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2′,5′-Oligoadenylates Chiral at Phosphorus: Enzymatic Synthesis, Properties, and Biological Activities of 2′,5′-Phosphorothioate Trimer and Tetramer Analogues Synthesized from (S_P)-ATPαS†

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ABSTRACT: The enzymatic synthesis and characterization of (R_P)-2′,5′-AMPS trimer and tetramer (S_P)-5′-*O*-(1-thiotriphosphates) from chirally substituted (S_P)-[α-^{35}S]ATPαS by 2′,5′-oligoadenylate synthetase from interferon-treated L cell extracts are described. The (R_P)-ATPαS isomer is not a substrate for the synthetase. The identification of the trimer and tetramer analogues (molar ratio 70:30) was accomplished by high-performance liquid chromatography and subsequent separation by charge using DEAE-cellulose thin-layer chromatography. The digestion of the analogue by snake venom phosphodiesterase I (SVPD) to [α-^{35}S]ATPαS and [^{35}S]AMPS but not by T2 RNase demonstrated the presence of the 2′,5′ linkage. The assignment of R_P configuration of the 2′,5′-phosphorothiodiester linkage was based on the highly specific stereoselectivity of SVPD for R_P diastereomers [Burgers, P. M. J., & Eckstein, F. (1978) *Proc. Natl. Acad. Sci. U.S.A. 75*, 4978–4800; Bryant, F. R., & Benkovic, S. J. (1979) *Biochemistry 18*, 2825–2828; Nelson, P. S., Bach, C. T., & Verheyden, J. P. H. (1984) *J. Org. Chem. 49*, 2314–2317]. This suggests that the synthesis of the phosphorothioate analogues proceeded via inversion of configuration at the chiral phosphorus of (S_P)-ATPαS. The putative (R_P)-2′,5′-AMPS tetramer (S_P)-5′-*O*-(1-thiotriphosphate) displaced the 2′,5′-p_3A_4[^{32}P]pCp analogue from 2′,5′-oligoadenylate-dependent endonuclease 5 times more efficiently than did equimolar concentrations of authentic 2′,5′-adenylate tetramer triphosphate. Furthermore, in studies using the calcium phosphate coprecipitation technique, the 2′,5′-phosphorothioate trimer and tetramer analogues inhibited protein synthesis better than did 2′,5′-adenylate trimer and tetramer triphosphates. Finally, the 2′,5′-phosphorothioate analogues are metabolically more stable than the naturally occurring 2′,5′-oligoadenylates. These phosphorothioate analogues chiral at phosphorus will be useful for the determination of the stereochemical course of the enzymes of the 2′,5′-adenylate system.

2′,5′-Oligoadenylate synthetase, found in lysates of rabbit reticulocytes and interferon-treated cells, produces 2′,5′-oligoadenylates from ATP in the presence of dsRNA (Kerr & Brown, 1978). There is strong evidence suggesting that the 2′,5′-oligoadenylates are important in the mechanism of antiviral action of interferon (Sen, 1982). The 2′,5′-oligoadenylates activate a latent 2′,5′-oligoadenylate-dependent endonuclease (RNase L) in mammalian cells. When 2′,5′-oligoadenylates bind to the 2′,5′-oligoadenylate-dependent endonuclease, mRNA is hydrolyzed, which then inhibits protein synthesis. However, 2′,5′-oligoadenylates are rapidly hydrolyzed by a 2′,5′-phosphodiesterase (Minks et al., 1979). Therefore, a structurally modified 2′,5′-oligoadenylate that would inhibit protein synthesis and yet be resistant to hydrolysis by 2′,5′-phosphodiesterase would be a useful biochemical probe. There have been several reports on the enzymatic and chemical syntheses of 2′,5′-oligoadenylate analogues and their biological properties (Doetsch et al., 1981; Hughes et al., 1983; Devash et al., 1984, and references 15–31 cited therein). Although a number of modifications have been made in the aglycon and ribosyl moiety as well as in the 5′-terminal phosphate of the 2′,5′-oligoadenylate molecule,

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relatively little is understood regarding the steric requirements of the enzymes involved in the 2′,5′-oligoadenylate system.

Phosphorothioate analogues of ATP have provided an experimental method of stereochemical analysis of nucleophilic substitution reactions at phosphorus catalyzed by nucleotidyltransferases, phosphotransferases, phosphohydrolases, and certain ATP-dependent synthetases (Knowles, 1980; Frey et al., 1982; Eckstein et al., 1982). By creating chiral phosphorus centers in which the nonbridging oxygen has been replaced by a sulfur at either the α or β phosphorus of ATP, the steric course of a large number of enzymes has been elucidated by the use of these chirally labeled phosphorothioates. The present study was undertaken to investigate the stereochemical requirements of three interferon-associated enzymes (e.g., 2′,5′-oligoadenylate synthetase, 2′,5′-phosphodiesterase, and 2′,5′-oligoadenylate-dependent endonuclease). We report the enzymatic synthesis of the chiral 2′,5′-phosphorothioate trimer and tetramer analogues of 2′,5′-oligoadenylate from the chirally substituted (S_P)-ATPαS[1] by 2′,5′-oligoadenylate synthetase and propose their application to the elucidation of the steric course of 2′,5′-phosphodiesterase and 2′,5′-oligoadenylate-dependent endonuclease.

Materials and Methods

Chemicals and Enzymes. (S_P)-[α-^{35}S]ATPαS (650 Ci/mmol), [α-^{32}P]ATP (410 Ci/mmol), 2′,5′-p_3A_4[^{32}P]pCp (3000 Ci/mmol), and [4,5-^{3}H]leucine (120 Ci/mmol) were purchased from Amersham; (R_P)-ATPαS and (S_P)-ATPαS were generous gifts from Dr. P. Frey; bacterial alkaline phosphatase (BAP) (orthophosphoric-monoester phosphohydrolase, type III from *Escherichia coli*, EC 3.1.3.1), creatine phosphokinase (ATP:creatine *N*-phosphotransferase, EC 2.7.3.2, from rabbit muscle), ribonuclease T2 (T2 RNase) (ribonuclease 3′-oligonucleotidohydrolase from *Aspergillus oryzae*, EC 3.1.4.23), and Nonidet P-40 (NP-40) were from Sigma; snake venom phosphodiesterase I (SVPD) (venom exonuclease from *Crotalus adamanteus* venom, EC 3.1.4.1) was from Worthington; DEAE-cellulose thin-layer chromatograms were from Brinkman; poly(rI)·poly(rC)–agarose, 2′,5′-p_3A_3, 2′,5′-p_3A_4, and 2′,5′-A_3 were from P-L Biochemicals; L929 cells and HeLa cells were maintained as monolayers in Eagle's minimum essential medium supplemented with 10% fetal bovine serum (GIBCO); mouse fibroblast interferon (1×10^5 units/mg) was from Calbiochem.

Synthesis of 2′,5′-Oligoadenylates and 2′,5′-Phosphorothioate Analogues. The synthesis of 2′,5′-oligonucleotides was carried out with the 2′,5′-oligoadenylate synthetase obtained from NP-40 extracts of L929 cells prepared following treatment of confluent monolayers with mouse fibroblast interferon (200 units/mL, 20 h) as described by Kimchi et al. (1979). Conditions for the synthesis were basically as described for 2′,5′-oligoadenylates (Suhadolnik et al., 1983). The reaction mixture (10 μL) contained 10 mM Hepes, pH 7.5, 2.5 mM $MgCl_2$, 60 mM KCl, 5% glycerol, 2.5 mM DTT, 3 units/mL creatine phosphokinase, 10 mM creatine phosphate, and 2.5 mM [α-^{32}P]ATP, (S_P)-[α-^{35}S]ATPαS, or (R_P)-ATPαS. The reaction mixture was added to poly(rI)·poly(rC)–agarose (30 μL) to which 2′,5′-oligoadenylate synthetase from L cell lysates was previously bound. The syntheses were at 30 °C for 20 h after which time the agarose was pelleted by centrifugation (3 min, 8000*g*, 25 °C). The synthetic products in the supernatant were analyzed by DEAE-cellulose column chromatography and HPLC. To determine substrate stereospecificity, competition assays were performed by varying concentrations (0.5, 1, 2, 3, 4, and 5 mM) of (S_P)-[α-^{35}S]ATPαS and (R_P)-ATPαS in the reaction mixture.

Isolation of Products by DEAE-cellulose Column Chromatography. The 2′,5′-[^{32}P]oligoadenylates and 2′,5′-[^{35}S]-phosphorothioate analogues were isolated by DEAE-cellulose chromatography as described by Doetsch et al. (1981). Following washing with 50 mL of 90 mM KCl buffer, the radioactive content in the material displaced with 350 mM KCl buffer (350-μL fractions) was determined. The peak fractions (2–4) were pooled, dialyzed (45 min, 4 L of H_2O, 4 times, 0 °C), and lyophilized for further characterization. The concentration of 2′,5′-oligonucleotides was calculated from the known specific activities of [α-^{32}P]ATP and (S_P)-[α-^{35}S]ATPαS.

Radiobinding Assays. Radiobinding assays were performed as described (Knight et al., 1981) with Ehrlich ascites tumor cell lysates as the source of the 2′,5′-oligoadenylate-dependent endonuclease.

Stability of 2′,5′-Oligoadenylates and 2′,5′-Phosphorothioate Analogues in Cell-Free Extracts. The stability of the 2′,5′-oligoadenylates and 2′,5′-phosphorothioate analogues was measured in NP-40 extracts prepared from HeLa cells and L cells as described (Weber et al., 1975). The 2′,5′-oligonucleotides displaced by 350 mM KCl buffer were dialyzed as above and lyophilized. Incubation mixtures (140 μL) contained 0.5 part of cell extract, 2.5 mM $Mg(OAc)_2$, 120 mM KCl, 20 mM Hepes, pH 7.4, 1 mM DTT, and 5 μM 2′,5′-[^{32}P]p_3A_n or [^{35}S]phosphorothioate analogue of 2′,5′-p_3A_n (n = 3 or 4). At the times indicated, 20-μL samples were withdrawn and heated for 3 min at 95 °C. The amount of unhydrolyzed 2′,5′-oligonucleotide was determined by DEAE-cellulose chromatography (Doetsch et al., 1981).

Determination of the Structure of 2′,5′-Phosphorothioate Analogues by HPLC and DEAE-cellulose TLC. HPLC analysis of the putative 2′,5′-[^{35}S]phosphorothioate analogues synthesized from (S_P)-[α-^{35}S]ATPαS that had been displaced with 350 mM KCl buffer from DEAE-cellulose columns was accomplished with a Waters model 6000 pump equipped with a model U6K injector and controlled by a model 660 solvent programmer. UV-absorbing material was detected with a Waters model 440 detector set at 254 nm and recorded on an LKB model 2210 recorder. Reverse-phase chromatography was done with a Waters C_{18} μBondapak analytical column (0.25 in. o.d. × 12 in.). For purification of the 2′,5′-phosphorothioates, a linear gradient (flow rate 1 mL min^{-1}) consisting of 50 mM ammonium phosphate, pH 7.0 (A), and methanol–water (1:1 v/v) (B) was used (t = 0 min, 0% B; t = 15 min, 100% B). At the end of the gradient, the final conditions (100% B) were maintained for an additional 10 min. Fractions of 0.5 mL were collected. Radioactivity was determined with ACS scintillant (Amersham) on a Beckman LS-100C liquid scintillation spectrometer. Two major peaks of radioactivity were observed. These two peaks were pooled separately and further purified by DEAE-cellulose TLC in 9

[1] Abbreviations: AMPS, adenosine 5′-*O*-phosphorothioate; 2′,5′-p_3A_3 and 2′,5′-p_3A_4, trimer and tetramer of adenylic acid with 2′,5′-phosphodiester linkages and a 5′-triphosphate; 2′,5′-A_3, 5′-dephosphorylated 2′,5′-p_3A_3; (R_P)- and (S_P)-ATPαS, the R_P and S_P diastereomers of adenosine 5′-*O*-(1-thiotriphosphate); 2′,5′-phosphorothioate trimer and tetramer analogues, (R_P)-2′,5′-AMPS trimer and tetramer (S_P)-5′-*O*-(1-thiotriphosphates); phosphorothiodiester, 2′-adenosine 5′-adenosine *O*,*O*-phosphorothioate; BAP, bacterial alkaline phosphatase; DEAE, diethylaminoethyl; DTT, dithiothreitol; Hepes, *N*-(2-hydroxyethyl)-piperazine-*N*′-2-ethanesulfonic acid; HPLC, high-performance liquid chromatography; NP-40, Nonidet P-40; SVPD, snake venom phosphodiesterase I; TLC, thin-layer chromatography; T2 RNase, ribonuclease T2; dsRNA, double-stranded ribonucleic acid.

M urea–0.1 M ammonium formate–1 mM Na_2EDTA for 2 h, followed by 9 M urea–0.2 M ammonium formate–1 mM Na_2EDTA for 2 h. This method identified the chain length by charge separation.

Enzymatic Digestion of 2′,5′-Phosphorothioate Analogues. 2′,5′-Phosphorothioate analogues obtained from the 350 mM KCl eluate from DEAE-cellulose columns and purified by HPLC were further characterized by digestion with BAP, SVPD, and T2 RNase as described by Minks et al. (1979). After enzymatic digestion, the samples were subsequently analyzed by HPLC as described above.

Inhibition of Cellular Protein Synthesis. 2′,5′-Oligoadenylates and 2′,5′-phosphorothioate trimer and tetramer analogues purified by HPLC were tested for their ability to inhibit protein synthesis in intact L cells by using the calcium phosphate coprecipitation technique as described by Lee & Suhadolnik (1983) with the exception that [4,5-^{3}H]leucine was used instead of [^{35}S]methionine.

Results and Discussion

The substrate specificity of 2′,5′-oligoadenylate synthetase was studied with two diastereomers of ATP, i.e., (S_P)-ATPαS and (R_P)-ATPαS. The 2′,5′-oligoadenylate synthetase from interferon-treated L cell extracts converted (S_P)-[^{35}S]ATPαS to putative 2′,5′-[^{35}S]phosphorothioate trimer and tetramer triphosphate analogues. The yield of the 2′,5′-phosphorothioate analogues was 20%, compared to a 22% yield for the 2′,5′-oligoadenylates. Similar yields have been obtained with lysates from rabbit reticulocytes and Daudi cell extracts (unpublished results). There was no detectable conversion of (R_P)-ATPαS to a 2′,5′-phosphorothioate analogue as determined by UV spectroscopy or HPLC. In competition assays with the 2′,5′-oligoadenylate synthetase in which the concentrations of (S_P)-ATPαS and (R_P)-ATPαS were varied from 0.5 to 5.0 mM, it was confirmed that (S_P)-ATPαS is the preferred substrate for the synthetase. Furthermore, (R_P)-ATPαS at high concentrations inhibited the synthetase; e.g., when 5 mM (R_P)-ATPαS was added to assays containing 5 mM (S_P)-[α-^{35}S]ATPαS, the synthesis of the 2′,5′-phosphorothioate analogues from (S_P)-[α-^{35}S]ATPαS was inhibited by 50%. These results demonstrate that the 2′,5′-oligoadenylate synthetase is similar to the majority of previously studied nucleotidyl- and phosphotransferases in that the (S_P)-ATPαS diastereomer, but not the (R_P)-ATPαS, is the substrate (Eckstein et al., 1982).

Separation of the products synthesized from (S_P)-ATPαS by the 2′,5′-oligoadenylate synthetase from L cell extracts was achieved by HPLC. Two major peaks were resolved. The elution times of the putative (R_P)-2′,5′-AMPS trimer and tetramer (S_P)-5′-*O*-(1-thiotriphosphates) were 15.0 (peak I) and 17.5 (peak II) min, respectively (Figure 1). The molar ratio of the 2′,5′-phosphorothioate trimer:tetramer analogues was 70:30. The amount of (S_P)-[^{35}S]ATPαS and [^{35}S]AMPS detected was negligible. The chain length of the 2′,5′-phosphorothioate analogues eluted at 15.0 and 17.5 min was confirmed by charge separation using DEAE-cellulose TLC. Peak I from the HPLC had a charge of 6– (equivalent to 2′,5′-adenylate trimer triphosphate); peak II had a charge of 7– (equivalent to 2′,5′-adenylate tetramer triphosphate). To demonstrate the formation of the 2′,5′ linkage, the 2′,5′-phosphorothioate analogues were treated with SVPD (2′,5′- and 3′,5′-diesterase) and T2 RNase (3′,5′-diesterase). The SVPD digestion and subsequent HPLC analysis of the 2′,5′-[^{35}S]phosphorothioate trimer analogue (peak I) yielded [^{35}S]AMPS and (S_P)-[α-^{35}S]ATPαS in an approximate molar ratio of 2:1 with elution times of 5.5 and 7.5 min, respectively. There was no hydrolysis of the 2′,5′-phosphorothioate analogue

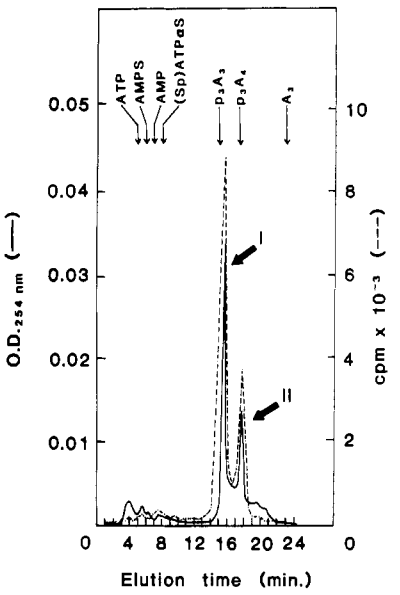

FIGURE 1: HPLC analysis of the 2′,5′-phosphorothioate analogue of 2′,5′-oligoadenylate synthesized from (S_P)-[α-^{35}S]ATPαS. HPLC analysis of the products (200-μL sample injected) synthesized from (S_P)-[α-^{35}S]ATPαS that were displaced from DEAE-cellulose columns with 350 mM KCl buffer was as described under Materials and Methods. Samples (10 μL) of each fraction (500 μL) were taken, and the radioactivity was determined. The elution times of standard ATP, AMP, (S_P)-ATPαS, AMPS, 2′,5′-p_3A_3, 2′,5′-p_3A_4, and 2′,5′-A_3 are indicated by arrows. The elution time of (R_P)-ATPαS was 13 min. Peak I, putative 2′,5′-phosphorothioate trimer analogue; peak II, putative 2′,5′-phosphorothioate tetramer analogue.

by T2 RNase. T2 RNase has been reported to cleave the 3′,5′-phosphorothiodiester linkages (Burgers & Eckstein, 1979). The digestion by SVPD to [^{35}S]AMPS and (S_P)-[α-^{35}S]ATPαS was complete in that no intact trimer analogue remained following digestion. This strongly suggests that the 2′,5′-phosphorothioate analogue has the R_P configuration at the chiral phosphate center of the 2′,5′ linkage because SVPD is known to selectively hydrolyze R_P diastereomers over S_P diastereomers (Burgers & Eckstein, 1978; Bryant & Benkovic, 1979; Nelson et al., 1984). The formation of the putative R_P configuration of the 2′,5′ internucleotide linkage in the 2′,5′-phosphorothioate analogue of 2′,5′-oligoadenylate would involve inversion of configuration of the chiral (S_P)-ATPαS during the reaction catalyzed by 2′,5′-oligoadenylate synthetase. This type of inversion is similar to that reported for DNA-dependent RNA polymerase, DNA-dependent DNA polymerase, RNA ligase, and tRNA nucleotidyltransferase (Eckstein et al., 1982). The formation of diastereomers with both R_P and S_P configurations in the 2′,5′ internucleotide linkages of the phosphorothioate analogue is highly unlikely in view of the fact that, with the aforementioned enzymes, (S_P)-ATPαS or (S_P)-dATPαS is utilized to produce only R_P, but not S_P, diastereomers. We are currently studying the absolute configuration of the 2′,5′-phosphorothioate internucleotide linkage by hydrolysis with SVPD in $H_2{}^{18}O$ as described by Brody & Frey (1981).

Hydrolysis of the 2′,5′-phosphorothioate trimer 5′-*O*-(1-thiotriphosphate) analogue by BAP produced (R_P)-2′,5′-AMPS trimer 5′-*O*-(1-thiophosphate) with a retention time of 19 min on reverse-phase HPLC. These data are in agreement with an earlier report that when the P═O of ATP was replaced by a P═S, the hydrolysis of ATPαS by BAP produced AMPS, not adenosine (Eckstein & Goody, 1976).

The biological properties of the 2′,5′-phosphorothioate analogues were examined in terms of binding to the 2′,5′-oligoadenylate-dependent endonuclease, metabolic stability,

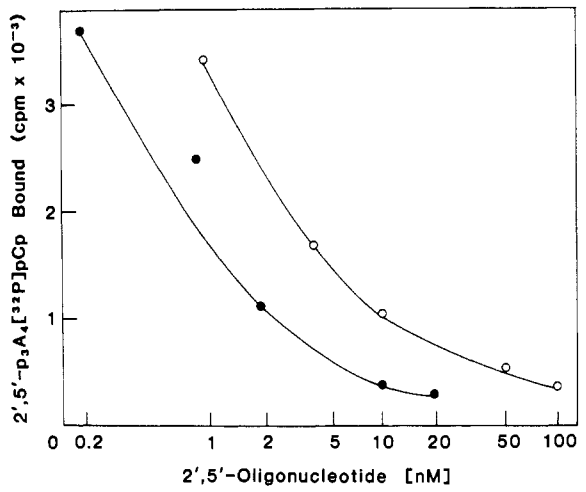

FIGURE 2: Radiobinding assay of the 2',5'-phosphorothioate analogue of 2',5'-p_3A_4 (●) and authentic 2',5'-p_3A_4 (○). The 2',5'-phosphorothioate tetramer analogue was purified by HPLC, and radiobinding assays were performed as described under Materials and Methods. Fifty percent binding of 2',5'-$p_3A_4[^{32}P]pCp$ (total cpm added = 8000) was achieved in the absence of 2',5'-oligonucleotides. Data represent an average of three experiments.

and inhibition of cellular protein synthesis. The ability of the 2',5'-phosphorothioate tetramer analogue to bind to the 2',5'-oligoadenylate-dependent endonuclease was studied by radiobinding assays where the 2',5'-phosphorothioate analogue was added to the endonuclease to which 2',5'-$p_3A_4[^{32}P]pCp$ had been previously bound. The 2',5'-phosphorothioate tetramer analogue displaced 2',5'-$p_3A_4[^{32}P]pCp$ from the endonuclease more efficiently than did authentic 2',5'-adenylate tetramer triphosphate (Figure 2). Seventy percent displacement was achieved with 2 nM 2',5'-phosphorothioate tetramer analogue, whereas 10 nM 2',5'-adenylate tetramer triphosphate was required to achieve equivalent displacement. These findings indicate greater affinity of the 2',5'-phosphorothioate analogue for the endonuclease than the naturally occurring 2',5'-oligoadenylate.

The stability of the 2',5'-phosphorothioate analogues was compared with that of 2',5'-oligoadenylate triphosphates in interferon-treated HeLa cell extracts. While the authentic 2',5'-oligoadenylate triphosphates were hydrolyzed 95% in 15 min, 45% of the 2',5'-phosphorothioate analogues remained after 60 min (Figure 3). There was slow hydrolysis of the phosphorothioate analogue for the initial 30 min, after which time no further degradation was detected. The fact that hydrolysis of the 2',5'-phosphorothioates did not proceed to completion appears to be due to the inactivation of degradative enzymes in the cell extracts after 30 min under these assay conditions; such inactivation was demonstrated by the lack of hydrolysis of authentic 2',5'-[^{32}P]oligoadenylates when added to cell extracts that had been preincubated for 30 min (data not shown). The unhydrolyzed 2',5'-phosphorothioate analogues were recovered by purification using DEAE-cellulose chromatography as described under Materials and Methods. The recovered 2',5'-phosphorothioates were still biologically active, as determined by radiobinding assays and the inhibition of protein synthesis in intact L cells as described below (data not shown). These results indicate that, unlike the 2',5'-phosphodiester bond in the naturally occurring 2',5'-oligoadenylates, the 2',5'-phosphorothiodiester bond is considerably more resistant to hydrolysis by cellular 2',5'-phosphodiesterase. The mechanism of resistance to hydrolysis of the 2',5'-phosphorothioate analogues is under further investigation in this laboratory.

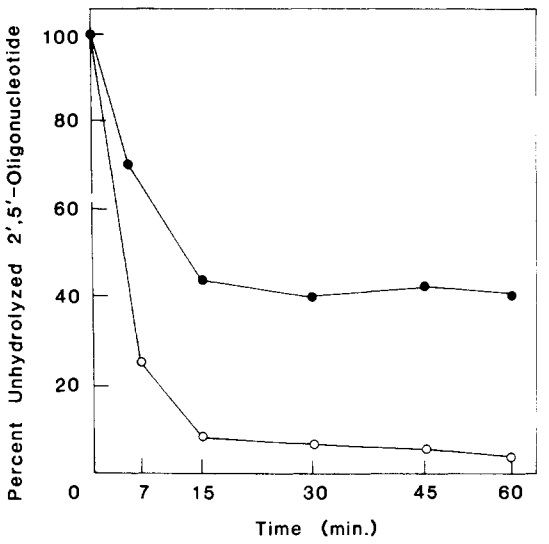

FIGURE 3: Stability of the 2',5'-phosphorothioate analogue of 2',5'-p_3A_n (●) and authentic 2',5'-p_3A_n (○) in HeLa cell extracts. The ^{35}S-labeled phosphorothioate analogue of 2',5'-p_3A_n and ^{32}P-labeled 2',5'-p_3A_n were prepared, and hydrolysis was performed as described under Materials and Methods. Degradation of 2',5'-oligonucleotides was monitored as a decrease of radioactive material eluting with 350 mM KCl buffer compared with the zero time point (100% unhydrolyzed). The same observations with respect to stability of the 2',5'-phosphorothioates were obtained with interferon-treated L cell extracts.

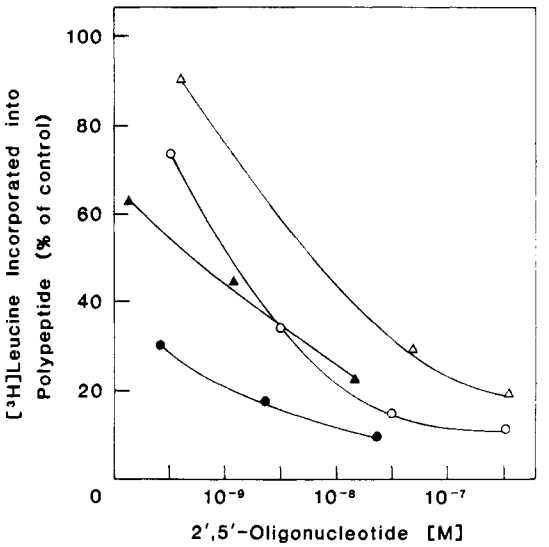

FIGURE 4: Inhibition of cellular protein synthesis by the 2',5'-phosphorothioate trimer and tetramer analogues and authentic 2',5'-p_3A_3 and 2',5'-p_3A_4 in mouse L929 cells. Inhibition of protein synthesis by the 2',5'-phosphorothioate trimer (●) and tetramer (▲) analogues was determined and compared with that of authentic 2',5'-p_3A_3 (○) and 2',5'-p_3A_4 (△) in intact L cells by using the calcium phosphate coprecipitation technique as described (Lee & Suhadolnik, 1983). Control cultures were treated with calcium phosphate, but without 2',5'-oligonucleotides. The incorporation of [^3H]leucine into control cultures was taken as 100%. (S_P)-ATPαS and AMPS were not inhibitory.

The ability of the 2',5'-phosphorothioate analogues to inhibit cellular protein synthesis was examined by using the calcium phosphate coprecipitation technique to introduce the 2',5'-phosphorothioate analogues into L cells. The putative (R_P)-2',5'-AMPS trimer and tetramer (S_P)-5'-*O*-(1-thiotriphosphates) inhibited cellular protein synthesis in a dose-dependent manner, as measured by the incorporation of [^3H]-leucine into trichloroacetic acid precipitable material (Figure 4). The inhibition of protein synthesis by the 2',5'-oligoadenylates is known to involve the activation of the 2',5'-

oligoadenylate-dependent endonuclease and degradation of mRNA and rRNA (Sen, 1982). Therefore, on the basis of the in vitro radiobinding assays reported here, it is likely that the inhibition of protein synthesis by the 2′,5′-phosphorothioate trimer and tetramer analogues is proceeding via the activation of the 2′,5′-oligoadenylate-dependent endonuclease. It is important to note that the inhibition of protein synthesis by the 2′,5′-phosphorothioate analogues was at least 1 order of magnitude greater than that of equimolar concentrations of authentic 2′,5′-oligoadenylate 5′-triphosphate; e.g., 75% inhibition was achieved by 1×10^{-8} M 2′,5′-phosphorothioate tetramer analogue compared to 75% inhibition by 5×10^{-7} M 2′,5′-adenylate tetramer triphosphate (Figure 4). The enhanced biological activity observed with the 2′,5′-phosphorothioate analogues might be attributed to the increased metabolic stability and/or a favorable stereospecific interaction between the chiral analogues and the endonuclease.

In conclusion, we have demonstrated that 2′,5′-oligoadenylate synthetase from L cells can utilize the chiral analogue of ATP, (S_P)-ATPαS, but not (R_P)-ATPαS, to synthesize 2′,5′-oligoadenylate analogues chiral at phosphorus. The effect of sulfur substitution at the α-phosphorus and formation of chirality in the 2′,5′-oligoadenylate molecule are reflected in the higher affinity of these 2′,5′-phosphorothioate analogues for the 2′,5′-oligoadenylate-dependent endonuclease, increased metabolic stability, and enhanced inhibition of cellular protein synthesis. Our studies have revealed the substrate stereospecificity of the 2′,5′-oligoadenylate synthetase and suggested a mechanism involved in the stereochemical course of the enzyme (i.e., inversion of configuration). Further elucidation of the stereochemistry of the synthetase is in progress. In addition, the availability of chiral phosphorothioate analogues of 2′,5′-oligoadenylate provides the basis for further study of the stereochemical course of reactions catalyzed by 2′,5′-phosphodiesterase, as well as the study of the steric requirements for the activation of the 2′,5′-oligoadenylate-dependent endonuclease.

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Registry No. RNase L, 76774-39-5; (S_P)-ATPαS, 58976-48-0; (R_P)-2′,5′-AMPS trimer (S_P)-5′-*O*-(1-thiotriphosphate), 94295-04-2; (R_P)-2′,5′-AMPS tetramer (S_P)-5′-*O*-(1-thiotriphosphate), 94324-69-3; 2′,5′-oligoadenylate synthetase, 69106-44-1.

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