Some general methods of preparing affinity columns

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ABSTRACT

Some general methods of covalent coupling of nucleotides, especially derivatized nucleotides, polynucleotides and cofactors to insoluble polymers are described in this paper. Wherever necessary individual methods also carry some information on the binding of enzymes to the same polymers to serve as a guide to the efficiency of the coupling methods.

INTRODUCTION

Affinity columns made by the covalent binding of nucleotides, derivatives of nucleotides and cofactors are finding increasing use in the purification of enzymes and proteins. Bioaffinity chromatography is a very efficient and powerful tool in the hands of a chemist. However, the affinity between the covalently bound ligand and the macromolecule to be purified is highly stereospecific, in that not only the interaction leading to a complex formation but also the subsequent displacement of the macromolecule from the complex will depend upon 1) the group and position on the ligand molecule through which it is coupled to the polymer 2) to some extent the chemical method by which the affinity column is made 3) the spacer distance between the ligand and the supporting polymer and 4) the three dimensional orientation of the bound ligand at the solid/liquid interface which forms part of the micro-environment.

The efficiency of covalent coupling of nucleotides and polynucleotides is governed by factors somewhat different from those of binding enzymes. In the case of nucleotides, it would appear that their small molecular weight should improve the coupling yield, in similarity to aminoacids and short chain polypeptides. However, the two major factors that influence

the coupling yield are the degree of helical structure and hydrogen bonding that exist in a given molecule. This could explain many of the conflicting claims that exist in the literature on nucleotide and polynucleotide coupling to polymers using different methods. In the case of proteins it is generally known how the isoelectric point, the globular nature of the protein, and in some cases the presence of subunits, bound metal ions and/or cofactors and non-protein parts such as carbohydrates could affect the efficiency of a coupling method.

Among the methods investigated in this study some of them, such as the cyanogen bromide (CNBr) method of coupling¹ and glutaraldehyde cross-linking, have become almost routine. However in spite of their being such facile and popular techniques, we do not yet know the precise chemistry involved in such complex reactions, nor why any one given chemical group such as for example the -NH₂ group should be more nucleophilic in one case than another, even though in pure chemical terms no rational explanation can be found for the different reactivities. This work was conceived as an attempt to clarify some of these interesting points.

Affinity columns of nucleotides and cofactors were prepared with a view to using them to study the wide variety of nucleic acid enzymes such as kinases, polymerases and esterases which catalyze very specific reactions. This specificity is demonstrated by the particular portions of a nucleotide molecule, such as the base, the sugar and the phosphate ester, to which the active sites of these enzymes bind to form activated complexes. In order to work with such a variety of enzymes, affinity columns will be required in which various positions on the nucleotide molecules are coupled to the polymers.

Unusual abbreviations used in this paper:

ATPyS = adenosine 5'-O-(1-thiotriphosphate)

AMP-PNP = adenylyl imidodiphosphate

⁶s ITP = 6-thioinosine-5'-triphosphate

⁶cl ITP = 6-chloropurine-5'-triphosphate

⁸br ATP = 8-bromoadenosine-5'-triphosphate

AE-cellulose = aminoethyl cellulose

CM-cellulose = carboxymethylcellulose

MATERIALS AND METHODS

AMP, ATP, AMP-PNP, Adenosine, NAD^+ , poly(I), poly(C), poly(U), tRNA, and DNA were obtained from Boehringer, Mannheim. The nucleotides $s^6\text{ITP}$ and $cl^6\text{ITP}$ and $br^8\text{ATP}$ were prepared from the monophosphates using the procedure described by Michelson². $\text{ATP}\gamma\text{S}$ was made using the method of Goody and Eckstein³. Polythiophosphoribocytidylic acid (poly(sC)) was made by the method described by Eckstein and Gindl⁴. The p-aminophenylester of ATP was made by the method of Berglund and Eckstein⁵. These compounds were prepared and supplied by Dr. Eckstein. Small molecular weight DNA from herring sperm was obtained from Serva, Germany. Commercial nylon 6 obtained from BDH Ltd. was mechanically ground in the presence of dry ice. AE-cellulose and CM-cellulose were supplied by Nutritional Biochemical Corporation, U.S.A. and Sigma chemical Company respectively. Sepharose 4 B was obtained from Pharmacia, Uppsala, Sweden. Aminoethyl Sepharose was made by coupling ethylenediamine to Sepharose by the cyanogen bromide method¹.

Bisimides were synthesized by the Pinner Synthesis method⁶ using the modifications of McElvain and Schroeder⁷.

Coupling to Sepharose by CNBr activation

This method essentially followed that devised by Axén, Porath and Ernback¹. The activated Sepharose was treated with the ligand at various pH values in an appropriate buffer as shown in Table 1. In most cases the CNBr-activated Sepharose supplied by Pharmacia was used. The gel was allowed to swell in cold 5 mM HCl for 15 minutes and washed (1 gram in 200 ml) in the same salt solution. The gel was then washed free of salt with ice cold water, filtered off in a Büchner funnel and transferred to the ligand solution made in a suitable buffer. After reaction over night (16-20 hours) at 4° C, the gel was washed initially with about thrice its volume of the coupling buffer. The washed buffer was collected for optical density measurement to calculate the amount of ligand bound by subtracting the amount left over after coupling from the initial amount.

After this initial wash the gel was washed with pH 4.5 acetate buffer (I = 0.1) and pH 8.5 borate buffer (I = 0.1)

alternately three times, using 50 ml each time per gram of gel, followed by a 100 ml wash with 1 M NaCl and finally removal of NaCl by a liberal washing with deionized water. The amount of ligand washed off in the subsequent washes was also determined by optical density measurement.

Table 1. Results of coupling nucleotides, polynucleotides and cofactors (by the CNBr method)

Nucleotide Polynucleotide	Polymer	pH Buffer	% Coupled
Adenosine	Sepharose 500 mg	8.0/HCO ₃ ⁻	38
AMP	" "	6, 8, 9	none
AMP-PNP	" "	" "	"
ATP	" "	" "	"
ATP γ S	" "	9/HCO ₃ ⁻	15
p-aminophenyl ATP	" "	" "	20
6-aminohexanoyl ATP	" "	" "	90
s ⁶ ITP	" "	8.5/HCO ₃ ⁻	43
NAD ⁺	" "	8.0/HCO ₃ ⁻	none
Poly(I)	" "	6.0/collidine (I = 0.06)	42
	" "	8.0/" "	less
Poly(C)	" "	6.0/collidine (I = 0.06)	20
Poly(sC)	0.5 mg wet packed volume	" "	33
tRNA	500 mg	9.0/HCO ₃ ⁻	15
DNA Calf thymus	" "	9.0/HCO ₃ ⁻ (0.08 M NaCl)	15
Small molecular weight DNA (denatured herring sperm)	" "	" "	25

In the case of DNA, contaminating protein was removed by phenol precipitation

Coupling to CM-cellulose by the classical mixed carbonic anhydride method

This method relies upon the reaction of a mixed carbonic anhydride generated on the insoluble polymer by the classical method using ethyl chloroformate and triethylamine⁸ (Table 2).

Table 2. Results of coupling by the mixed carbonic anhydride method

Nucleotide, Cofactor or Enzyme	Time Hours	pH Buffer	Amount Coupled	%
Adenosine	24	8.5 Borate	63 μ moles	50
ATP	22.5	8.5 Borate	5.95 "	16
NAD ⁺	22.5	9.2 Borate	1.83 "	12
Poly (U)	24.0	8.0 Borate	30 O.D.	12.5
tRNA	26	8.0 Borate	6.75 mg	13.5
DNA Calf Thymus	22.5	8.0 0.08 M NaCl	69.4 O.D.	42
Urease	2.0	7.0 Phosphate	4.75 mg	24

All the above experiments were conducted with 500 mg CM-cellulose

In a typical experiment 0.5 gram of CM-cellulose is suspended in 8 ml of cold tetrahydrofuran, to which is added with stirring 1 ml of ethylchloroformate. Then 1.5 ml of triethylamine is added dropwise over 10 minutes, and reaction allowed to continue for 15 minutes. Precaution must be taken not to let the temperature drop below freezing, since otherwise the cellulose clumps and the mixed anhydride formation is not efficient. The CM-cellulose is then washed on a suction funnel with cold tetrahydrofuran, followed by cold ethanol and finally ether. The activated CM-cellulose is transferred to the ligand solution prepared usually in pH 8 borate buffer ($I = 0.1$) at 4° C and stirred for various lengths of time. After reaction is finished the supernatant is retained, and the cellulose washed with thrice its volume of coupling buffer which is then pooled

with the supernatant for optical density measurement.

The same method may be used for other celluloses and for CM-Sephadex. Table 2 lists results for nucleotides, cofactors and enzymes coupled by this method.

Coupling of substituted nucleotide by reaction with polymer bearing an amino group

A halogen derivative of a nucleotide may be allowed to react with a polymer bearing an amino group, such as an amino substituted Sepharose, AE-cellulose and partially hydrolyzed nylon, to effect covalent binding of the nucleotide to the polymer.

In a given experiment the polymer is equilibrated with borate buffer of pH 10.0 ($I = 0.1$) to which a solution of the nucleotide in the same buffer is added and allowed to react for various lengths of time. A temperature of 4°C was usually preferred even though the reaction may be carried out at room temperature. Since reaction times were long in many cases the possible lability of some of the nucleotides at room temperatures precludes carrying out the reactions at temperatures higher than 4°C . Table 3 summarizes the results obtained with this method of coupling.

Table 3. Coupling yields of reaction of halogen-substituted nucleotide with NH_2 -polymer.					
Nucleotide	Polymer	pH	Time Hours	μmoles Coupled	%
br^8ATP 5 μm	AE-Cellulose 200 mg	10.08 (THF:H ₂ O) 9:1	20	2.0	40
"	"		22	2.7	54
"	"	10.08 (H ₂ O)	44	4.26	86
cl^6I 86 μm	"	10.08 (THF:H ₂ O) 9:1	19.5	37.0	43
cl^6I 18.9 μm	Nylon 100 mg	10.0	9	7.1	37
cl^6ITP 50 μm	AE-Cellulose 300 mg	10.0	19.5	36	72
cl^6ITP 50 μm	AE-Sephadex 500 mg	10.0	20	24	48

Two methods of coupling using bifunctional cross-linking agents were used, the first a symmetrical dialdehyde and the second a bisimide.

Coupling by the glutaraldehyde method

Coupling by this method followed the two step procedure used for the coupling of enzymes to amino polymers described by Sundaram and Hornby⁹. The aminopolymer is suspended in a 2% glutaraldehyde solution in pH 9.4 bicarbonate buffer ($I = 0.1$) for about 40 minutes and is then filtered in a suction funnel. The excess glutaraldehyde is washed off the polymer with plenty of deionized water. The glutarated polymer is at this stage ready for the coupling of the ligand. The amino-group-bearing nucleotide is suspended in bicarbonate buffer pH 8.5 ($I = 0.1$) along with the glutarated polymer and reaction allowed to proceed for the desired length of time. After coupling is over the supernatant is recovered and pooled with the effluent from washing the coupled product with 1 M NaCl. The nucleotide content of the pooled solutions is optically determined. Results of this method of coupling may be found in Table 4.

Table 4. Coupling yields of ligands by the glutaraldehyde method			
Nucleotide	Polymer	<u>Amount %</u> <u>Coupled</u>	
ATP	400 mg AE-Cellulose	67.68 μ moles	18.8
ATP	1 Gram AH-Sepharose	1.339 μ moles	15.5
ATP	500 mg AE-Sepharose	4.1 μ moles	20

Imide method of coupling

Symmetrical bisimides are good bifunctional cross-linking agents that react with amino groups to form amidines. Hartman and Wold¹⁰ first used symmetrical bisimides to cross-link ribonuclease intramolecularly through the $-NH_2$ groups of the

lysine residues. The same principle is used in coupling NH_2 -group bearing nucleotides and enzymes to NH_2 -group bearing polymers such as AE-cellulose, AE-Sepharose and nylon. The polymer is first activated by coupling one of the imido groups of the bisimides to the polymer followed by coupling of the nucleotide or the enzyme to the imide-activated polymer.

In a typical experiment, 500 mg of AE-cellulose was equilibrated in 30 ml of a 90% ethanol solution containing 10% of 0.1 M phosphate solution, pH 10.5. 75 mg of the solid imide was added slowly, and the reaction allowed to proceed for an hour at room temperature. After the reaction is completed the cellulose is washed with ice cold ethanol and filtered in a suction funnel. The filtered polymer is added to the nucleotide or enzyme in 20 ml pH 9.0 phosphate ($I = 0.1$) and allowed to react at 4°C for about 2-3 hours in the case of enzymes and longer in the case of nucleotides. Solutions for enzyme coupling are made up at 2 mg/ml concentration.

Table 5. Coupling yields of ligands/enzyme by bisimide cross-linking			
<u>Nucleotide</u> <u>Enzyme</u>	<u>Polymer</u>	<u>Amount</u> <u>Coupled</u>	<u>%</u>
Adenosine	AE-Cellulose 500 mg	2.52 μmoles	25
AMP	" "	2.8 μmoles	20
ATP	" "	2.5 μmoles	22
Urease	" "	10.2 mg	48

Coupling to derivatized nylon

Nylon 6 was derivatized by direct methylation¹¹ using dimethyl sulphate. The methoxy group

is vulnerable to attack by a nucleophile such as an amino group. The same method may be used to activate polyacrylamide. Powdered nylon of average size less than $75\ \mu$ is suspended in dimethyl sulphate and on a boiling water bath for 5 minutes and then immediately emptied into methanol at ice temperature. The nylon is filtered and washed thoroughly with further quantities of cold methanol. The nylon is activated and ready for coupling at this stage. The methylated nylon is washed once in the coupling buffer before being added to the ligand solution. It may be directly exposed to a solution of nucleotide or enzyme made up in pH 7 to 8.5 buffer. In a typical experiment a nucleotide solution was made up in pH 8.0 borate buffer ($I = 0.1$) and the filtered and washed nylon added. The mixture was allowed to react for a few hours at 4°C before being filtered in a suction funnel and washed thoroughly with the coupling buffer, 1 M NaCl and deionized water. The coupled product was stirred in a suitable buffer.

The activated nylon may also be derivatized after the methylation step by first reacting it with lysine or polylysine which may then be coupled to a ligand through glutaraldehyde or any other suitable cross-linking agent. Table 6 summarizes the coupling yields obtained by this method.

Table 6. Coupling yields of ligand/enzyme to derivatized nylon by the methylation procedure			
Nucleotide Enzyme	Polymer	Amount Coupled	%
ATP	Nylon 6 Powder 200 mg	4 μ moles	80
Urease	" "	8 mg	85

RESULTS AND DISCUSSION

The results presented in Tables 1 to 5 show that it is possible to couple nucleotides successfully to different kinds of polymers through various positions on the base, the sugar or the phosphate ester moieties.

In all the experiments the amount of nucleotide, oligonucleotide or cofactor bound to the polymer has been ascertained optically by the difference in the optical density of the starting solution and that of the pooled washes at the end of the coupling. Control experiments were always performed with the supporting polymer being subjected to the same treatment in each method. In the case of enzymes, protein determination by the Lowry method¹² was also used. There are two other alternatives available in the case of the nucleotides. The one, which in most cases will not be in quantitative agreement with the optical measurement, is to subject the final coupled product to enzymic cleavage by a suitable nuclease or snake venom phosphodiesterase and determine the amount of the nucleotide cleaved. However, the enzymes do not cleave all of the bound nucleotide due to steric hindrance on the polymer surface. There is also the possibility, in the case of Sepharose, of subjecting the coupled product to alkaline or acid hydrolysis and measuring spectrophotometrically the amount of bound nucleotide.

My experience with this method has been that some of these polymers such as Sepharose and AE-cellulose can give spectra very closely resembling those of some of the nucleotides.

The CNBr-mediated method of coupling has been one of the most exploited methods in the preparation of affinity columns. Even though the columns thus prepared have been successfully employed in the purification of enzymes, there is still a controversy as to which groups of the nucleotide molecule actually take part in the covalent bond with the polymer. De Xifra et al.¹³ claim to have succeeded in binding ATP to Sepharose by the CNBr-method, even though this has been disputed by many laboratories including ours. De Xifra et al. conclude, on circumstantial evidence, that the 6-amino group must be

involved in the binding whereas Mosbach et al.¹⁴ believe that the sugar hydroxyl must be involved in ester bond formation. Dean et al.^{15, 16} also believe that coupling occurs through the 6-amino group. Our results (Table 1) show that AMP, ATP and AMP-PNP do not bind, whereas p-aminophenyl ATP couples to about the same extent as ATP γ S. It can be argued that the p-aminophenyl ATP couples through the p-amino group⁴. ATP γ S and s⁶ITP (of which the latter gives a better coupling yield) probably couple through their -SH groups. Adenosine binds quite well whereas NAD⁺ does not couple at all. This could mean that the 5'-OH in adenosine takes part in coupling to the Sepharose. Wagner et al.¹⁷ hypothesize as a result of their pH data that different groups may be involved in the coupling of poly(I) and poly(C). Poonian et al.¹⁸ found that single-stranded RNA and DNA couple more effectively than the double-stranded molecules. We also find that denatured DNA couples more effectively by this method.

Table 2 lists the efficiency of the mixed carbonic anhydride method for coupling various molecules to CM-cellulose. The three different ways in which a mixed carbonic anhydride could react with an ATP molecule, for example, are 1) an amide linkage with the 6-amino group 2) an ester bond with the sugar hydroxyls and 3) an acyl phosphate ester bond with the phosphate end of the molecule. Of these the last possibility may be ruled out because even if such a bond forms it will be too unstable to survive. The data in Table 2 shows that adenosine couples better than ATP. This could mean that the triphosphate part of the molecule acts as a shield preventing an efficient coupling through the amino group on the base. Poly(U) tends to leak out if washed with 1 M sucrose, but otherwise even high salt concentration such as 1 M NaCl washes off only the ionically bound ligand.

Reaction of halogen-substituted nucleotides with NH₂ groups on the polymer is slow. However, this is a simple method (Table 3) and works well with various polymers. If it is desired to slow down the reaction even further, the presence of a nonpolar solvent will do so.

Cross-linking with glutaraldehyde is widely used for coupling enzymes. A two-step coupling process is more efficient and prevents the formation of loose sheaths of intermolecularly linked ligand which can slowly leak. Table 4 shows some results obtained by this method, surprisingly, however, NAD could not be bound by this method to AE-cellulose.

Imidates offer a nice series of cross-linking agents of various chain lengths. The results are presented in Table 5 of experiments done with dimethylsuberimide. Dimethyladipimide and diethylmalonimide also give satisfactory results.

The imidates are easily hydrolyzed in aqueous medium, especially at pH values below 9. Hartman and Wold¹⁰ found that in a completely aqueous medium the imidates act up to 60% mono-functionally. This presents a problem if these compounds are to be used as cross-linking agents. This can be achieved by carrying out the first step in the presence of minimum water and at as high a pH as possible even if this means a slow reaction.

Coupling ligands to derivatized nylon by direct reaction of the ligand to the methylated nylon can lead to very high coupling yields. However, the coupled ligand will be very close to the surface of the polymer which will not make it an efficient affinity column for separation purposes. Alternatively, lysine or polylysine coupled to the methylated nylon will give rise to polymers which may then be cross-linked to the ligand. Well over 70% may be coupled by direct binding to the methylated nylon. Enzymes give even better results.

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