Covalent Attachment of Nucleic Acids to Agarose for Affinity Chromatography*

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ABSTRACT: The covalent coupling of deoxy- and ribonucleic acids to the agarose derived matrix activated with cyanogen bromide has been investigated. It has been found that whereas single-stranded DNA can be easily attached to agarose, double-stranded DNA binds with very low efficiency. However, introduction of single-stranded ends into a double-stranded molecule permits covalent attachment to agarose. Similarly, the covalent binding of single-stranded RNA to agarose can be effectively accomplished. tRNA which contains double-stranded regions, attaches very poorly by this technique. However, the predominantly double-stranded RNA obtained from reovirus was bound with high efficiency. Experiments with DNA polymerase obtained from HeLa cells showed that DNA attached to agarose was about 50-fold more efficient in binding the enzyme than a corresponding DNA-cellulose matrix.

In recent years, the concept of "affinity chromatography" has utilized the unique biological property of proteins and polypeptides to bind to certain ligands specifically and reversibly (Cuatrecasas *et al.*, 1968; Kato and Anfinson, 1969). In these studies, a clear advantage of this novel tool over the conventional procedure for protein purification has been demonstrated. Small molecule ligands such as substrate analog inhibitor, effector, cofactor, vitamin, and substrate have been used for effecting covalent linkage to the solid insoluble support like agarose derivatives and polyacrylamide (Cuatrecasas and Anfinson, 1971).

Previously DNA has been covalently attached to a cellulose support and used as a substrate for purification of DNA polymerase (Litman, 1968). Gilham (1968) has also reported on the synthesis of celluloses containing covalently bound nucleotides, polynucleotides, and nucleic acids.

Since agarose derivatives such as Sepharose have been found to possess advantageous properties of high capacity and good flow rates when used in columns, the availability of Sepharose-DNA and Sepharose-RNA substrates would be a useful tool for the isolation and purification of proteins which bind to nucleic acids.

Materials and Methods

Sepharose 4B is an agarose product of Pharmacia, Uppsala, Sweden. Cyanogen bromide was purchased from Eastman Kodak Co., Rochester, N. Y. Polyriboadenylic acid was obtained from Miles Laboratories, Inc., Elkhart, Ind. Radioisotopes were routinely obtained from New England Nuclear, Chicago, Ill. *Escherichia coli* K 12 cells came from General Biochemicals. Both *E. coli* B tRNA and yeast tRNA were from Schwarz BioResearch Inc. Samples of reovirus were generously provided by Drs. A. J. Shatkin and A. K. Banerjee. BSM[1] has VBM salts (Vogel and Bonner, 1956), 0.5% glycerol, 0.1% CasAmino acids, 5×10^{-4} M Mg^{2+}, 0.01% yeast extract, and a trace of ferric chloride. PEG is a product of General Biochemicals. The solution used for radioactivity counting in the scintillation counter contained per liter: toluene (667 ml), Triton X-100 Beckman (337 ml), PPO (5.5 g), and POPOP (100 mg). λ phage assays were performed by soft agar overlayer technique using *E. coli* K12 or *E. coli* C as indicator cultures for K12 or *E. coli* C grown phage, respectively (Adams, 1959).

E. coli DNA. DNA from *E. coli* K12 cells was prepared by the Marmur technique (Marmur, 1961). For tritium-labeled DNA, a culture of bacterial strain *E. coli* CR_{34} leu^-,thr^-,thy^-,B_1^- was grown in BSM supplemented with 60 μmoles/l. of thymine, 1 mCi/l. of tritiated thymidine, and 4 μg/ml of vitamin B_1. At cell concentrations of $4–5 \times 10^8$/ml, the culture was chilled in ice and the cells collected by centrifugation. The procedure for extraction of DNA was same as referred to in the preparation of unlabeled DNA.

Preparation of Phage λ and Extraction of DNA. Cultures of *E. coli* C grown to 1×10^8 cells/ml in tryptone broth were infected at multiplicity of 0.1 from a stock of phage λ_{CI}. The infected cultures were incubated at 37° for 4 hr and the progress of lysis followed by measurement of optical density at 650 mμ. After completion of lysis, the lysate was cooled to 4° and sodium chloride added to a final concentration of 0.5 M. The lysate was centrifuged to remove bacterial debris (10,000 rpm, 10 min) and the supernatant fluid was supplemented with PEG (mol wt 5700–6700) at a final concentration of 10% (Yamamoto *et al.*, 1970). Further centrifugation of the PEG supplemented supernatant fluid (10,825*g*, 10 min, 3°) concentrated the phage in a pellet which was resuspended in 0.02 M Tris containing 10^{-3} M Mg^{2+} (pH 7.4). Assays for phage λ at various steps showed that approximately 98% of the original viable phage was obtained in the final pellet. Further purification of phage was achieved by isopycnic centrifugation (ρ 1.5 g/cm^3) in cesium chloride in a Spinco ultracentrifuge (35,000 rpm, 50 Ti rotor, 4°, 18 hr). The phage solution was dialyzed and then treated with an equal volume of phenol (saturated with 0.02 M Tris, pH 7.4, and 10^{-3} M EDTA). The aqueous layer containing DNA was withdrawn and dialyzed against 0.015 M NaCl and 0.0015 M sodium citrate. The tritiated thymidine-labeled λ DNA was prepared from the phage obtained by thermal induction at

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[1] Abbreviations used, but not listed in *Biochemistry* 5, 1445 (1966), are: BSM, basic synthetic media; PEG, polyethylene glycol; DTT, dithiothreitol; TEA, triethylamine; RSB, 10^{-2} M Tris–10^{-2} M KCl–1.5×10^{-3} M $MgCl_2$; POPOP, 1,4-bis[2-(4-methyl-5-phenyloxazoyl)]-benzene; PPO, 2,5-diphenyloxazole; SDS, sodium dodecyl sulfate.

41° of a culture of Hfr 1858 (λ C_{I857}) thy^-,B_1^- grown to 2–3 × 10^8 cells/ml in BSM supplemented with 60 μmoles/l. of thymine and 4 μg/ml. of vitamin B_1. Tritiated thymidine at 1 mCi/800 ml was added immediately after the temperature was raised for induction of prophage. The procedures for isolation and purification of phage and for extraction of its DNA were similar to the one described above.

HeLa DNA. A modification of a previous procedure (Schildkraut and Maio, 1969) was used. HeLa cells (3 g) were suspended in RSB (30 ml) for 10 min. The cell suspension was treated with ten strokes of a tight pestle in a dounce homogenizer and nuclei centrifuged at 1000*g*. The pellet was suspended in 0.02 M Tris (pH 8.5) containing 10^{-2} M EDTA and incubated at 37° with 0.1% SDS and pronase (1 mg/ml) for 6 hr. The concentration of SDS was then increased to 1% and sodium perchlorate added to a final concentration of 1 M. It was shaken twice with chloroform–isoamyl alcohol (24:1, v/v) and precipitated with two volumes of ethanol and collected by spooling. The precipitate was dissolved in 20 ml of a 0.05 M Tris buffer (pH 7.4) containing EDTA at 0.01 M. The solution was incubated with RNase (20 μg/ml) at 37° for 30 min, shaken three-times with $CHCl_3^-$ isoamyl alcohol, and precipitated with ethanol by spooling. The precipitate was dissolved in a solution of NaCl (0.015 M) and sodium citrate (0.0015 M) (20 ml). To the solution was added 3 M potassium acetate and 10^{-3} M EDTA (pH 7.0, 10% of total volume) and precipitated again with 0.54 volume of 2-propanol. The precipitate of DNA was finally dissolved in NaCl (0.015 M) and sodium citrate (0.0015 M) solution. The treatment with pronase, deproteinization with $CHCl_3$–isoamyl alcohol, and precipitation with 2-propanol was repeated once again. Procedures for preparation of reovirus and the extraction of its RNA have previously been published (Banerjee and Shatkin, 1970).

Treatment of DNA with λ Exonuclease. This reaction was used to generate single-stranded ends on double-stranded DNA species. The description is given for the λ-exonuclease reaction. Tritium-labeled λ DNA, A_{260} 43.4 and 2 × 10^4 cpm/A_{260}, was incubated with $MgCl_2$ (0.0075 M), DTT (0.001 M), TEA (pH 9.0, 0.036 M), and 0.25 ml of purified λ DNase (Amberlite XE-64 fraction containing 6 units[2]/ml) (Korn and Weissbach, 1963) in a total of 10.4 ml of solution at 37°. Aliquots were withdrawn at various intervals and assayed for acid-soluble counts as previously described (Weissbach and Korn, 1963). After 3.5 hr the percentage of acid-soluble counts was found to be 25.8% (about 13% from each end) and the reaction was stopped by chilling the reaction mixture in ice. In order to deactivate the enzyme, the incubation mixture was heated at 60° for 5 min and then dialyzed in the cold against 0.05 M potassium phosphate buffer (pH 8.0) prior to the Sepharose-coupling reaction. For the preparation of HeLa DNA containing single-stranded ends, an exonuclease directed by φ21 (Pricer and Weissbach, 1966) was used under similar conditions. DNA polymerase assays were carried out as previously described (Berns *et al.*, 1969).

Sepharose Activation and Ligand-Coupling Reaction (Cuatrecasas, 1970). A slurry of a known volume of Sepharose 4B was obtained by mixing it with an equal volume of water. The slurry container was surrounded with a jacket of iced water to control temperature below 20° during the reaction. The pH of the reaction was determined by keeping the electrode of the pH meter in the reaction mixture all through the reaction. A calculated amount (200–500 mg/ml of Sepharose) of well powdered cyanogen bromide was added in one lot to the gently stirred slurry and dropwise addition of sodium hydroxide (2–5 M) was started immediately so as to maintain pH at 11. Cessation of the falling of the pH below 11 and simultaneous disappearance of solid cyanogen bromide was indicative of completion of the activation reaction. The slurry of activated Sepharose was at once cooled by adding ice and then filtered and washed three times with 0.05 M potassium phosphate (pH 8.0) under suction without ever letting the activated Sepharose go dry. The activated complex was added to the nucleic acid ligand in 0.05 M potassium phosphate (pH 8.0) whose volume was the same as that of packed Sepharose used in reaction and stirred gently at 4° for a period of 16–48 hr. The reaction slurry was then put on an appropriate column and washed with 0.05 M potassium phosphate (pH 8.0) until no more nucleic acid material appeared in wash (as determined by radioactivity and/or $A_{260\,m\mu}$). To ascertain complete removal of noncovalently attached material a final wash was generally given with 0.5 M potassium phosphate (pH 8.0). Quantitation of the results was based upon (a) the difference of input and wash radioactive counts and/or A_{260} (b) in case of radioisotope-labeled material direct counts were also taken on coupled Sepharose by making a slurry in appropriate buffer. Agreement between the two values was good.

Results and Discussion

The results of coupling of various DNA and RNA species to activated Sepharose are presented in Table I. With the exception of poly(d(A-T)), the other DNA species measured do not undergo a significant coupling reaction when present in a native double-stranded form. In contrast, when a double-stranded species is denatured to the single-stranded form, extensive binding occurs. Thus 20% of the *E. coli* DNA when denatured can be attached to the agarose whereas none of the original native DNA binds under these conditions. Similarly, the introduction of single-stranded ends into a double-stranded DNA permits attachment of the nucleic acid to occur. As shown in Table I native λ DNA, a double-stranded structure containing complementary single-stranded 5′ ends of 12 nucleotides in length (Wu and Kaiser, 1968) does not bind to Sepharose. If 5′ ends of the λ DNA are degraded 12.5% on each side (about 6000 nucleotides) with the λ exonuclease 40% of the DNA can be attached. It is important to note that the digestion of double-stranded DNA by the λ-type exonucleases to yield single-stranded ends must be carried out at high pH (9.5) where the enzyme acts in a random fashion. At lower pH values, the enzyme acts progressively, preferentially digesting the molecule it initially binds to (Carter and Radding, 1970; Little *et al.*, 1967). The dependency of binding on single strandedness is further illustrated by the results with HeLa DNA where the per cent of DNA which can be attached to agarose increases as the amount of single-stranded ends increases. However, the amount of DNA which the Sepharose can bind is apparently limited since at high DNA/Sepharose ratio (10 *A* units/ml of Sepharose) the efficiency of attachment seems to decrease. In case of poly(d(A-T)) the pH of the solution (pH 8.0) is probably enough to "soften" the double-stranded hydrogen bonding and allow the coupling to proceed.

The coupling of various RNA species tested here may also obey these rules. Polyriboadenylic acid binds with high efficiency to Sepharose while the tRNA of yeast and *E. coli*

[2] A unit is defined as 10 mμmoles of nucleotide solubilized in 30 min at 37°.

TABLE I: Attachment of Nucleic Acids to Sepharose.[a]

Nucleic Acid	Packed Sepharose Vol (ml)	Amt of CNBr (g)	Amt of Nucleic Acid Used		Amount Coupled to Sepharose		
			A_{260}	cpm	A_{260}/ml	Total A_{260}	%
[^{14}C]Poly((dA-T))	5	1	0.91	2.2×10^5	0.136	0.68	75
^{3}H *E. coli* DNA (native)	5	1.3	29.6	1.5×10^7	0	0	0
^{3}H *E. coli* DNA (native) (sonicated)	5	1.2	8.7	5×10^5	0	0	0
^{3}H *E. coli* DNA (native) (coupling performed at pH 10.65)[b]	5	1.3	4.3	4×10^5	0.017	0.085	2
^{3}H *E. coli* DNA (denatured)	5	1.3	11.05	4×10^5	0.461	2.3	20.9
^{3}H λ DNA (native)	5	2.26	6.0	1×10^5	0		0
^{3}H λ DNA (12.5% single-stranded) end on each side)	10	5.0	29.8	5×10^5	1.19	11.9	40
^{3}H HeLa DNA (native)							
2.2% single-stranded end on each side	5	1.8	5.0	5×10^5	0.043	0.215	4.3
3.8% single-stranded end on each side	10	4.95	39.0	5×10^5	0.663	6.63	17
8.3% single-stranded end on each side	5	1.5	2.0	2×10^5	0.192	0.96	24
^{3}H HeLa DNA (denatured)	30	9.0	300	1×10^6	0.90	27	9.0
Polyadenylic acid	10	2	77.0		7.5	75	98
Reovirus [^{3}H]RNA	5	2	4.2	8×10^5	0.722	3.62	86
Yeast tRNA	10	5.0	76.6		0.433	4.33	5.7
E. coli B tRNA	10	5.0	49.5		0	0	0

[a] All coupling reactions were carried out in 0.05 M sodium or potassium phosphate buffer (pH 8.0) unless otherwise indicated. [b] 0.05 M sodium carbonate buffer was used.

TABLE II: Binding of HeLa Cell DNA Polymerase by DNA-Sepharose and DNA-cellulose.

Matrix	DNA Polymerase Activity[d]			Enzyme Units Adsorbed/A_{260} Unit of DNA Matrix	Enzyme Units Eluted/A_{260} Unit of DNA Matrix
	Enzyme Units Applied	Units Adsorbed	Units Eluted		
Cellulose *a*	6.9×10^3	2.9×10^3	1×10^3	20.4	7
b	9.9×10^2	8.5×10^2	5.4×10^2	40.5	25.3
Sepharose *c*	9.0×10^3	7.6×10^3	3.3×10^3	1100	470
	3.4×10^3	3.3×10^3	3.1×10^3	482	442
	3.75×10^2	3.6×10^2	2.45×10^2	51.4	35

[a] 8 g of cellulose containing 140 A_{260} units of HeLa DNA was prepared by the method of Litman (1968). Volume of packed column was 27 ml. [b] 1 g of cellulose containing 21 A_{260} units of HeLa DNA was prepared by the method referred to in footnote *a* above. Volume of packed column was 4 ml. [c] 10 ml of Sepharose 4B containing 7 A_{260} units of HeLa DNA with 3.8% of each end single stranded was prepared as described in text. [d] A crude sonicate of HeLa cells (1 g/10 ml of 0.02 M potassium phosphate, pH 8.5) was centrifuged 3 hr. at 155,000*g* and the supernatant fluid was applied to the column. After washing the column with 0.02 M potassium phosphate (pH 8.0) the enzyme was eluted with 0.1 M potassium phosphate buffer (pH 8.0). No further enzymatic activity is eluted by higher salt concentrations up to 0.5 M. A unit of enzyme activity is defined as 0.4 mμmole of deoxynucleotide triphosphate incorporated into an acid-insoluble form in 30 min.

B which have much of the structure in a double-stranded form show little or no binding. However, reovirus RNA seems to show some unexplained binding. The reovirus RNA is known to be a double-stranded species and yet binds reasonably well to the Sepharose. This result would not be surprising if substantial single-stranded stretches were present somewhere within or on the ends of the double-stranded segments of reovirus genome. A recent report has presented evidence in favor of short single-stranded ends consisting mainly of purines on each segment of the reovirus RNA (Millward, 1970).

The binding of double-stranded DNA to Sepharose after

the introduction of single-stranded ends permits one to attach a relatively intact native DNA to the matrix for affinity chromatography. This has been confirmed by the binding of DNA polymerase from HeLa cells to HeLa DNA-Sepharose columns as shown in Table II. Passage of a crude soluble enzyme preparation from sonicated HeLa cells through a HeLa DNA-Sepharose column resulted in a retention of a significant proportion of the enzyme activity. The enzymatic activity eluted from the column is purified approximately 3-fold over the crude sonicated supernatant fluid applied to the column. The capacity by which the DNA-Sepharose column can absorb the HeLa DNA polymerase is 30–60-fold better than a corresponding DNA-cellulose column when calculated per unit of DNA fixed to the matrix (Table II). In addition, the Sepharose columns exhibit faster flow rates than do corresponding cellulose columns and like other columns of this type can be used repeatedly. The limited ability of DNA-cellulose matrices prepared by the Litman procedure (Litman, 1968) to absorb protein may be a reflection of the type of linkage introduced by the ultraviolet irradiation used in this technique.

The decision about the pH at which the reactions presented here were performed was aided by the knowledge that aromatic amino functionalities couple best at pH 8–9 (Cuatrecasas, 1970). The presence of three such groups on the base moieties of nucleotides adenosine, guanosine, and cytidine supported the promise of success of coupling. Although the relative coupling efficiencies of these three amino groups is not known, the best results obtained in case of polyriboadenylic acid points to the fact that a high concentration of probably any one of these bases should increase the binding manyfold. The three parameters which probably control the efficiency of coupling reation are (a) structure and nature of nucleic acid ligands, (b) concentration of cyanogen bromide, and (c) the concentration of nucleic acid ligand. Factors b and c have not as yet been systematically investigated.

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