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Immobilization of DNA for affinity chromatography and drug-binding studies

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A method is described in which double-stranded DNA is alkylated with 4-bis-(2-chloroethyl)amino-L-phenylalanine and the product immobilized on an insoluble support via the primary amino group of the phenylalanine moiety. The DNA is hence irreversibly bound to the matrix by both strands at a limited number of points.

Double-stranded DNA immobilized on a support suitable for chromatography is useful for the isolation of DNA-binding proteins (Weissbach & Poonian, 1974) and for screening for drugs that bind to DNA (Inagaki & Kageyama, 1970). Ideally the DNA should be irreversibly immobilized and retain its native structure. Early methods for immobilization of DNA include adsorption on to cellulose acetate/agar (Bautz & Hull, 1962) and on to cellulose (Alberts *et al.*, 1968; Litman, 1968). Entrapment of double-stranded DNA in a polyacrylamide matrix has also been reported (Cavalieri & Carroll, 1979) but relatively low amounts of DNA are immobilized, and shearing of the DNA will occur. Entrapment of single-stranded DNA in agar (Bendich & Bolton, 1968) and in agarose (Schaller *et al.*, 1972) has also been described. All of the above methods have the disadvantage that the DNA is not covalently bound and so will be leached from the matrix.

Covalent binding of the DNA to the support is preferable, and several such methods have been reported. Denatured DNA has been bound to bisoxirane-activated Sepharose (Potuzak & Dean, 1978) and sheared DNA has been attached to Sephadex by using carbodi-imide (Rickwood, 1972). Native DNA has been bound both to Sepharose, with CNBr (Poonian *et al.*, 1971; Arndt-Jovin *et al.*, 1975; Kempf *et al.*, 1978) and to carboxymethylcellulose (Potuzak & Wintersberger, 1976), and a very stable preparation has been obtained by cyanuric chloride activation of cellulose before binding of the DNA (Biagioni *et al.*, 1978). All these methods have the disadvantage that they are non-specific and there is no control of the extent of attachment of the DNA to the matrix; the mechanism of attachment is uncertain, presumably being via single-stranded regions of the DNA.

An elegant alternative method has been developed by Jovin & Kornberg (1968) which involves enzymic construction of a poly(dA) region at the end of a single-stranded DNA, then base pairing of this region to poly(dT) immobilized on cellulose, and finally completion of a strand complementary to the now immobilized DNA. Although this method gives a known method of attachment, again only one strand is attached and the procedure is not facile.

In all the above methods, the DNA is coupled directly to the matrix. As an alternative approach, the use of *p*-aminobenzoic acid as an extender arm (being attached to the DNA via reaction of the latter with *p*-diazobenzoic acid) has been reported, the complex being immobilized via reaction of the matrix with the carboxylic acid group of the linker unit (Dickermann *et al.*, 1978). Again only one strand is attached.

For use of immobilized DNA in affinity chromatography, and especially in drug-binding studies, it is necessary that both strands are irreversibly immobilized via a small number of linkages, that the DNA substantially retains its native conformation, and that the chemical nature of the linking group is known. We report here the alkylation of DNA with 4-bis-(2-chloroethyl)amino-L-phenylalanine (an alkylating agent, which will cross-link DNA) followed by coupling of the primary amino group of the phenylalanine unit to a matrix (in this case the polyacrylamide support, Enzacryl). The advantages of coupling a ligand to the DNA prior to coupling to the matrix are firstly, that the number of attachment points can be controlled and secondly, that the ligand will act as an extender arm.

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Materials and methods

Enzacryl AH was purchased from Koch-Light Laboratories; calf thymus DNA type 1, deoxyribonuclease I (EC 3.1.21.1 and ethidium bromide were obtained from Sigma; 4-bis-(2-chloroethyl)-amino-L-phenylalanine was supplied by The Wellcome Foundation, Crewe, Cheshire, U.K.; and daunomycin hydrochloride by Farmitalia, Milan, Italy.

Alkylation of DNA

A sample (10 μl) of a solution of 4-bis-(2-chloroethyl)amino-L-phenylalanine in dimethyl sulphoxide ($10\,\mu g \cdot ml^{-1}$) was added to 12 ml of a solution of DNA in distilled water ($1.0\,mg \cdot ml^{-1}$). The mixture was agitated occasionally at 20°C for 3 h and then dialysed against 500 ml of distilled water for 12 h to remove excess alkylating agent. This dialysis procedure was repeated three times. The concentration of DNA was calculated from the absorbance of the non-diffusable material at 260 nm by using the value $\varepsilon(P) = 6500\,litre \cdot mol^{-1} \cdot cm^{-1}$. The bulked diffusate was analysed for 4-bis-(2-chloroethyl)amino-L-phenylalanine.

Binding of alkylated DNA to Enzacryl

The conventional coupling procedure was used. Hydrochloride acid (2 M, 10 ml) was added to Enzacryl AH (100 mg) and the mixture was cooled to 0°C. Ice-cold $NaNO_2$ solution (4% w/v, 4 ml) was added with swirling, and after 15 min additional stirring, the Enzacryl was washed four times with ice-cold Clark & Lubs's (Dawson *et al.*, 1969) borate buffer (pH 8.4). A solution of alkylated DNA (8 ml) was then added to the activated Enzacryl and the mixture was allowed to stand at 4°C for 72 h. Then 0.008 M-Tris/HCl/0.05 M-NaCl buffer (pH 7.4, 10 ml) was added. After a further 16 h at 4°C, the supernatant was decanted and the Enzacryl was washed with the Tris/NaCl buffer and then with 2 M-NaCl until the washings showed no absorbance at 260 nm. The supernatant and washings were bulked and assayed, at 260 nm, for residual alkylated DNA. The amount of alkylated DNA bound to the Enzacryl was then calculated by difference.

Enzymic determination of alkylated DNA bound to Enzacryl

Deoxyribonuclease I (2 mg) was dissolved in acetate buffer (1.0 M, pH 5.0, 5 ml) and $MgSO_4$ solution (0.1 M, 2 ml) was added. The mixture was made up to 25 ml with distilled water and added to a sample of DNA immobilized on Enzacryl. After 16 h at 4°C, the absorbance of the supernatant at 260 nm was determined and the amount of DNA cleaved was calculated by comparison with a control solution of DNA treated in an identical manner.

Binding of ethidium to immobilized DNA

Ethidium bromide solution (5×10^{-5} M in 0.008 M-Tris/HCl/0.05 M-NaCl buffer, pH 7.4; 10 ml) was added to DNA–Enzacryl equivalent to 5.0 mg of DNA. After swirling, the absorbance of the supernatant at 480 nm was determined, and the concentration of unbound ethidium was calculated. Aliquots (10 μl) of appropriate concentrations of ethidium bromide in buffer were then added sequentially to give total concentrations of 8×10^{-5} M-, 1×10^{-4} M-, 2×10^{-4} M- and 5×10^{-4} M-ethidium bromide. The unbound concentration of ethidium was determined for each as above.

Solutions containing DNA (free in solution) and ethidium bromide were prepared to contain identical amounts of ethidium and DNA in 10 ml, as in the five tests with immobilized DNA. The absorbance reading at 480 nm was determined for each solution and the molar absorption coefficient for ethidium was calculated for each sample. The unbound concentration of ethidium in each sample was then calculated from the fraction unbound, which is equal to $1-(\varepsilon_f-\varepsilon_{obs.}/\varepsilon_f-\varepsilon_b)$ where $\varepsilon_{obs.}$ is the observed molar absorption coefficient, ε_f is the molar absorption coefficient for free (unbound) ethidium and ε_b that for bound ethidium (the latter two having been found from independent determinations). The concentration of unbound ethidium was then compared with that from the experiment with DNA–Enzacryl, for each pair of samples.

Results and discussion

We have attempted to produce a defined, covalently immobilized DNA preparation for use in affinity chromatography and drug-binding studies. Alkylation of DNA with 4-bis-(2-chloroethyl)amino-L-phenylalanine at about 1:3 molar ratio (assuming a mol.wt. of 10^8 for the DNA) gave over 95% binding of the alkylating agent to the DNA. Although the degree of binding was found by a difference method, and so will include any drug bound to the apparatus, a substantial fraction of the alkylating agent will have bound to the DNA giving at least 2 residues/DNA molecule. Coupling of the alkylated DNA to the activated Enzacryl AH was found to give 70–80% attachment to the support, hence at least 5 mg of DNA/100 mg of support. This was determined by a 'washings' method in which (after reaction) excess DNA solution was decanted, and the resin washed until no absorbance at 260 nm by the washings was detectable. The amount of alkylated DNA not bound to the support was then calculated from the absorbance of the bulked recovered solutions at 260 nm. The amount not bound was then subtracted from the amount added to give the amount bound. One sample of DNA–Enzacryl, for which the amount of DNA bound,

calculated by this method, was 1.69 mg, was treated with deoxyribonuclease I to give a direct estimation of the amount of DNA bound. This enzymic degradation method showed 1.33 mg of DNA to be bound. This lower figure is undoubtedly due to some of the immobilized DNA being inaccessible to the enzyme, and so it can be concluded that the washings method does give a reasonable estimate of the amount of DNA immobilized.

Several controls were performed to show whether the attachment to the matrix is via the phenylalanine group, as intended. When alkylated DNA was added to unactivated Enzacryl (and the procedure was performed in the same manner as for activated Enzacryl) 5.90 mg of the 6.00 mg of alkylated DNA was recovered. Next, DNA (rather than alkylated DNA) was carried through the procedure with activated Enzacryl and with unactivated Enzacryl; of 6.00 mg of DNA added in each case, 5.97 mg and 5.98 mg respectively were recovered. These controls therefore show that only coupling of alkylated DNA to activated Enzacryl gives a substantial degree of immobilization, verifying that immobilization is via the phenylalanine unit. A column was packed with the DNA–Enzacryl preparation and eluted with buffers of varying pH. The preparation was found to give no loss of material absorbing at 260 nm over the pH range 1.0–9.0. It is not stable at highly alkaline pH.

In order to be of use in affinity chromatography and drug-binding studies, the remaining active groups on the Enzacryl must be inactivated in some way. Preliminary studies, which attempted to block the remaining groups with glycine, were unsuccessful, but it was found that washing with Tris buffer gave substantial inactivation of the remaining sites. This was shown by using ethidium as a probe for residual active groups. Ethidium bromide solution was added first to activated Enzacryl that had not been washed with Tris buffer and NaCl solution, secondly to activated Enzacryl that had been washed with Tris buffer and NaCl solution, and thirdly to unactivated Enzacryl. Unbound ethidium assayed in the supernatant corresponded to 15%, 84% and 90% respectively of the added ethidium. This shows that the active acid azide groups can be blocked by washing with Tris buffer. It also shows that even with unactivated Enzacryl there is some adsorption of ethidium on to the matrix.

It was now necessary to determine whether the immobilized DNA retains its double-helical structure: this was assessed by using intercalating probes. It is known that, when daunomycin intercalates into double-stranded DNA, its absorption spectrum in the visible region shows a red shift, and the phenolic groups can no longer be ionized (Plumbridge & Brown, 1977). Consequently a solution of daunomycin (5×10^{-5} M in the Tris buffer) was added to unactivated Enzacryl and to a sample of the DNA–Enzacryl preparation and the colour of the matrix was observed. With the unactivated Enzacryl, the colour remained orange, whereas with the DNA–Enzacryl preparation, the matrix showed the red colour typical of intercalated daunomycin. On alkalinization, the matrix of the unactivated Enzacryl turned purple, due to ionization of adsorbed daunomycin, whereas with the DNA–Enzacryl, the red colour of DNA-bound daunomycin was retained: this is typical of intercalated daunomycin, confirming the double-helical nature of the immobilized DNA. A similar effect was seen with ethidium; the matrix showed the colour typical of intercalated ethidium on addition of a solution of ethidium to the immobilized DNA preparation. A comparison was then made between the binding of ethidium to immobilized DNA and to an equivalent amount of DNA in solution. Assuming that the affinity of ethidium for immobilized DNA is equal to its affinity for DNA in solution, an estimation of the 'apparent' amount of DNA in the immobilized DNA was made from a comparison of the free and bound concentrations in the immobilized and soluble DNA samples. This 'apparent' amount of DNA was found to be 64% of the actual DNA present; hence well over half of the immobilized DNA is available for intercalation of ethidium.

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