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Comparative Study on the Biological Properties of 2′,5′-Oligoadenylate Derivatives with Purified Human RNase L Expressed in *E. coli*[1]

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An endoribonuclease, RNase L, which is activated in the presence of 2′,5′-linked oligoadenylates, $p_{1-3}A(2'p5'A)_{>2}$, is the terminal factor of the anti-viral action of interferon. Activation of RNase L results in inhibition of viral proliferation along with induction of apoptosis. Attempts to acquire more effective activators, 2-5A derivatives, have been made for the development of antiviral or anticancer agents. However, the ability of 2-5A derivatives to activate RNase L could not simply be compared due to the diversity of the assay methods used. We have now developed a facile method for assaying the activity of RNase L involving the use of non-fusion RNase L expressed in *Escherichia coli* and yeast 5S ribosomal RNA as a substrate. Using this method, several 2-5A derivative species have been revaluated. The results suggest that 2-5A molecules modified at the 8-position of the third (from the 5′ terminus) adenine ring cause effective dimerization of RNase L and thus increase the ability of RNase L activation.

Key words: antiviral drug, interferon, 2′,5′-oligoadenylate, ribonuclease L.

The defensive effect of interferon against viral infections is mediated by the 2-5A system (*1*). The mechanism is as follows: (i) Interferon induces the expression of 2-5A synthetases and thereby increases the intracellular levels of 2′,5′-linked oligoadenylates, $pppA(2'p5'A)_{>1}$ (2-5A); (ii) The 2-5As convert RNase L into a catalytically active homodimer by binding to it; (iii) The activated RNase L cleaves the dyad sequences of UU, UA, AU, AA, and UG (*2*); (iv) The RNA degradation results in inhibition of protein synthesis and thereby inhibition of viral replication.

There is evidence that RNase L is concerned in defense against viral infections and induction of apoptosis. Overexpression of wild-type RNase L suppressed the replication of several viruses, such as the encephalomyocarditis virus (*3*). Conversely, the antiviral effect of interferon was suppressed by overexpression of a catalytically inactive mutant of RNase L in murine cells (*3*). When RNase L was overexpressed, cell death, with morphological and biochemical characteristics of apoptosis, was also caused (*4*). The expression of the inactive mutant of RNase L blocked staurosporine-induced apoptosis (*5*). Furthermore, enlarged thymuses resulted from suppression of apoptosis in RNase L −/− gene–knockout mice (*6*).

Recently, a 2-5A linked to an antisense-oligodeoxyribonucleotide, called 2-5A-antisense, was developed for regulation of the expression of a specific gene. The strategy is based on the accelerated degradation of a target RNA by both RNase L and RNase H. Using this strategy, sequence-specific degradation of mRNA for protein kinase R has been achieved in HeLa cells (*7*). In addition, the treatment of prostate cancers with 2-5A-antisense targeting telomerase mRNA reduced the numbers of cancer cells *in vitro* and *in vivo* (*8*). Linking of the antisense moiety to the 2′-terminal of 2-5A, however, brought about a one- or two-log decrease in the ability to activate RNase L, compared with the 2-5A molecule itself (*9*). To overcome this disadvantage of 2-5A-antisense, chemical modification is necessary to obtain 2-5As possessing a high potential to activate RNase L.

In order to prepare more effective 2-5As and to understand the structure–activity relationships of 2-5As, a variety of 2-5A derivatives with base, sugar and/or linkage modifications were synthesized (*10*). We previously reported the synthesis of 2- and 8-substituted 2-5As, and showed the importance of the syn/anti-conformation of the base moiety for the RNase L activation (*11–13*). However, it is hard to directly compare the activation among these derivatives because of the differences in the evaluation conditions.

The present paper describes a facile strategy for evaluation of the biological properties of 2-5As as to activation of RNase L with bacterially expressed non-fusion RNase L and yeast 5S ribosomal RNA as a substrate. Using this strategy, revaluation of 2- or 8-substituted 2-5As was performed.

MATERIALS AND METHODS

Materials—*Escherichia coli* JM105, expression vector pKK223-3, SP-Sepharose FF, Red-Sepharose, and Sephacryl S200 were from Amersham Pharmacia. The POROS® HS/M column was from PE Biosystems. 2′,5′-Linked oligoadenylates were previously synthesized in our laboratory.

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Abbreviations: 2-5A, 2′,5′-oligoadenylate; DMS, dimethyl suberimidate.

Expression vectors for *E. coli* chaperones GroESL (pT-groE) and thioredoxin (pT-Trx) (*14*) were kindly provided by Dr. S. Ishii. Purified 5S ribosomal RNA (rRNA) of *Candida utilis* (*15*) was a generous gift from Professor K. Nishikawa of Gifu University.

Construction of an Expression Plasmid—cDNA encoding human RNase L was amplified by reverse transcription-polymerase chain reaction (RT-PCR) from total RNA of HepG2 cells. Reverse transcription was performed using SuperScriptII® reverse transcriptase (Gibco) according to the manufacturer's instructions. PCR was carried out using *Pfu* turbo DNA polymerase (Stratagene) and oligonucleotide primers (sense, 5′-CCGAATTCATGGAGAGCAGGGAT-3′ containing an *Eco*RI site; antisense, 5′-GGGTAAAGCTTATGGACTAGTGTAGTCTGGG-3′ containing a *Hin*dIII site). The PCR product was ligated into the *Eco*RI and *Hin*dIII sites of the pKK223-3 expression vector (Amersham Pharmacia) after digestion with *Eco*RI and *Hin*dIII. The nucleotide sequence of the cDNA in the resulting vector (pKKRNL) was subsequently verified by dideoxy sequencing.

Expression of RNase L—E. coli JM105 harboring pKKRNL was grown at 30°C overnight in 40 ml of LB medium containing 50 μg/ml ampicillin. The culture was transferred to 4 liters of the same medium and then incubated on a rotary shaker at 30°C. When OD_{600} reached 0.6, expression was induced with 0.5 mM isopropyl-1-thio-β-D-galactopyranoside. The bacterial cells were collected after an additional 5 h cultivation by centrifugation at 5,000 ×*g* for 10 min and stored at −20°C.

Purification of Recombinant RNase L—All steps were carried out at 4°C. Frozen cells were thawed and then homogenized in 200 ml of buffer A [25 mM Tris-HCl (pH 7.5), 50 mM KCl, 10% glycerol, 1 mM EDTA, 5 mM $MgCl_2$, 0.1 mM ATP, 14 mM 2-mercaptoethanol] by sonication disruption. Cell debris was removed from the homogenate by centrifugation at 12,000 ×*g* for 15 min at 4°C. The cell extract was loaded onto a SP-Sepharose column (2.4 × 13 cm) that had been preequilibrated with buffer A at the flow rate of 50 ml/h. The column was washed with buffer A until the absorbance at 280 nm had decreased to 0.1. RNase L was eluted with 500 ml of buffer A containing a linear gradient of NaCl, from 0 to 1 M. Each fraction was subjected to SDS-PAGE and the fractions containing RNase L were pooled. The pooled fractions were diluted 3-fold with buffer A and then loaded onto a Red-Sepharose column (2.4 × 6 cm) preequilibrated with buffer A at the flow rate of 30 ml/h. After extensive washing of the column with buffer A, the enzyme was eluted with 300 ml of buffer A containing a linear gradient of NaCl, from 0 to 1.5 M. The fractions containing RNase L were pooled and concentrated to 6 ml with a YM-10 ultrafiltration membrane. A sample was then subjected to gel filtration on a Sephacryl S200 column (1.6 × 90 cm) that had been preequilibrated with buffer A at the flow rate of 30 ml/h. The fractions obtained on gel filtration were subsequently separated with a fast protein liquid chromatography system equipped with a POROS® HS/M cation-exchange column (0.46 × 10 cm). Elution was carried out with 40 ml of buffer A containing a linear gradient of NaCl, from 0 to 0.8 M, at the flow rate of 1.0 ml/min.

Assaying of RNase Activity—Purified RNase L [0–100 ng (1.2 pmol)] was preincubated with various amounts (0–100 pmol) of 2-5As in 18 μl of buffer C [22.2 mM Tris-HCl (pH 7.5), 11.1 mM magnesium acetate, 8.9 mM 2-mercaptoethanol, 0.11 M KCl, 0.11 mM ATP] at 0°C for 30 min for complete binding with 2-5A. The RNase reaction was initiated by the addition of 2 μl of yeast 5S rRNA (1 μg) to the 2-5A/RNase L mixture at 30°C and terminated by the addition of 20 μl of a loading solution (9 M urea, 1 mM EDTA, 0.02% xylene cyanol, 0.02% bromophenol blue). An aliquot (20 μl) of the mixture was electrophoresed on a 10% polyacrylamide/7 M urea/1× TBE gel, followed by staining with ethidium bromide. The RNA signal corresponding to the intact 5S rRNA was quantified using a computer program, NIH Image 1.61.

Assaying of Dimerization of RNase L—The chemical cross-linking method described by Dong and Silverman (*16*) was used for the detection of RNase L dimerization. The purified enzyme (1 μg in 4 μl) in buffer B [25 mM Hepes-NaOH (pH 7.4), 0.15 M KCl, 14 mM 2-mercaptoethanol, 50 μM ATP, 5 mM $MgCl_2$, 1 mM EDTA, 10% glycerol] was incubated with each 2-5A (0.75–3.0 μM in 4 μl) on ice for 1 h. Then, 4 μl of a dimethyl suberimidate solution (12 mg/ml) in 0.4 M triethanolamine-HCl (pH 8.5) was added to the reaction mixture, followed by incubation at room temperature for 1 h. After separation by SDS/7.5% polyacrylamide gel electrophoresis (SDS-PAGE), proteins were transferred to a Fluorotrans® poly (vinylidene difluoride) membrane. RNase L on the membrane was probed with 2 μg/ml of a polyclonal antibody raised in a rabbit against bacterially expressed RNase L, and detected with goat-antirabbit IgG-alkaline phosphatase (Bio-Rad). The intensities of signals on the membrane were quantified using NIH Image 1.61.

RESULTS

Expression and Purification of Non-Fusion Human RNase L—Cells harboring pKKRNL were cultured at temperatures between 25 and 37°C. The growth of the bacteria was especially slow, as mentioned by Diaz-Guerra *et al.* (*17*). The recombinant RNase L was mostly produced as inclusion bodies at 37°C. Although the amount of inclusion bodies decreased as the culture temperature was lowered, proteins other than RNase L increased simultaneously. The increases in other proteins complicated the following purification. Co-expression of the thioredoxin or GroESL chaperones did not affect the amount of the inclusion bodies containing RNase L (data not shown). Attempts to renature the inclusion bodies after solubilization with either 8 M urea or 6 M guanidinium hydrochloride were also unsuccessful. Eventually, a cell extract of *E. coli* harboring pKKRNL grown at 30°C was found to be the best source of recombinant RNase L. Recombinant human RNase L (1.5 mg) (Fig. 1) was obtained from the cell extract of a 4-liter culture. The N-terminal sequence of the purified protein was identical with that of human RNase L (*18*).

Assaying of RNase Activity and Its Validity—The RNase activity of the purified RNase L was measured by quantification of the remaining intact substrate. With the assay system used, RNase activity was only observed in the presence of a 2-5A and degradation of the substrate comprised a pseudo first order reaction (Fig. 2A). Besides, the rate of degradation depended on the amount of RNase L (Fig. 2B). These findings showed that the RNA degradation was caused by the 2-5A–activated RNase L.

To determine the enzymatic properties of the bacterially expressed RNase L, the EC_{50} and C_{rel} values for well-characterized 2-5As, *i.e.* 2-5A trimer with 5′-triphosphate (pppApApA), tetramer with 5′-monophosphate (pApApApA), trimer lacking 5′-phosphoryl groups (ApApA), and dimer with 5′-triphosphate (pppApA), were determined (Fig. 3A). EC_{50} was defined as the concentration of a 2-5A required to degrade 50% of the RNA substrate, and C_{rel} as the relative (compared to pppApApA) EC_{50} value. For pppApApA and pApApApA, the EC_{50} values were the same at a low concentration (4 nM). Although the absolute values were about 15 times larger than those reported by Dong and Silverman (*16*), due to the use of a different assay system, the C_{rel} values were identical. In addition, the considerably high EC_{50} values for ApApAp and pppApA also agreed with the previous report. Therefore, one or more 5′-phosphoryl groups and three adenylyl residues were

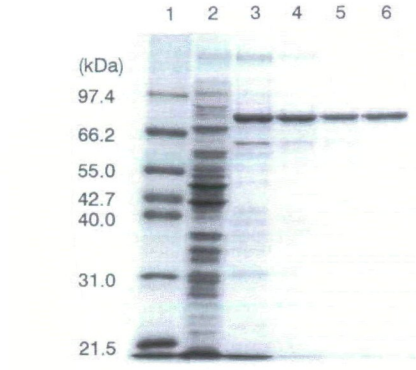

Fig. 1. **Purification of bacterially expressed RNase L.** Proteins at each purification stage were subjected to 10% SDS-PAGE and then stained with Coomassie brilliant blue G250. Lanes: 1, size markers; 2, homogenate of *E. coli* harboring pKKRNL (18 μg); 3, SP-Sepharose FF (7.5 μg); 4, Red-Sepharose (3 μg); 5, Sephacryl S200 (1 μg); 6, POROS HS/M (1 μg).

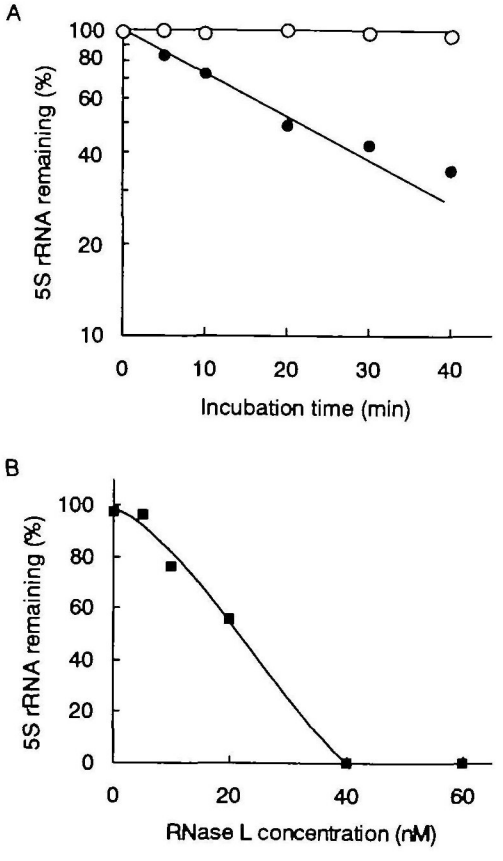

Fig. 2. **RNase activity of bacterially expressed RNase L.** A: Time-course of RNA degradation. The substrate RNA (1 μg) was digested in a volume of 20 μl at 30°C by RNase L (50 ng, 30 nM) preincubated with (●) or without (○) pApApA (100 pmol, 5 μM) at 0°C for 30 min. B: Enzyme-dependency of RNA degradation. Different amounts of preactivated RNase L (as above) were incubated with substrate RNA (1 μg) at 30°C for 30 min.

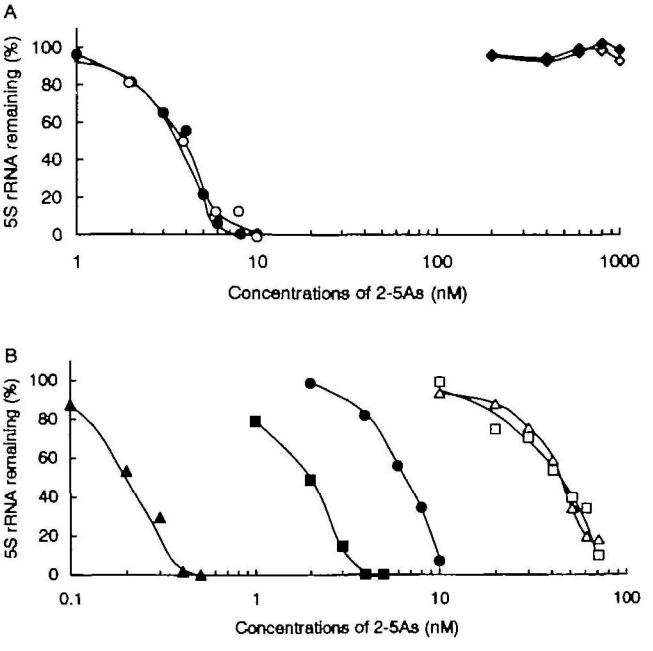

Fig. 3. **Structural requirement of 2-5As for activation of recombinant RNase L.** Each 2-5A was used to activate RNase L (60 nM) at the indicated concentrations. RNA was digested at 30°C for 30 min. A, pppApApA (●), pApApApA (○), ApApA (■), and pppApA (□). B, pApApA (●), pApAp(me8A) (■), pApAp(me2A) (□), pApAp(br8A) (▲), and pApAp(br2A) (△).

TABLE I. **Abilities of 2-5As to activate RNase L.**

2-5As	EC_{50}[a] (nM)	C_{rel}[b]	EC_{50}[c] (nM)	C_{rel}
pppApApA	3.9	1	0.27	1
pApApApA	4.0	0.98	0.27	1
ApApA	>1,000	–	110	0.0025
pppApA	>1,000	–	No activity	–
pApApA	6.6	0.59	ND	–
pApAp (br2A)	42	0.09	ND	–
pApAp (me2A)	43	0.09	ND	–
pApAp (br8A)	0.2	19.5	ND	–
pApAp (me8A)	1.9	2.05	ND	–

[a]2-5A concentration required for the degradation of 50% of 1 μg of 5S rRNA by 60 nM RNase L in 20 μl at 30°C for 30 min. [b]C_{rel} is the relative (compared to pppApApA) EC_{50} value. A C_{rel} value of more than 1 indicates the greater potency of the 2-5A derivative than that of pppApApA. [c]The concentration of a 2-5A required to achieve a 50% reduction in intact poly(rU)-[^{32}P]pCp using 24 nM RNase L at 30°C for 30 min (*16*). ND = not determined.

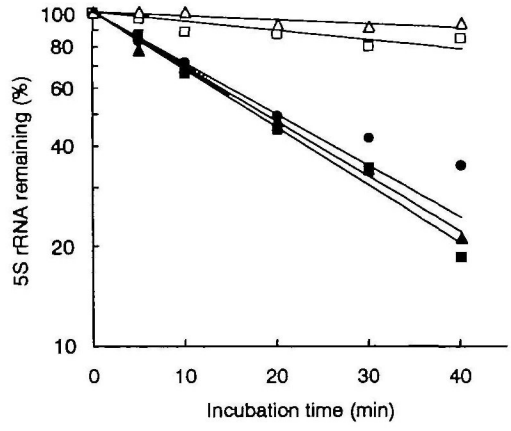

Fig. 4. **Time course of RNA degradation.** Intact 5S rRNA (1 μg) was digested with 30 nM purified RNase L in the presence of 5 μM 2-5As (pApApA (●); pApAp(me8A) (■); pApAp(me2A) (□); pApAp(br8A) (▲); pApAp(br2A) (△)).

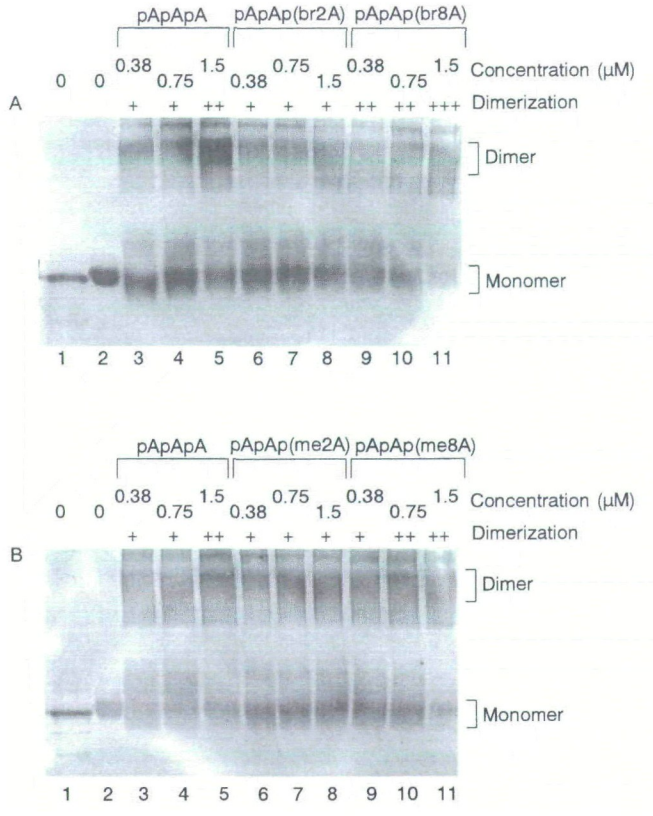

Fig. 5. **2-5A dependent dimerization of RNase L.** RNase L was cross-linked with DMS after incubation in the presence of 2-5As (as indicated). The level of dimer formation was determined as the ratio (r = [dimer]/[monomer]) of the intensities of signals: +, $0 < r < 1$; ++, $1 \leq r < 2$; +++, $2 \leq r$.

required for maximal activation of RNase L. These requirements of a 2-5A for the activation of RNase L coincided with those of the human RNase L from a baculovirus expression system (*16*).

Activation of RNase L by 2- or 8-Substituted 2-5As—The abilities of base-modified 2-5As to activate RNase L were determined by means of two different functional assays: an RNA degradation assay and a chemical cross-linking assay for detecting RNase L dimerization (*16*). In the RNA degradation assay (Fig. 3B), 2-substituted 2-5As, *i.e.*, pApAp(br2A) and pApAp(me2A), were required at a 6.5-fold greater concentration to obtain the same effect as that of pApApA (Table I). On the other hand, 8-substituted 2-5As, *i.e.*, pApAp(br8A) and pApAp(me8A), activated RNase L more efficiently than pApApA. In particular, the introduction of bromine at the 8-position of the third adenine ring of a 2-5A caused approximately 33-fold enhancement of the degradation of 5S rRNA, compared to with pApApA (Table I). The degradation pattern of the substrate RNA was not affected by the activator species.

Reaction Rate Assay—To examine the effects of base modifications on the reaction rate of RNase L, time courses were followed in the presence of excess (5 μM) 2-5As (Fig. 4). Under such conditions, the differences in binding affinity between RNase L and the activators should be negligible, since the 2-5A binding site of the enzyme was saturated by the activators. The results interestingly showed the same slope for pApApA and 8-substituted 2-5As, whereas the slope for 2-substituted 2-5As was *ca.* 3.6-fold less than that for pApApA. Therefore, the high potency of 8-substituted 2-5As could not be accounted for by acceleration of the reaction rate.

Dimerization of RNase L—RNase L was activated less effectively by 2-substituted 2-5As. This tendency was also observed with the chemical cross-linking assay. Although the results of the cross-linking assay were qualitative, differences between 2-substituted and 8-substituted 2-5As were observed (Fig. 5). The addition of 1.5 μM 8-substituted 2-5As converted almost all the RNase L to the dimeric form, whereas the addition of 2-substituted 2-5As induced only partial dimerization of RNase L.

DISCUSSION

In this study, we have revaluated the abilities of 2- and 8-substituted 2-5As under definite conditions, and revealed the effect of substitutions at the third adenosine of the 2-5A trimer. As for 8-substituted 2-5As, which are thought to take on the syn form (Fig. 6) due to the steric hindrance of the substituent (*19*), their abilities as RNase L activators were examined by using murine RNase L in the early studies (*20*). On the other hand, purified recombinant human RNase L from insect cells was previously used for estimation of 2-substituted 2-5As, which were expected to take on the anti form (*21*). To determine the effects of the substitutions, the abilities of 2- and 8-substituted 2-5As must be compared under definite conditions.

The catalytic activity of RNase L has been determined by several methods, such as the protein synthesis inhibition assay (*22*), 18S and 28S rRNA degradation assay (*23*), poly (U) degradation assay (*24*), chemically synthesized-oligo RNA cleavage assay (*25*), and fluorescence resonance energy transfer (FRET) analysis (*26*). In this study, we adopted a new method because of its simplicity. The method requires neither chemical synthesis of substrate RNA nor special equipment. Moreover, the use of a short homogeneous RNA as the substrate gives semi-quantitative results.

According to previous reports (*11, 12*), substitution at the 2-position of the adenine ring at the third (from the 5′ ter-

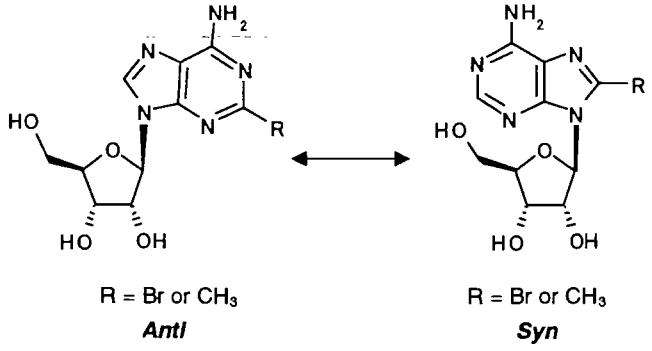

Fig. 6. **Syn- and anti-conformations of adenosine.**

minus) nucleotide residue of the 2-5A trimer has a slightly negative effect on RNase L activation, *i.e.*, the C_{rel} value (the value was normalized as to EC_{50} of pApApA instead of that of pppApApA) of pApAp(me2A) was 0.9 when a labeled poly (U) was used as the substrate (*12*). The results also demonstrate the negative effect of substitution at the 2-position of the adenine ring, whereas the ability of 2-substituted 2-5As was ca. 6.5-fold lower than that of pApApA regardless of the substituent (Table I). The discrepancy might be due to the difference in the substrate. On the other hand, the effect of substitution at the 8-position of the third nucleotide of the 2-5A trimer had not hitherto been reported for human RNase L. The effect of the substitution was determined by using murine RNase L, which is not activated by the 5′-monophosphate 2-5A trimer (pApApA) but by the 5′-triphosphate 2-5A trimer (pppApApA). As for murine RNase L, pppApAp(me8A) and pApAp(br8A) resulted in 10- and 0.25-fold abilities compared to in the case of pppApApA, respectively. Although 8-brominated 2-5A is a monophosphate, this derivative has the ability to activate RNase L. Therefore, the ability of 8-brominated 2-5A can be assumed to be enhanced. The data suggest a positive effect of substitution at the 8-position of the third adenosine of a 2-5A. Our data obviously demonstrated the enhancing effect of substitution at the 8-position of the adenine ring of a 2-5A on human RNase L for the first time. The C_{rel} values for pApAp(br8A) and pApAp(me8A) were 19.5 and 2.05, respectively.

The time course of RNA degradation revealed that the reaction rates of the enzyme activated by 2-substituted 2-5As were lower than those of the enzyme activated by 8-substituted 2-5As or pApApA. RNase L requires two steps for expression of its activity, namely, binding of 2-5A and subsequent dimerization (*27*). Therefore, the following two factors can be assumed to influence the reaction rate of RNase L: (i) binding affinity of the activator to the enzyme; and (ii) dimerization ability of the enzyme–activator complex. The former factor could, however, be ruled out as the reason for the lower rates with the 2-substituted 2-5As. The earlier studies proved that the affinity of 2- or 8-substituted 2-5As to RNase L was superior to that of pApApA (*11–13, 20*). The latter is likely to account for the effect of 2-substituted 2-5As. The chemical cross-linking assay in this study (Fig. 5) indicated the poor ability of 2-substituted 2-5As to dimerize RNase L, even at high concentrations; and thus the RNase activity was not fully exerted. On the other hand, 8-substituents might not affect the dimerization, since the time course for the enzyme activated by 8-substituted 2-5As was the same as in the case of pApApA. The enhanced ability of 8-substituted 2-5As should be due to the high affinity to RNase L.

In this report, we have demonstrated the effect of base modifications at the third (from the 5′ terminus) nucleotide of the 2-5A trimer using bacterially expressed human RNase L and yeast 5S rRNA as a substrate. The observations suggest that interference with the dimerization of RNase L occurs when 2-substituted 2-5As are used as activators. This finding provides a clue for the molecular design of 2-5As possessing high ability to activate RNase L.

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