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Mechanism of Polynucleotide Phosphorylase†

Michael Sulewski,‡ Silvio P. Marchese-Ragona,§ Kenneth A. Johnson,§ and Stephen J. Benkovic*,‡
Department of Chemistry and Department of Molecular and Cell Biology, The Pennsylvania State University, University Park, Pennsylvania 16802
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ABSTRACT: The de novo polymerization of RNA initiated by polynucleotide phosphorylase from nucleoside diphosphates was examined. End group analysis performed under conditions designed to specifically end label the polymer revealed no evidence for a 5′-pyrophosphate-terminated polymer. However, we observed preferential incorporation of the ADPαS(R_P) diastereomer into the 5′ end (Marlier & Benkovic, 1982) in chain initiation, suggesting that the enzyme incorporates a nucleoside diphosphate specifically into the 5′ end of the product, with subsequent enzymatic removal of the polyphosphate linkage. No evidence could be obtained for a covalent adduct between the enzyme and the 5′ end of the polymer chain, despite the high processivity of the polymerization reaction. Gel electrophoretic analysis showed the polymer to be highly disperse, varying from 1 to 30 kb. Scanning transmission electron microscopy supported this product analysis and further suggested that (i) each subunit can produce an RNA polymer and (ii) both 5′ and 3′ ends of the RNA can be bound simultaneously to the same or differing enzyme molecules.

The primer-independent form of polynucleotide phosphorylase (PNPase)[1] from *Micrococcus leuteus* catalyzes the reversible polymerization of nucleoside diphosphates to polyribonucleotides and inorganic phosphate. These polynucleotides are synthesized de novo in the 5′ → 3′ direction, resulting in high molecular weight polymers (Godefroy-Colburn & Grunberg-Manago, 1972). Polymerization by form I enzyme is stimulated only slightly by oligonucleotides (Moses & Singer, 1970). Since its discovery by Grunberg-Manago and Ochoa (1955), PNPase has been studied in great detail as a tool for the synthesis of model nucleic acids (Engel & Davidson, 1978; Mackey & Gilham, 1971; Littauer & Soreq, 1982). However, the elementary steps in the de novo initiation mechanism have not been established and a well-defined biological function has remained elusive.

Recently, the first purification of the form I PNPase from *M. luteus* free of contaminating activities was reported (Barbehenn et al., 1982). The availability of contaminant-free PNPase has made possible the first unambiguous determination of several aspects of the de novo polymerization mechanism (Harvey & Grunberg-Manago, 1970; Marlier & Benkovic, 1982). The earlier report of Barbehenn indicated that a homogeneous population of polymeric products was formed, apparently by a highly processive mechanism (Barbehenn et al., 1982). Data bearing on the nature and origin of the 5′ end group of the polymeric products will be presented in this paper. In addition, experiments utilizing rapid quench techniques and scanning transmission electron microscopy were performed to quantitate the degree of processivity and the distribution of products formed, leading us to suggest an unusual mechanism of de novo polymerization.

Experimental Procedures

Materials. Polynucleotide phosphorylase (PNPase) was obtained from Dr. Claude Klee (NIH) or purified from *M. luteus* cells (ATCC 4698). The *M. luteus* cells were grown

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* Author to whom correspondence should be addressed.
‡ Department of Chemistry.
§ Department of Molecular and Cell Biology.

[1] Abbreviations: AMPS, adenosine 5′-*O*-(thiophosphate); ADPαS(R_P) and ADPαS(S_P), diastereomers of adenosine 5′-*O*-(1-thiodiphosphate); ApA, adenylyl(3′–5′)adenosine; HEPES, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; MOPS, 3-(*N*-morpholino)-propanesulfonic acid; pApA, 5′-phosphoadenylyl(3′–5′)adenosine; PNPase (form I), polynucleotide phosphorylase, primer-independent form; PNPase (form T), polynucleotide phosphorylase, primer-dependent form; poly(A), poly(adenylic acid); poly(I), poly(inosinic acid); poly(U), poly(uridylic acid); poly(N)$_{ox}$, any polyribonucleotide with both a 5′- and a 3′-phosphate; ppApA, 5′-diphosphoadenylyl(3′–5′)adenosine; p(S)Ap(S), adenosine 5′,3′-bis(thiophosphate); p(S)Ap(O), adenosine 5′-(thiophosphate) 3′-phosphate; pp(S)Ap(S), adenosine 5′-*O*-(1-thiodiphosphate) 3′-*O*-(thiophosphate); STEM, scanning transmission electron microscopy; TEAB, triethylammonium bicarbonate; Tris, tris(hydroxymethyl)aminomethane.

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in a 90-L fermenter (Beers, 1955). The enzyme was purified with only slight modification of the procedure of Barbehenn et al. (1982), 1 μg of DNase/L of cell hydrolysate was added prior to the streptomycin sulfate precipitation step, and 1.5 mL of packed resin/4 A_{280} units was used in the DE-52 chromatography step.

Pyruvate kinase, phosphoenolpyruvate, ADP, ATP, adenylyladenosine, Sigmacote, poly(uridylic acid), poly(inosinic acid), ribonuclease, and adenylic acid deaminase were all obtained from Sigma (St. Louis, MO) and used without further purification. All organic solvents were purified according to the method of Perrin et al. (1980). Agarose was of Ultra-Pure DNA grade from Bio-Rad (Richmond, CA). Formaldehyde for the denaturing agarose gels was deionized by passing it over a column of mixed-bed ion-exchange resin (Bio-Rad AG-501-X8).

[2,8-^{3}H]Adenosine monophosphate and diphosphate were obtained from New England Nuclear (Boston, MA). Specific activities were 11.2 and 28.2 Ci/mmol, respectively. Carrier-free inorganic [^{32}P]phosphate was also from New England Nuclear.

Polynucleotide kinase was from United States Biochemical Corp. (Cleveland, OH). PNPase (form I), commercial grade, was from P-L Biochemicals. Nuclease P1, proteinase K, *Escherichia coli* 16S and 23S rRNAs, and 27S MS-2 phage RNA were from Boehringer Mannheim (Indianapolis, IN).

All Eppendorf tubes were siliconized with Sigmacote and then autoclaved for 30 min at 115 °C. All other materials and glassware were either autoclaved or treated with a diethyl pyrocarbonate solution to prevent ribonuclease contamination.

Buffers. TMN refers to a standard buffer that contains 100 mM Tris-HCl, pH 9.0, 5 mM $MgCl_2$, and 50 mM NaCl. TEN refers to a standard buffer that contains 200 mM Tris-HCl, pH 9.0, 10 mM EDTA, and 100 mM NaCl.

Chromatography. TLC on PEI-cellulose plates was done by ascending chromatography with 1.2 M LiCl or 0.75 M KH_2PO_4, pH 3.5.

Analytical HPLC employed an Altex Ultrasphere ODS column (5 μm, 4.6 × 250 mm), eluted isocratically with 1% $NH_4H_2PO_4$ (w/w) and 10 mM tetrabutylammonium hydroxide, pH 5.9, containing 15% CH_3CN (v/v). Semipreparative HPLC utilized a Whatman Partisil 10 ODS-3 Magnum 9 column (9.4 × 500 mm), eluted with 1% $NH_4H_2PO_4$ (w/w) and 10 mM tetrabutylammonium hydroxide, pH 5.9, containing 13% CH_3CN.

Enzyme Assays. Previous preparations of *M. luteus* polynucleotide phosphorylase were found to contain various contaminating activities (Craine & Klee, 1976; Marlier & Benkovic, 1981). The deoxynucleotide kinase activity reported by Craine and Klee (1976) is inactive above pH 8.0 and was not assayed. All studies in this paper were carried out at pH 9.0, the pH optimum for polymerization.

The reported polyphosphate kinase activity (Marlier & Benkovic, 1981) was assayed by the adenylic acid deaminase assay of Murray and Atkinson (1968) in a buffer of 0.09 M potassium cacodylate, pH 6.5, containing 0.4 M KCl. The amount of AMP produced was calculated from the decrease of 265 nm, utilizing a $\Delta\epsilon$ of 8100 M^{-1} cm^{-1} (Murray & Atkinson, 1968). The preparations of PNPase used in these studies contained polyphosphate kinase activity at the level of 0.9% relative to PNPase activity.

Preparations of the enzyme have also been noted to catalyze the exchange of the β-phosphorus of ADP with inorganic [^{32}P]phosphate (Chou & Singer, 1971). The enzyme used in these studies was assayed for exchange activity as follows. A 20-μL reaction mixture containing 1.4 μg/mL PNPase (5 nM), 2 mM P_i (500 cpm/pmol), and 20 mM ADP in TMN buffer was incubated at 37 °C for 15 min. Aliquots of 10 μL were analyzed by TLC on PEI-cellulose developed in 0.75 M KH_2PO_4, pH 3.5. The TLC was then autoradiographed at room temperature with Kodak X-Omat film. The film showed no incorporation of ^{32}P into the ADP.

The polymerization activity of the enzyme was assayed by monitoring the incorporation of [^{3}H]ADP into polymer in TMN buffer containing 20 mM ADP; the amount of enzyme is specified in the figure legends. Reactions were carried out at 21 °C, unless specified otherwise. The reaction was followed by taking aliquots at the desired time intervals and quenching them into an EDTA solution to give a final concentration of 25 mM EDTA. Samples were then analyzed for incorporation of [^{3}H]ADP into polymer by the method of Brutlag and Kornberg (1972). The quenched solutions were spotted on 2.5-cm circles of Whatman DE-81 paper, which had been prespotted with 10 μL of 0.25 M EDTA and allowed to dry. The papers were washed in 0.3 M ammonium formate, pH 8.0, followed by three to five 25-mL washes until the wash solution contained background levels of radiation. Two 100-mL washes with 95% ethanol and one 100-mL wash with diethyl ether serve to dehydrate the papers. After air-drying, the papers were placed in 5 mL of a standard toluene scintillation fluid, and radiolabel was counted.

Agarose Gel Electrophoresis. RNA samples were heated in 2.2 M formaldehyde/50% formamide buffer for 5 min at 65 °C (Lehrach et al., 1977) and then loaded onto gels in 50% glycerol/0.4% bromophenol blue/2.2 M formaldehyde buffer. No differences in results were observed between gels run in 0.018 M Na_2HPO_4/0.022 M NaH_2PO_4 (Lehrach et al., 1977) or those run in 40 mM MOPS–acetate, pH 7.0, buffer (Maniatis et al., 1982). The formaldehyde (37% solution) was added to a heated solution of agarose to give a final concentration of 2.2 M formaldehyde. The gel was immediately poured into a slab apparatus and allowed to set for at least 1 h.

Gels that contained 5 M urea were run according to the method of Long and Dawid (1979). RNA was visualized by staining with a solution of acridine orange (33 μg/mL) in 10 mM phosphate buffer, pH 7.0, for 1 h. Destaining was in 10 mM phosphate buffer, pH 7.0 (McMaster & Carmichael, 1977).

Preparation of Poly(N)$_{ox}$ Trap. Both traps [poly(A)$_{ox}$ and poly(U)$_{ox}$] were prepared by a method based on that of Webb and Trentham (1980). A solution containing 20 mg of poly(U) (0.2 μmol of 3′ ends) in 5 mL of 20 mM $NaHCO_3$/NaOH buffer, pH 10.5, was added to a 50-mL plastic Corning tube. To this solution was added 1.1 equiv of $NaIO_4$ (1 equiv is the amount of 3′ ends present) in the same buffer. The mixture was shaken at room temperature for 10 min and then quenched with ~2.3 mL of β-mercaptoethanol. The quenched solution was then incubated at 50 °C for 35 min. Product was precipitated by adding solid NaCl to 0.25 M and 2.5 volumes of ethanol at −20 °C. The precipitate was collected by centrifugation, then dissolved in 1 mL of ice-cold TMN buffer, and immediately loaded onto a Sephadex G-50 column (1 × 50 cm) equilibrated with TMN buffer. The column was eluted with TMN buffer at 4 °C while the eluant was monitored at 254 nm. The peak eluting in the void volume was collected and concentrated to a volume of 1 mL in a collodian bag apparatus (molecular weight cutoff 25 000).

To minimize the possibility of contamination by unreacted poly(I), the solution was made 10 mM in inorganic phosphate

and incubated with 20 μg of purified PNPase for 2 h at 37 °C. The protein was removed by phenol/chloroform extraction as described by Maniatis et al. (1982). Final purification of the trap was with Sephadex G-50 as described above. The final yield was 17 mg of polymer as determined by UV spectroscopy.

Preparation of ppApA. Polynucleotide kinase was added to a 3.5-mL reaction mixture containing 4.7 mM adenylyladenosine, 10 mM ATP, 29 mM $MgCl_2$, 3 mM DTT, 30 μg/mL bovine serum albumin, and 50 mM HEPES, pH 8.0 (300 units). After incubation at 37 °C for 36 h, analysis by HPLC indicated complete transfer of the phosphorus had taken place. To facilitate purification, phosphoenolpyruvate (5.7 mM) and pyruvate kinase solution were added (0.05 mg/mL) to catalyze complete conversion of the byproduct ADP to ATP (after 4 h of incubation at 37 °C). The pApA product from this reaction was then purified by semipreparative HPLC, described above. This isolated pApA product was loaded onto a small (0.2 × 5 cm) column of DEAE-Sephadex A-25 in 10 mL of water. The column was then washed with 0.1 M TEAB, pH 7.8, until the effluent was devoid of inorganic phosphate as measured by the Lanzetta assay (Lanzetta et al., 1979). The pure pApA product was then eluted with 0.6 M TEAB.

The pApA was converted to ppApA by a procedure based on that of Hoard and Ott (1965). Conversion to the pyridinium salt was effected by passing the triethylamminium salt over a 1 × 5 column of Dowex AG 50W-X8 (pyridinium form). The pyridinium salt was converted to the tributylammonium salt by the addition of 2 equiv of tributylamine, followed by repeated evaporation from anhydrous pyridine and DMF. The anhydrous tributylammonium salt was suspended in DMF (0.5 mL) followed by the addition of 5 equiv of 1,1′-carbonyldiimidazole in 0.2 mL of DMF. The mixture was shaken vigorously for 30 min and then stirred vigorously overnight under argon. Anhydrous methanol was added to destroy the excess 1,1′-carbonyldiimidazole, and the mixture was allowed to react for 30 min. A 5-fold excess of tributylammonium phosphate was then added in 1.0 mL of anhydrous DMF and the solution stirred vigorously under argon for 24 h at room temperature. The reaction was diluted with an equal volume of methanol, and the solution was evaporated under vacuum to give a yellow syrup. The residue was taken up in 15 mL of 0.01 M TEAB and loaded onto a Sephadex A-25 column (2 × 20 cm) equilibrated in 0.01 M TEAB. The column was then washed with 0.1 M TEAB, pH 7.8, until the effluent showed no inorganic phosphate (Lanzetta et al., 1979). The column was then eluted with a linear gradient of 0.1–0.85 M TEAB (800 mL each). This gave three partially resolved peaks. Final resolution was effected by semipreparative HPLC (see above for conditions). The ppApA product was characterized by enzymatic degradation by nuclease P1 to the known compounds ADP and AMP. Only ADP and AMP were observed (in a ratio of 0.85:1.0 for ADP:AMP) after incubation with nuclease P1. Purity was checked by running a sample in both TLC systems (see above) and an alternative HPLC system utilizing an ion-exchange column (Cashmore et al., 1980). Both reverse-phase and ion-exchange HPLC indicated the compound was >94% pure.

ppApA Assay. The dinucleotide triphosphate ppApA was tested for substrate activity in a solution containing 1.7 μM PNPase and 2 mM ppApA incubated at 37 °C in TMN buffer and [^{3}H]ADP. All reactions were initiated by addition of enzyme. Aliquots of 10 μL were quenched at the desired time intervals with 25 mM EDTA. The quenched solutions were then assayed for polymerization by the DE-81 assay described earlier.

End Group Analysis for 5′-Pyrophosphate. End group analysis utilizing ADPαS(S_P) and [^{3}H]ADPαS(R_P) was performed according to the procedure of Marlier and Benkovic (1982).

Incorporation of [^{3}H]AMP into Polymer. The reaction mixture for this analysis consisted of 20 mM ADP, 4.3 μM (1.2 mg/mL) PNPase, and 50 μCi of [^{3}H]AMP (11.8 Ci/mmol) in TMN buffer. The final specific activity of [^{3}H]AMP was 33 cpm/pmol, relative to the entire nucleotide pool. The reaction was initiated by the addition of an ADP/[^{3}H]AMP mixture. After a 30-min incubation at 37 °C, the reaction was quenched with EDTA to a final concentration of 10 mM in EDTA. The entire reaction mixture was then diluted to 1 mL with TEN buffer to reduce the viscosity of the sample. The entire sample was then loaded onto a Sephadex G-150 column (1 × 40 cm) that had been equilibrated with TEN buffer. The peak eluting in the void volume was collected, made 0.3 M in KOH, and incubated at 37 °C for 18 h. The procedure of Marlier and Benkovic (1982) was then followed.

Enzyme Labeling with [^{3}H]ADP. A reaction mixture containing 4 μM PNPase (1.1 mg/mL) and 20 mM [^{3}H]ADP (12.5 cpm/pmol) in TMN buffer was incubated at 37 °C for 30 min. After 30 min, the solution was brought to 40 mM in Na_2HAsO_4 and then incubated at 37 °C for 7 h. The entire sample (200 μL) was then diluted to 500 μL with ice-cold TMN buffer and chromatographed on Sephadex G-150 in TMN buffer at 4 °C. Fractions containing protein were identified by SDS–PAGE (Laemmli, 1970) using a silver stain (Merril et al., 1981). Each fraction was counted in 10 mL of Scintiverse II.

In a second experiment, ribonuclease (250 000 units/mg) was added instead of arsenate to a final concentration of 2 μg/mL (100 units added). This mixture was then incubated for 4 h at 37 °C. The reaction was diluted to 500 μL with ice-cold TMN buffer and analyzed as described above.

Determination of Primer Fate. A reaction solution composed of labeled dinucleotide [5′-^{32}P]pApA (34 cpm/pmol), 2 mM ADP, and 1.16 μM (320 μg/mL) PNPase in TMN buffer was incubated at 37 °C for 60 min, and then half the reaction was quenched in ice-cold EDTA (25 μL of 50 mM). The remaining solution was quenched in like fashion at 90 min. The quenched solutions were evaporated under vacuum to a volume of 2–3 μL and then diluted with sequencing gel loading buffer (Maniatis et al., 1982). The samples were heated for 10 min in a water bath at 65 °C and then loaded into the wells of a 10% polyacrylamide sequencing gel. The gel had been preelectrophoresed at 1800 V for 2 h to increase the temperature of the gel to 50 °C, thereby avoiding problems from secondary structural effects. Electrophoresis was carried out at 1800 V for 90 min. Autoradiography used two intensifier screens and Kodak X-Omat film.

Distribution of Polymerization Products. The reaction was initiated by the addition of [α-^{32}P]ADP to obtain a solution containing 113 nM (31.2 μg/mL) PNPase and 200 μM [α-^{32}P]ADP (443 cpm/pmol). The reaction mixture was incubated at 37 °C. Aliquots of 10 μL were quenched with EDTA (final concentration 25 mM) at selected time intervals ranging from 2 to 30 min. The quenched solutions were quickly frozen in liquid nitrogen and stored at −70 °C until all aliquots were quenched. The samples were then evaporated under vacuum to a volume of 3 μL. Each sample was brought to 5 μL with sequencing gel loading buffer (Maniatis et al., 1982) and loaded into the wells of a 7% polyacrylamide (0.4 × 400 mm)

sequencing gel. The gel had been warmed, by 2-h preelectrophoresis, to 50 °C. The gel was run at 1700 V for 95 min at 50 °C. Autoradiography was carried out by using two intensifier screens and Kodak X-Omat film at −70 °C.

Polymer Trapping Reactions. PNPase (40.8 nM) (11.3 μg/mL) was preincubated in TMN buffer at 21 °C. Preincubation did not affect the results obtained in the time range 30 s to 10 min. Reactions were initiated by the addition of [^{3}H]ADP (specific activity 5 cpm/pmol) in TMN buffer to give a total volume of 100 μL. The final polymerization solutions contained 20 mM ADP and 15.9 nM PNPase in TMN buffer. Polymer trap was added at the times indicated to a final concentration of 15 μg/mL. Controls were run in which trap and [^{3}H]ADP were added simultaneously or by preincubating the enzyme with trap before adding the [^{3}H]-ADP. All reactions were quenched by adding aliquots to ice-cold EDTA to give a final concentration of 25 mM EDTA. The quenched solutions were then applied to DE-81 papers and analyzed for polymer formation as described (see polymerization assay).

Inhibition of PNPase by Hydroxylamine. A series of polymerization reactions were run to test the effect of adding hydroxylamine to the reaction mixture. All assays were performed at 37 °C. The [^{3}H]ADP used in all assays had a specific activity of 5 cpm/pmol. Controls were run to check for the reaction of hydroxylamine with ADP by TLC (on PEI-cellulose with pH 3.5, 0.75 M KH_2PO_4) and with the $FeCl_3$ test for hydroxamic acid formation (Jencks, 1958). Both of these controls proved negative. To disrupt the rate of synthesis of actively polymerizing enzyme, the reaction was started with 41.3 nM PNPase and 10 mM [^{3}H]ADP in TMN buffer and allowed to proceed for 20 min. Then hydroxylamine was added to a final concentration of 1 mM, and at various times, aliquots were removed and assayed by the DE-81 filter assay described above.

STEM Analysis. High-resolution electron microscopy was carried out at Brookhaven National Laboratory with a dedicated STEM (Wall, 1979). The procedures for the preparation of carbon films, specimen application, and freeze-drying are described in detail elsewhere (Woodcock et al., 1980; Mosesson et al., 1981). Samples of PNPase were diluted to 5 μg/mL in TMN buffer and applied to a carbon film by injection of 2.5 μL into a 2.5-μL droplet of buffer. The sample was allowed to adsorb to the grid for 1 min and was then washed ten times with 20 mM ammonium acetate, pH 7.0. Tobacco mosaic virus was added as a standard with the final wash step in water. The grids were then frozen in nitrogen slush (−210 °C) and freeze-dried at constant temperature (−110 °C) and pressure (10^{-6} torr) (Wall, 1979) and then transferred under vacuum to the microscope stage. Solutions containing enzyme and substrate (10 mM ADP) were first quenched with 25 mM EDTA before sample preparation was begun.

Mass Analysis. STEM data were stored digitally as intensity of electron scattering as a function of position over the grid. Mass analysis was accomplished by integration of electron scattering intensities over an area bounded by the particle minus a background obtained by averaging scattering from areas not containing particles. TMV was used as an internal standard. The dose of electrons for the single scan required to record the data was <2e/Å and was sufficiently low to keep mass loss to <2% (Wall, 1979). The use of the internal TMV standard largely compensated for the small degree of mass loss (Mosesson et al., 1981). Mass per unit length measurements were also conducted with TMV as a standard. Magnifications were controlled by attenuating the scanning currents, which gave a <0.1% error in length measurement.

Results

End Group Analysis. Previous analyses of the 5′ end group of the polymer products from PNPase had implicated a 5′-monophosphate rather than the 5′-pyrophosphate predicted from the simple polymerization of nucleoside diphosphates (Harvey & Grunberg-Manago, 1970; Marlier & Benkovic, 1982). However, these previous results may have been compromised by the later discovery of a contaminating polyphosphate kinase activity. An end group analysis using highly purified PNPase has been repeated according to the protocol of Marlier and Benkovic (1982).

The polymeric product generated from [^{3}H]ADPαS(R_P) and ADPαS was collected in the void volume from a Sephadex G-50 column and was found to contain about 19 000 cpm of tritium label. The products of an overnight alkaline hydrolysis were separated by ion-exchange chromatography on a column of DEAE-Sephadex A-25. The radioactive products were identified by coelution with unlabeled marker compounds that had been added to the sample and eluted in the expected ionic charge order: adenosine, adenosine cyclic 2′,3′-phosphate, AMPS, and p(S)Ap(O). The last radioactive peak was due to the 5′-terminus and is nearly coincident with the unlabeled marker used, p(S)Ap(O). If a 5′-pyrophosphate linkage had been present, it would not have been cleaved by this analytical procedure (Harvey & Grunberg-Manago, 1970). Under our conditions, pp(S)Ap(S) would have eluted after p(S)Ap(S) and would have been detectable at a level 15–20% of the p(S)Ap(S) observed. These data demonstrate that the 5′ end group is a monophosphate.

The possibility that the origin of the 5′-monophosphate group was a contaminating nucleoside monophosphate was investigated by adding a large amount (50 μCi) of [^{3}H]AMP to a polymerization assay and then doing a similar end group analysis. The specific activity of the [^{3}H]AMP used in this experiment was sufficient to have detected the production of 0.1 tritium-labeled polymer per PNPase molecule. No significant amount of radiolabel was associated with any of the individual hydrolysis products.

Labeling with [^{3}H]Adenosine Diphosphate. The possibility that PNPase becomes covalently attached to growing polymer chains was investigated in two separate experiments. Each was designed to gain evidence that the 5′ end of a polymer chain had become covalently attached to PNPase via the first nucleotide in the chain. Such an attachment would suggest that the observed 5′-monophosphate in the product results from attack by an enzyme nucleophile on the α-phosphorus of the first nucleoside diphosphate incorporated into a chain, followed by hydrolysis of this linkage.

The initial experiment involved adding ribonuclease to an actively polymerizing solution to degrade the polymer. This method of degradation affords a gentle way of degrading the polymer chains to mononucleotides that are no longer substrates for PNPase. The steric bulk of the ribonuclease should also prevent it from entering the active site. The protein was isolated free of small molecule contaminants in the void volume of a Sephadex G-150 column. An analysis of the contents of this peak consisted of two parts. An aliquot of the peak fraction was analyzed by SDS–PAGE and showed only the presence of PNPase that migrated in parallel to an authentic sample of PNPase. An analysis by liquid scintillation counting of each fraction in the elution profile revealed only the presence of background amounts of radioactivity in all fractions con-

taining protein. If there had indeed been radioactivity associated with the protein peak, the conditions would have allowed detection of 0.05–0.1 mol of [^{3}H]adenosine/mol of PNPase molecules.

A second experiment employed an observation by Singer that arsenate ion could replace inorganic phosphate in the phosphorolysis reaction (Singer, 1958, 1963). Singer also showed that arsenate could participate in arsenolysis reactions at rates similar to those of phosphorolysis and that arsenate could act catalytically (Singer, 1963). As in the ribonuclease experiment described above, arsenate was added to an actively polymerizing enzyme reaction at a concentration that was about ten times the reported K_m for arsenate (final concentration 40 mM). After arsenolysis, the protein was isolated by Sephadex G-150 chromatography and examined by SDS–PAGE and liquid scintillation counting. No tritium counts were found associated with the protein-containing fractions. Due to the inability of arsenate ion to arsenolyze the dinucleotide pApA (Singer, 1963), the inherent sensitivity of this experiment would have been limited by the presence of a pApA moiety attached to PNPase, if it had been present. Under the conditions employed here, the reliable limit of detection would have corresponded to the attachment of 0.02–0.05 [^{3}H]pApA moiety per PNPase molecules. In both experiments described here >95% of the tritiated material was recovered in the inclusion volume of the column.

Effects of ppApA. The dinucleotide 5′-diphosphate ppApA was synthesized to determine if it can initiate de novo synthesis. PNPase does not incorporate AMP into the 5′ end of a polymer. Furthermore, a dinucleotide 5′-phosphate, pNpN, is not a substrate for the phosphorolysis reaction, whereas longer oligonucleotides and long-chain polymers are substrates (Chou & Singer, 1970). Under the conditions employed here (see Experimental Procedures), the dinucleotide 5′-diphosphate did not participate in PNPase-catalyzed hydrolysis, phosphorolysis, or ppApA-primed polymerization, at times up to 24 h. Control experiments showed that the enzyme maintains substantial activity over such long time periods. The methods used (UV absorbance at 259 nm) would have detected a 1% change in the ppApA levels under the conditions employed here. However, ppApA acts as an activator of PNPase-catalyzed polymerization similar to the effects of dinucleotides and higher oligonucleotides (Moses & Singer, 1970). This interaction with PNPase probably takes place at a separate oligonucleotide binding site (Godefroy-Colburn & Grunberg-Manago, 1972).

The inability to detect a covalent linkage between PNPase and the polymer chain could be due to its instability toward hydrolytic or nucleophilic attack as would be expected if a mixed anhydride between a carboxylic acid and a phosphate residue was formed. Attempts to trap the putative mixed anhydride intermediate (Jencks, 1958; Pickart & Jencks, 1979) with 1 mM hydroxylamine that was added to an actively polymerizing solution were unsuccessful. Within experimental error (~10%), no change was observed in the rate of incorporation of [^{3}H]ADP into polymer. This observation argues strongly against the formation of a mixed anhydride in any significant concentration subject to the caveat that hydroxylamine might not have access to the polymerization site.

Enzyme Trapping. A processive cycle is defined as the number of bases added by the enzyme each time it encounters a polymerization substrate and is a function of the kinetic partitioning of enzyme between dissociation from the nascent chain and continued polymerization (McClure & Chow, 1980). In order to examine PNPase processivity, we required an unreactive polymer capable of trapping enzyme. Polyribonucleotides with both 5′- and 3′-monophosphate ends were found to act as challenger RNA to allow us to measure processivity.

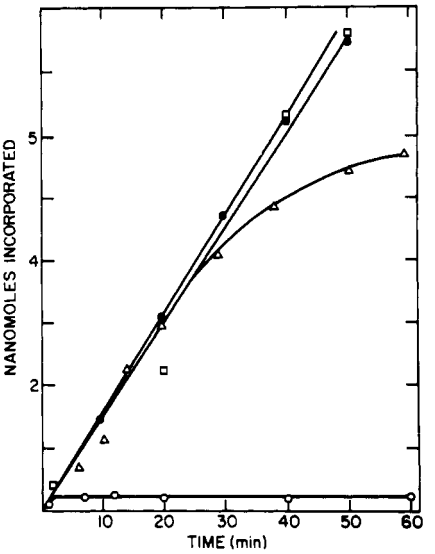

FIGURE 1: Trapping of PNPase with poly(I)$_{ox}$: curve A (●), a linear incorporation control, no trap added; curve B (□), trap added at 30 min; curve C (△), trap added at 14 min; curve D (○), trap added at 2 min. All assays were performed at 21 °C. Conditions are as stated under Experimental Procedures.

The result of such an experiment using poly(I)$_{ox}$ as an enzyme trap is shown in Figure 1. When the polymerization was initiated with [^{3}H]ADP in the absence of trap RNA, the normal uninterrupted polymerization time course was observed (Figure 1, curve A). The simultaneous addition of [^{3}H]ADP and trap RNA to PNPase or the preincubation of enzyme with trap RNA prior to the addition of [^{3}H]ADP resulted in no significant incorporation (data not shown). These experiments demonstrate the effectiveness of the trap RNA. Under the conditions of these two experiments, no RNA (except trap) was observed in an agarose gel of the reaction mixture. No incorporation of [^{3}H]ADP into polymer was observed when the trapping reagent was added at 2 min (Figure 1, curve D). However, addition of the trap at 14 min caused a progressively slower rate of tritium incorporation some 15 min later (Figure 1, curve C). At 30 min the addition of the trapping agent was ineffective (Figure 1, curve B). The limit of detection in this experiment was a processive cycle of about 17 bases per enzyme.

In order to test the possibility that the trap was a base-specific phenomenon, a parallel set of experiments using poly(U)$_{ox}$ as challenger RNA was carried out. The results of this experiment were analogous to those seen in the experiment using poly(I)$_{ox}$ except for slight differences in the timing of the response to the addition of trapping reagent.

Polymer Sizing. The size of polymeric products was estimated by agarose gel electrophorsis in the presence of 2.2 M formaldehyde (Maniatis et al., 1982). Samples taken from polymerization reactions were often viscous or gellike even after relatively short incubation periods (>8 min). It was also observed that, even in low-percentage (0.5%) agarose gels, a significant amount of acridine orange stained material often remained in the wells of the gel, prompting us to suspect the presence of extremely large polymers (or enzyme–polymer complexes). Although the pretreatment by proteinase K destroyed the PNPase activity, controls showed there to be extremely protease-resistant fragments of PNPase. Consequently, the samples were extracted with phenol/chloroform

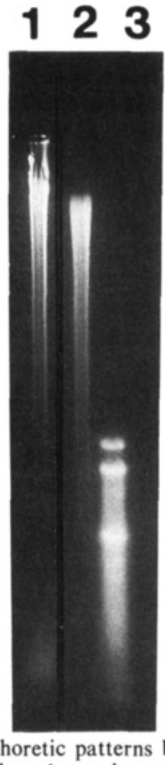

FIGURE 2: Gel electrophoretic patterns before and after phenol/chloroform extraction: lane 1, a polymerization reaction that was proteinase K treated but not phenol/chloroform extracted; lane 2, a phenol/chloroform-extracted polymerization reaction; lane 3, RNA standards 27S, 23S, and 16S.

to remove remaining protein, leading to a typical migration pattern shown in Figure 2 that is independent of proteinase K treatment. The observed pattern was very broad with polymer ranging in size from 21–27 to <1 kb. No material was present in the wells in any samples that had been phenol/chloroform extracted. Control experiments using homogeneous RNA standards gave no indication that this broad range of sizes was due to shearing of the large RNAs taking place during the extraction process. These RNA standards also served as markers against which the poly(A) molecules were measured. The 27S MS-2 phage genome and the *E. coli* 16S and 23S rRNAs served as the standards. Similar patterns of distribution were observed in polyacrylamide systems and in composite polyacrylamide–agarose gels (Maniatis et al., 1982; Peacock & Dingman, 1968).

Distribution of Label in Polymer. In light of the broad distribution of polymer sizes observed in agarose gel electrophoresis, it was important to measure the time course of production of the large polymers. To assist in analyzing the results, samples were run in a sequencing gel (Maniatis et al., 1982) for high-resolution electrophoresis. Previous results, described above, utilizing modified RNAs to trap free enzyme raised the possibility that PNPase polymerization reactions are characterized by a finite length of polymer that when exceeded leads to highly processive synthesis. This experiment was designed to observe and quantitate the size class of molecules that characterize the switch from synchronous to processive synthesis. The use of [α-^{32}P]ADP in this experiment introduced label in proportion to the size of the polymer formed. We therefore expected to observe more intense bands with increasing size of the polymer.

Samples collected at times ranging from 2 to 30 min were loaded onto a 7% acrylamide gel. After autoradiography it was found that large polymers (>400 bases) had begun to accumulate already at the earliest time point (Figure 3). Another population of molecules was also evident in the autoradiogram that corresponded to much smaller polymers (<60 bases). The amount of polymer in this size class diminished drastically after their length exceeded ~60 bases.

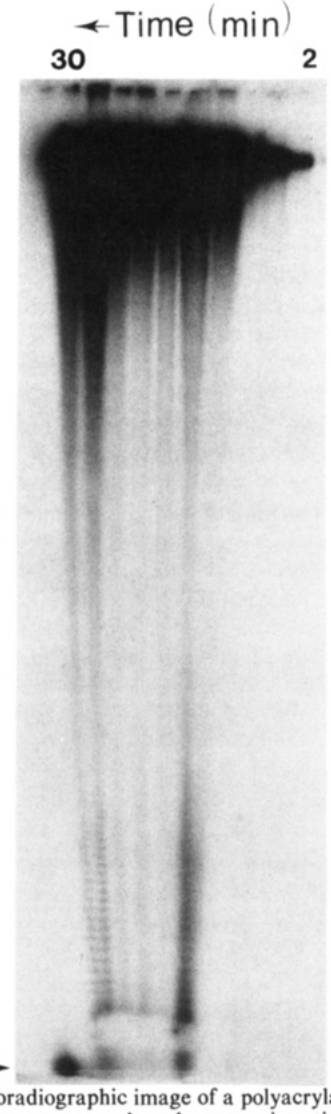

FIGURE 3: Autoradiographic image of a polyacrylamide sizing gel. The ten lanes represent samples taken at various times from 2 to 30 min of reaction. The arrow at bottom left points to the position of an oligo(dT) marker (16 bases).

Parallel experiments with labeled primer, [5′-^{32}P]pApA, demonstrated that this material is not converted into the high molecular weight size class even upon prolonged incubation with the enzyme. Thus, it appears that the small size class observed is due to either addition of ADP to indigenous oligonucleotides or the slow production of short chains by the small amount of primer-dependent PNPase (form T) that is known to be present in the preparation (form I to form T ratio of 8:1). If each PNPase molecule had produced a single labeled chain once during the lengthy incubation, it would have been readily detectable. Separate controls using [^{3}H]ADP indicated that the enzyme used remained fully active during the time course of this experiment.

STEM Analysis. Analysis of the polymerization reaction products by STEM was conducted by applying EDTA-quenched polymerization reactions to carbon films and immediately freeze-drying the unstained specimens for morphological and mass analyses as described under Experimental Procedures. Figure 4 shows a micrograph of a sample representative of images obtained at either zero time or 30 s; no significant difference was observed between samples quenched

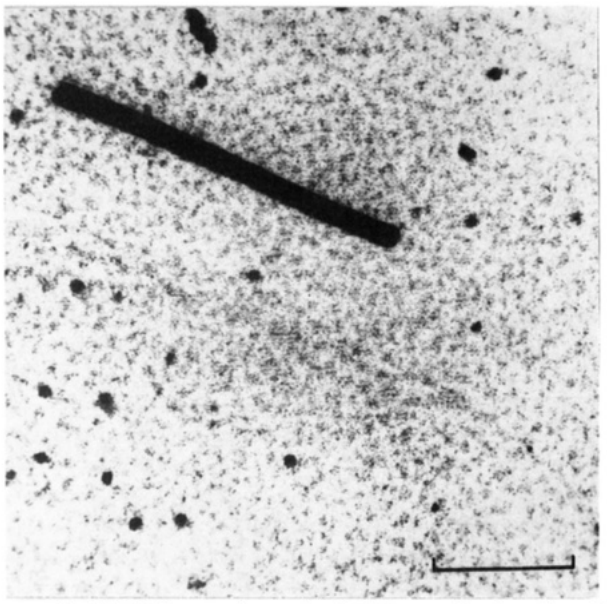

FIGURE 4: STEM image of unstained PNPase molecules. Samples were diluted to 5 μg/mL, applied to a carbon film, and freeze-dried as described under Experimental Procedures. The rod is a TMV particle used as an internal standard for the mass analysis.

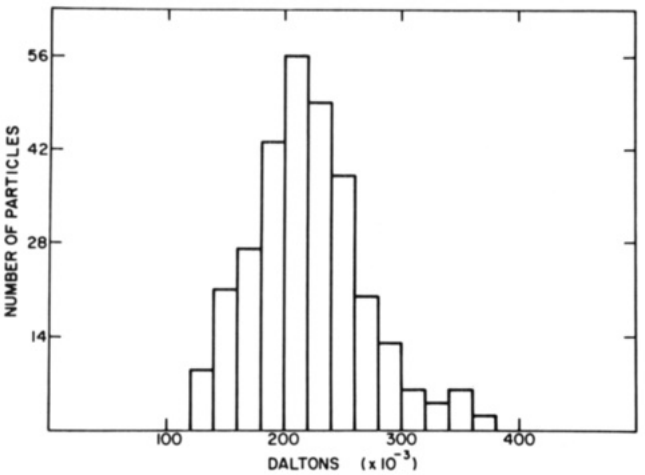

FIGURE 5: Mass of individual PNPase particles. The mass of isolated particles was determined by integration of electron scattering intensities over each particle. The histogram shows the mass distribution for 304 particles. The mean particle mass is 229.5 kDa (error of means 3.6 kDa).

at zero time and at 30 s, each showing only the individual PNPase molecules. The particles appeared quite uniform in size, as demonstrated by the mass measurements of the individual particles, which provided a mean mass of 229.5 kDa (error of mean 3.6 kDa, n = 304) (Figure 5).

In the samples that were quenched after 7.5 min of polymerization, some of these individual PNPase molecules are still present. However, there are also striking examples of the large polymers that were produced by this polymerization system. The polyribonucleotides were observed to be in a broad range of states of compactness (Figure 6). In addition, it was frequently observed that an RNA polymer would have a PNPase molecule attached to both ends of the polymer, rather than the anticipated observation of attachment at only one end (Figure 7). Further, PNPase was never found in a position that would indicate it was bound to the interior portions of the polymer.

In order to gain insight into the number of RNA strands in each of the observed filaments, measurements were carried out to ascertain the mass per unit length of these filaments.

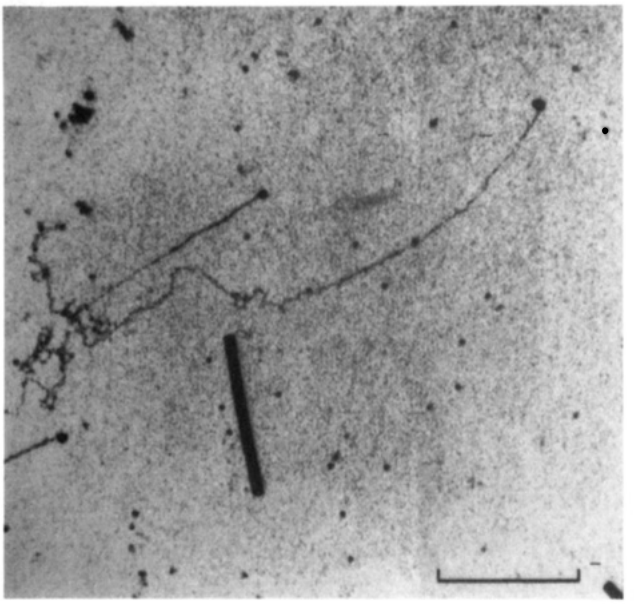

FIGURE 6: Unstained freeze-dried image of polymerization products from PNPase. PNPase molecules are seen singly and attached to long RNA filaments. The RNA is seen to exist in particularly compact form near the left side of the figure. Magnification bar = 0.2 μM.

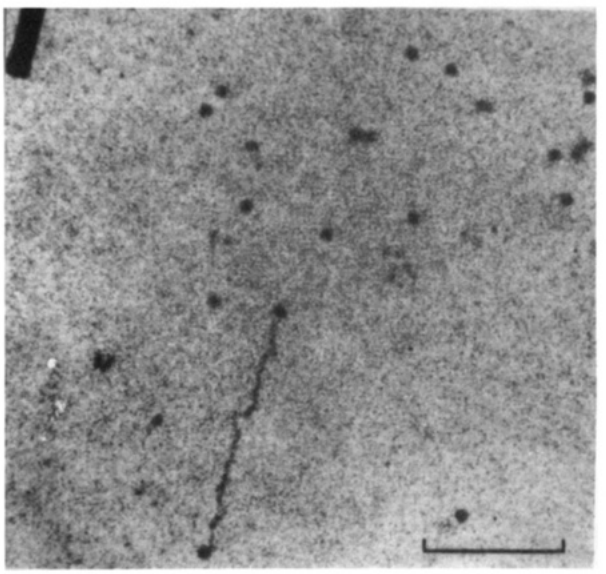

FIGURE 7: STEM image of unstained freeze-dried PNPase with associated RNA. Lower center of image shows an RNA filament with a PNPase molecule attached to both ends. Magnification bar = 0.1 μM.

The analyses were made on apparently straight sections of these filaments in an effort to minimize the problems associated with interpretation of filaments of widely varying conformation. The data yielded an average of 790.4 Da/Å (error of mean = 23.0, n = 22) (Figure 8). In a manner similar to that used for individual PNPase molecules, mass measurements were carried out on entire RNA–protein complexes. Such analyses yielded a broad range of molecular masses ranging from 2.39 to 11.3 MDa (6.5–30.5 kb).

Discussion

The mechanism of de novo polymerization by PNPase has been studied extensively because of its uniqueness and its possible role in a general scheme for nucleic acid polymerizing systems. We have addressed the following aspects of the PNPase polymerization mechanism: (1) the nature and origin

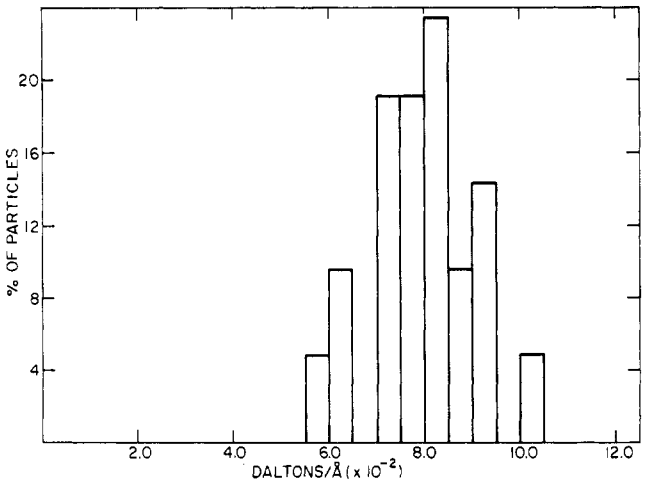

FIGURE 8: Mass distribution in RNA filaments produced by PNPase. Mass/unit length measurements were made only on straight portions of filaments. The histogram shows the frequency of occurrence (as percent of particles) of mass distribution for 22 filaments measured. TMV was used as the internal standard.

of the initiating nucleotide by 5- end analysis, (2) the length of the polymer and the processivity of the polymerization reaction, and (3) the number of active sites per enzyme trimer.

Nature and Origin of the 5′ End. The results of experiments reported here strongly support the proposal that the first nucleotide in the chain originates directly from the nucleoside diphosphate pool. However, end group analysis performed under conditions designed to specifically end label the polymer revealed no evidence for a 5′-pyrophosphate-terminated polymer. Consequently, initiation of a polymer chain with a contaminating nucleoside monophosphate offered a simple alternative to a proposed mechanism involving enzyme-catalyzed cleavage of the 5′-pyrophosphate moiety. However, no incorporation of [^{3}H]AMP into polymer was observed. However, we observed preferential incorporation of the ADPαS(R_P) diastereomer into the 5′ end (Marlier & Benkovic, 1982) in chain initiation. These results argue strongly that the enzyme is incorporating a nucleoside diphosphate specifically into the 5′ end of the product.

Enzymatic removal of the polyphosphate linkage raises the possibility of a covalent adduct between the enzyme and the polymer chain. Experiments using [^{3}H]ADP were conducted to determine whether an E–polynucleotide covalent complex participated as an intermediate in the reaction. Conditions were chosen in an attempt to stabilize any labile linkages that had formed. In one experiment arsenate ion was used to degrade the polymer chains, so that the failure to observe the enzyme–nucleotide complex might result from the attack of arsenate at the site of the proposed E–pN linkage. No such argument is tenable, however, in the parallel experiment using RNase to degrad the nascent chains. The steric bulk of the RNase would severely limit direct contact with an E–pN present in the reaction. Such protection has been used to advantage by Galluppi and Richardson (1980) in determining how many bases of an RNA chain were protected by binding to *E. coli* ρ protein. Therefore, these experiments as well as the failure to trap with hydroxylamine argue against an E–polynucleotide intermediate.

A related question in the de novo polymerization mechanism of PNPase concerns the timing of pyrophosphate cleavage relative to the formation of the first and subsequent internucleotide linkages. One plausible pathway would be the formation of a dinucleotide diphosphate as the initial polymerization product, before cleavage of the pyrophosphate linkage. However, the results obtained showed ppApA did not participate in enzyme-catalyzed phosphorolysis, hydrolysis, or ppApA-primed polymerization. Therefore, a dinucleotide diphosphate is not a competent intermediate in the PNPase polymerization pathway. Polymerization assays containing the dinucleotide did show that ppApA is recognized in much the same way as oligonucleotide primers, which have been shown to be activators (Godefroy-Colburn & Grunberg-Manago, 1972), apparently binding at a separate activator site (Marlier & Benkovic, 1982; Chou & Singer, 1970). The collective evidence presented here is clearly in favor of enzymatic removal of the pyrophosphate linkage without an intervening E–pN intermediate. However, there are examples of labile linkages with short lifetimes (Prasher et al., 1982) so that an E–pNpN... type intermediate might be present only for the short length of time it would take to produce a chain that would bind to PNPase tightly enough to make the off rate of the oligonucleotide from the enzyme slower than the forward rate of polymerization.

Polymer Product. A second unique feature of PNPase is its apparent ability to polymerize nucleoside diphosphates with an extremely high degree of processivity (Barbehenn et al., 1982; Moses & Singer, 1970). The report of Barbehenn suggested that a novel termination mechanism must be operating to generate a very homogeneous polynucleotide (Barbehenn et al., 1982). Our studies show the product to be polydisperse, 1–27 kb (Figure 2). This broad range of sizes became apparent only when protein was removed by phenol/chloroform extraction and the RNA samples were run in the highly denaturing electrophoresis system of Lehrach et al. (1977). Although no polymerization activity could be detected, we believe that substantial fragments of PNPase were present in samples that were proteinase K treated but not phenol/chloroform extracted. The support for this proposal is 2-fold; PNPase was shown to have protease-resistant fragments (Sulewski, unpublished observations), and a large change in the pattern of migration was observed upon gel electrophoresis of the RNA samples after phenol/chloroform extraction of protein. Proteinase K resistant fragments have also been found for a DNA processing enzyme, with resistance to proteolysis being increased in the presence of nucleic acid (Roth et al., 1984). It is possible that these differences in experimental protocols led to the appearance of a homogeneous product, since the 5 M urea–agarose system of Long and Dawid (1979) may not be fully denaturing for such large polymers. In addition, polyacrylamide gels that do not contain a strong denaturant are likely to be nonlinear with respect to the variety of molecular masses contained in a given RNA sample. Such nonlinearity is particularly pronounced in the first centimeters of a gel, thereby causing a wide variety of molecular masses to appear similar (Lehrach et al., 1977).

The polymerization mechanism of PNPase was postulated to occur by a completely processive mechanism (Barbehenn et al., 1982; Moses & Singer, 1970). Processivity is defined here as the number of bases added per enzyme per encounter with a polymerization substrate. By use of challenger RNA to trap free enzyme, it was found that the number of bases added before releasing a polymer varied with the time the trap was added and was independent of the chemical nature of the trap. However, it is unlikely that challenger RNA simply acts to trap free enzyme, since the short-chain products (<60 bases) found at early times are not further elongated (Figure 3). The curves shown in Figure 1 suggest that initially PNPase produces polymers that can be displaced from the enzyme often and are not effective competitors for enzyme, relative to the

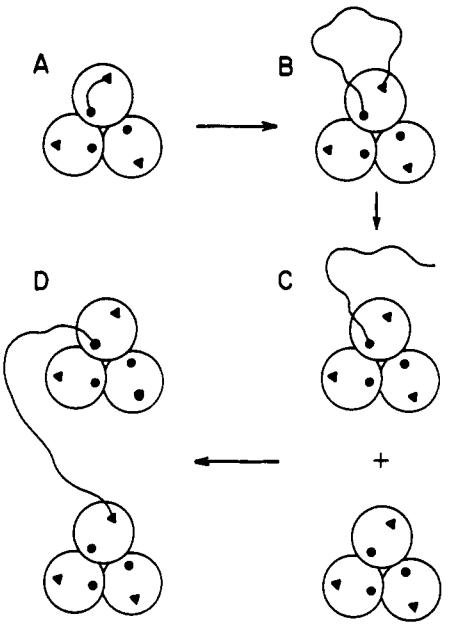

FIGURE 9: Schematic model of the PNPase reaction.

trap. They also imply that, at some finite length of polymer, dissociation or displacement from the enzyme becomes a rare event leading to large polymers (Figure 3).

Number of Active Sites. STEM analysis has been shown to provide accurate mass measurements on individual biological macromolecules (Wall, 1979). Data reproduced here show several uses of this technique (Figures 4–8). The products seen in actively polymerizing solutions (Figure 6) clearly agree with the results from agarose gel electrophoresis discussed earlier. Mass measurements of the whole particle (RNA plus protein) ranged from 6 to 30 kb. Smaller polymers were not observed; however, the range of sizes clearly indicates that the polymers produced are not homogeneous. Agreement with the interpretation of the trapping experiments is also seen in the STEM photos. PNPase molecules can be clearly seen as dots at the end expected from an enzyme that is polymerizing in a highly processive fashion (i.e., chains are rarely, if ever, released). Additional measurements with the STEM included a measurement of mass per unit length (Figure 8), which showed a mean value of 790.4 Da/Å. On the basis of a value of 125 Da/Å for a single strand of RNA derived from 2.8-Å axial rise per residue of RNA double helix (Saenger, 1984), this mean value corresponds to 6.3 single strands of RNA. It seems unlikely that six single strands of RNA would be produced by a single PNPase trimer. We feel it is more likely that an arrangement of three single strands doubled back on themselves or three single strands in a less than fully extended conformation are the source of the large mass/unit length value. In either case, however, it is clear from the data on which these measurements were based that the conformation of the RNA strands is fairly extended, leading to the strong possibility that PNPase is producing one polymer strand per subunit, rather than one per trimer as had previously been assumed.

Another measurement taken with the STEM was of individual PNPase molecules. Figure 5 shows the result of these measurements, which yielded a mean mass of 230 kDa. This measurement agrees very well with the value of 237 ± 24 kDa obtained by Barbehenn by sedimentation equilibrium (Barbehenn et al., 1982). It is, however, in contrast to the mass of 92 kDa per subunit obtained by SDS–PAGE and the value of 280 kDa obtained by cross-linking studies (Barbehenn et al., 1982). The basis for this disparity is not known, although systematic errors are common in molecular weights estimated by SDS–PAGE.

Conclusions

The data reported here support a mechanism for de novo polymerization by PNPase in which an enzyme nucleophile is responsible for the removal of the 5′-pyrophosphate linkage that would be expected to be present if only the 3′-OH function of ribose moieties were acting to release phosphate from the growing polymer chain. Experiments using a dinucleotide diphosphate further suggest that the pyrophosphate linkage may be broken prior to or concomitant with the formation of the first internucleotide linkage. No evidence was found for an enzyme-nucleotide bond; however, a short-lived or otherwise unstable covalent intermediate cannot be ruled out. Upon initiation, PNPase apparently proceeds to make large polymers rapidly in an increasingly processive manner. This high degree of processivity with long polymers results in a highly disperse polymer product ranging from ~1 to 30 kb. STEM analysis supports the conclusion that PNPase binds tightly to both ends of the growing polymer chain and that more than one polymer is produced per trimer. In support of this, Figure 7 shows a representative example of a particle that has a PNPase molecule attached to both ends. Such an arrangement may represent the continued attachment of PNPase to the 5′ end while the 3′ end may begin to undergo phosphorolysis.

A possible explanation for the large value obtained in the mass/unit length measurement and the possible origin of particles with a PNPase at both ends is shown in Figure 9. At the time of initiation, the 5′ and 3′ ends are in close proximity to each other (A). As the reaction progresses, the intervening distance along the chain becomes very long (B). It is at this stage that the measurement of mass along the chain would, at any length, appear to be twice the mass expected for a single chain. Release of the 3′ end of the polymer chain (C) may represent the stage at which a second PNPase molecule could attach to the 3′ end of the polymer to account for the morphology observed in Figure 7. The extremely long RNA product could lead to the entanglement of the type postulated by Harvey to explain the anomalously high viscosity (Harvey et al., 1969) and which may explain the ineffectiveness of the trapping reagent at long times.

Acknowledgments

We thank Joseph S. Wall and James Hainfeld of Brookhaven National Laboratory for providing the STEM analysis.

Registry No. PNPase, 9014-12-4; poly(U), 27416-86-0; ppApA, 10209-67-3; ApA, 2391-46-0; ATP, 56-65-5; pApA, 3593-47-3.

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Dynamic Interaction between Actin and Myosin Subfragment 1 in the Presence of ADP†

M. A. Geeves
Department of Biochemistry, School of Medical Sciences, University of Bristol, Bristol, BS8 1TD, U.K.
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ABSTRACT: The equilibrium and dynamics of the interaction between actin, myosin subfragment 1 (S1), and ADP have been investigated by using actin which has been covalently labeled at Cys-374 with a pyrene group. The results are consistent with actin binding to S1·ADP (M·D) in a two-step reaction, $A + M{\cdot}D \overset{K_1}{\rightleftharpoons} A{-}M{\cdot}D \overset{K_2}{\rightleftharpoons} A{\cdot}M{\cdot}D$, in which the pyrene fluorescence only monitors the second step. In this model, $K_1 = 2.3 \times 10^4\ M^{-1}$ ($k_{+1} = 4.6 \times 10^4\ M^{-1}\ s^{-1}$) and $K_2 = 10$ ($k_{+2} \leq 4\ s^{-1}$); i.e., both steps are relatively slow compared to the maximum turnover of the ATPase reaction. ADP dissociates from both M·D and A–M·D at $2\ s^{-1}$ and from A·M·D at $\geq 500\ s^{-1}$; therefore, actin only accelerates the release of product from the A·M·D state. This model is consistent with the actomyosin ATPase model proposed by Geeves et al. [(1984) *J. Muscle Res. Cell Motil. 5*, 351]. The results suggest that A–M·D cannot break down at a rate $>4\ s^{-1}$ by dissociation of ADP, by dissociation of actin, or by isomerizing to A·M·D. It is therefore unlikely to be significantly occupied in a rapidly contracting muscle, but it may have a role in a muscle contracting against a load where the ATPase rate is markedly inhibited. Under these conditions, this complex may have a role in maintaining tension with a low ATP turnover rate.

The cyclical interaction between actin and myosin driven by ATP hydrolysis forms the molecular basis of the crossbridge cycle which results in force generation in muscle. During each ATP hydrolysis cycle, the nature of the interaction between actin and myosin alternates between two defined states (Eisenberg & Greene, 1980; Geeves et al., 1984; Eisenberg & Hill, 1985). In the absence of nucleotide, actin is tightly bound to myosin ($K_d \simeq 0.1\ \mu M$), and the rate at which actin dis-

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