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Thermostable polynucleotide phosphorylases from Bacillus stearothermophilus and Thermus aquaticus

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ABSTRACT

Polynucleotide phosphorylase from Bacillus stearothermophilus has been purified to homogeneity. Polyacrylamide gel electrophoresis run under denaturing conditions indicates that the enzyme is a tetramer with subunits of apparent molecular weight 51,000 daltons. A partial purification of polynucleotide phosphorylase from Thermus aquaticus has also been effected. The two enzymes show similar catalytic properties, which differ little from those of mesophilic polynucleotide phosphorylases. The use of thermostable polynucleotide phosphorylases for in vitro nucleic acid synthesis is discussed.

INTRODUCTION

Polynucleotide phosphorylases (E.C.2.7.7.8.) of the thermophilic organisms Bacillus stearothermophilus and Thermus aquaticus have been studied in the hope of easily polymerising modified nucleotide diphosphates. Although the substrate specificities of the two enzymes are similar to those reported for mesophilic enzymes, their thermal stability does present advantages, for instance in the synthesis of polyriboguanylic acid from GDP or in the copolymerisation of GDP with other nucleotides to give polymers of base composition similar to the input ratio of nucleotides[1]. Preliminary structural studies have been carried out on the homogenous B.stearothermophilus enzyme, which can be readily obtained as a single active protein species. In contrast, purification of the enzyme from other sources e.g. Micrococcus luteus usually leads to the isolation of a number of different active species, presumably as a result of proteolysis of the original enzyme.[2]

MATERIALS

B.stearothermophilus (NCIB 8924) and *T.aquaticus* were supplied as freeze dried pastes by MRE Porton, and stored at - 20°C until used. Polynucleotides were supplied by P.L. Biochemicals Inc. Nucleotide diphosphates and alkaline phosphatase (E.C.3.1.3.1.) were supplied by Boehringer Corporation. Other enzymes were supplied by BDH, radioactive materials were obtained from the Radiochemical Centre, Amersham. Dimethyl suberimidate was synthesised as described by Davies and Stark[3].

METHODS

Protein was assayed by the Biuret or Lowry methods.[4] Purified samples of proteins were assayed spectrophotometrically[5]. Polymerisation assays utilised the colorimetric determination of phosphate released with time[6], and phosphorolysis was assayed by the method of Klee.[7] One unit of enzyme activity is defined as that which will polymerise one µmole of ADP per hour. Polymerisation assays were carried out at 60°C in a mixture containing 50mM Tris-HCl pH 9.2, 20mM ADP, 1mM DTT, 1mM EDTA, 0.6M KCl, 7.5mM $MgCl_2$, and 100µM oligo (A) primer. Phosphorolysis assays were carried out at 60°C in a mixture containing 50mM Tris-HCl pH 8.2, 1mM DTT, 1mM EDTA, 0.6M KCl, 100µM poly A and 10mM [^{32}P]-labelled KH_2PO_4 with a specific activity of about 10^5 cpm per µmole.

Oligo (A) primer was prepared by treating poly (A) with 0.1 M NaOH at 25°C for 3 hr., bringing the solution to pH 8.0 with acetic acid and treating with alkaline phosphatase at 37°C for one hr. (50 units of enzyme per mg. of poly (A)). The solution was then deproteinised with isoamyl alcohol:chloroform (5:2 v/v), and fractionated on a Sephadex G10 column equilibrated with 50mM Tris-HCl pH 8.2, 0.2M KCl. Samples were stored at - 20°C.

ENZYME PURIFICATION

B.stearothermophilus was grown under the growth conditions described by Sargent.[8] *T.aquaticus* growth conditions were as described by Brock and Freeze[9] with the exception of the substitution of tris(hydroxymethyl)aminomethane for nitrilotriacetic acid, resulting in a considerable slowing of the growth rate. Freshly harvested or freeze dried *B.stearothermophilus* (25g) was suspended in Buffer A (Tris-HCl 50mM pH 8.2, 1mM EDTA, 1mM DTT (100 ml) and broken by treatment with lysozyme and EDTA.[10] Cell debris was centrifuged off, and saturated ammonium sulphate solution

added to precipitate those proteins insoluble between 35 and 55% ammonium sulphate. This fraction was isolated by centrifugation then resuspended in buffer A and applied to a TEAE-cellulose column (30 x 2.5 cms) which was eluted with a linear gradient of 0 to 0.5 M KCl in buffer A. The active fractions, eluting at a concentration of about 0.28M KCl were pooled, concentrated and desalted in a Bio-Rad hollow fibre device. The protein was then applied to a DEAE-Sephadex A50 column (40 x 1.5 cms), and eluted with a linear gradient of 0 to 0.5 M KCl in buffer A. Active fractions were pooled, precipitated with ammonium sulphate, and applied in a minimal volume of buffer A to an LKB AcA 34 Ultrogel column (60 x 2.5 cms). Active fractions were combined and stored at - 20°C. The purification of the T.aquaticus enzyme was essentially the same, except that cell lysis was effected by ultrasonication at full power for thirty seconds in an MSE 150 watt ultrasonicator. Material purified to the stage after TEAE-cellulose chromatographs was used for preliminary characterisation. The presence of nucleases, phosphatases and adenyl kinase activity in purified preparations was assessed. Nuclease activity was detected by measuring the production of TCA-soluble material from [^{14}C]-labelled poly(A) or poly(C)in a phosphorolysis assay mixture containing no inorganic phosphate. Phosphatase activity was measured under polymerisation assay conditions, with no metal co-factor present. Adenyl kinase activity was measured using a polymerisation assay mixture containing [^{14}C]ADP. Reaction products at various time intervals were chromatographed on Whatman 4 paper in an isobutyric acid/NH_4OH/H_2O (66/1/33) solvent system. The ratio of counts co-chromatographing with ADP to those co-chromatographing with AMP was determined.

Approximate molecular weights for native B.stearothermophilus and T.aquaticus enzymes were determined by gel exclusion chromatography on an LKB AcA 34 Ultrogel column (80 x 2.5 cms) equilibrated with buffer A, using lysozyme, haemoglobin and glutamate dehydrogenase as molecular weight markers. Comparison of molecular weights with E.coli polynucleotide phosphorylase was carried out by density gradient centrifugation in a 5 to 20% sucrose gradient at 30,000 rpm for 22 hr. in an MSE 3 x 25 ml rotor. Samples were run separately or as admixtures.

Standard polyacrylamide gel electrophoresis was carried out in phosphate, Tris-glycine or borate-acetate buffer systems, using 7.5% gels. Denaturing gels were run in the presence of SDS and mercaptoethanol[12], molecular weights being computed by comparison with bovine serum albumin, yeast mitochondrial F1 ATPase and lysozyme as markers.

Isoelectric focussing was carried out by the method of O'Farrell[13] and N-terminal determinations were carried out by the method of Weber.[14]

RESULTS

Enzyme preparations were carried out on bacteria harvested in the late logarithmic phase of growth. However, the specific activity of the enzyme was highest in the early logarithmic phase, and varied with growth in a manner analogous to that observed for E.coli.[15] The specific activity of the T.aquaticus enzyme was also twice as high in the early stages of growth.

Crude extracts of the B.stearothermophilus and T.aquaticus enzymes were primer independent. Early B.stearothermophilus enzyme preparations using A25 Sephadex produced primer independent enzyme, which could be converted to primer dependency by gel filtration on a Sephadex G200 column. However, Sephadex A50 chromatography produced primer dependent enzyme as a broad peak with the leading and trailing edges showing different requirements for primer. A four fold stimulation of CDP polymerisation was achieved when an oligo (A) primer was added to the trailing fractions of the peak whereas a fifty fold stimulation was achieved with the leading fractions. This suggests that an easily dissociable factor is responsible for conferring primer independency on the enzyme. As the factor is present in the same fractions as polynucleotide phosphorylase eluted from an A25 column, but is separated from the enzyme by A50 Sephadex chromatography, a molecular weight of between 1 and 2 x 10^5 daltons for the factor is suggested. By recombining fractions treated with nucleases or proteases with primer dependent enzyme, we attempted to determine the nature of this factor. However, no reconstitution of primer independency could be observed using this approach. The T.aquaticus enzyme also showed primer dependency on purification past the stage of TEAE-cellulose chromatography. Purified polynucleotide phosphorylase from B.stearothermophilus ran as a single band on polyacrylamide gel electrophoresis. The protein had a specific activity of 600 units per mg, an extinction coefficient of $E^{1\%}_{280} = 5.68$

and a 280/260nm absorbance ratio of 1.68, indicating the absence of contaminating nucleic acids.[4] No phosphatase, nuclease or adenyl kinase activity could be detected in the final preparation. The T.aquaticus enzyme purified to the stage of TEAE-cellulose chromatography was contaminated with nucleic acid and showed low levels of adenyl kinase activity.

The molecular weight of the B.stearothermophilus enzyme was 2.1×10^5 daltons as judged by behaviour on gel filtration and density gradient centrifugation, the behaviour of the enzyme being similar to that of the E.coli polynucleotide phosphorylase purified by a similar procedure. Impure T.aquaticus enzyme had a molecular weight of more than 4×10^5 daltons as judged by gel filtration. Gel electrophoresis of B.stearothermophilus polynucleotide phosphorylase under denaturing conditions showed a single protein band of molecular weight 5.1×10^4 daltons. Cross-linking of the protein with dimethyl suberimidate[3] prior to electrophoresis led to the appearance of three more bands of lower mobility in positions corresponding to a dimer, trimer, and tetramer of the monomeric subunit. We therefore consider the enzyme to be tetramer of subunits of molecular weight 5.1×10^4 daltons. N-terminal analysis by the method of Weber[12] showed the presence of a single dansylated amino acid, identified as methionine by comparison with standards. This observation provides evidence for the identical nature of the subunits. Isoelectric focussing in polyacrylamide gels[13] showed the iselectric point of the pure enzyme to be 4.1.

CATALYTIC STUDIES

Both enzymes showed pH optima for polymerisation of 9.2 and for phosphorolysis of 8.2. The optimal nucleotide to magnesium ratio is about 3:1 for all nucleotides with the exception of GDP which is polymerised optimally at a ratio of 1:1. In these aspects both enzymes resemble polynucleotide phosphorylases previously characterised from other sources[16]. However, the optimal temperature for crude or pure preparations of the B.stearothermophilus enzyme is 69°C, whilst crude T.aquaticus enzyme functions optimally at about 80°C, although the partially purified material has an optimum of 73°C. In common with other thermostable enzymes, B.stearothermophilus polynucleotide phosphorylase was activated by salt although

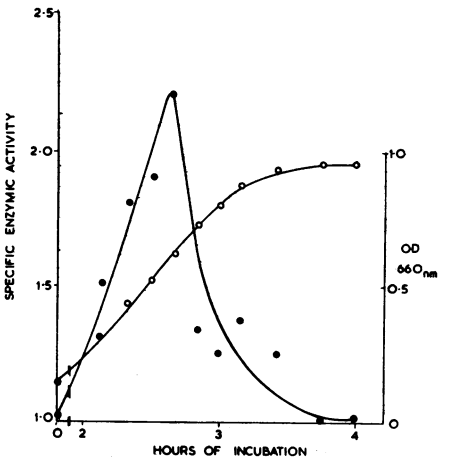
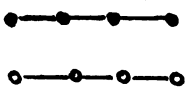


FIGURE I

VARIATION OF SPECIFIC ACTIVITY OF B.STEAROTHERMOPHILUS POLYNUCLEOTIDE PHOSPHOROLYSIS WITH GROWTH

Cell numbers were estimated from the turbidity at 660 nm. Aliquots of cell suspension were lysed ultrasonically and assayed for both phosphorolysis activity and protein concentration as described in the text.



phosphorolysis activity per mg protein

turbidity

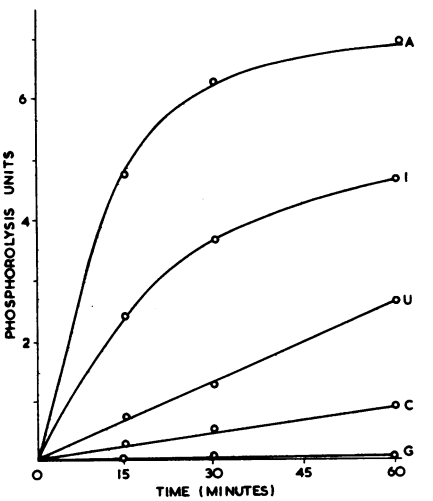


FIGURE 2

PHOSPHOROLYSIS OF POLYNUCLEOTIDES UNDER STANDARD CONDITIONS BY B.STEAROTHERMOPHILUS POLYNUCLEOTIDE PHOSPHORYLASE

the crude T.aquaticus enzyme was not. We observed an interesting effect of Ca^{++} ions on the polymerisation activity of the B.stearothermophilus enzyme, which may be of some physiological significance. Although at millimolar concentrations Ca^{++} ions inhibit both phosphorolysis and polymerisation assays, at concentrations of about 5μM, there is a selective activation of polymerisation by about three-fold. By analogy with the levels found in E.coli[17], intracellular concentrationsof Ca^{++} ions might be of this order.

The substrate specificities of the two enzymes varied through the purification procedure, becoming more selective with purification. Phosphorolysis data are presented in Figures 2 and 3. Neither enzyme showed polymerisation activity with the modified nucleotides br^8ADP[18], br^8GDP[18] or 8-azido ADP[19] even in the presence of Mn^{++} ions, and all were found to be strong competitive inhibitors. Attempts to degrade processively sheared calf thymus or T4 DNA which had been previously treated with alkaline phosphatase were also unsuccessful. Hence the enzymes from thermophilic bacteria show a rather narrower range of substrate specificities compared with polynucleotide phosphorylases from mesophilic organisms.[16] Poly (A) is synthesised and degraded rapidly by the enzyme from B.stearothermophilus and GDP is polymerised smoothly in high yield. Copolymers were obtained from GDP and other nucleoside diphosphates in which the base ratios

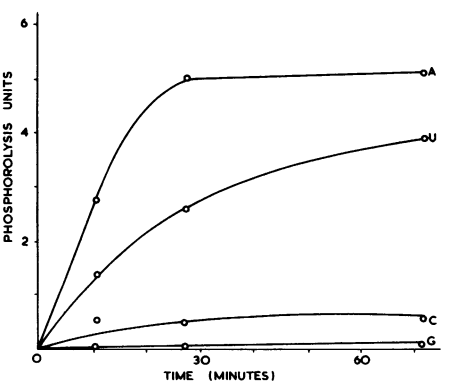


FIGURE 3
PHOSPHOROLYSIS OF POLYNUCLEOTIDES UNDER STANDARD CONDITIONS BY T.AQUATICUS POLYNUCLEOTIDE PHOSPHORYLASE

TABLE 1

PURIFICATION OF POLYNUCLEOTIDE PHOSPHORYLASES FROM THERMOPHILIC ORGANISMS

a) B.stearothermophilus

Step	Specific Activity	Purification	Yield (%)
Cell lysate	1.2	1	100
35 - 55% ammonium sulphate	6.5	5	82
TEAE-cellulose	59	49	74
Sephadex A50	283	203	41
AcA 34 Ultrogel	603	500	41

b) T.aquaticus

Step	Specific Activity	Purification	Yield (%)
Cell lysate	0.8	1	100
35 - 55% ammonium sulphate	4	5	57
TEAE-cellulose	25	31	21

TABLE 2

KINETIC PARAMETERS FOR B.STEAROTHERMOPHILUS POLYNUCLEOTIDE PHOSPHORYLASE

a) Polymerisation

Substrate	K_m (mM)	$\%V_{max}$
ADP	2.5	100
UDP	2.9	30
CDP	2.9	27.5
GDP*	2.6	11

b) Inhibition

Substrate	K_i (mM)
br^{8}ADP*	0.21
8-azido ADP*	0.12
pyrophosphate	0.04
methylene diphosphate	0.28

Polymerisation and inhibition studies were carried out under conditions as described in text.

*Using Mn^{++} as metal cofactor, all others with Mg^{++}.

TABLE 3

COPOLYMERISATION OF [^{3}H]-GDP AND [^{14}C]-UDP

GDP:UDP* Input Ratio	(G:U) Base Ratio of High Mol. Wt. Product
10	18
20	19
30	38
40	65

*Specific radioactivities [^{3}H]-GDP 24,000 cpm/mg, [^{14}C]-UDP 26,000 cpm/mg. Reactions were carried out overnight under polymerisation assay conditions with final substrate concentrations of 10mM. Pancreatic RNase (50 µg/ml) and alkaline phosphatase (200 µg/ml) were then added and the mixture incubated at 37° for 3 hr. Deproteinisation with isoamyl alcohol: chloroform (5:2 v/v) followed by chromatography of the aqueous layer on Sephadex G25 with elution by 0.1% SDS and 0.2M KCl solution gave the products in the void volume.

correspond approximately to the input ratios of the component nucleotides. Unexplained anomalies between the input ratio and the subsequent composition of the polymer have been observed using E.coli polynucleotide phosphorylase (1), but we have not observed this with the B.stearothermophilus enzyme. Using a modification of the method of Tazawa et al,[20] we have prepared oligomers with a composition of 20 G: 1U, using [^{3}H]-GDP and [^{14}C]-UDP.

DISCUSSION

Purine nucleoside diphosphates in which the purine and the ribose are in the syn-conformation are poor substrates for polynucleotide phosphorylase[18]. As the temperature of a solution of such a purine nucleoside diphosphate is increased, rotation about the glycosidic bond should be facilitated and hence it may be that the rate of polymerisation by a thermostable polynucleotide phosphorylase

would increase with temperature. We have found that 8-bromoadenosine and 8-bromoguanosine diphosphates, which are in the syn-conformation at room temperature[18], are not substrates for polynucleotides phosphorylases from B.stearothermophilus or T.aquaticus at elevated temperatures. This lack of activity may be the result of a more rigid tertiary structure of the enzymes which might be necessary to avoid denaturation at the operating temperatures of the microorganisms in vivo. However the structural integrity of polynucleotide phosphorylases from thermophilic organisms makes them useful for physical studies. Proteolytic degradation products which contaminate most preparations from mesophilic sources have not been observed in the purification of the B.stearothermophilus enzymes, making structural studies considerably easier to accomplish. The gross amino acid composition of this enzyme does not differ markedly from those reported for E.coli and M.luteus enzymes, there being similar proportions of hydrophobic and hydrophilic amino acids. Our evidence for a tetrameric structure for B.stearothermophilus polynucleotide phosphorylase contrasts with recently published data on the quaternary structure of the E.coli enzyme[21]. Nevertheless we consider that B.stearothermophilus polynucleotide phosphorylase offers advantages for structural analysis which may well be of relevance to mesophilic enzyme.

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