INHIBITION OF POLYNUCLEOTIDE KINASE BY AGAR, DEXTRAN SULFATE AND OTHER POLYSACCHARIDE SULFATES

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SUMMARY

In certain preparations of lambda DNA, the phosphorylation of their 5'-ends as catalyzed by polynucleotide kinase was inhibited. The inhibitor appeared to be due to traces of agar left in the DNA preparation. Of the several polysaccharides and their sulfuric acid esters tested for their inhibitory activity, all the polysaccharide sulfates are found to be strong inhibitors of the polynucleotide kinase whereas the non-sulfated polysaccharides are non-inhibitory. Since agar is sometimes used in growing the phages and dextran sulfate is often used in purifying the phages or DNA preparations, these potential inhibitors must be carefully removed from the final DNA preparations before a polynucleotide kinase reaction can be successfully carried out. However, the effect of small amounts of these inhibitors on the kinase can be counteracted by the addition of either polylysine or spermine.

Polynucleotide kinase is an enzyme which catalyzes the transfer of orthophosphate from ATP to the 5'-hydroxyl termini of polynucleotides. This enzyme was independently discovered by Richardson (1) and by Novagrodsky and Hurwitz (2,3) in T4 phage and T2 phage infected Escherichia coli B, and it has been widely used in studying the 5'-terminal structure of various nucleic acids as well as in making useful substrates for monitoring other reactions.

Inorganic phosphate (1,3) and pyrophosphate (3) have been reported to be inhibitors of polynucleotide kinase. In this communication, polysaccharide sulfates were found to be approximately 10,000 times more potent than inorganic phosphate or sulfate as inhibitors of the kinase, on a weight basis.

PREPARATIONS

The strains of lambda phage employed were lambda ind$^-$cI857 (4) and lambda c71 (5). The phages were grown and purified, and their DNA extracted and purified as described previously (4,6,7).

Polynucleotide kinase was purified according to the procedure of Richardson (1) with slight modification (7). The enzyme used for the experiments reported here had a specific activity of 20,000 to 30,000 units per mg.

RESULTS

Phosphorylation of the 5'-termini of lambda DNA - Purified lambda DNA was treated with alkaline phosphatase to remove the 5'-phosphate (7) and was used as substrate in a polynucleotide kinase catalyzed reaction. Results in Table I show that whereas the lambda DNA preparation 1 (DNA-1) accepted the theoretical amounts of ^{32}P, DNA-2 accepted none. Assay of the biological activity of the DNA by an infectivity assay (not given here) showed that both DNA preparations gave a comparable number of plaques per A_{260} of DNA indicating that the 5'-terminated cohesive ends are present in both preparations (6,7,8). The presence of an inhibitor of polynucleotide kinase in DNA-2 was suspected and this was confirmed by the observation that the phosphorylation of DNA-1 was strongly inhibited after the addition of small amounts of DNA-2 (Expt. 2). DNA-1 differs from DNA-2 in two aspects. First, the genetic background of the phages from which these DNA preparations were extracted were different. Second, the method of growing the phages was different: DNA-1 was extracted from phage lambda cI857 grown in liquid medium and DNA-2 was extracted from phage lambda c71 grown in agar containing solid medium. Since the main difference in the composition of the two growth media was agar, it was suspected as the inhibitor. Indeed, addition of small amounts of agar to the polynucleotide kinase reaction mixture resulted in strong inhibition of the phosphorylation of both DNA-1 and a partial digest of salmon sperm DNA (Expt. 3 and 4). It may be estimated that approximately 1500 μg of DNA-2 was obtained from a phage preparation starting with 500 ml of soft agar containing medium, it may be calculated that approximately 0.003% of the original agar was present in the final DNA-2 preparation.

Effect of polysaccharides and polysaccharide sulfates on polynucleotide kinase - Agar is a sulfuric acid ester of a complex polysaccharide, composed

TABLE I

Phosphorylation of the 5'-Termini of DNA and Inhibition of Polynucleotide Kinase by Agar

The reaction mixture (0.3 ml) contained 70 mM Tris buffer, pH 7.6, 12 mM $MgCl_2$, 10 mM 2-mercaptoethanol, 0.03 mM [γ-^{32}P] ATP (1 x 10^9 cpm per μmole), and DNA as indicated. Ten units of polynucleotide kinase were used for Experiments 1, 2, 3, and 0.1 unit for Experiment 4. After incubation at 37° for 60 min. the labeled DNA was precipitated with perchloric acid, washed, filtered and counted (7).

Expt.	DNA preparation*	μg DNA per tube	μg agar added	Moles of ^{32}P incorp/ 10^5 moles DNA-P**	% inhibition
1	DNA-1	50		2.2	0
	DNA-2	50		0	100
2	DNA-1 + DNA-2	50 + 5		1.0	55
	DNA-1 + DNA-2	50 + 15		0.4	82
3	DNA-1	50	-	2.1	0
	"	50	0.3	0.9	57
	"	50	1.0	0.3	86
4	Salmon DNA digest	10	-	1200	0
	"	10	0.3	370	69
	"	10	1.0	60	95

* Lambda DNA preparation 1 (DNA-1) was purified from lambda cI857 phage and lambda DNA preparation 2 (DNA-2) was purified from lambda c71 phage. Both preparations had been treated with alkaline phosphatase to remove the 5'-phosphate. The salmon DNA digest used in Experiment 4 was prepared by digesting native salmon sperm DNA with micrococcal nuclease until 30% of the DNA was converted to acid-soluble products (1). The DNA digest was extensively dialyzed against 1 M KCl and then against 0.02 M KCl before use.

** The molecular weight lambda DNA was taken as 3.3 x 10^7, therefore, 1 mole of lambda DNA contains 10^5 moles of DNA-P (or 10^5 moles of mononucleotides). From the phosphorylation data, the average molecular weight of DNA in the salmon DNA digest was calculated to be 6 x 10^4. The values for incorporation have been corrected for blank values obtained in the absence of the kinase (7).

mainly of galactose units. Since commercial agar is not highly purified, it was of interest to determine whether polysaccharide sulfate was responsible

for the inhibition of the polynucleotide kinase and if so, whether the polysaccharide portion or the sulfate group was primarily responsible. Agar can be separated into agaropectin and agarose components and the latter contains almost no sulfate groups (10,11). Agarose (with less than 0.1% S) is much less inhibitory than agar (with 0.84% S, from Difco Company) suggesting that it is the sulfate group of agar which is responsible for the inhibition of polynucleotide kinase (Table II). This interpretation is confirmed by comparing the effect of highly purified dextran sulfate and dextran on the kinase. Dextran sulfate contains approximately twenty times as much sulfur and possesses approximately twenty times stronger inhibitory activity on the kinase as compared to agar, whereas dextran contains no sulfur and is non-inhibitory even at much higher concentrations. Heparin has a high sulfur content and it is a strong inhibitor of the kinase. In fact, the extent of inhibition appears to be approximately proportional to the sulfur content of these polysaccharide sulfates, independent of their molecular weights (2×10^4 for heparin and 3.6×10^7 for amylopectin sulfate).

Since these polysaccharide sulfates are all polyanions at pH 7.6, the pH of the kinase reaction mixture, other polyanions were tested for their action on the kinase. Hyaluronic acid and polygalacturonic acid at much higher concentrations showed little or no inhibition on the kinase activity. Lipopolysaccharide isolated from Salmonella typhimurium (gift of Drs. Falaschi and Kornberg, see reference 14) was not inhibitory at a concentration of 10 μg per ml.

Comparison of the inhibitory action of dextran sulfate with inorganic sulfate or phosphate - It was concluded from the data just presented that it is the sulfate group on the polysaccharide rather than the polysaccharide itself which is responsible for the inhibition of polynucleotide kinase. Inorganic sulfate was then tested as a possible inhibitor. Although Na_2SO_4 is an inhibitor of polynucleotide kinase it is much less inhibitory than dextran sulfate. At least 10,000 times higher level (on weight basis) of Na_2SO_4

TABLE II

Effect of Polysaccharides and Polysaccharide Sulfates on Polynucleotide Kinase

The incubation conditions were the same as in Table I, except that the specific activity of labeled ATP was lowered to 1 x 10^8 cpm per μmole. In each tube 8 μg of the salmon DNA digest and 0.1 unit of polynucleotide kinase were used. Incubation was at 37° for 30 min.

Additions	% Sulfur	μg per ml	Counts/min incorporated	% inhibition
None	-	-	10,100	0
Agar	0.84	1	5,100	50
		3	1,800	82
Agarose	<0.1	3	9,600	4
		20	8,300	17
Dextran sulfate*	17	0.06	2,000	80
		0.3	240	99
Dextran*	0	20	10,000	0
Amelopectin sulfate*	14.7	0.06	2,800	72
		0.3	500	95
Amelopectin*	0	3	9,900	0
Heparin**	13-15	0.06	3,200	68
		0.3	1,300	87
Hyaluronic acid*	0	3	9,000	10
Polygalacturonic acid**	0	3	9,500	5

* Gifts of Dr. P.S. Cammarata (12). Dextran sulfate and dextran were also purchased from Pharmacia Co. The average molecular weight of amylopectin sulfate is 3.6 x 10^7, that of the two samples of dextran sulfate is 2 x 10^6 and 5 x 10^5. The % inhibition by 0.02 μg of dextran sulfate is the same with both samples.

** Heparin and polygalacturonic acid was purchased from Sigma Co., and Mann Research Laboratories, respectively.

(K_i = 600 μg/ml) than dextran sulfate (K_i = 0.05 μg/ml) was needed to produce the same inhibition. Therefore, the attachment of the sulfate group to the

TABLE III

Reversal of Agar or Dextran Sulfate Inhibition of Polynucleotide Kinase by Poly-L-Lysine or Spermine

The incubation conditions were the same as in Table II. The order of addition is as follows: Buffer, cofactors and salmon DNA digest in a mixture; compound listed under First Addition; compound listed under Second addition; polynucleotide kinase.

First Addition	μg per ml	Second Addition*	μg per ml	Counts/min incorporated	% inhibition
None	-			8,000	0
Dextran sulfate	0.15			1,100	86
"	0.15	Polylysine	0.15	2,600	67
"	0.15	"	0.30	7,800	3
"	0.15	Spermine	0.90	4,600	43
"	0.15	"	1.50	7,400	7
None	-			8,400	0
Agar	3.0			900	90
"	3.0	Poly-L-lysine	1.0	6,200	26
"	3.0	"	3.0	7,600	9

* Poly-L-lysine and spermine were purchased from Sigma Co. After the addition of these compounds, the reaction mixture was preincubated at 37° for 15 min. to allow them to react with the inhibitor before polynucleotide kinase was added. The polyamines added separately to the enzyme assay did not affect the polynucleotide kinase activity.

dextran or other polysaccharide molecules resulted in a much more potent inhibitor of the kinase. It is likely that the dextran portion of the dextran sulfate participates in increasing the affinity between the sulfate groups

and the enzyme molecule. A second difference between dextran sulfate and Na_2SO_4 is that the former is a non-competitive inhibitor with respect to both DNA and ATP whereas the latter is non-competitive with respect to DNA but is a competitive inhibitor with respect to ATP.

The K_i for inorganic phosphate as an inhibitor of the kinase as well as the mode of inhibition appears to be similar to that of inorganic sulfate ($K_i = 4 \times 10^{-3}$ M).

DISCUSSION

Polysaccharide sulfates are found to be potent inhibitors of polynucleotide kinase. Since microgram levels can give strong inhibition of the polynucleotide kinase, it is essential that the DNA preparations be sufficiently free of the polysaccharide sulfates. If the phage was concentrated with the dextran sulfate-polyethylene glycol two-phase system (13), or if the DNA was purified with dextran sulfate-methylcellulose system (13), the dextran sulfate must be removed. However, the effect of small amounts of these inhibitors on polynucleotide kinase can be counteracted by the addition of either polylysine or spermine (Table III). Since these inhibitors are polyanions at neutral pH, it is not surprising that their inhibitory effect can be reversed by the addition of cationic compounds such as polylysine and spermine. A similar neutralization effect of polyamines on a lipopolysaccharide inhibitor of a DNA methyl transferase has been reported (14).

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