Plant Oligoadenylates: Enzymatic Synthesis, Isolation, and Biological Activities†

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ABSTRACT: An enzyme that converts [^{3}H,^{32}P]ATP, with a ^{3}H:^{32}P ratio of 1:1, to oligoadenylates with the same ^{3}H:^{32}P ratio was increased in plants following treatment with human leukocyte interferon or plant antiviral factor or inoculation with tobacco mosaic virus. The enzyme was extracted from tobacco leaves, callus tissue cultures, or cell suspension cultures. The enzyme, a putative plant oligoadenylate synthetase, was immobilized on poly(rI)·poly(rC)–agarose columns and converted ATP into plant oligoadenylates. These oligoadenylates were displaced from DEAE-cellulose columns with 350 mM KCl buffer, dialyzed, and further purified by high-performance liquid chromatography (HPLC) and DEAE-cellulose gradient chromatography. In all steps of purification, the ratio of ^{3}H:^{32}P in the oligoadenylates remained 1:1. The plant oligoadenylates isolated by displacement with 350 mM KCl had a molecular weight greater than 1000. The plant oligoadenylates had charges of 5– and 6–. HPLC resolved five peaks, three of which inhibited protein synthesis in reticulocyte and wheat germ systems. Partial structural elucidation of the plant oligoadenylates has been determined by enzymatic and chemical treatments. An adenylate with a 3′,5′-phosphodiester and/or a pyrophosphoryl linkage with either 3′- or 5′-terminal phosphates is postulated on the basis of treatment of the oligoadenylates with T_2 RNase, snake venom phosphodiesterase, and bacterial alkaline phosphatase and acid and alkaline hydrolyses. The plant oligoadenylates at 8×10^{-7} M inhibit protein synthesis by 75% in lysates from rabbit reticulocytes and 45% in wheat germ cell-free systems. Unlike the mammalian 2′,5′-oligoadenylate triphosphates, the plant oligoadenylates inhibited protein synthesis *without* activating the 2′,5′-A-dependent endonuclease from lysates of rabbit reticulocytes and *without* competing for 2′,5′-p_3A_4[^{32}P]pCp binding to the endonuclease. In addition, the plant extracts *do not* contain any binding protein as determined by lack of binding of 2′,5′-p_3A_4[^{32}P]pCp. These results suggest that the plant oligoadenylates differ substantially from the mammalian 2′,5′-oligoadenylates.

The antiviral state in mammalian tissues is dependent on the induction of the double-stranded RNA (dsRNA)-dependent 2′,5′-adenylate synthetase and a protein kinase by interferon (Pestka, 1981a,b). The synthetase converts ATP in the presence of dsRNA into 2′,5′-adenylate 5′-triphosphates (2′,5′-p_3A_n, n = 2–15)[1] (Ball, 1982) which activate a latent 2′,5′-A-dependent endonuclease to degrade cellular and viral RNA (Wreschner et al., 1981). Recent reports demonstrate that the 2′,5′-A-dependent endonuclease and the 2′,5′-A synthetase can be induced as cells approach the confluent state (Creasey et al., 1983; Etienne-Smekens et al., 1983; Jacobson et al., 1983a,b). Lysates from phenylhydrazine-injected rabbits have high levels of 2′,5′-A synthetase and 2′,5′-A-dependent endonuclease, whereas these enzymes cannot be detected in the wheat germ cell-free system (unpublished results).

Tobacco plants carrying the N gene for virus localization respond to tobacco mosaic virus (TMV) infection by producing an antiviral factor (AVF) that resembles interferon [for a review, see Sela (1981)]. We previously reported that AVF-treated tobacco plant tissue extracts exhibit a discharging factor (DF) activity which discharges histidine from aminoacylated TMV RNA (Devash et al., 1981). The activity of an enzyme similar to the mammalian 2′,5′-adenylate synthetase was also stimulated in TMV-infected or AVF-treated tobacco tissues. This enzyme converts ATP in the presence of dsRNA to plant oligoadenylates which stimulate the transient DF activity (Devash et al., 1981). Recently, we reported that human interferons, mammalian 5′-dephosphorylated 2′,5′-adenylate trimer and its 2′,5′ analogues, and plant oligonucleotides synthesized in vivo or plant oligoadenylates synthesized in vitro inhibit TMV replication in tobacco leaf disks (Devash et al., 1982, 1984; Orchansky et al., 1982; Reichman et al., 1983). In this paper, we describe the enzymatic synthesis of plant oligoadenylates from ATP. We provide the first evidence of their inhibition of protein synthesis in reticulocyte lysates and in the wheat germ cell-free systems.

Materials and Methods

Materials. Lysates from rabbit reticulocytes were obtained from Clinical Convenience, Madison, WI; wheat germ was from General Mills, Inc., Minneapolis, MN; recombinant human leukocyte interferon (IFN-α) was from Dr. S. Pestka, Roche Institute of Molecular Biology, Nutley, NJ; poly(rI)·poly(rC)–agarose was from P-L Biochemicals, Inc.; DEAE-cellulose (DE-52) and GF/A glass fiber filters (2.3 cm)

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[1] Abbreviations: 2′,5′-p_3A_n, oligomer of adenylic acid with 2′,5′-phosphodiester linkages and a triphosphate at the 5′ end; 2′,5′-A_n, 5′-dephosphorylated 2′,5′-adenylate triphosphates, also referred to as cores; AVF, antiviral factor; DF, discharging factor; IFN-α, human leukocyte interferon; TMV, tobacco mosaic virus; VSV, vesicular stomatitis virus; HPLC, high-performance liquid chromatography; HCP, hydrated calcium phosphate; Hepes, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; BAP, bacterial alkaline phosphatase; SVPD, snake venom phosphodiesterase; TLC, thin-layer chromatography; EDTA, ethylenediaminetetraacetic acid.

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were from Whatman; [8-^{3}H]ATP (23 Ci/mmol), [α-^{32}P]ATP (410 Ci/mmol), [4,5-^{3}H]leucine (147 Ci/mmol), and [U-^{14}C]leucine (330 mCi/mmol) were from Amersham; 2′,5′-p_3A_3, 2′,5′-p_3A_4, 2′,5′-pA_4, 2′,5′-A_3, and 2′,5′-A_4 were from P-L Biochemicals, Inc.; oligo(dT)–cellulose (type 2) was from Collaborative Research, Inc.; thin-layer chromatograms were from Eastman (Chromagram, 13254 cellulose) and Brinkmann (Polygram CEL 300, DEAE). All other chemicals were of the highest grade commercially available.

Enzymatic Synthesis of Plant Oligoadenylates. The dsRNA-dependent plant oligoadenylate synthetase was prepared from plant leaves of *Nicotiana glutinosa* or *Nicotiana tabacum*, from tobacco callus tissue cultures, or from tobacco cell suspensions. Plant tissues were immersed in buffer or in the various antiviral agents [i.e., mock AVF, AVF, DE-0.65 fraction (10 ng of protein/mL) (Devash et al., 1981), or IFN-α (1 unit/mL)]. Plant leaves or tobacco callus tissue cultures (10 g) were homogenized in a blender in 10 mL of 0.01 M sodium phosphate buffer, pH 7.6, at 4 °C. The plant cell suspensions (20 mL of packed cells) were washed 3 times with 50 mL of 0.01 M sodium phosphate buffer, pH 7.6, at 4 °C, and broken by passage through a French pressure cell (5000 psi). The homogenates were filtered through cheesecloth and centrifuged (10000*g*, 10 min, 4 °C). The fractions of the plant extracts that did not adsorb to hydrated calcium phosphate (HCP) were used for further purification (Devash et al., 1981). The plant proteins were chromatographed on DEAE-cellulose columns (1 × 20 cm) in 0.01 M sodium phosphate buffer, pH 7.6, at room temperature. The proteins that did not adsorb to the column (fraction DE-0, adjusted to 500 ng of protein/mL, 5 mL) (Devash et al., 1981) served as the source of plant oligoadenylate synthetase. Poly(rI)·poly(rC)–agarose columns (0.5 × 2 cm) bound with plant oligoadenylate synthetase were prepared by adding 5 mL of fraction DE-0 (500 ng of protein/mL) as described (Suhadolnik et al., 1981). In each oligoadenylate synthesis with ATP, a poly(rI)·poly(rC)–agarose column with no addition of enzyme served as one control, and a second poly(rI)·poly(rC)–agarose column bound with 2′,5′-A synthetase prepared by adding 1.2 mL of lysate from rabbit reticulocytes served as the standard for the enzymatic conversion of ATP to 2′,5′-p_3A_n.

The plant oligoadenylate synthetase and the mammalian 2′,5′-A synthetase bound to the poly(rI)·poly(rC)–agarose column were incubated at room temperature for 17 h with 2.5 mM ATP plus [^{3}H]ATP (10 μCi) and [α-^{32}P]ATP (10 μCi). After 17 h, ATP and oligoadenylates were eluted from the columns with 2 mL of buffer A [0.02 M Hepes–KOH, pH 7.5, 0.1 M KCl, 0.002 M $Mg(OAc)_2$, 0.002 M dithiothreitol, and 10% glycerol], adjusted to 90 mM KCl, applied to DEAE-cellulose columns (0.6 × 2.1 cm), and washed with 100 mL of 90 mM KCl in buffer A. The oligoadenylates were displaced with 350 mM KCl in buffer A. The formation of oligoadenylates was determined as described (Suhadolnik et al., 1981). The oligoadenylates displaced with 350 mM KCl were dialyzed (SpectraPor 6, molecular weight cutoff 1000) in 4 L of H_2O replaced each hour for 4 h at 0 °C.

In Vitro Translation Assays. The inhibition of protein synthesis by the plant oligoadenylates was determined in lysates from rabbit reticulocytes as reported from this laboratory (Doetsch et al., 1981; Suhadolnik et al., 1983). The inhibition of protein synthesis by plant oligoadenylates in the wheat germ cell-free system was determined according to Roberts & Patterson (1973).

Radiobinding Assays. The binding of 2′,5′-p_3A_4[^{32}P]pCp to the 2′,5′-A-dependent endonuclease present in lysates from rabbit reticulocytes and its competition by 2′,5′-p_3A_4 or plant oligoadenylates were analyzed as described (Knight et al., 1981). The presence of 2′,5′-p_3A_4[^{32}P]pCp binding protein was determined as described (Cayley et al., 1982).

Hydrolysis of Vesicular Stomatitis Virus (VSV) [^{3}H]-mRNA by Activated 2′,5′-A-Dependent Endonuclease. Reaction conditions for hydrolysis of VSV [^{3}H]mRNA (40 000 dpm), partial purification of 2′,5′-A-dependent endonuclease, and reisolation of poly(A) mRNA by oligo(dT)–cellulose column chromatography were as described (Suhadolnik et al., 1983).

Structural Characterization. (*A*) *Acid Hydrolysis.* Plant [^{3}H,^{32}P]oligoadenylates (3500 dpm), displaced from DEAE-cellulose columns by 350 mM KCl buffer and dialyzed, were heated in 0.1 M HCl for 60 min at 95 °C. Reactions (10 μL) were terminated by neutralization with 10 μL of 0.1 M NaOH. Reaction mixtures were applied to cellulose thin-layer chromatograms (Eastman) and developed in H_2O. Adenine, adenosine, AMP, ADP, and ATP were used as UV markers (R_f = 0.30, 0.55, 1, 1, and 1, respectively). Each chromatogram was cut into 1 cm^2 pieces, and the radioactivity was determined.

(*B*) *Alkaline Hydrolysis.* Plant oligoadenylates (400 nM) or 2′,5′-p_3A_4 (1 μM) was treated with 0.3 M KOH as described (Suhadolnik et al., 1983).

(*C*) *Enzymatic Digestions.* Enzymatic digestions with bacterial alkaline phosphatase (BAP), snake venom phosphodiesterase (SVPD), and T_2 RNase were as described (Suhadolnik et al., 1983).

Determination of the Net Charge of Plant Oligoadenylates. TLC was performed by using DEAE-cellulose (Brinkmann). The running solvent was 0.2 M ammonium formate/9 M urea/1 mM Na_2EDTA, after a short prerun in H_2O (Konarska et al., 1981). ^{3}H,^{32}P-Labeled plant oligoadenylates and UV markers were applied to the chromatograms. UV markers migrated as follows: adenosine, R_f = 0.81; AMP and 2′,5′-A_3, R_f = 0.62; ADP and 2′,5′-A_4, R_f = 0.47; ATP, R_f = 0.30; 2′,5′-pA_4, R_f = 0.32; 2′,5′-p_3A_3, R_f = 0.27; 2′,5′-p_3A_4, R_f = 0.21 (Suhadolnik et al., 1983).

Analysis of Plant [^{3}H,^{32}P]Oligoadenylates by High-Performance Liquid Chromatography (HPLC). The HPLC system used was composed of a μBondapak C_{18} column, two 6000A pumps, a 660 solvent programmer, a U6K injector, and a fixed-wavelength (254 nm) spectrometric detector (Waters Associates) linked to a double-pen recorder (LKB Model 2210). A linear gradient consisting of solvent A (50 mM ammonium phosphate, pH 7.0) and solvent B (1:1 v/v methanol:H_2O) was employed. Elution (1 mL/min) was with a linear gradient of 0–45% solvent B in 20 min.

Results

It has previously been demonstrated that AVF and human leukocyte interferon stimulate oligoadenylate synthetase activity in *N. glutinosa* and *N. tabacum* (Devash et al., 1981; Reichman et al., 1983). TMV infection was stimulatory only in *N. glutinosa* which carries the N gene, presumably due to the induction of AVF which is associated with this gene (Sela, 1981). However, AVF was stimulatory in *Nicotiana* plants whether or not they carry the N gene (Devash et al., 1981). The appearance of oligoadenylates was followed in these studies by their activity as DF stimulators or by their antiviral activity (Devash et al., 1981; Reichman et al., 1983).

Enzymatic Synthesis and Partial Purification of Oligoadenylates by the Plant Oligoadenylate Synthetase. In the present study, the enzymatic conversion of ATP to oligoadenylates was measured in three systems. Leaves were either

Table I: Enzymatic Synthesis of Oligoadenylates by Putative Plant Oligoadenylate Synthetase

enzyme source	conversion of ATP to oligoadenylates[a] nmol[b]	%
noninfected *N. glutinosa* leaves[c]	1.10	0.22
TMV-infected *N. glutinosa* leaves	2.04	0.41
mock AVF-treated tobacco leaves	1.00	0.20
AVF-treated tobacco leaves	1.45	0.30
IFN-α-treated tobacco leaves	1.55	0.31
tobacco callus cultures	1.40	0.28
mock AVF-treated tobacco callus cultures	1.50	0.30
AVF-treated tobacco callus cultures	1.95	0.39
IFN-α-treated tobacco callus cultures	2.00	0.40
tobacco cell suspension	1.45	0.29
IFN-α-treated tobacco cell suspension	2.20	0.44
2′,5′-A synthetase from lysates of rabbit reticulocytes	67.50	13.50

[a] Conversion of ATP to oligoadenylates was determined by the amount of radioactivity displaced from the DEAE-cellulose columns with 350 mM KCl buffer divided by the total radioactivity recovered. [b] The amount of ATP in each enzyme assay was 500 nmol (100%). [c] Five milliliters of DE-0 fraction containing 500 ng of protein/mL.

inoculated with TMV (5 μg/mL) or immersed in AVF (DE-0.65, 10 ng of protein/mL) (Devash et al., 1981) or in IFN-α (1 unit/mL) for 60 min followed by immersion for 24 h in 0.01 M phosphate buffer, pH 7.6, and extraction of the oligoadenylate synthetase. Tobacco callus tissue cultures and cell suspensions were similarly treated. The oligoadenylate synthetase in each reaction mixture (DE-0, 500 ng of protein/mL) was partially purified as described under Materials and Methods and was incubated by immobilization on poly(rI)·poly(rC)–agarose with 500 nmol of ATP and [^{3}H,α-^{32}P]ATP (ratio of ^{3}H:^{32}P = 1:1). At the end of the incubation, the oligoadenylates which were not displaced from DEAE-cellulose columns by 90 mM KCl buffer, but were displaced by 350 mM KCl buffer, were collected (500-μL fractions), radioactivity was determined, and fractions were dialyzed. The radioactivity and yields following dialysis were determined. The ^{3}H:^{32}P ratio in the oligoadenylates was 1:1 throughout all purification steps.

The enzymatic synthesis of oligoadenylates by adenylate synthetases in TMV-, AVF-, and IFN-α-treated leaves, callus tissue cultures, and tobacco cell suspensions clearly demonstrates the conversion of ATP to plant oligoadenylates (Table I). TMV infection of *N. glutinosa* and AVF and IFN-α treatment of the various plant systems stimulated this synthetase activity. It is also apparent that extracts from untreated plants are capable of converting ATP enzymatically to oligoadenylates. Under the assay conditions described here, the conversion of ATP into plant oligoadenylates by the plant oligoadenylate synthetase was considerably lower than by the IFN-induced mammalian 2′,5′-adenylate synthetase.

Kinetics of Plant Oligoadenylate Synthesis. The kinetics of synthesis of plant oligoadenylates were determined as described under Materials and Methods with modification as follows: The poly(rI)·poly(rC)–agarose columns bound with the synthetase were transferred to sterilized microcentrifuge tubes (1.5 mL). At 0, 5, and 17 h, 30-μL aliquots were removed, and product formation was determined. The rate of ATP conversion of plant oligoadenylates increased with time (Figure 1). The plant oligoadenylate synthetase present in the plant extracts is stimulated by AVF and IFN-α (▲, □) but not by mock AVF or no treatment (△, ○). The nanomoles of ATP converted to 2′,5′-oligoadenylates by the mammalian synthetase (75 nmol; Figure 1, ■) was 32-fold higher than the

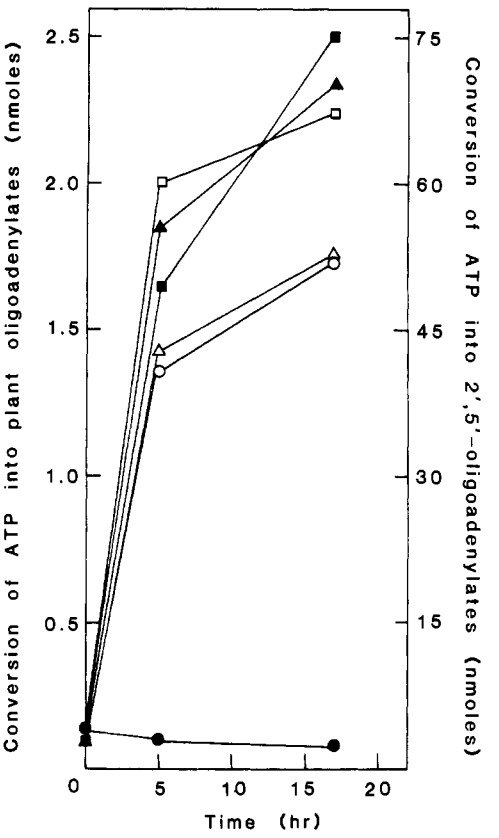

FIGURE 1: Kinetics of plant oligoadenylate synthesis. At the times indicated, 30-μL aliquots were removed from standard reaction mixtures containing DE-0 fractions purified from tobacco callus cultures. Product formation was determined by the radioactivity displaced from the DEAE-cellulose columns with 350 mM KCl divided by the total radioactivity recovered and multiplied by the total nanomoles of ATP added (Doetsch et al., 1981). The amount of ATP converted to oligoadenylates by the plant oligoadenylate synthetase after treatment with mock AVF (△), AVF (▲), and IFN-α (□) was compared to untreated tobacco callus culture (○), to mammalian 2′,5′-oligoadenylate synthetase (■), and to reactions to which no plant extract was added (●).

amount of ATP converted to plant oligoadenylates by the plant synthetase (2.35 nmol). However, at this time, it is not possible to compare product formation on the basis of specific activities in the mammalian and plant synthetases because neither enzyme has been purified to homogeneity. Furthermore, as is known in the mammalian 2′,5′-A synthetase system, an endogenous 2′,5′-phosphodiesterase degrades the 2′,5′-oligoadenylates. A similar degradative enzyme or inhibitor(s) may be present in the plant cell-free extracts.

Inhibition of Protein Synthesis by Plant Oligoadenylates. Tobacco cell suspensions were incubated with 1 unit/mL IFN-α for 24 h prior to extraction of the oligoadenylate synthetase and in vitro synthesis of plant oligoadenylates. The plant oligoadenylates used in these assays were from the fractions displaced from DEAE-cellulose columns by 350 mM KCl buffer and dialyzed. Aliquots were added to a rabbit reticulocyte protein synthesizing system. The plant oligoadenylates inhibited protein synthesis in a dose-dependent manner (Figure 2). In contrast to the mammalian 2′,5′-adenylate triphosphates, which inhibit protein synthesis only in mammalian cells, the plant oligoadenylates inhibited protein synthesis both in lysates from rabbit reticulocytes and in the wheat germ cell-free system (Figures 2 and 3). At 8×10^{-7} M, the plant oligoadenylates inhibited protein synthesis in rabbit reticulocyte lysates and wheat germ cell-free extracts by 75% and 45%, respectively (Figure 2, △; Figure 3, △),

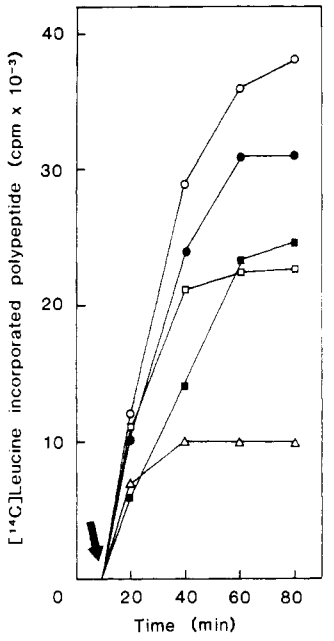

FIGURE 2: Inhibition of protein synthesis in lysates from rabbit reticulocytes by plant oligoadenylates. Assays were done as described under Materials and Methods. Tobacco cell suspensions were incubated with 1 unit/mL IFN-α for 24 h prior to extraction of the oligoadenylate synthetase and in vitro synthesis of plant oligoadenylates. The resultant plant oligoadenylates were purified on DEAE-cellulose, dialyzed, and added to the reticulocyte lysates. Reaction mixtures were incubated for 10 min, and then master mix (arrow) was added to initiate protein synthesis. The inhibition of protein synthesis by plant oligoadenylates at 2×10^{-7} (●), 4×10^{-7} (■), and 8×10^{-7} M (▲) was compared to the inhibition at 1×10^{-8} M by 2′,5′-p_3A_4 (□) and to control assays (○).

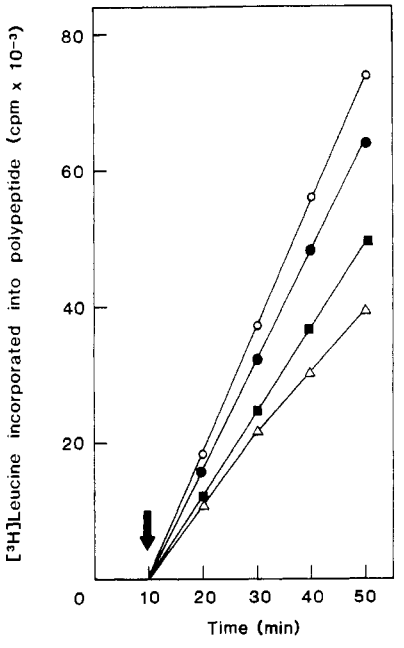

FIGURE 3: Inhibition of protein synthesis in wheat germ cell-free system by plant oligoadenylates. Plant oligoadenylates, obtained as in Figure 1, were tested for their ability to inhibit translation in a wheat germ cell-free system. Reaction mixtures were incubated for 10 min; then master mix (arrow) was added to initiate protein synthesis. The inhibition of protein synthesis by plant oligoadenylates at 2×10^{-7} (●), 4×10^{-7} (■), and 8×10^{-7} M (▲) was compared to control assays (○).

whereas the mammalian 2′,5′-p_3A_4 at 1×10^{-8} M inhibited protein synthesis in rabbit reticulocyte lysates by 43% (Figure 2, □). Similar results were obtained with plant oligoadenylates prepared with plant oligoadenylate synthetase from either

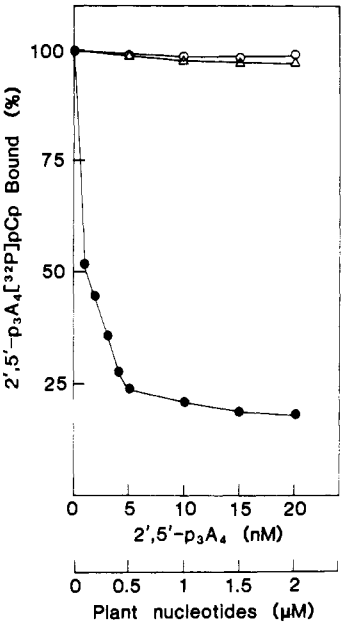

FIGURE 4: Radiobinding assays. Assays were performed with reticulocyte lysates as described under Materials and Methods. The ability of plant oligoadenylates, synthesized by plant oligoadenylate synthetase obtained from AVF-treated tobacco callus tissue cultures (○) or from TMV-infected *N. glutinosa* leaves (▲), to compete with the binding of 2′,5′-p_3A_4[^{32}P]pCp to the 2′,5′-A-dependent endonuclease in lysate from rabbit reticulocytes was compared to that of the mammalian 2′,5′-p_3A_4 (●).

TMV-infected *N. glutinosa* leaves or AVF-treated tobacco callus cultures. Fractions to which no plant extract was added (Figure 1, ●) did not inhibit protein synthesis. However, as described in the next two sections, the inhibition of protein synthesis in the reticulocyte lysates by plant oligoadenylates did not proceed via activation of the latent 2′,5′-A-dependent endonuclease and subsequent hydrolysis of RNA as occurs with the mammalian 2′,5′-adenylate triphosphates.

Inability of the Plant Oligoadenylates To Bind to the 2′,5′-A-Dependent Endonuclease. Competition of the 2′,5′-p_3A_4[^{32}P]pCp analogue with plant oligoadenylates was used to determine if the plant oligoadenylates bind to the 2′,5′-A-dependent endonuclease from rabbit reticulocyte lysates. Whereas the mammalian 2′,5′-p_3A_4 competed with and displaced the [^{32}P]pCp analogue, the plant oligoadenylates prepared with synthetase from either TMV-infected *N. glutinosa* leaves, AVF-treated tobacco callus cultures, or IFN-α-treated tobacco cell suspensions did not compete with or displace the [^{32}P]pCp analogue from 2′,5′-A binding sites on the mammalian 2′,5′-A-dependent endonuclease (Figure 4). The [^{32}P]pCp analogue *did not bind* to crude extracts of *N. glutinosa* leaves (infected or uninfected), demonstrating a lack of 2′,5′-oligoadenylate binding proteins in plants. Similarly, when the [^{32}P]pCp analogue was added to DE-0, DE-0.3, and DE-0.65 protein fractions, there was no binding (data not shown).

Inability of Plant Oligoadenylates To Activate the Partially Purified 2′,5′-A-Dependent Endonuclease. VSV [^{3}H]mRNA was incubated with partially purified 2′,5′-A-dependent endonuclease isolated from lysates of rabbit reticulocytes in the presence or absence of 2′,5′-p_3A_3 or 2′,5′-p_3A_4 or plant oligoadenylates from TMV-infected *N. glutinosa*. The reaction mixtures were incubated and applied to oligo(dT)–cellulose columns, and the hydrolysis of VSV [^{3}H]mRNA was determined. 2′,5′-p_3A_3 and 2′,5′-p_3A_4 activated the partially purified mammalian 2′,5′-A-dependent endonuclease to hydrolyze VSV [^{3}H]mRNA; however, the plant oligoadenylates at concen-

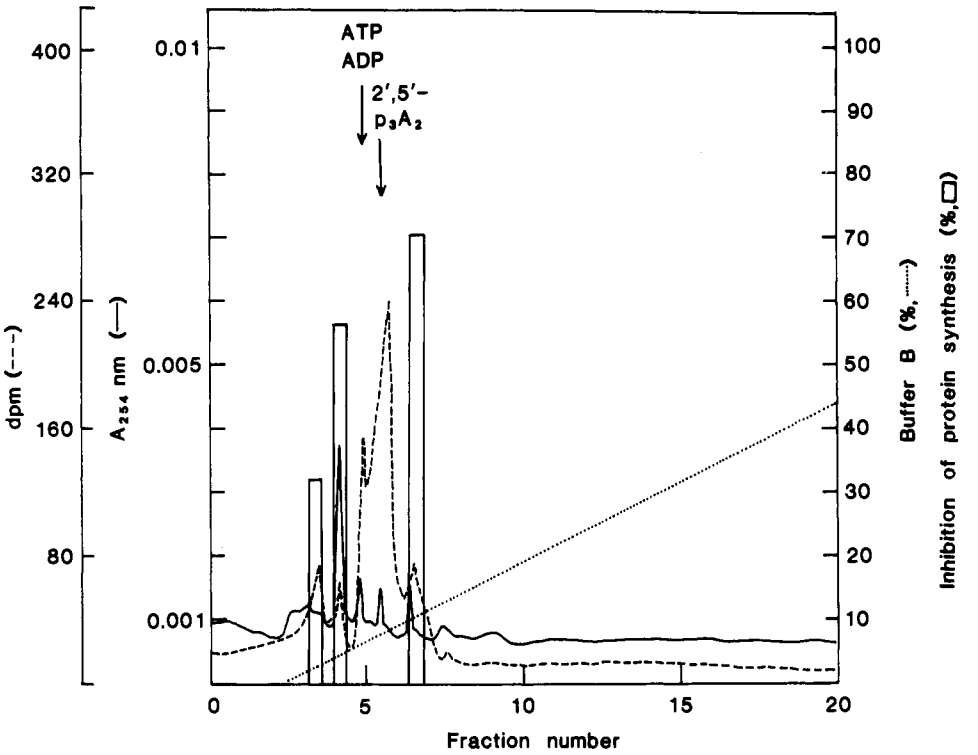

FIGURE 5: HPLC profile of plant oligoadenylates synthesized by plant oligoadenylate synthetase purified from IFN-α-treated tobacco cell suspensions. Plant oligoadenylates were separated by HPLC as described under Materials and Methods. Retention times of ADP, ATP, and 2′,5′-p_3A_2 are indicated (arrows). The ability of the various UV-radioactive peaks to inhibit protein synthesis in lysates from rabbit reticulocytes is indicated by the open bars. (All UV-radioactive peaks were dialyzed prior to the protein synthesis inhibition assays.)

Table II: DEAE-cellulose Thin-Layer Chromatography of 2′,5′-Adenylate Trimer and Tetramer Triphosphates and Plant Oligoadenylates following Enzymatic Digestions

	% net charge of digestion products[b]					
treatment[a]	2−	3−	4−	5−	6−	7−
2′,5′-[^{3}H]oligoadenylate trimer and tetramer triphosphates enzymatically synthesized from lysates of rabbit reticulocytes						
no treatment	0	0	0	0	73	27
T_2 RNase	0	0	0	0	73	27
BAP	74	26	0	0	0	0
SVPD	100	0	0	0	0	0
^{3}H,^{32}P-labeled oligoadenylates synthesized from AVF-treated tobacco callus cultures						
no treatment	0	0	0	46	54	0
T_2 RNase	0	46	54	0	0	0
BAP	100	0	0	0	0	0
SVPD	100	0	0	0	0	0

[a] Enzymatic digestions were as described under Materials and Methods, using 3000 dpm of mammalian 2′,5′-[^{3}H]adenylate trimer and tetramer triphosphates or plant [^{3}H,^{32}P]oligoadenylates. [b] Percent net charge of product (average of four experiments) was calculated as (dpm of product/total dpm) × 100.

trations *100 times greater* than the mammalian 2′,5′-adenylate triphosphates did not hydrolyze VSV [^{3}H]mRNA (data not shown).

Structural Studies of the Plant Oligoadenylates. The HPLC reverse-phase elution pattern of the plant oligoadenylates synthesized by the interferon-induced plant oligoadenylate synthetase is shown in Figure 5. The substrate, ATP, was doubly labeled with ^{3}H and α-^{32}P (1:1 ratio), and the oligoadenylates were purified by chromatography on DEAE-cellulose and dialysis prior to HPLC. The plant oligoadenylates which were inhibitory to protein synthesis in lysates from rabbit reticulocytes were equivalent to peaks retained for 3.6, 4.1, and 6.5 min. The ^{3}H:^{32}P ratio in the plant oligoadenylates purified by HPLC was 1:1. Specific HPLC-purified plant oligoadenylates (4×10^{-8} M) inhibited protein synthesis; HPLC peaks retained for 3.6, 4.1, and 6.5 min inhibited protein synthesis by 33%, 55%, and 70%, respectively. HPLC peaks retained for 4.9 and 5.65 min (62% of the total plant oligoadenylates synthesized) did not inhibit protein synthesis. The plant oligoadenylates synthesized by the interferon-induced tobacco plant oligoadenylate synthetase were chromatographed on DEAE-cellulose TLC plates and were resolved to have charges of 5− and 6− (Table II). The plant oligoadenylates were degraded by SVPD to AMP. They were partially degraded by T_2 RNase to products with charges of 3− and 4− (Table II). Bacterial alkaline phosphatase (BAP) dephosphorylated the plant oligoadenylates to products with a charge of 2−. A mixture of mammalian 2′,5′-[^{3}H]adenylate trimer and tetramer triphosphates was completely degraded by SVPD to [^{3}H]AMP and was not degraded by T_2 RNase. Bacterial alkaline phosphatase dephosphorylated the 2′,5′-[^{3}H]adenylate trimer and tetramer triphosphates to the corresponding 2′,5′-adenylate trimer and tetramer cores (with charges of 2− and 3−, respectively. Following acid hydrolysis of the plant oligoadenylates, [^{3}H]adenine was released. Alkaline hydrolysis of the plant oligoadenylates as described by

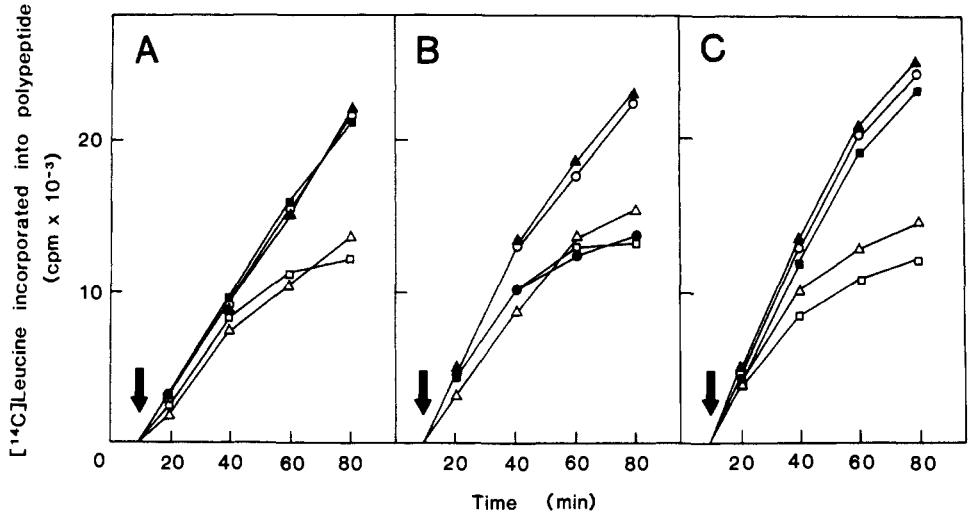

FIGURE 6: Inhibition of protein synthesis in lysates from rabbit reticulocytes by plant oligoadenylates following enzymatic and chemical digestions. Plant oligoadenylates (synthesized by plant oligoadenylate synthetase obtained from AVF-treated *N. tabacum* leaves) were treated with SVPD, T_2 RNase, or alkali as described under Materials and Methods. After the incubations, the enzymes or alkali were neutralized by either heat or dialysis. The treated samples were added to lysates from rabbit reticulocytes and incubated as in Figure 1. (A) Inhibition of protein synthesis by plant oligoadenylates following digestion with SVPD. The inhibition of protein synthesis by 400 nM plant oligoadenylates (△) and 100 nM 2′,5′-p_3A_4 (□) was compared to that of 400 nM plant oligoadenylates after SVPD digestion (▲), 100 nM 2′,5′-p_3A_4 after SVPD digestion (■), and control assays (○). (B) Inhibition of protein synthesis by plant oligoadenylates following digestion with T_2 RNase. The inhibition of protein synthesis by 400 nM plant oligoadenylates (△) and 100 nM 2′,5′-p_3A_4 (□) was compared to that of 400 nM plant oligoadenylates after T_2 RNase digestion (▲), 100 nM 2′,5′-p_3A_4 after T_2 RNase digestion (●), and control assays (○). (C) Inhibition of protein synthesis by plant oligoadenylates following alkaline hydrolysis. All samples were dialyzed 45 min in 4 L of H_2O at 0 °C prior to addition to reactions. The inhibition of protein synthesis by 400 nM plant oligoadenylates (△) and 100 nM 2′,5′-p_3A_4 (□) was compared to that of 400 nM plant oligoadenylates after alkaline hydrolysis (▲), 100 nM 2′,5′-p_3A_4 after alkaline hydrolysis (■), and control assays (no addition of oligoadenylates) (○).

Samanta et al. (1980) and Suhadolnik et al. (1981) and digestion with T_2 RNase and SVPD result in degradation products that no longer inhibit protein synthesis in lysed rabbit reticulocytes (Figure 6A–C).

Discussion

In an earlier report, we demonstrated that plants treated with either AVF or human interferon showed an increase in cell-free extracts of an enzyme that converted ATP to oligoadenylates (Reichman et al., 1983). At the time, it was not known if the putative plant oligoadenylate synthetase converted ATP to 2′,5′-oligoadenylates with the same structure which occurs in the mammalian interferon-treated cell. It also was not known if the role of the plant oligoadenylates in the plant antiviral system was similar to the mammalian 2′,5′-oligoadenylates as mediators of the interferon system. In this study, we report that there are similarities with respect to substrate specificity and inhibition of protein synthesis between the plant oligoadenylates and the mammalian 2′,5′-oligoadenylates. However, there are marked differences with respect to the structure and biological activity of the mammalian and plant oligoadenylates in the inhibition of protein synthesis. Induction of the plant oligoadenylate synthetase, like induction of the mammalian synthetase in interferon-treated mammalian cells, increases in plant cells treated with plant AVF or interferon. Furthermore, the plant oligoadenylate synthetase, like the mammalian 2′,5′-oligoadenylate synthetase, converts ATP to oligoadenylates when the enzyme is immobilized on dsRNA (Figure 1). However, whereas ATP is converted to mammalian 2′,5′-oligoadenylates with a 5′-triphosphate terminus, the plant oligoadenylate synthetase produces oligoadenylates with a structure unlike the mammalian 2′,5′-oligoadenylates. In addition, the plant oligoadenylates are known to activate an enzyme which hydrolyzes histidine from histidinyl–TMV–RNA (Devash et al., 1981; Reichman et al., 1983). The activation of this plant enzyme is somewhat analogous to the activation of the mammalian 2′,5′-A-dependent endonuclease by the mammalian 2′,5′-oligoadenylates which then hydrolyzes mRNA and rRNA.

Partial purification of the plant oligoadenylates was accomplished by their displacement with 350 mM KCl buffer from DEAE-cellulose columns followed by dialysis. The ^3H:^{32}P ratio before and after dialysis was 1:1, the same ratio as the ATP used in the incubation mixture. Several interesting biological properties have been attributed to the plant oligoadenylates. First, the plant oligoadenylates inhibit protein synthesis in rabbit reticulocyte lysates (which contain the latent 2′,5′-A-dependent endonuclease) and in wheat germ cell-free systems (which do not contain the 2′,5′-A-dependent endonuclease) (Figures 2 and 3). Second, the inhibition of protein synthesis in rabbit reticulocyte lysates by the plant oligoadenylates is not analogous to the mammalian system. For example, the plant oligoadenylates do not compete with 2′,5′-p_3A_4[^{32}P]pCp for binding sites on the 2′,5′-A-dependent endonuclease. Furthermore, unlike mammalian cells which are known to contain multiple 2′,5′-oligoadenylate binding proteins (St. Laurent et al., 1983), plant extracts do not contain binding proteins or binding sites for the mammalian 2′,5′-oligoadenylates (Figure 4). Moreover, the 2′,5′-A-dependent endonuclease in reticulocyte lysates is not activated by the plant oligoadenylates. This may explain why Cayley et al. (1982) were not able to detect 2′,5′-oligoadenylates in plants by the 2′,5′-A binding assays.

When the plant oligoadenylates were further purified by HPLC, five radioactive, UV-absorbing peaks were resolved. Three of these peaks inhibited protein synthesis in the reticulocyte lysate system. The ^3H:^{32}P ratio in all fractions was 1:1. The two peaks displaced from the column at 4.9 and 5.65 min did not inhibit protein synthesis; however, they were radioactive. Similarly, when the dialyzed plant oligoadenylates were purified by urea/NaCl/DEAE-cellulose gradient chromatography (Doetsch et al., 1981), four peaks were resolved.

The ^3H:^{32}P ratio again was 1:1. All four peaks inhibited protein synthesis when added to the reticulocyte lysate system (data not shown). The physical and chemical properties of the plant oligoadenylates isolated by this gradient procedure were identical with the properties of the plant oligoadenylates isolated by HPLC and 350 mM KCl elution from DEAE-cellulose columns.

Partial structural elucidation of the plant oligoadenylates has been completed by means of chemical, physical, and enzymatic digestions. The plant oligoadenylates contain AMP residues that are covalently linked. The plant oligoadenylates are hydrolyzed by SVPD, alkaline phosphatase, and T_2 RNase (Table II, Figure 6). Acid hydrolysis releases [^{3}H]adenine, and alkaline hydrolysis results in a product that no longer inhibits protein synthesis in the reticulocyte lysate or wheat germ cell-free systems. A combination of covalently linked 3′,5′- and 2′,5′-phosphodiester bonds is unlikely in view of the report by Lesiak et al. (1983) in which this type of mixed oligoadenylate was able to displace the 2′,5′-p_3A_4[^{32}P]pCp analogue in binding assays at concentrations of 1×10^{-8} to 10^{-6} M. The plant oligoadenylates differ from the 2′,5′-oligoadenylates or mixtures of 2′,5′- and 3′,5′-oligoadenylates in that they *do not* compete with the pCp analogue at concentrations up to 2×10^{-6} M (Figure 4) nor do the plant oligoadenylates activate the 2′,5′-A-dependent endonuclease. Further studies are under way using the techniques of mass spectrometry (fast atom bombardment) and ^{1}H and ^{31}P NMR to elucidate the structure of the plant oligoadenylates. We are also studying the mechanism by which the plant oligoadenylates inhibit protein synthesis that does not involve activation of the mammalian 2′,5′-A-dependent endonuclease. Studies will be aimed at determining if there is a biochemical link between the antiviral state of the interferon/mammalian system and the AVF/plant systems.

Registry No. ATP, 56-65-5; oligoadenylate synthetase, 9026-30-6; poly(A), 24937-83-5.

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