Polynucleotide Phosphorylase Covalently Bound to Cellulose and Its Use in the Preparation of Homopolynucleotides.

Carl H. Hoffman, Elbert Harris, Susan Chodroff, Stuart Michelson, John W. Rothrock, Elwood Peterson and William Reuter

Merck Sharp & Dohme Research Laboratories
Division of Merck & Co., Inc.
Rahway, New Jersey 07065

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Summary: A readily prepared, crude concentrate of polynucleotide phosphorylase (PNPase) has been covalently bound by the method of Porath to cyanogen bromide-activated cellulose; this is the first report of a synthesizing enzyme bound to a solid support. The bound enzyme had good activity and stability; more than forty consecutive polymerization cycles of nucleoside diphosphates have been carried out with a single preparation to give consistently good yields of stable polymers, and these were readily separated from the bound phosphorylase without tedious phenol extractions.

The discovery that poly (I:C) is a potent inducer of interferon (1) has generated a demand for large amounts of the required high molecular weight homopolymers. They may be prepared by enzymatic polymerization of the nucleoside-5'-diphosphates with PNPase[a]. When large amounts of these homopolymers are required the purification of the enzyme and separation of the product becomes a formidable task.

This paper describes a simple procedure for the isolation of the crude enzyme and its attachment to cellulose by the Porath cyanogen bromide technique (2). The resulting bound enzyme has shown high activity and stability and has afforded homopolymers of excellent quality.

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[a] Polynucleotide phosphorylase, (systematic name: polyribonucleotide: orthophosphate nucleotidyl transferase, EC 2.7.7.8) is abbreviated PNPase.

Experimental

Assays - PNPase activity[a] was measured by the incorporation of ^{14}C-labeled ADP into 5% trichloroacetic acid-precipitable polynucleotides after incubation for 15 min at 37°. The unit was defined as equivalent to 1 micromole of polymerization per minute; this is fifteen to sixty-fold larger than some others which have been used (3). Protein and inorganic phosphate were determined by conventional methods (4, 5).

Enzyme - Isolation was carried out below 5° except during lysis. M. luteus[b] cell slurry, about 100 g dry weight/ l in 0.5% NaCl solution at pH 8, was treated[c] with 3.5×10^6 units of lysozyme/l for 12 min at 37°, cooled rapidly, mixed with one-half volume of a cold saturated $(NH_4)_2SO_4$ solution and centrifuged; the supernatant was dialyzed overnight against PNP-buffer[d]. The retentate was treated with 0.66 volume of 2BA ethanol[e] which had been pre-cooled below -20°. After aging overnight, the precipitate was separated by centrifugation, resuspended in 100 ml of PNP-buffer, and dialyzed against the same buffer; the retentate was then clarified by centrifugation. This supernatant could be kept in the frozen state for many weeks. A batch from cells of good activity produced approximately 100 units from 100 g (dry wt. equiv.) of cells.

Enzyme Binding - All operations were carried out under sterile conditions and all substrate solutions contained 0.02% NaN_3 as bacteriostat (6). Whatman Cellulose Powder CF-1 (83 g) was swollen overnight in 5 M sodium

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[b] ATCC 4698, formerly known as Micrococcus lysodeikticus.
[c] We are indebted to Mr. W. Subjack for suggesting that lysis could be done on a large scale and supervising the isolation in the Pilot Plant.
[d] PNP-buffer is 0.01 M tris chloride, pH 8.2, 10^{-3} M $MgCl_2$, 10^{-3} M mercaptoethanol.
[e] 2BA ethanol is 0.5% benzene by volume in absolute ethanol.

hydroxide and washed exhaustively with water by decantation. The slurry (50 g dry weight) was treated at 10° for 45 min with an aqueous solution of 100 g of cyanogen bromide maintained at pH 10.5 by the intermittent addition of sodium hydroxide. After transfer to six 500 ml centrifuge bottles, the slurry was washed free of excess reagent and then rinsed with PNP-buffer by centrifugation and decantation at 5°.

Enzyme stock solution (500 units, 12.5 g of protein, specific activity 0.04 u/mg) was sterilized by Millipore filtration and magnetically stirred with the cellulose in the six centrifuge bottles overnight at 5°. Following centrifugation, the protein-free supernatant was discarded and the residue washed repeatedly with cold PNP-buffer before use.

Polymerization - A solution of inosine diphosphate (120 mmoles by uv absorption) in two liters of sterile 0.15 M tris buffer, pH 8, 0.01 M in $Mg(OAc)_2$, 0.001 M in EDTA, and containing 0.02% NaN_3, was distributed among the six bottles and incubated at 37° with magnetic stirring. After 2.5 hrs, when phosphate analysis indicated about 60% polymerization, the bottles were chilled, centrifuged, decanted and charged with a second cycle of substrate. This supernatant from the initial cycle was routinely discarded because it was thought that it might contain foreign RNA introduced with the enzyme extract. Subsequent cycles were filtered, dialyzed against pH 7 sodium citrate and EDTA, then water, and lyophilized. The product contained no detectable foreign bases (7). A typical polymerization cycle afforded 22.5 g (55 mmoles of base residues, 46% of theory) of poly I, S_{20} = 15, uv max = 248 nm, $E^{1\%}_{1\ cm}$ = 255 (in 0.15 M NaCl, 0.006 M phosphate pH 7.0).

Polymerization of CDP in a similar way gave an average of 26.7 g (68 mmoles of base residues, 57% of theory) of poly C per cycle, uv max = 268 nm, $E^{1\%}_{1\ cm}$ = 165.

Discussion - Mercerized cellulose was chosen as the support for the enzyme since the less open matrix of most cross-linked dextrans might interfere with diffusion of the long polymer chains.

The bound enzyme retained 26% of the activity of the starting free enzyme, as measured by the rate of inorganic phosphate release. This activity remained undiminished and the quality of the polymers it synthesized remained unchanged over a period of six months during which the enzyme was stored cold and intermittently employed in a total of forty polymerization cycles. As with free enzymes (8), short time cycles afforded polymers of high molecular weight (S_{20} = 12-15) while overnight incubations effected a redistribution of chain lengths and reduced the average sedimentation coefficient to 6 or 7.

The sedimentation coefficients of the poly I and poly C, and the T_m, hyperchromicity, viscosity, sedimentation characteristics and biological activity of the complexes prepared from these homopolymers were indistinguishable from those of polymers prepared with conventional PNPase.

Poly A, S_{20} = 14.0, and poly U, S_{20} = 13.7, have been prepared with bound enzyme in a similar way. Active PNPase bound to cellulose has also been prepared from purified M. luteus preparations and partially purified E. coli fractions.

The facile separation of the enzyme from the formed polymers by simple centrifugation, rather than tedious, emulsion-forming phenol extractions, is a major advantage of this new approach. Polymers prepared in this way showed the same stability of sedimentation coefficient and uv absorbance after incubation for 20 hr at 37° as samples of poly I and poly C prepared in the conventional manner with highly purified free enzyme and worked up by exhaustive phenol extraction.

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