Phosphorothioate Analogues of 2′,5′-Oligoadenylate. Activation of 2′,5′-Oligoadenylate-Dependent Endoribonuclease by 2′,5′-Phosphorothioate Cores and 5′-Monophosphates†

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Received February 3, 1987; Revised Manuscript Received June 2, 1987

ABSTRACT: The preceding paper in this issue described the synthesis and structural elucidation of the phosphorothioate analogues of 2′,5′-oligoadenylate (2-5A) dimer and trimer cores [Karikó, K., Sobol, R. W., Jr., Suhadolnik, L., Li, S. W., Reichenbach, N. L., Suhadolnik, R. J., Charubala, R., & Pfleiderer, W. (1987) *Biochemistry* (preceding paper in this issue)]. In this report, the binding and activation processes of 2-5A-dependent endoribonuclease (RNase L) have been examined by using four diastereomeric 2′,5′-phosphorothioate trimer core analogues and their 5′-monophosphates. These 2′,5′-phosphorothioates have revealed a distinct separation of the structural parameters that govern binding vs activation of RNase L. Radiobinding assays have demonstrated that extensive stereochemical modification of the internucleotide linkages of 2-5A is possible without adversely affecting its ability to bind to RNase L. However, a marked difference was observed in the *activation* of RNase L by the stereochemically modified 2-5A molecules as determined in core–cellulose and rRNA cleavage assays. Three of the four 2′,5′-phosphorothioate trimer cores (with R_PR_P, S_PR_P, and R_PS_P internucleotide linkages) are the *first 2-5A core* molecules able to *activate RNase L*. For example, the R_PR_P, S_PR_P, and R_PS_P diastereoisomers activate RNase L to hydrolyze poly(U)-3′-[^{32}P]pCp 65%, 20%, and 15%, respectively, at 5×10^{-5} M. The S_PS_P diastereomer cannot activate RNase L. The order of RNase L activation was the same for the core analogues and their 5′-monophosphates ($R_PR_P > S_PR_P > R_PS_P$). The binding/activation ability of the 2′,5′-phosphorothioate cores could result from the combined effect of stereoconfigurational and electronic alterations brought about by the replacement of oxygen by sulfur in the internucleotide linkages. The S_PS_P and pS_PS_P 2′,5′-phosphorothioates are capable of binding to RNase L as well as the corresponding authentic A_3 and pA_3 but are unable to activate the enzyme at concentrations as high as 10^{-3} and 10^{-5} M, respectively. The S_PS_P and pS_PS_P analogues *inhibit* the activation process as shown in competition assays. This new 2-5A analogue (pS_PS_P) is the most effective inhibitor of RNase L reported to date and will be useful in in vivo studies. The results reported here are consistent with the hypothesis that RNase L is a functionally stereoselective enzyme and that the binding process is independent of the activation process. RNase L cannot distinguish between R_P and S_P chirality in the internucleotide linkages of 2-5A for binding but can distinguish between different stereoconfigurations for activation. The binding and activation processes are discussed in terms of their spatial homology and their accommodation in the binding domain of RNase L.

The activation of RNase L[1] by the enzymatically synthesized 2′,5′-phosphorothioate *dimer* 5′-triphosphate with the R_P stereoconfiguration in the 2′,5′-internucleotide linkage [as shown in the preceding paper (Karikó et al., 1987)] prompted us to expand our application of phosphorothioate substitution in the 2-5A molecule. We have used R_P and S_P chirality, introduced into the 2-5A molecule in the internucleotide linkages, to further define the regions of the 2-5A molecule that are strategically involved in binding to and activation of its target enzyme, RNase L. Altered stereoconfiguration in the 2-5A backbone combined with altered ionic properties following substitution of the P–O with the P–S group has been examined. Because nucleoside transferases produce exclusively the inverted stereoconfiguration when (S_P)-ATPαS is the substrate, 2-5A molecules containing phosphorothioate groups of the S_P configuration in the internucleotide linkages cannot be synthesized enzymatically (Eckstein et al., 1982; Lee & Suhadolnik, 1985). Therefore, we have chemically synthesized, separated, and fully characterized four 2′,5′-phosphorothioate trimer cores with R_P and/or S_P stereoconfigurations in the internucleotide linkages. In this paper, we analyze the binding and activation of RNase L by these 2′,5′-phosphorothioate trimer core diastereomers.

† This study was supported in part by a research grant from the National Science Foundation (DMB84-15002) awarded to R.J.S., U.S. Public Health Service Grant P01 CA-29545 from the NCI, and Federal Work Study awards (R.W.S.).

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[1] Abbreviations: pR_PR_P, pS_PR_P, pR_PS_P, and pS_PS_P trimers, 5′-monophosphorylated trimer phosphorothioate analogues of A_3 with R_P and S_P stereoconfigurations in the two chiral centers with assignment of configuration from the 5′-terminus to the 2′/3′-terminus; DTT, dithiothreitol; HPLC, high-performance liquid chromatography. All other abbreviations used were as described in the preceding paper in this issue (Karikó et al., 1987).

0006-2960/87/0426-7136$01.50/0

Our aim was to determine if separate structural parameters in the 2-5A molecule govern binding vs activation of RNase L. The R_PR_P, S_PR_P, R_PS_P, and S_PS_P diastereoisomers provide excellent model molecules to elucidate the effect of stereochemical modification on action of RNase L. In addition, these molecules have supplied new information on the role of the P–O group in the natural 2′,5′-phosphodiester bond in either ionic interaction or hydrogen bonding. By introduction of R_P or S_P chirality via P–S substitution, these physicochemical studies are now possible. Furthermore, in the studies described here we clearly demonstrate the importance of chirality/charge interactions between 2-5A and the RNase L in the formation of a productive complex that is essential for the hydrolysis of the substrates, poly(U) or rRNA. Further, by the use of our stereochemically modified trimer cores, we demonstrate for the first time that all four 2′,5′-phosphorothioate trimer cores can bind to RNase L and three of the trimer cores have the correct stereoconfiguration to activate RNase L. On the basis of these dramatic results and the known increased binding ability of authentic 5′-monophosphates of 2-5A compared to the corresponding cores (Haugh et al., 1983), we reasoned that 5′-monophosphorylation of the 2′,5′-phosphorothioate trimer core diastereomers would provide 2-5A species capable of binding to RNase L at physiological concentrations equivalent to p_3A_n. Data are presented that show a 1000-fold increase in the binding to RNase L by the 5′-monophosphates of the four 2′,5′-phosphorothioate trimer core diastereomers.

One of our aims in this study was to develop a 2-5A molecule that would be metabolically stable and not activate RNase L at physiological concentrations equivalent to p_3A_3. Such a molecule has been an elusive goal in the evaluation of the role of RNase L in the interferon-induced biological cascade. Introduction of chirality via phosphorothioate substitution in the 2-5A molecule has provided us with an analogue inhibitor (i.e., the pS_PS_P trimer) which selectively inhibits the activation of RNase L at physiological concentrations and has the added feature of being metabolically stable to specific and nonspecific phosphodiesterases. Therefore, phosphorothioate substitution has produced a class of 2-5A analogues with profoundly differing stereochemical, physicochemical, and biological properties. The use of these 2′,5′-phosphorothioates in the further elucidation of the interactions between the 2-5A molecule and RNase L in the binding and activation processes is discussed.

Experimental Procedures

All experimental procedures were as described in the preceding paper in this issue (Karikó et al., 1987). In addition, the 2′,5′-phosphorothioate trimer cores were 5′-phosphorylated with T_4 polynucleotide kinase (Bethesda Research Laboratories) as described by Oshevski (1982) in a reaction mixture (total volume = 20 μL) containing the following: [γ-^{32}P]ATP (0.5 μCi, 4500 Ci/mmol, ICN) or [γ-^{35}S]ATPγS (1.2 μCi, 600 Ci/mmol, Amersham), A_3 or 2′,5′-phosphorothioate trimer core (0.15 mM), T_4 polynucleotide kinase (500 units/mL) (in 0.05 M Tris-HCl, pH 7.6, 10 mM $MgCl_2$, 2.85 mM DTT, 0.1 mM EDTA), and 0.5 mM ATP. Incubations were allowed to proceed for 48 h at 37 °C. The progress of the reaction was monitored by reverse-phase HPLC as described in the preceding paper (Karikó et al., 1987) using the following solvent system: solvent A = 50 mM ammonium phosphate, pH 7.0; solvent B = methanol/H_2O (1:1 v/v); solvent system 4, linear gradient (beginning at 1 min) (t = 1 min, 10% B; t = 31 min, 70% B). Fractions (500 μL) were collected, and the radioactivity was determined. 5′-Nucleotidase (Sigma) reactions were as described by Sulkowski et al. (1963) for 1 h at 37 °C.

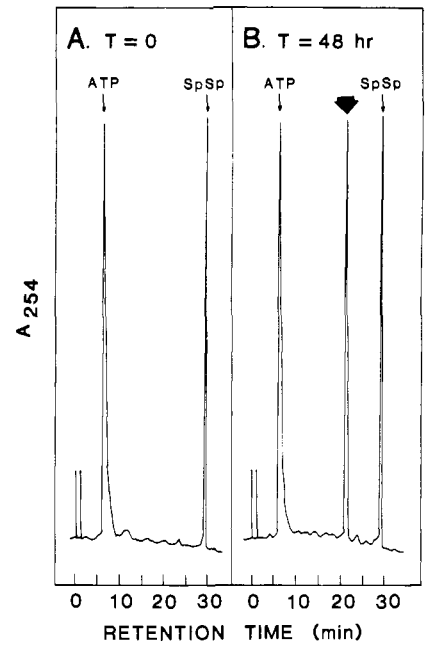

FIGURE 1: 5′-Monophosphorylation of S_PS_P trimer core by T_4 polynucleotide kinase. Reaction was for 48 h as described under Experimental Procedures followed by HPLC analysis in solvent system 4. Elution times of standard ATP and S_PS_P trimer core at T = 0 h (panel A) and T = 48 h (panel B) are indicated. Bold arrow indicates elution time of product (i.e., pS_PS_P).

Table I: Transfer of [γ-^{32}P]Phosphate from [γ-^{32}P]ATP or [γ-^{35}S]Thiophosphate from [γ-^{35}S]ATPγS by T_4 Polynucleotide Kinase to the 5′-Hydroxyl of 3′,5′- and 2′,5′-Oligoadenylates

	transfer from			
	[γ-^{32}P]ATP		[γ-^{35}S]ATPγS	
oligonucleotide	dpm × 10^{-3}	%[a]	dpm × 10^{-3}	%[a]
3′,5′-A_2	665	76	435	12.1
3′,5′-A_3	194	20.5		
2′,5′-A_2	122	14	0	0
2′,5′-A_3	52.1	5.8	0	0
S_PS_P trimer core	100	11.5	0	0
R_PR_P trimer core	22.9	2.5	0	0
S_PR_P trimer core	50.2	5.3	0	0
R_PS_P trimer core	285	29.0	0	0

[a] Percent conversion based on total dpm recovered from HPLC.

Results

To understand more clearly the binding and activation processes by which RNase L hydrolyzes RNA, stereochemical modifications have been made in the 2-5A molecule. The results of studies utilizing these 2′,5′-phosphorothioates have further elucidated the regions of the 2-5A molecule involved in binding to and activation of RNase L.

5′-Monophosphorylation of 2′,5′-Phosphorothioate Trimer Cores with T_4 Polynucleotide Kinase. The 5′-monophosphates of the 2′,5′-phosphorothioate trimer cores have been synthesized with ATP and T_4 polynucleotide kinase. The formation of the 5′-monophosphates was determined by HPLC analyses (Figure 1). Identical experimental HPLC retention times and charge separations were obtained with the chemically and enzymatically phosphorylated 2′,5′-phosphorothioate trimer 5′-monophosphates. Subsequent hydrolysis to cores was accomplished with 5′-nucleotidase. The phosphorylation of the four 2′,5′-phosphorothioate trimer cores was dependent on the stereoconfiguration in the phosphodiester backbone (Table I). 5′-Phosphorylation was more effective on oligomers

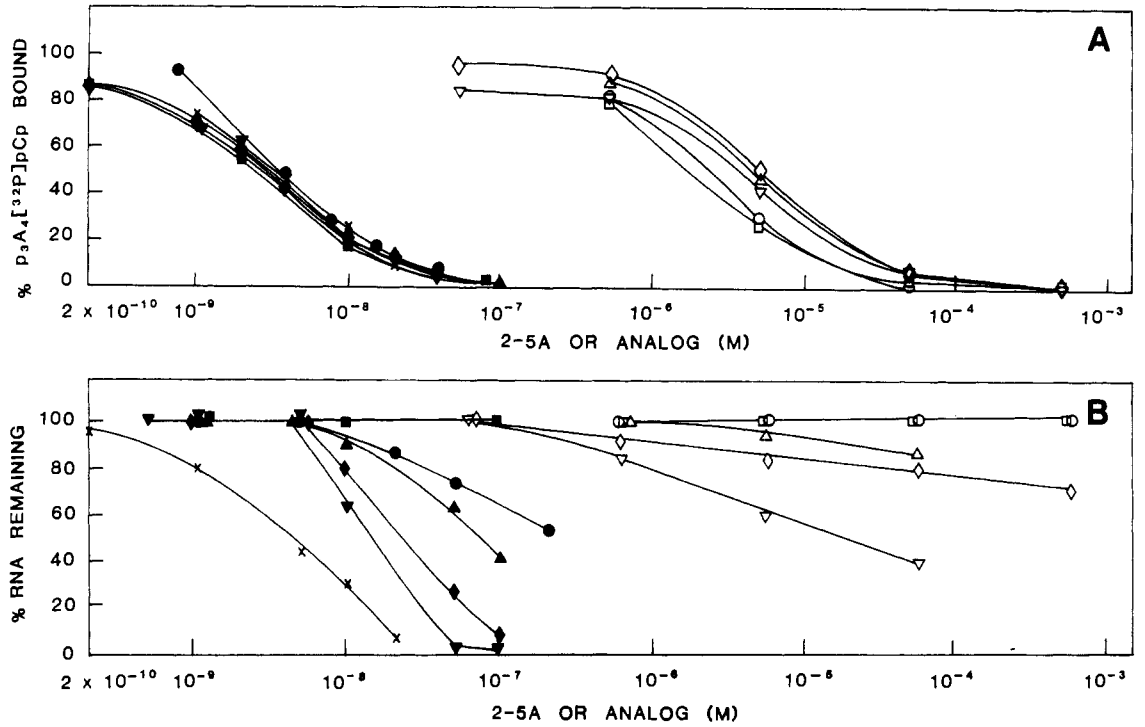

FIGURE 2: (A) Ability of 2′,5′-phosphorothioate trimer cores and 5′-monophosphates to compete with $p_3A_4[^{32}P]pCp$ probe for binding to the RNase L in L929 cell extracts in the radiobinding assay (Knight et al., 1981). Approximately 60% of the $p_3A_4[^{32}P]pCp$ was bound in the absence of added oligonucleotide (total dpm = 23 000). (B) Ability of the 2′,5′-phosphorothioates to activate the partially purified RNase L from L929 cell extracts as determined by the hydrolysis of poly(U)-3′-[^{32}P]pCp in the core–cellulose assay (Silverman, 1985). Activation of RNase L was determined by conversion of poly(U)-3′-[^{32}P]pCp to acid-soluble fragments. 100% represents 30 000 dpm of labeled poly(U)-3′-[^{32}P]pCp bound to glass fiber filters. A_3 (○); p_3A_3 (×); pA_3 (●); R_PR_P (▽); S_PR_P (◇); R_PS_P (△); S_PS_P (□); pR_PR_P (▼); pS_PR_P (◆); pR_PS_P (▲); pS_PS_P (■).

with 3′,5′- than 2′,5′-internucleotide linkages. In addition, the dimers were better substrates than the trimers. Although it had been previously reported by Bryant et al. (1981) and Oshevski (1982) that the γ-phosphorothioate of ATPγS was transferred to 3′,5′-oligonucleotides by T_4 polynucleotide kinase, the 2′,5′-oligoadenylates did not undergo thiophosphorylation (Table I).

Binding Affinity of 2′,5′-Phosphorothioate Trimer Cores and 5′-Monophosphates to RNase L. The ability of the 2′,5′-phosphorothioate trimer cores and 5′-monophosphates to bind to RNase L was determined by the radiobinding assay (Knight et al., 1981). The 2′,5′-phosphorothioate trimer cores and authentic A_3 core were able to displace the $p_3A_4[^{32}P]pCp$ probe from RNase L from L929 cell extracts in a concentration-dependent manner (Figure 2A); IC_{50}'s varied from 2 × 10^{-6} to 5 × 10^{-6} M. However, the 5′-monophosphorylated trimers had 1000-fold higher binding affinity to RNase L than their respective cores (i.e., IC_{50}'s ranged from 2 × 10^{-9} to 5 × 10^{-9} M) (Figure 2A).

Activation of RNase L by 2′,5′-Phosphorothioate Trimer Cores and 5′-Monophosphates. (A) Hydrolysis of Poly(U)-3′-[^{32}P]pCp. The activation of partially purified RNase L by the 2′,5′-phosphorothioates was measured in the core–cellulose assay (Silverman, 1985). A most striking finding is that three of the four 2′,5′-phosphorothioate trimer *cores* were able to activate RNase L to cleave poly(U)-3′-[^{32}P]pCp in the core–cellulose assay. The order of activation for the trimer cores was $R_PR_P > S_PR_P > R_PS_P$. The R_PR_P timer core activated RNase L to cleave poly(U)-3′-[^{32}P]pCp 50% at 10^{-5} M (Figure 2B); however, the S_PS_P trimer core did not activate RNase L, even at a concentration of 10^{-3} M. As was observed in the binding assay (Figure 2A), there was a 1000-fold increase in the *activation* of RNase L by the 5′-monophosphates of the 2′,5′-phosphorothioate trimers compared to their respective cores. When the activation of RNase L by the 5′-monophosphate trimers was examined, the same order of decreasing activation ability was seen (i.e., $pR_PR_P > pS_PR_P > pR_PS_P$). The pR_PR_P trimer activated RNase L to cleave poly(U)-3′-[^{32}P]pCp by 35% at 10^{-8} M (Figure 2B).

(B) Cleavage of Ribosomal RNA. Activation of RNase L by the 2′,5′-phosphorothioate trimer cores and 5′-monophosphorylated analogues was measured in the rRNA cleavage assay using L929 cell extracts. The R_PR_P and S_PR_P trimer cores activated RNase L to cleave 28S and 18S rRNA to specific cleavage products at 10^{-5} M (Figure 3, lanes 2 and 3). However, the R_PS_P and S_PS_P trimer cores did not activate RNase L at concentrations as high as 10^{-4} M (lanes 4 and 5). Degradation of poly(U) by the R_PS_P trimer core was detectable and reproducible by the core–cellulose assay (Figure 2); however, the rRNA cleavage assay was not sensitive enough to detect activation of RNase L by R_PS_P trimer core. Under the experimental conditions used, authentic A_3 core was also inactive (lane 1), which is in agreement with previous reports (Haugh et al., 1983). The pR_PR_P, pS_PR_P (at 10^{-8} M), and pR_PS_P trimers (at 10^{-7} M) activated RNase L to cleave 28S and 18S rRNA (Figure 4, lanes 3–5); authentic pA_3 was active at 10^{-6} M (lane 7). However, incubations with the pS_PS_P trimer, even as high as 10^{-5} M, did not result in any detectable rRNA degradation (lane 6).

Inhibition of Activation of RNase L by S_PS_P Trimer Core and Its 5′-Monophosphate. In order to establish the potential of the S_PS_P and pS_PS_P phosphorothioate analogues as effective inhibitors of RNase L, it was essential to demonstrate that these 2′,5′-phosphorothioates were indeed inhibiting RNase L activation by binding to the enzyme, and not by another mechanism. Incubations of L929 cell extracts showed the well-established dose-dependent activation of RNase L by p_3A_3 (5 × 10^{-10}–1 × 10^{-8} M) (Figure 5, lanes 1, 3, 5, and 7). This

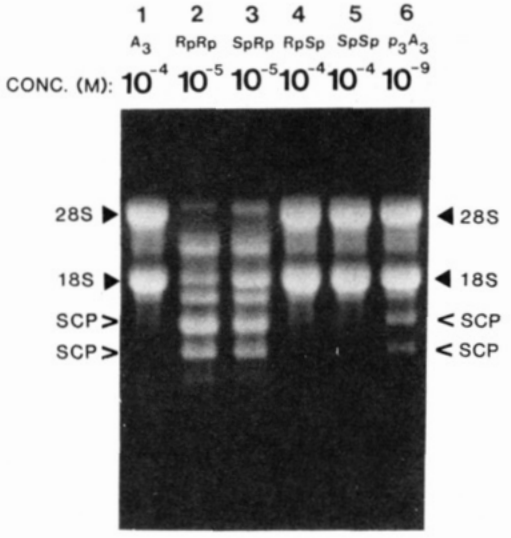

FIGURE 3: Ribosomal RNA cleavage assay with 2′,5′-phosphorothioate trimer cores. Incubations were for 1 h at 30 °C, and extraction and electrophoretic analysis of the RNA was as described under Experimental Procedures of the preceding paper (Karikó et al., 1987). Incubations were in the presence of R_PR_P and S_PR_P at 10^{-5} M (lanes 2 and 3), R_PS_P and S_PS_P at 10^{-4} M (lanes 4 and 5), authentic A_3 at 10^{-4} M (lane 1), or p_3A_3 at 10^{-9} M (lane 6). The positions of 28S and 18S rRNA are shown; the arrows indicate the positions of specific cleavage products (SCP).

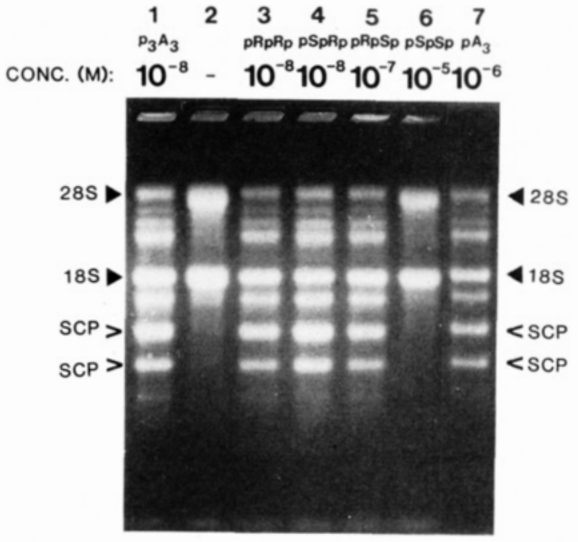

FIGURE 4: Ribosomal RNA cleavage assay with 2′,5′-phosphorothioate trimer 5′-monophosphates. All experimental conditions were as described in Figure 3. Incubations were in the absence (lane 2) or presence of p_3A_3 at 10^{-8} M (lane 1), pR_PR_P and pS_PR_P at 10^{-8} M (lanes 3 and 4), pR_PS_P at 10^{-7} M (lane 5), pS_PS_P at 10^{-5} M (lane 6), or pA_3 at 10^{-6} M (lane 7).

activation of RNase L was inhibited when pS_PS_P trimer (at 5×10^{-7} M) was added prior to incubation (Figure 5, lanes 2, 4, 6, and 8). Similar inhibition of RNase L activation was observed when S_PS_P trimer core (2×10^{-4} M) was added to L929 cell extracts incubated in the presence of R_PR_P trimer core (5×10^{-7}–5×10^{-5} M) (Figure 5, lanes 10, 12, and 14). Similar findings were obtained in the core–cellulose assay with S_PS_P trimer core and pS_PS_P trimer. For example, a 5-fold excess of p_3A_3 was required in the presence of 5×10^{-7} M pS_PS_P trimer to achieve equivalent hydrolysis of poly(U) as was achieved with p_3A_3 alone (Figure 6). S_PS_P trimer core (2×10^{-4} M) inhibited activation of RNase L by R_PR_P trimer core in the core–cellulose assay in the same manner (data not shown).

DISCUSSION

The 2′,5′-phosphodiester backbone is universally acknowledged as a critical factor in 2-5A action in terms of antigenic determinants and its specific recognition by RNase L. The initial generally accepted wisdom was that the minimum structure required for activation of RNase L in cell-free systems was two 5′-terminal phosphates and three adenylate or adenylate-like residues in the 2-5A molecule (Baglioni et al., 1981). This dogma was altered to include the 5′-monophosphate as an active 2-5A species (Haugh et al., 1983). The nonphosphorylated and therefore less highly charged 2-5A "core" is accepted to be unable to activate RNase L (Haugh et al., 1983). In the first paper of three in this issue (Karikó et al., 1987), we have demonstrated that the 2′,5′-phosphorothioate *dimer* with a 5′-*O*-(1-thiotriphosphate) can bind to and activate RNase L. Our observation that enzymatically synthesized 2′,5′-phosphorothioates with phosphorothioate-substituted internucleotide linkages of the R_P stereoconfiguration are able to bind to and activate RNase L raised the question as to how a phosphorothioate group influences the binding and activation processes of RNase L. Our aims in this study concerned (i) the elucidation of the minimal structural requirements for binding to and activation of RNase L and (ii) the development of a 2-5A analogue inhibitor that could shut off selectively the RNase L system. To accomplish these aims, we used four chemically synthesized diastereomeric 2′,5′-phosphorothioate trimer cores and their 5′-monophosphates (refer to the preceding paper in this issue for their synthesis and characterization). The 5′-monophosphates of the 2′,5′-phosphorothioate trimer cores were prepared by T_4 polynucleotide kinase. We present here several heretofore unknown aspects of the 2-5A molecule with respect to its activation of RNase L.

The stereoconfiguration of the 2′,5′-phosphodiester bond of the 2′,5′-phosphorothioates does not affect binding to RNase L (Figure 2A). These findings compare with the reported binding studies of oligonucleotide analogues where changes in 2-5A structure in either the adenyl or the ribosyl moieties or in the 2′,5′-internucleotide linkages caused a significant decrease in binding affinity (Imai et al., 1985; Sawai et al., 1985; Lesiak et al., 1983). Most striking is our observation that although binding to RNase L is not affected by R_P or S_P chirality in the 2′,5′-phosphodiester linkages, the activation process is markedly affected by stereoconfiguration. Three of the 2′,5′-phosphorothioate trimer cores (i.e., those with R_PR_P, S_PR_P, and R_PS_P internucleotide linkages) can *activate* RNase L (Figures 2B and 3). This is the first report of the activation of RNase L by *core* analogues of 2-5A. On the basis of the activation of RNase L in the core–cellulose assays (Figure 2B), we can exclude the possibility that the activation of RNase L by the three 2′,5′-phosphorothioate trimer cores is due to rephosphorylation, because factors responsible for phosphorylation are removed during the purification of the RNase L on the 2-5A_4 core–cellulose (Krause et al., 1986).

In view of the dramatic observation of the activation of RNase L by the cores and the known increased binding ability of authentic pA_3 compared to A_3, we focused our efforts on the 5′-monophosphorylation of the trimer core analogues. This was accomplished with T_4 polynucleotide kinase (Table I). Haugh et al. (1983) reported that the affinity of the 5′-monophosphate of trimer 2-5A core to RNase L in mouse L929 cell extracts is approximately 1000 times greater than that of the corresponding trimer core. This increase in affinity may be attributed to the ability of the 5′-monophosphate of

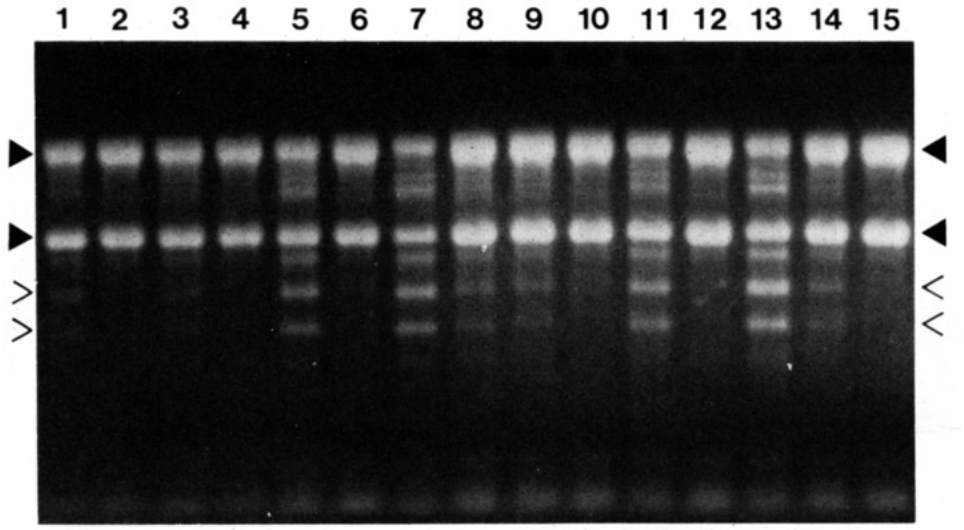

FIGURE 5: Inhibition of RNase L activation by phosphorothioate analogue of 2-5A trimer core and its 5′-monophosphate as determined by rRNA cleavage assay. Extracts of L929 cells were incubated in the absence (lane 15) or presence of p_3A_3 at 5×10^{-10} M (lanes 1 and 2), 1×10^{-9} M (lanes 3 and 4), 5×10^{-9} M (lanes 5 and 6), 1×10^{-8} M (lanes 7 and 8) without (lanes 1, 3, 5, and 7) or with (lanes 2, 4, 6, and 8) 5×10^{-7} M pS_PS_P trimer. Lanes 9–14 were as follows: incubations in the presence of R_PR_P trimer core at 5×10^{-7} (lanes 9 and 10), 5×10^{-6} (lanes 11 and 12), and 5×10^{-5} M (lanes 13 and 14) without (lanes 9, 11, and 13) or with (lanes 10, 12, and 14) 2×10^{-4} M S_PS_P trimer core. Extraction and analysis of the RNA were as described in Figure 3.

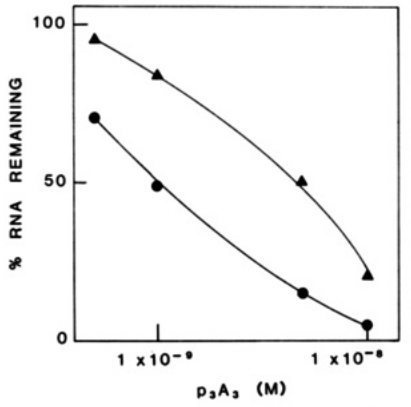

FIGURE 6: Inhibition of p_3A_3-mediated poly(U)-3′-[^{32}P]pCp hydrolysis by the pS_PS_P trimer. Inhibition of RNase L activation was determined in the core–cellulose assay by measuring the conversion of poly(U)-3′-[^{32}P]pCp to acid-soluble fragments incubated with p_3A_3 in the absence (●) or presence (▲) of 5×10^{-7} M pS_PS_P trimer. One hundred percent represents 4000 dpm of poly(U)-3′-[^{32}P]pCp bound to glass fiber filters.

2-5A to "anchor" the 2-5A molecule to RNase L more effectively because of increased polarity of the activator. Indeed, we have observed that all of the 2′,5′-phosphorothioate trimer monophosphates have 1000-fold increased binding affinity to RNase L compared to their corresponding cores (Figure 2A).

Modification of the 2′,5′-phosphodiester bond by replacement of the nonbridging oxygen with sulfur did *not* influence the binding affinity of the 2′,5′-phosphorothioate trimer cores or their 5′-monophosphates to RNase L. Sulfur substitution, however, did result in profound differences in activation of RNase L. The experiments described here reveal that RNase L is a highly *functionally* stereoselective enzyme. Unlike results from radiobinding assays (Figure 2A), not all the 2′,5′-phosphorothioate analogues were able to activate RNase L to the same extent. However, a similar pattern of activation was observed for the 2′,5′-phosphorothioate trimer core analogues and their 5′-monophosphates (Figures 2B, 3, and 4). The order of activation of RNase L was the same for the 5′-monophosphates as was observed for the cores (i.e., $R_PR_P > S_PR_P > R_PS_P$). Most interesting, the (S_PS_P)-2′,5′-phosphorothioate 5′-monophosphate was unable to active RNase L (Figures 2B and 4, lane 6) as was demonstrated for the corresponding S_PS_P trimer core (Figures 2B and 3, lane 5). These observations suggest that replacement of the nonbridging oxygen with sulfur in the 2′,5′-internucleotide linkages can alter the authentic 2-5A molecule to the highly efficient (R_PR_P and pR_PR_P) and moderately efficient (S_PR_P, pS_PR_P, R_PS_P, and pR_PS_P) as well as inefficient (S_PS_P and pS_PS_P) forms of the activator needed for RNase L activation.

The configuration of the phosphorothioate group in the phosphodiester backbone determines the stereoconfiguration of the 2′,5′-phosphorothioate analogues. However, in addition, there are also important contributions from the electronic properties of the P–S group (Frey & Sammons, 1985; Cosstick & Eckstein, 1985). If the RNase L activation process involves ionic interactions or hydrogen bonding between the nonbridging oxygens in the internucleotide linkages of 2-5A and amino acyl residues of RNase L, then replacement of the nonbridging oxygen of phosphorus with sulfur would alter the ability of the 2-5A molecule to activate RNase L. Within a phosphodiester bond, there is a delocalization of charge between the two nonbridging oxygens (Suggs & Taylor, 1985). However, in the phosphorothioate internucleotide linkage, the anionic charge is highly localized on sulfur (Frey & Sammons, 1985). In addition, the replacement of the oxygen by sulfur introduces chirality into the phosphodiester bond. The resulting 2′,5′-phosphorothioate trimer diastereomers would thus exhibit a localization of charge (on the sulfur) in four different stereoconfigurations. Introduction of sulfur results in changes in hydrogen bonding, degree of hydration, and ionic interactions. On the basis of these variables, it is possible that introduction of sulfur into the trimer core molecule in the R_PR_P configuration results in a stereoisomer in which the physicochemical changes facilitate the formation of a productive complex between RNase L, the 2-5A activator, and the substrate (RNA) to form a productive complex such that hydrolysis of RNA occurs. This compares with the S_PS_P isomer, which *inhibits* productive complex formation with RNase L, and hydrolysis of RNA does not occur. Furthermore, introduction of the 5′-monophosphate into the S_PS_P trimer core (i.e., pS_PS_P) is not sufficient to result in the formation of a prod-

uctive complex. This observation indicates that the R_P stereomer facilitates formation of a productive complex with RNase L, whereas the S_P conformer does not.

In addition to differences in the physicochemical properties in the P–S bond compared to the P–O bond, there are conformational characteristics of the 2′,5′-phosphodiester bond that differ markedly from the 3′,5′-phosphodiester bond. Numerous theoretical, stability, and stereochemical restriction studies have been completed on 2′,5′- and 3′,5′-linked oligo- and polynucleotides. The conformation of the dinucleoside monophosphate has been demonstrated to be critically dependent on the position of the phosphodiester linkage. Thus the adenine–ribose in the adenyl moiety of 2′,5′-adenylate dimer has the anti,anti right-handed asymmetrical stack (Kondo et al., 1970; Yathindra & Sundaralingam, 1973). It is known that the combined interactions of the bases with 2′- and 5′-phosphate groups limit the rotational freedom of the 2′,5′ chain (Srinivasan & Olson, 1986). Thus, the 5′-phosphate may serve not only to attach the 2′,5′-phosphorothioates on the binding/activation site of RNase L, thereby explaining the 1000-fold increased affinity of the 2′,5′-phosphorothioate 5′-monophosphates compared to the cores (Figure 2), but also to influence the activation process. In contrast to 3′,5′-linked nucleotides where both phosphate oxygens hydrogen bond with the C2′ hydroxyl group, only one of the phosphate oxygens hydrogen bonds to the C3′ hydroxyl group in the 2′,5′-nucleotides (Anukanth & Ponnuswamy, 1986). Consequently, when sulfur substitution occurs in either the R_P or S_P stereoconfiguration in the internucleotide linkages, intramolecular hydrogen bonding could be dramatically affected, thereby profoundly affecting the configuration of the 2-5A molecule. Although these physicochemical differences are present in all 2′,5′-linked molecules, the 2′,5′-phosphorothioates are model molecules to test the relative contributions of these various parameters.

It may be that the interaction between 2-5A and RNase L is similar to the interaction reported with cyclic AMP and the cyclic AMP dependent protein kinase. The observation that the S_PS_P or pS_PS_P 2′,5′-phosphorothioate trimer analogues can bind to but cannot activate RNase L may be analogous to the finding that the R_p isomer cyclic AMPS binds to the regulatory subunit of protein kinase but cannot induce its dissociation from the catalytic subunit. However, the S_P isomer can bind to and will dissociate the catalytic subunit (deWit et al., 1982; O'Brian et al., 1982; Rothermel et al., 1983). It has been suggested that the activation of RNase L by 2-5A causes a conformational change in the enzyme as the result of binding of 2-5A to the allosteric site of the enzyme (Krause et al., 1986). On this basis, it is reasonable that the phosphorothioate substitution in the phosphodiester backbone alters the configuration of the oligonucleotide in such a way as to facilitate its ability to cause a conformational change in RNase L and thus activation. The biological activity of the phosphorothioate analogues supports this possibility. This is most dramatically illustrated when authentic A_3 trimer core is compared with R_PR_P trimer core. Even at a concentration of 10^{-3} M, authentic A_3 cannot activate RNase L, whereas 10^{-6} M R_PR_P trimer core was able to activate the enzyme (Figure 2). Similarly, authentic dimer 5′-triphosphate (p_3A_2) is devoid of activity, whereas the $p_3A_2\alpha S$ activates RNase L at 10^{-6} M (see preceding paper, Figure 6). The increased biological activity of the 2′,5′-phosphorothioates (compared to their naturally occurring counterparts with the 2′,5′-phosphodiester backbone) may be attributed to their ability to adopt a conformation that mimicks that of 2-5A at a *transition* stage of RNase L activation. Such a mechanism has been hypothesized by Cheng and co-workers in the interaction of deoxynucleoside triphosphates and HSV DNA polymerases (Frank et al., 1985).

A 2-5A molecule capable of inhibiting RNase L activation is critical to permit a precise assessment of the involvement of RNase L in the interferon-induced antiviral and antiproliferative cascade. One of our aims in this study was to develop a 2-5A molecule that would shut off selectively RNase L activation. Not only do we now have access to such an inhibitor which acts at physiological concentrations (i.e., the pS_PS_P timer), but this inhibitor is the *most stable* 2-5A analogue tested [please refer to preceding paper (Karikó et al., 1987)]. The significantly increased stability of the pS_PS_P isomer makes it most attractive for in vivo studies. On the basis of the results reported here, we are currently in the process of synthesizing the trimer 2′,5′-phosphorothioates with sulfur substitution on the α-phosphorus of the 5′-terminus. It is anticipated that these substituted trimers will compare favorably to the 5′-monophosphates with respect to increased resistance of 5′-monophosphorothioates to hydrolysis by cellular phosphatases (Eckstein et al., 1982; Gratecos & Fischer, 1974; Lee & Suhadolnik, 1985; Karikó et al., 1987).

The analogue inhibitors described here will help to determine the role of the 2-5A/RNase L system in the normal cell cycle and in virus replication. Introduction of 2-5A cores and their 5′-monophosphates into intact cells by the calcium phosphate technique has proven difficult (Stenberg et al., 1982). However, several other suitable vectors for the intracellular delivery of functional 2-5A to intact cells have been demonstrated. The antiviral effects of 2-5A have been demonstrated following microinjection into HeLa cells (Bayard et al., 1984; Defilippi et al., 1986). 2-5A and analogues have also been successfully encapsulated in unilamellar liposomes and delivered with the aid of monoclonal antibodies to cells (Bayard et al., 1985). Poly(L-lysine) has been described as a versatile membrane carrier for 2-5A and other macromolecules (Bayard et al., 1986). Finally, reconstituted Sendai virus envelopes have been successfully used to deliver RNA and DNA to cells (Arad et al., 1986). These vectors show great promise for the introduction of the 2′,5′-phosphorothioates into cells as a means to evaluate the biological role of 2-5A and its role in antiviral and antineoplastic chemotherapy.

Acknowledgments

We thank Dr. Perry A. Frey for kindly providing phosphorothioate analogues of ATP and Dr. Robert H. Silverman for the generous supply of core–cellulose.

Supplementary Material Available

Chemical synthesis and ^{1}H NMR spectra of the four 5′-monophosphate phosphorothioate trimers (4 pages). Ordering information is given on any current masthead page.

Registry No. **11A**, 110356-00-8; **11B**, 110415-98-0; **12A**, 110415-99-1; **12B**, 110416-00-7; **13A**, 110356-01-9; **13A** free base, 110416-01-8; **13B**, 110453-79-7; **13B** free base, 110416-02-9; **14A**, 110453-80-0; **14A** free base, 110416-03-0; **14B**, 110453-81-1; **14B** free base, 110416-04-1; R_PR_p, 102282-14-4; S_PR_p, 102282-17-7; R_PS_p, 102282-15-5; S_PS_p, 102282-16-6; 3′,5′-A_2, 2391-46-0; 3′,5′-A_3, 917-44-2; 2′,5′-A_2, 2273-76-9; 2′,5′-A_3, 70062-83-8; RNase L, 76774-39-5; p-$O_2NC_6H_4(CH_2)_2OH$, 100-27-6; 1,2,4-triazole, 288-88-0; 2,5-dichlorophenylphosphorodichloridate, 53676-18-9; polynucleotide kinase, 37211-65-7.

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