Ribonuclease L (RNase L) is an endoribonuclease that is activated upon binding of adenosine oligomers linked 2' to 5', RNase L degrades viral and cellular mRNAs, leading to an inhibition of protein synthesis in virally infected cells (1, 2). It has been demonstrated by gel filtration, chemical cross-linking (3), and analytical ultracentrifugation (4) that RNase L exists as a monomer in solution but is dimerized in the presence of an activator, suggesting that the catalytically active form of RNase L is activated upon binding of adenosine oligomers linked 2' to 5'.

Fluorescent conjugates of 2',5'-linked adenosine trimer with 7-hydroxycoumarin have been prepared. The coumarin emission anisotropy shows a large increase upon binding to RNase L. Analysis of anisotropy titrations yields values of $K_a$ and $K_d$ close to those obtained by sedimentation. The sedimentation parameters for unmodified 2',5'-linked adenosine trimer also agree with those obtained by enzyme kinetic methods (Carroll, S. S., Cole, J. L., Viscount, T., Geib, J., Gehman, J., and Kuo, L. C. (1997) J. Biol. Chem. 272, 19193–19198). Thus, the data presented here clearly define the energetics of RNase L activation and support the minimal activation model.

RNase L is an endonuclease involved in the interferon pathway. Upon activation by adenosine oligomers linked 2' to 5', RNase L degrades viral and cellular mRNAs, leading to an inhibition of protein synthesis in virally infected cells (1, 2). It has been demonstrated by gel filtration, chemical cross-linking (3), and analytical ultracentrifugation (4) that RNase L exists as a monomer in solution but is dimerized in the presence of an activator, suggesting that the catalytically active form of RNase L is a homodimer. Under stoichiometric binding conditions, dimerization and activation of RNase L require the binding of one activator molecule per ribonuclease L monomer (4). However, in the absence of an activator, only monomer is observed up to a protein concentration of at least 18 μM, indicating that unliganded enzyme is unable to dimerize, or the association is extremely weak. These observations support a minimal model for RNase L activation depicted in Scheme 1. In the context of this model, the energetics of activation of RNase L are defined by two equilibrium dissociation constants, $K_a$ and $K_d$. Note that this model is a subset of the full reaction scheme (5, 6). We have also extended this model to include the substrate binding step and employed it to compare the activation of RNase L by analogs of the native activator (7).

Under the conditions of our previous sedimentation equilibrium measurements (4 °C), the enzyme concentrations were much higher than either $K_a$ or $K_d$. Thus, the stoichiometry of the interaction was defined, but the association constants were inaccessible. However, activator binding is highly temperature-dependent, such that at 20 °C association is weaker and directly accessible by biophysical measurements for several activators containing a 5'-OH group. In the present study, we have characterized the binding of several activators with both sedimentation equilibrium and fluorescence anisotropy measurements. The results are compared with those obtained by enzyme kinetic methods in the accompanying paper (7).

**MATERIALS AND METHODS**

Human RNase L was expressed and purified as described previously (8) and stored in 40% glycerol, 25 mM HEPES, pH 7.5, 100 mM KCl, 5.8 mM MgCl₂, and 5 mM DTT. To reduce the UV absorbance due to oxidized DTT, the sample buffer (11 mM HEPES, pH 7.5, 104 mM KCl, 5.8 mM MgCl₂) was purged of oxygen by bubbling with argon prior to adding 2 mM DTT. The enzyme was equilibrated into the sample buffer using Bio-Rad Biospin 6 spin columns. Protein concentration was measured spectrophotometrically using a molar extinction coefficient at 280 nm of 8.41 ± 0.87 × 10⁴ M⁻¹ cm⁻¹ (4).

The 2',5'-linked adenosine trimer (HO-2',5'-A₃-NH₂) was obtained from the Midland Certified Reagent Company or from Sigma. The activators containing a free 2'-amino group, HO-2',5'-A₃-2'-NH₂ and p-2',5'-A₃-2'-NH₂, were obtained from NAPS GmbH, Göttingen, Germany. The oligornucleotide concentrations were determined spectrophotometrically using ε₂₈₀ nm = 1.53 × 10⁶ M⁻¹ cm⁻¹/adenosine. The succinimidyl ester of 7-hydroxycoumarin-3-carboxylic acid (NHS-7HC) was obtained from Molecular Probes.

Fluorescence activators were prepared by reacting NHS-7HC with HO-2',5'-A₃-2'-NH₂ or p-2',5'-A₃-2'-NH₂. NHS-7HC was dissolved in N,N-dimethylformamide at a concentration of 5 mM. The conjugation reactions were performed in 50 mM sodium phosphate, pH 7.65, at a concentration of 20 μM oligonucleotide, 200 μM NHS-7HC at room temperature for 2 h. The conjugate was purified by reverse phase HPLC using a Vydac C18 column with a linear gradient of acetonitrile (0.5–30% in 30 min, flow rate = 1 ml/min) in 50 mM aqueous triethylammonium acetate, pH 6.8. Representative elution times were: NHS-7HC, 12.95 min; HO-2',5'-A₃-2'-NH₂, 10.13 min; HO-2',5'-A₃-7HC, 15.92 min.

Equilibrium analytical ultracentrifugation were performed using six-channel (1.2-cm path) charcoal-Epon cells with a Beckman XL-A centrifuge and an An-60 Ti rotor at a speed of 14,000 rpm and a temperature of 20 °C unless otherwise indicated. Samples of 110-μl volume were loaded under argon. Scans were recorded at 230 and 260 nm using 0.001-cm point spacing and averaging 10 readings at each point. Equilibrium was judged to be achieved by the absence of systematic deviations in a plot of the difference between successive scans taken 4 h apart.

For fluorescence experiments, 120-μl samples containing fluorescent activator and RNase L were prepared in sample buffer and allowed to

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1 The abbreviations used are: DTT, dithiothreitol; HO-2',5'-A₃, 2',5'-linked adenosine trimer; p-2',5'-A₃, 2',5'-linked adenosine trimer with a 5'-monophosphate; NHS-7HC, 7-hydroxycoumarin-3-carboxylic acid, succinimidyl ester; HPLC, high performance liquid chromatography; HO-2',5'-A₃-7HC, conjugate of HO-2',5'-A₃-2'-NH₂ and 7-hydroxycoumarin-3-carboxylic acid, succinimidyl ester; Me₆POP, 1,4-bis(4-methyl-5-phenoxazoyl)-benzene; r.m.s., root mean square.
equilibrate at least 2 h prior to measurement. Data were collected at
20 °C in 10 × 2-mm Hellma microcuvettes using an ISS K2 fluorometer
at an excitation wavelength of 404 (16 nm bandpass), and emission was
collected using a Schott KV-470 long pass glass filter. Steady-state
anisotropy was measured in the t-form, and instrument G-factors
were measured with the excitation polarizer in the horizontal position. Data
for both the sample and buffer blank were collected for 2 s in each
orientation, and at least three measurements were averaged. Fluorescence
lifetime measurements were performed using the phase modulation
method over a frequency range of 5–190 MHz. The lifetime reference
was MePOPOP in ethanol, (τ = 1.45 ns) (9).

Sedimentation equilibrium data were globally fit using the Mar-
quardt-Levenberg least-squares algorithm with the SAS software pack-
age (SAS Institute, Cary, NC). Within the context of Scheme 1, the
concentrations of E and A_E were expressed in terms of the dissocia-
tion constants K_s and K_A and the concentrations of E and A.

\[
[EA] = \frac{[E][A]}{K_s}
\]  
(Eq. 1)

\[
[A_E] = \frac{[E][A]}{K_A}
\]  
(Eq. 2)

\[
[A] = \frac{[EA][A][E]}{[E][A]} + \frac{1}{K_s} \left( \frac{[E][A]}{K_s} \right)^2
\]  
(Eq. 3)

Our method to determine the dissociation constants relies on the
differences in the absorption spectra of E and A and is similar to the
approach described by Lewis and co-workers (10) to characterize pro-
tein-DNA interactions. We can express the absorbance at any wave-
length as the sum of the contributions from each of the species partici-
pating in the equilibrium

\[
A(\lambda) = \varepsilon_s[A] + \varepsilon_A[A] + (\varepsilon_s + \varepsilon_A)[EA] + 2(\varepsilon_s + \varepsilon_A)[E][A]
\]  
(Eq. 4)

Finally, in the presence of a centrifugal field we can express the ab-
sorbance at wavelength \( \lambda \) and radius \( r \), \( A(r,\lambda) \), as

\[
A(\lambda,r) = \delta_s + \varepsilon_sC_{\text{diff}}e^{-\delta_s r} + \varepsilon_A C_{\text{diff}}e^{-\delta_s r} + (\varepsilon_s + \varepsilon_A)C_{\text{diff}}[A][E]e^{-2\delta_s r}
\]  
(Eq. 5)

where \( \delta_s \) is the baseline offset at wavelength \( \lambda \), \( C_{\text{diff}} \) is the molar concentra-
tion of E at the reference distance \( r_0 \), \( C_{\text{diff}} \) is the reduced molecular
weight of E, and \( \xi = (r^2 - r_0^2)/2C_{\text{diff}} \) and \( \delta_s \) are defined analogously.
The natural logarithms of \( K_s \) and \( K_A \) are the fitted quantities to constrain
the dissociation constants to be positive.

The value of \( \sigma_{\text{diff}} \) was experimentally measured by sedimentation
equilibrium of RNase L alone at 45,000 rpm, and \( \sigma_{\text{diff}} \) was obtained for
each activator by sedimentation equilibrium at 45,000 rpm. Because of
the low molecular weight of the activators, \( \delta_{\text{diff}} \) could not be obtained by
overspeeding following the run. Therefore, we used a mass conservation
method in which \( \delta_{\text{diff}} \) was measured as the difference in the integrated
absorption at 260 nm at equilibrium at 14,000 rpm and the absorbance
at 260 nm at the beginning of the run. Because the activators do not
contribute much absorbance at 230, \( \delta_{\text{diff}} \) was measured by overspeeding
to 45,000 rpm following the centrifugation experiment at 14,000 rpm.
For each experiment three channels containing different activator
concentrations were analyzed globally at 230 and 260 nm where \( C_{\text{diff}} \) and
\( C_{\text{diff}} \) were treated as local fitting parameters, and \( K_s \) and \( K_A \) were global
parameters.

The fluorescence anisotropy titration data were fit using the Mar-
quardt-Levenberg algorithm within the IGOR Pro software package

\[
[\lambda] = \left( \frac{[E]_0 + 1}{K} \right) + \frac{8 \cdot [E] [A]}{4 \cdot [E][A] R}
\]  
(Eq. 6)

where \( r_i \) is the anisotropy of the free activator A, and \( r_i \) is anisotropy of
the bound activator in the species \( E_A \). We assign the anisotropy of EA
to be equal to \( r_i \); the justification is presented below. Equation 7 in-
cludes a quenching factor \( R \), which is the ratio of the quantum yields of
the free and bound forms (9).

The titrations of RNase L were performed at four activator concen-
trations under conditions where \( [A]_t \ll [E]_t \) one can make the approximation that \( [E] \sim [E]_t \). The observed anisotropy \( r_{obs} \) is given as the sum of the anisotropies of
the individual species weighted by the molar concentration of A in each
species

\[
r_{obs} = \left( A_i [A] + [EA]_i [E] + 2[E_A]_i A \right) R
\]  
(Eq. 7)

where \( r_i \) is the anisotropy of the free activator A, and \( r_i \) is anisotropy of
the bound activator in the species \( E_A \).

RESULTS

Sedimentation Equilibrium—Our previous studies of activa-
tor-induced dimerization of RNase L by sedimentation equilib-
rium were performed at 4 °C, using conditions where \( [E] \gg K_s \)
and \( K_d \), so that the stoichiometry could be characterized (4). In
contrast, for the weaker activators, such as HO-2,5'-A, activa-
tor-induced dimerization is not stoichiometric at the higher
temperature of 20 °C. Thus, sedimentation equilibrium data
obtained under these conditions may be used to extract \( K_s \) and
\( K_A \). The radial absorbance profiles contain contributions from
each of the species participating in the equilibrium: \( E, A, EA, \) and
\( E_A ; \) these data can be fit to Equation 5 to obtain \( K_s \) and
\( K_A \). To define the contributions of RNase L and activator at
each radial position we have taken advantage of differences in
their absorption spectra. Fig. 1 shows that at enzyme and activator
concentrations typically used in our experiments the
230 nm absorbance is dominated by the contribution from the

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enzyme, whereas the 260 nm absorbance is dominated by the activator. Thus, we record data for each channel at both of these wavelengths and fit using a single value of $C_{\text{Enz}}$ and $C_{\text{AO}}$. To further constrain the fits, data can be obtained at several intervals. The scale for the residuals is obtained at a rotor speed of 14,000 rpm and a temperature of 20 °C. The three activator concentrations are sufficient to define concentrations of enzyme and activator. We have found that to further constrain the fits, data can be obtained at several intervals. To further constrain the fits, data can be obtained at several intervals.

Fig. 2 shows the sedimentation equilibrium data obtained at 230 and 260 nm for samples loaded at a concentration of 0.5 μM RNase L and 1 (○), 3 (□), and 10 (●) μM activator. The data have been fit to Equation 5; the solid lines are the best fit to the data, the insets are the residuals, and the results are summarized in Table I. There is a weak positive systematic deviation in the residuals at 230 nm for the data obtained at 1 and 10 μM and a negative deviation for the data obtained at 3 μM. These deviations are likely due to errors in the baseline offset term $\delta_{\text{base}}$. However, outside of these deviations the fit is quite good. Although baseline errors result in higher r.m.s. deviations, in the range of 0–0.02 OD they do not affect the fitted values of $K_a$ or $K_d$ significantly as defined by the 1 S.D. confidence intervals.  

Fig. 3 shows a contour plot of the fit error surface for the data in Fig. 1 as a function of ln$K_a$ and ln$K_d$. The values of ln$K_a$ and ln$K_d$ were fixed, and the other parameters were allowed to adjust to the best fit values. The inner contour is drawn at 1 S.D., and the outer contour is at 2 S.D. joint confidence intervals. The validity of this fitting procedure was also verified using simulated data. Extinction coefficients and reduced molecular weights were fixed at the experimental values for RNase L and HO-2’,5’-A$_9$. Data were simulated according to Scheme 1 with a loading concentration of 0.5 μM enzyme and 1, 3, and 10 μM activator. Random Gaussian noise of amplitude 0.0062 r.m.s. was added to corresponding to typical data obtained with our XL-A centrifuge. With values of $K_a$ and $K_d$ fixed at the experimental determined values from Table I, the fitting procedure recovers the correct values with 1 S.D. joint confidence intervals of 1.31–1.89 μM for $K_a$ and 15.1–21.8 nM for $K_d$. The slightly greater width of the experimental confidence intervals presumably reflects the contributions of small systematic errors in the fixed parameters.

Table I also shows values of $K_a$ and $K_d$, and r.m.s. errors obtained from fitting the same sedimentation data to an alternative association model in which E dimerizes to form E$_2$. This is then competent to bind two activator molecules to form E$_2$A$_2$ (Scheme 2). In this case, $K_a$ is found to be 7 orders of magnitude lower than in the previous fit, and $K_d$ is significant.
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To compare directly the sedimentation equilibrium and fluorescence anisotropy methods, we characterized one of the fluorescent activators, HO-2,5'-A3-7HC, using the sedimentation equilibrium protocol. Initial measurements indicated that $K_d$ for the activator is significantly lower than for HO-2,5'-A3. Therefore we have obtained sedimentation equilibrium data at a lower enzyme concentration of 0.2 μM and three activator concentrations of 0.2, 0.6, and 2 μM. As shown in Table I, the best fit parameters for HO-2,5'-A3-7HC are $K_a = 2.67 \text{ μM}$ and $K_d = 1.72 \text{ nm}$. The joint confidence intervals for this fit are much broader than for HO-2,5'-A3, so that the change in $K_a$ relative to HO-2,5'-A3 is not statistically significant. However, the decrease in $K_d$ is significant. As in the case of HO-2,5'-A3, simulations were performed with the experimentally derived parameters and Gaussian noise of 0.0062 OD r.m.s. amplitude. Again, the correct values of $K_a$ and $K_d$ are recovered from the fitting procedure with joint confidence intervals of 1.50–8.62 μM for $K_a$ and 0.19–4.35 nm for $K_d$. Thus, the increased breadth in the confidence intervals for the fit of HO-2,5'-A3-7HC are also reflected in the simulations.

Fluorescence Anisotropy—A fluorescence activator binding assay was developed to characterize independently the equilibria depicted in Scheme 1. Fluorescent activators, synthesized from 2',5'-oligoadenylate precursors containing a single 2'-NH2 moiety, were prepared by standard amine coupling reactions. We found that activator conjugates with the NHS ester of NHS-7HC exhibited very useful fluorescence properties. Coupling reactions were characterized by HPLC and spectrophotometry. The product was identified based on the appearance of an HPLC peak with retention time different from either un-conjugated oligonucleotide or the reactive fluorophore. In all cases, a single predominant product was formed in the reaction. No new HPLC peaks were formed in a reaction of NHS-7HC with HO-2',5'-A3 lacking a 2'-NH2 group. Thus, the chemistry is specific for the 2'-NH2 group. The identity of the product was confirmed by absorption spectrophotometry. In aqueous solution, the NHS-7HC exhibits one predominant absorption band at 415 nm without appreciable absorption near 260 nm. In contrast, the product p-2',5'-A3-7HC exhibits two absorption bands at 405 and 260 nm. Thus, the absorption spectral features are consistent with the proposed structure.

The fluorescence emission parameters of p-2',5'-A3-7HC are summarized in Table II. The emission maximum is at 446 nm (uncorrected). Addition of a slight molar excess of RNase L results in about 60% quenching in the fluorescence amplitude and a slight shift in the emission maximum to 451 nm. A dramatic effect is observed in steady-state fluorescence anisotropy, which increases from 0.05 to 0.33 upon binding RNase L. Further addition of RNase L does not result in appreciable increase in the fluorescence changes. Table II also shows that the fluorescence lifetime is 3.5 ns in the absence and 3.4 ns in the presence of RNase L. Because there is no marked change in the lifetime, the observed increase in anisotropy of p-2',5'-A3-7HC observed upon binding to RNase L is clearly due to an increase in the rotational correlation time.3 Similar spectral perturbations and anisotropy increases are observed for HO-2',5'-A3-7HC, which lacks a 5'-phosphate.4 Because the anisotropy increase is very large upon binding, we have chosen to use this parameter for quantitative characterization of the dissociation constants.

The interaction of p-2',5'-A3-7HC with RNase L is too strong to be readily characterized by fluorescence anisotropy experiments, where the lowest accessible fluorophore concentration is about 2 nM. However, the values of $K_a$ and $K_d$ for HO-2',5'-A3-7HC, which lacks a 5'-phosphate, are accessible to fluorescence measurements. Fig. 4 shows a series of RNase L titrations performed at four activator concentrations ranging from 5 to 50 nM. At each activator concentration the anisotropy shows a pseudo-hyperbolic increase with increasing enzyme concentration. The plateau value of the anisotropy observed at the highest enzyme concentration of 2 μM decreases with decreasing activator concentration. In contrast, the half-maximum point is not strongly dependent on activator concentration. We have globally fit these data to Equations 6 and 7. To reduce the number of floating parameters it is necessary to constrain the value of the emission anisotropy of EA, $r_E$. The two limiting cases we have considered are $r_{EA} = r_f$ and $r_{EA} = r_0$. Fig. 4 shows a fit of the data assuming $r_{EA} = r_f$ and the fitted parameters are in Table III. The fitted curves overlay the data quite well (r.m.s. deviation of 0.0131), and the best fit values of $K_a = 1.32 \text{ μM}$ and $K_d = 1.09 \text{ nm}$ agree well with those determined by sedimentation; however, the joint confidence intervals are much narrower for the fluorescence results. A much poorer fit is obtained assuming $r_{EA} = r_0$ (r.m.s. deviation of 0.0229), and the values of $K_a = 44.1 \text{ μM}$ and $K_d$ near 1 ps are not compatible with the sedimentation data. We have also considered the intermediate model in which $r_{EA} = r_f/2$. The quality of this fit is intermediate between the two former cases (r.m.s. deviation of 0.0204), and the values of $K_a$ and $K_d$ are close to that determined assuming $r_{EA} = r_f$. In all of these cases we have also assumed that the 60% quenching only occurs upon dimerization of EA to form $E_2A_2$. However, the values of the equilibrium constants and the fit quality are not very sensitive to the extent of quenching of EA. We conclude that the simplest model capable of fitting the data is that the anisotropy of HO-2',5'-A3-7HC is not strongly changed

\[ \frac{1}{K_d} = \frac{1}{K_a} + \frac{1}{K_d} \]

Table II

| Sample | $\lambda_{max}^a$ | Intensity$^b$ | Anisotropy$^c$ | Lifetime$^d$ |
|--------|------------------|--------------|----------------|-------------|
|        | (nm)             | (%)          |                | (ns)        |
| 50 nM Activator | 446             | 100          | 0.050          | 3.5         |
| + 60 nM RNase L | 451             | 36           | 0.327          | 3.4         |
| + 200 nM RNase L | 451             | 39           | 0.332          | 3.4         |

$^a$ Fluorescence emission maximum. Excitation was at 404 nm (16 nm bandpass).

$^b$ Intensity at 446 nm.

$^c$ Emission anisotropies were measured in the-l-configuration using a KV-470 Schott long pass filter with experimentally determined G-factors.

$^d$ Fluorescence lifetimes were measured by phase modulation from 5 to 190 MHz. Emission was collected using a KV-470 Schott long pass filter.

3 For a simple system with a single fluorescence lifetime ($\tau$) and a single rotational correlation time ($\phi$), the steady-state anisotropy is given by $r = r_f/(1 + (\tau/\phi))$, where $r_f$ is the intrinsic anisotropy in the absence of rotation. Thus, a change in the value of $r$ can be ascribed to change in $\phi$ only if independent fluorescence lifetime measurements indicate that $\tau$ is constant.

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FIG. 4. Anisotropy titration of HO-2,5'-A3-7HC with RNase L. Activator concentrations: ○, 5 nM; □, 10 nM; △, 20 nM; ●, 50 nM. The solid lines are a global fit of the data to Scheme 1, and the results are summarized in Table III.

TABLE III

| Model | $K_a$ | $K_d$ | r.m.s. |
|-------|-------|-------|--------|
| $r_{EA} = r_{t}$ | 1.32 (1.04, 1.76) | 1.09 (0.63, 1.71) | 0.0131 |
| $r_{EA} = r_{b}$ | 44.1 | 9.65 × 10−4 | 0.0229 |
| $r_{EA} = r_{b}/2$ | 0.87 | 0.75 | 0.0204 |

a The data were fit according to Equations 6 and 7 using three different assumptions regarding the anisotropy of the EA species. For details, see “Results.”

b Root mean square deviation of the fit in anisotropy units.

upon complexation to EA but increases dramatically upon dimerization of EA.

DISCUSSION

The sedimentation equilibrium and fluorescence data presented here define the energetics of RNase L activation by the 2',5'-linked adenosine trimers HO-2',5'-A3 and HO-2',5'-A3-7HC and provide support for the minimal activation model presented in Scheme 1. For these activators, $K_a$ is in the low micromolar range, and $K_d$ is in the low nanomolar range. The value of $K_a$ is decreased by about 10-fold for the latter activator relative to HO-2',5'-A3. Despite the correlation of the $K_a$ and $K_d$ parameters in fitting the fluorescence data, global analysis of data obtained over a range of activator and/or enzyme concentrations gives rise to well defined fit minima and joint confidence intervals. For HO-2',5'-A3-7HC, the lower value of $K_d$ gives rise to broad confidence intervals for the sedimentation fitting parameters. However, the confidence intervals for the anisotropy parameters are quite narrow, and there is good agreement between the best fit values of $K_a$ and $K_d$ for this activator determined by the two independent methods.

The values of $K_a$ and $K_d$ obtained by sedimentation equilibrium may also be compared with those determined by kinetic methods (7). Using a model that explicitly accounts for substrate binding, we have found that for a given activator, $K_a$ and $K_d$ are dependent on the identity of the substrate: $K_a$ is about 3-fold higher for C11U2C7 than for C11UC8, whereas $K_d$ is about 5-fold lower. The parameters obtained by sedimentation measurements for HO-2',5'-A3 agree more closely with the kinetic data obtained with poorer substrate C11UC8, such that $K_a$ and $K_d$ determined by the two methods are within a factor of 2.

The sedimentation equilibrium data are also capable of distinguishing the activation model presented in Scheme 1 from the alternative model in Scheme 2. In the latter case, the enzyme monomer is capable of dimerization prior to binding activator. Although both models are consistent with our earlier stoichiometry measurements, the absence of any detectable dimer in the absence of activator at enzyme concentrations up to at least 18 μM strongly suggested that activator binding precedes dimerization (4). In the present study, the data fit well to the model in Scheme 1, but the large increase in r.m.s. for the alternative model (Scheme 2) indicates that it does not describe the data adequately. Most importantly, the deduced value of $K_d = 138$ ns is not consistent with the absence of measurable dimerization of free enzyme at micromolar concentrations. Thus, Scheme 1 represents the minimal model capable of describing the sedimentation equilibrium data.

The fluorescence anisotropy data are also consistent with the model in Scheme 1, but they cannot easily be used to distinguish different models because of the need to constrain the value of $r_{EA}$. The binding data obtained at several activator concentrations fit well to Scheme 1 if it is assumed that the anisotropy increase only occurs upon dimerization of EA and fits less well to models that invoke an increase in anisotropy upon binding of A to E to form EA. This observation is surprising, since one might expect that the mobility of the fluorophore would be at least partially restricted upon binding, especially given the short linker between the oligoadenylate moiety and the fluorophore. It is likely that some anisotropy increase upon formation of EA does actually occur but is masked by the very large increase observed upon dimerization to $E_2A_2$. In any case, the fitted values of $K_a$ and $K_d$ are not strongly dependent on the value of $r_{EA}$.

It is noteworthy that RNase L is monomeric at protein concentrations up to at least 18 μM, whereas in the presence of activator the $K_d$ for dimerization is in the nanomolar range. Thus, activator binding increases dimerization affinity by a factor of at least 10^5–10^6, corresponding to strong thermodynamic linkage. The structural basis of this linkage is not yet clear. The activator binding site may be distant from the dimerization interface and modulate dimerization via a long range conformational change. Alternatively, activator binding may occur at or near the dimerization interface and directly modify the interactions that govern dimerization. There is precedence for strong linkage between dimerization and ligand binding in the *Escherichia coli* Rep helicase system. In the absence of DNA, Rep exists as a monomer at concentrations of at least 8 μM (13). However, dimerization is induced upon binding DNA with a $K_d$ of about 5 nM, indicating an enhancement of dimerization of at least 10^4-fold (14).

The strong coupling of enzyme dimerization to activator binding presumably represents a physiological mechanism to control RNase L activity. The enzyme kinetic data suggest that monomeric RNase L is inactive (7), and the ability of various oligoadenylates to activate RNase L correlates with enzyme dimerization (3). Synthesis of 2',5'-linked oligoadenylates is performed by a family of double-stranded RNA-activated synthetases which are induced by interferon treatment (15). Although there are low, basal levels of RNase L expression in most mammalian cells (less than one part in 500,000 of the protein in mouse liver), it is also induced during interferon treatment (16). According to Scheme 1, formation of $E_2A_2$, and therefore the specific activity of RNase L, is dependent on the concentrations of both E and A. Coordinate induction of 2',5'-
linked oligoadenylate synthetases and RNase L by interferon would serve to prime the system for a high level of RNase L activity upon activation of the synthetases by double-stranded RNA.

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