POLYNUCLEOTIDE CELLULOSE AS A SUBSTRATE FOR A POLYNUCLEOTIDE LIGASE INDUCED BY PHAGE T4*

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Enzymes which catalyze the covalent joining of interrupted deoxyribopolynucleotide strands of a bihelix have been identified and purified on the basis of several different techniques. The change in sedimentation after the closure of a cohered λ DNA circle (Gellert, 1967), the change in chromatographic properties of a non-covalent λ DNA dimer after sealing of the interruption (Zimmerman, Little, Oshinsky, and Gellert, 1967), and the protection of a ^{32}P-labeled 5'-phosphoryl end of a DNA strand from attack by bacterial alkaline phosphatase (Weiss and Richardson, 1967; Olivera and Lehman, 1967a; Becker, Gefter, and Hurwitz, 1967) have all been used. We wish to report the discovery of a joining enzyme using a distinctive assay method involving the linking of one polynucleotide strand to another attached to a solid matrix of cellulose. Inasmuch as this enzyme, purified from extracts of Escherichia coli infected with phage T4, appears to be identical to

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the ligase described by Weiss and Richardson, this brief report will emphasize the use of polynucleotide cellulose (Jovin and Kornberg, 1967) and will describe the purification and properties of the enzyme in only limited detail.

The assay is rapid and sensitive and directly measures the linkage of two polynucleotide strands. The starting material is the interrupted homopolymer pair of dI : dC[1] schematically depicted in Fig. 1a.

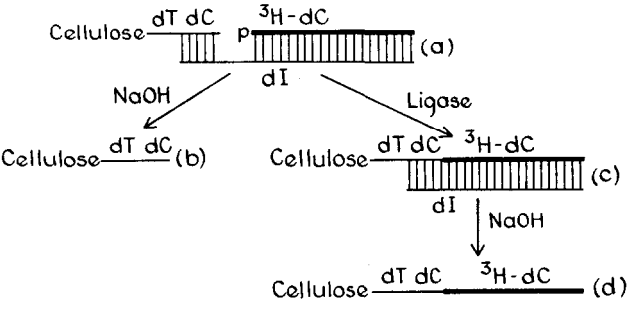

Fig. 1. Schematic representation of the assay for polynucleotide ligase. The substrate (a) consists of dT, an oligomer of 5 to 10 deoxythymidylate residues esterified through its 5'-phosphoryl terminus to one of the glucose hydroxyl groups of the cellulose. The dT is in turn joined by phosphodiester linkage to dC, a polymer of 100-200 deoxycytidylate residues with a free 3'-hydroxyl group at its terminus. The dC and ^{3}H-dC, a strand of about 1800 deoxycytidylate residues with free 5'-phosphoryl and 3'-hydroxyl termini, are hydrogen bonded to dI, a strand of about 1800 deoxyinosinate residues. Alkaline denaturation of (a) leads to (b), in which ^{3}H-dC and dI are removed from the cellulose complex. Ligase action joins ^{3}H-dC covalently to cellulose-dTdC to produce (c), alkaline denaturation of which leads to (d), in which ^{3}H-dC is retained in the cellulose complex.

[1] Abbreviations used are: poly dI, poly dT, and poly dC for deoxyinosinate, deoxythymidylate, and deoxycytidylate homopolymers; dI : dC for the hydrogen-bonded homopolymer pair of poly dI and poly dC; oligo dT-cellulose for oligodeoxythymidylate in phosphodiester linkage to cellulose; and poly dC, oligo dT-cellulose for a block copolymer formed by the extension of oligo dT-cellulose by a homopolymeric region of poly dC.

One strand of poly dC is ^{3}H-labeled and the other is covalently joined to a cellulose particle. Alkaline denaturation removes the labeled poly dC from the cellulose (Fig. 1b) unless the interruption has been enzymatically repaired (Fig. 1c, 1d). Labeled strands covalently attached to cellulose are collected by filtration of the alkaline mixture and provide a measure of the number of successful strand unions; the results are amplified in proportion to the length of the labeled poly dC chain.

MATERIALS AND METHODS

Preparation of the Substrate (Fig. 1a). Terminal deoxynucleotidyl transferase from calf thymus (Kato, Gonçalves, Houts, and Bollum, 1967) was used to extend oligo dT-cellulose[1] (Gilham, 1964) with deoxycytidylate residues to form poly dC, oligo dT-cellulose. The reaction mixture contained in 0.23 ml, 20 mg of oligo dT-cellulose (about 1.5 μmoles of nucleotide residues), 200 mM potassium cacodylate, pH 7.0, 35 mM KCl, 1 mM $CoCl_2$, 1 mM β-mercaptoethanol, 1 mM dCTP, and 9 μg of purified terminal transferase (generously provided by Dr. F. J. Bollum). The product, containing from 4 to 8 mμmoles of deoxycytidylate residues/mg of cellulose, was washed with 50-ml portions of 10 mM EDTA, 300 mM NaCl, 50 mM NaOH, water, ethanol, and ether and dried in vacuo. Poly dI and ^{3}H-labeled poly dC, prepared according to the method of Chamberlin and Patterson (1965), were sequentially annealed to the poly dC, oligo dT-cellulose. Forty mg of poly dC, oligo dT-cellulose were mixed with 1.24 ml of 1.5 mM EDTA, 0.19 mM poly dI, and 20 mM NaOH to destroy any pre-existing secondary structure. The mixture was brought to pH 7.8 with 20 mM sodium phosphate

buffer, and NaCl was added to a final concentration of 110 mM. After agitation for 2 hours at 25° and 2 hours at 21°, the product was washed 3 times with Buffer A: 10 mM sodium phosphate, pH 7.8, 100 mM NaCl, and 0.5 mM EDTA. The cellulose pellet was resuspended in 0.83 ml of Buffer A containing 0.11 mM ^{3}H-labeled poly dC (7.6×10^{7} cpm/μmole of nucleotide). After 4 hours of agitation at 21°, about 60% of the poly dC had been annealed. The product was washed with Buffer A and stored at 4° at a cellulose concentration of 10 mg/ml Buffer A; this is the substrate described in Fig. 1a.

Assay of Polynucleotide Ligase. The Buffer A suspension of substrate was stirred vigorously with a magnetic stirrer in order to obtain 0.05-ml aliquots for distribution into centrifuge tubes. After centrifugation, the supernatant fluid was discarded and the cellulose resuspended in a final volume of 0.15 ml containing 20 mM Tris-HCl, pH 8.0, 6.7 mM $MgCl_2$, 1 mM ATP, and 0.02 ml of enzyme diluted in a solution composed of 25 mM Tris-HCl, pH 8.0, 10 mM β-mercaptoethanol, and 1 mg/ml of bovine plasma albumin. The reaction was carried out at 21° for 12 min with agitation sufficient to maintain a uniform suspension of cellulose. After addition of 2 ml of cold 10 mM EDTA and 0.2 ml of 1 M NaOH, the reaction mixture was filtered on 24 mm Whatman GF/C glass filters, washed with 50 mM NaOH and then with ethanol, dried, and counted. The blank value obtained in the absence of enzyme was less than 0.3% of the input radioactivity. One unit of enzyme is defined as the amount catalyzing the joining of 1 mμmole of nucleotide residues of poly dC to cellulose per min. The reaction was proportional to added enzyme between 2 and 12×10^{-4} unit. The enzyme activity with different batches of substrate varied within a factor of two.

RESULTS

The purified enzyme[2] requires ATP and Mg^{++} for activity (Table 1).

Table 1

Requirements of Polynucleotide Ligase

Components	Nucleotide joined to cellulose
	μμmoles
complete	22
plus 0.016 μmole dCTP	23
minus $MgCl_2$	<0.5
minus ATP	<0.5
minus ATP + 0.15 μmole DPN	<0.5
minus enzyme	<0.5
substrate heated 65°, 15 min; cooled rapidly	<0.5
substrate prepared without poly dI	<0.5

Standard assay conditions were used as described in Materials and Methods.

[2] The ligase was purified from an extract of E. coli B infected for 75 min with T4 am N82, a mutant which allows the sustained synthesis of early enzymes (Epstein et al., 1963). Cells infected by a gene 30 mutant, T4 am H39, shown by Fareed and Richardson (1967) to lack polynucleotide ligase, as well as uninfected cells, contained less than 3% of this activity. Forty ml of extract (940 units, 2.3 units/mg protein) were prepared in 50 mM glycylglycine buffer, pH 7.0, 2 mM EDTA, and 2 mM reduced glutathione by blending 10 gm of infected cells with glass beads. All other buffers contained 10 mM β-mercaptoethanol and 0.5 mM EDTA. The supernatant fluid after addition of 10 ml of 5% streptomycin sulfate was fractionated between 35 and 51% saturated $(NH_4)_2SO_4$. After dialysis against 20 mM potassium phosphate, pH 7.6, this fraction (780 units, 6.9 units/mg) was applied to a 2.2 cm by 9 cm DEAE-cellulose column at the same pH and eluted with a linear phosphate gradient (610 ml) between 20 mM and 250 mM. The peak fractions, near 80 mM potassium phosphate (490 units, 22 units/mg), were dialyzed against 40 mM potassium phosphate, pH 6.5, applied to a 1.4 cm by 9.5 cm phosphocellulose column equilibrated with the same buffer. The adsorbent was eluted successively with 15 ml of 40 mM, 50 ml of 100 mM, 125 ml of 250 mM, and 100 ml of 400 mM potassium phosphate, pH 6.5. The bulk of the enzyme was usually eluted by the last buffer. The purified enzyme (430 units) had a specific activity of 1,000 units/mg protein; this represents a 430-fold purification and a yield of 46%.

DPN, the cofactor for the joining enzyme in uninfected cells (Olivera and Lehman, 1967b; Zimmerman et al., 1967), did not replace ATP. The reaction also requires the helical, oriented secondary structure of the substrate inasmuch as denaturation or the absence of poly dI abolished all activity (Table 1). The addition of dCTP had no effect.

Demonstration that the product of ligase action contains a 5'-3'-phosphodiester bond between ^{3}H-poly dC and the poly dC, oligo dT-cellulose utilized the known specificity of the micrococcal (Cunningham, Catlin, and de Garilhe, 1956) and Bacillus subtilis nucleases (Kerr, Chien, and Lehman, 1967) in degrading DNA to nucleoside 3'-phosphates. Polynucleotide kinase was used to label the 5'-phosphoryl terminus of ^{3}H-poly dC with ^{32}P after prior treatment with bacterial alkaline phosphatase (Richardson, 1965). The product contained 12 mμmoles of deoxycytidylate residues and 6.7 μμmoles of ^{32}P-phosphate (3 x 10^{9} cpm/μmole) or a number average chain length of 1800. The cellulose substrate (3 mg) was then formed and contained 30% of the input poly dC. Ligase action resulted in the joining to the cellulose of 450 μμmoles of deoxycytidylate residues and 0.78 μμmole of ^{32}P-phosphate; the number average chain length of 580 indicates a preferential reaction of the smaller molecules of poly dC. The ligase product was degraded to mononucleotides by the nucleases, yielding 90% of the ^{32}P as 3'dCMP as judged by sensitivity to 3' nucleotidase (Becker and Hurwitz, 1967). The remainder appeared as inorganic phosphate. In a control experiment without ligase, more than 90% of the ^{32}P was resistant to the nucleotidase.

DISCUSSION

The enzyme induced by phage T4 am N82 reported here appears to be the same as that purified by Weiss and Richardson (1967) from cells infected with wild type T4. Both enzymes are purified by a similar pro-

cedure, have the same cofactor requirements, are present only in phage-infected cells, and are lacking in cells infected with a phage bearing a mutation in gene 30. In addition, a ligase preparation kindly supplied by Dr. Richardson also utilized the cellulose substrate.

Joining of poly dC strands by the ligase does not require DNA polymerase for filling any gap that interrupts the strands. There is no requirement for, or stimulation by, added dCTP. Although the purified enzyme contains DNA polymerase activity (1,600 units/mg protein) when assayed under optimal conditions for the T4 polymerase (Goulian, Lucas, and Kornberg, in preparation), fewer than two residues of ^{32}P-dCTP were incorporated per strand of poly dC product under standard assay conditions for the ligase. Since it is unlikely that the poly dC strands in the substrate are perfectly aligned without any gap, it may be inferred that these strands can probably slip along the poly dI until they are fixed by the ligase in covalent linkage (cf. Kornberg, Bertsch, Jackson, and Khorana, 1964; Olivera and Lehman, 1967a).

A hydrogen-bonded bihelical structure has proved to be a common requirement of the substrates for the polynucleotide joining enzymes (Table 1; Gellert, 1967; Weiss and Richardson, 1967; Olivera and Lehman, 1967a; Becker et al., 1967). Whether the function of the polynucleotide joining enzymes be in DNA replication, repair, or recombination, these in vitro results suggest the existence of a hydrogen-bonded intermediate in the relevant process (cf. Tomizawa and Anraku, 1964; Frankel, 1966). The source of such structures in vivo and the identification of enzymes that form these intermediates may now be more approachable problems with the ligase in hand. An assay which measures the joining of labeled

dI : dC in solution to dI : dC fixed to cellulose may in the presence of an added quantity of ligase prove useful in the search for new factors involved in replication, repair, or recombination.

SUMMARY

An enzyme which joins polydeoxynucleotide strands has been identified in and purified from extracts of Escherichia coli infected with phage T4 am N82 and appears to be the same as the ligase described by Weiss and Richardson. A novel method for detecting and assaying such enzymes involves the joining of a strand to one that is covalently linked to cellulose.

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