SPACER ARMS IN AFFINITY CHROMATOGRAPHY: USE OF HYDROPHILIC ARMS TO CONTROL OR ELIMINATE NONBIOSPECIFIC ADSORPTION EFFECTS

P. O'CARRA, S. BARRY and T. GRIFFIN
Department of Biochemistry, University College, Galway, Ireland

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1. Introduction

In previous communications [1–4] we have shown that failure to distinguish adequately between biospecific adsorption (bioaffinity) and nonbiospecific adsorption has seriously affected the validity and usefulness of much published affinity chromatographic work. In some cases nonbiospecific interference is so severe that it eclipses any element of biospecificity in the chromatography.

While some of this interference may be attributable to ion-exchange effects (see, e.g., refs. [5–10]), evidence suggests that the most serious and intractable interference is attributable to the commonly-used, hydrocarbon spacer arms which themselves promote nonspecific adsorption of a wide range of enzymes. This adsorption appears to be associated with the hydrophobic nature of these spacer arms and it was postulated that the interfering, nonspecific adsorption results mainly from hydrophobic interactions between these and the enzymes [1,2,4,11]. It was suggested that replacement of the currently-used arms by less hydrophobic ones might lessen or eliminate the interference [1].

We describe here the preparation and characteristics of more hydrophilic spacer arms, and we show that the use of such arms in the construction of affinity gels does indeed eliminate most of the interfering adsorption effects. Our previous conclusions regarding the source and nature of the interference are largely verified by these results.

2. Experimental

2.1. *Materials and methods*

1,3-Diaminopropanol was obtained from Ralph Emanuel Ltd., Wembley, Middlesex, England. All other chemicals were obtained as previously described [4,7,12,13]. The sources of the enzymes, the methods used for their assay and the chromatographic procedures were also as described in previous papers [4,7, 12,13].

2.2. *Construction of the hydrophilic arms*

The mode of construction of the hydrophilic spacer arms is illustrated in fig. 1. The agarose matrix (Sepharose 4B) is first activated with cyanogen bromide and coupled with 1,3-diaminopropanol (procedure 1. fig. 1), following the general procedure for coupling of amines described by Cuatrecasas [14,15].

The aminopropanol arm is lengthened and provided with a variety of terminal functional groups by addition of bromoacetyl and further diaminopropanol units as indicated, the procedures used for these steps being as follows (the numbering of the procedures corresponds to the numbering in fig. 1).

Procedure 2. The *N*-hydroxysuccinimide ester of bromoacetic acid is prepared and used to bromoacetylate the terminal amino group following the general procedure described by Cuatrecases [14,15].

Procedure 3. Diaminopropanol is condensed with the terminal bromoacetyl group by mixing the bromoacetylated gel with an equal volume of 0.1 M sodium

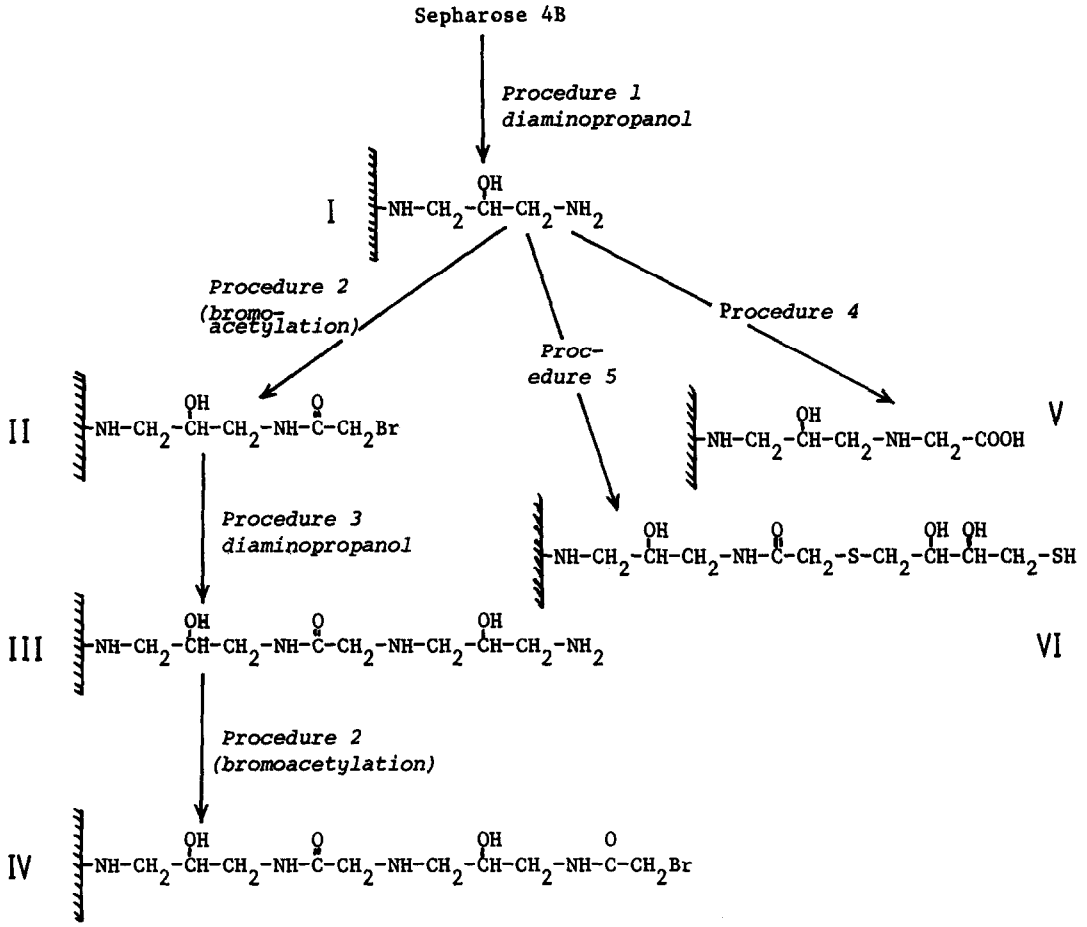

Fig. 1. Derivatization of Sepharose with hydrophilic spacer arms. (See experimental section for details of the synthetic procedures.)

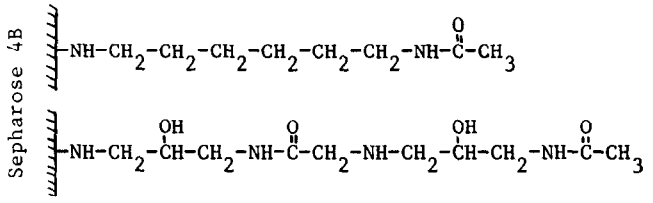

Fig. 2. 'Control gels' with no attached biospecific ligand. The terminal amino group of each spacer is acetylated to eliminate its ion-exchange capacity. The upper, hydrophobic control gel was prepared from aminohexyl-Sepharose 4B [14] by acetylation with the *O*-acetyl derivative *N*-hydroxysuccinimide [14,15] and the lower, hydrophilic gel was prepared by similar treatment of derivative III (fig. 1).

bicarbonate containing 0.4 M 1,3-diaminopropanol adjusted to pH 9, and incubating the mixture for 3 days at room temperature (about 15°C) with gentle stirring. The gel is then washed with 0.1 M saline (about 40 vol) and distilled water (about 10 vol) and any remaining unreacted bromoacetyl groups are blocked by incubation of the gel in 0.2 M mercaptoethanol in 0.1 M bicarbonate buffer, pH 9, at about 15°C for 24 hr, followed by washing of the gel as before.

Procedure 4. (To provide a carboxyl-ended arm). The amino-terminating gel is incubated for 3 days at about 15°C with an equal volume of 0.3 M bromoacetate in 0.1 M bicarbonate buffer adjusted to pH 9. The gel is washed as in procedure 3.

Procedure 5. (To provide a thiol-ended arm). The bromoacetylated gel is incubated with an equal volume of 20 mM dithiothreitol (Cleland's reagent) in 0.1 M bicarbonate buffer, pH 9, for 2 hr at about 15°C and then washed as in procedure 3.

2.3. *Attachment of ligands*

The hydrophilic arms posed no special problems as regards ligand attachment. Coupling of ligands to the derivatized gels was carried out exactly as previously described for the corresponding gels with hydrophobic spacer-arms.

The gels whose structures are illustrated in fig. 3 were prepared as follows: derivative A by the procedures described in ref. [12] and derivatives B and C by the same procedure applied to matrix-arm assemblies I and III (fig. 1); derivative D by the procedure described in ref. [4] and derivatives E and F by the same procedure applied to Derivatives I and III (fig. 1); derivative G by the solid-phase modular ap-

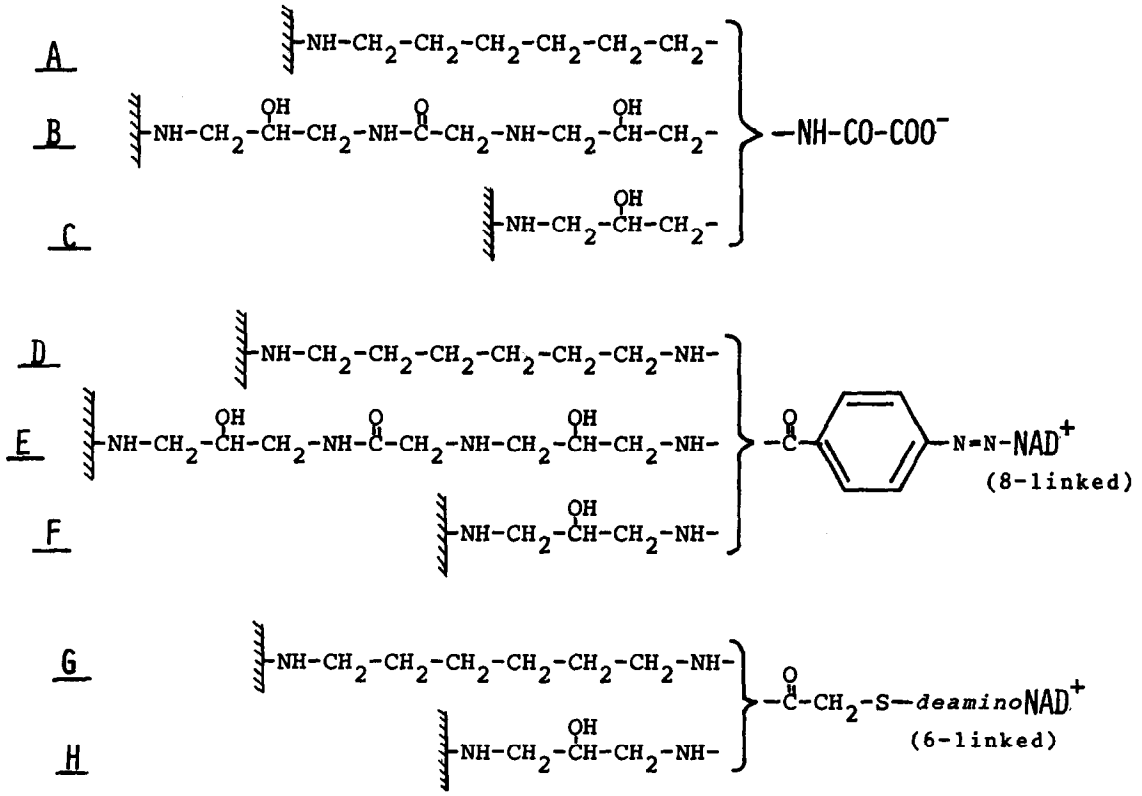

Fig. 3. Three selected affinity ligands discussed in the text immobilized through hydrophobic (A, D and G) and hydrophilic (B, C, E, F, and H) spacer arms. (A–C, immobilized oxamate; D–F, 8-linked azo-NAD^+; G and H, 6-linked 6-mercaptopurine analogue of NAD^+). The hatched portions on the left represent the Sepharose 4B matrix.

proach described in ref. [13] and derivative H by the same approach applied to assembly I of fig. 1.

3. Results and discussion

3.1. *Rationale behind the spacer-arm construction*

The chief aim governing the design and construction of the spacer arms illustrated in fig. 1, was the elimination of hydrophobic regions by ensuring that at least every alternate atom along the length of the arms formed part of a hydrophilic grouping such as an amide, secondary amino or carbinol grouping.

3.2. *Control experiments*

The clearest evidence of nonspecific adsorption involving hydrocarbon spacer arms has been provided by control experiments in which enzymes were chromatographed on control gels consisting of the agarose matrix substituted with commonly-used hydrophobic spacer arms to which no biospecific ligand was attached – for example the upper gel illustrated in fig. 2. Such control gels strongly adsorb a wide range of enzymes, the adsorption being quite unrelated to the biospecificity of the enzymes (but disturbingly similar in some cases to 'affinity chromatographic' behaviour described for a number of the same enzymes on affinity gel incorporating the same hydrophobic spacer-arms) [1,2,4]. This nonspecific adsorption onto the hydrophobic spacer-arms seems most likely to be attributable to hydrophobic interactions of the enzymes with the arms, but the interfering adsorption can be overcome in some cases. (e.g. lactate dehydrogenase) by raising the ionic strength of the irrigating buffer to a high level [4]. In other cases extreme dissociating conditions, such as extreme pH or concentrated urea, are required. Examples of such strongly-adsorbed ('sticky') enzymes are β-galactosidase, alcohol dehydrogenase and xanthine oxidase [1,2,4].

Similar control experiments were performed to ascertain whether such nonspecific adsorption was eliminated when the hydrophilic spacer arms of fig. 1 were used. Again control gels bearing no biospecific ligand were studied, the lower gel illustrated in fig. 2 being a typical example.

Such gels displayed none of the strong adsorption characteristics of the analogous hydrophobic control gels. Neutral buffers of low ionic strength (e.g. 0.05 M phosphate buffer, pH 7) were sufficient to prevent any significant adsorption of any of the enzymes tested – including the 'sticky' enzymes mentioned above.

3.3. *Effects on some selected affinity systems*

An affinity system for lactate dehydrogenase, which we have reported previously [7] was based on derivative A (fig. 3). This is an immobilized oxamate derivative which lactate dehydrogenase recognises as a pyruvate analogue. Owing to its ordered kinetic mechanism, the enzyme binds biospecifically to this derivative only in the presence of NADH and it is eluted when NADH is discontinued in the irrigating buffer. However, high salt concentrations must be continually maintained in the irrigant to avoid interference due to nonbiospecific adsorption [7,12]. With lightly substituted gels, 0.3 to 0.5 M KCl is sufficient for this purpose and this creates no great problem. However, much more severe interference is observed with more highly substituted gels (approx. 10 μmoles of ligand-arm assembly per ml of gel) whose higher capacity would otherwise be desirable for large-scale purifications.

Replacement of the hexyl spacer arm (A, fig. 3) by a more hydrophilic one, as in derivatives B and C (fig. 3), readily eliminated the interference. Lactate dehydrogenase is strongly bound by both these derivatives in the presence of NADH and is cleanly eluted, even at low salt concentrations, when the NADH is discontinued in the irrigant.

The H isoenzymic form of lactate dehydrogenase displays a weak bioaffinity for oxamate in the presence of NAD^+ (as contrasted with the strong bioaffinity in the presence of NADH). With the original type of gel (deriv. A, fig. 3) the weak NAD^+-induced bioaffinity could be usefully strengthened by carefully adjusting the salt concentration to a threshold level just above the level at which nonbiospecific adsorption begins to cause noticeable interference [12]

It has been suggested that the strengthening of the weak bioaffinity results from a synergistic compounding of the bioaffinity with marginal nonbiospecific interactions involving the hexyl spacer arm, to produce a comparatively strong 'compound affinity' [2,4,12]. In agreement with this interpretation, replacement of the hydrophobic hexyl spacer arm by

hydrophilic ones eliminates the reinforcement effect. For example, with derivatives B and C (fig. 3) no noticeable strengthening of the weak NAD^+-promoted retardation is observable even at very low salt concentrations (e.g. dilute phosphate buffer containing no additional salt).

The chromatographic behaviour of lactate dehydrogenase on the immobilized NAD^+ derivative D (fig. 3) has also been interpreted in terms of compound affinity [4]. Here again, strong nonbiospecific adsorption predominates at low ionic strength e.g. in buffers containing 0.1 M KCl and the enzyme is not competitively elutable from the gel by addition of soluble biospecific counter ligands, such as soluble NADH, to the irrigant. At high ionic strength (e.g. 0.5 M KCl) the adsorption is biospecific but weak, the enzyme being only marginally retarded by columns of the gel. At a threshold salt concentration of about 0.2 M, this weak biospecific adsorption is considerably strengthened; the enzyme is retained by the gel while the column is irrigated with several column-volumes of irrigant but is cleanly eluted when soluble NAD^+ or NADH is added to the irrigant.

When the aliphatic hexyl protion of the spacer arm is replaced by more hydrophilic groups, as in derivatives E and F (fig. 3), the reinforcing effect is diminished rather than completely lost in this case. At the previously-used threshold salt concentration the reinforcement disappears and the retardation is now only marginal, but at very low ionic strengths a reinforcement effect is again observed. This is attributed to the presence of the hydrophobic phenylazo grouping adjacent to the attached NAD^+ ligand. This grouping could not be eliminated or replaced since it is necessary for linkage of the NAD^+.

Glyceraldehyde-3-phosphate dehydrogenase is strongly adsorbed on derivative in an apparently biospecific fashion, but this biospecific adsorption is almost completely eclipsed unless a salt concentration of about 0.2 M or higher is maintained in the irrigant. Even this permits biospecific elution of only a low yield (approx. 20%) of the enzyme on addition of soluble NAD^+ to the irrigant and increasing the salt concentration to much higher levels does not improve the yield in this case [4].

Replacement of the spacer arm by a hydrophilic one, as in derivatives E and F (fig. 3) solves the problem, however. The glyceraldehyde-3-phosphate dehydrogenase was strongly and biospecifically adsorbed by both these derivatives and it was cleanly and almost quantitatively elutable by soluble NAD^+. The results with derivatives E and F were not significantly affected by variation of the KCl concentration between 0.1 and 0.8 M.

Alcohol dehydrogenase (from both horse liver and yeast) is one of the 'sticky' enzymes mentioned above. It is strongly adsorbed by derivative D (fig. 3) but even at very high salt concentrations it can not be biospecifically eluted with soluble NAD^+ or NADH [4]. By contrast, this enzyme is not significantly retarded by columns of derivatives E and F even at low ionic strength. This supports the contention [4] that the alcohol dehydrogenase has little biospecific affinity for the 8-linked, azo-substituted NAD^+ residue and that the adsorption on derivative D is almost entirely attributable to hydrophobic interaction with the spacer arm.

By contrast, adsorption of horse liver alcohol dehydrogenase on derivative G, containing a 6-linked NAD^+ analogue, is at least partly biospecific [13] and the enzyme is also effectively adsorbed when the spacer arm is changed to a hydrophilic one as in derivative H. However, the enzyme is much more cleanly eluted with soluble NADH from derivative H, even at low salt concentrations, than it is from derivative G at high salt concentrations – reflecting the positive interference from the hexyl spacer arm which is eliminated by the introduction of the hydrophilic arm in derivative H.

3.4. *General comments and conclusions*

Replacement of hydrophobic spacer arms by hydrophilic ones makes some affinity chromatographic systems more convenient to operate in the sense that the need to counteract nonbiospecific adsorption by maintaining high salt concentrations in the irrigant is largely eliminated. More important benefits accrue from a change of spacer arm where the nonbiospecific interference is not amenable to control by adjustment of the ionic strength or other parameters of the irrigating buffer and also in cases where high salt concentrations tend to interfere with the bioaffinity of the enzyme for the immobilized ligand. On the other hand, 'compound affinity' systems, which are dependent on a marginal level of nonbiospecific interactions to reinforce the bioaffinity are not necessarily im-

proved by changing to a hydrophilic spacer arm.

Certain strongly adsorbing 'affinity chromatographic' systems of doubtful biospecificity lose their strong adsorptive capacity when the spacer-arm is made hydrophilic, confirming the suspicion that at least some of these systems are mainly nonbiospecific in operation and are largely dependent on hydrophobic adsorption by the spacer arms. For example, systems developed for β-galactosidase [16] and sialidase [17] appear to be in this category [1,2,10].

The latter systems, and some others like them, are largely responsible for the view that long spacer arms are necessary to allow effective interaction between enzyme and ligand [14,15,18]. A reassessment of this view is now clearly necessary, since it now seems likely that the dependence of such systems on the long hydrocarbon spacer arms derives largely from the fact that the spacer arms rather than the attached ligands represent the dominant adsorbing species. In cases where the 'compound affinity' effect operates, the hydrophobic spacer arm also makes a positive, though much less overpowering, contribution to the adsorption, and it is possible that this positive reinforcing effect is more important than the passive spacer effect.

The hydrophilic arms clearly play a role much closer to that of passive spacer groups, and it is therefore notable that, to date, we have found no affinity system in which a long hydrophilic spacer arm (such as those of derivatives B and E (fig. 3), was noticeably more effective than a comparatively short one (such as those of derivatives C and F, fig. 3). We have not investigated analagous systems containing no spacer arm, since the spacer arms in most systems also constitute essential linking groups providing suitable termini for attachment of the ligands.

Our results cast considerable doubt on the appropriateness of the current preoccupation with ionic groups introduced onto the matrix by side reactions of the CNBr activation and ligand coupling processes. There has been a tendency to regard such side products as the most serious potential source of interfering adsorption. As a consequence it seems to have been generally accepted that ligand-arm assemblies should be pre-assembled by orthodox free-solution organic chemistry and carefully purified before being linked to the agarose matrix in a single coupling step to provide 'well-defined' affinity gels. However, it has been our experience that any nonbiospecific adsorption effects caused by immobilized side-products of the coupling procedures are negligible by comparison with the interference caused by the hydrophobic spacer arms. We have found it far more beneficial to modify the arms, or to control interference by adjusting the ionic strength, than to abandon convenient modular solid-phase synthesis of ligand-arm assemblies in favour of pre-assembly approaches which are nearly always much more technically difficult. For example, derivative H (fig. 3) was built up by solid-phase assembly on the agarose matrix in four separate, but technically easy, coupling steps. The resulting gel displays negligible nonbiospecific adsorption effects when compared with many of the 'presynthesized' assemblies embodying hydrophobic spacer arms.

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