Stoichiometry of 2',5'-Oligoadenylate-induced Dimerization of Ribonuclease L

A SEDIMENTATION EQUILIBRIUM STUDY*

(Received for publication, September 26, 1995, and in revised form, December 6, 1995)

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Ribonuclease L is an endoribonuclease that is activated by binding of 2',5'-linked oligoadenylates. Activation of ribonuclease L also induces dimerization. Here, we demonstrate using equilibrium sedimentation that dimerization requires the binding of one 5'-monophosphate 2',5'-adenosine trimer (2,5A3,5'), its 5'-monophosphate derivative, or RNA oligonucleotide substrates. Adenosine triphosphate or RNA oligonucleotide substrates do not induce dimerization. The observed stoichiometry supports a model for ribonuclease L dimerization in which activator binding to the monomer induces dimerization.

Ribonuclease L is an enzyme involved in the interferon pathway (1). The enzyme is activated upon binding of adenosine oligomers linked 2' to 5' to cleave viral and cellular RNAs at the 3'-side of UpNp sequences (2, 3). Human ribonuclease L has been cloned and overexpressed in a baculovirus system (4). The enzyme is activated upon binding of adenosine triphosphate or RNA oligonucleotide substrates and RNA oligonucleotide substrates do not induce dimerization. The observed stoichiometry supports a model for ribonuclease L dimerization in which activator binds to monomer, which subsequently dimerizes.

Ribonuclease L is an enzyme involved in the interferon pathway (1). The enzyme is activated upon binding of adenosine oligomers linked 2' to 5' to cleave viral and cellular RNAs at the 3'-side of UpNp sequences (2, 3). Human ribonuclease L has been cloned and overexpressed in a baculovirus system (4). Recently, it was demonstrated by biochemical methods that ribonuclease L exists as a monomer in solution but is dimerized in the presence of activator, suggesting that the catalytically active form of ribonuclease L is a homodimer (5, 6). However, the stoichiometry and affinity for the activator-induced dimerization are not known. As an initial step in the development of a thermodynamic model for the activation of ribonuclease L, we have employed equilibrium sedimentation to define the stoichiometry of activator-induced dimerization of the enzyme.

MATERIALS AND METHODS

Oligonucleotides were obtained from The Midland Certified Reagent Company. Human ribonuclease L was expressed and purified as described previously (7) and stored in 40% glycerol, 25 mM HEPES, pH 7.5, 100 mM KCl, 5.8 mM MgCl2, and 5 mM DTT (8). In order 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 1 mM DTT.

Ribonuclease L was equilibrated into the sample buffer using a Bio-Rad Biospin 6 spin columns. Protein concentration was measured spectrophotometrically. The molar extinction coefficient at 280 nm was determined by amino acid analysis to be 8.41 ± 0.87 × 10^4 M^-1 cm^-1 (average of 4 determinations). The concentrations of 2',5'-adenosine triphosphate (2,5A3), and p2,5A5, its 5'-monophosphate derivative, were measured spectrophotometrically using an extinction coefficient of 7.287 ± 0.87 × 10^4 M^-1 cm^-1 with an estimated standard deviation of 5%. The uncertainties in the concentrations of enzyme and activator were propagated in the final calculation of the uncertainty of the stoichiometry of activator binding. The partial specific volume of ribonuclease L, v, was calculated to be 0.725 at 25°C using the method of Cohn and Edsall (7) and adjusted for temperature (8). The solvent density, ρw, was measured to be 1.0066 at 4°C using an Antoun PAAR DGA 48 density meter. Samples were loaded into 6-channel (1.2-cm path) or 2-channel (0.3-cm path) centrifuge cells under argon, and equilibrium analytical centrifugation was performed at 4°C using a Beckman XL-A centrifuge. Scans were taken at 230 or 276 nm. At 230 nm there is negligible contribution to the absorbance from RNA and 2',5'-oligoadenylate derivatives. Equilibrium was judged to be achieved by the absence of systematic deviations in a plot of the difference between successive scans. Molecular weights were obtained by fitting the data to the expression,

\[ C_m = C_0 \exp \left[ \frac{M_w (1-\eta)^2}{2RT} \left( r_2^2 - r_0^2 \right) \right] \]  

where \( C_m \) is the total protein concentration, \( C_0 \) is the protein concentration at the reference distance \( r_0 \), \( M_w \) is the weight average molecular weight, and \( \eta \) is the angular velocity. Data analysis was performed using the nonlinear least-squares programs NONLIN (9) and Kaleidograph (Abelbeck Software).

For activity measurements ribonuclease L (50–200 nM) was incubated on ice with p2,5A5 (50–600 nM) in buffer containing 11.5 mM HEPES, pH 7.6, 104 mM KCl, 5.8 mM magnesium acetate, 5 mM DTT, and 0.2% polyethylene glycol 8000 for 30 min. Reactions (100 μl) were initiated by addition of an aliquot of the incubation solution to a reaction mixture containing 2 μM 5'-[32P]C11UC8 as the RNA substrate in the same buffer used in the incubation plus 1.2 mM ATP but without any additional p2,5A5. Aliquots (8 μl) were quenched after a 2-min reaction time with an equal volume of gel load buffer. Products were separated by denaturing gel electrophoresis and were quantified using a Molecular Dynamics PhosphorImager.

RESULTS AND DISCUSSION

Fig. 1 shows the concentration profiles of ribonuclease L (233 nM loading concentration) in the absence and presence of 400 nM p2,5A5. The data for the sample in the absence of activator fit well to a single ideal species model with a molecular weight of 83,800 ± 4,500, in excellent agreement with the molecular weight of 83,400 deduced from the amino acid sequence. Addition of excess activator results in an increase of the molecular weight to 162,000 ± 8,000, which is close to the value expected for a dimer of ribonuclease L (166,800). Thus, the enzyme is monomeric in the absence and dimeric in the presence of excess p2,5A5. These results confirm an earlier report (5) that activators induce dimerization of the ribonuclease L. The present results also demonstrate that close to 100% of the ribonuclease L in our preparation is competent for dimerization. In separate experiments we observe that in the absence of activator ribonuclease L remains completely monomeric up to protein concentrations of 18 μM (data not shown), indicating that ribonucleotide 2',5'-adenosine triphosphate 2',5A5, 2',5'-adenosine monophosphate, 2',5A5, and 2',5'-adenosine tetramer.
Dimerization of Ribonuclease L

Fig. 1. Sedimentation equilibrium of ribonuclease L. A, absorption profiles (230 nm) of 233 nm ribonuclease L sediments at 14,000 rpm, 4 °C in the absence (○) and in the presence (□) of 400 nm p2,5A3. Solid lines are nonlinear least-squares fit to the data to Equation 1. The molecular weights are 83,800 ± 4,500 kDa in the absence and 162,000 ± 8,000 in the presence of activator. B, residuals for the fits in A.

Ribonuclease L cannot dimerize in the absence of activator, or $K_d$ for dimerization is significantly greater than 20 μM. Conversely, in the presence of saturating activator, ribonuclease L is fully dimerized at a protein concentration as low as 100 nm (data not shown), indicating that $K_d$ for fully liganded ribonuclease L is much less than 100 nm. Analytical ultracentrifugation experiments cannot readily be performed at lower ribonuclease L concentrations because of limited UV absorption of the enzyme.

It is important to define the stoichiometry of activator binding to ribonuclease L in order to develop a thermodynamic model for the activation/dimerization process and to interpret results from enzyme kinetics studies. In kinetic experiments, the dissociation constant for p2,5A3, $K_a$ has been estimated to be less than 10 nm under conditions similar to those employed for the sedimentation experiments.8 Thus, the stoichiometry for activator binding-induced dimerization can be obtained by characterizing the dependence of the dimerization on the molar ratio of activator to ribonuclease L monomers under conditions where the concentration of enzyme is held much higher than $K_a$ and $K_d$. In the case of a monomer-dimer equilibrium, the relevant parameter to characterize the stoichiometry of ligand-induced dimerization is the weight fraction of dimer, $F_d$, which is given by,

$$F_d = \frac{W_d}{W_m + W_d}$$

where $W_d$ is the weight concentration of dimer and $W_m$ is the weight concentration of monomer. For a monomer-dimer system, $M_w$ is given by,

$$M_w = \frac{W_m \cdot M_1 + W_d \cdot 2M_1}{W_m + W_d}$$

where $M_1$ is the monomer molecular weight. Thus, $F_d = (M_1 / M_1) - 1$. Values of $M_w$ were obtained by fitting sedimentation equilibrium profiles obtained at various activator to ribonuclease L ratios using Equation 1. The value of $M_1$ was fixed at the

$$M_1 = \frac{2 M_w}{M_w + 1}$$

where $M_4$ is the weight concentration of dimer and $M_5$ is the weight concentration of monomer. For a monomer-dimer system, $M_w$ is given by,

$$M_w = \frac{W_m \cdot M_1 + W_d \cdot 2M_1}{W_m + W_d}$$

where $M_1$ is the monomer molecular weight. Thus, $F_d = (M_1 / M_1) - 1$. Values of $M_w$ were obtained by fitting sedimentation equilibrium profiles obtained at various activator to ribonuclease L ratios using Equation 1. The value of $M_1$ was fixed at the

$$M_1 = \frac{2 M_w}{M_w + 1}$$

3 S. S. Carroll, unpublished observations.
binding of substrate does not induce dimerization. Either substrates do not bind in the absence of activators or we have found that ATP up to a concentration of 50 mM does not influence the dimerization as in the incubation. The rates are shown relative to the average of the rates obtained at stoichiometries of activator:ribonuclease L subunits greater than 1.0. The data were fit to Equation 5, which indicated that the maximal activity was reached at a ratio of 1.15 ± 0.14 activator to ribonuclease L monomer.

Fig. 3. Stoichiometry of activation of ribonuclease L by p2,5A3. Ribonuclease L (200 nM) was incubated with p2,5A3 (50–600 nM) on ice in reaction buffer as described under "Materials and Methods." Cleavage reactions were initiated by addition of 2 µM 5'-[32P]C11UC8 as substrate but did not include additional p2,5A3 so that the same ratio of activator to enzyme was maintained in the cleavage reaction as in the incubation. The rates are shown relative to the average of the rates obtained at stoichiometries of activator:ribonuclease L subunits greater than 1.0. The data were fit to Equation 5, which indicated concentrations of 2,5A3 and analyzed by sedimentation equilibrium under the same conditions as in Fig. 1. The solid line is a nonlinear least fit of the two data sets to Equation 5 giving a value of R = 1.10 ± 0.14 activator:ribonuclease L binding ratio. In contrast, Dong and Silverman (5) have suggested that the stoichiometry depends on the identity of the activator. The origin of this discrepancy is not clear.

Adenosine triphosphate is found to enhance the activity of ribonuclease L in the presence of activators (10). However, we have found that ATP up to a concentration of 50 mM does not induce dimerization and does not influence the dimerization induced by binding of 2,5A3. Carroll et al. (4) have recently kinetically defined several synthetic oligoribonucleotide substrates. The effect of ribonuclease L substrates on the dimerization was tested. Addition of 1 µM of the substrate 5'-C11UC8-3' (K_m = 205 nM) to ribonuclease L does not induce dimerization. Thus, either substrates do not bind in the absence of activators or binding of substrate does not induce dimerization.

The data presented here provide constraints on the mechanism of activation of ribonuclease L. Dimerization of ribonuclease L may proceed via three possible routes as shown in Scheme I, where E is ribonuclease L monomer and A is activator. In (a) dimerization occurs prior to activator binding, whereas in (b) and (c) dimerization requires prior binding of either one or two activators, respectively. Note that these mechanisms are not mutually exclusive. We have not found any evidence for the unliganded dimer, E_2. However, we cannot completely exclude mechanism (a), since the dimerization constant could be extremely weak. The observed stoichiometry of 1:1 for ligand-induced dimerization indicates that E_A does not accumulate to a significant extent, which would tend to reduce R toward 0.5. Thus, if mechanism (a) were operative, then the binding of the two activators would have to be a highly cooperative. Similarly, mechanism (b) would require that binding of the second activator molecular to E_A occurs much more readily than to the monomer. Taken together, these data suggest that dimerization of ribonuclease L likely proceeds via mechanism (c).

\[
\begin{align*}
E + E & \rightarrow E_2 & (a) \\
E + EA & \rightarrow E_A & (b) \\
EA + EA & \rightarrow E_2A_2 & (c)
\end{align*}
\]

Ligand-linked oligomerization of proteins is a commonly observed biological regulatory mechanism which is analogous to allosteric (6). However, an additional feature in oligomerizing systems is the dependence on protein concentration. Thus, ligand binding measurements as a function of enzyme concentration will be useful to define the coupled equilibria depicted in Scheme I. In concert with enzymatic activity measurements, these studies will allow a detailed description of the relationship between the ligation/association states of ribonuclease L and catalysis.

Acknowledgments—We thank Tracy Viscount for growing baculovirus-infected insect cells and James Geib for purification of ribonuclease L. We also thank members of the Reversible Associations in Structural and Molecular Biology group for suggesting methods to maintain low UV buffer absorption in samples containing DTT.

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J. Biol. Chem. 1996, 271:3979-3981.
doi: 10.1074/jbc.271.8.3979

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