in the connection subdomain, L289 is located in the thumb subdomain. It is, at present, not clear how the p66 thumb is involved in dimerization (Goel et al., 1993).

In the search for inhibitors of HIV-1 RT dimerization, we decided to establish an assay that would allow us to estimate the dimerization state of the enzyme in the presence of various inhibitors. After chemically crosslinking the RT subunits with dimethylsuberimidate (DMS), we resolve the crosslinked species in a denaturing gel. As far as the crosslinking occurs intramolecularly, the ratio of oligomers to monomers can inform us about the dimerization state of the enzyme.

Results

Chemical crosslinking of HIV-1 RT

Alkylimidates are known to react exclusively with primary amino groups of a protein, resulting in the formation of an amidine. Bis-imidates such as DMS have been used as crosslinking reagents to produce amidine crosslinks among the protomers of oligomeric proteins (Davies & Stark, 1970). We have applied this technique to study the oligomerization state of HIV-1 reverse transcriptase, starting from the reaction conditions described previously in the study of the oligomerization of bacteriophage T7 primase/helicase (Patel & Hingorani, 1993). In our assays, RT was present at a concentration of only 110 nM to avoid aspecific protein aggregation. After separating the crosslinked protein species by 4–15% denaturing PAGE, detection was performed by western blotting using monoclonal or polyclonal antibodies, because immunostaining proved more sensitive than silver staining (data not shown). Incubation of a heterodimeric preparation of HIV-1 RT in the presence of 10 mg/mL DMS resulted in the formation of covalently linked oligomers, as depicted in Figure 1A.

We estimated the molecular mass of the distinct reaction products from the plot shown in Figure 1B. The change in molecular mass caused by the addition of the chemical crosslink is

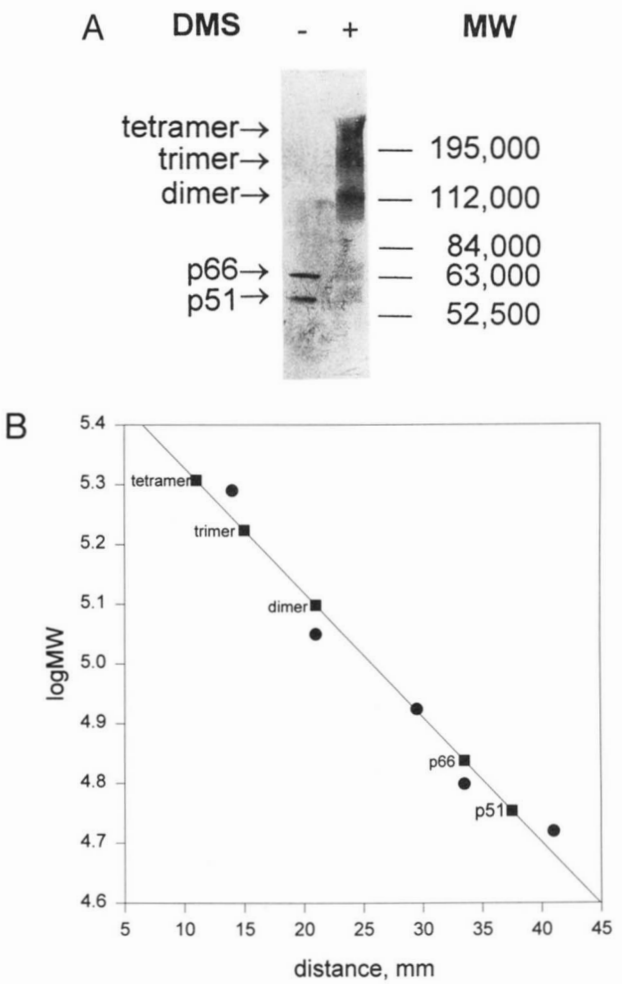

Fig. 1. Chemical crosslinking of HIV-1 RT. Heterodimeric HIV-1 RT (110 nM) was incubated in the presence of DMS (10 mg/mL) for 15 min at 37 °C. The reaction was stopped by the addition of 0.5 M glycine and SDS sample buffer. After 5 min of boiling, samples were run in a 4–15% ready-made polyacrylamide gel under denaturing and reducing conditions. Prestained molecular weight markers were run in parallel. Proteins were subsequently transferred onto PVDF membranes and immunostained with monoclonal antibodies directed against HIV-1 RT. **A:** Results are shown for 1 μL of quenched reaction mixture in the absence of DMS (lane 1) and in the presence of DMS (lane 2). **B:** MW determination of the observed protein species (■) is based on the plot shown, which represents the linear relationship of the logarithm of the molecular masses of the standard proteins (●) versus their distance of migration.

negligible. The three major crosslinked species have an estimated molecular mass of 125 kDa, 166 kDa, and 207 kDa, respectively. Theoretically, the following molecular masses for crosslinked RT subunits could be expected: dimers ranging from 102 (p51/p51) to 132 kDa (p66/p66), with a mass of 117 kDa for crosslinked heterodimeric p66/p51; trimers ranging from 153 (p51/p51/p51) to 198 kDa (p66/p66/p66); and tetramers ranging from 204 to 264 kDa. Based on the accordance with the experimentally obtained molecular weights, we assume that the respective bands represent crosslinked heterodimers, trimers, and tetramers. The smearing of the observed bands is probably due to the presence of different combinations of crosslinked subunits and/or different crosslinks between the same combination of RT subunits. Intrasubunit crosslinks are likely to occur as well. They may also cause aberrant SDS gel mobilities and fuzzy bands. Intrasubunit crosslinks within the p51 subunit are likely to occur, because the carboxy- and amino-terminal domains are in close contact. It is possible that the protein species that behaves as a trimer in the SDS gel is in fact an incompletely unfolded, internally crosslinked tetramer.

The two minor bands that accompany the band representing the heterodimer (clearly visible in Fig. 2A, lane 3) are most likely homodimers of p51 (lower band) and p66 (upper band). The relative contribution of crosslinked homodimers was estimated at 24% for p51/p51 and 30% for p66/p66, whereas the heterodimeric form makes up 47% of crosslinked dimers. The predominant species accumulating after 16 min of incubation has a molecular weight of 207,000 and is presumably tetrameric. Oligomers with a higher molecular weight were not observed even after prolonged incubation (up to 16 h), although the resolution of the 4–15% gels might limit their detection.

Omission of Mg^{2+}, DTT, NaCl or polyuridylic acid from the reaction mixture resulted in a decrease in the efficiency of the crosslinking of the RT subunits into HMW oligomers, whereas the addition of 20 mM EDTA diminished the crosslinking efficiency for dimers and HMW oligomers (Table 1). Consistent with these findings are the results of studies on the dimerization of HIV-1 RT using protein fluorescence (Divita et al., 1993) that demonstrated an increase in dimer stability in the presence of Mg^{2+} ions or template/primer.

In order to describe the kinetics of the crosslinking process, we performed time-course experiments. HIV-1 RT was incubated in the presence of DMS for various lengths of time. An immunoblot of the reaction products (Fig. 2A) and a quantitative representation (Fig. 2B) are shown. The presence of the monomeric forms decreased with time, whereas the presence of the oligomeric forms increased concomitantly. Moreover, the emergence of the different crosslinked forms appeared to be ordered: the dimers emerged first, and the tetramers last (Fig. 2B). These results seem to indicate a progressive crosslinking of HIV-1 RT subunits, whereby a longer incubation is required to produce the multiple crosslinks of the HMW oligomers. The observed time dependence (0–15 min) for the appearance of crosslinked products has been reported and is likely due to the relatively slow reaction of the intermediate protein *N*-alkyl imidates (Peters & Richards, 1977). The crosslinking agent DMS will crosslink lysyl side chains that are separated 11 Å from each other, whereas dimethyl adipimidate (DMA) requires lysyl residues separated

Table 1. *Effect of reaction components on the efficiency of chemical crosslinking*

	Relative efficiency of crosslinking (%)[a]	
	All forms[b]	HMW forms[c]
Complete reaction	100	100
No Mg^{2+}	80 ± 9	66 ± 10
No DTT	105 ± 25	54 ± 3
No NaCl	105 ± 23	78 ± 8
No poly(U)	91 ± 37	45 ± 12
+ EDTA 20 mM	64 ± 1	52 ± 15

[a] Crosslinking efficiency relative to the efficiency of the complete reaction mixture. Quantification and calculations are explained in the Materials and methods. Results given are mean values ± SD for two to three separate crosslinking experiments.
[b] Efficiency for crosslinking dimers, trimers, and tetramers.
[c] Efficiency for crosslinking trimers and tetramers.

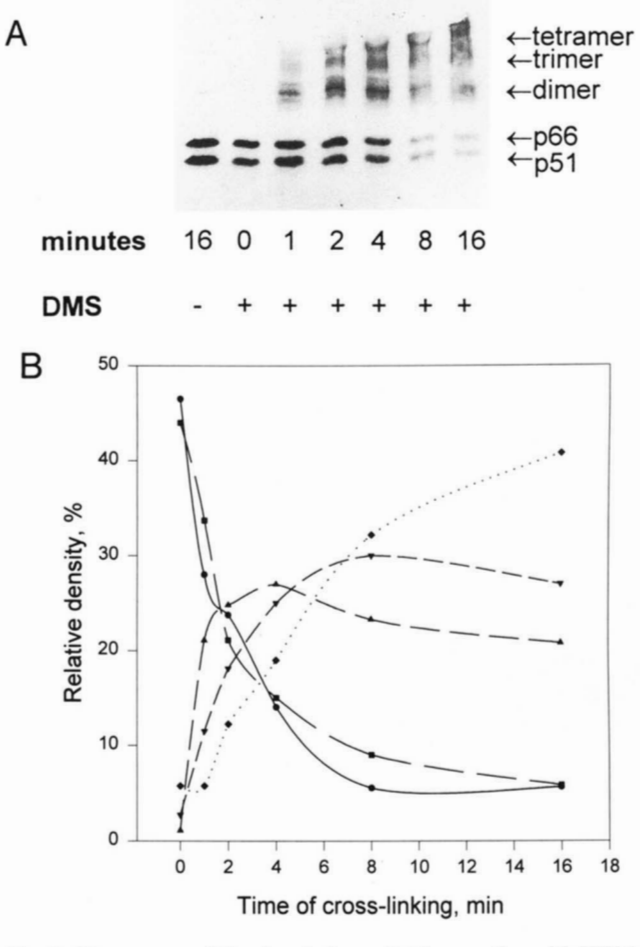

Fig. 2. Time course of the chemical crosslinking reaction with HIV-1 RT. A heterodimeric HIV-1 RT (110 nM) was incubated under optimal reaction conditions in the presence of DMS (10 mg/mL) at 37 °C for various lengths of time. **A:** Analysis involved SDS PAGE and western blotting. **B:** Quantitation of the obtained bands, carried out with the Bioquant Image Analysing System. Various protein species are indicated as follows: ■, p51; ●, p66; ▲, dimers; ▼, trimers; and ◆, tetramers.

no further than 9 Å from each other. When DMA was used as crosslinking agent, a similar crosslinking pattern was observed, although the rate of crosslinking was significantly decreased (data not shown).

In order to investigate the effect of the amidinations on RT activity, RT was incubated in the typical crosslinking reaction mixture in the absence of template. After various time intervals, aliquots were withdrawn and assayed for RT activity with poly(C)·oligo$(dG)_{12\text{-}18}$ as template/primer. A time-dependent inactivation of RT activity was observed upon incubation in the presence of DMS. After an incubation of 15 min in the presence of DMS, RT retained only 2.5% of its original enzymatic activity (data not shown).

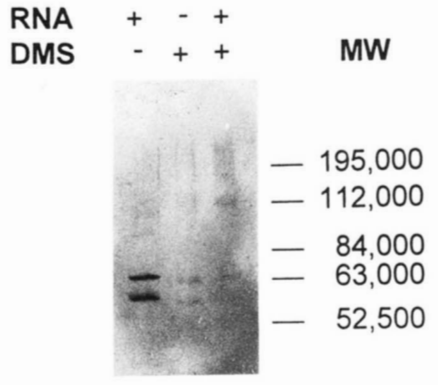

Fig. 3. Template-induced oligomerization. Standard crosslinking reaction mixture (150 μL) contained the separately expressed subunits of HIV-1 RT (p66 and p51), each at a concentration of 10 nM. Three samples were incubated for 2 min at 37 °C to promote dissociation of preformed homodimers, before the addition of polyuridylic acid at a final concentration of 350 nM to the first and third samples. After 2 min at 37 °C, 10 mg/mL DMS was added to the second and the third sample and all samples were incubated for 30 min at 37 °C. Reactions were stopped by the addition of 0.5 M glycine, and the mixtures subsequently concentrated 30-fold by spin filtration. Thereafter, SDS-sample buffer was added, and samples were analyzed by SDS-PAGE in 4–15% gradient gels followed by immunoblotting with monoclonal RT antibodies. Left lane, 2 μL of the sample without DMS; center lane, 2 μL of the sample with DMS but without poly(U); right lane, 2 μL of the sample with DMS and poly(U). A molecular weight marker was run in parallel.

Intramolecular versus intermolecular crosslinking

Chemical crosslinking of pure, recombinant HIV-1 RT apparently results in the formation of high molecular weight (HMW) oligomers. The question remains whether the amidinations take place between the subunits of one complex oligomer (intramolecular crosslinks) or are the result of the collision between two individual dimers (intermolecular crosslinks). Theoretically, it is also possible that aggregates of the recombinant protein may get crosslinked. It should be noticed that, in our crosslinking reactions, RT is present at 110 nM and that aggregation at this protein concentration is not very likely. Although we cannot totally exclude that aggregation took place in our protein stock (2.2 μM), or during prior expression/purification of HIV-1 RT, the number of subunits that are crosslinked appears to be limited to four, even after prolonged incubation (see Fig. 2). In an aggregate, we would expect more extensive ladders of crosslinked protein species.

The following experiments were performed to address the issue of intramolecular versus intermolecular oligomerization. Previously, we have shown that, at nanomolar concentrations of HIV-1 RT, addition of the RNA template promotes the oligomerization of the separately expressed p66 and p51 subunits (Debyser et al., 1992). We have now performed crosslinking experiments with an equimolar mixture of p66 and p51 at a final concentration of 10 nM in the absence and in the presence of an excess of polyuridylic acid. The dilution of HIV-1 RT is believed to promote the dissociation of the preformed homodimers. After concentration, the samples were analyzed by SDS-PAGE and immunoblotting (Fig. 3). The presence of the RNA appears to be required for chemical crosslinking of the RT subunits into dimers and oligomers at the diluted concentrations of RT.

In a more definitive experiment to examine whether the observed crosslinks are the result of collision-induced complex formation or represent time-independent stable complexes of RT, we investigated the distribution of the reaction products as a function of protein concentration (Fig. 4). At a concentration of 110 nM, we observe predominantly HMW crosslinked protein species. At lower concentrations, the proportion of crosslinked species seems even higher (Fig. 4B), although the sensitivity of the technique may not be high enough to produce reproducible results at the lowest concentration. Increasing the RT concentration results in a moderate increase in the relative proportion of monomers and a concomitant decrease in the proportion of HMW proteins. Apparently, formation of the trimeric and tetrameric crosslinked species is not promoted by increasing the protein concentration, which would be expected for collision-induced complex formation. These results seem to indicate the existence of protein–protein interactions between HIV-1 RT dimers that result in the formation of a tetrameric RT molecule.

Crosslinking reaction with various RT preparations

In order to rule out the possibility that the observed oligomerization is an artifact due to our RT preparation, we performed chemical crosslinking experiments with various RT preparations (data not shown). A similar crosslinking pattern was obtained when using purified heterodimeric RT composed of subunits that are expressed by two different expression plasmids in one bacterial cell. Crosslinking experiments with two purified p66 preparations (one from yeast, one from *Escherichia coli*) revealed an analogous ladder, representing dimers, trimers, and tetramers, whereas crosslinking of purified p51 resulted in the appearance of crosslinked dimers but apparently no HMW oligomers (data not shown).

Inhibition of the crosslinking reaction

The ultimate goal of our studies on the oligomerization of HIV-1 RT is the identification of novel RT inhibitors. We assume that the chemical crosslinking depends on the oligomerization state of HIV-1 RT and that inhibitors of oligomerization will interfere with the appearance of the crosslinked protein species in our gels. We realize that the crosslink will trap the oligomers that are normally in a dynamic equilibrium with the monomeric forms. Therefore, this assay will not give a true quantitative estimation of the actual oligomerization state of the enzyme.

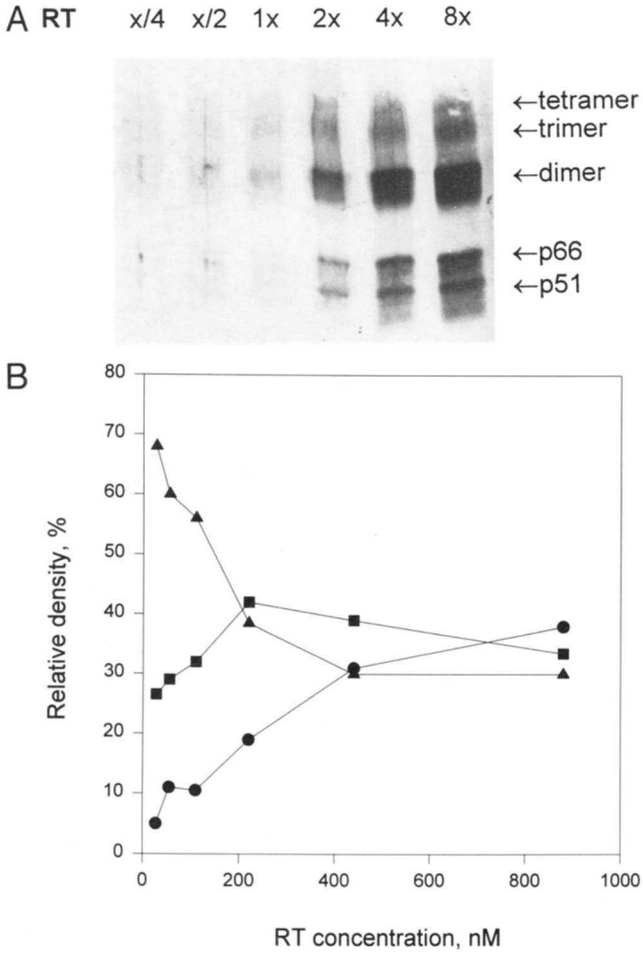

Fig. 4. Crosslinking at various protein concentrations. Various concentrations of heterodimeric HIV-1 RT were incubated under optimal reaction conditions in the presence of DMS (10 mg/mL) at 37 °C for 15 min. **A:** Analysis involved SDS-PAGE and western blotting with polyclonal antibodies directed against HIV-1 RT. **B:** Quantitation of this titration experiment. The relative density of monomeric (●), dimeric (■), and HMW forms (i.e., trimers and tetramers) (▲) at the different concentrations of RT is presented.

Table 2. *Crosslinking efficiency in the presence of various inhibitors of oligomerization*

	Relative efficiency of crosslinking (%)[a]	
	All forms[b]	HMW forms[c]
No addition	100	100
+ SDS 2%	0	0
+ Triton X-100 1%	84	24
+ Ethylene glycol 1%	99	100
+ DMSO 5%	92	100
+ KCl 200 mM	105	65
+ BSA 100 μg/mL	109	91
+ Suramin 4 mg/mL	107	93
+ TIBO R82150 20 μg/mL	100	104

[a] Crosslinking efficiency in the presence of the indicated concentration of inhibitor relative to the efficiency in the absence of inhibitor. Reaction mixtures were incubated for 2 min at 37 °C before addition of DMS. Quantification and calculations are explained in the Materials and methods. Results are mean values for two to three separate crosslinking experiments.
[b] Efficiency for crosslinking dimers, trimers, and tetramers.
[c] Efficiency for crosslinking trimers and tetramers.

We have initiated the search for dimerization inhibitors by examining the susceptibility of the chemical crosslinking reaction to inhibition by detergents (Table 2; Fig. 5). In the presence of 2% SDS, no chemical crosslinking took place, whereas a specific inhibition of the higher-order oligomerization was seen in the presence of the nonionic detergent Triton X-100. There was a small decrease in the higher-order crosslinking in the presence of 200 mM KCl, but no inhibition was observed in the presence of ethylene glycol or DMSO at the concentrations used. Neither suramin, a polyanionic compound that inhibits RT activity by interfering with the template/primer binding, nor TIBO, a nonnucleoside RT inhibitor (Pauwels et al., 1990; Debyser et al., 1991), showed any inhibitory effect in the assay.

Two amino acid repeat motifs located in the central tether domain (residues 252–428 of p66) are believed to be involved in the hydrophobic protein–protein interactions within the heterodimer (Baillon et al., 1991). The first motif is a leucine zipper-like motif and is located between positions 282 and 310. The second repeat motif consists of a repeat of five tryptophan residues separated from each other by three amino acids and is located between positions 398 and 414 (Baillon et al., 1991). We obtained two sets of three overlapping peptides that correspond to parts of the two repeat motifs and investigated whether they are capable of interfering with the dimerization of HIV-1 RT as analyzed by the chemical crosslinking assay (Table 3). A clear inhibition was observed in the presence of 200 μg/mL of the peptide RT-80, which includes four of the five tryptophan residues of the second repeat motif. The inhibition was concentration dependent and not due to the solvent (2% DMSO) (Fig. 6). In the presence of 200 μg/mL of RT-80, the RNA-dependent DNA polymerase activity of HIV-1 RT was also inhibited by 50% (Ta-

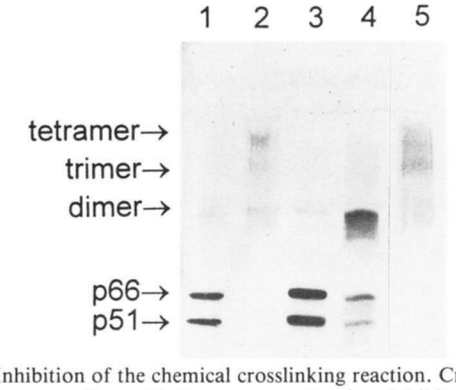

Fig. 5. Inhibition of the chemical crosslinking reaction. Crosslinking reactions were performed in the presence of various inhibitors at 37 °C for 15 min. Reaction mixtures were preincubated for 2 min at 37 °C before the addition of DMS. The immunoblots are presented. Lane 1, 1 μL of reaction mixture in the absence of DMS; lanes 2–5, 1 μL of reaction mixture in the presence of 10 mg/mL DMS. The following inhibitors were present: lane 3, 2% SDS; lane 4, 1% Triton X-100; lane 5, TIBO R82150 at 40 μg/mL.

Table 3. *Crosslinking efficiency in the presence of peptide inhibitors*

Peptide	Position[a]	Sequence[b]	Relative crosslinking efficiency (%)[c]	Relative RT activity (%)[d]
RT-57	283–297	LRGTKA**L**TEVIPL**T**E	85	79
RT-58	288–302	A**L**TEVIPL**T**EEAELE	80	85
RT-59	293–307	IPL**T**EEAEL**E**LAENR	80	97
RT-78	388–402	KFKLPIQKET**W**ETW**W**	ND	106
RT-79	393–407	IQKET**W**ETW**W**TEY**W**Q	25	83
RT-80	398–412	**W**ETW**W**TEY**W**QATW**I**P	12	55

[a] Positions are referred to HIV-1 LAI RT.

[b] Leucine (or threonine) and tryptophan residues of the repeat motifs are indicated in bold.

[c] Crosslinking efficiency in the presence of 200 μg/mL of peptide relative to the efficiency in the absence of inhibitor. Reaction mixtures were incubated in the presence of the inhibitor for 2 min at 37 °C before addition of DMS. All samples contained 2% DMSO. Quantification and calculations are explained in the Materials and methods. Results are mean values for two to three separate crosslinking experiments. ND, not done. Apparent crosslinking of RT-78 to HIV-1 RT prevents correct quantification.

[d] RT activity was determined with recombinant heterodimeric RT and poly(C)·oligo(dG)$_{12\text{-}18}$ as template/primer.

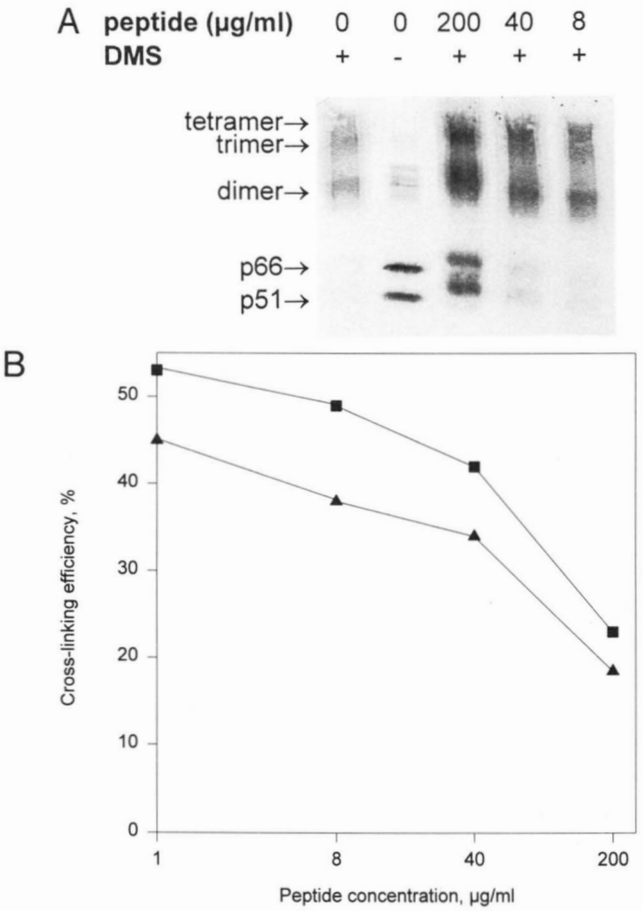

Fig. 6. Inhibition of crosslinking by a peptide homologous to the RT dimerization domain. Crosslinking reactions were performed in the presence of varying concentrations of the peptide RT-80 at 37 °C for 15 min. Reaction mixtures were preincubated in the presence of RT-80 for 2 min at 37 °C before the addition of DMS. **A:** Immunoblot. **B:** Quantification of the concentration-dependent inhibition of the crosslinking efficiency for dimers, trimers, and tetramers (■) and for HMW forms only (▲).

ble 3). Although 200 μg/mL of RT-79 inhibited the crosslinking efficiency by 75%, there was only a minor decrease in RT activity. The crosslinking assay is thus able to detect inhibitors of dimerization that are without apparent effect on the measured enzymatic activity. This may be due to inherently different kinetics of the crosslinking and the enzymatic reaction. It may also be more difficult to dissociate an actively replicating RT molecule. The peptides that were homologous to the leucine zipper motif were inhibitory in neither the crosslinking nor the RT assay.

Discussion

In this report, we describe a method for studying the oligomerization of HIV-1 reverse transcriptase by using the chemical crosslinking agent DMS. This method is a classic approach used to identify the number of protomers in an oligomer (Davies & Stark, 1970). The bifunctional reagent DMS produces covalent bonds between lysyl side chains that are separated by at most 11 Å from each other (Fig. 1B) (Peters & Richards, 1977). When the lysine residues are located on separate subunits, amidination will result in a covalent bond between the subunits. Denaturing PAGE will subsequently resolve the principal species of crosslinked oligomers, the number of bands being identical with the number of protomers in the homo-oligomer. When the oligomer consists of distinct subunits, as is the case with the p66 and p51 subunits of HIV-1 RT, the number of theoretically possible crosslinked species rises significantly. This can make their identification more complicated.

The technique of chemical crosslinking has recently helped in the elucidation of the hexameric nature of bacteriophage T7 primase/helicase (Patel & Hingorani, 1993). When similar reaction conditions were used to crosslink HIV-1 RT, a time-dependent crosslinking of the HIV-1 RT subunits was observed. The efficiency of crosslinking is high (more than 80%) as judged by the disappearance of monomers and the appearance of various crosslinked species. After 16 min of incubation, three major crosslinked species are visible, with estimated molecular

masses of 125, 166, and 207 kDa, respectively. Based on their accordance with the theoretical molecular weights for the different combinations of crosslinked subunits, we assume that the respective bands represent crosslinked dimers, trimers, and tetramers. However, the band corresponding to the trimeric form may be the result of intrasubunit crosslinking of p51 and thus represent incompletely unfolded tetramer. The two minor bands that accompany the band representing the heterodimer are most likely homodimers of p51 (lower band) and of p66 (upper band). After 16 min of incubation, the predominant accumulating species is the tetrameric form.

Although it is difficult to deny the existence of HMW species of HIV-1 RT after DMS crosslinking, the obvious unanswered question is whether the covalent bonds are made intramolecularly (i.e., between subunits of an oligomeric RT) or depend on the formation of collision complexes (intermolecular crosslinking between two dimers). We have tried to address this issue in several ways. At first, our chemical crosslinking studies were performed with low (110 nM) concentrations of RT. Moreover, increasing the protein concentration did not give rise to more extensive oligomerization, as would be expected from collision-induced crosslinking. In fact, at higher RT concentrations, the proportion of HMW species appears to decrease. This is not due to high protein concentration as such, because addition of an excess of bovine serum albumin (BSA) did not interfere with HMW crosslinking (Table 2). DMS cannot be limiting because the crosslinking agent DMS is used in a large molar excess (30 mM) even with the highest RT concentrations used (880 nM). We have no explanation for the decrease in HMW species at the highest RT concentrations. At dilute RT concentrations (10 nM), oligomerization is promoted in the presence of an RNA template. Because the template is used in a large molar excess over protein, the effect cannot be due to a promotion of intermolecular crosslinking by trapping dimeric molecules in close vicinity on the same RNA molecule. Template-induced dimerization of HIV-1 RT has been demonstrated previously (Debyser et al., 1992; Divita et al., 1993).

Denaturation of HIV-1 RT by addition of 2% SDS abolishes chemical crosslinking. The inhibition of the higher order oligomerization of HIV-1 RT by Triton X-100 might point to the importance of hydrophobic interactions. Previous kinetic studies with heterodimeric HIV-1 RT revealed sigmoidal plots when the enzyme activity was plotted at various template/primer concentrations. Interestingly, in the presence of 0.05% Triton X-100, RT activity increased 10-fold, but the kinetics displayed Michaelis-Menten characteristics (Debyser et al., 1992). Both phenomena might be related: in the presence of the nonionic detergent, weak higher-order interactions may be abolished, and the heterodimeric enzyme will obey Michaelis-Menten kinetics.

Supporting evidence for the higher-order oligomerization of HIV-1 RT comes from the recently published analytical ultracentrifugation data of Lebowitz et al. (1994). Optimal fitting of their sedimentation equilibrium data with HIV-1 RT required monomer-dimer-trimer or monomer-dimer-tetramer associations. These results are consistent with a higher degree of association beyond the dimeric state. Is there any evidence from the resolved crystal structures of heterodimeric HIV-1 RT that supports the higher-order oligomerization state of the enzyme? With the help of a molecular modeling software program and on the basis of the available coordinates of two crystal structures, we looked for possible interactions between RT heterodimers in the crystals. In both crystals, protein-protein contacts between heterodimers do exist. Interestingly, dimer-dimer interactions in the crystal of Jacobo-Molina et al. (1993), which includes a Fab fragment, are different from those in the nevirapine-RT crystal of Kohlstaedt et al. (1992). In the latter crystal, there are close contacts between the connection domain of the p66 subunit of a first molecule and the connection domain of the p51 subunit of a second molecule. The software allows estimation of lysyl residues that are candidates for chemical crosslinking (11 Å distance). More than 10 possible lysyl-lysyl linkages may be identified between both subunits of one heterodimer, whereas the number of candidate lysyl residues that are positioned appropriately to link two heterodimers covalently seems limited. We are planning to verify whether these regions are involved in the higher-order oligomerization in later experiments. Addition of competing homologous peptides in the crosslinking assay and site-directed mutagenesis of the interacting amino acids should prevent tetramerization.

A definite answer on the authenticity of the observed oligomerization of recombinant HIV-1 reverse transcriptase awaits the elucidation of the molecular architecture of the protein complex that is carrying out reverse transcription in the infected cell. Undoubtedly, the next step would be to perform chemical crosslinking studies with permeabilized HIV-1 virions that are capable of carrying out the so-called endogenous HIV-1 reverse transcription step. In fact, a model that has been recently proposed to accommodate all reaction steps during reverse transcription requires the existence of a tetrameric RT complex that consists of two heterodimers (Cook, 1993).

The described crosslinking assay allows the identification of inhibitors of HIV-1 RT oligomerization. Using this assay, we present evidence that a peptide that contains four of five tryptophan residues of the tryptophan repeat motif inhibits HIV-1 RT dimerization. During our studies, a method for studying the HIV-1 RT dimerization process based on measuring intrinsic tryptophan fluorescence has been reported (Divita et al., 1994). This approach has led to the identification of a peptide inhibitor (corresponding to residues 389-407) that partially overlaps with the peptide RT-80 and contains three of the five tryptophan residues. The two assays are complementary. Although the fluorescence assay allows the determination of rate constants, it involves dissociation of preformed dimers with organic solvents such as acetonitrile. Also, the inhibitory effect of the peptide has to be measured indirectly by following RT activity, because of interference of the tryptophan-rich peptide with fluorescence measurement. No actively induced RT dissociation or RT inhibition was observed, albeit at lower peptide concentrations than used in our assay. Finally, the chemical crosslinking assay makes possible direct visualisation of the inhibition of dimerization in a gel-based assay that allows quantitation of the distinct protein species.

Materials and methods

Materials

Heterodimeric HIV-1 reverse transcriptase, produced in *E. coli* and purified to near homogeneity, was a kind gift from R. Bhikhabhai and B. Strandberg (Uppsala University, Uppsala, Sweden) (Bhikhabhai et al., 1992). In the expression system, the entire HIV-1 *pol* gene, encoding for protease, RT, and integrase,

is expressed (Unge et al., 1990); the p51 subunit is believed to originate through proteolytic cleavage by the coexpressed viral protease (Unge et al., 1990). This RT preparation has a specific activity of 0.03 s^{-1} on poly(C) templates (Debyser, 1994). Aliquots of the enzyme (at 2.2 μM) were stored at −80 °C until used. Separately expressed and purified p51 and p66 subunits were a kind gift from P.J. Barr (Chiron). Those enzymes were expressed in *Saccharomyces cerevisiae* (Bathurst et al., 1990). A third RT preparation was made at our institute and is the result of coexpressing both RT subunits on different plasmids in the same *E. coli* cell (Jonckheere et al., 1994). Polyuridylic acid was from Pharmacia. DMS and DMA were from Sigma. TIBO R82150 was obtained from the Janssen Research foundation (Beerse, Belgium). The overlapping HIV-1 RT peptides RT-57 (residues 283–297), RT-58 (288–302), RT-59 (293–307), RT-78 (388–402), RT-79 (393–407), and RT-80 (398–412) were obtained through the MRC Reagent Project from Drs. Graham and H. Marsden. Spin filtration was carried out in low binding cellulose filters (MW cut-off 30,000) from Millipore.

Chemical crosslinking

The bifunctional crosslinking reagent DMS (Davies & Stark, 1970) was used for protein crosslinking studies with HIV-1 RT. DMS was prepared immediately before use by dissolving 10 mg in 180 μL of ice-cold triethanolamine (TEA)-HCl (0.15 M, pH 8.2). The pH of the DMS solution was readjusted to 8.2 by addition of 20 μL of 1 M NaOH. Crosslinking was initiated by the addition of 2 μL DMS (final concentration 10 mg/mL) to 8 μL of reaction mixture at 4 °C. The final reaction mixture (45 mM TEA buffer, pH 8.2) contained 110 nM HIV-1 RT, 650 nM of polyuridylic acid, 10 mM $MgCl_2$, 100 mM NaCl, and 1 mM DTT. Incubation was performed for various time intervals at 37 °C. The crosslinking reaction was stopped by adding an equal volume of 1 M glycine to the aliquots. The quenched samples were subsequently mixed with 6 μL of a 4× SDS sample buffer (10% SDS/20% 2-mercaptoethanol/0.04% bromophenol blue in TE buffer, pH 8), boiled for 5 min, and then frozen at −20 °C until analyzed by western blotting.

Denaturing electrophoresis and western blotting

SDS-PAGE analysis was done on PhastSystem (Pharmacia) using 4–15% ready-made gradient gels. The following electrophoresis conditions were used: prerun at 10 mA for 10 V h; loading at 1 mA for 2 V h; and run at 10 mA for 120 V h. The voltage was 400 V and the temperature was 15 °C. Electroblotting from SDS-PAGE gels onto polyvinylidene difluoride (PVDF) membranes was performed with PhastTransfer (Pharmacia). The transfer buffer contained 25 mM Tris-HCl and 192 mM glycine, pH 8.3, and 20% (v/v) methanol. Transfer required 15 min at a current of 25 mA/gel. Thereafter, PVDF membranes were blocked in 4% milk powder in Tris-buffered saline (20 mM Tris-HCl, pH 7.4, 500 mM NaCl) for 45 min. Blots were incubated overnight with monoclonal mouse antibody that recognizes both p51 and p66 subunits (NEN research products, DuPont de Nemours), diluted 100-fold in Tris-buffered saline. Alternatively, polyclonal rabbit antibodies were generated in collaboration with Dr. F. Vandesande (Leuven, Belgium) by immunization with a purified recombinant HIV-1 RT preparation. In this preparation, the p51 and p66 subunits are coexpressed from two different plasmids (Jonckheere et al., 1994). Polyclonal antibodies recognized both subunits and were used at a 1,000-fold dilution. As secondary antibodies, biotinylated rabbit anti-mouse or goat anti-rabbit immunoglobulins (1:500, Dakopatts, Prosan, Ghent, Belgium) were used. Detection was achieved with alkaline phosphatase-conjugated avidin (1:200, Prosan), 0.33 mg/mL 4-nitrobluetetrazolium salt, and 0.165 mg/mL 5-bromo-4-chloro-3-indolylphosphate (both from Sigma) in alkaline phosphatase buffer (100 mM Tris-HCl, pH 9.5, 100 mM NaCl, and 5 mM $MgCl_2$). Biotinylated or prestained SDS molecular weight markers were used (both from Sigma).

Quantitation of crosslinking efficiency

Quantitation of the proportion of crosslinked RT was carried out by means of a Bioquant Image Analyzing System (R & M Biometrics, Nashville, Tennessee) in combination with a video camera. Rectangles of identical size were drawn around the bands representing monomeric p51 and p66 and the different crosslinked species. The background density of a rectangle drawn in between the p66 band and the band representing crosslinked dimers was subtracted from the densities of the rectangles. The relative proportion of each band to the total density in the lane (obtained by adding the densities of the five rectangles) was calculated. Assuming that all monomeric and crosslinked RT species are immunostained to the same extent, we define the crosslinking efficiency in a particular experiment as the sum of the proportional densities corresponding to crosslinked oligomers. Under our conditions of denaturing electrophoresis, we consistently observe faint bands at the position of RT oligomers even in the absence of DMS (Fig. 2, lane 1). We assume that those bands correspond to incompletely denatured oligomers of HIV RT. Therefore, when calculating the crosslinking efficiency, we correct for the relative amount of oligomers present in the absence of the crosslinking agent.

Reverse transcriptase assay

The reverse transcriptase assays were performed as described previously (Debyser et al., 1991).

Molecular modeling

Molecular modeling based on the coordinates of the HIV-1 RT crystal structures deposited in the Protein Data Bank was done with the "What If" software program (Vriend, 1990). We analyzed both the 1hmi (Jacobo-Molina et al., 1993) and the 3hvt (Kohlstaedt et al., 1992) structure.

Acknowledgments

These studies were supported by grants from the Belgian Nationaal Fonds voor Wetenschappelijk Onderzoek, Fonds voor Geneeskundig Wetenschappelijk Onderzoek, and Geconcerteerde Onderzoeksacties. We thank Dr. R. Bhikhabhai and Dr. B. Strandberg (Uppsala University), Dr. P. Barr (Chiron) and H. Jonckheere (our institute) for providing us with the various recombinant HIV-1 RT preparations used throughout these studies. We acknowledge the collaborative effort with Dr. F. Vandesande and L. Grauwels (Zoological Institute, Leuven) for producing polyclonal antibodies directed against HIV-1 RT, and we thank Dr. B. Wroblowski (Laboratory for Chemical and Biological Dynamics, Leuven) for computer analysis of the coordinates of the HIV-1 RT crystal structures. Helpful discussions with Dr. V. Baekelandt (Zoological Institute, Leuven) are greatly appreciated.

References

Baillon JG, Nashed NT, Kumar A, Wilson SH, Jerina DM. 1991. A leucine zipper-like motif may mediate HIV reverse transcriptase subunit binding. *New Biol 3*:1015–1019.

Bathurst IC, Moen LK, Lujan MA, Gibson HL, Feucht PH, Pichuantes S, Craik CS, Santi DV, Barr PJ. 1990. Characterization of the human immunodeficiency virus type 1 reverse transcriptase produced in yeast. *Biochem Biophys Res Commun 171*:589–595.

Becerra SP, Kumar A, Lewis MS, Wide SG, Abbotts J, Karawya EM, Hughes SH, Shiloach J, Wilson SH. 1991. Protein–protein interactions of HIV-1 reverse transcriptase: Implications of central and C-terminal regions in subunit binding. (Lewis MS. Appendix: Ultracentrifuge analysis of a mixed association.) *Biochemistry 30*:11707–11719.

Bhikhabhai R, Jackson T, Unge T, Strandberg B, Carlsson T, Lövgren S. 1992. Purification, characterization and crystallization of recombinant HIV-1 reverse transcriptase. *J Chromatogr 604*:157–170.

Chandra A, Gerber T, Kaul S, Wolf C, Demirhan I, Chandra P. 1986. Biochemical heterogeneity of reverse transcriptase purified from the AIDS virus. *FEBS Lett 200*:327–323.

Cook PR. 1993. A model for reverse transcription by a dimeric enzyme. *J Gen Virol 74*:691–697.

Davies GE, Stark GR. 1970. Use of dimethylsuberimidate, a crosslinking reagent, in studying the subunit structure of oligomeric proteins. *Proc Natl Acad Sci USA 66*:651–656.

Debyser Z. 1994. Studies on HIV-1 reverse transcriptase and reverse transcriptase inhibitors. In: *Viral DNA synthesis*. Leuven University Press. *Acta Biomedica Lovaniensia 77*:43–46.

Debyser Z, Pauwels R, Andries K, Desmyter J, Engelborghs Y, Janssen PAJ, De Clercq E. 1992. Allosteric inhibition of HIV-1 reverse transcriptase by TIBO compounds. *Mol Pharmacol 41*:203–208.

Debyser Z, Pauwels R, Andries K, Desmyter J, Kukla M, Janssen PAJ, De Clercq E. 1991. An antiviral target on reverse transcriptase of human immunodeficiency virus type 1 revealed by tetrahydroimidazo[4,5,1-jk][1,4]benzodiazepin-2(1H)one and -thione derivatives. *Proc Natl Acad Sci USA 88*:1451–1455.

De Clercq E. 1992. HIV inhibitors targeted at the reverse transcriptase. *AIDS Res Human Retrovir 8*:119–134.

Di Marzo-Véronese F, Copeland TD, DeVico AL, Rahman R, Oroszlan S, Gallo RC, Sarngadharan MG. 1986. Characterization of highly immunogenic p66/p51 as the reverse transcriptase of HTLV-III/LAV. *Science 231*:1289–1291.

Ding J, Jacobo-Molina A, Tantillo C, Lu X, Nanni RG, Arnold E. 1994. Buried surface analysis of HIV-1 reverse transcriptase p66/p51 heterodimer and its interaction with dsDNA template/primer. *J Mol Recognition 7*:157–161.

Divita G, Müller B, Immendörfer U, Gautel M, Rittinger K, Restle T, Goody RS. 1993. Kinetics of interaction of HIV reverse transcriptase with primer/template. *Biochemistry 32*:7966–7971.

Divita G, Restle T, Goody RS, Chermann JC, Baillon JG. 1994. Inhibition of human immunodeficiency virus type 1 reverse transcriptase using synthetic peptides derived from the connection domain. *J Biol Chem 269*: 13080–13083.

Goel R, Beard WA, Kumar A, Casas-Finet JR, Strub MP, Stahl SJ, Lewis MS, Bebenek K, Becerra SP, Kunkel TA, Wilson SH. 1993. Structure/function studies of HIV-1 reverse transcriptase: Dimerization-defective mutant L289K. *Biochemistry 32*:13012–13018.

Goff SP. 1990. Retroviral reverse transcriptase: Synthesis, structure and function. *J AIDS 3*:817–831.

Hansen J, Schultze T, Mellert W, Moelling K. 1988. Identification and characterization of HIV-specific RNase H by monoclonal antibody. *EMBO J 7*:239–243.

Ho DD, Neumann AU, Perelson AS, Chen W, Leonard JM, Markowitz M. 1995. Rapid turnover of plasma virions and CD4 lymphocyte in HIV-1 infection. *Nature 373*:123–126.

Hoffman AD, Banapour B, Levy JA. 1985. Characterization of the AIDS-associated retrovirus reverse transcriptase and optimal conditions for its detection in virions. *Virology 147*:326–335.

Jacobo-Molina A, Arnold E. 1991. HIV reverse transcriptase structure–function relationships. *Biochemistry 30*:6351–6361.

Jacobo-Molina A, Ding J, Nanni R, Clark AD, Lu X, Tantillo C, Williams R, Kamer G, Ferris AL, Clark P, Hizi A, Hughes S, Arnold E. 1993. Crystal structure of human immunodeficiency virus type 1 reverse transcriptase complexed with double-stranded DNA at 3.0 Å resolution shows bent DNA. *Proc Natl Acad Sci USA 90*:6320–6324.

Johnson MA, McClure MA, Feng DF, Gray J, Doolittle RF. 1986. Computer analysis of retroviral *pol* genes: Assignment of enzymatic functions to specific sequences and homologies with nonviral enzymes. *Proc Natl Acad Sci USA 83*:7648–7452.

Jonckheere H, Taymans JM, Balzarini J, Velazquez S, Camarasa MJ, Desmyter J, De Clercq E, Anné J. 1994. Resistance of HIV-1 reverse transcriptase against TSAO derivatives is determined by the mutation Glu^{138}→Lys on the p51 subunit. *J Biol Chem 269*:25255–25258.

Kohlstaedt LA, Wang J, Friedman JM, Rice PA, Steitz TA. 1992. Crystal structure at 3.5 Å resolution of HIV-1 reverse transcriptase complexed with an inhibitor. *Science 256*:1783–1790.

Larder BA. 1993. Inhibitors of HIV reverse transcriptase as antiviral agents and drug resistance. In: Skalka AM, Goff SP, eds. *Reverse transcriptase*. Cold Spring Harbor, New York: Cold Spring Harbor Laboratory Press. pp 205–222.

Lebowitz J, Kar S, Braswell E, McPherson S, Richard DL. 1994. Human immunodeficiency virus-1 reverse transcriptase heterodimer stability. *Protein Sci 3*:1374–1382.

Le Grice SFJ. 1993. Human immunodeficiency virus reverse transcriptase. In: Skalka AM, Goff SP, eds. *Reverse transcriptase*. Cold Spring Harbor, New York: Cold Spring Harbor Laboratory Press. pp 163–191.

Lightfoote NN, Coligon JE, Folks TM, Fauci AS, Martin MA, Venkatesan S. 1986. Structural characterization of reverse transcriptase and endonuclease polypeptides of the acquired immunodeficiency syndrome retrovirus. *J Virol 60*:771–775.

Merluzzi VJ, Hargrave KD, Labadia M, Grozinger K, Skoog M, Wu JC, Shih CK, Eckner E, Hattox S, Adams J, Rosenthal AS, Faanes R, Eckner RJ, Koup RA, Sullivan JL. 1990. Inhibition of HIV-1 replication by a non-nucleoside reverse transcriptase inhibitor. *Science 250*:1411–1413.

Pantaleo G, Graziosi C, Demarest JF, Butini L, Montroni M, Fox CH, Orenstein JM, Kotler DP, Fauci AS. 1993. HIV infection is active and progressive in lymphoid tissue during the clinically latent stage of disease. *Nature 362*:355–358.

Patel SS, Hingorani MM. 1993. Oligomeric structure of bacteriophage T7 DNA primase/helicase proteins. *J Biol Chem 268*:10668–10675.

Pauwels R, Andries K, Desmyter J, Schols D, Kukla MJ, Breslin HJ, Raeymaeckers A, Van Gelder J, Woestenborghs R, Heykants J, Schellekens K, Janssen MAC, De Clercq E, Janssen PAJ. 1990. Potent and selective inhibition of HIV-1 replication in vitro by a novel series of TIBO derivatives. *Nature 343*:470–474.

Peters K, Richards FM. 1977. Chemical crosslinking: Reagents and problems in studies of membrane structure. *Annu Rev Biochem 46*:523–551.

Restle T, Müller B, Goody RS. 1990. Dimerization of human immunodeficiency virus type 1 reverse transcriptase. *J Biol Chem 265*:8986–8988.

Unge T, Ahola H, Bhikhabhai R, Bäckbro K, Lövgren S, Fenyö EM, Honigman A, Panet A, Gronowitz JS, Strandberg B. 1990. Expression, purification, and crystallization of the HIV-1 reverse transcriptase (RT). *AIDS Res Human Retrovir 6*:1297–1303.

Vriend G. 1990. A molecular modeling and drug design program. *J Mol Graph 8*:52–56.

Wei X, Ghosh SK, Taylor ME, Johnson VA, Emini EA, Deutsch P, Lifson JD, Bonhoeffer S, Nowak MA, Hahn BH, Saag MS, Shaw GM. 1995. Viral dynamics in human immunodeficiency virus type 1 infection. *Nature 373*:117–122.