Solution Oligomerization of the rev Protein of HIV-1: Implications for Function

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ABSTRACT: rev is an RNA-binding protein of human immunodeficiency virus-1 and is required for the expression of incompletely spliced viral transcripts. Oligomerization of rev is thought to be associated with RNA binding and rev function. Here, we have characterized the oligomerization of rev using equilibrium analytical centrifugation. rev is predominantly monomeric at low concentrations, but reversibly polymerizes to produce large aggregates at higher concentrations. The data fit well to an unlimited isodesmic self-association model in which the association constants for the addition of a monomer to each aggregate are equal [$K = 1.08 \times 10^6$ M^{-1} at 4 °C]. The association constant is essentially independent of monovalent salt concentration from 0.15 to 2 M at pH 6–9. Thermodynamic parameters derived from the temperature dependence of the association constant over the limited range of 0–30 °C reveal that the primary contribution to the free energy of oligomerization is a large negative enthalpy. Binding of rev to the rev-responsive element of RNA was characterized under the same conditions as the centrifugation experiments using a nitrocellulose filter assay. rev binds to the RRE at a protein concentration where rev is predominantly monomeric, suggesting that solution multimerization of rev is not required for rev function.

Replication of the human immunodeficiency virus type 1 (HIV-1)[1] requires the 13-kDa viral regulatory protein, rev [for reviews, see Green and Zapp (1989), Karn et al. (1991), and Cullen (1992)]. Retroviruses such as HIV-1 express various proteins by alternative splicing of the genomic mRNA. rev mediates a progression in HIV-1 gene expression from multiply spliced transcripts encoding regulatory proteins, such as rev and tat, early in the infection to expression of singly spliced env mRNA, and finally to the unspliced gag–pol polyprotein mRNAs. It has been suggested that rev directly inhibits splicing, possibly by preventing formation of the spliceosome (Chang & Sharp, 1989; Kjems et al., 1991a), or indirectly inhibits splicing by accelerating transport of the unspliced and singly spliced mRNAs from the nucleus—where splicing occurs—to the cytosol. These two possibilities are not mutually exclusive (Green & Zapp, 1989).

rev binds to a highly structured 234-nt region of RNA located within rev-responsive transcripts known as the RRE (Daly et al., 1989; Zapp & Green, 1989). Chemical and RNAase protection experiments (Kjems et al., 1991b) and mutational analysis (Heaphy et al., 1990; Malim et al., 1990; Holland et al., 1990) defined a 66-nt domain, referred to as stem II, that is sufficient for high-affinity rev binding *in vitro* and a for weak *in vivo* rev response (Huang et al., 1991). Recently, several groups have identified a minimal element in stem II consisting of a purine-rich "bubble" within a double-helical stem–loop that is capable of specific, high-affinity rev binding (Heaphy et al., 1991; Bartel et al., 1991; Tiley et al., 1992).

It is generally believed that oligomerization of rev is crucial for the activation of a rev response. Gel filtration (Nalin et al., 1990) and chemical cross-linking studies (Zapp et al., 1991) have led to the suggestion that rev is predominantly tetrameric in solution. At high protein concentrations, rev appears to form extended filamentous structures observable by electron microscopy in both the absence and presence of RNA (Wingfield et al., 1991; Heaphy et al., 1991). However, the relationship between oligomerization and RNA binding remains controversial. Some studies suggest that oligomerization in solution is a prerequisite for binding (Olsen et al., 1990; Zapp et al., 1991), whereas others have suggested that monomeric rev binds to the RRE and subsequently oligomerizes (Malim & Cullen, 1991; Cook et al., 1991; Iwai et al., 1992).

In the present study, we have employed analytical equilibrium centrifugation to define the mechanism and equilibrium constants for rev oligomerization. Equilibrium centrifugation is a thermodynamically rigorous technique for accurate measurements of macromolecular associations. We observe that rev oligomerization is well described by an isodesmic association model. We have also employed nitrocellulose filter assays of rev binding to RRE to demonstrate that RNA binding occurs at protein concentrations where rev is predominantly monomeric in solution. Thus, rev is capable of binding to the RRE as a monomer.

EXPERIMENTAL PROCEDURES

Purification and Characterization of rev. The rev protein of HIV-1 was expressed in *Escherichia coli* strain XA-90 from plasmid pZ76-Rev. The cells were induced at mid-log phase with IPTG for 3 h. The cells were harvested and lysed in 20 mM HEPES, 200 mM KCl, 2 mM EDTA, 5 mM DTT, 5 mM benzamidine, and 1 mM PMSF, pH 7.9. Cell debris was removed by centrifugation, and the supernatant was adjusted to 500 mM KCl and loaded onto a heparin Sepharose column equilibrated in 20 mM HEPES, 2 mM EDTA, 1 mM DTT, and 500 mM KCl, pH 7.9 (buffer B). The column was washed extensively (15 column vol) with buffer B, and rev was eluted with a 0.5–3 M KCl gradient. The protein was dialyzed against buffer B and stored at −70 °C until use. Sepharose 4B (Pharmacia) was activated by cyanogen bromide (March et al., 1974) and was coupled to heparin (Sigma,

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[1] Abbreviations: CD, circular dichroism; DTT, dithiothreitol; EDTA, ethylenediaminetetraacetic acid; HEPES, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; HIV-1, human immunodeficiency virus-1; IPTG, isopropyl β-D-thiogalactopyranoside; nt, nucleotide; PMSF, phenylmethanesulfonyl fluoride; rms, root mean square; RRE, rev-responsive element.

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porcine mucosa, grade I) in 200 mM $NaHCO_3$ while shaking overnight at 4 °C.

The amino acid composition of the purified rev and its N-terminal sequence (20 residues) were found to be as predicted from the gene sequence. The N-terminal methionine was absent. The molecular mass of the purified rev determined by laser desorption mass spectrometry (Finnigan MAT lasermat) agreed with the mass predicted using the amino acid composition deduced from the gene sequence (12 919 Da experimental, 12 935 Da predicted). In equilibrium centrifugation in guanidine hydrochloride, rev sedimented with a molecular mass of 12 500 ± 1600 Da. The rev molar extinction coefficient measured by quantitative amino acid analysis using a scattering correction on the absorption spectrum was found to be ϵ_{280} = 8900 ± 900 M^{-1} cm^{-1}.

RNA Preparation. Full-length rev-responsive element RNA corresponding to positions 7758–8022 of the HXB-2 sequence was synthesized by *in vitro* transcription of plasmid pRL401 using an SP6-driven system. Uniformly [α-^{32}P]UTP-labeled (800 Ci/mmol, Amersham) RRE was prepared using the Promega Riboprobe Gemini kit. RNA was purified by electrophoresis in 8% polyacrylamide gels containing 8 M urea. The RNA was electroeluted from the gel with an Elutrap device (Schleicher and Schuell) and stored at −20 °C.

Analytical Centrifugation. Equilibrium analytical centrifugation measurements were performed at various rotor speeds and temperatures using a Beckman XL-A centrifuge with six-channel 12-mm charcoal-filled epon centerpieces. Scans were taken at 280 nm at 2-h intervals after 12 h, and equilibrium was judged to be achieved by the absence of systematic deviations in the plot of the difference between successive scans. Solvent densities were calculated according to Laue et al. (1993). The partial specific volume of rev, $\bar{v}$, was calculated to be 0.725 at 20 °C using the method of Cohn and Edsall (1943) and was adjusted for temperature using the following equation (Durschlag, 1986):

$$\bar{v}_T = \bar{v}_{20} + 4.25 \times 10^{-4}(T - 293.15) \quad (1)$$

where $\bar{v}_T$ is the partial specific volume at temperature T (in Kelvin) and $\bar{v}_{20}$ is the partial specific volume at 20 °C calculated from the Cohn and Edsall data.

For data analysis using discrete self-association models, we employed the nonlinear least-squares program NONLIN (Johnson et al., 1981) using the equation

$$C(r) = \delta + C_{10} \exp\{\sigma\xi\} + \sum_{N>1} C_{10}^N K_N \exp\{N\sigma\xi\} \quad (2)$$

where $C(r)$ is the total concentration (or absorption) at a given radius r, δ is the base-line offset, C_{10} is the monomer concentration at the arbitrary reference distance r_0, σ is the reduced molecular weight of the monomer, $\xi = (r^2 - r_0^2)/2$, N is the stoichiometry of the association, and K_N is the equilibrium constant for the formation of N-mer. σ is defined as

$$\sigma = \frac{M_1(1 - \bar{v}\rho)\omega^2}{RT} \quad (3)$$

where M_1 is molecular weight of the monomer, $\bar{v}$ is the partial specific volume, ρ is the solvent density, and ω is the angular velocity of the rotor. In the case where an average molecular weight is obtained by a fit to the first two terms of eq 2, then it is the z-average molecular weight, M_z, that is determined as long as a base-line offset is included and the entire solution column is visible (Correia et al., 1993; Arakawa & Yphantis, 1987).

The data were also analyzed by an unlimited isodesmic association model (Adams & Lewis, 1968; Van Holde & Rosetti, 1967) in which the equilibrium constants for the addition of monomer to any aggregate are equal.

$$M_{N-1} + M_1 \overset{K}{\rightleftarrows} M_N \qquad N \geq 2 \quad (4)$$

Provided that the product $kC_1 < 1$, where k is the isodesmic association constant expressed in units of $(g/L)^{-1}$ and C_1 is the monomer concentration, the total concentration, C_T, can be expressed as

$$C_T = \frac{C_1}{(1 - kC_1)^2} \quad (5)$$

The radial distribution of the monomer is given by

$$C_1(r) = C_{10} \exp\{\sigma\xi\} \quad (6)$$

Thus, the radial distribution of the total concentration can be obtained by substituting eq 6 into eq 5, and the experimental data were fit using the expression

$$C(r) = \delta + \frac{C_{10} \exp\{\sigma\xi\}}{(1 - kC_{10} \exp\{\sigma\xi\})^2}$$

with the NLIN procedure within the SAS software package (SAS Institute, Cary, NC). As in NONLIN, this program allows multiple data sets at several loading concentrations to be simultaneously fit to a single equilibrium constant.

Filter-Binding Assay. rev binding to RRE was measured with a nitrocellulose filter-binding assay similar to that reported by Daly et al. (1989). [α-^{32}P]UTP-labeled RRE at a concentration of 6 pM and variable concentrations of rev were incubated in 20 mM HEPES (pH 7.9) buffer containing 1 mM DTT, 2 mM EDTA, 20 units/mL RNasin (Promega), and variable concentrations of KCl. Triplicate 200-μL samples were incubated at 4 °C for at least 15 min, filtered through a 0.45 μm pore nitrocellulose filter on a 96-well manifold (Schleicher and Schuell), and washed twice with 100-μL aliquots of buffer. For measurements of the rate of dissociation of rev from the RRE, rev was preincubated with 10 pM [α-^{32}P]-labeled RRE for 15 min, and dissociation was initiated with the addition of 50 nM unlabeled RRE. Aliquots were withdrawn from the reaction tube at the indicated time points and filtered as above. Filter-bound radioactivity was quantitated using a Molecular Dynamics Model 425E phosphorimager.

RESULTS

Equilibrium Sedimentation. Sedimentation equilibrium measurements of rev were performed over the concentration range 0.025–0.5 mg/mL at three different rotor speeds. Figure 1 shows the z-average molecular weights (M_z/M_1) of rev plotted as a function of protein concentration from 0.025 to 0.5 mg/mL. It is apparent that M_z/M_1 increases continuously with rev concentration and that this increase is independent of rotor speed. These results indicate that rev self-associates under these experimental conditions and that the association is a reversible mass-action equilibrium. At the lower concentrations, M_z/M_1 value approaches 2–3, indicating that rev does not fully dissociate even at a concentration of 0.025 mg/mL. At 0.5 mg/mL, M_z/M_1 approaches a value of 15–20. Thus, rev reversibly polymerizes into large oligomeric assemblies in solution under these experimental conditions.

In order to define whether rev fully dissociates into monomer under dilute conditions, sedimentation equilibrium experiments

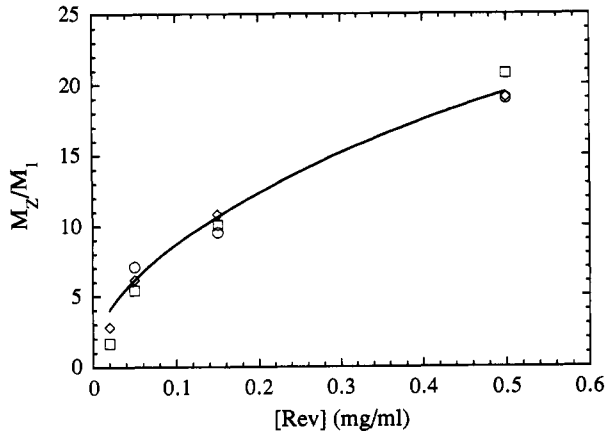

FIGURE 1: z-average molecular weight of the rev protein as a function of protein loading concentration. The z-average molecular weights were determined as described in Experimental Procedures. The rotor speeds are 10 000 (○), 15 000 (□), and 20 000 (◇) rpm. The temperature is 4 °C in all cases. Also shown are the theoretical z-average molecular weights calculated using the isodesmic model with $K = 1.08 \times 10^6$ M^{-1} (solid line).

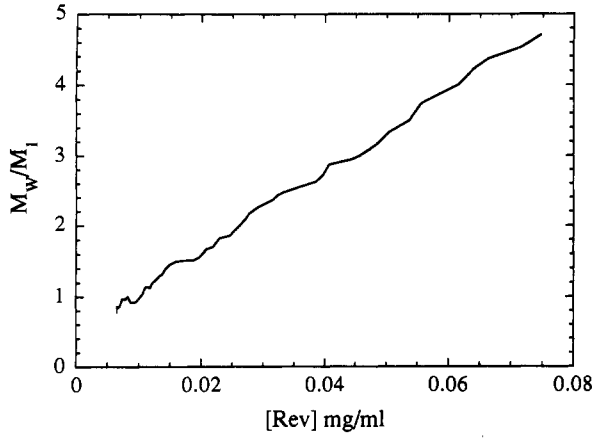

FIGURE 2: Weight-average molecular weight of rev as a function of protein concentration. The sample was loaded at a concentration of 0.05 mg/mL and centrifuged at 26 000 rpm at 4 °C. The weight-average molecular weight was calculated on a point by point basis from measurements of d ln C/dr^2 across the cell.

were performed at higher rotor speeds to fully deplete the meniscus region of higher oligomeric species. The data were analyzed by calculating weight-average molecular weights on a point by point basis across the cell. Figure 2 shows a plot of the weight-average molecular weight (M_w/M_1) as a function of total protein concentration taken from a single sample channel loaded at 0.05 mg/mL rev and centrifuged at 26 000 rpm. Although these data display a high level of noise owing to the very low absorbance near the meniscus, it is obvious that, at the lower protein concentrations, M_w/M_1 is close to unity, indicating that rev completely dissociates to monomer.

Equilibrium Constants. The results depicted in Figures 1 and 2 suggest that rev is capable of forming large oligomers with monomer as the fundamental assembly unit. In order to accurately define the mechanism of rev self-association and to obtain the association constants, the equilibrium centrifugation data were analyzed using nonlinear least-squares procedures. Two classes of models were considered: discrete self-association involving one or more stoichiometrically defined monomer to *n*-mer equilibria, and unlimited self-association with no bound to the stoichiometry of rev oligomers. In both cases, M_1 was fixed at the rev monomer molecular weight of 12 935 Da. Data from cells loaded at concentrations of 0.05, 0.15, and 0.5 mg/mL were analyzed simultaneously, with the constraint of a single set of equilibrium constants common to all of the cells.

Table I: Analysis of Sedimentation Equilibrium Data[a]

model	association constants[b]	rms (OD)[c]
monomer–16-mer	$K_{16} = 4.27 \times 10^{70}$ M^{-15}	0.0135
monomer–20-mer	$K_{20} = 2.04 \times 10^{85}$ M^{-19}	0.0102
monomer–24-mer	$K_{24} = 8.33 \times 10^{100}$ M^{-23}	0.0129
isodesmic	$K = 1.08$ (1.07–1.10) $\times 10^6$ M^{-1}	0.0052

[a] Calculated by a simultaneous fit to data acquired at 0.05, 0.15, and 0.5 mg/mL loading concentrations in 0.5 M KCl, 20 mM HEPES, 2 mM EDTA, and 1 mM DTT (pH 7.90) at a rotor speed of 15 000 rpm and a temperature of 4 °C. See Experimental Procedures for details of the analysis methods. [b] Values in parentheses refer to 65% confidence intervals. [c] Root mean s quare deviation of the fit in units of optical density.

The simplest discrete self-association model considered is a monomer to *N*-mer equilibrium, where *N* is fixed at several integer values. The best fit occurs at a value of $N = 20$. However, the quality of the fit is relatively insensitive to the value of *N* over a range of 16–24, and even for $N = 20$ the rms error is significantly larger than the noise level of the data (~0.005 OD). The results are given in Table I. The fact that the best fit occurs at $N = 20$ is likely fortuitous, in that the highest concentration of rev included in the data set has a value of M_z/M_1 near 20. The fit was significantly improved by including a second equilibrium constant for the formation of a larger aggregate, but again the rms deviations were not sensitive to the stoichiometry (data not shown). Inclusion of a third equilibrium slightly improved the fit.

Several proteins have been found to undergo unlimited polymerization in solution (Adams & Lewis, 1968; Reisler et al., 1970). Many of these systems are well described by a simple isodesmic model (Van Holde & Rosetti, 1967), in which the equilibrium constants for the addition of monomer to each aggregate are equal. This model has an exact solution (see Experimental Procedures). Figure 3 shows a plot of the rev data fitted to the isodesmic model, and the results are also summarized in Table I. The isodesmic model fits the data well at all three loading concentrations, and the rms error approaches the noise level in the data. We note that there is no need to include a nonideality term to fit the data adequately.

The isodesmic model is a significantly better description of the data than the discrete models, in that it requires only a single equilibrium constant to account for the data and does not require the inclusion of arbitrary stoichiometries. However, given the limited resolution of the equilibrium centrifugation technique, more complex association models cannot be strictly excluded. Using the association constant derived from the isodesmic model and the definition

$$M_z = \frac{\sum_i N_i M_i^3}{\sum_i N_i M_i^2}$$

M_z/M_1 was calculated as a function of loading concentration and overplotted with the experimental data in Figure 1. The theoretical points closely overlay the data, indicating internal consistency for the isodesmic analysis.

Effects of Ionic Strength, pH, and Temperature on rev Association. Figure 4A shows the dependence of the rev isodesmic association constant on the ionic strength of the buffer. For each determination of an equilibrium constant, three data channels at different loading concentrations were simultaneously fit to the isodesmic model as described above.

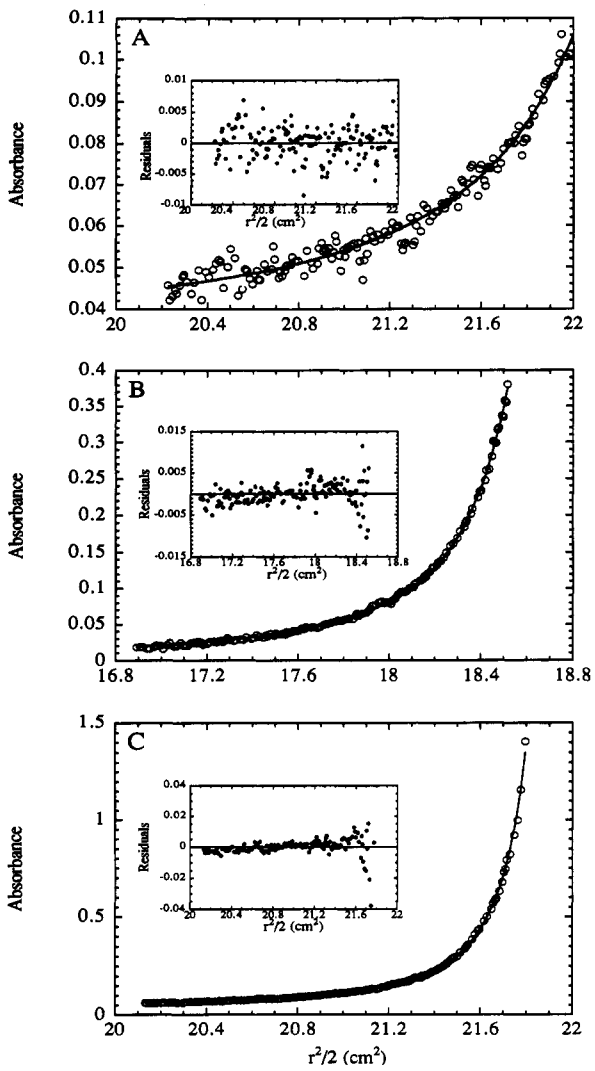

FIGURE 3: Isodesmic fit to the concentration distributions of rev at a rotor speed of 15 000 rpm and a temperature of 4 °C. The loading concentrations are (A) 0.05, (B) 0.15, and (C) 0.5 mg/mL. The open circles are the experimental data, and the solid line is the simultaneous fit of all three data channels using the isodesmic model. The insets show the residuals, and the results of the fit are given in Table I.

The rev association is essentially independent of salt concentration. We observed no effect from the addition of 25 mM SO_4^{2-} or 10 mM Mg^{2+} on the association constant of rev in 0.5 M KCl.

Figure 4B shows the pH dependence of rev oligomerization. The association constant increased slightly with increasing pH. However, this is a weak effect: over a $[H^+]$ range spanning 3 orders of magnitude, the isodesmic association constant varies by less than 2-fold.

The thermodynamic parameters for rev self-association were obtained from the temperature dependence of the equilibrium constant over the range 0–30 °C. The results are presented in the form of a van't Hoff plot in which ln K is plotted against the reciprocal of temperature (Figure 5). The plot is linear, indicating that the enthalpy change is temperature-independent in this range. Therefore the data were analyzed according to the following relationships:

$$\Delta G^\circ = -RT \ln K \quad (8)$$

$$\Delta G^\circ = \Delta H^\circ - T\Delta S^\circ \quad (9)$$

ΔH° was obtained from the slope of the plot and ΔS° from the y-intercept of a linear least-squares fit to the data. The

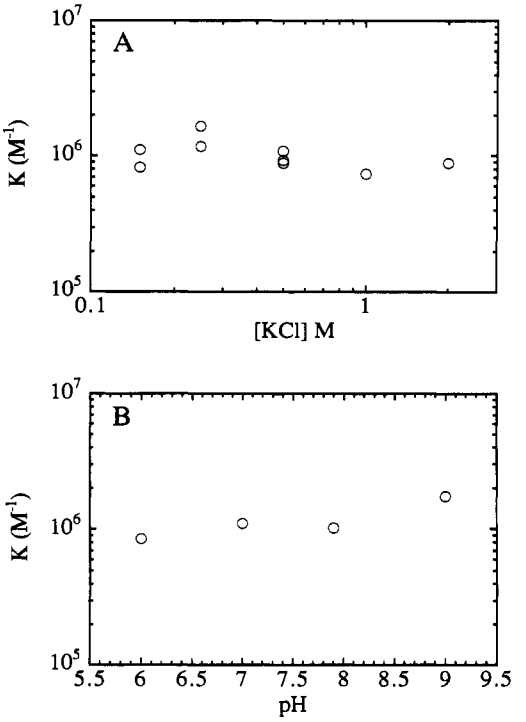

FIGURE 4: Dependence of the isodesmic association constant on monovalent salt concentration (A) and pH (B). Each point represents the results of simultaneous fitting of three channels at different loading concentrations, 15 000 rpm rotor speed, and a temperature of 4 °C to a single isodesmic binding constant. (A) For measurement of the effect of the ionic strength, rev was suspended in buffers containing 20 mM HEPES, 2 mM EDTA, 1 mM DTT, and 0.25–2 M KCl at pH 7.9 and concentrations of 0.05, 0.15, and 0.5 mg/mL. The low solubility of rev in 0.15 M KCl limited the accessible protein concentration to ≤0.2 mg/mL, and samples were prepared at 0.05, 0.1, and 0.2 mg/mL. (B) For measurements of the effect of pH, samples were dialyzed into buffers containing 0.5 M KCl, 2 mM EDTA, 1 mM DTT, and 20 mM bistris propane (pH 9), HEPES (pH 7–7.9), or MES (pH 6). Irreversible precipitation of protein precluded measurements at pH <6. Three samples at 0.05, 0.15, and 0.5 mg/mL were prepared at each pH.

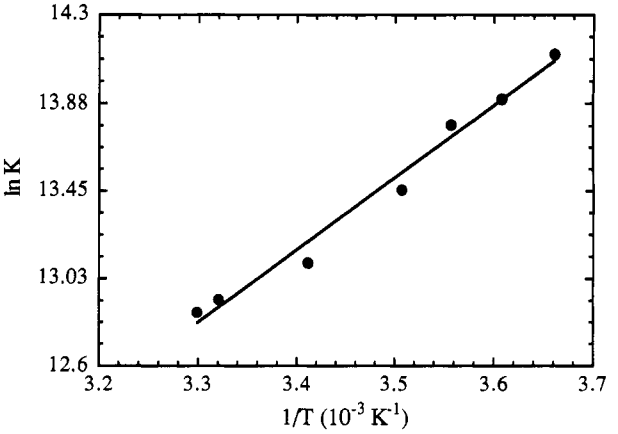

FIGURE 5: van't Hoff plot for the self-association of rev over a temperature range of 0–30 °C. For each temperature, fresh samples were prepared at loading concentrations of 0.05, 0.15, and 0.5 mg/mL in 0.5 M KCl, 20 mM HEPES, 2 mM EDTA, and 1 mM DTT at pH 7.9 and centrifuged at 15 000 rpm. The isodesmic association constants at each temperature were obtained from simultaneously fitting the data at three loading concentrations. The solid line is the linear least-squares fit to the data.

thermodynamic parameters are $\Delta G^\circ = -7.65$ kcal/mol (at 4 °C), $\Delta H^\circ = -6.97 \pm 0.42$ kcal/mol, and $\Delta S^\circ = +2.45 \pm 1.45$ cal/mol·K. The self-association reaction is primarily driven by a negative enthalpy change. The entropy change is positive,

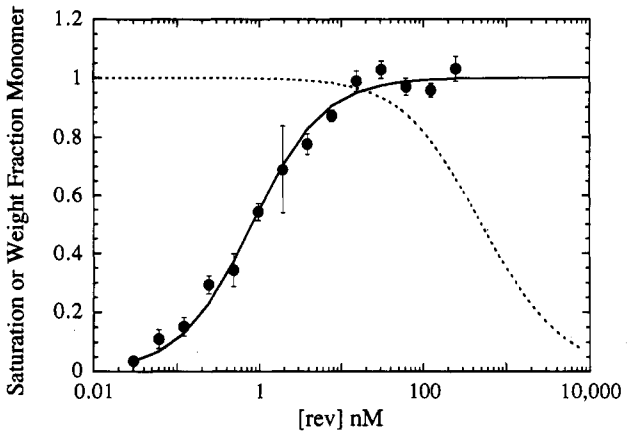

FIGURE 6: Equilibrium binding of rev to the RRE RNA. rev binding to the full-length RRE was assayed by a nitrocellulose filter-binding assay. The assay was performed at 4 °C in 20 mM HEPES, 2 mM EDTA, 1 mM DTT, and 0.25 M KCl at pH 7.9 with [RRE] = 6 pM. The error bars are the 65% confidence intervals for assays run in triplicate. The solid line is the best fit to the data with a hyperbolic binding model. The K_d from the fit is 0.86 nM. The dotted line is the weight fraction of monomeric rev in solution calculated from the isodesmic fit the equilibrium centrifugation data at 0.25 M KCl.

but represents only a minor contribution to the overall free energy change.

rev–RRE Binding. rev binding to the RRE was characterized using the same conditions as those employed for the equilibrium centrifugation in order to elucidate the relationship between rev oligomerization in solution and binding to RNA. Figure 6 shows a binding isotherm for rev interaction with the full-length RRE using a nitrocellulose filter retention assay at 0.25 M KCl. The curve can be fit as a simple bimolecular reaction with a K_d of 0.86 nM, which agrees with previous estimates of the affinity of rev for the full-length RRE [e.g., Daly et al. (1989) and Heaphy et al. (1991)].[2] We have observed that the affinity of rev for the RRE is essentially independent of [KCl] from 0.1 to 0.25 M, but decreases dramatically at higher salt concentrations (data not shown). We note that the binding of rev to the RRE is essentially saturated at protein concentrations ≥10 nM. Overlaid with the binding isotherm is the calculated weight fraction of monomeric rev in solution obtained by solving eq 5 for C_1/C_T and using the self-association constant for rev oligomerization at 0.25 M KCl:

$$\frac{C_1}{C_T} = \frac{2C_Tk + 1 - \sqrt{4C_Tk + 1}}{2C_T^2k^2} \qquad (10)$$

This curve indicates that rev is predominantly monomeric in solution up to a concentration of 10 nM, whereupon binding of rev to the RRE is essentially complete.

The kinetics of dissociation of rev from the RRE was measured by preincubating 1.5–5 nM rev with 10 pM [α-^{32}P]-labeled RRE at 0.25 M KCl followed by adding of 50 nM unlabeled RRE. A dissociation rate of 0.003 ± 0.001 s^{-1} was determined by fitting the decrease in filter-bound radioactivity with time to a first-order decay function.

[2] Although multiple rev molecules are known to bind to the full-length RRE, a simple interpretation of the shape of the binding curve in terms of cooperativity parameters is not justified. In filter retention assays one does not actually measure binding saturation, but rather retention of the nucleic acid. The shape of the binding curve will be influenced by the number of protein molecules required for retention (Freeman & Jones, 1967). This fact was not always recognized in earlier filter-binding studies.

DISCUSSION

Our sedimentation equilibrium data define a model for the mechanism of rev association in solution and provide insights into the relationship between multimerization and RNA binding. Our observations qualitatively confirm earlier work that suggested that rev self-associates in solution (Olsen et al., 1990; Nalin et al., 1990; Daly et al., 1991; Wingfield et al., 1991; Zapp et al., 1991). However, in contrast with two earlier reports (Nalin et al., 1990; Zapp et al., 1991), we find that rev does not form a stable tetramer, but instead polymerizes to form a continuum of oligomeric forms. This polymerization occurs over a wide range of salt concentration, pH, and temperature, which covers the range employed by earlier researchers. Thus, the discrepancy likely is not due to differences in experimental conditions. The evidence presented by Nalin et al. (1990) for tetramer formation is based on gel filtration elution volumes. The shape of the rev gel filtration elution profiles is characteristic of an oligomeric protein in which dissociation/association is rapid on the time scale of the column run (Wingfield et al., 1991). We have observed that the elution volume depends on the loading concentration (J. L. Cole, unpublished results). Thus, gel filtration at a single loading concentration is not a reliable method for characterization of the rev stoichiometry.

Zapp et al. (1991) employed chemical cross-linking to determine that rev is a tetramer free in solution. The cross-linking experiments may be sensitive to the kinetics of the reaction of the cross-linker with rev or to the accessibility of reactive moieties on rev in the multimer, and they do not necessarily reflect an equilibrium distribution of oligomeric forms in solution. Thus, the higher order multimeric species may not have been detected. In contrast, equilibrium centrifugation is a classical, thermodynamically rigorous technique for the characterization of protein self-association reactions. Our sedimentation equilibrium results also suggest that, at low protein concentrations, rev dissociates to monomer. On the basis of sedimentation velocity measurements, Wingfield et al. (1991) suggested that rev is dimeric at, and probably below, 0.1 mg/mL. Their method for the determination of the limiting rev stoichiometry at low concentrations was based on sedimentation velocity measurements extrapolated to zero protein concentration using the assumption that rev is globular with a normal level of hydration. In contrast to hydrodynamic methods, sedimentation equilibrium measurements do not require any shape information to obtain molecular weights.

Our observation that rev is capable of binding to the RRE when it is predominantly monomeric provides the first direct physical evidence that multimerization of rev in solution is not required for binding to the RRE and, presumably, for *in vivo* activity. One may postulate that the <1% of multimer is the species active for binding; however, in order to fit the experimental isotherm, this would require an unreasonably low K_d for the multimer–RRE interaction of ~1 pM. This is much lower than the dissociation constants of other proteins which bind RNA [K_d for tat protein–TAR RNA, ~5 nM (Weeks et al., 1990); K_d for bacteriophage coat proteins–RNA operator, ~0.4–2.5 nM (Witherall et al., 1991)]. The dissociation rate of rev from RRE is 0.003 s^{-1}. Combined with a K_d of 1 pM, this would require an association rate constant near 3×10^9 M^{-1} s^{-1}, which is greater than that expected for a diffusion controlled reaction.

Although solution oligomerization of rev is required for interaction with the RRE, multimerization of rev subsequent to binding to the full-length RRE may be physiologically significant. Cook et al. (1991) employed a gel mobility shift

assay to determine a binding stoichiometry of 1 rev/stem IIb RRE fragment. In gel shift experiments with the full-length RRE, several slower migrating bands develop upon incubation with higher concentrations of rev (Kjems et al., 1991b; Daly et al., 1989; Heaphy et al., 1990; Malim & Cullen, 1991), suggesting that several rev molecules sequentially bind to the RRE. Multiple bands are also observed in stem IIb constructs that contain flanking heterologous double-stranded RNA (Iwai et al., 1992), supporting a model in which monomer rev first binds to a high-affinity nucleation site followed by polymerization on adjacent duplex RNA. Inactive mutants of rev have been identified which bind to the RRE but do not elicit multiple bands in the gel mobility assay, suggesting that multimerization of bound rev may be required for activity (Malim & Cullen, 1991). The oligomerization of rev following binding to the RRE may be governed by the same protein–protein contacts that drive the solution oligomerization reaction. However, direct physical measurements are required to clearly define the mechanism of oligomerization of rev on the RRE.

The lack of a systematic ionic strength and pH dependence of the rev association suggests that the micromolar association constant for rev oligomerization found in this study is likely to be relevant to physiological conditions where rev is active. The pH and ionic milieu within the mammalian nucleus—where rev is functional—is not well characterized and depends on the metabolic state of the cell (Vanden Broeck et al., 1992). In addition, the local ionic environment where rev is active may be quite different from the bulk medium. Nonetheless, the effective ionic strength and pH in nuclei in HIV-1-infected cells is likely to be within the range investigated in this study. Because the concentration of rev within the nucleus of an HIV-1-infected cell has not been measured, the physiological aggregate state of rev cannot be assessed with certainty.

The lack of a systematic ionic strength and pH dependence also suggests that electrostatic interactions do not significantly affect the energetics of rev self-association. On the basis of its amino acid sequence, rev is expected to carry a substantial net positive charge near neutral pH, which would result in repulsive interactions. The positive charges are clustered within the arginine-rich motif of this protein. This domain must be distant from the rev interaction sites in order to shield electrostatic repulsion.

We have not observed that low concentrations of divalent ions influence rev oligomerization. It has been reported that the addition of greater than 2 mM K_2SO_4 inhibited temperature-induced aggregation of rev (Wingfield et al., 1991). However, we do not observe any effect of 25 mM SO_4^{2-} or 10 mM Mg^{2+} on the reversible self-association of rev in solution.

The thermodynamic parameters characterizing rev oligomerization provide further insight into the forces governing the assembly of rev oligomers. The association is characterized by a strongly negative $\Delta H°$ and a weakly positive $\Delta S°$ and is thus enthalpically driven. rev is somewhat unusual in that most protein self-association reactions are associated with a negative $\Delta H°$ and a negative $\Delta S°$ (Ross & Subramanian, 1981). Hydrophobic interactions give rise to both positive $\Delta H°$ and positive $\Delta S°$, whereas the formation of hydrogen bonds in a medium of low dielectric or van der Waals contacts is associated with both negative $\Delta H°$ and negative $\Delta S°$ (Ross & Subramanian, 1981). The strong negative $\Delta H°$ for rev self-association indicates that, in contrast to earlier suggestions (Wingfield et al., 1991; Heaphy et al., 1991), the reaction is not controlled by hydrophobic interactions; hydrogen-bonding and/or van der Waals contacts must provide substantial contributions to the energetics of rev association.

The isodesmic model for rev association is compatible with earlier reports of the formation of hollow filaments about 15–20 nm in diameter with a regular repeat of ~4 nm upon incubation of rev at high protein concentrations (Wingfield et al., 1991; Heaphy et al., 1991). The isodesmic model requires that rev contain two interaction sites, and the angle between these sites relative to the center of mass of the monomer will determine the geometry of the rev oligomer. The diameter and repeat size observed in electron micrographs are compatible with the helical polymerization of rev monomers.

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REFERENCES

Adams, E. T., Jr., & Lewis, M. S. (1968) *Biochemistry 7*, 1044–1053.

Arakawa, T., & Yphantis, D. A. (1987) *J. Biol. Chem. 262*, 7484–7485.

Bartel, D. P., Zapp, M. L., Green, M. L., & Szostak, J. W. (1991) *Cell 67*, 529–536.

Chang, D. D., & Sharp, P. A. (1989) *Cell 59*, 789–795.

Cohn, E. J., & Edsall, J. T. (1943) in *Proteins, Amino Acids and Peptides as Ions and Dipolar Ions*, p 157, Rheinhold, New York.

Cook, K. S., Fisk, G. J., Hauber, J., Usman, N., Daly, T. J., & Rusche, J. R. (1991) *Nucleic Acids Res. 19*, 1577–1583

Correia, J. J., & Yphantis, D. A. (1993) in *Analytical Ultracentrifugation in Biochemistry and Polymer Science* (Harding, S., & Rowe, A., Eds.) Royal Society for Chemistry, London (in press).

Cullen, B. R. (1992) *Microbiol. Rev. 56*, 375–394.

Daly, T. J., Cook, K. S., Gray, G. S., Maione, T. E., & Rusche, J. R. (1989) *Nature 342*, 816–819.

Daly, T. J., Cook, K. S., Fisk, G., Jensen, D., Hauber, J., Jaksche, H., & Rusche, J. R. (1991) in *Genetic Structure and Regulation of HIV* (Haseltine, W. A., & Wong, Staal, F., Eds.) pp 135–142, Raven, New York.

Durchschlag, H. (1986) *Thermodynamic Data for Biochemistry and Biotechnology* (Hinz, H. J., Ed.) p 45, Springer-Verlag, New York.

Freeman, E. J., & Jones, O. W. (1967) *Biochem. Biophys. Res. Commun. 29*, 45–52.

Green, M. R., & Zapp, M. L. (1989) *Nature 338*, 200–201.

Heaphy, S., Dingwall, C., Ernberg, I., Gait, M. J., Green, S. M., Karn, J., Lowe, A. D., Singh, M., & Skinner, M. A. (1990) *Cell 60*, 685–693.

Heaphy, S., Finch, J. T., Gait, M. J., Karn, J., & Singh, M. (1991) *Proc. Natl. Acad. Sci. U.S.A. 88*, 7366–7370.

Holland, S. M., Ahmad, N., Maitra, R. K., Wingfield, P., & Venkatesan, S. (1990) *J. Virol. 64*, 5966–5975.

Huang, X., Hope, T. J., Bond, B. L., McDonald, D., Grahl, K., & Parslow, T. G. (1991) *J. Virol. 65*, 2131–2134.

Iwai, S., Pritchard, C., Mann, D. A., Karn, J., & Gait, M. J. (1992) *Nucleic Acids Res. 20*, 6465–6472.

Johnson, M. L., Correia, J. J., Yphantis, D. A., & Halvorson, H. R. (1981) *Biophys. J. 36*, 575–588.

Karn, J., Dingwall, C., Gait, M. J., Heaphy, S., & Skinner, M. A. (1991) in *Nucleic Acid and Molecular Biology* (Eckstein, F., & Lilley, D. M. J., Eds.) Vol. 5, pp 194–218, Springer-Verlag, Heidelberg, Germany.

Kjems, J., Frankel, A. D., & Sharp, P. A. (1991a) *Cell 67*, 169–178.

Kjems, J., Brown, M., Chang, D. D., & Sharp, P. A. (1991b) *Proc. Natl. Acad. Sci. U.S.A. 88*, 683–687.

Laue, T. M., Shah, B. D., Ridgeway, T. M., & Pelletier, S. L. (1993) in *Analytical Ultracentrifugation in Biochemistry and Polymer Science* (Harding, S., & Rowe, A., Eds.) Royal Society for Chemistry, London (in press).

Malim, M. H., & Cullen, B. R. (1991) *Cell 65*, 241–248.

Malim, M. H., Tiley, L. S., McCarn, D. F., Rusche, J. R., Hauber, J., & Cullen, B. R. (1990) *Cell 60*, 675–683.

March, S. C., Parikh, I., & Cuatrecasas, P. (1974) *Anal. Biochem. 60*, 149–152.

Nalin, C. M., Purcell, R. D., Antelman, D., Mueller, D., Tomchak, L., Wegrzynski, B., McCarney, E., Toome, V., Kramer, R., & Hsu, M.-C. (1990) *Proc. Natl. Acad. Sci. U.S.A. 87*, 7593–7597.

Olsen, H. S., Cochrane, A. W., Dillon, P. J., Nalin, C. M., & Rosen, C. A. (1990) *Genes Dev. 4*, 1357–1364.

Reisler, E., Pouyet, J., & Eisenberg, H. (1970) *Biochemistry 9*, 3095–3102.

Ross, P. D., & Subramanian, S. (1981) *Biochemistry 20*, 3096–3102.

Tiley, L. S., Malim, M. H., Tewary, H. K., Stockley, P. G., & Cullen, B. R. (1992) *Proc. Natl. Acad. Sci. U.S.A. 89*, 758–762.

Vanden Broeck, J., De Loof, A., & Callaerts, P. (1992) *Int. J. Biochem. 23*, 1907–1916.

Van Holde, K. E., & Rossetti, G. P. (1967) *Biochemistry 6*, 2189–2194.

Weeks, K. M., Ampe, C., Schultz, S. C., Steitz, T. A., & Crothers, D. M. (1990) *Science 249*, 1281–1285.

Wingfield, P. T., Stahl, S. J., Payton, M. A., Venkatesan, S., Misra, M., & Steven, A. C. (1991) *Biochemistry 30*, 7527–7534.

Witherall, G. W., Gott, J. M., & Uhlenbeck, O. C. (1991) *Prog. Nucleic Acid Res. Mol. Biol. 40*, 185–220.

Zapp, M. L., & Green, M. R. (1989) *Nature 342*, 714–716

Zapp, M. L., Hope, T. J., Parslow, T. G., & Green, M. R. (1991) *Proc. Natl. Acad. Sci. U.S.A. 88*, 7734–7738.