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Human immunodeficiency virus type 1 (HIV-1) recombinant reverse transcriptase

Asymmetry in p66 subunits of the p66/p66 homodimer

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Abstract

A recombinant p66 form of human immunodeficiency virus type 1 (HIV-1) reverse transcriptase (RT) can be obtained [(1991) Biotechnol. Appl. Biochem. 14, 69–81] from crude *Escherichia coli* extracts by immobilized metal affinity chromatography (IMAC). We have analyzed the p66 HIV-1 RT, isolated in the presence of 0.3 M imidazole, by gel permeation HPLC on Superose 12. The results show that it contains two major distinct p66 forms (24.1 min and 28.3 min peaks) which are distinguishable from the purified homodimeric (p66/p66) HIV-1 RT (22.2 min peak). Protein peak 1 (24.1 min) is converted to a 22.3 min peak upon storage for 20 h at 4°C. Under identical conditions, the isolated peak 2 (28.3 min) appeared as a conformationally heterogeneous mixture elaborated by peaks at 22.3 min and 25.9 min. The protein species thus obtained were active in the RNA-dependent DNA polymerase and RNase H activity assays and produced heterodimeric HIV-1 RT upon incubation with the HIV-1 protease. When the IMAC-purified, imidazole-free homodimeric (p66/p66) form of the enzyme was incubated with 0.3 M imidazole for 16 h at 4°C, protein peaks at 28.3 min (peak A) and 30.5 min (peak B) were isolated by gel permeation HPLC. While both of these p66-containing species were stable and displayed identical RNA-dependent DNA polymerase activities, the protein in peak B was only 50% active in RNase H function compared with the protein from peak A. These imidazole-mediated dissociation studies support the hypothesis of partial unfolding of one of the RNase H domains of the p66/p66 homodimer, suggesting that the p66 subunits are asymmetric in the native enzyme.

Key words: HIV-1; Reverse transcriptase; p66/p66 homodimer; Ribonuclease H; Protein asymmetry

1. Introduction

Human immunodeficiency virus type 1 (HIV-1) reverse transcriptase (RT) is encoded by the *pol* gene in the HIV-1 genome and is the multifunctional viral enzyme essential for proviral DNA synthesis from viral RNA (for reviews, see [1,2]). It is found as a heterodimer (p66/p51) when isolated from virions or infected cells [3]. The heterodimer is thought to arise from viral protease-mediated processing of the gag–pol fusion protein, generating a p66/p66 homodimer from which only one p66 subunit is further cleaved to produce p51 [3]. The RT open reading frame codes for 560 amino acids and its expression in *E. coli* yields p66 HIV-1 RT [4] and a p66/p51 heterodimer in which one of the RNase H domains is apparently processed by host enzymes or HIV-1 protease [5–10].

The crystal structure of heterodimeric (p66/p51) HIV-1 RT has been solved [11,12]. These studies show that the heterodimeric (p66/p51) HIV-1 RT is asymmetric and the conformation of the polymerase domain (p51) in p66 is different from that of the p51 domain. The structural asymmetry of heterodimeric HIV-1 RT raises the possibility of a similar asymmetry in p66/p66 homodimers [6,13,14]. Alternatively, the heterodimer may undergo a conformational rearrangement only after proteolysis of one p66 subunit of the p66/p66 homodimer. In this paper we provide direct biochemical evidence for conformational asymmetry in the p66 subunits of homodimeric HIV-1 RT in terms of partial unfolding of one of the RNase H domains.

2. Materials and methods

2.1. Chemicals

General laboratory chemicals were purchased from Sigma. Protein assay and gel reagents were obtained from Bio-Rad. Poly(rA)·oligo(dT), deoxythymidine triphosphate (dTTP), IDA–Sepharose, and Superose 12 columns were purchased from Pharmacia. [^{3}H]dTTP and [^{32}P]ATP were obtained from DuPont New England Nuclear. Glass microfiber filter papers (GF/C) were purchased from Whatman, Maidstone Great Britain. The scintillation fluid used

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Abbreviations: HPLC, high pressure liquid chromatography; HIV-1, human immunodeficiency virus type 1; RT, reverse transcriptase, RNase H, ribonuclease H; IMAC, immobilized metal affinity chromatography; SDS, sodium dodecylsulfate; DTT, dithiothreitol; IDA, iminodiacetic acid; TCA, trichloroacetic acid; BSA, bovine serum albumin.

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was Ultima Gold from Packard. Both the p66/p66 homodimeric and p66/p51 heterodimeric HIV 1-RT were expressed and purified as described earlier [4,5].

2.2. Gel permeation HPLC on Superose 12

Gel permeation HPLC was performed on a Varion 5060 HPLC attached to an HP3396A integrator. A Superose 12 sizing HPLC column (1.0 × 30 cm) was equilibrated with 20 mM Tris, 1 M NaCl, pH 8.0. Protein samples (50–200 μl) were applied and eluted isocratically at a flow rate of 0.4 ml/min. Eluted samples were collected by monitoring relative absorbance at 280 nm on a chart recorder. The collected fractions under each peak were pooled, concentrated, and analyzed for purity and enzymatic activities.

2.3. SDS-polyacrylamide gel electrophoresis

SDS-PAGE was carried out with 12% polyacrylamide gels as described by Laemmli [15] and stained with Commassie blue R-250.

2.4. Protein determination

Concentration of purified protein samples was determined by Bio-Rad protein assay using BSA as the standard.

2.5. RNA-dependent DNA polymerase and RNase H activity assays

These enzymatic assays were carried out as reported in Chattopadhyay et al. [5].

2.6. In vitro processing by HIV-1 protease

The isolated protein peak 1 (975 μg/ml) and peak 2 (932 μg/ml), after storage for 20 h at 4°C, were treated with purified HIV-1 protease. The incubation was carried out in a final volume of 50 μl containing 10 μl protein sample, 29 μl buffer (0.2 M Tris, 1 M NaCl, pH 7.2), 1.5 μl DTT (0.1 M), and 9 μl HIV-1 protease (1 μM). Appropriate controls with and without HIV-1 protease were also run using the purified p66/p66 homodimer. All samples were incubated at 37°C for 60 min. The reaction was stopped by adding 40 μl SDS buffer (2 ×) and the samples were analyzed by SDS-PAGE.

3. Results and discussion

The recombinant p66 form of HIV-1 RT, from crude *E. coli* extracts, was isolated by imidazole elution from an IMAC column followed by overnight dialysis at 4°C [4]. As expected [5], the precursor protein, p66 HIV-1 RT, was predominantly unprocessed. Heterodimeric (p66/p51) HIV-1 RT was produced by in vitro processing of this p66 HIV-1 RT with recombinant HIV-1 protease [5]. Subsequent gel permeation HPLC of the purified heterodimeric HIV-1 RT on Superose 12 sizing column showed that it corresponds to a molecular mass of 120 kDa, with a retention time of 22.4 min [16]. When the IMAC-purified precursor protein, obtained by dialysis of imidazole, was subjected to the Superose 12 sizing column under identical experimental conditions, a similar elution profile with a major peak retention time of 22.2 min was observed (Fig. 1). These results led us to conclude that heterodimers were produced by processing of the p66/p66 homodimers, and not by p66 monomers, obtained by IMAC followed by dialysis of the imidazole.

It was assumed initially that the p66/p66 homodimers, isolated in the presence of 0.3 M imidazole vs. those obtained upon dialysis of imidazole, were identical. Therefore, it was with some surprise that the two preparations, identical in amino acid sequence, were found to be markedly different on a gel permeation HPLC column (Fig. 2). The HIV-1 RT isolated in the presence of 0.3 M imidazole is a mixture of two major protein species represented by peak 1 (24.1 min) and peak 2 (28.3 min). These peaks are distinguishable from the p66/p66 homodimeric RT peak (22.2 min) shown in Fig. 1.

The protein peaks of Fig. 2 were collected and analyzed by SDS-PAGE. As shown in Fig. 3, both the isolated peaks represent predominantly unprocessed HIV-1 RT with a molecular mass of 66 kDa. It is concluded that the two p66 protein species are conformational isomers of the same protein. Our results agree with a report [17] suggesting that p66 RT is not conformationally homogeneous.

Although the IMAC-purified p66 sample that was loaded to the sizing HPLC column contained 0.3 M imidazole, the gel permeation experiment was performed in a buffer without imidazole. Thus imidazole from the protein sample was effectively eliminated during the gel permeation HPLC. It was, therefore, of interest to see if the isolated peaks remained stable in the absence of imidazole. Fig. 4 shows the HPLC sizing profile of each isolated peak after storage for 20 h at 4°C. As shown,

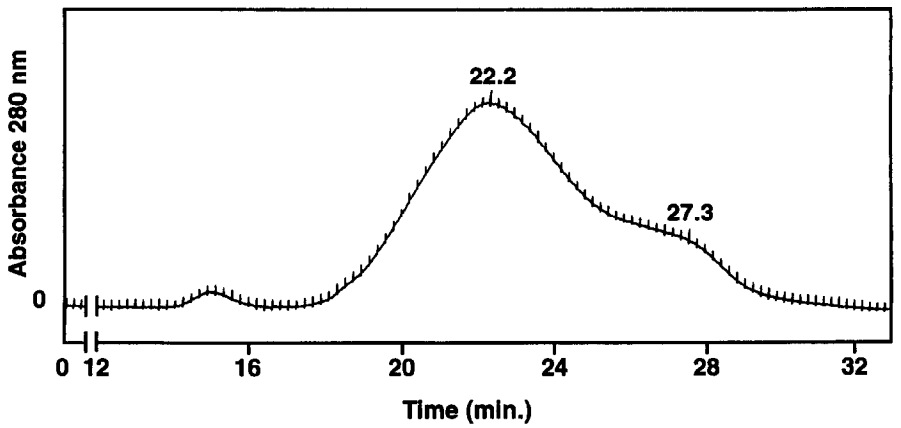

Fig. 1. Superose 12 gel permeation HPLC profile (absorbance vs. time) of purified p66/p66 homodimeric HIV-1 RT. 60 μg of the protein was applied to the column in a volume of 50 μl. The column was equilibrated with 20 mM Tris, 1 M NaCl, pH 8.0, and run at a flow rate of 0.4 ml/min.

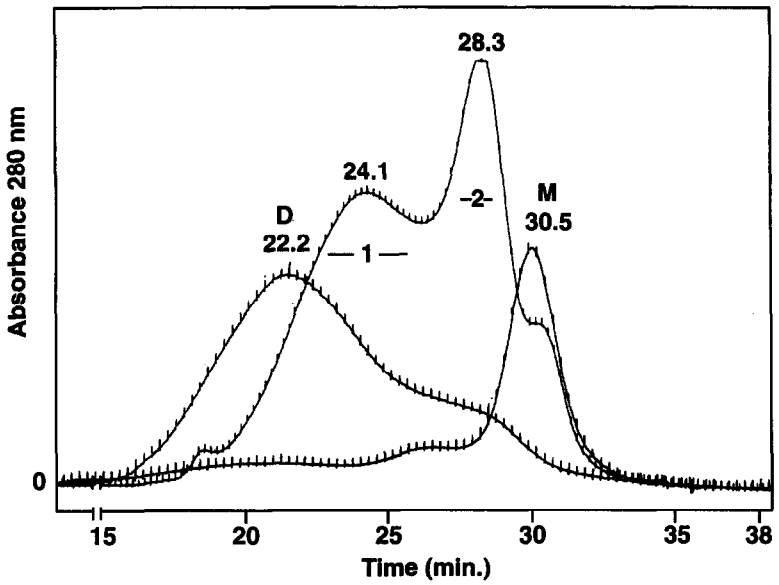

Fig. 2. Superose 12 gel permeation HPLC profile (absorbance vs. time) of p66 HIV-1 RT eluted from an IMAC column in the presence of 0.3 M imidazole. 120 μg of the protein was applied to the column in a volume of 100 μl. Two internal protein markers, homodimeric HIV-1 RT (peak D) and monomeric (peak M) HIV-1 RT [16], are also shown. Other details are the same as in Fig. 1.

both the isolated peaks tend to undergo dimerization, although they differ in the extent of dimerization under defined experimental conditions. It seems from these results that 0.3 M imidazole is sufficient to prevent dimerization of the p66 subunits. The isolated p66 subunits, in the absence of imidazole, undergo slow association to form p66/p66 homodimers. This is consistent with studies which show that p66 forms a homodimer with a dissociation constant higher than that of p66 with the p51 subunit [18]. We conclude that the p66 form of HIV-1 RT can exist in two conformations, and each form can independently undergo association to give rise to a p66/p66 homodimer.

We were also interested in determining whether the stored, isolated peaks 1 and 2 (Fig. 4), represented by different conformers of p66, were active with regard to RNA-dependent DNA polymerase and RNase H functions. As shown in Table 1, both the p66/p66 homodimers, produced from conformationally different p66 subunits, displayed activities that were indistinguishable from each other. Moreover, Fig. 5 shows that the isolated protein peaks of Fig. 4 can be processed in vitro by HIV-1 protease to give rise to heterodimeric (p66/p51) HIV-1 RT. The appearance of a p51-like cleavage product in the control lane 2 is attributed to overloading of the purified unprocessed p66 HIV-1 RT known to possess associated microheterogeneity [5]. As noted, no associated microheterogeneity was observed and there were equimolar amounts of both the p66 and p51 subunits, suggesting that all the p66 molecules in each peak were competent with regard to cleavage of the protease-sensitive Phe^{440}-Tyr^{441} bond [5]. Previously, two possible hypotheses [6,13,14] have been put forward to explain processing of p66/p66 homodimers into the asymmetric heterodimer seen in the crystal structure [11]: (i) the two subunits in p66/p66 are asymmetric or (ii) the p66/p66 homodimers are symmetrical and undergo a conformational rearrangement after proteolysis of one of the subunits. Our studies favor the first hypothesis in that they show that 0.3 M imidazole prevents dimerization in HIV-1 RT and that the two p66 subunits are not identical in conformation. Elimination of imidazole by overnight dialysis results in dimerization, presumably by reassociation of the two p66 subunits. Therefore, we conclude that the p66/p66 homodimers of HIV-1 RT are conformationally asymmetric.

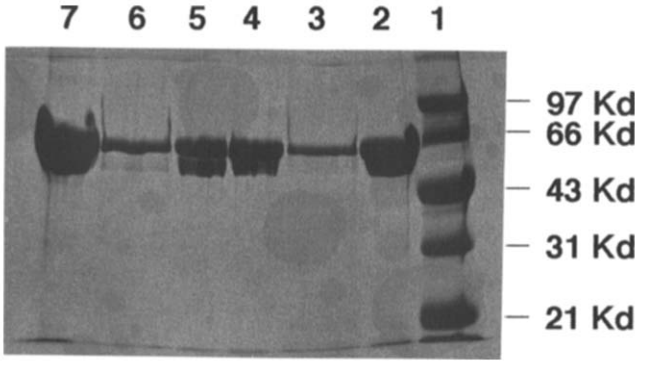

Fig. 3. SDS-PAGE (12%) of isolated protein peaks shown in Fig. 2. Lane 1, molecular weight markers; lane 2, 15 μg purified p66/p66 homodimer; lane 3, 5 μg of protein peak 1 (Fig. 2); lane 4, 15 μg of protein peak 1 (Fig. 2); lane 5, 15 μg of protein peak 2 (Fig. 2); lane 6, 5 μg of protein peak 2 (Fig. 2); lane 7, 15 μg of purified p66/p66 homodimeric HIV-1 RT.

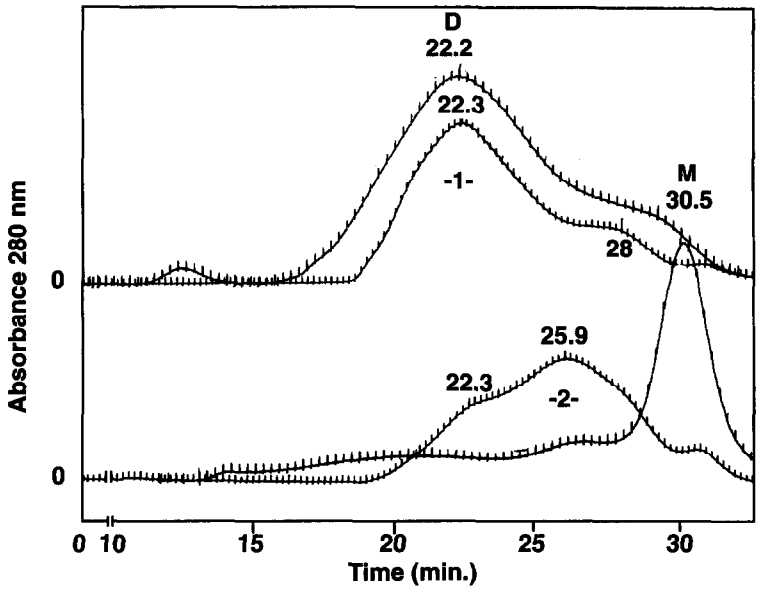

Fig. 4. An absorbance vs. time profile obtained during re-chromatography on a Superose 12 HPLC column of isolated peaks (Fig. 2) after 20 h of storage at 4°C. (Top traces) Re-chromatography of 50 μg of isolated peak 1 at 975 μg/ml (Fig. 2). (Bottom traces) Re-chromatography of 50 μg of isolated peak 2 at 932 μg/ml (Fig. 2). Two internal protein markers, homodimeric HIV-1 RT (peak D) and monomeric (peak M) HIV-1 RT [16], are also shown. Other details are the same as in Fig. 1.

The underlying molecular events in identical sequence-containing p66 proteins of HIV-1 RT which are manifested in different HPLC gel permeation profiles are not known. However, the HPLC sizing technique is sensitive enough to permit the differentiation of p66 proteins. Therefore, we studied de-dimerization of purified p66/p66 homodimeric HIV-1 RT in the presence of 0.3 M imidazole. Fig. 6 shows protein peaks obtained upon incubation of purified p66 homodimeric RT with 0.3 M imidazole. Apparently, each of these peaks behave like monomeric HIV-1 RT [16]. These results suggest that one of the p66 subunits (24.1 min, peak 1 of Fig. 2) upon dialysis of the imidazole undergoes a conformational change before association with the 28.3 min (peak 2 of Fig. 2) to give rise to the p66/p66 homodimer. This change is reflected in the de-dimerization studies in the presence of 0.3 M imidazole resulting in peaks A and B (Fig. 6). Again, the gel permeation HPLC technique turned out to be a useful tool to register the subtle conformational differences among the p66 protein species.

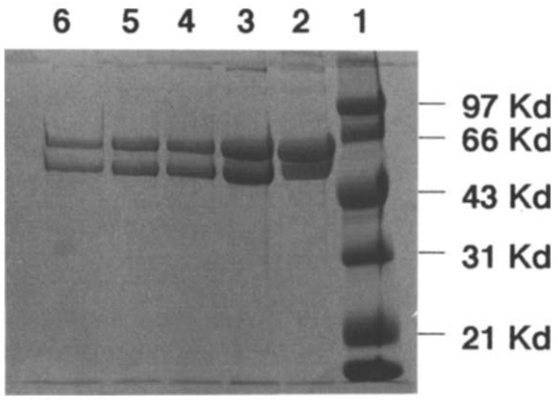

Fig. 5. SDS-PAGE (12%) of isolated peaks (Fig. 2) after HIV-1 protease treatment. Lane 1, molecular weight markers; lane 2, purified p66/p66 homodimer without HIV-1 protease treatment; lane 3, heterodimeric HIV-1 RT produced from p66/p66 homodimers (lane 2) by HIV-1 protease treatment; lane 4, peak 1 in the presence of HIV-1 protease; lane 5, peak 2 in the presence of protease; lane 6, the 31 min shoulder peak (Fig. 2) in the presence of HIV-1 protease. For other details, refer to section 2.

Interestingly, re-chromatography of the peaks isolated from Fig. 6 showed that the two protein species, generated by in vitro dissociation of the p66/p66 homodimers by 0.3 M imidazole, are stable for at least 48 h at 4°C. These p66 protein species were analyzed for both RNA-dependent DNA polymerase and RNase H activities. As shown in Table 2, both forms possess polymerase activities that are indistinguishable from the polymerase activ-

Table 1
Relative enzymatic activities of p66 HIV-1 RT before and after gel permeation HPLC

Enzyme	Specific activity (U/mg)[a]	
	Polymerase[b]	RNase H[c]
p66/p66[d]	24,890 ± 535	702 ± 115
Peak 1	22,540 ± 2124	943 ± 33
Peak 2	24,690 ± 2655	772 ± 44

[a] Data represent the mean ± S.D. (n = 3).
[b] 1 U = 1 mol radiolabeled dTMP incorporated into poly(rA)·oligo(dT) in 1 h at 37°C.
[c] 1 U = 1 mol of TCA-soluble radiolabeled adenylate released in 1 h at 37°C.
[d] IMAC-purified. The specific activities vary from batch to batch (see Table 2).

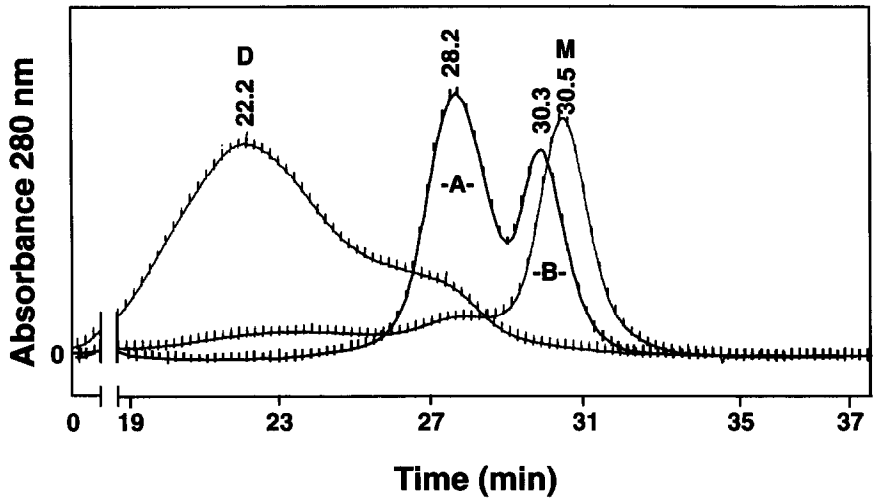

Fig. 6. Superose 12 gel permeation HPLC profile (absorbance vs. time) of products obtained during dissociation of purified p66/p66 homodimers with 0.3 M imidazole. 200 μg of p66/p66 homodimers (1 mg/ml) were incubated for 16 h at 4°C with 0.3 M imidazole in 20 mM Tris, 1 M NaCl, pH 8.0. The sample (100 μl) was loaded to Superose 12 HPLC under conditions described in Fig. 1. Two internal protein markers, homodimeric HIV-1 RT (peak D) and monomeric (peak M) HIV-1 RT [16], are also shown.

ity of the p66/p66 homodimer before and after gel permeation HPLC. In contrast, the RNase H activity of the peak B was approximately 50% of that observed for peak A. As shown for the p66/p66 homodimers, there appears to be a 30% loss in RNase H activity during the HPLC step. This would explain why the specific activity of peak A (235 ± 8 U/mg) is about 25% lower than the p66/p66 homodimer (306 ± 14 U/mg), since peak A has undergone re-chromatography. The RNase H activity results for peak B after re-chromatography (115 ± 9 U/mg) compared with RNase H activity of peak A (235 ± 8 U/mg) thus most likely reflects a structurally disordered RNase H domain in the p66 promoter manifested by peak B. The isolated protein peak B was sensitive to HIV-1 protease, while protein peak A was relatively resistant to cleavage by this enzyme (data not shown). From the combined results of Figs. 2 and 6 and Table 2, we conclude that the two subunits in p66/p66 homodimer are not symmetrically related, presumably as a result of partial unfolding of the C-terminal p15 RNase H domain in one of the p66 protomers. This conclusion is consistent with our recent comparative studies on the susceptibility of p66/p66 homodimer and the active p15 RNase H domain to digestion by HIV-1 protease [19].

Table 2
Dissociation of p66/p66 homodimer by imidazole: relative enzymatic activities of protein peaks isolated and re-chromatographed using gel permeation HPLC

Enzyme	Specific activity (U/mg)[a]	
	Polymerase[b]	RNase H[c]
p66/p66[d]	15,930 ± 625	430 ± 5
p66/p66[e]	13,490 ± 248	306 ± 14
Peak A[f]	15,170 ± 326	235 ± 8
Peak B[f]	15,870 ± 334	115 ± 9

[a] Data represent the mean ± S.D. (n = 3).
[b] 1 U = 1 mol radiolabeled dTMP incorporated into poly(rA)·oligo(dT) in 1 h at 37°C.
[c] 1 U = 1 mol of TCA-soluble radiolabeled adenylate released in 1 h at 37°C.
[d] IMAC-purified. The specific activities vary from batch to batch (see Table 1).
[e] IMAC-purified, subjected to HPLC.
[f] Isolated from gel permeation HPLC column and re-chromatographed.

The origin of conformational differences in the two p66 subunits, as elaborated by peaks 1 and 2 of Fig. 2 and peaks A and B of Fig. 6, may be an inherent flexible property of the polypeptide sequence related to the p51 and p15 RNase H domains. In addition, dimerization may provide some of the energy required to unfold the RNase H domain of one of the p66 subunits [1]. Thus, the slow dimerization of p66 subunits associated with a conformational change and unfolding of one of the RNase H domains could be the rate-limiting step for p66/p66 homodimer formation. This is consistent with the experimental data (Table 2) in which one of the isolated p66 subunits, generated by dissociation of purified p66/p66 homodimer, is much less active with respect to RNase H function. Our results support the view [6,13,14,19] that the asymmetric nature of the heterodimeric HIV-1 RT is more likely due to processing of the asymmetric p66/p66 homodimer and is unlikely to be the result of clipping-off of the RNase H domain from one of the symmetric p66 subunits followed by a conformational change. The potential biological implications concerning the question of why the p66/p66 homodimer may acquire asymmetry prior to processing by HIV-1 protease remains to be answered.

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