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Characterization of the dimerization process of HIV-1 reverse transcriptase heterodimer using intrinsic protein fluorescence

G. Divita, T. Restle and R.S. Goody

Max-Planck-Institut für medizinische Forschung, Abteilung Biophysik, Jahnstraße 29, 6900 Heidelberg, Germany

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Intrinsic protein fluorescence has been used to study dimerization of the HIV-1 reverse transcriptase (RT). We observed a 25% increase of the tryptophan fluorescence of the enzyme during dissociation of the subunits induced by the addition of acetonitrile. Upon reassociation of the separated subunits, the original fluorescence emission of the heterodimer is restored. A two-state transition model for the RT dimerization process in which the dimers are in equilibrium with folded monomers is proposed. The free energy of dissociation was determined to be 12.2 (± 0.2) kcal/mol. In the absence of Mg^{2+} ions a decrease of this value was observed, whereas the addition of a synthetic primer/template (18/36mer) results in an increase of dimer stability. Analyzing the effect of Mg^{2+} on the establishment of the binding equilibrium, a dramatic effect with a 100-fold acceleration of the association by the divalent ion was observed.

HIV; Reverse transcriptase; Dimerization; Intrinsic protein fluorescence

1. INTRODUCTION

HIV-1 reverse transcriptase consists of two polypeptides with molecular weights of 66 kDa and 51 kDa [1,2] forming a stable heterodimer [3,4], which represents the biologically relevant form [5]. As suggested previously [4], this dimeric organization offers an interesting target for chemotherapeutic intervention against the acquired immunodeficiency syndrome (AIDS). However, as a prerequisite to developing inhibitors of the dimerization process, it is necessary to characterize this protein–protein interaction in more detail.

The 3.5 Å resolution crystal structure of Steitz and coworkers provides a very good view of the overall arrangement of the two subunits within the asymmetric dimer [6]. The p66 subunit has a large cleft carrying the polymerase active site, whereas in the small subunit this site is not accessible due to an intramolecular rearrangement of the so-called connection domain. Although the significance of the dimeric character of the retroviral enzyme for its catalytic properties has become more and more clear during recent years, the process of dimer formation and the physico-chemical parameters involved in the protein–protein interaction need further investigation.

Correspondence address. G. Divita, Max-Planck-Institut für medizinische Forschung, Abteilung Biophysik, Jahnstraße 29, 6900 Heidelberg, Germany. Fax: (49) (6221) 486 437.

Abbreviations: Gdn-HCl, guanidinium hydrochloride; HIV, human immunodeficiency virus; HPLC, high-performance liquid chromatography; RT, reverse transcriptase.

Recently, we have presented data on HIV reverse transcriptase dimerization, including a kinetic description of the process, identification of interface domains and a characterization of the enzymatic properties of the different monomeric, homodimeric and heterodimeric forms of the enzyme (for a review, see [5]). Here we describe a more detailed study of this process using intrinsic protein fluorescence as a signal for the subunit interaction. In particular, we present data on the stability of the RT heterodimer in terms of free energy calculations and the effects of substrate and Mg^{2+} ions on this intermolecular interaction.

2. MATERIALS AND METHODS

2.1. *Materials*

Nucleotides were purchased from Boehringer Mannheim, acetonitrile (gradient grade) and Gdn-HCl (recrystallized) from Merck (Darmstadt, Germany). Buffers were filtered and degassed before use.

2.2. *Enzyme preparation*

Recombinant HIV-1 RT was expressed in *Escherichia coli* and purified as described [7]. A highly homologous preparation of the heterodimeric form of the enzyme resulting from co-expression of the 66 kDa and 51 kDa subunits was used. The protein concentration was determined according to Bradford [8], using a gravimetrically prepared solution of RT as standard.

2.3. *Oligonucleotides*

Oligodeoxynucleotides were synthesized on an Applied BioSystems 380B DNA synthesizer and purified by HPLC reverse-phase chromatography on a Hypersil C18 column as described by Müller et al. [9]. Primer and template oligonucleotides were annealed by heating an equimolar mixture of both in 20 mM Tris-HCl, pH 7.5, for 15 min at 70°C followed by cooling to room temperature over a period of 2 h in a water bath. Routinely, an 18/36mer oligonucleotide primer/template was used.

2.4 *Determination of the polymerase activity of RT*

Polymerase activity was measured by a standard assay using poly(rA) $(dT)_{15}$ as primer/template [4]. The RT preparations used showed a specific activity of about 10,000 units/mg, where one unit of enzyme catalyzes the incorporation of 1 nmol of TMP in 10 min at 37°C to acid-insoluble material.

2.5. *HPLC size exclusion chromatography*

Chromatography was performed using two HPLC columns in series (Bio-Rad TSK-250 followed by Bio-Rad TSK-125; both 7.5 × 300 mm). The columns were eluted with 200 mM potassium phosphate, pH 6.5, at a flow rate of 1 ml/min [4].

2.6. *Fluorescence measurements*

Fluorescence measurements were performed at 25°C using a SLM Smart 8000 spectrofluorometer (Colora, Lorch, Germany) equipped with a PH-PC 9635 photomultiplier, using spectral bandwidths of 2 nm and 8 nm for excitation and emission, respectively. All measurements were corrected for wavelength dependence of the exciting light intensity by the use of the quantum counter rhodamine-B in the reference channel. The intrinsic fluorescence emission of RT was measured using a quartz cuvette with 0.5 cm path-length to minimize the inner filter effect in a total volume of 0.7 ml of fluorescence buffer containing 50 mM Tris-HCl, pH 8.0, 10 mM $MgCl_2$, 50 mM KCl and 1 mM dithiothreitol. Analyzing the effect of magnesium, the same buffer without $MgCl_2$ was used and 1 mM EDTA was added to chelate Mg^{2+} traces. The enzyme was routinely excited at 295 nm and the emission spectrum was integrated between 315 and 420 nm. All data were corrected for background intensity of the buffer, for dilution effects and oligonucleotide inner filter effects as described previously (Divita et al. (1993) Biochemistry, in press). The Raman scatter contribution was also removed by subtracting buffer blank.

2.7. *Studies on the dissociation and association of the RT heterodimer under equilibrium conditions*

Dissociation of the HIV-1 RT heterodimer achieved by addition of acetonitrile (up to 25%) was monitored under equilibrium conditions by measuring the relative changes of the intrinsic fluorescence emission of the protein or by the decrease of the polymerase activity using a standard assay [4]. All experiments were performed at 25°C with an enzyme concentration of 0.5–2 μM in a total volume of 0.7 ml of appropriate buffer containing the required concentration of acetonitrile.

2.8. *Analysis of the rate constants of association and dissociation*

The establishment of the dimerization equilibrium was followed in a time-dependent manner by monitoring the change of the intrinsic fluorescence of the enzyme. Excitation was routinely performed at 295 nm and the emission was measured at the wavelength of greatest intensity change (340 nm). For dissociation experiments, a protein concentration of 2 or 10 μM and for association experiments a protein concentration of 0.5 or 5 μM were used. The time course of the dissociation of the heterodimer was fitted by a first-order equation with a single rate constant. To analyze the dimerization process, heterodimeric RT was completely dissociated by the addition of 17% acetonitrile to the buffer as described [4]. Association of the subunits was induced by a 10-fold dilution of the sample with an organic solvent free buffer resulting in a final concentration of 1.7% acetonitrile. The experimental data were evaluated as a second order reaction.

2.9. *Data analysis*

Data were transferred to a personal computer and evaluated using the commercially available fitting program GRAFIT (Erithacus software), which allows the user to define his own equations.

The experimental data from the fluorescence measurements and from the measurements of the polymerase activity of RT during dissociation or association of the heterodimer were transformed to give the relative fraction of the monomers. For each value of f_{obs} the monomeric fraction of RT (M_a) was calculated applying the following equation [10,11]:

$$M_a = (f_{obs} - f_D)/(f_m - f_D) \quad (1)$$

where f_D and f_m are the fluorescence intensities (or the polymerase activity) of heterodimeric (obtained in the absence of dissociating agent) and monomeric RT (obtained at high concentration of dissociating agent), respectively.

Assuming a one-step model for the reversible dimerization of the HIV-1 RT heterodimer the process can be described by Eqns 2 and 3:

$$D \overset{K_d}{\longleftrightarrow} 2\,M \quad (2)$$

$$K_d = [M]^2/[D] \quad (3)$$

where D represents the heterodimeric form and M the monomeric forms of RT.

The total concentration of monomers (M_t) at any concentration of acetonitrile can be described in terms of the fraction of monomeric protein (M_a) as obtained from the fluorescence measurements by Eqn. 4:

$$M_t = 2D + M = (1 - M_a)M_t + M_a \cdot M_t \quad (4)$$

From Eqns 3 and 4, the K_d can be expressed in terms of the measurable values M_t and M_a:

$$K_d = 2M_t(M_a)^2/(1 - M_a) \quad (5)$$

For the determination of the free energy of HIV-1 RT heterodimer dissociation we have used the two-state denaturation model according to Pace et al. [10], where the free energy of dissociation is defined as a linear function of the concentration of the dissociating agent acetonitrile:

$$\Delta G_d = \Delta G_d^{H_2O} + m[U] = -RT \ln K_d \quad (6)$$

where m is the slope of the plot of ΔG_d versus [U]. [U] is the concentration of the dissociating agent and R and T are the gas constant and the absolute temperature, respectively. ΔG_d was calculated via the K_d at the corresponding concentrations of acetonitrile using Eqn. 4. $\Delta G_d^{H_2O}$ is the extrapolated free energy of monomerisation in the absence of a dissociating agent

3. RESULTS

3.1. *Studies on the dissociation of the RT heterodimer by the use of acetonitrile under equilibrium conditions*

As reported previously ([12], Divita et al. (1993) Biochemistry, in press), HIV-1 RT shows a typical intrinsic tryptophan fluorescence with a maximal excitation at 282 nm and a maximal emission spectrum centered at 338 nm (Fig. 1). The heterodimeric enzyme carries a total of 37 tryptophanyl residues (18 on the p51- and 19 on the p66-subunit). The fluorescence properties of these aromatic amino acids can be used as a signal to investigate conformational changes of proteins [13,14]. For protein–protein interactions, the magnitude of fluorescence changes induced by dissociation of the complex can be relatively high, since a separation of the subunits tends to change the solvent environment and accessibility of these residues [11,15,16].

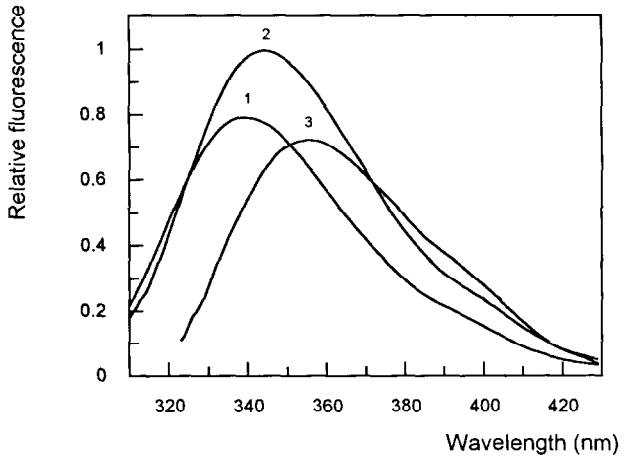

Fig. 1. Intrinsic fluorescence emission spectra of HIV-1 heterodimeric RT under different conditions. For each experiment, 2 μM enzyme was incubated in the standard fluorescence buffer (curve 1), standard fluorescence buffer containing either 17% acetonitrile (curve 2) or 6 M Gdn-HCl (curve 3), corresponding to the native heterodimeric form, folded monomeric forms and denatured monomeric forms, respectively. The excitation was performed at 295 nm. The experimental data were corrected as described in section 2.

The heterodimeric HIV-1 RT can be reversibly dissociated by organic solvents without denaturating the subunits, but resulting in a complete (reversible) loss of enzymatic activities [4,17]. This process has been studied by gel filtration and by monitoring the enzymatic activities, as described. Here we have used the fluorescence approach as mentioned above and the acetonitrile-induced dissociation of the dimer to analyze the thermodynamics and kinetics of the protein–protein interaction. Full dissociation of the dimer to folded monomers was achieved at a concentration of 20% acetonitrile, and is accompanied by a 25% increase of the intrinsic fluorescence with a 7 nm red shift of the emission spectrum from 338 to 345 nm, as shown in Fig. 1. The separate folded monomers, p66 and p51, show emission spectra with maxima at 335 nm and 346 nm, respectively. The absolute emission fluorescence intensity of the p51 subunit was 1.6-fold higher than for the p66 subunit. The sum of the two spectra gave a maximal emission at 343 nm, which was similar to the value of the dissociated heterodimer. In contrast, denaturation of the subunits by 6 M Gdn-HCl was characterized by a 30% fluorescence quenching and a large red shift of the emission spectrum to 352 nm (14 nm), which corresponds to an increase of the solvent exposure of the buried-tryptophan residues, altering their fluorescence characteristics [11,15].

The correlation between the acetonitrile concentration and the dissociation of HIV-1 RT heterodimer using the standard fluorescence buffer at 25°C was followed by the decrease of the polymerase activity as well as by the fluorescence changes described above, as shown in Fig. 2A. For both types of analysis a sharp

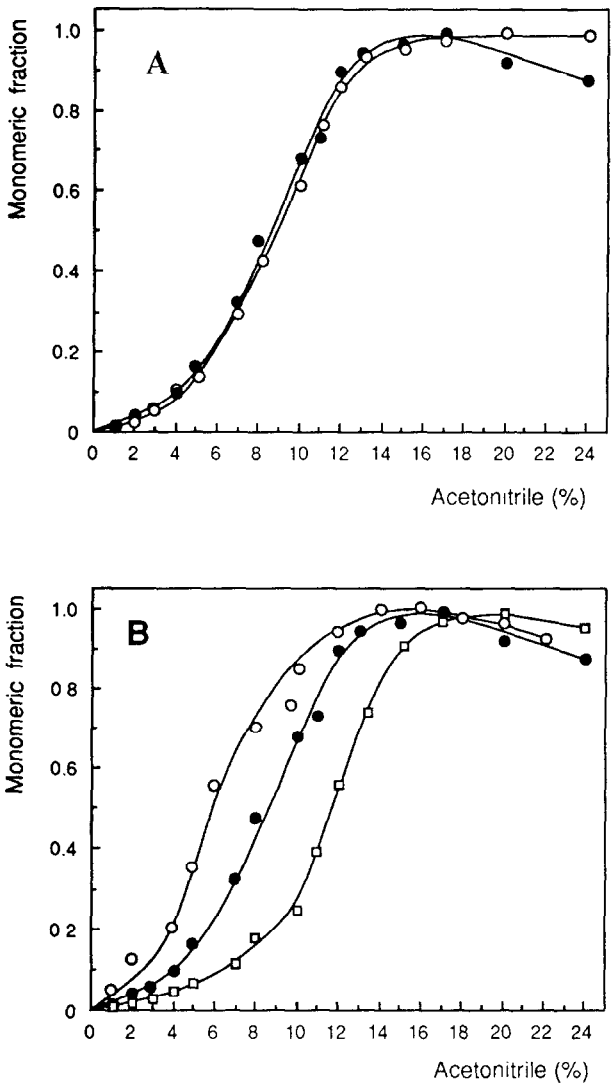

Fig. 2. Correlation between the applied acetonitrile concentration and the degree of dissociation of the HIV-1 RT heterodimer. 2 μM enzyme was incubated in the standard fluorescence buffer. (A) Acetonitrile-induced dissociation of the heterodimeric protein analyzed by the intrinsic fluorescence change (closed circles) or by measuring the polymerase activity (open circles) applying a standard assay. (B) Effect of Mg^{2+} and primer/template (18/36mer) on the stability of the RT heterodimer followed by intrinsic fluorescence. (Open circles) Minus Mg^{2+}; (closed circles) plus 10 mM Mg^{2+}; and (squares) plus primer/template (1 μM) in the presence of Mg^{2+}.

single sigmoidal transition was observed between the initial and final states from 5% to 14% acetonitrile, suggesting a single phase dissociation process. The previously established correlation between dissociation and loss of activity [4] completes the correlation between the fluorescence change and dissociation. The 50% value of the transition from the dimeric to the monomeric state corresponds to 8.5% acetonitrile and complete dissociation was achieved with 17% acetonitrile. As a control the samples were checked by HPLC gel filtration, revealing that the results are fully consis-

tent (data not shown; see section 2). For acetonitrile concentrations above 18% a small decrease of the fluorescence emission was observed, which probably arises from protein precipitation (less than 5%).

We have used this procedure to investigate the influence of divalent ions (especially Mg^{2+}) and substrate (primer/template) on dimer stability (Fig. 2B). Removing Mg^{2+} from the incubation buffer results in a distinct decrease of dimer stabilty with a midpoint for the transition at 6% and an end value at 14% acetonitrile. On the other hand, addition of primer/template (18/36mer) at saturating concentrations (1 μM) induces a stabilization of the protein–protein interaction, suggesting that both subunits may participate in nucleic acid binding. A sharp transition was observed between 9% and 16% with a midpoint at 12% acetonitrile. The nucleotide dTTP showed no effect.

As reported, the acetonitrile induced dissociation of the HIV RT heterodimer is fully reversible, restoring the original enzymatic activities observed before dissociation [4]. Re-association is achieved by reducing the concentration of acetonitrile by 10-fold dilution of the sample. This process can also be studied by the fluorescence approach using the 25% decrease of intrinsic fluorescence, and a 7 nm emission spectrum blue shift during association of the subunits and restoring the situation before dissociation.

3.2. *Analysis of the rate constants for association and dissociation of the HIV-1 RT heterodimer*

A limitation of the HPLC gel filtration method for monitoring the time course of dimerization is that reactions occurring in less than ca. 30 min are not amenable to investigation. However, the described method of intrinsic protein fluorescence as a signal for the subunit interaction offers the possibility to study this process in a time-dependent manner with a resolution of a few seconds.

For analyzing the rate constant of dimer dissociation we used the same conditions as for the equilibrium studies (see above). The dissociation was induced by adding acetonitrile to a final concentration of 17%. The degree of dissociation was checked by gel filtration and by assaying the RNA-dependent DNA-polymerase activity. The two independent approaches revealed that full dissociation takes place under the chosen conditions (data not shown). The experimental data were evaluated using a first-order equation (Fig. 3). Using the standard fluorescence buffer we obtained a value for k_{diss} of 4.5×10^{-3} s^{-1}. This value was not dramatically affected by removing the magnesium ions, which results in a rate constant of 6.1×10^{-3} s^{-1}. However, in the presence of primer/template the dissociation rate constant was reduced three-fold to 2.0×10^{-3} s^{-1}, consistent with the described dimer stabilization by primer/template under equilibrium conditions.

On reducing the acetonitrile concentration, the re-

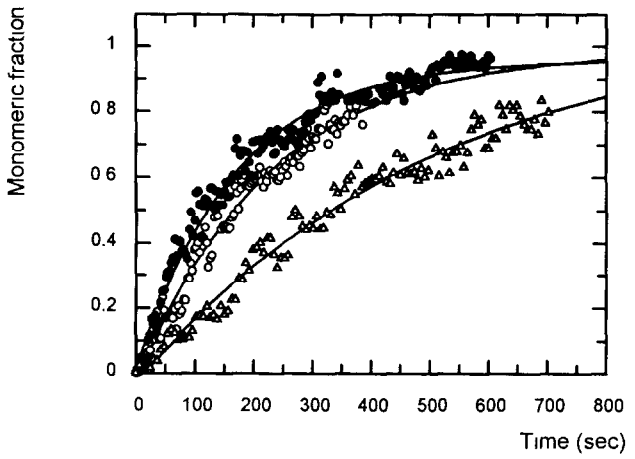

Fig. 3. Time-resolved analysis of acetonitrile-induced dissociation of the HIV-1 RT heterodimer. 2 μM enzyme was incubated in the standard fluorescence buffer (open circles), standard fluorescence buffer without Mg^{2+} (closed circles) or standard fluorescence buffer plus 2 μM primer/template (triangles) at 25°C. The reaction was started by the addition of acetonitrile to give a final concentration of 17%. The changes in intrinsic protein fluorescence were measured at 340 nm with an excitation wavelength of 290 nm. The curves show the best fit to a first-order equation. The fitted k_{diss} values are: 6.1×10^{-3} s^{-1} (without Mg^{2+}), 4.5×10^{-3} s^{-1} (10 mM Mg^{2+}) and 2.0×10^{-3} s^{-1} (2 μM 18/36mer).

association of the dissociated heterodimer can be analyzed. This process occurs in the same manner as the association of separately purified subunits (p51 and p66) which have never been in contact with an organic solvent, indicating that the acetonitrile treated subunits are in a properly folded conformation [4]. The re-association of acetonitrile-treated RT under different conditions is shown in Fig. 4. The experimental data were analyzed in terms of a second-order equation. In the standard fluorescence buffer, we obtained a value of 1.4×10^4 $M^{-1} \cdot s^{-1}$ for the association rate constant. In the presence of primer/template a 2-fold increase of k_{ass} was obtained. This value was lower than expected from the results presented above but could be explained by a lower affinity of the separated subunits for primer/template compared to the entire heterodimer (T. Restle, unpublished observation). In the absence of Mg^{2+} ions, the rate of dimerization was reduced dramatically (by a factor of 100) to a value of 7.5×10^2 $M^{-1} \cdot s^{-1}$.

3.3. *Free energy calculations of RT heterodimer dissociation*

Transforming the experimental data shown in Fig. 2 according to Pace et al. [10,11] to give a linear plot of ΔG_d versus the acetonitrile concentration we have extrapolated, according to the Eqn. 6 as described in section 2, the free energy of monomerisation of the HIV-1 RT heterodimer to 12.2 (± 0.2) kcal/mol at 25°C. The addition of primer/template gave a value of 13.5 (± 0.5) kcal/mol, whereas the removing of $MgCl_2$ results in a

decrease of the $\Delta G_d^{H_2O}$ value to 10.65 (± 0.3) kcal/mol. In each case, the best fit obtained is consistent with a two- state transition model for the RT heterodimer dissociation process in which the dimers were in equilibrium with folded monomers as shown in Eqn. 2. This model is further supported by the following findings: (i) the dissociation is fully reversible (Restle et al. (1990); this report); (ii) both subunits exist in a defined three-dimensional conformation even after acetonitrile treatment. This conformation can be destroyed by the addition of Gdn-HCl resulting in a further change of intrinsic fluorescence (see Fig. 1; Divita et al., manuscript in preparation).

Using the determined $\Delta G_d^{H_2O}$ values and Eqn. 6, we have calculated the apparent equilibrium dissociation constant (K_d) for the dimerization of the HIV-1 heterodimer. For the standard buffer we obtained a K_d of 4×10^{-10} M. Without Mg^{2+} we found a value of 6.8×10^{-9} M, which is in very good agreement with our earlier findings of at least 10^{-9} M using HPLC gel filtration to investigate this process [4]. And finally, as expected from the results presented above, addition of primer/template induces an increase of the subunit affinity, K_d now having a value of 5×10^{-11} M.

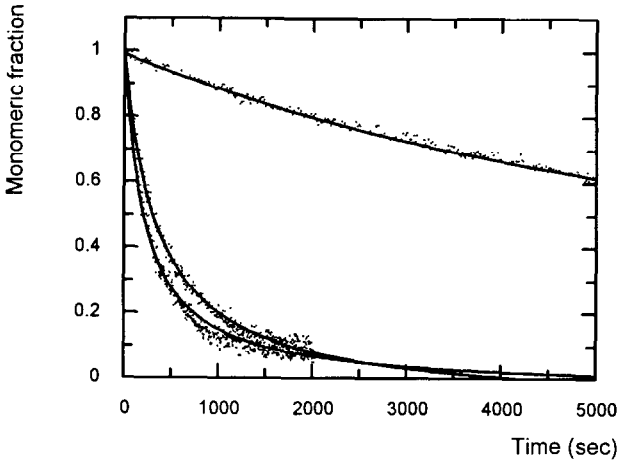

Fig. 4. Time-resolved analysis of association of the HIV-1 RT heterodimer. 5 μM heterodimeric RT was dissociated by 17% acetonitrile. The re-association process was started by a 10-fold dilution of the sample with the standard fluorescence buffer to give a final concentration of 1.7% acetonitrile and 0.5 μM enzyme. The fluorescence measurements were performed under the conditions given in Fig. 3. The curves show the best fit to a second-order equation with k_{ass} values of 7.5×10^2 $M^{-1} \cdot s^{-1}$ (without Mg^{2+}), 1.4×10^4 $M^{-1} \cdot s^{-1}$ (10 mM Mg^{2+}) and 2.8×10^4 $M^{-1} \cdot s^{-1}$ (plus primer/template)

4. DISCUSSION

We have shown that intrinsic protein fluorescence is a powerful approach to investigate the process of dimerization of the HIV-1 RT heterodimer, as reported for numerous other dimeric proteins (for a review, see [18]). Using HPLC gel filtration and activity measurements, it could be demonstrated that the results of these three independent methods are fully consistent. The observed relatively large change (25%) of the intrinsic protein fluorescence during association or dissociation of the heterodimer implies that dramatic rearrangements of the subunit conformation take place, as proposed by Anderson and Coleman [19], who applied circular dichroism to study this process. It seems reasonable to conclude that many tryptophan residues are directly involved in the interaction. There is a cluster of 7 tryptophan residues at position 398–414 in the primary sequence of RT already proposed to participate in the protein–protein interaction [2,20,21], which is consistent with the recently solved crystal structure [6].

In interpreting the presented transition curves for association and dissociation of the heterodimer, we propose a single-step model for dimerization, but we cannot rule out that there is another step involved, which is not detected by the fluorescence method. It is conceivable that an intramolecular rearrangement of one or both subunits occurs before association takes place. In this context, it is of interest to note that Mg^{2+} increases the association rate of the heterodimer by 100-fold, suggesting that the divalent ion induces significant conformational changes of the enzyme or that it is directly involved in subunit interactions. In the crystal structure of the RNaseH domain, two metal binding sites were identified which are in close contact to the His^{539}-containing loop [6,22]. This loop has been identified by the use of a monoclonal antibody to be involved in protein–protein interaction [5]. Furthermore, three other antibodies also recognizing residues in this region of the enzyme have been found to accelerate the association of the dimer by a factor of up to 5-fold. Taking all these findings together, one can speculate that the binding of magnesium results in a conformational change of this domain which favors a rapid association of the subunits. On the other hand, we have clear indications that the dissociated subunits are in a defined three-dimensional conformation as revealed through a further change of the intrinsic fluorescence by the addition of the denaturing agent Gdn-HCl.

The effect of primer/template on the stability of the dimer is not surprising if we assume that both subunits participate in nucleic acid binding, with this interaction providing additional energy for the intermolecular protein interaction. This result might explain the finding of Andreola et al. [23], that tRNA induces a dramatic increase of the enzymatic activities of a p66 preparation. This effect is likely to be due to promotion of homodimerization, which is a pre-requisite for the expression of the activity.

The higher dimer stability in the presence of Mg^{2+}, with a K_d value of 4×10^{-10} M and an increased value for the free energy of dissociation of 12.1 (± 0.2) kcal/mol versus 10.65 (± 0.3) kcal/mol in the absence of the divalent ion, arises mainly from the higher association

rate constant, since the rate of dissociation is only weakly affected by magnesium. Without Mg^{2+}, we found a K_d of 6.8×10^{-9} M confirming our earlier results which were obtained in a Mg^{2+} free buffer using HPLC gel filtration to determine the binding equilibrium [4]. This value seems to be in contrast to the K_d value of 4.9×10^{-5} M reported by Beccera et al. [21]. This can be partially explained by the different pH values of 7.8 versus 6.5 applied for the experiments, since the dimer stability is strongly affected by the pH. The dimer is most stable at pH values of 7.5–8.0 (T. Restle, unpublished observation). Another aspect is the extremely slow establishment of the binding equilibrium, taking more than 100 h at μM concentrations and at temperatures below 5°C in the absence of Mg^{2+}, which may make the ultracentrifuge method unsuitable. On the other hand, at 25°C in the presence of Mg^{2+} the association of the heterodimer is performed within 30 min at a concentration of 0.5 μM, as shown in Fig. 4, which makes the fluorescence approach presented here a useful tool for large scale screening of potential dimerization inhibitors.

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