AFFINITY CHROMATOGRAPHY ON AN HOMOLOGOUS SERIES OF IMMOBILISED N^6-ω-AMINOALKYL AMP. THE EFFECT OF LIGAND–MATRIX SPACER LENGTH ON LIGAND–ENZYME INTERACTION

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1. Introduction

The successful purification of a protein by affinity chromatography requires that the macromolecule should interact with the immobilised ligand in a manner which reflects the free solution interaction. To attain this objective, ligands are usually immobilised via an extension arm (spacer molecule) to the inert matrix. Similarly, the capacity of an affinity absorbent can be related to the length of the spacer molecule [1]. Cuatrecasas [2] has proposed that the extension arm negates the steric hindrance of the matrix backbone and allows an increased flexibility and mobility to the ligand. Lowe et al. [3] examining the affinity of several dehydrogenases and kinases for a series of insolubilised nucleotides, NAD^+ and ATP, showed that a marked increase in affinity was apparent when the ligand was immobilised at a distance of 5–10 Å from the matrix. It was concluded in this study that steric considerations are more important with high M.W. proteins and systems of low affinity; and further suggested that the extension arm enables the ligand to traverse a barrier imposed by the microenvironment of the hydrophilic polymer.

In the present communication this effect has been re-examined with several dehydrogenases on an homologous series of N^6-ω-aminoalkyl-adenosine 5′-monophosphate matrices in an attempt to avoid the effects of unsubstituted spacer arms encountered in previous studies and to re-examine the effect of varying spacer arm length using molecules of very similar chemical composition.

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2. Materials and methods

Enzymes and substrates were purchased from Boehringer (Mannheim), Germany. Cyanogen bromide, 1,5-diamino-pentane and 1,7-diaminopheptane were obtained from R.N. Emanual Ltd., Wembley, Middlesex, U.K. Ethylene diamine, 1,3-diaminopropane and 1,6-diaminohexane were purchased from Eastman Chemicals, Kodak, N.Y., U.S.A.; 1,4-diaminobutane, 1,8-diaminoctane and 1,10-diaminodecane were purchased from Aldrich Chemical Co., Inc., Milwaukee, Wisc., U.S.A. and 1.9-diaminononane was purchased from K. and K. Labs, Plainview, N.Y., U.S.A. The diamines C_2–C_7 were purified by distillation prior to use. 6-Mercaptopurine riboside 5′-phosphate was obtained from P-L Biochemicals Inc., Milwaukee, Wisc., U.S.A. Sepharose 4B was purchased from Pharmacia (GB) Ltd., London. W.5., U.K. Cellulose-coated aluminium plates were purchased from E. Merk, Darmstadt, Germany. All other chemicals were of the highest purity obtainable from BDH Chemicals Ltd., Poole, Dorset, U.K.

Enzymes: Lactate dehydrogenase (EC 1.1.1.27); D-glucose 6-phosphate dehydrogenase (EC 1.1.1.49); Malate dehydrogenase (EC 1.1.1.37); D-glyceraldehyde 3-phosphate dehydrogenase (EC 1.2.1.12).

2.1. *General methods*

The 100 MHz nuclear magnetic resonance (NMR) spectra were recorded on a Varian HA-100 instrument using the frequency sweep mode. Samples (~ 56mg/ml) were dissolved in D_2O and the pH adjusted to 10.5 by addition of anhydrous K_2CO_3. The mass spectrum of an exhaustively tri-methylsilylated sample [4] of N^6-(6-aminohexy)-AMP was recorded on an AEI MS 12 instrument at 70 eV using direct insertion (ion source temperature of 250°C) and an accelerating voltage of 7 kV.

Thin-layer chromatography was carried out on aluminium plates precoated with cellulose (0.1 mm thick) in iso-butyric acid : 0.880 NH_4OH : water (66:1:33 v/v). The products were located by ultra violet absorption (254 nm) or by spraying with group specific reagents. Phosphate determinations were carried out by the method of Meun and Smith [5]. The gels were treated by the method of Craven et al. [6] prior to the phosphate analyses. Protein was assayed by the method of Warburg and Christian [7], and enzymes were assayed by methods described by Barman [8].

2.2. *Preparation of the immobilised homologous series*

An homologous series of N^6(-ω-aminoalkyl)-AMP derivatives (fig. 1, $n = 2 \rightarrow 10$) were prepared from the corresponding α,ω-diaminoalkanes following the method of Craven et al. [6]. Coupling of the products to Sepharose was achieved by the method of Axen, Porath and Ernbäck [9]. Cyanogen bromide-activated Sepharose (5 g moist weight) was added to each AMP derivative (8.5 μmoles) in 0.1 M NaHCO, pH 10.0

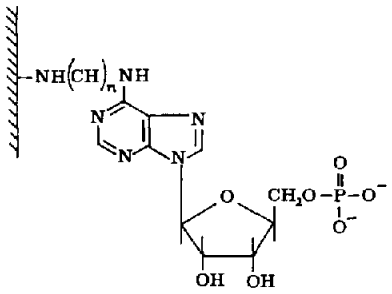

Fig. 1. Structure of immobilised N^6-ω-aminoalkyl-AMP-derivative n = 2 to 10.

(5 ml), and treated as described by Craven et al. [6]. In each case between 95 and 100% coupling was observed by ultra violet absorption (267 nm) difference. Phosphate analysis [5] indicated a final ligand concentration of 1.70 ± 0.04 μmoles N^6-ω-aminoalkyl-AMP per g moist weight Sepharose.

2.3. *Chromatography on the immobilised homologous series*

The modified gels (1 g moist weight) were packed into glass columns (5 × 50 mm) and equilibrated at 4°C in 10 mM KH_2PO_4-KOH at pH 7.5 containing 0.02% sodium azide.

A mixture of enzyme and bovine serum albumin (BSA) was dialysed overnight against equilibration buffer at 4°C. The enzyme–protein sample was run into a moist bed of each polymer and developed by washing with several bed volumes of equilibration buffer, a linear gradient of KCl (0→1.0 M; 20.0 ml total volume) followed by a 200 μl 'pulse' of 5 mM NADH applied to the column in the same way as the enzyme–protein mixture. The column flow rate was maintained at 8.0 → 10.0 ml per hour and 1.4 ml fractions were collected. The binding (β) of the enzyme to a polymer is defined as the molarity of KCl, conductivity measured at 20°C, coincident with the peak of enzyme activity in the eluant.

3. Results

Thin layer chromatography of the products on cellulose yielded a single ultraviolet absorbing spot: the R_f value was dependent on the number of methylene groups in the extension arm. No measurable increase in R_f was observed for compounds where n (fig. 1) was greater than 7 ($R_f = 0.78$) however, below $n = 7$ the R_f value decreased by 0.06 with each decreasing methylene group such that at $n = 2$ the R_f was 0.48.

Ninhydrin confirmed the presence of the terminal amino group and the Hanes–Isherwood reagent [10] demonstrated the presence of organic phosphate. The higher chain length derivatives where $n = 8 \rightarrow 10$ (fig. 1) were characterised by following the increase in the NMR resonance signal assigned to the inner methylene protons of the side chain at 8.7 τ. The 100 MHz nuclear magnetic resonance spectrum of N^6-(6-aminohexyl)-AMP is shown in fig. 2. The signals at 1.55

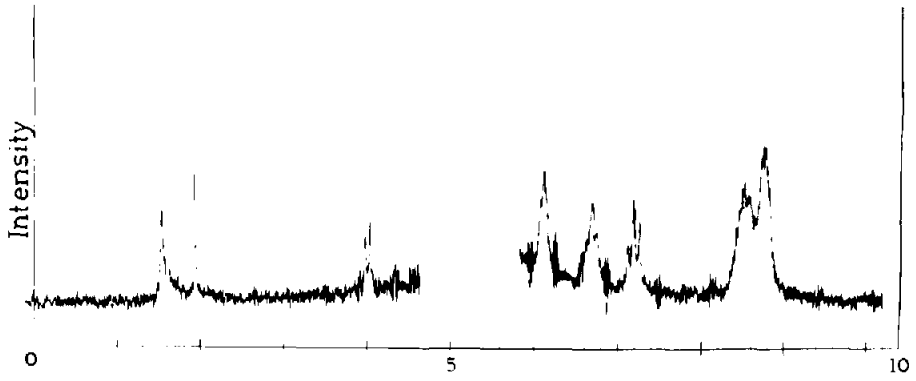

Fig. 2. 100 MHz nuclear magnetic resonance spectrum of N^6-(6-aminohexyl) adenosine 5'-monophosphate. Experimental conditions are given in text.

and 1.95 τ have been assigned to the aromatic protons. The doublet at 4.00 τ is the signal associated with the anomeric proton and the resonance signal at 6.12 τ to the H_5'. The triplets at 6.70 τ and 7.2 τ are due to the α and ω methylene protons and the strong broad doublet at 8.7 τ has been assigned to the other methylene proton resonances.

3.1. *Enzymic studies with the immobilised homologous series*

Fig. 3 illustrates the chromatography of a mixture of lactate dehydrogenase (rabbit skeletal muscle) and bovine serum albumin on two polymers, N^6-(2-aminoethyl) and N^6-(6-aminohexyl)-AMP-Sepharose. The enzyme bound strongly to the latter polymer, elution being effected by a nucleotide 'pulse' (200 µl 5 mM NADH). In comparison, the binding to the former polymer, in which the ligand is spaced by only two methylene units from the matrix, was considerably less. In this case, elution occurred at 200 mM KCl: bovine serum albumin was eluted in the void volume (fig. 3).

Fig. 4 summarises the effect of increasing the length of the spacer arm on the binding (β) of several

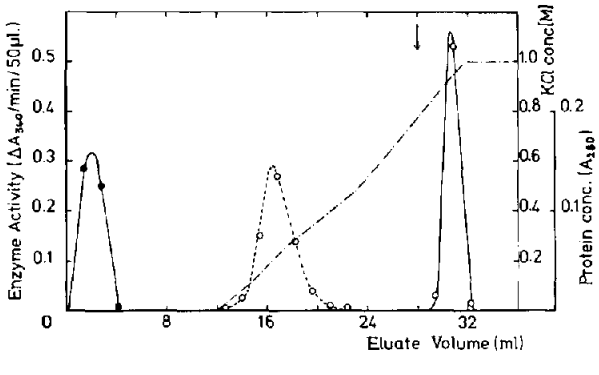

Fig. 3. Chromatography of lactate dehydrogenase-M_4 (○) on N^6 (2-aminoethyl)-5'-AMP-Sepharose (——) and N^6-(6-aminohexyl)-5'-AMP-Sepharose (———). Experimental procedure is given in the text, the enzyme sample (50 µl) contained LDH-M_4 (4 U) and bovine serum albumin (0.75 mg) (●———●). KCl concentration (----) was monitored at 20°C, (↓) indicates the addition of 5 mM NADH (200 µl).

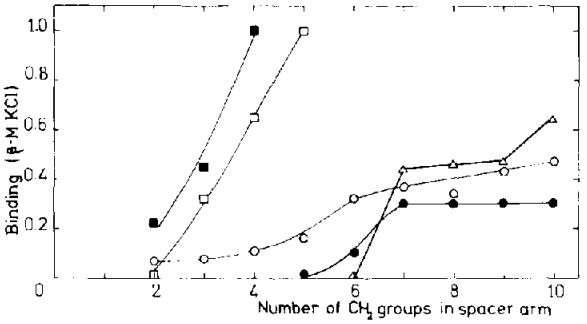

Fig. 4. Effect of spacer arm length on the binding of several dehydrogerases to N^6-ω-aminoalkyl-AMP-Sepharose. Expermental procedure is given in the text; each enzyme sample (50 µl) contained bovine serum albumin (0.75 mg). Binding (β) represented the concentration of KCl (20°C) required to elute the enzyme. Lactate dehydrogenase-H_4 (4 U, □); lactate dehydrogenase-M_4 (4 U, ■); D-glucose 6-phosphate dehydrogenase (2 U, ●); malate dehydrogenase (4 U, ○) and D-glyceraldehyde 3-phosphate dehydrogenase (2.5 U, △).

dehydrogenases to these polymers. The binding (β) of the two isoenzymes of lactate dehydrogenase increased rapidly from $n = 2$ to $n = 5$, further extensions of the spacer arm necessitated the use of NADH pulses to effect elution. The binding of malate dehydrogenease, D-glucose 6-phosphate dehydrogenase and D-glyceraldehyde 3-phosphate dehydrogenase is significantly lower than that of the lactate dehydrogenase isoenzymes. However, in all cases, the binding of the enzymes in polymers where $n > 7$ did not appear to change significantly.

4. Discussion

It is becoming increasingly difficult to make generalisations about the effect of spacer-arm length on enzyme binding in affinity chromatography. As the ligand is extended further from the matrix the binding approaches a maximum (Cuatrecasas [2]; Lowe et al. [3]). After the point of maximum interaction is reached, however, two distinct types of behaviour have been reported. Lowe et al. [3] have found that in the chromatography of various dehydrogenases and kinases on an homologous series of ω-aminoalkanoyl-NAD^+ (or ATP)-Sepharose, the binding approaches a maximum and in some instances then declines. The latter phenomenon has been explained by the suggestion that the chain folds back on itself resulting in a decrease in the spacing of the ligand as the series is ascended. Cuatrecasas [2], on the other hand, has found that the efficiency of a specific adsorbent of staphylococcal nuclease increased to a maximum which was maintained and showed no decline on any further extension of the spacer arm.

These two distinct types of behaviour may reflect, in part, the nature of the spacer arm used. Previous results from this laboratory were limited by the undefined nature of the polymers and the presence of unsubstituted spacer arms [3]. In the present study, the polymers were defined and also since measurements were made at constant ligand concentrations, the only variable is the addition of methylene residues to the spacer arm. The present results agree with those of Cuatrecases in that no decline in binding after the maximum was observed. The chemical difference between the spacer arms use by Lowe et al. [3] and those used in this study is the absence of any carbonyl function adjacent to the ligand in our polymers.

The greater binding observed for the skeletal muscle isoenzyme of lactate dehydrogenase may reflect an electrostatic contribution to the binding of the enzyme which will be positively charged at the pH of the experiment. It has been shown recently [11,12] that specific retention of enzymes by columns substituted with spacer arms alone is possible and that these columns serve as effective 'affinity' adsorbents. This suggests that the spacer-arm contributes to the binding of the enzyme. Preliminary work in this laboratory suggests that the binding properties of the spacer arm are amplified on isolubilisation in the case of N^6-(6-aminohexyl)-AMP.

Inspection of fig. 4 shows that suitable choice of the polymethylene extension arm-length has obvious practical applications to specific enzyme separation. This parameter can be combined with other variables, important to the affinities of individual enzymes to such matrices, such as ligand concentration [13], temperature [14], and pH [15].

Subsequent to the completion of this work, it has become clear that the isourea structure associated with amine immobilisation to CNBr activated polysaccharides is the predominant species and this group is charged (pK 8) [16]. Furthermore, Wilchek has proposed that the combination of this charged group and polymethylene spacer arms has not only potential ion exchange capacity, but also has an additional 'detergent' effect. This effect can produce very pronounced binding of a number of enzymes [12] and probably explains many of the observations relating to the 'importance' of spacer arms in affinity chromatography. Similar conclusions have recently been drawn by O'Carra et al. [11] in a re-examination of the original β-galactosidase study conducted by Steers et al. [17].

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