Activation of RNase L by 2',5'-Oligoadenylates

KINETIC CHARACTERIZATION*

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Ribonuclease L (RNase L), the 2',5'-oligoadenylate-dependent ribonuclease, is one of the cellular antiviral systems with enhanced activity in the presence of interferon. A reaction scheme has been developed to model the sequence of steps necessary for the activation of RNase L (Cole, J. L., Carroll, S. S., Blue, E. S., Viscount, T., and Kuo, L. C. (1997) J. Biol. Chem. 272, 19187–19192). The model comprises three sequential binding steps: the binding of activator to enzyme monomer, the subsequent dimerization of the activated monomer to form the active enzyme dimer, followed by the binding of substrate prior to catalysis. The model is used to evaluate the activation of RNase L by several synthetic analogs of the native activator. The 5'-phosphate of the activator has been determined to be an important structural determinant for the efficient activation of RNase L, and its loss caused a loss of activator affinity of 2-3 orders of magnitude. The length of activator is not an important determinant of activator potency for the activator analogs examined. The specific activity of the enzyme under conditions of saturation of activator binding and complete dimerization of the activated monomers varies only by about a factor of 3 for the activators examined, indicating that once dimerized in the presence of any of these activators, the enzyme exhibits a similar catalytic activity.

Interferons induce the expression of 2',5'-oligoadenylate synthetases (1), which, in the presence of double-stranded RNA, cause growth arrest, suggesting a role for the RNase L (6). Transfection of cells (2, 3) with a plasmid expressing RNase L which can then degrade viral and cellular mRNAs, leading to an inhibition of protein synthesis in virally infected cells (2, 3).

RNase L activity has been shown to be an important antiviral defense mechanism. Overexpression of 2',5'-oligoadenylate synthetase leads to the inhibition of picornavirus replication (4, 5). Introduction of an inactive mutant of RNase L causes an increased susceptibility to infection by picornavirus and a loss of the inhibition of cell growth caused by interferon treatment (6). Transfection of 2',5'-oligoadenylate into cells (2) or the expression of 2',5'-oligoadenylate synthetase (5) has been found to cause growth arrest, suggesting a role for the RNase L system in the regulation of cell growth.

The activation of RNase L on binding of 2',5'-oligoadenylates is correlated with the dimerization of enzyme monomers. Dimerization has been detected with chemical cross-linking and gel filtration techniques (7) and with the use of analytical ultracentrifugation (8). Activator-induced dimerization occurs with a stoichiometry of one activator/RNase L subunit (8). The extent of dimerization correlates with the fraction of activated enzyme, suggesting that most if not all of the enzymatic activity results from the dimeric form of the enzyme (8). A quantitative description of the relationship among activator concentration, enzyme concentration, and enzymatic activity is required to make valid comparisons of the potency of activation by different analogs of the native activator, ppp-2',5'-A<sub>9</sub>. This work extends the model (9) describing the dimerization of the enzyme on binding of the activator to include the binding of substrate. The activation of RNase L by analogs of the native activator is determined in terms of the activation model. Comparisons are made between the activation parameters as determined from the rates of the RNase L-catalyzed reactions in this work and the equilibrium measurements of the binding of the activator and the dimerization of enzyme monomers as detected with sedimentation equilibrium and fluorescence anisotropy in the accompanying report (9).

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1 The abbreviations used are: ppp-2',5'-A<sub>9</sub>, a trimer of adenosines linked 2' to 5' with a 5'-triphosphate; p-2',5'-A<sub>9</sub>, a trimer of adenosines linked 2' to 5' with a 5'-monophosphate; 2,5A, an oligomer of adenosine linked via 2',5'-phosphodiester groups; AC<sub>50</sub>, concentration at which half-maximal activation is obtained under specified reaction conditions; HO-2',5'-A<sub>9</sub>, a trimer of adenosines linked 2' to 5' with a 5'-hydroxyl; HO-2',5'-A<sub>9</sub>-7HC, a conjugate of HO-2',5'-A<sub>9</sub>-2'-NH<sub>2</sub> and 7-hydroxy-coumarin-3-carboxylic acid, succinimidyl ester.
Characterization of RNase L

Equation 7 was carried out using the commercial fitting program, Kaleidograph, which is based on the algorithm of Marquardt (12).

RESULTS

Enzyme Activation—RNase L activity has been monitored by following the cleavage of radiolabeled synthetic oligoribonucleotides after separation of substrate and product on denaturing polyacrylamide gels, as described (11). The activation of RNase L by 2,5A analogs is allowed to reach equilibrium during a preincubation step that included activator and enzyme in reaction buffer at 22 °C for at least 20 min. Control reactions indicate that 20 min is a sufficient time to allow the association of activator and enzyme and the formation of enzyme dimers to reach equilibrium. The activity of the preactivated enzyme stock is stable for at least 60 min. Substrate is added to the enzyme-activator solution in a volume equal to 0.10 of the final reaction volume to initiate the reaction. Addition of the substrate in a small volume is designed to minimize the effect of volume increase on the equilibria of activator binding and enzyme inhibition.

At low enzyme concentrations (<0.3 nM) after a 20-min preincubation with activator, a lag in the reaction time course was observed lasting ~1 min followed by a linear phase. The lag in the time course is not evident at high enzyme concentrations (>1 nM). Longer preincubation times (up to 1 h) do not eliminate the lag phase at low enzyme concentrations. The lag is consistent with the binding of substrate to dimers which would serve to increase the concentration of enzyme dimers under low enzyme concentrations where a higher fraction of the enzyme exists as activated monomers. Care has been taken to measure reaction rates during the final linear phase of the reaction time

\[
K_c = [E][A]/[E_A] \text{ or } [E_A] = [E][A]/K_c
\]

\[
K = [E_A][S]/[E_A] \cdot S \text{ or } [E_A] \cdot S = [E_A][S]/K
\]

where \(E\) is enzyme monomer, \(A\) is activator, \(EA\) is enzyme with activator bound, \(E_A\) is the active dimer with two activator molecules bound, \(S\) is the substrate, and \(E_A\cdot S\) is the active dimer with substrate bound. The equation describing the total concentration of enzyme species is given by,

\[
[E] = [E]_0 + [E_A] + 2[E_A] \cdot S
\]

\[
K_a = [E_A][S]/[E_A] \cdot S
\]

\[
K_d = [E_A][S]/[E_A] \cdot S
\]

where \([E]_0\) is the original concentration of enzyme monomers, and \([E]\) is the concentration of monomers at equilibrium. Rearranging Equations 1–3 to solve for \([E_A]\), \([E_A]_0\), and \([E_A] \cdot S\) and substituting into Equation 4 followed by rearrangement gives a quadratic equation in terms of \([E]\). A solution is reached when:

\[
[E] = [-b \pm (b^2 - 4ac)^{1/2})/2a
\]

where: \(a = 2[A]/K_a[K]/[A], b = [1 + K_a/[A]], \) and \(c = 1-|E|/K_a/[A]|.

In practice only the positive operator in Equation 5 gives a physically meaningful result. The rate equation is determined by substituting Equation 5 into Equation 1 to yield a description of \([E]A\) which may then be substituted into Equation 2 to give an equation describing \([E_A]\). The rate will be proportional to the total concentration of dimeric species,

\[
v = 2v_c(2[E_A]/[E_A] \cdot S + [S]/K_a) = 2v_c((1 + [S]/K_a)/[E]_0
\]

where \(v_c\) is the specific activity of the activated dimeric enzyme under the defined reaction conditions written in terms of \([E]_0\). Therefore the rate equation is:

\[
v = 2v_c(1 + [S]/K_a)(-b \pm (b^2 - 4ac)^{1/2})/2a
\]

with \(a, b, c\) as described for Equation 5.

Data Analysis—Reaction data were fit to Equation 7 in which the concentration of active dimeric RNase A at equilibrium is expressed in terms of the initial concentration of enzyme monomers, the concentration of substrate, the concentration of activator in the reaction, and the equilibrium dissociation constants associated with the model of activation which is presented under “Results.” Fitting of the data to Equation 7 was carried out using the commercial fitting program, Kaleidograph, which is based on the algorithm of Marquardt (12).

Reaction time courses were followed by quenching an aliquot (5 µl) of the reaction with an equal volume of electrophoresis gel load buffer containing 90% formamide and 10 mM EDTA. Substrates and products were separated on the gel containing 20% acrylamide, 7 M urea gels and were quantified with the use of a PhosphorImager (Molecular Dynamics).

Enzyme (50 pM–8 nM in monomers) was preincubated with a

The effect of the dilution of enzyme on the rate of

Activator Complex—

Ki

is the competitive inhibition constant,

Kd

is enzyme monomer, \(A\) is activator, \(EA\) is enzyme with activator bound, \(E_A\) is the active dimer with two activator molecules bound, \(S\) is the substrate, and \(E_A\cdot S\) is the active dimer with substrate bound. The equation describing the total concentration of enzyme species is given by,

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Course in reactions containing low concentrations of the enzyme.

Reversibility of Activation—The reversibility of the activation process has been investigated with the use of dilution experiments. In one set of experiments, enzyme is preincubated with 20 nM p-2',5'-A3. The concentrations of the enzyme and activator are then decreased 20-fold by dilution, and the reaction time course is monitored. The results shown in Fig. 1 indicate that the activation of the enzyme is reversible as evidenced by a slow decrease in reaction rate after dilution of activator compared with a reaction in which the enzyme was diluted from 20 nM to 1 nM but the activator concentration remained at 20 nM. The observed rate of activity loss is 0.004 min⁻¹, indicating a slow reequilibration under the conditions of the reaction.

In another set of experiments enzyme is preactivated at a concentration that is 40-fold (1 nM) higher or at a concentration that was 1.1-fold (27.5 pm) higher than the final reaction concentration. A cleavage reaction is initiated by the addition of substrate to the preincubation containing enzyme at 27.5 pm or by the addition of an aliquot of the 40-fold concentrated enzyme solution to a reaction mixture. In each experiment the concentration of activator, p-2',5'-A3, remains constant (500 nm). As shown in Fig. 2 the reaction in which enzyme is diluted 40-fold initially shows a higher rate than that of a reaction preincubated at 1.1-fold higher than the final concentration, but the rate then decreases to approximately the same rate as the reaction in which enzyme is diluted 1.1-fold, indicating that the specific activity of the enzyme decreases upon 40-fold dilution of the enzyme to a final concentration of 25 pm.

Model for Activation of RNase L—The following model (Scheme I) has been developed as a basis for the quantitative description of the kinetics of catalysis by RNase L.

\[
\begin{align*}
E + A & \rightleftharpoons EA & K_a \\
EA + EA & \rightleftharpoons E_2A_2 & K_d \\
E_2A_3 + S & \rightleftharpoons E_2A_2 \cdot S & K_c
\end{align*}
\]

Scheme I

E represents enzyme monomer, A is the activator, EA is the activated monomer, E2A2 is the enzyme dimer, and S and E2A2-S are substrate and the enzyme dimer-substrate complex, respectively. (9) \(K_a\) represents the dissociation constant for activator binding to enzyme monomers, \(K_d\) is the dissociation constant for enzyme dimers, and \(K_c\) is the dissociation constant for substrate binding to enzyme dimers.

The first two steps of this binding scheme represent a subset of the complete coupled ligand binding-enzyme dimerization scheme (13, 14) and are minimally required to explain the observed dependence of the reaction rate on both activator concentration and enzyme concentration. It is necessary to extend the model developed in the preceding paper (9) by including the binding of substrate because substrate binding influences the equilibrium concentrations of activated monomer (EA) and dimer (E2A2). The model assumes that only activated dimer binds substrate. Under “Experimental Procedures,” a derivation is given of the rate equation (Equation 7) dictated by the model in terms of enzyme monomer concentration, activator concentration, substrate concentration, and the dissociation constants \(K_a\), \(K_d\), and \(K_c\).

Saturation of Reaction Rate with Activator—As shown in Fig. 3, the rate of cleavage of the substrate C11U12C13 is saturable with increasing concentrations of p-2',5'-A3. The concentration of p-2',5'-A3 at which half-maximal activation is obtained (AC₅₀) under the reaction conditions is 10 nM. A previous determination of AC₅₀ for p-2',5'-A₅ at preincubation of enzyme and activator at 0°C was 1 nM, indicating a temperature dependence of AC₅₀ (11). The increase in the rate of the reaction with increasing concentrations of activator could be due to a change in \(k_{cat}\) or \(K_m\) or both. The value of \(K_m\) for substrate C11U12C13 has been determined at a near saturating concentration of activator p-2',5'-A₅ (500 nM) and at a subsaturating concentration of p-2',5'-A₅ (4 nM). The \(K_m\) values are 230 nM and 200 nM, respectively, indicating that the concentration of activator does not have a significant effect on the \(K_m\) for substrate.

Rate saturation data have been fit to Equation 7 to determine \(K_d\) and \(K_c\) to give an approximate value for \(K_a\). After
The function of activator concentration was fit to Equation 7 with the reaction time courses monitored. The rate of the reaction as a function of enzyme concentration were fit to Equation 7 divided by enzyme concentration to determine the value of \( K_d \). For activation in the presence of 500 nM p-2,5'-A3, the maximal specific activity was determined to be 2 nM, and the maximal specific activity was determined to be 11 nM.

The value of \( K_a \) parameters of substrate \( C_{11}U_3C_7 \) in reactions containing from 0 to 1,800 nM \( C_{11}U_3C_7 \) and a near saturating concentration of the enzyme. Reaction rate data are fit to a competitive mechanism, and the values for \( K_s \) shown in Table I are determined from a replot of the slopes of the double-reciprocal plots. The values for \( K_a \) shown in Table II for substrate \( C_{11}UC_8 \) as an alternate substrate inhibitor of the cleavage of substrate \( C_{11}U_3C_7 \) have been determined in a similar manner.

**Effect of Substrate Sequence on \( K_a \) and \( K_d \)—**The activation parameters \( K_a \) and \( K_d \) have been determined for the activators p-2,5'-A3 and HO-2,5'-A3 using \( C_{11}UC_8 \) as substrate in a manner similar to that used to determine the same parameters with substrate \( C_{11}U_3C_7 \). As shown in Table II, the values of \( K_a \) for the activators p-2,5'-A3 and HO-2,5'-A3 determined using \( C_{11}UC_8 \) as substrate are both 3-fold lower than the values of \( K_d \) determined using \( C_{11}U_3C_7 \) (Table I). The values for \( K_d \) determined in reactions included \( C_{11}UC_8 \) (Table II) in the case of activation by p-2,5'-A3 or by HO-2,5'-A3 are 5-fold higher than the \( K_d \) determined in reaction containing substrate \( C_{11}U_3C_7 \) (Table I).

**Activation by Analogs of 2',5'-A**—The activation of RNase L by structural analogs of 2,5A has been analyzed according to Scheme I. The results are shown in Table I. The most potent activator of RNase L examined in this study is a synthetic version of the native activator, ppp-2,5'-A3, which activates the enzyme with a \( K_a \) of 1.1 nM. The least potent activator characterized is the HO-2,5'-A3, which has \( K_a \) of 7.7 \( \mu \)M. The values determined for the dimer dissociation constant, \( K_d \), also show variation depending on the structure of the activator, although the \( K_d \) values show less variation than do the \( K_a \) values. The values for \( K_d \) which are equal to the equilibrium binding constant for the substrate, \( K_d \), are independent of the nature of the activator with the exception of activation by p-2',5'-A5 which has a 4-fold increased \( K_d \). For 5'-monophos-
phate activators the optimal length in terms of the lowest value for $K_a$ is four adenosines with slight increases in $K_a$ for activation by the oligonucleotide with five adenosines. The maximal specific activity, the activity of the enzyme under conditions of saturating activator and complete dimerization, does not show a significant dependence on the structure of the activators shown in Table I, varying by 3-fold maximally.

**DISCUSSION**

Reaction rate kinetics have been used to measure the kinetic parameters governing the activation of RNase L. A model for the activation of the enzyme was developed (9) as the basis for the quantitative evaluation of activation by structural analogs of the native activator. The rates of cleavage reactions catalyzed by RNase L show saturation kinetics as a function of the concentration of the activator in the reaction (Fig. 3). The specific activity of the enzyme decreases at low concentrations of enzyme consistent with the dissociation of active enzyme dimers at low enzyme concentrations (4). Both the binding of activator and the dimerization of the enzyme are reversible processes as evidenced by a slow loss of activity on dilution of either the activator below $K_a$ or dilution of the enzyme to a concentration well below $K_a$. These observations are consistent with the proposed model for activation wherein enzyme monomers first bind activator to yield activated monomers, followed by dimerization of the monomers to give the active dimeric enzyme species (9).

Experimental support for the model comes from sedimentation equilibrium experiments, which fail to detect any dimerization of enzyme monomers in the absence of activator at concentrations up to 18 μM (8). Further support for the proposal that the primary active species is the enzyme dimer comes from the observation that the extent of dimerization of the enzyme at high enzyme concentrations (greater than $K_a$) is a linear function of the stoichiometry of activator to enzyme monomers. Because the stoichiometry of dimerization closely parallels the stoichiometry of activation, most if not all of the enzyme activity results from the dimeric form of the enzyme (8). The binding of substrate is included in the model for activation since substrate binding can influence the extent of dimerization by binding solely or preferentially to the dimer. The $K_a$ of the substrates used in reactions for determination of $K_a$ and $K_d$ as alternate substrates for the cleavage of the substrate $C_{11}{U_C}C_7$ has been determined experimentally because the $K_a$ of the competitive inhibitor will be equivalent to the binding constant of the substrate $K_a$.

Prior activation conditions involved preincubation of enzyme and activator at 0 °C (11). Because both $K_a$ and $K_d$ show a temperature dependence, preincubation at 0 °C gives a greater degree of dimerization and higher activity than does preincubation at the same concentrations of activator and enzyme at 22 °C, the conditions used in this work. Further support for a decrease in $K_a$ and $K_d$ with lower temperatures comes from sedimentation equilibrium measurements of enzyme dimerization (9).

A change in the structure of the activator can cause changes in the activation parameters. Three main conclusions may be drawn from the data presented in Table I. First, comparison of activation by $p$-pentaphosphate to activation by $p$-pentaphosphate indicates that loss of the 5'-diphosphate causes a 10-fold increase in $K_a$ and only minor changes in $K_d$ and $K_a$. Further loss of the 5'-phosphate in the case of $H_2$, 5'-A3 causes another 700-fold increase in $K_a$ and minor increases in $K_d$ and $K_a$, indicating that the 5'-phosphate moiety is an important determinant of the potency of activator binding. Second, the values of $K_a$ for the series of activators with a 5'-monophosphate and lengths 3, 4, or 5 adenosines show only minor variations, indicating that the length of these activators is not a strong determinant for potency of activation. However, addition of a fourth base in the 5'-OH analogs is associated with a significant decrease in $K_a$, from 7.7 μM for the trimer to 210 nM for the tetrameric activator. Third, despite the greater than 3 orders of magnitude variation in $K_a$, the maximal specific activity of the enzyme, under conditions of saturating concentrations of activator and complete dimerization, does not show a large dependence on the structure of the activator, indicating that once the enzyme has dimerized it retains a similar efficiency of catalysis.

These values of $K_a$ are consistent with activator binding as determined with competitive binding affinity measurements. The binding constant of $p$-pentaphosphate with the murine enzyme has been reported variously as 40 pm (15) or 1 nM (16), with the differences probably being due to differences in experimental conditions and the inherent difficulties of measuring so tight a binding interaction. Competitive binding measurements with $H_2$, 5'-A3 indicate a binding constant in the low micromolar range (17), a value consistent with the $K_a$ determined in this work using $C_{11}{U_C}C_7$ (2.8 μM, Table I).

The activation parameters $K_a$, $K_d$, and $K_a$ have been determined for activation by $p$-pentaphosphate and $H_2$, 5'-A3 in reactions containing two different substrates, $C_{11}{U_C}C_7$ ($k_{cat}/K_m = 1.9 \times 10^{-8} \text{ M}^{-1} \text{s}^{-1}$) and $C_{11}{U_C}C_7$ ($k_{cat}/K_m = 8 \times 10^7 \text{ M}^{-1} \text{s}^{-1}$). The value of $K_a$ for each activator decreases by about a factor of 3 in reactions containing the substrate $C_{11}{U_C}C_7$ relative to reactions with substrate $C_{11}{U_C}C_{7}$ (Tables I and II). The values for $K_d$ determined with each activator also show a 5-fold increase in reactions containing substrate $C_{11}{U_C}C_7$ relative to the $K_d$ determined in reactions with the substrate $C_{11}{U_C}C_7$. These changes in $K_d$ and $K_a$ with the sequence of the substrate may indicate that the binding of substrate produces an effect on the conformation of the enzyme such that the activation parameters are intrinsically changed slightly from those of the enzyme in the absence of substrate. Comparison of the $K_d$ determined by measurement of reaction kinetics with activation by $H_2$, 5'-A3, for example (5 and 24 nM for reactions containing $C_{11}{U_C}C_7$ and $C_{11}{U_C}C_7$, respectively), with the $K_d$ determined by analytical ultracentrifugation experiments (17 nM; Ref. 9) supports the possibility of a decrease in $K_a$ in the presence of the better substrate, $C_{11}{U_C}C_7$. The small change in the energy of binding (0.6–1 kcal/mol) for the activator with different substrates is not unreasonable.

In general, a good agreement exists between the values for $K_a$ and $K_d$ as determined with enzyme kinetics for HO-2, 5'-A3, using substrate $C_{11}{U_C}C_7$ with $K_a$ and $K_d$ as determined with the use of sedimentation equilibrium methods (9). The activation by a fluorescent analog of HO-2, 5'-A3, HO-2, 5'-A3-5'H, described in the previous paper, was also examined. The values for $K_a$ and $K_d$ as determined by examination of enzyme kinetics (0.8 μM, 1.1 nM, respectively, Table I) are in reasonable agreement with $K_a$ and $K_d$ as determined with fluorescence anisotropy (1.8 μM, 1.1 nM, respectively; Ref. 9).

The proposed model for the activation of RNase L takes into account the three equilibria that lead to the formation of the activated complex. The model should prove useful for the quantitative evaluation of the activation of RNase L by different activators.

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