Reverse transcriptases from both human immunodeficiency viruses type 1 and 2 are obligatory dimers. A tryptophan-rich repeat motif that is highly conserved between these proteins, as well as in the reverse transcriptase from simian immunodeficiency virus, has been postulated to be involved in hydrophobic subunit interactions. A synthetic 19-mer peptide covering part of this tryptophan repeat motif was recently shown to inhibit human immunodeficiency viruses type 1 reverse transcriptase subunit dimerization (Divita, G., Restle, T., Goody, R. S., Chemermann, J.-C., and Baillon, J. G. (1994) J. Biol. Chem. 269, 13080-13083). In the present study, we show that the same peptide can also inhibit human immunodeficiency virus type 2 reverse transcriptase subunit dimerization, suggesting that the same inhibitors might be used as agents against both viruses as well as against variants of human immunodeficiency virus type 1 that differ from the variant against which they were developed. Under appropriate experimental conditions, e.g. at acidic pH, this peptide is also able to induce the dissociation of the enzyme from human immunodeficiency virus type 1.

A significant effort has been made over the past few years toward improving or finding new and more potent and specific inhibitors of reverse transcriptase (RT) of human immunodeficiency virus (HIV), the etiologic agent responsible for the development of AIDS. Unfortunately, despite the high potency of some of these compounds, resistance to these inhibitors arises very rapidly both in cell culture experiments and during treatment of patients (Larder and Kemp, 1989; St. Clair et al., 1991; Nunberg et al., 1991). This corresponds to the emergence of point mutations within the RT sequence and arises from the hypermutability of HIV (Preston et al., 1988; Roberts et al., 1988).

Recent crystallographic data obtained with HIV-1 RT (Kohlstaedt et al., 1992; Jacob-Molina et al., 1993; Rodgers et al., 1995; Ren et al., 1995) suggest that there are ways other than the current use of small molecules, i.e. nucleoside analogs or nonnucleoside inhibitors to inhibit enzyme activity (for review, see Nanni et al. (1993)). As originally proposed by Restle and co-workers in 1990 (Restle et al., 1990), dimerization of RT might be a good target for therapeutic intervention in AIDS. This hypothesis was based on the observation that only dimeric forms of the enzyme are active (Restle et al., 1990; 1992). The dimer interface is largely dominated by interactions between the two connection subdomains (Nanni et al., 1993; Wang et al., 1994). These protein-protein interactions have been shown to be highly hydrophobic (Becerra et al., 1991; Müller et al., 1991; Divita et al., 1995). Sequence comparison (Baillon et al., 1991), as well as intrinsic protein fluorescence studies (Divita et al., 1993) have focused on a tryptophan repeat motif that is highly conserved between HIV-1 and HIV-2 RTs, as well as in simian immunodeficiency virus RT and that was suggested to be involved in RT subunit interactions. Indeed, a synthetic 19-mer peptide, corresponding to residues 389-407 from the BH-10 molecular clone of HIV-1 and covering part of this tryptophan repeat motif was recently shown to inhibit RT subunit dimerization (Divita et al., 1994).

In the present paper, we show that the same peptide can also inhibit HIV-2 RT subunit reassociation, although it is slightly less efficient than in the case of HIV-1 RT. This peptide appears to act on the first step of the two-step process for HIV-1 and HIV-2 RT heterodimer formation described recently (Divita et al., 1995), the rapid association of the monomers into a heterodimeric form devoid of polymerase activity, and not on the second phase of the process, which consists of a slow isomerization leading to the "mature" active form of RT. Under appropriate experimental conditions, P1 is also able to induce the dissociation of HIV-1 RT.

EXPERIMENTAL PROCEDURES

Materials—Peptides 1 (P1, KFLPIQETWETWTRYET) and 2 (P2, TEYWQATWPEWE) were synthesized by Chiron Mimotopes Pty Ltd, (Clayton, Australia) and were provided 95% pure as determined by HPLC analysis. Peptides 3 (P3, QKOGQGWTRYQYQEP) and 4 (P4, DVQLTEAVKITTIES) were provided by Dr. R. Franck and Dr. H. Gausepohl and were purified by reversed-phase HPLC using an acetonitrile-water linear gradient containing 0.1% trifluoroacetic acid. All peptides were solubilized in a 30% Me2SO solution at 3.8 mm. The maximal concentration of the peptides in the cell-free assays was 100 μM, which corresponds to a final concentration of less than 1% Me2SO. It was verified that at this concentration, Me2SO has no effect on enzyme stability and activity. Acetoni triol (gradient grade) was purchased from Merck and [3H]dTTTP was from Amersham Corp.

Enzyme Preparation—Recombinant HIV-2R10 and HIV-2D10 reverse transcriptases were expressed in Escherichia coli and purified as described previously (Müller et al., 1989, 1991). Highly homogeneous preparations of the heterodimeric forms of the enzymes resulting from
co-expression of the 66- and 51-kDa subunits for HIV-1 RT (68 and 54 kDa for HIV-2 RT) were used. For convenience, and despite the slight difference in the molecular mass of the subunits between the two enzymes we use the nomenclature of “p66” and “p51” subunits for HIV-2 RT by analogy to the related peptides from HIV-1 RT. Enzyme concentrations were routinely determined according to Bradford (1976) using a standard assay using poly(rA)-olig(dT)12 as template-primer (Restle et al., 1990).

Polymerase RT Assay—Polymerase activity was measured by a standard assay using poly(rA)-olig(dT)12 as template-primer (Restle et al., 1990). The RT preparations used showed a specific activity of about 10,000 units/mg, where 1 unit of enzyme catalyzes the incorporation of 1 nmol of TMP in 10 min at 37°C into acid-insoluble material. The kinetics of formation of the native RT were monitored on aliquots containing 50 ng of protein after defined incubation times. In this case, enzymatic activity was measured for 5 min at 37°C, this short time being chosen in order to limit the possibility of further activation by dimerization.

HPLC Size Exclusion Chromatography—Chromatography was performed using two 7.5 × 300-mm HPLC columns in series (Bio-Rad TSK-250 followed by Bio-Rad TSK-125). The columns were eluted with 200 mM potassium phosphate, pH 6.5, at a flow rate of 0.8 ml/min (Restle et al., 1990).

Fluorescence Experiments—Fluorescence measurements were performed at 25°C, using a SLM Smart 8000 spectrofluorometer (Colora, Longmont, CO) equipped with a PHS-PC 9635 photomultiplier. The spectral bandwidths were 2 and 8 nm for excitation and emission, respectively. The fluorescence of RT (0.5-5 μM of protein) was measured in a total volume of 0.7 ml of fluorescence buffer containing 50 mM MOPS-HCl, pH 6.5, 10 mM MgCl2, 50 mM KCl and 5% glycerol. The excitation was routinely performed at 290 nm, and fluorescence emission intensity was measured at 340 nm. All measurements were corrected for the buffer blank and for the wavelength dependence of the exciting light intensity through the use of the quantum counter rhodamine B in the reference channel.

Dissociation and Association of the RT Heterodimer Using Organic Solvent—Dissociation of HIV-1 and HIV-2 heterodimers was performed at 25°C by the addition of 12% acetonitrile in the fluorescence buffer described above. The monomer formation was monitored under equilibrium conditions by measuring the relative increase of the intrinsic fluorescence emission of the protein, by the loss of polymerase activity, and by size-exclusion HPLC (Divita et al., 1993, 1995). The association of the subunits was induced by a 12-fold dilution of the sample with an organic solvent-free buffer resulting in a final concentration of 1.4% acetonitrile in the absence or presence of increasing amounts of peptides. The establishment of the dimerization equilibrium was followed in a time-dependent manner using the same parameters. For association and dissociation experiments, protein concentrations of 0.5 and 5 μM were used. Data were collected using a personal computer and evaluated with the commercially available fitting program Grafit (Erithacus software). According to our previously described two-step mechanism for the association process of RT (Divita et al., 1995), the HPLC and the fluorescence experimental data were analyzed as a second-order reaction, and the polymerase activity was analyzed as a single exponential term.

RESULTS

Peptides Derived from the Connection Domain of HIV-1 RT as Inhibitors of HIV-2 RT Dimerization—We have recently proposed that the formation of the native heterodimeric form of both HIV-1 and HIV-2 RTs occurs in a two-step process, the first corresponding to the association of the two subunits to give an inactive intermediate, which then slowly isomerizes to the “mature” RT (Divita et al., 1995). We have also shown that peptides derived from the connection domain of HIV-1 RT can be used as inhibitors of the dimerization process of isolated subunits of the enzyme (Divita et al., 1994). In order to generalize this approach and considering the high sequence homology between HIV-1 and HIV-2 RTs in that region (Baillon et al., 1991), the same peptides derived from the connection domain of HIV-1 RT have been tested as potential inhibitors of the dimerization process of isolated subunits of HIV-2 RT. As already shown, both RTs can be reversibly dissociated at pH 6.5, 25°C, by acetonitrile treatment, and either dissociation or association processes can be followed in a time-dependent manner using three complementary methods: intrinsic fluorescence of the protein, enzymatic activity, and size exclusion HPLC, as observed previously for HIV-1 RT at pH 8.0 (Divita et al., 1993, 1995). In the following experiments, for a direct comparison with our previous data obtained on HIV-1 RT (Divita et al., 1994), and in order to limit the effect of Me2SO, the solvent of the peptides, a concentration of 10 μM of each peptide was used. The kinetics were fitted as described under “Experimental Procedures,” and the rate constants derived from these experiments are reported in Table I. As shown in Fig. 1A, the presence of 10 μM P1 in the dimerization buffer strongly reduced (~18-fold) the rate of formation of active heterodimeric HIV-2 RT from 1.4 h⁻¹ under standard conditions to 0.075 h⁻¹, which is still faster than that obtained for HIV-1 RT (0.025 h⁻¹) under the same conditions (Table I).

In order to identify which step of the dimerization process is affected by the presence of the peptides, this dimerization process was also monitored by size-exclusion HPLC (Fig. 1B). As shown in this figure, the monomer-association step is greatly affected by the presence of 10 μM P1 and reduced by a factor of ~44, with a second-order rate constant value (kassoc) of 0.9 × 10⁴ M⁻¹ s⁻¹ in the case of HIV-2 RT. In contrast, when the heterodimer intermediate form of RT is previously formed by a 40-min incubation in an acetonitrile-free buffer and then incubated at pH 8.0 in the presence of 10 μM P1, the first-order rate constant of maturation is only reduced by 10–15%. This is the result of partial dissociation of the intermediate form of RT, which takes place even in the absence of peptide, as observed by an HPLC size exclusion experiment performed after 40 h of incubation (not shown).

Two other peptides (P3 and P4), which were also derived from the connection domain of HIV-1 RT, inhibit the association of HIV-2 RT subunits, although these two peptides show a less marked effect than that observed for P1. As observed for P1, both peptides affected the monomer-association step, as revealed by the correlation between Fig. 1, A and B, with association rate constant values of 1 × 10⁴ M⁻¹ s⁻¹ and 0.7 × 10⁴ M⁻¹ s⁻¹ and isomerization rate constant values of 0.8 h⁻¹ and 0.28 h⁻¹ for P3 and P4, respectively (Table I). In contrast, peptide P2, which corresponds to the carboxyl-terminal end of the tryptophan cluster of the connection domain did not affect HIV-2 RT dimerization (not shown), as previously observed in the case of HIV-1 RT (Divita et al., 1994).

As shown in Fig. 2, the inhibition of HIV-1 and HIV-2 RT dimerization is dependent on the peptide concentration, and analysis of these curves leads to the determination of apparent dissociation constant values for each peptide. Essentially complete inhibition of HIV-2 RT dimerization was observed for a concentration of 20 μM P1, and an apparent Kd value of 2.7 ± 0.6 μM was determined, which is ~2-fold higher than the value of 1.2 ± 0.7 μM obtained for HIV-1 RT. In the case of P3 and P4,
it was not possible to obtain complete inhibition of HIV-2 RT dimerization, even at the highest concentration used (100 μM, not shown). However, apparent K_d values could be estimated and led to values of 25.7 ± 4 μM for P4 and >100 μM for P3, with respect to HIV-2 RT dimerization inhibition, and 21 ± 5 μM for P3 and >100 μM for P4, with respect to HIV-1 RT dimerization inhibition.

Peptide-induced RT Dissociation—At pH 8.0, 25 °C, none of these peptides actively induced dissociation nor altered the polymerase activity of heterodimeric HIV-1 and HIV-2 RTs, which is not very surprising considering the very high stability of the heterodimeric forms of both enzymes (Müller et al., 1991; Divita et al., 1993). However, the presence of 100 μM P1 increased significantly the rate of acetonitrile-induced dissociation by 55-fold (HIV-1 RT) and 10-fold (HIV-2 RT) at pH 6.5 (not shown). This prompted us to investigate the effect of the most efficient peptide (P1) alone on enzyme dissociation at acidic pH. HIV-1 RT (2 μM) was incubated in the presence of 100 μM P1, at pH 7.5, 6.8, and 6.2, and the residual fraction of active RT was monitored by both the polymerase activity assay (Fig. 3A) and determination of the ratio of monomer/dimer by size exclusion HPLC (Fig. 3B) in a time-dependent manner. In this range of pH, it was checked that HIV-1 RT is quite stable in the absence of peptide. At the lowest pH used (6.2), only 10–15% of polymerase activity is lost after 24 h of incubation at 25 °C in the absence of peptide. After a 40-h incubation with P1, only 10% of HIV-1 RT remains dimeric and active instead of 60% at pH 6.8, and >90% at pH 7.5. The dissociation rate constant value was determined to be 0.08 h⁻¹ at pH 6.2 in the presence of peptide 1 (100 μM). The effect of P3 (100 μM) on HIV-1 RT at pH 6.2 was less marked, with only 60% monomeric form observed after a 40-h incubation, and P4 did not induce significant dissociation of RT at this pH and at the same concentration (not shown). In the case of HIV-2 RT, P1 induced a relatively low dissociation at a concentration of 100 μM (80% of RT remains dimeric and fully active after a 40-h incubation at pH 6.2), but it was not possible to induce enzyme dissociation using P3 and P4.

**DISCUSSION**

Heterodimeric HIV-1 and HIV-2 RTs represent the biologically active relevant forms found in infectious virions (Chandra et al., 1986; Di-Marzo Veronese et al., 1986; Lightfoot et al., 1986; DeVico et al., 1989), and their dimeric nature presents an interesting target for the design of new antiviral agents (Restle et al., 1990; Divita et al., 1994). Recently, we have proposed a two-step mechanism for the dimerization process of both HIV-1 and HIV-2 RTs, which involves first an interaction between the connection subdomains of the two subunits leading to an inactive intermediate followed by a slower conformational change, which corresponds to the stacking of the thumb subdomain of...
ever, it is still possible to reversibly dissociate the heterodimeric RT (Divita et al., 1995). Here we have demonstrated that peptide (P1) also constitutes a powerful inhibitor of dimerization of HIV-2 RT subunits, which is not very surprising since this region is highly conserved in HIV-1, HIV-2, and simian immunodeficiency virus RTs (Table II and Baillon et al., 1991). P1 can effectively block the dimerization process of both enzymes at a concentration of 25 μM, and exhibits relatively high affinities for the isolated subunits of HIV-1 (1.2 μM) and HIV-2 (2.7 μM) RTs.

The correlation between polymerase activity and HPLC size exclusion data leads to the conclusion that for both RTs, the peptide directly targets the first step of the dimerization process, i.e. the monomer-monomer association. The fact that P1, which is derived from the connection subdomain of HIV-1 RT, is able to inhibit the association process of HIV-2 RT subunits suggests that at least part of the dimerization domains of the two enzymes exhibit some similarities. This observation might be significant, considering the relatively high variance between HIV-1 and 2 isolates. The first step in the association process between subunits might be similar in HIV-1 and HIV-2 RTs, via part of the conserved tryptophan cluster corresponding to α-helix L and/or β-sheet 19 in the three-dimensional structure of HIV-1 RT, since P1 exhibits almost the same effect on the subunit association of both enzymes. This is experimentally confirmed here by the observation that the association rate constants are quite similar for the two enzymes, both in the absence of peptide (2–4 × 10^4 M^-1 s^-1) or in the presence of P1 (Table I and Divita et al., 1995). Interestingly, the projection of α-helix L of p51 subunit reveals that all of the well conserved aromatic amino acid residues are located on the external side of the helix and interact directly with the p66 subunit (Wang et al., 1994).

Although P3 and P4, which are also derived from the connection subdomain of HIV-1 RT, show inhibitory properties toward HIV-2 RT dimerization (Table I), the effect of these peptides on the two enzymes is slightly different. In the case of HIV-1 RT, the inhibitory effect of P3 is greater than that of P4, while the opposite is true in the case of HIV-2 RT.

At pH 8.0, none of the peptides used can induce RT dissociation. In fact, this is not surprising considering the relatively low affinity of P1 for the subunits (micromolar range) in comparison with the apparent dissociation constant between the subunits (nanomolar range) (Restle et al., 1990; Divita et al., 1993). In contrast, HIV-1 RT dissociation induced by P1 at acidic pH is in good agreement with the fact that the stability of both HIV-1 and HIV-2 RTs are highly dependent on the pH, being strongly reduced at low pH (Restle et al., 1990; Becerra et al., 1991).
remains dimeric after a 40-h incubation with 100 μM P1. In comparison, this peptide has a relatively low effect on HIV-2 RT at the same concentration. One can speculate that at least part of the additional contacts between subunits, other than those present at the level of the tryptophan cluster, are highly dependent on the pH in the case of HIV-1 RT and less so in the case of HIV-2 RT. Current efforts are directed toward the identification of these additional contacts between subunits in both enzymes.

Inhibition of enzymes by the use of peptidic inhibitors of obligatory dimeric enzymes has been proposed as an alternative to the use of small molecules or substrate analogs (Cohen et al., 1986; Dutia et al., 1990; Restle et al., 1992). However, so far, such inhibitors have been considered mostly as academic tools due to their relatively low potency in comparison with more classical small inhibitors and also to the problems of delivery and targeting of these compounds. A very recent report shows that their use may not be limited to the laboratory. In this report, it is shown that a peptidomimetic inhibitor derived from such an inhibitory peptide by individually optimizing each amino-acid of the parental peptide can exhibit a tremendous improvement of potency over the starting material (Liuzzi et al., 1994). The optimized peptidomimetic inhibitor, whose function is to block the dimerization of Herpes simplex ribonucleotide reductase, exhibited a subnanomolar IC_{50} and showed antiviral activity in vivo. This result emphasizes the need to proceed with the study of such inhibitors to identify substances of high potency, which should be more specific than most currently used inhibitors and, perhaps more importantly, should lower the risk of emergence or selection of resistant mutants.

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