Preparation, Proteolysis and Reversible Oxidation of Highly Purified *Azotobacter vinelandii* Polynucleotide Phosphorylase

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1. A new method has been developed for the preparation in good yield of highly purified *Azotobacter vinelandii* polynucleotide phosphorylase in its reduced form. 2. Aging or digestion with trypsin causes the enzyme to develop a primer requirement that is not eliminated by β -mercaptoethanol. 3. The development of a primer requirement is accompanied by marked changes of the electrophoretic mobility of the enzyme in polyacrylamide gels. 4. The enzyme is inactivated by aerial oxidation or thiol-specific reagents. The lost activity is restored by β -mercaptoethanol, but not by oligonucleotide primers.

We have described (Gajda & Fitt, 1969, 1970) the previously unsuspected reversible oxidation of *Azotobacter vinelandii* polynucleotide phosphorylase (nucleoside diphosphate–polynucleotide nucleotidyltransferase, EC 2.7.7.8). The enzyme had a greatly decreased activity in its oxidized form and could be readily reactivated with β -mercaptoethanol.

The discovery afforded a probable explanation for the lack of reproducibility (Ochoa & Mii, 1961; Ochoa, Krakow & Basilio, 1963) and low yield (Thang, 1967) of the published purification procedures and made possible the development of an effective method for the isolation in good yield of highly purified *A. vinelandii* polynucleotide phosphorylase in its reduced form.

The present paper describes the purification procedure and studies of the effects of aging and proteolysis on the electrophoretic properties and primer requirement of the enzyme. We have also examined further the effects of oxidation and thiol-specific reagents on the highly purified enzyme.

EXPERIMENTAL

Materials. Intermediates and enzymes were purchased from the following suppliers: [^{14}C]ribonucleoside diphosphates and [^{32}P]orthophosphate, Amersham–Searle Corp., Don Mills, Ont., Canada; sodium dodecyl sulphate (specially pure), B.D.H. (Canada) Ltd., Toronto, Ont., Canada; ApA, Gallard–Schlesinger Chemical Manufacturing Corp., Carle Place, N.Y., U.S.A.; frozen *A. vinelandii* cells and poly A, Miles Chemical Co., Elkhart, Ind., U.S.A.; DEAE-Sephadex A-50, QAE-Sephadex A-50 and Sephadex G-200, Pharmacia (Canada) Ltd., Montreal, P.Q., Canada; unlabelled ribonucleoside diphosphates, P-L Biochemicals Inc., Milwaukee, Wis., U.S.A.; bovine serum

albumin, hyaluronidase (bovine testis) and *Streptomyces griseus* protease (pronase), Sigma Chemical Co., St Louis, Mo., U.S.A.; deoxyribonuclease, ribonuclease and lysozyme, Worthington Biochemical Corp., Freehold, N.J., U.S.A. Coomassie Brilliant Blue R250 was a gift from Canadian Industries Ltd., Montreal, P. Q., Canada.

Preparation of *A. vinelandii* polynucleotide phosphorylase. All steps in the purification were carried out at 0–4°C. The $(\text{NH}_4)_2\text{SO}_4$ concentrations were calculated by the procedure of Dixon & Webb (1964). Concave gradients were prepared by using three chambers of a Varigrad variable-gradient mixer (Buchler Instruments Inc., Fort Lee, N.J., U.S.A.): the first two chambers each contained 0.25 M-NaCl in buffer (500 ml) and the third chamber contained 0.45 M- or 0.5 M-NaCl in buffer (500 ml), as indicated below. During Sephadex G-200 gel filtration, the hydrostatic pressure was always less than 10 cm.

Frozen *A. vinelandii* cells (100 g) were thawed, suspended in 3 vol. of 0.1 M-tris-HCl buffer (pH 8.2)–10 mM-EDTA–10 mM- β -mercaptoethanol, and disrupted by sonication (Bronwill Biosonik II, 15 min). The suspension was centrifuged at 28000g for 30 min. The pellet was resuspended in the same buffer and the process repeated. The supernatants were pooled to give the crude extract (Table 1) which was fractionated by $(\text{NH}_4)_2\text{SO}_4$ precipitation. The protein insoluble between 25 and 60% saturation was redissolved in 0.1 M-tris-HCl buffer (pH 8.2)–10 mM-EDTA–10 mM- β -mercaptoethanol and dialysed overnight against two changes of 10 mM-tris-HCl buffer (pH 8.2)–1 mM-EDTA–1 mM- β -mercaptoethanol (referred to below as 'buffer'). NaCl was added to a final concentration of 0.1 M, and the solution passed through a column (2.5 cm $\times$ 80 cm) of DEAE-Sephadex A-50 previously equilibrated with 0.1 M-NaCl in buffer. Elution was performed at 30 ml/h successively with (i) 0.1 M-NaCl in buffer (400 ml), (ii) a linear gradient of 0.1–0.25 M-NaCl in buffer (1000 ml), (iii) 0.25 M-NaCl in buffer (400 ml) and (iv) a concave gradient of 0.25–0.5 M-NaCl in buffer (1500 ml). The active fractions, eluted between 0.28 M- and 0.32 M-NaCl, were combined and solid

(NH_4)₂SO₄ was added to 60% saturation. The precipitate was dissolved in buffer to give the DEAE I fraction (Table 1). This solution, without prior dialysis, was passed at 8 ml/h through a column (2.5 cm $\times$ 95 cm) of Sephadex G-200 previously equilibrated with buffer. The active fractions, which emerged between 143 and 187 ml, were combined to give the G-200 fraction (Table 1). The NaCl concentration was adjusted to 0.25 M and the enzyme adsorbed to a column (1.5 cm $\times$ 20 cm) of DEAE-Sephadex A-50 previously equilibrated with 0.25 M-NaCl in buffer. The column was washed with 0.25 M-NaCl in buffer (100 ml), and the enzyme eluted with a concave salt gradient, 0.25–0.45 M-NaCl in buffer (1500 ml). The active fractions, which emerged between 0.29 M- and 0.31 M-NaCl, were combined and dialysed against buffer to give the DEAE II fraction (Table 1). The NaCl concentration was adjusted to 0.25 M and the enzyme was adsorbed to a column (1.5 cm $\times$ 20 cm) of QAE-Sephadex A-50 previously equilibrated with 0.25 M-NaCl in buffer. The column was washed with 0.25 M-NaCl in buffer (100 ml) and elution was performed with a linear gradient of 0.25–0.4 M-NaCl in buffer (1000 ml). The active fractions emerged at about 0.3 M-NaCl. They were combined, dialysed against buffer and concentrated in a Sartorius Collodion Bag Apparatus [B. D. H. (Canada) Ltd.] to give the final QAE fraction (Table 1).

Purification of trypsin-digested *A. vinelandii* polynucleotide phosphorylase. The digestion mixture (final volume 3 ml) contained: tris-HCl buffer, pH 8.2, 12 μ mol; EDTA, 1.2 μ mol; β -mercaptoethanol, 1.2 μ mol; trypsin, 15 μ g; and purified reduced polynucleotide phosphorylase (QAE fraction; 380 μ g). The polynucleotide phosphorylase had been stored for several weeks at 4°C and its ADP-incorporation activity was stimulated 30% by added ApA. Incubation was at 37°C. Samples (0.4 ml) were withdrawn at intervals and the progress of the digestion followed by the procedure described in Fig. 3. The reaction was stopped after 1 h by addition of an ice-cold solution of soya-bean trypsin inhibitor (2.6 ml; 58 μ g/ml). A sample (5 ml) of this mixture was applied to a column (1.5 cm $\times$ 20 cm) of DEAE-Sephadex A-50 previously equilibrated with buffer and the enzyme was eluted with a linear gradient of 0.1–0.8 M-NaCl in buffer (500 ml). The active fractions were combined, dialysed against buffer and concentrated in a Sartorius Collodion Bag Apparatus to give the purified digested enzyme.

Assay procedures. Polynucleotide phosphorylase was assayed by the following procedures.

(a) **Incorporation of nucleoside diphosphates.** This assay depends on the incorporation of [¹⁴C]nucleoside diphosphate into an acid-insoluble form (Fitt & Fitt, 1967; Littauer & Kornberg, 1957). The reaction mixture (final volume 0.1 ml) contained: tris-HCl buffer, pH 9.0, 15 μ mol; MgCl₂, 1.0 μ mol; [¹⁴C]nucleoside diphosphate (about 3000 c.p.m./ μ mol), 4 μ mol; EDTA, 0.04 μ mol; ApA, 100 μ g, where appropriate; bovine serum albumin, 10 μ g; enzyme. Incubation was at 37°C for 10 min. The reaction was stopped by addition of 0.1 ml of 7% (w/v) HClO₄ and the mixture was kept at 0°C for 10 min. The precipitate was collected on a Whatman GF/C glass-fibre filter, washed four times with 1% (w/v) HClO₄ (2 ml) and once with 50% (v/v) ethanol (2 ml), dried and the radioactivity counted. One unit was defined as the amount of enzyme catalysing incorporation of 1 μ mol of [¹⁴C]ADP/h

into an acid-insoluble form. All specific enzymic activities are in terms of units of ADP-incorporation activity/mg of protein unless otherwise indicated.

(b) **Phosphorolysis.** This assay measures the formation of [³²P]ADP during the phosphorolysis of poly A in the presence of [³²P]P_i (Fitt & Fitt, 1967; Grunberg-Manago, Ortiz & Ochoa, 1956). The reaction mixture (0.1 ml) contained: tris-HCl buffer, pH 8.2, 10 μ mol; MgCl₂, 0.5 μ mol; EDTA, 0.03 μ mol; [³²P]P_i (10 000–100 000 c.p.m./ μ mol), 1 μ mol; poly A, 50 μ g; bovine serum albumin, 5 μ g; enzyme. Incubation was at 37°C for 10 min. The reaction was stopped with 1% (w/v) acid-washed charcoal in 2.3% (w/v) HClO₄ (1 ml). The suspension was allowed to stand for 10 min at 0°C, with occasional mixing, and the charcoal was then collected on a Whatman GF/C glass-fibre filter. The filter was washed three times with 10 ml of cold water. It was then placed upside-down in a planchet, dried and the radioactivity counted. The specific radioactivity of [³²P]P_i was determined by counting a suitable dried portion of the solution over which a GF/C filter had been placed. One unit was defined as the amount of enzyme that catalysed the formation of 1 μ mol of [³²P]ADP/h in these conditions.

(c) **Exchange.** This assay measures the exchange of [³²P]P_i with the terminal phosphate of ADP (Grunberg-Manago *et al.* 1956; Thang, 1967). The reaction mixture (0.1 ml) contained: tris-HCl buffer, pH 8.0, 10 μ mol; MgCl₂, 0.4 μ mol; EDTA, 5 nmol; ADP, 0.8 μ mol; [³²P]P_i (10 000–100 000 c.p.m./ μ mol), 2 μ mol; ApA, 100 μ g, where appropriate; bovine serum albumin, 10 μ g; enzyme. Incubation was at 37°C for 10 min. The reaction was stopped with a charcoal-HClO₄ suspension (1 ml) and the charcoal was collected, washed and the radioactivity counted as described for the phosphorolysis assay. The amount of phosphate incorporated was calculated from the expression (Grunberg-Manago *et al.* 1956): μ mol of P_i incorporated = c.p.m. incorporated (μ mol of P_i + μ mol of ADP)/c.p.m. in reaction. One unit was defined as the amount of enzyme that catalysed the incorporation of 1 μ mol of P_i/h into ADP in these conditions.

(d) **Radioactivity assay.** Radioactivity was determined in a Nuclear-Chicago low-background thin-window counter. Appropriate controls were carried out in all cases and their radioactivity was subtracted from the assay values.

(e) **Protein assay.** Protein was determined spectrophotometrically (Warburg & Christian, 1942).

(f) **Nucleic acid assay.** Total nucleic acid was determined spectrophotometrically (Warburg & Christian, 1942), and RNA by the orcinol reaction (Dische & Schwarz, 1937).

Analytical polyacrylamide-gel electrophoresis. Analytical polyacrylamide-gel electrophoresis was performed in a twelve-gel Buchler Polyanalyst (Buchler Instruments Inc.). The procedure of Fitt, Fitt & Wille (1968) was used with the following modifications: (i) the gels contained 0.2 M-tris-borate buffer and 4 mM-EDTA; (ii) the electrode compartments contained 0.05 M-tris-borate buffer-1 mM-EDTA; (iii) the pH of the buffers was 9.0 except where indicated; (iv) the gel concentration was 7% (w/v) except where indicated; (v) electrophoresis was performed at 0.5 mA/gel for 15 min, then at 1.5 mA/gel for 2 h, by using a constant current power supply. Protein was (i) stained with Aido Black or (ii) fixed with 12.5% (w/v) trichloro-

acetic acid and stained for not more than 1 h with 0.1% (w/v) Coomassie Blue in 12.5% (w/v) trichloroacetic acid (Chrambach, Reisfeld, Wyckoff & Zaccari, 1967). To locate the enzyme, gels were incubated for 1–2 h in a reaction mixture for nucleoside diphosphate polymerization and the polynucleotide formed was stained with Acridine Orange (Fitt *et al.* 1968). Gels stained with Amido Black or Acridine Orange were destained overnight in running tap water. Those stained with Coomassie Blue were destained overnight with 12.5% (w/v) trichloroacetic acid and the position of the bands recorded immediately after destaining, since prolonged exposure to dilute trichloroacetic acid caused the gels to shrink. Nucleic acids were stained with 0.2% (w/v) Methylene Blue in 0.4 M-sodium acetate buffer, pH 4.7 (Peacock & Dingman, 1967) and the gels destained with running tap water.

Preparative polyacrylamide-gel electrophoresis. These experiments were performed with a Shandon Preparative Acrylamide Electrophoresis Apparatus (kindly loaned by Dr C. M. Johnson). The equipment was operated according to the manufacturer's instruction manual, using a 7% (w/v) gel containing 0.2 M-tris-borate buffer (pH 9)–4 mM-EDTA. The electrode compartments contained 0.05 M-tris-borate buffer (pH 9)–1 mM-EDTA and buffer of the same composition was used for elution. The annular running gel (above the elution chamber) was 3.5 cm long and approx. 3 mm across. After preparation of the gel and assembly of the apparatus, the equipment was placed in a cold-room and cold water was passed through the outer jacket and inner 'cold-finger'. The flow of buffer through the elution chamber was started and maintained at 5 ml/h for the duration of the experiment. After an initial 1 h pre-run at 200 V (about 14 mA), the enzyme sample, containing 10% (w/v) sucrose and 10 mM-GSH, was run on to the surface of the gel under the upper electrode buffer. Electrophoresis was performed at (i) 100 V for 1½ h and (ii) 200 V for 24 h; 3 ml fractions were collected and assayed for protein, nucleic acid and enzymic activity.

Isoelectric focusing. These experiments were performed with an LKB 8100-10 (110 ml) Electrofocusing Column operated according to the manufacturer's instructions. LKB Ampholine carriers were used: pH 3–10 (LKB 8141) for preliminary experiments and pH 3–6 (LKB 8142) for later experiments. The current was turned on and the voltage raised from 300 V to 1000 V over several hours so that the power never exceeded 0.5 W. Electrophoresis

was continued until equilibrium (zero current) was attained after approx. 48 h. The power supply was then disconnected and the Ampholine solution was run out of the column. Fractions (2 ml) were collected and their pH and enzymic activity determined immediately.

RESULTS

Purification of *A. vinelandii* polynucleotide phosphorylase. The results of a typical purification by the procedure described in the Experimental section are summarized in Table 1. The final product was purified 2900-fold, in a yield of 66% of the units present in the crude extract. A total of 17 000 units were discarded in less pure side fractions at various stages, so the overall recovery was about 90%. It should be noted that some increase in activity always occurs after the first DEAE-Sephadex column step, probably through removal of degradative enzymes.

Although the ammonium sulphate fractionation and subsequent dialysis do not lead to any significant purification, they are essential. If they are omitted, the enzyme is only partially adsorbed to the DEAE-Sephadex column during the next step.

Approximately 95% of the protein of the DEAE II fraction migrates as a single sharp band containing all the enzymic activity during analytical polyacrylamide-gel electrophoresis in the conditions described in the Experimental section. Enzyme of this purity was used in the studies described by Gajda & Fitt (1969).

The final product after QAE-Sephadex chromatography was subjected to analytical polyacrylamide gel electrophoresis in 5, 7 and 10% (w/v) gels, in each case at three different pH values (7.4, 8.2 and 9.0). In all these conditions, the fresh, fully reduced enzyme appeared to migrate as a single band of protein corresponding exactly to the band of enzymic activity, provided Amido Black was used for detection of the protein. Coomassie Blue staining revealed a second, minor, slower-moving component equivalent to less than 1% of the total

Table 1. *Purification of A. vinelandii* polynucleotide phosphorylase

The enzyme was purified and assayed for ADP-incorporation activity as described in the Experimental section.

Fraction	Total protein (mg)	E_{280}/E_{260}	ADP-incorporation activity		
			Total units	Specific activity (units/mg)	Relative purification
Crude extract	21 200	0.65	62 400	3.0	1.0
(NH ₄) ₂ SO ₄ 25–60%	10 800	0.61	62 300	5.8	1.9
DEAE I	264	0.96	69 200	262	87
G-200	40.2	0.86	50 200	1250	417
DEAE II	7.03	0.75	50 000	7000	2300
QAE	4.73	0.75	41 200	8700	2900

sample placed on the gel. This minor component was inactive and was still present in samples digested exhaustively before electrophoresis with trypsin, pronase (which completely eliminated the polynucleotide phosphorylase band), deoxyribonuclease, ribonuclease, hyaluronidase or α -amylase. The two bands could be separated cleanly by preparative gel electrophoresis (see the Experimental section). Unfortunately, the method was of no preparative value, because only 18% of the protein or enzymic activity applied to the gel could be recovered, even when pre-electrophoresis was performed for 1 h before application of the sample and the latter contained GSH, which has been shown to improve the recovery of some enzymes from polyacrylamide gels (Ray, Troisi & Rappaport, 1969). If the pre-electrophoresis and GSH were omitted, less than 5% of the original enzymic activity was recoverable. (It should be noted that the recoveries of incorporation and phosphorolysis activities were identical.) The separated enzyme and 'anomalous' component retained their original mobilities during subsequent analytical polyacrylamide-gel electrophoresis. The isolated 'anomalous' band substance was unaffected by digestion with the enzymes mentioned above or by lysozyme and its nature is as yet uncertain.

The purified enzyme (QAE fraction) was closely associated with nucleic acid. The E_{280}/E_{260} ratio was 0.75 and the orcinol reaction confirmed the presence of 7% (w/w) of RNA. Methylene Blue staining showed that this RNA, or a significant part of it, migrated with the active protein during analytical gel electrophoresis and the electrophoretically purified enzyme obtained by preparative gel electrophoresis had an extinction ratio of 0.83 corresponding to 6% (w/w) of nucleic acid. Although the extinction ratio rose to 1.2–1.3 after the gel filtration step during some purifications, the final value for the QAE fraction was always 0.7–0.8. The enzyme-associated nucleic acid could not be detected on polyacrylamide gels with Acridine Orange unless very large samples were used, so it did not interfere with the localization of the enzymic activity.

The pI (isoelectric point) of the purified enzyme was determined several times by isoelectric focusing and found reproducibly to be between pH 4.1 and 4.2. The enzyme precipitates at this pH, which may therefore only be an upper limit for the pI; it is also probable that it corresponds to the isoelectric point of the nucleic acid–enzyme complex. The method could not be used for purification, since considerable irreversible losses of activity occur during electrofocusing, probably as a result of the prolonged exposure of the enzyme to pH values below 8.2.

A study of the stability of the enzyme showed that it lost activity rapidly at pH 7.4 and was most

stable at pH 8.2 or higher. The final purified product was comparatively stable when stored in 10 mM-tris-HCl buffer (pH 8.2)–1 mM-EDTA–1 mM- β -mercaptoethanol at a protein concentration of 0.4 mg/ml. Incubation of the QAE fraction at 37°C for 12 h caused a 40% loss of activity, measured with or without ApA, without any detectable change in electrophoretic mobility of the enzyme in polyacrylamide gels. Freezing and thawing of the enzyme solution caused a rapid loss of activity, but after 5 weeks at 4°C only 15% of the original ADP-incorporation activity, measured in the presence of ApA, was lost. However, prolonged storage leads to considerable changes in the primer requirement and electrophoretic mobility of the fully reduced enzyme (see below).

Effect of aging on the activity and primer requirement of reduced highly purified A. vinelandii polynucleotide phosphorylase. Table 2 shows that ADP polymerization and ADP- P_i exchange catalysed by the fresh, fully reduced enzyme were not stimulated by ApA. However, CDP incorporation was stimulated 30–40% by added primer, and it should be noted that we have always detected a primer requirement for optimum CDP polymerization even with fresh preparations that require no primer for ADP polymerization (the latter are referred to below as primer-independent enzymes). No explanation can be given for this difference at present. Fig. 1 shows that a primer requirement developed steadily during prolonged storage, so that after 72 days at 4°C ADP incorporation was stimulated 45% by added ApA. A parallel increase in primer requirement for CDP incorporation took place, with a rise in stimulation by ApA from 30–40 to 160%. The addition of β -mercaptoethanol to the assay medium did not affect the need for oligonucleotide primer, thus confirming that the enzyme was still fully reduced.

The increase in primer requirement was accom-

Table 2. *Comparison of the activities of highly purified A. vinelandii polynucleotide phosphorylase*

The ADP- and CDP-incorporation activities, poly A phosphorolysis activity and ADP- P_i exchange activity of the QAE fraction (Table 1) were measured as described in the Experimental section. All assay mixtures contained 0.13 μ g of enzyme and, where appropriate, ApA, 100 μ g.

Assay	Specific activity (units/mg)	
	Without ApA	With ApA
ADP-incorporation	8700	8950
CDP-incorporation	5500	7900
ADP- P_i exchange	4600	4100
Phosphorolysis	2140	—

panied by changes in mobility during analytical gel electrophoresis (Fig. 2). The fresh enzyme migrated as a single band of protein and activity, apart from the faint, anomalous, slow-moving band discussed above. As the preparation aged, a new, slightly more mobile, band of protein and activity developed. The intensity of this band steadily increased with time, while that of the original band diminished. Neither of the two bands of enzymic activity appeared to have an absolute primer requirement. The sensitive Methylene Blue staining procedure (Peacock & Dingman, 1967) suggested that nucleic acid was still present in both bands, but the latter were not resolved by this method, which, in our hands, often gave less sharp bands than the procedures used for detecting protein and enzymic activity.

These changes in primer requirement and electrophoretic mobility occurred with all partially or highly purified *A. vinelandii* polynucleotide phosphorylase preparations. However, the rate at which the primer requirement developed varied from one batch of enzyme to another and appeared greater with preparations that contained relatively little nucleic acid. It therefore seems that the development of a primer requirement is related to the association of nucleic acids with the enzyme and preliminary experiments suggest that it occurs faster at pH 9 than at 8.2.

Effect of trypsin digestion on *A. vinelandii* polynucleotide phosphorylase. Treatment of the purified, reduced enzyme with trypsin caused a rapid loss of the ADP- and CDP-incorporation activities without primer (Fig. 3). The corresponding activities with

Fig. 1. Change in primer requirement for ADP incorporation during aging of *A. vinelandii* polynucleotide phosphorylase. The purified enzyme was stored at 4°C in 10mM-tris-HCl buffer (pH 8.2)-1mM-EDTA-1mM- β -mercaptoethanol. Samples were withdrawn at the indicated times and assayed for ADP incorporation by the standard method (see the Experimental section) with and without ApA. The results are expressed as the ratio of the activity with primer to that without.

Fig. 2. Effect of aging on the electrophoretic mobility of polynucleotide phosphorylase. Analytical polyacrylamide-gel electrophoresis of the enzyme (3-8 μ g/gel) used for the experiment described in Fig. 1 was performed by the standard procedure (see the Experimental section). Protein was stained with Coomassie Blue. (The broken line indicates the position of the faint, anomalous band discussed in the Results section.) The enzymic activity was located by the standard method and corresponded in all cases to the main band or bands of protein.

Fig. 3. Time-course of the proteolysis of *A. vinelandii* polynucleotide phosphorylase by trypsin. The digestion medium (final volume 1ml) contained: tris-HCl buffer, pH 8.2, 4 μ mol; EDTA, 0.4 μ mol; β -mercaptoethanol, 0.4 μ mol; trypsin, 5 μ g; polynucleotide phosphorylase, 128 μ g. Incubation was at 37°C. Samples (0.1ml) were withdrawn at the indicated times and added to ice-cold soya-bean trypsin inhibitor (0.1ml; 0.5mg/ml). The samples were assayed for ADP- and CDP-incorporation with and without ApA (100 μ g) as described in the Experimental section. The residual activity is expressed as a percentage of that of a similar, non-incubated control to which the inhibitor was added before the trypsin. Identical results were obtained with and without added 10mM- β -mercaptoethanol. ■—■, ADP incorporation; ■---■, ADP incorporation with ApA; ▲—▲, CDP incorporation; ▲---▲, CDP incorporation with ApA; ○—○, phosphorolysis.

Fig. 4. Analytical polyacrylamide-gel electrophoresis of trypsin-digested *A. vinelandii* polynucleotide phosphorylase. Samples of the digested enzyme obtained during the experiment described in Fig. 3 were analysed by gel electrophoresis. Separate electrophoretograms with 150 μ l and 10 μ l of each sample were run simultaneously and were stained for protein (p) with Coomassie Blue or for enzymic activity (a), respectively, as described in the Experimental section. The broken line indicates the position of the faint anomalous component described in the text. The broad, fast-moving band was shown in separate experiments to be due to soya-bean trypsin inhibitor.

ApA were lost more slowly, so that a large increase in primer requirement took place. However, there was little or no differential effect on the incorporation activities with these substrates, in contrast with the selective increase in primer requirement for CDP- and UDP-incorporation observed during controlled trypsin digestion of *Micrococcus lysodeikticus* polynucleotide phosphorylase (Fitt & Fitt, 1967; Fitt *et al.* 1968). The phosphorolytic activity was lost at a rate intermediate between the rates of loss of the incorporation activities with and without ApA.

Fig. 4 shows that the loss of activity and increase in primer requirement of the enzyme during proteolysis were accompanied by changes in its electrophoretic mobility in polyacrylamide gels. Initially, the enzyme was partially converted into a second, slightly more mobile, form in a manner similar, but presumably not identical, to the change observed during aging (Fig. 2). Both components were then destroyed, the less mobile one more rapidly than the new, faster-moving form of the enzyme. The end product was a single component that migrated more rapidly than either of the other two bands. All three bands, including this final form, stained for protein, nucleic acid and enzymic activity with and without ApA (provided sufficient material was placed on the gel). Longer treatment with trypsin caused further loss of activity without any additional change in the electrophoretic mobility of the protein. No difference was observed

Table 3. *Effect of β -mercaptoethanol and ApA on ADP incorporation by trypsin-digested reduced A. vinelandii polynucleotide phosphorylase*

The digestion medium (final volume 0.25 ml) contained: tris-HCl buffer, pH 8.2, 0.5 μ mol; EDTA, 0.05 μ mol; β -mercaptoethanol, 0.05 μ mol; trypsin, 1.25 μ g; polynucleotide phosphorylase, 16 μ g. Incubation was at 37°C. Samples (50 μ l) were withdrawn at the indicated times and added to an ice-cold aqueous solution of soya-bean trypsin inhibitor (50 μ l; 0.25 mg/ml). The control contained, in addition, soya-bean trypsin inhibitor (62.5 μ g) and the corresponding samples were added to cold water (50 μ l). Portions of the digested and control samples were assayed for ADP-incorporation activity by the standard assay (i) without additions, (ii) with β -mercaptoethanol (10 mM) and (iii) with ApA (100 μ g). The residual activity of the digested samples is expressed as a percentage of the activity of the zero-time control sample assayed without additions (no change occurred in the activity of the incubated controls).

Digestion time (min) ... Addition	Residual activity (%)		
	0	5	10
None	100	14	6
β -Mercaptoethanol (10 mM)	98	14	6
ApA (100 μ g)	106	33	29

between the final results of trypsin digestion of the fresh and aged enzymes.

At no time during the tryptic hydrolysis did any β -mercaptoethanol requirement develop (Table 3).

This observation is similar to that of Fitt & Wille (1969) in their study of the proteolysis of *Clostridium perfringens* polynucleotide phosphorylase, but differs from the results obtained with the *M. lysodeikticus* enzyme (Klee, 1968; Klee & Singer, 1968; Fitt *et al.* 1968) when it was found that the increase in primer requirement that occurred during trypsin digestion could be reversed by thiols.

A sample of the enzyme was extensively digested with trypsin and purified by DEAE-Sephadex chromatography. Table 4 shows that the product was stimulated 23-fold by ApA, whereas the untreated enzyme, which was several weeks old, was only stimulated 30% by added primer. The activities were unchanged by addition of β -mercaptoethanol to the assay medium. The purified, digested enzyme migrated as a single band of protein and activity during polyacrylamide-gel electrophoresis, but the faint anomalous band was still present.

Finally, preliminary studies (Table 5) of the effect of trypsin digestion on purified oxidized *A. vinelandii* polynucleotide phosphorylase also suggest that the state of oxidation of the enzyme does not influence the stimulation by added oligonucleotides in a major way. It can be seen that ADP incorporation in the presence of the partially digested oxidized enzyme was stimulated to a similar extent by ApA as was ADP incorporation in the presence of the same sample after it had been reduced, even though the actual incorporation in the presence of β -mercaptoethanol was at least six times that in its absence.

Effect of aerial oxidation and thiol-specific reagents on reduced highly purified A. vinelandii polynucleotide phosphorylase. Highly purified oxidized polynucleotide phosphorylase can be prepared easily

from the reduced enzyme by the method described in Table 6. This method is difficult to control, since channelling occurs during passage of air through the ion-exchanger, so the degree of oxidation varies from experiment to experiment. In the experiment described in the Table, the ADP-incorporation activity of the oxidized enzyme in the absence of β -mercaptoethanol was only 38% of that measured in the presence of the thiol. However, the assay with β -mercaptoethanol showed that all the original enzyme was still present, and complete reduction of the oxidized enzyme (Table 6, oxidized and reduced enzyme) proved that oxidation and reduction gave a product whose enzymic properties were closely similar to those of the untreated enzyme. The oxidized enzyme became progressively more oxidized during storage until the stimulation by β -mercaptoethanol was sixfold. The oxidized enzyme was very rapidly reactivated by β -mercaptoethanol and preincubation with the thiol was unnecessary, provided its concentration in the assay medium was at least 1 mM.

Oxidation caused no detectable change in the electrophoretic mobility of the enzyme during polyacrylamide-gel electrophoresis, so the reaction must be intramolecular. The optimum ADP and Mg^{2+} concentrations of the oxidized enzyme were somewhat lower than those of the reduced enzyme, but

Table 4. *Stimulation by ApA of untreated and trypsin-digested reduced A. vinelandii polynucleotide phosphorylase*

Purified, reduced polynucleotide phosphorylase was digested with trypsin and the product was purified by DEAE-Sephadex chromatography as described in the Experimental section. Samples of the digested enzyme before and after purification and of the undigested enzyme were assayed with and without ApA (100 μ g) in the standard ADP-incorporation assay. It should be noted that the untreated enzyme had acquired a primer requirement during storage (30% stimulation by ApA) and that identical results were obtained with 10 mM- β -mercaptoethanol in the assay medium.

Enzyme	ADP incorporation with ApA	
	ADP incorporation without ApA	
Untreated	1.3	
Digested, before purification	14.0	
Digested, purified	23.0	

Table 5. *Effect of trypsin-digestion on the primer requirement of purified, oxidized A. vinelandii polynucleotide phosphorylase*

Purified, oxidized polynucleotide phosphorylase (1 ml; 5.63 units of ADP-incorporation activity/ml, assayed without β -mercaptoethanol or ApA) in 1 mM-EDTA-10 mM-tris-HCl buffer, pH 8.2, was incubated at 37°C for 3 min. Trypsin (3 μ g/ml, 50 μ l) was added and incubation continued. Samples (0.2 ml) were withdrawn at the indicated times, mixed with 20 μ l of soya-bean trypsin inhibitor (65 μ g/ml.) and cooled in ice. They were assayed for ADP-incorporation with and without ApA (100 μ g) in the standard incorporation assay (see the Experimental section) in the presence and absence of 10 mM- β -mercaptoethanol. The zero-time sample was obtained separately by adding enzyme to an appropriate mixture of trypsin and inhibitor. The results are expressed as the ratio of the ADP incorporation with ApA to that without ApA determined in the same conditions. The preparation of oxidized polynucleotide phosphorylase by aerial oxidation of the purified, reduced enzyme is described in Table 6.

Digestion time (min)	ADP incorporation + ApA	
	ADP incorporation - ApA	
	-thiol	+thiol
0	1.35	1.12
0.33	1.06	1.32
5	1.58	1.47
20	2.08	1.91

Table 6. *Effect of aerial oxidation on the β -mercaptoethanol and ApA requirements of *A. vinelandii* polynucleotide phosphorylase*

Purified, reduced polynucleotide phosphorylase (QAE fraction, 1800 units) was adsorbed on a column (0.5 cm $\times$ 3 cm) of DEAE-Sephadex A-50. The column was washed with 0.25 M-NaCl-1 mM-EDTA-10 mM-tris-HCl buffer, pH 8.2, to remove β -mercaptoethanol, and air was then sucked through it at about 360 ml/h for 5 h. The enzyme was eluted with 0.8 M-NaCl-1 mM-EDTA-10 mM-tris-HCl buffer, pH 8.2 (40 ml). The volume of the eluate was adjusted to 50 ml with water and the solution was divided into two equal portions. One portion was dialysed overnight against 2 l of 1 mM-EDTA-10 mM-tris-HCl buffer, pH 8.2, to give the 'oxidized enzyme'; the second was dialysed against 2 l of the same buffer containing in addition 1 mM- β -mercaptoethanol to give the 'oxidized and reduced enzyme'. Finally, a further sample of the enzyme was treated as above except that air was not sucked through the column and the elution and dialysis buffers both contained 1 mM- β -mercaptoethanol: this gave the 'control enzyme'. The activities of the three enzyme fractions were determined in the ADP-incorporation assay in the presence and absence of β -mercaptoethanol and ApA and in the phosphorolysis assay in the presence and absence of β -mercaptoethanol. (It should be noted that the assays in the presence of β -mercaptoethanol showed that all the original units placed on each of the columns were recovered.) Abbreviation: BME, β -mercaptoethanol.

Enzyme	ADP-incorporation activity (relative)				Phosphorolysis activity (relative)	
	No addition	+BME	+ApA	+BME +ApA	No addition	+BME
Control	1.00	0.90	0.98	0.97	1.00	1.09
Oxidized	1.00	2.63	1.08	2.66	1.00	1.22
Oxidized and reduced	1.00	1.02	1.08	1.08	1.00	1.08

the pH optimum was unchanged. In no case did oxidation cause any significant increase in primer requirement.

The reduced enzyme was inactivated by both *p*-chloromercuribenzoate (Gajda & Fitt, 1969) and mercuric chloride. All the lost activity was restored by 10 mM- β -mercaptoethanol in the assay medium and neither reagent caused any increase in primer requirement.

DISCUSSION

The method of purification of *A. vinelandii* polynucleotide phosphorylase described in this paper has been readily reproducible in our hands and has the additional advantage that the yield of purified enzyme has consistently been better than 50% of the units in the crude extract. The product is at least 99% pure as judged by analytical polyacrylamide-gel electrophoresis in several different conditions of pH and gel concentration. The success of the method appears to depend on a combination of four main factors: (1) the maintenance of the enzyme in the fully reduced form, which we have found to be much easier to purify than the oxidized enzyme; (2) the avoidance of cellulose ion-exchangers or calcium phosphate gel, which for some unexplained reason yield unstable polynucleotide phosphorylase even though they are often effective in the purification itself; (3) the use of very shallow salt gradients for elution of the enzyme from ion-exchange columns; (4) the use of pH values above 8 at all times during the purification.

Ribonucleic acid appears to be very closely associated with the enzyme, in agreement with the conclusions of Ochoa & Mii (1961) based on experiments with comparatively impure preparations of *A. vinelandii* polynucleotide phosphorylase. This nucleic acid migrated together with the enzyme during polyacrylamide-gel electrophoresis even after partial proteolysis with trypsin, and the nucleic acid content of the highly purified enzyme was not changed significantly by preparative gel electrophoresis.

The readily reversible oxidation of *A. vinelandii* polynucleotide phosphorylase and its reversible inactivation by thiol-specific reagents indicate that the enzyme contains one or more thiol groups essential for activity. However, the state of oxidation of the enzyme has no major effect on its primer requirement.

In contrast, trypsin digestion of the fully reduced enzyme does lead to a very large primer requirement accompanied by a progressive loss of overall activity. This effect is paralleled by a considerable increase in the electrophoretic mobility of the active protein. A similar but less pronounced requirement for oligonucleotide primers develops during prolonged storage of the reduced enzyme. The electrophoretic mobility in polyacrylamide gels also increases in this case, but the new form is only slightly more mobile than the primer-independent enzyme. It is possible that the gradual change in primer requirement during storage is due to a slow hydrolysis of an endogenous tightly bound primer, an explanation favoured by the observation that the primer requirement develops more rapidly at pH 9 than

at pH 8.2. However, gel electrophoresis shows that nucleic acid is still present in the partially active species obtained after prolonged trypsin digestion. At no stage, either during storage or as a result of trypsin digestion, did any requirement for β -mercaptoethanol develop. The primer-requiring enzyme was evidently fully reduced and could not be converted into a primer-independent form by added thiols.

These results are quite different from those observed with trypsin-digested *M. lysodeikticus* polynucleotide phosphorylase. Partial digestion of this enzyme yields a primer-requiring form (Fitt & Fitt, 1967; Klee, 1967, 1969) whose activity can be partially restored by thiols (Klee, 1968; Klee & Singer, 1968; Fitt *et al.* 1968) as well as by oligonucleotides. It is clear that in the case of the *A. vinelandii* enzyme the development of a primer requirement is not related to the state of oxidation of the enzyme. This conclusion is confirmed by the effects of trypsin digestion on the oxidized *A. vinelandii* polynucleotide phosphorylase; a primer requirement also developed in this case, but the stimulation of the product by ApA was approximately the same for the digested oxidized enzyme and its corresponding reduced form. Thus the effect of the primer was not altered significantly by a change in the oxidation state of the partially digested enzyme.

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