



## STABILITY OF THE HUMAN IMMUNODEFICIENCY VIRUS-1 REVERSE TRANSCRIPTASE HETERODIMER\*

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### Abstract

The characterization of a heteroassociating system is described in this article:

- The thermodissociation is studied using band sedimentation analysis;
- The electrostatic dissociation is studied using boundary sedimentation analysis.

The heterodimer stability of human immunodeficiency virus-1 (HIV-1) reverse transcriptase (RT) was examined recently<sup>(1)</sup> by sedimentation equilibrium and velocity methods as a function of temperature and ionic strength. RT completely dissociates at 37°C in 50 and 100 mM Tris, pH 7.0, based on sedimentation equilibrium measurements. To obtain the temperature transition data for dissociation, we used band sedimentation velocity in D<sub>2</sub>O, which allows determination of the sedimentation coefficient,  $s_{20,w}$  using approximately 60 µg of RT. The dissociation of RT as a function of increasing temperature from 20°C to 37°C was monitored by measuring the decrease in sedimentation velocity. Different time periods of temperature incubation for RT generated different thermal dissociation curves that allow estimates of dissociation rate constants and respective equilibrium times for dissociation. To examine the effect of ionic strength on p66p51 association, we determined the changes in  $s_{20,w}$  as a function of NaCl concentration. There is a sharp decrease in  $s_{20,w}$  between 0.10 and 0.5 M NaCl, leading to apparent complete dissociation. The above results support a major role for electrostatic interactions in the stabilization of the RT heterodimer.

### Introduction

Numerous questions remain unanswered regarding the components of the human immunodeficiency virus and their interactions. Many HIV-1 viral components can be expressed in large amounts using DNA recombination methods. Consequently, it has become possible to characterize binding interactions *in vitro* in order to gain a greater understanding of how these interactions may control different steps in the viral replication cycle.

Analytical ultracentrifugation represents a powerful methodology for carrying out this type of solution interaction analysis.<sup>(2)</sup> In conjunction with appropriate data analysis methodologies, analytical ultracentrifugation can be used to examine sample purity, detect and characterize conformational changes, determine subunit stoichiometries, characterize assembly and disassembly mechanisms of macromolecular complexes, and measure equilibrium constants and thermodynamic parameters of associating systems.<sup>(2)</sup> No other solution technique provides such a broad range of information for the characterization of hydrodynamic and thermodynamic properties of macromolecular systems. The evaluation of binding interactions as a function of temperature and ionic strength provides important information regarding the type of forces stabilizing association. For an excellent in-depth introduction to the evaluation of association constants for self-associating systems, the reader is referred to McRorie and Voelker.<sup>(3)</sup> Sedimentation velocity measurements strongly complement sedimentation equilibrium analysis, and this report describes the application of band and boundary sedimentation velocity analysis of the dissociation of RT to characterize the stability of HIV-1 reverse transcriptase heterodimers.<sup>(1)</sup>

\* This article is a condensation of the following published paper: Lebowitz, J., Kar, S., Braswell, E., McPherson, S., Richard, D. L. Human immunodeficiency virus-1 reverse transcriptase heterodimer stability. *Protein Sci.* 3, 1374-1382 (1994).

The viral reverse transcriptase (RT) with its associated DNA polymerase and RNase H activities has been a major target for the treatment of acquired immunodeficiency syndrome (AIDS), since it is a crucial enzyme in the viral replication cycle. Structural and biochemical evidence strongly support a heterodimeric (p66p51) active form for HIV-1 reverse transcriptase.<sup>(4-6)</sup> The p66 subunit contains both the DNA polymerase and RNase H functional domains, whereas the smaller polypeptide, p51, lacks the carboxyl-terminal RNase H domain.<sup>(7)</sup> A viral protease is responsible for the asymmetric removal of an RNase H domain from a presumptive p66 homodimer to generate a p66p51 heterodimer; however, this model has not been proven. It has been suggested that agents that can bind at the dimer interface and prevent subunit association could represent a novel therapeutic approach to stem the progression of AIDS.<sup>(6, 8, 9)</sup> To accomplish this objective it is extremely important to establish the strength and nature of the subunit-subunit interactions involved in stabilizing the p66p51 heterodimer.

This article will focus exclusively on the sedimentation velocity characterization of the dissociation of RT. The application of band centrifugation in D<sub>2</sub>O for obtaining thermal dissociation curves will also be highlighted. Band sedimentation velocity allows investigators to explore the effects of temperature using small amounts of protein and should prove valuable for assessing whether intermediate conformational states occur upon dissociation or association.

## Methods

### *HIV-1 Reverse Transcriptase Preparation*

Recombinant HIV-1 RT heterodimer for these studies was prepared by mixing *E. coli* cultures separately expressing the p66 and p51 subunits.<sup>(1)</sup>

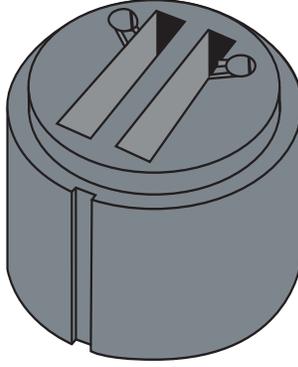
### *Band Sedimentation Velocity Analysis in D<sub>2</sub>O*

Band sedimentation<sup>(10)</sup> of RT was used to evaluate the changes of  $s_{20,w}$  as a function of temperature. This technique provides estimates of the sedimentation coefficient in a comparatively short amount of time using relatively small amounts of material. Moving molecules are always contained within bands, or zones, and a complete physical separation of bands will occur given a sufficient difference in the sedimentation coefficients of the components in a mixture. This physical separation will eliminate the Johnston-Ogston effect due to macromolecular solute interactions that may occur in boundary sedimentation velocity experiments.<sup>(11)</sup>

A double-sector band-forming centerpiece was used for this analysis (Figures 1 and 2). In this cell, macromolecules are transferred, upon the initiation of centrifugation, through a channel from a small well to the sample sector solution, which contains a solution of significantly greater density than the macromolecular solution. D<sub>2</sub>O containing 50 mM Tris, pH 7.0, was used in this study. The sample was spun at 55,000 rpm\*. A wavelength between 290 nm and 300 nm was selected depending on the concentration of RT. The sedimentation velocity of RT as a function of temperature was performed as follows:

1. The band well was filled with 20  $\mu$ L (~3 mg/mL) of RT in 50 mM Tris, pH 7.0, and the cell assembled.
2. D<sub>2</sub>O (50 mM Tris, pH 7.0) was then loaded in both sectors of the centerpiece.
3. The centrifuge cell and rotor were incubated in a constant temperature incubator, and upon reaching a temperature close to the value desired, the rotor was then loaded into the analytical ultracentrifuge.
4. Centrifugation was initiated upon reequilibration to the desired temperature. This experimental setup required approximately 70 minutes.

\* Please reference Beckman Coulter product literature for proper operating guidelines



**Figure 1.** The double-sector band-forming centerpiece.

Tracings of band movement were recorded as a function of time, and the radial position of the symmetric midpoint of the Gaussian profile of the zone was measured at each time interval. The standard correction equation for converting experimental (uncorrected)  $s^*$  values to  $s_{20,w}$  values was used with viscosity and density values for  $D_2O$  at the run temperatures calculated using respectively: 1) a polynomial equation (Eq. 1) that we developed for the viscosity of  $D_2O$  in millipoise units as a function of temperature from data extracted from Kirshenbaum<sup>(12)</sup> and; 2) an equation (Eq. 2) for the density of  $D_2O$  as a function of temperature extracted from Laue *et al.*<sup>(13)</sup>

$$\eta_{D_2O} = 23.524 - 0.80619T + 1.565 \times 10^{-2} T^2 - 1.3647 \times 10^{-4} T^3 \quad \text{1}$$

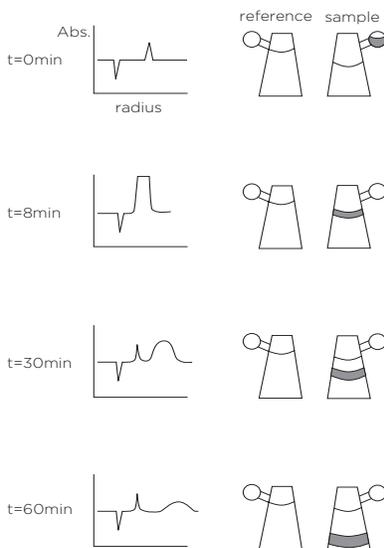
$$\rho_{D_2O} = 1.10602 + \left[ 1 - 1.0555 \times 10^{-6} (T - 11.24)^2 \times \left( 1.74224 + \frac{482.502}{T + 66.621} \right) \right] \quad \text{2}$$

where  $T$  is the temperature in °C. It is possible to estimate the value of  $\bar{v}$  fairly accurately from amino acid composition data.<sup>(14)</sup> This was found to be 0.717 mL/g for the heterodimer. The partial specific volume of RT was not corrected for the effects of deuteration or changes in temperature.

Good agreement of  $s_{20,w}$  values was obtained for RT between band and boundary analysis using the standard correction equation in accord with the original band centrifugation analyses of Vinograd *et al.*<sup>(10)</sup> However, it should be pointed out that Belli<sup>(15)</sup> found that the standard correction overestimated  $s_{20,w}$  by 17% for the band centrifugation of ribosomes in  $D_2O$ . A comparable overestimate of  $s_{20,w}$  was obtained by us (unpublished results) for  $\beta$ -amylase in  $D_2O$  relative to the boundary  $s_{20,w}$  value.<sup>(16)</sup> The reason for the  $s_{20,w}$  variation in the above examples is not clear. Consequently, we advise investigators to compare the  $s_{20,w}$  value from band centrifugation in  $D_2O$  with the boundary  $s_{20,w}$  value in  $H_2O$  buffer to ensure that agreement has been obtained, and if there is a discrepancy, one can empirically correct the  $s_{20,w}$  value.

### **Boundary Sedimentation Velocity Analysis**

Boundary sedimentation velocity of RT was used to evaluate the changes of  $s_{20,w}$  as a function of NaCl concentration. A 5.0 M NaCl stock solution, 50 mM Tris, pH 7.0, was used to titrate an RT solution in 50 mM Tris, pH 7.0. The sample was spun at 56,000 rpm. Sedimentation coefficients were determined at the indicated salt concentrations shown below. The density and viscosity corrections for different concentrations of NaCl were made using the polynomial equations and tables of coefficients for these polynomials in Laue *et al.*<sup>(13)</sup>



**Figure 2.** Operation of the band-forming (*Type I*) centerpiece. In band sedimentation, a small volume of sample solution is layered onto a solvent having fluid density that is significantly higher than that of the sample. A self-generating density gradient caused by diffusion of the solvent into the sample layer forms a region of increasing density ahead of the sedimenting macromolecules. With proper selection of operating conditions, the gradient continues to move ahead of the sedimenting macromolecules by diffusion. Since the sedimenting material moves in a region of essentially constant composition, sedimentation constants are computed from the rate of movement of the band center in a fashion exactly analogous to that used for boundary experiments. The diagrams above show the sample layer moving radially through the solvent at different times from the perspective of the centerpiece and the corresponding absorbance plot. Note: the well on the reference side does not need to be filled, but can be used to subtract any refractive effects created from the initial band from the sample well at the very early stages of a run.

## Results

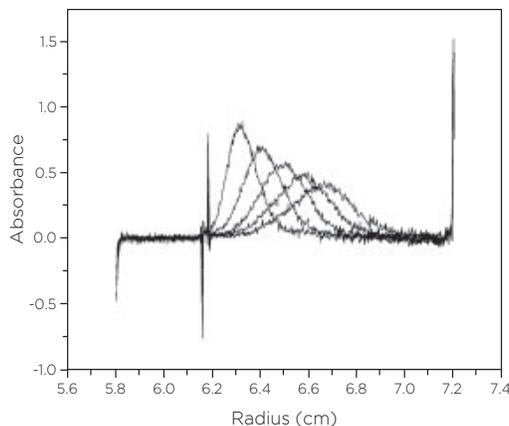
### ***Sedimentation Velocity Analysis of Heterodimer Stability using Band Centrifugation in D<sub>2</sub>O*** ***Characterization of Thermal Dissociation***

Based on sedimentation equilibrium data, the interactions between RT subunits appear to be completely disrupted at 37°C with the generation of subunit monomers that behave ideally (see Ref. 1). To support and extend the sedimentation equilibrium results, we determined a temperature transition curve for RT dissociation. One can predict the sedimentation velocity coefficients for spherical proteins by combining the Svedberg equation and Stokes equation for the frictional coefficient to obtain Eq. 3.<sup>(17)</sup>

$$s = 0.010M^{2/3} \left( \frac{1 - \bar{v}\rho}{\bar{v}^{1/3}} \right) \quad \text{3}$$

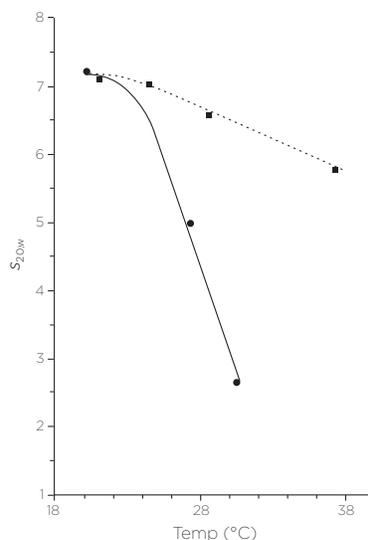
where  $M$  equals the molecular weight, and  $\bar{v}$  and  $\rho$  are the partial specific volume of the protein and density of the sedimentation solvent, respectively. Using Eq. 3 we predict  $s_{20,w}$  values of 7.44 S and 4.68 S for the heterodimer and monomers (weight average), respectively. Within experimental error, the measured sucrose density gradient  $s_{20,w}$  values (results not shown) were in good agreement with the hydrodynamic behavior anticipated for essentially spherical heterodimer and monomer molecules based on Eq. 3.

Band sedimentation velocity measurements in D<sub>2</sub>O allowed us to explore the effects of temperature on the extent of dissociation using minimal amounts of enzyme (approximately 60 µg per experiment). [The original analysis was performed using Model E scanner data. However, for the purposes of allowing the reader to visualize an overlay of band profiles as a function of time, we present a more recent band sedimentation velocity of RT that was performed in a Proteomelab XL-A analytical ultracentrifuge (Figure 3)]. Band sedimentation velocity analysis of RT from 21°C to 37°C revealed the progressive dissociation of the heterodimer as monitored by a decrease in  $s_{20,w}$  from approximately 7.10 S to 5.65 S (dashed curve, Figure 4). Based on an anticipated 4.7 S weight average  $s_{20,w}$  value for monomers, full dissociation of RT did not appear to have been obtained at 37°C. However, the RT samples were only incubated at each indicated temperature for approximately 70 min and equilibrium may not have been achieved during this time, compared to 36 h for the sedimentation equilibrium analysis. To test this hypothesis, we incubated RT samples at



**Figure 3.** Band sedimentation of RT in D<sub>2</sub>O. This is a general overlay scan from a band sedimentation experiment. The plots are spaced 16 min apart.  $s^*$  was determined from the radial movement of the midpoint of the Gaussian profile as a function of time.

20°C, 27°C, and 30°C for 36 h, and band sedimentation velocity experiments were performed at each of the indicated temperatures (solid line curve, Figure 4). It is evident that 36-h temperature incubation leads to a sharp decrease in sedimentation velocity. An  $s_{20,w}$  value of 2.56 S was obtained at 30°C, which is well below the  $s_{20,w}$  value for monomers, indicating that complete dissociation and some monomer unfolding had occurred during the long temperature incubation period. No degradation of enzyme was evident from an SDS-gel electrophoresis analysis of the 30°C RT sample (results not shown). From the 37°C nonequilibrium  $s_{20,w}$  value of 5.65 S, we estimate that the fraction of dimers present at 70 min was 0.40 using the values of 7.1 S and 4.7 S for complete association and dissociation, respectively. From first order dissociation kinetics  $[-\ln(0.40/4200)]$ , we calculate a dissociation rate constant of  $2.2 \times 10^4 \text{ s}^{-1}$  and a  $t_{1/2}$  of 53 min at 37°C. It would require 5.8 h to achieve 99% dissociation. From the 27°C nonequilibrium  $s_{20,w}$  value of 6.5 S, the fraction of dimers present at 70 min was 0.75, and we estimate a dissociation rate constant of  $6.85 \times 10^{-5} \text{ M}^{-1}\text{s}^{-1}$ . The fraction of dimers at 27°C, after 36 h, is estimated to be 0.08 from the  $s_{20,w}$  value of 4.92 S. From the above 27°C rate constant, this level of subunit dissociation would require 10.3 h. The above rough estimates of dissociation rate constants point out that the 36-h incubation period was far in excess of the time needed for full dissociation. The results presented in Figure 3 and the sedimentation equilibrium data provide conclusive evidence for temperature-induced dissociation of HIV-1 RT in 50 and 100 mM Tris buffer at pH 7.0.



**Figure 4.** Thermodissociation transition for HIV-1 RT. The dashed and solid curves represent RT temperature incubation at the indicated temperatures for approximately 70 min and 36 h, respectively.

### Boundary Sedimentation Velocity Analysis of the Dissociation of RT as a Function of NaCl Concentration

Boundary sedimentation velocity analysis was used to examine the effect of ionic strength on heterodimer stability. A single RT solution was titrated with increasing NaCl and  $s_{20,w}$  values repeatedly measured at 20°C. It is evident from Figure 5 that there is a sharp decrease in  $s_{20,w}$  between 0.1 and 0.5 M NaCl. SDS-gel analysis<sup>(1)</sup> shows that the polypeptide integrity of RT is maintained during the salt treatment. Consequently, the decrease in  $s_{20,w}$  can only be accounted for by a dissociation of RT into monomers.

### Discussion

The combined use of sedimentation equilibrium and velocity analysis provides a rigorous, self-consistent thermodynamic and hydrodynamic characterization of the association state of RT in solution. Based on the temperature- and ionic-strength-induced dissociation of RT, electrostatic and hydrogen bond interactions appear to provide very substantial contributions to heterodimer stability

Comparison of  $s_{20,w}$  vs. temperature for different incubation times gave different thermal transition curves for dissociation. With a knowledge of the  $s$ -values of monomer and oligomer, this data can be used to provide estimates of dissociation rate constants from the fractional change in the oligomer.

How can we account for an active enzyme at physiological temperature if free RT appears to completely dissociate into subunits at 37°C? Measurement of RT dissociation rates from primer/templates can be made by trapping free RT with an excess competitor. Using the dissociation rate constant and  $K_d$  values from kinetic measurements, binding rate constants in the range of  $10^6$  to  $10^7$  M<sup>-1</sup>s<sup>-1</sup> are obtained.<sup>(18-20)</sup> Given the slow rate of dissociation of free RT heterodimers to monomers, an estimated  $t_{1/2}$  of 53 min at 37°C, the primer/template binding step is kinetically isolated. The stability of the RTprimer/ template complex and its rapid rate of formation accounts for enzymatic activity at 37°C.

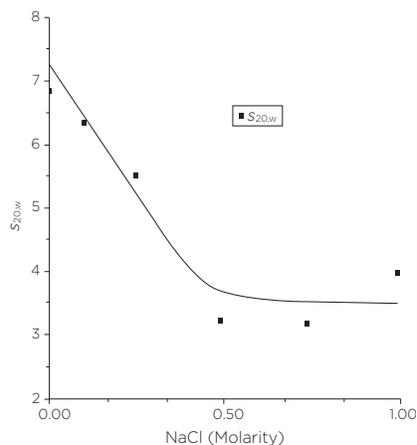


Figure 5. Salt-induced dissociation of HIV-1 RT.

### References

1. Lebowitz, J., Kar, S., Braswell, E., McPherson, S., Richard, D. L. Human immunodeficiency virus-1 reverse transcriptase heterodimer stability. *Protein Sci.* 3, 1374-1382 (1994)
2. Hansen, J. C., Lebowitz, J., Demeler, D. Analytical ultracentrifugation of complex macromolecular systems. *Biochemistry* 33, 13155-13163 (1994)
3. McRorie, D. K., Voelker, P. J. *Self-Associating Systems in the Analytical Ultracentrifuge*. Beckman Instruments, Fullerton, California, 1993.
4. Kohlstaedt, L. A., Wang, J., Friedman, J. M., Rice, P. A., Steitz, T. A. Crystal structure at 3.5 Å resolution of HIV-1 reverse transcriptase complexed with an inhibitor. *Science* 256, 1783-1790 (1992)
5. Jacobo-Molina, A., Ding, J., Nanni, R. G., et al. Crystal structure of human immunodeficiency virus type 1 reverse transcriptase complexed with double-stranded DNA at 3.0 Å resolution shows bent DNA. *Proc. Nat. Acad. Sci.* 90, 6320-6324 (1993)

6. Restle, T., Muller, B., Goody, R. S. Dimerization of human immunodeficiency virus type 1 reverse transcriptase. *J. Biol. Chem.* 265, 8986-8988 (1990)
7. Hansen, J., Schulze, T., Mellert, W., Moelling, K. Identification and characterization of HIV specific RNase H by monoclonal antibody. *EMBO J.* 7, 239-243 (1988)
8. Babe, L. M., Rose, J., Craik, C. S. Synthetic "interface" peptides alter dimeric assembly of the HIV 1 and 2 proteases. *Protein Sci.* 1, 1244-1253 (1992)
9. Divita, G., Restle, T., Goody, R. S., Chermann, J.-C., Baillon, J. G. Inhibition of human immunodeficiency virus type 1 reverse transcriptase dimerization using synthetic peptides derived from the connection domain. *J. Biol. Chem.* 269, 13080-13083 (1994)
10. Vinograd, J., Bruner, R., Kent, R., Weigle, J. Band centrifugation of macromolecules and viruses in self-generating density gradients. *Proc. Nat. Acad. Sci.* 49, 902-910 (1963)
11. Johnston, J. P., Ogston, A. G. A boundary anomaly found in the ultracentrifugal sedimentation of mixtures. *Trans. Faraday Soc.* 42, 789-799 (1946)
12. Kirschenbaum, I. *Physical Properties and Analysis of Heavy Water*, pp. 1-41. New York, McGraw-Hill, 1951.
13. Laue, T. M., Shah, B. D., Ridgeway, T. M., Pelletier, S. L. Computer aided interpretation of analytical sedimentation data for proteins. *Analytical Ultracentrifugation in Biochemistry and Polymer Science*, pp. 90-125. Edited by S. Harding, A. Rowe, and J. Horton. Cambridge, UK, Royal Society of Chemistry, 1992.
14. Cohn, E. J., Edsall, J. T. *Proteins, Amino Acids and Peptides as Ions and Dipolar Ions*, pp. 370- 381. New York, Reinhold, 1943.
15. Belli, M. The evaluation of standard sedimentation coefficient in band sedimentation of macromolecules. *Biopolymers* 12, 1853-1864 (1973)
16. Englard, S., Singer, T. P. Physicochemical studies on  $\beta$ -amylase. *J. Biol. Chem.* 187, 213-219 (1950)
17. Teller, D. C., Swanson, E., DeHaen, C. The translation friction coefficient of proteins. *Methods Enzymol.* 61, 103-124 (1979)
18. Kati, W. M., Johnson, K. A., Jerva, L. F., Anderson, K. S. Mechanism and fidelity of HIV reverse transcriptase. *J. Biol. Chem.* 267, 25988-25997 (1992)
19. Hsieh, J.-C., Zinnen, S., Modrich, P. Kinetic mechanism of the DNA-dependent DNA polymerase activity of human immunodeficiency virus reverse transcriptase. *J. Biol. Chem.* 268, 24607-24613 (1993)
20. Beard, W. A., Wilson, S. H. Kinetic analysis of template-primer interactions with recombinant forms of HIV-1 reverse transcriptase. *Biochemistry* 32, 9745-9753 (1993)