INSOLUBLE DERIVATIVES OF POLYNUCLEOTIDE PHOSPHORYLASE

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1. Introduction

The polymerisation of nucleoside 5′-diphosphates using polynucleotide phosphorylase (PNPase) is a common method for the preparation of single stranded polynucleotides [1]. However, the polymerisation of nucleoside diphosphates which contain atypical bases often proceeds sluggishly or not at all [2]. Extensive degradation of high molecular weight polymer can occur due to phosphorolysis when prolonged reaction times are employed leading to heterogeneous products. We have investigated the properties on PNPase which has been attached to a number of insoluble supports and found that the properties of the enzyme have been altered to such an extent that polymerisation can occur smoothly at a pH when phosphorolysis is negligible. The amount of enzyme bound varies with the support used.

2. Materials and methods

PNPase was isolated from *Micrococcus luteus* (NCTC 2665 [3] or *Escherichia coli* [4] and was bound to either mercerised cellulose or Sepharose 4B in the following manner. The support (100 mg wet weight) was added with vigorous stirring to a solution of cyanogen bromide (100 mg) in water (4 ml), the pH being maintained at 11.0 by the addition of 2 M NaOH. After 6–8 min the suspension was filtered through a sintered glass filter, the Sepharose washed with cold 0.1 N $NaHCO_3$ (300 ml) and then added to a solution of PNPase (3.3 mg/ml) in 0.01 M collidine-HCl buffer pH 8.2 (5.0 ml). The suspension was stirred at 4° for 16 hr and filtered; the Sepharose was then washed with 500 ml portions of cold 0.1 M $NaHCO_3$, 1 M NaCl and finally water. The insolubilised PNPase was stored at 4° in water which contained 0.02% NaN_3.

Aminoalkylsilane glass beads (2 g), prepared and activated with glutaraldehyde as described by Robinson et al. [5], were added to a solution of PNPase (35 mg) in 0.01 M collidine-HCl buffer pH 8.2 (10 ml). The suspension was stirred at 4° for 2 hr, then filtered and the beads washed with water. The insolubilised enzyme was stored at 4° as above.

The amount of protein bound to insoluble support was determined by hydrolysis in 6 N HCl at 110° followed by the estimation with ninhydrin of the total amino acids liberated [6]. Phosphorolysis was assayed by following the incorporation of ^{32}P-labelled phosphate into ADP [7]; polymerisation was assayed by the incorporation of ^{14}C-labelled ADP into poly A as described previously [1]. Sedimentation constants of polynucleotides were estimated by gel electrophoresis on 3% acrylamide gels by comparison with known samples.

3. Results

PNPase has been bound to cyanogen bromide-activated cellulose in earlier work [3]. However, this reaction was carried out in Tris buffer and it is probable that the amino group of the tris-(hydroxymethyl)-aminomethane would react with the activated cellulose in competition with the enzyme, thereby reducing the amount of PNPase bound to the cellulose. We have found that an increased amount of PNPase is bound to cellulose if a collidine-HCl buffer is used in the

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Table 1
Properties of insolubilised PNPase.

Enzyme derivative	Protein content (mg/g support)	pH Optima		$10^4 K_m$ (M) ADP polymerisation
		Phosphorolysis	Polymerisation	
a) *M. luteus*				
Free	–	8.1	9.0	1.0
Cellulose (Tris)	43	7.7	9.25	1.0
Cellulose (collidine)	121	7.7	9.25	1.0
Sepharose	475	8.0	9.25	4.0
Glass	68	8.5	9.4	0.5
b) *E. coli*				
Free	–	7.8	9.0	1.25
Sepharose	430	7.8	9.25	1.67

All polymerisation and phosphorolysis assays were carried out in a jacketed, stirred vessel at 30° in 2 mM $MgCl_2$, 0.5 mM EDTA, 0.15 M Tris buffer.

coupling reaction in place of a Tris buffer. Steric hindrance of the nitrogen atom in collidine by the 2- and 6-methyl groups would prevent the base from reacting with the activated cellulose and an increased binding of PNPase to the support would result. If Sepharose is used in place of cellulose as a support,

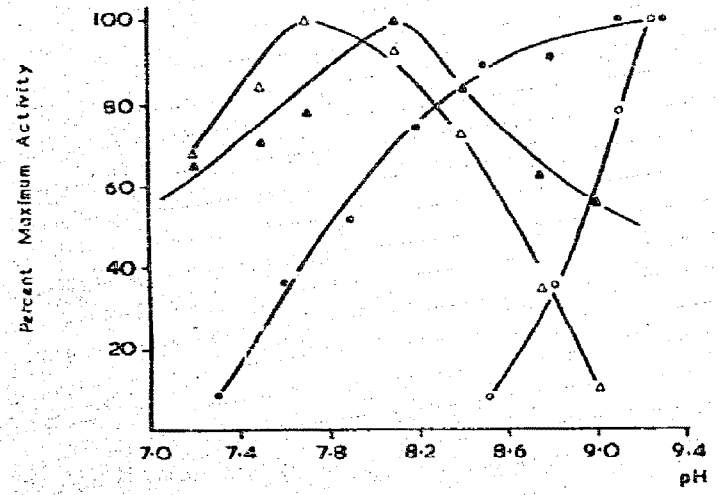

Fig. 1. pH Profiles for polymerisation and phosphorolysis reactions catalysed by PNPase from *M. luteus*. Polymerisation: free enzyme (●—●—●), cellulose bound enzyme (○—○—○). Phosphorolysis: free enzyme (▲—▲—▲), cellulose bound enzyme (△—△—△).

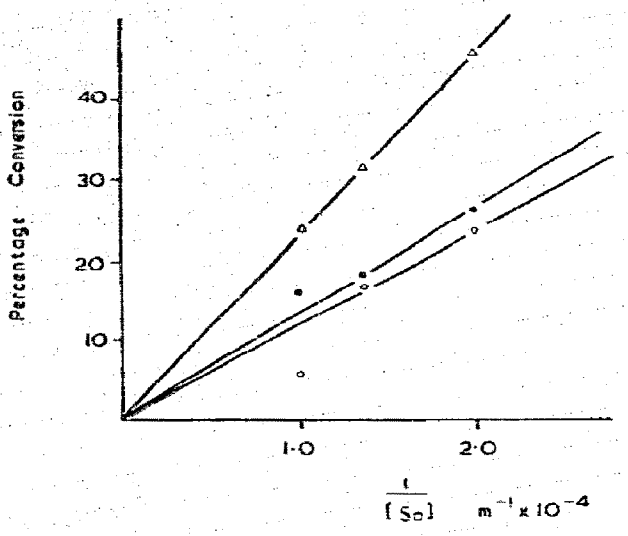

Fig. 2. Polymerisation of nucleotides by a packed bed of Sepharose bound *E. coli* PNPase as described in text (flow rate 0.5 ml/min).

the amount of bound protein increases considerably. The properties of PNPase attached to various supports are given in table 1.

With soluble PNPase from either *E. coli* or *M. luteus* the pH profiles for the polymerisation and phosphorolysis reactions are broad with maxima occurring around pH 9 and 8, respectively. On attachment to either cellulose or Sepharose the pH profiles are sharpened, enabling conditions to be found when polymerisation of ADP takes place smoothly and phosphorolysis of poly A was negligible (fig. 1). This change in shape of the pH profiles did not occur to an appreciable extent when the *M. luteus* enzyme was bound to glass.

The values of the apparent Michaelis constant of cellulose bound PNPase was similar to that for the soluble enzyme while that for the glass bound enzyme was lower. One factor in the latter case may be that charged groups on the surface of the enzyme affect the approach of the substrate to the PNPase [8]. In the case of the Sepharose derivatives, gradients of substrate concentration could be established in the Sepharose particle thereby affecting the K_m.

One problem with the preparation of polynucleotides on a large scale with insolubilised PNPase is that mechanical shear of the polymer can occur if the reaction mixture is stirred too vigorously. We have found that this problem can be overcome if a packed bed of

insolubilised PNPase is used and mechanical shear of the polymer is prevented if the eluate from each cycle through the bed is retained by means of an Amicon PF-12 ultrafilter cell with an XM-50 membrane. Polymerisation reactions were typically carried out in a jacketed column (6.7 ml Sepharose) at 30°. The substrate nucleoside diphosphate in 10 mM $MgCl_2$, 5 mM EDTA, 0.15 M Tris buffer were passed through the column and the polynucleotides could be recovered by dialysis against 0.1 M NaCl/0.001 M EDTA, 0.001 M EDTA and then water. In this manner poly A (8 S), poly C (12 S) and poly 5-chloro C (6 S) [1] were prepared in high yield after one cycle through the bed (fig. 2). There is an inverse relationship between substrate concentration and percentage conversion; furthermore, the flow rate through the packed bed is also an important factor [9]. Further studies on this polymerisation reaction are in progress.

References

[1] M.A.W. Eaton and D.W. Hutchinson, Biochemistry 11 (1972) 3162.
[2] M. Ikehara, I. Tazawa and T. Fukui, Biochemistry 8 (1969) 736.
[3] C.H. Hoffman, E. Harris, S. Chodroff, S. Michelson, J.W. Rothrock, E. Peterson and W. Reuter, Biochim. Biophys. Res. Commun. 41 (1970) 710.
[4] K.H. Scheit, private communication.
[5] P.J. Robinson, P. Dunnill and M.D. Lilly, Biochim. Biophys. Acta 242 (1971) 659.
[6] E.M. Crook, K. Brocklehurst and C.W. Wharton, Methods in Enzymology 19 (1970) 963.
[7] F.R. Williams and M. Grunberg-Manago, Biochim. Biophys. Acta 89 (1964) 66.
[8] W.E. Hornby, M.D. Lilly and E.M. Crook, Biochem. J. 107 (1968) 669.
[9] M.D. Lilly, W.E. Hornby and E.M. Crook, Biochem. J. 100 (1966) 718.