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ALTERATION OF PROCESSIVE MECHANISM OF NATIVE POLYNUCLEOTIDE PHOSPHORYLASE BY FIXATION TO SOLID MATRIX

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SUMMARY

Native E.coli polynucleotide phosphorylase can be covalently bound to BrCN activated Sepharose. The Sepharose bound enzyme retains 70 % of its initial activity in polymerisation of nucleoside diphosphate. The Km of the enzyme for the polymerisation reaction in comparison to the soluble enzyme is not affected by its linkage to a solid matrix. The phosphorolysis of an hexanucleotide by the Sepharose-bound enzyme is not affected either. However, the rate of phosphorolysis of a long chain polynucleotide is dramatically altered. The Km values for poly(A) or poly(U) are increased by two orders of magnitude. The decrease of affinity for polymeric substrate is accompanied by a significant modification of the known processive mechanism characteristic of the native soluble enzyme.

INTRODUCTION

During the recent years, studies on the insoluble matrix bound enzymes have been largely developped (1-2). The fixation of polynucleotide phosphorylase to a solid support and its application to the synthesis of polynucleotides have also been investigated (3-5). Nevertheless, the mechanism of action of the insoluble enzyme was not investigated. Soluble native polynucleotide phosphorylase is known to catalyse the phosphorolysis of a long polynucleotide chain or the polymerisation of nucleoside diphosphates to a polynucleotide in a processive mechanism (6-9). That is to say that the enzyme does not dissociate from the polymeric substrate during the degradation reaction or during the elongation process. On the other hand, when polynucleotide phosphorylase is altered by proteolytic degradation, the

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mechanism of the enzyme is no more processive (10-11). The modification of the processive mechanism observed with the degraded enzyme has been shown to be the result of the loss of the polynucleotide binding site whereas the catalytic center does not seem to be altered (12). Since the agarose bound polynucleotide phosphorylase keeps its activity for both polymerisation and phosphorolysis, it is interesting to see whether the covalent fixation of the enzyme to a solid matrix could modify its mode of action. The findings of the present work show that the mechanism of phosphorolysis of long polynucleotide chain is indeed modified whereas the polymerisation reaction does not seem to be affected.

MATERIALS AND METHODS

Chemicals were obtained from the following sources : nucleoside diphosphates (ADP, UDP) from Yamasa (SHOYU, Japan) ; polyribonucleotides (poly(A) poly(U)) from CHOAY Laboratories (Paris, France) ; cyanogen bromide activated Sepharose 4B from Pharmacia (Uppsala, Sweden) ; (^{32}P) inorganic orthophosphate from CEN Saclay (France) ; (^{3}H) CDP from Radiochemical center (Amersham, Great-Britain) ; bovine pancreatic ribonuclease and bacterial alkaline phosphatase from Biochemical Corporation.

E.coli polynucleotide phosphorylase was purified as previously described (13). The specific activity of the enzyme was 700 units/mg of protein (1 unit corresponds to 1 µmole ADP polymerised at pH 8.0, 37°C during 60 minutes).

Polynucleotide phosphorylase was assayed by polymerisation and phosphorolysis. Polymerisation of nucleoside disphosphates was followed by the liberation of inorganic phosphate determined by the micromethod of Chen et al. (14).

Phosphorolysis of polynucleotide was followed by the measurement of radioactive nucleoside diphosphate released in the presence of (^{32}P) phosphate. These two compounds were separated by solvants phase separation under conditions described (15).

Poly(A) (^{3}H)C was prepared as described (6) by combining the action of three enzymes : polynucleotide phosphorylase, pancreatic RNase and alkaline phosphatase ; these two last enzymes being covalently bound to BrCN activated agarose (A. Guissani and M.N. Thang, unpublished). The poly(A) (^{3}H)C obtained was purified on a Sephadex G50 column. The polymer did not contain internal CMP according to the analysis of nucleotide composition after alkaline hydrolysis. The average chain length determined by the ratio of ADP/CDP (calculated from the $A_{259\ nm}$ and the specific radioactivity) was 300. The polymer was designed as $(A)_{300}(^{3}H)C$.

Fixation of polynucleotide phosphorylase to the Sepharose

The BrCN activated Sepharose was swollen and prewashed with 1 mM HCl to remove the protecting Dextran before coupling. About 185 units of purified

polynucleotide phosphorylase in 0.1 M sodium bicarbonate buffer, pH 8.9 containing 0.25 M NaCl were mixed with a suspension of activated Sepharose (an equivalence of 500 mg dry weight) in the same bicarbonate NaCl buffer to make a final volume of 3 ml. The mixture was gently stirred overnight at 4°C. The remaining active groups on the Sepharose were neutralized with ethanolamine, 1 M, pH 8.6 for 2 hours. The enzyme-Sepharose was washed with the bicarbonate buffer containing 1 M NaCl, then with 0.1 M Tris-HCl buffer, pH 8. The Sepharose was recovered by filtration on cintered glass filter over vacuum. In those conditions of fixation, no free proteins, nor soluble enzymatic activity were found in the supernatant after overnight mixing. It was concluded that 100 % of the enzyme was covalently linked to the activated Sepharose. On this basis, 70 % of initial units were recovered (see Fig. 1) after fixation. The preparations contained usually one unit per 10 mg wet weight of Sepharose.

RESULTS

Polymerisation of nucleoside diphosphates

The Sepharose bound enzyme, when incubated under standard conditions described for polymerisation of nucleoside diphosphates by soluble polynucleotide phosphorylase, catalysed the synthesis of homo and co-polymers from monomeric substrates without primer. The reaction started without a significant lag phase as in the case of native enzyme. When the same amount of enzyme, expressed as protein content was used, the rate of ADP (or UDP) polymerisation catalysed by Sepharose bound enzyme was about 70 % of that observed with soluble enzyme from the same batch used for fixation (Fig. 1). However, the plateau of the kinetic curve of the matrix-bound enzyme attained 70 % of input substrate as compared to a value of around 50 % usually obtained with soluble enzyme under the conditions used.

The fact to bind covalently the enzyme to an insoluble matrix did not markedly change the kinetic constants in the polymerisation reaction. As shown in Table 1, the apparent Km values for ADP and for UDP are the same for both soluble and bound enzyme.

The chain lengths of polynucleotides synthesized with Sepharose-bound polynucleotide phosphorylase were comparable (but not necessarily identical) to that made by soluble enzyme according to the S_W values of a great number of polymer batches synthesized during several years in this laboratory and in others. These values ranges from 8S to 12S for poly(A), 7S to 12S for poly(I), 6.5S to 8S for poly(C) and 6S to 8S for poly(U).

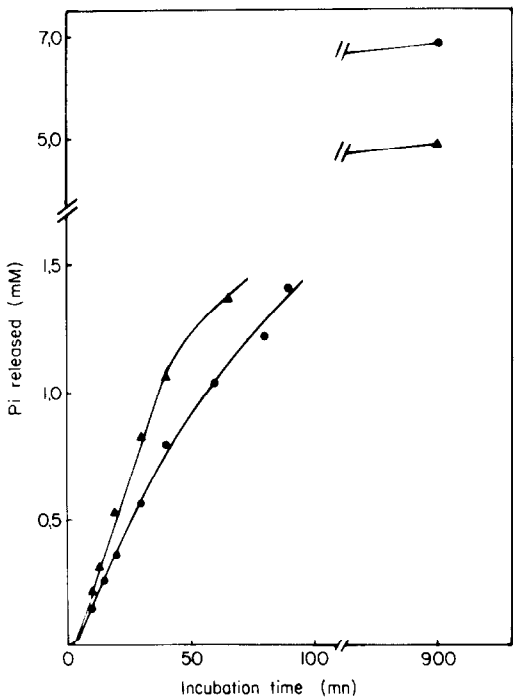

Figure 1. Polymerisation activity of polynucleotide phosphorylase bound on Sepharose. The reaction mixture (1 ml) contained : 100 mM Tris-HCl, pH 8, 10 mM ADP, 5 mM $MgCl_2$, 0.1 mM EDTA and 2 µg of soluble enzyme or 13 mg of enzyme-Sepharose corresponding to 2 µg of the same soluble polynucleotide phosphorylase with a specific activity of 700 u/mg. The mixture was incubated at 37°C. Aliquots were withdrawn at intervals indicated. The reaction was followed by the liberation of phosphate, determined according to Chen et al. (14). ●—● Sepharose polynucleotide phosphorylase ; ▲—▲ soluble polynucleotide phosphorylase.

TABLE 1

Apparent Michaelis constants of polymerisation of ADP and UDP by soluble and matrix bound polynucleotide phosphorylase

Substrate	Km	
	soluble enzyme	Sepharose-enzyme
ADP	2×10^{-3} M	2×10^{-3} M
UDP	6.7×10^{-3} M	6.7×10^{-3} M

Polymerisation assays were performed as described under Fig. 1. The concentrations of nucleotides varied from 0.3 mM to 20 mM by keeping a constant ratio of nucleotide/Mg^{++} = 2. The concentration of soluble enzyme was 1 µg/ml and that of Sepharose bound enzyme was 20 mg Sepharose/ml. The Km values were determined from the Lineweaver-Burk plots.

The activity of the bound enzyme is quite stable. No significant loss of polymerising activity was observed after recycling four to five times

the same batch of Sepharose-bound enzyme in synthesis medium. The enzyme kept in "wet form" at 2°C in the presence of 0.02 % azide, retained more than 50 % of its initial activity after three months of storage.

Phosphorolysis

The fixation of polynucleotide phosphorylase to Sepharose did affect, on the contrary, the kinetic behaviour of the enzyme in the phosphorolysis of polynucleotides. The Michaelis constants for $(A)_{500}$ and $(U)_{300}$ were 1.2×10^{-8} M and 2×10^{-8} M for the soluble enzyme (Table 2). However, the phosphorolysis of oligonucleotides was not affected. The apparent Km for $U(pU)_5$ is the same (8.3×10^{-4} M) in the two cases.

The dramatic change of affinity for the polymeric substrate, reflected by an increase of two orders of magnitude of the Km, might indicate an alteration of the mechanism of the enzyme in the degradative reaction of high molecular weight polynucleotides. As we initially suggested (6) the synchronous or the processive mechanism could be easily distinguished by following the phosphorolysis of the homopolynycleotide terminated in the 3'OH end with another single nucleotide labelled with radioactive element, a poly(A) (^{3}H)C for instance. The relation between the percentage of ADP

TABLE 2

Comparison of apparent Michaelis constants of phosphorolysis for long chain polynucleotides and oligonucleotides

	Km	
	soluble enzyme	Sepharose-bound enzyme
$U(pU)_5$	1.3×10^{-4} M	1.3×10^{-4} M
$(A)_{\overline{500}}$	1.2×10^{-8} M	1.6×10^{-6} M
$(U)_{\overline{300}}$	1.2×10^{-8} M	1.3×10^{-6} M

The rate measurements were drawn from kinetic curves of phosphorolysis assayed as described in Methods. The oligonucleotide concentrations varied from 0.02 to 1 mM (in monomer). The polymer concentrations varied from 0.01 mM to 0.5 mM (in monomer). The enzyme concentrations were 1 µg/ml for soluble enzyme and 20 mg Sepharose/ml for Sepharose-bound enzyme. The Km values were determined from the Lineweaver-Burk plots.

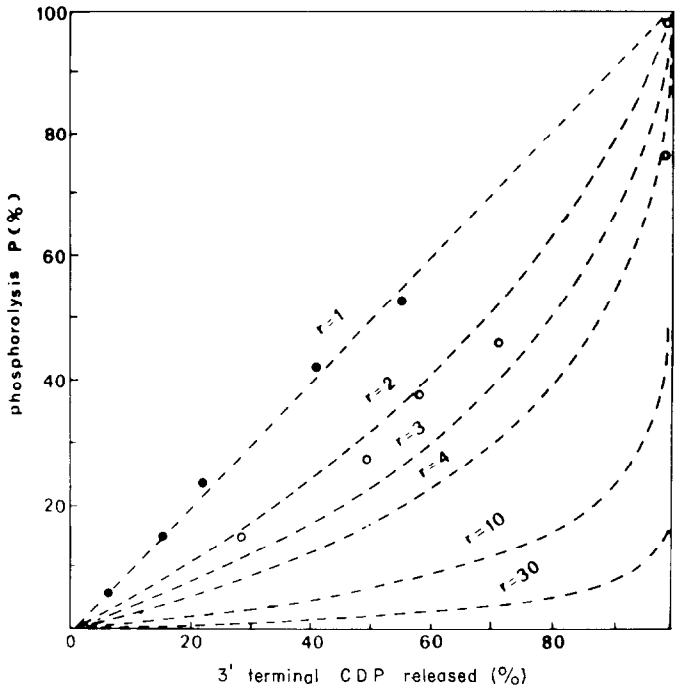

Figure 2. The basic incubation mixture contained 100 mM Tris-HCl pH 8.0, 0.1 mM EDTA, 1 mM $MgCl_2$, 0.4 mM (A)$_{300}$-(^{3}H)C (in mononucleotide concentration) and 10 mM PO_4^{3-}. To 1 ml of this mixture, 0.4 units of soluble enzyme or 1.5 unit of Sepharose-bound enzyme were added. Each ml reaction mixture was then divided into two egal parts. One part of 500 ml received (^{32}P)PO_4^{3-} made the final specific activity of 1.10^6 cpm/µmole PO_4^{3-} in order to label the nucleoside diphosphate released by phosphorolysis. The counterpart 500 µl without addition of (^{32}P) was used to follow the liberation of the (^{3}H)CDP. Incubation was carried out at 37°C and aliquots were withdrawn at intervals of 4, 6, 10, 15 and 30 minutes. Determination of (^{32}P) nucleotide and (^{3}H)CDP was performed as described previously (6). The percentage of phosphorolysis was defined as the ratio of (^{32}P) nucleotide released to initial concentration of polymer, i.e. 0.4 mM. The percentage of terminal C liberated is the ratio of soluble radioactivity to the initial total tritium counts. The percentage of phosphorolysis was plotted against the percentage of CDP released (11). • soluble polynucleotide phosphorylase ; o Sepharose-polynucleotide phosphorylase.

and CDP released determined during the course of phosphorolysis, indicates the degree of progressiveness or the synchronicity of the mechanism. The results of such experiments are plotted in Figure 2 using the representation proposed by A. Guissani (11). In this representation, curves were obtained by plotting the total number of nucleotides released (% of phosphorolysis) against the number of 3' terminal nucleotide released, for each chosen value of r, which is the number (or times) of removal of the enzyme from its substrate during the complete phosphorolysis.

It is apparent that the soluble native enzyme, degraded the poly(A) (^{3}H)C in a processive manner since the experimental points were alined on the mediane of the diagram with r = 1. The bound enzyme, however, is no more processive since the experimental data are located in between the theoretical curves with r = 2 and 3. The drop of the affinity of the matrix bound enzyme for long chain polymers was thus accompanied with a loss of the processiveness of its mode of action. Nevertheless, the mechanism does not become completely synchronous.

DISCUSSION

As it can be seen from our results, the fixation of polynucleotide phosphorylase to a solid matrix such as Sepharose reveals very useful for the manufacture of polynucleotides. The high field of synthesis (70 %), the facility in separation of polymers from enzyme after polymerisation, the possibility in recycling the same preparation of enzyme in multiple times of synthesis and the high stability of enzymatic activity are certainly the advantages of this kind of matrix-bound enzyme. The fixation of the enzyme to BrCN activated Sepharose is complete. The recovery of the enzyme units is favorable since 70 % of the initial activity are conserved. The loss of about one third of the total units might be due to the inactivation of part of enzyme molecules during fixation process or/and to the change in accessibility of substrates to the active center induced by the presence of solid matrix on part of the protein. The fact that the kinetic parameters in the polymerisation and in the phosphorolysis of small oligonucleotides catalyzed by agarose-bound enzyme are quite similar to those observed with the native enzyme suggests that the active center and the oligonucleotide binding site are not the area of fixation to the agarose beads.

On the contrary, the Michaelis constants in phosphorolysis of high molecular weight polynucleotides, poly(A) and poly(U) were increased by two orders of magnitude as compared to those obtained constantly with native

soluble enzyme. The decrease of the affinity for polymeric substrate, consequent to the fixation of polynucleotide phosphorylase on Sepharose matrix, and the loss of processiveness in its mode of action, remind the behaviour of the proteolysed polynucleotide phosphorylase (11,12).

It has been inferred that proteolytic degradation of the native enzyme by E.coli protease I or by trypsine (12) affected mainly the polypeptide domain corresponding to the polynucleotide binding site. Alteration of this domain resulted in the loss of the complete processiveness (or in the increase of synchronicity) and the decrease of affinity for polynucleotide. By analogy, we postulate that the polypeptide area by which the enzyme is linked to Sepharose could be either overlapped or in the vicinity of the polynucleotide binding site which then becomes inaccessible to the polynucleotide. Such steric hindrance introduces a modification of the enzymatic mechanism in phosphorolysis as does the degradation of this polypeptide domain.

A difference however subsists between the proteolytic modification of this polynucleotide binding site and the fixation of the enzyme to Sepharose (visualized as an occupation of this domain) since a long lag phase in the polymerisation was observed with the proteolysed enzyme whereas the Sepharose-bound enzyme started the reaction like the native enzyme with a very short lag phase. We infer that degradation by proteases alters the protein to an extent in such a way that area nearby the active center could be affected. In opposition, the fixation site of the enzyme might be limited outside the active center and the oligonucleotide site. Since the fixation of the protein to the BrCN activated agarose is random, the preservation of the active center, evidenced by the recovery of 70 % of the polymerising units, indicates that this site is in some way hidden, in agreement with the model of the 3 subunits arrangement, a triangle profile with a central hole in which the active center might be located (16,17). Such model is strengthened by the previous finding (18) which indicated a free access of nucleoside diphosphate to the active center, when the polynucleotide phosphorylase linked the blue dextran Sepharose through its polynucleotide binding site.

Very recently, Yang et al. (19) from Microbiology Institute, Peking, reported that E.coli polynucleotide phosphorylase coupled to diazotized p-aminobenzene sulphonylethyl (ABSE) agarose had a Km value for ADP egal to 1.31×10^{-3} M as compared to 1.28×10^{-3} M obtained with the soluble enzyme. Yet, these authors did not compare the behaviour of both enzymes on phosphorolysis.

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