BINDING OF PROTEINS TO CNBr-ACTIVATED SEPHAROSE 4B

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1. Introduction

CNBr-activated Sepharose is finding increasing use in the preparation of specific adsorbents, and with the advent of a simplified activation procedure [1] the popularity of Sepharose-conjugated materials will undoubtedly increase.

The concentrations of coupled ligand relative to Sepharose bead volume (referred to as 'bead concentration') has been demonstrated to be quite important in determining the ultimate specific activity of the ligand. For example, we found a decrease of about 10–15% in the antigen-binding capacity of rabbit anti-fetal calf serum globulin when we increased the concentration of bound globulin from 6.6 mg to 10 mg of globulin per ml of Sepharose 4B. Furthermore, the specific activity of a coupled enzyme can be drastically altered by changing its bead concentration [2].

While on a gross scale one can correlate the activity of a Sepharose-bound molecule with its bead concentration, it would seem that a more accurate correlation can be derived only from consideration of the microenvironmental concentration of that species. In other words, the distribution of coupled ligand within the bead must be considered. It is conceivable that varying the coupling procedure, such as by altering the amount of CNBr during the activation step, or by using aminoethyl-Sepharose or carboxymethyl-Sepharose and carbodiimide, may cause considerable differences in ligand distribution.

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It is generally assumed that if one couples to Sephadex or Sepharose a molecule sufficiently small to penetrate the matrix, coupling will take place throughout the gel, while a molecule too large to diffuse into the gel will be localized on the surface [3,4]. Lasch et al. [5] have presented data which confirmed this assumption, using fluorescein-conjugated leucine aminopeptidase. Ten-micron sections of Sepharose 6B exhibited fluorescence throughout the bead matrix, while the authors stated that sections of Sephadex G-100 showed fluorescence only on the surface. Fluorescence in the latter case was apparently too weak to photograph.

While working with Sepharose-bound bovine lactoperoxidase, results obtained during the enzymatic iodination of polyoma virus suggested that perhaps most of the lactoperoxidase resided at the surface of the Sepharose bead (G. S. David and T. Friedmann, unpublished data). It was felt that the iodination efficiency was considerably higher than would be expected if the enzyme were distributed throughout the bead. If the above hypothesis were correct one would expect lactoperoxidase (M.W. = 78 000) to be distributed throughout the Sepharose bead (molecular exclusion limit 20×10^6 molecular weight) and consequently only a small fraction would be accessible to the polyoma virus (M.W. = 25×10^6), which should not have penetrated the bead.

Similarly, Axén et al. were surprised at the levels of caseinolytic activity of Sephadex-bound chymotrypsin [6]. If the rate of hydrolysis were diffusion-limited, they reasoned, a longer time should be necessary for substrate and enzyme to come into proximity and acquire the appropriate orientation for catalysis to take place. In view of the data presented below, the

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results of Axén et al. might have been less surprising if one assumed that the chymotrypsin was localized at the surface of the Sephadex beads.

Data presented here suggest (a) that radioautography is a far more sensitive means of determining the localization of a conjugated species within a Sepharose bead than is fluorescent labeling, and (b) it should not be assumed that a low molecular weight ligand will necessarily be distributed throughout a CNBr-activated bead.

2. Materials and methods

Sepharose 4B (Pharmacia) was activated with CNBr (4 g/15 ml beads) according to the method of Cuatrecasas [3], except that the activation was carried out in an ice bath over a period of approximately 1–1 1/2 hr rather than for a much shorter time at room temperature [2]. Bovine lactoperoxidase (Sigma) and bovine serum albumin (Pentex) were coupled to the activated beads as previously described [2]. The lactoperoxidase was iodinated with ^{125}I to a specific activity of 11.7 μCi/μg using the solid state lactoperoxidase method [2,7].

Radioautography was carried out using Kodak NTB-2 emulsion. Exposure times were approximately 0.1 hr-μCi/bead (assuming a bead diameter of roughly 120 μ). For example, a preparation of beads exhibiting a calculated specific activity of 0.001 μCi per bead would have been exposed for 100 hr.

3. Results and discussion

To determine the effect of total protein in the coupling reaction mixture on the bead distribution of the coupled product, approximately 4.5 μg of [^{125}I]LP* were bound to about 100–300 μl of CNBr-activated Sepharose 4B in the presence of carrier BSA ranging in concentration from 0–5.5 mg per ml of beads. In all cases, at least 90% coupling efficiency was obtained. The beads were sectioned into 10 μ slices and the distribution of [^{125}I]LP was determined by radioautographic analysis.

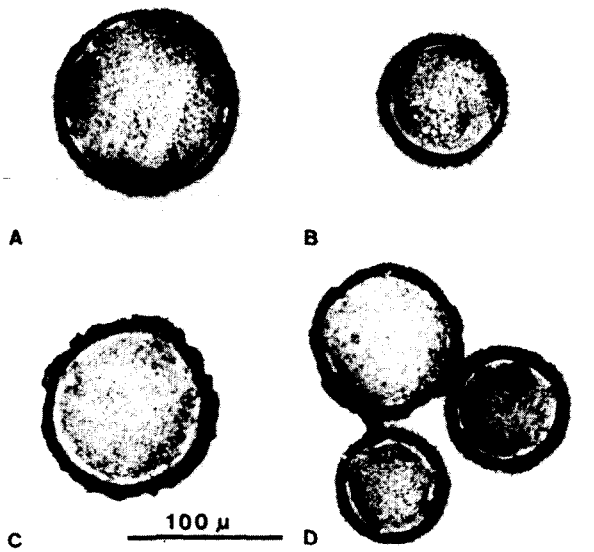

Fig. 1. Radioautographs of 10 μ sections of CNBr-activated Sepharose 4B beads to which have been coupled (A) 55 μg [^{125}I]LP per ml of beads, (B) 26 μg [^{125}I]LP + 210 μg BSA per ml of beads, (C) 27 μg [^{125}I]LP + 1120 μg BSA per ml of beads and (D) 27 μg [^{125}I]LP + 5540 μg BSA per ml of beads.

In fig. 1 are shown the results of four different preparations of beads containing 0.055, 0.24, 1.14 and 5.6 mg protein (BSA + [^{125}I]LP) per ml of packed beads. As the radioautographs indicate, even at a total protein concentration of 5.6 mg per ml of beads, virtually no [^{125}I]LP was localized on the inside of the beads. All of the coupling appears to have taken place at the surface of the beads, forming a 'shell' of Sepharose-bound protein.

An interesting effect of this 'shell' is that the protein seems to be providing cross-linking of the Sepharose matrix, leading to some degree of stabilization of the agar in the area of protein binding. As the radioautographs suggest, the drying process may have caused some shrinkage of the inside of the beads, while the outer shell retained most of its configuration.

The discrepancy between the results presented here and those presented by Lasch et al. [5] may have been due to differences in the bead activation procedure or to the nature or concentration of the ligand. Perhaps

* Abbreviations: LP, lactoperoxidase; BSA, bovine serum albumin.

the fluorescein labeling of the leucine aminopeptidase inhibited the coupling reaction (by reducing the number of primary amino groups) sufficiently to allow diffusion into the Sepharose bead, although this seems unlikely, since only four fluorescein residues were incorporated per enzyme of mol.wt. 326 000.
The high concentration of protein (25 mg per ml of beads) used by Lasch et al. [5] may have been at least partially responsible. Furthermore, these authors used Sepharose 6B rather than Sepharose 4B and an enzyme with a molecular weight of 326 000 rather than the 78 000 MW lactoperoxidase molecule. These differences may have led to a decrease in accessibility of CNBr-activated sites within the Sepharose 6B bead matrix and may thus have allowed a greater penetration of a molecule into the bead before it 'found' an activated site. Finally, differences in CNBr activation procedures may have allowed for differences in concentration of activated sites on the Sepharose beads and thus differences in the rate of the coupling reaction. A slower coupling reaction should allow greater penetration of the bead by the ligand.

Data presented here suggest that one should not assume that the microenvironmental concentration of a ligand within a CNBr-activated Sepharose bead is necessarily in direct relationship to the bead concentration of that ligand. A number of factors may influence the bead distribution of that ligand, and if such considerations are important for a given experimental design, the bead distribution should be ascertained under the exact activation and coupling conditions used for that experiment. Radioautography, used previously to determine the distribution of growing ^{3}H-labeled peptide chains within a Merrifield resin bead [8], provides a relatively simple and sensitive tool for that purpose.

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