PROTEIN BINDING BY AGAROSE CARRYING HYDROPHOBIC GROUPS IN CONJUNCTION WITH CHARGES

B.H.J. Hofstee
With the technical assistance of N. Frank Otillio

Biochemistry Division, Palo Alto Medical Research Foundation
Palo Alto, California 94301

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SUMMARY: The binding of a series of proteins to agarose (Sepharose 4B) substituted with n-alkylamines of varying C-chain length (C_1, C_4 or C_8) has been investigated. At pH 8 and 0.05 ionic strength the negatively charged proteins (chymotrypsinogen X, serum albumin, ovalbumin and β-lactoglobulin), in contrast to the positively charged species (chymotrypsinogen A, α-chymotrypsin, and lysozyme) had strong affinity for the adsorbents with the longer C-chains (C_4, C_8). The binding appears to depend on cooperation between hydrophobic and electrostatic forces, the latter involving positive charges on the adsorbent which are introduced by the substitution process.

INTRODUCTION: Many biological processes depend on accessible hydrophobic groups on protein molecules. This holds true, for example, for the interaction of many enzymes with their substrates and modifiers (1-3), for the affinity of antibodies for antigens and haptens (4,5) or for the binding and transport of metabolites (and drugs) by serum albumin (6,7).

Due to the low solubility of strongly hydrophobic ligands, study of the hydrophobic effect per se is often difficult in aqueous solution. However, molecular dispersion of such ligands, even in the absence of solubilizing agents, can be obtained through their attachment to an insoluble hydrophilic material such as beaded agarose.

It has been shown for several proteins that hydrophobic interaction is rather nonspecific. For instance, the affinity of serum albumin for certain hydrophobic ligands appears to be determined merely by their lyophilic character as estimated from the octanol-water partition coefficients (8,9). Thus the possibility exists that certain unrelated proteins, endowed with accessible hydrophobic sites, might bind to a hydrophobic moiety likely to occur in a ligand designed to have specific affinity for a particular biologically active protein.

Previous results (10) showed that, at an ionic strength of 0.05 and pH 8, several negatively charged proteins, as well as the positively charged α-chymotrypsin, are strongly bound by agarose substituted with 4-phenylbutylamine (PBA). Chymotrypsinogen A, also positively charged,

showed little affinity for this adsorbent. A specific effect, based on complementarity of molecular contours, may be assumed to be involved in the interaction of the active site of the enzyme with the phenyl group of the substituting ligand (see ref. 11). The present adsorbents, which were obtained through substitution of Sepharose 4B with simple aliphatic $-NH(CH_2)_nCH_3$ groups of varying C-chain length, did not show this specific effect. These adsorbents, at least the ones with the larger hydrophobic groups (C_4, C_8), showed a strong affinity only for the negatively charged proteins that were tested, including a negatively charged carbamylated derivative of chymotrypsinogen A. The data indicate that strong binding may result through the cooperation of hydrophobic and electrostatic forces, even in the absence of "specific" interaction.

EXPERIMENTAL: Chymotrypsinogen A (CTg-A), α-chymotrypsin (CT), lysozyme (LYS), bovine serum albumin (BSA), ovalbumin (OV) and β-lactoglobulin (β-LG) were highly purified crystalline preparations (Worthington, Nutritional Biochemical Corporation, Sigma). Chymotrypsinogen X (CTg-X), a carbamylated fully biologically active derivative of CTg-A was the same preparation as applied previously (12). Sepharose 4B was obtained from Pharmacia, methylamine hydrochloride was purchased from Baker, n-butylamine hydrochloride and n-octylamine hydrochloride were from Eastman. Cyanogen bromide (CNBr) was obtained from Aldrich, dimethylformamide from Matheson Coleman and Bell and ethylene glycol was 'Baker analyzed.'

Activation of the Sepharose by CNBr and coupling with each of the amines (dissolved as the hydrochlorides in 50 per cent DMF and adjusted to pH 10 - 10.5) was carried out by the procedure of Cuatrecasas (13). Since the amines buffer at the applied pH, no further buffer was added. The resulting methyl-, n-butyl- and n-octyl-derivatives, referred to as the C_1-, C_4-, and C_8- adsorbents respectively, were washed exhaustively with 50 per cent dimethylformamide and with 50 per cent ethylene glycol, both solutions containing 1 M NaCl, and subsequently were washed with water. At least for the case of serum albumin, the protein binding capacities in 0.05 M Tris-HCl, pH 8, were several mg per ml of slurry of the C_4- and of the C_8-derivatives (see below). Further experimental details are described in the figure legends.

RESULTS: Fig. 1-A shows that in 0.05 M Tris-HCl buffer at pH 8, CTg has little or no affinity for C_1-substituted agarose. Although the protein is slightly retarded on the C_4- and to a somewhat greater extent

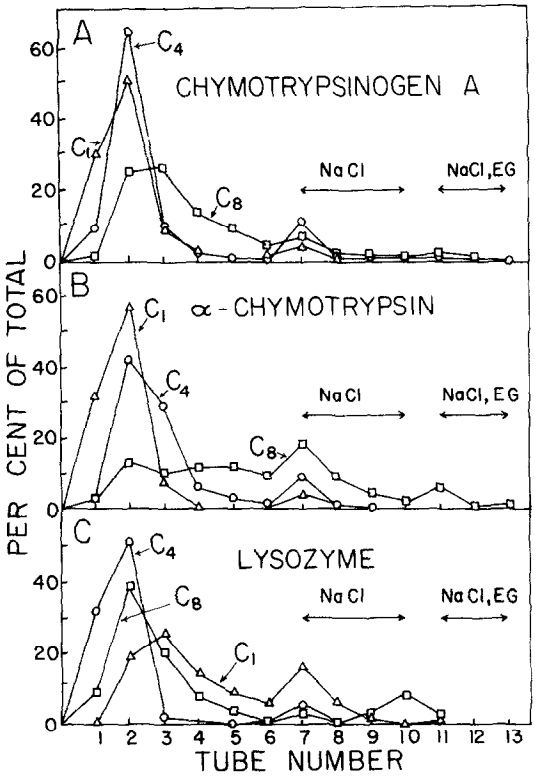

Fig. 1. Effects of the addition of salt (1 M NaCl) and of a polarity reducing agent (50 per cent ethylene glycol, EG) on the binding of chymotrypsinogen A (part A), α-chymotrypsin (part B) and lysozyme (part C) on 1 ml columns of Sepharose 4B substituted with either methyl (C_1)-, n-butyl(C_4)- or n-octyl(C_8)amine. The columns were equilibrated at ≅ 5° with 0.05 M Tris-HCl buffer, pH 8, loaded with 1 mg of protein in the buffer and washed successively with 2 ml portions of the buffer alone, buffer containing 1 M NaCl, and buffer containing 1 M NaCl plus 50 per cent (v/v) ethylene glycol. The amount of protein in the eluate (per cent of total applied) was determined from the light absorbance at 280 nm.

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on the C_8-adsorbent, the data indicate that the zymogen can be eluted from all three adsorbents by the buffer alone. The same is true for CT (Fig. 1-B) and for LYS (Fig. 1-C). However, Fig. 2-A indicates that, under the same conditions, CTg-X is strongly held by the C_4- and C_8- materials, although not by C_1. It should be noted that at the applied pH the A-zymogen (as well as CT and also LYS) carries a positive overall charge, whereas the X-zymogen is negatively charged under the experimental conditions (12). From the results of Fig. 2-B it can be seen that the behavior of OV, which is also negatively charged at the applied pH, is similar to that of CTg-X. Likewise, negatively charged BSA and β-LG (Fig. 3) have little or no affinity for C_1 but are strongly bound by C_4

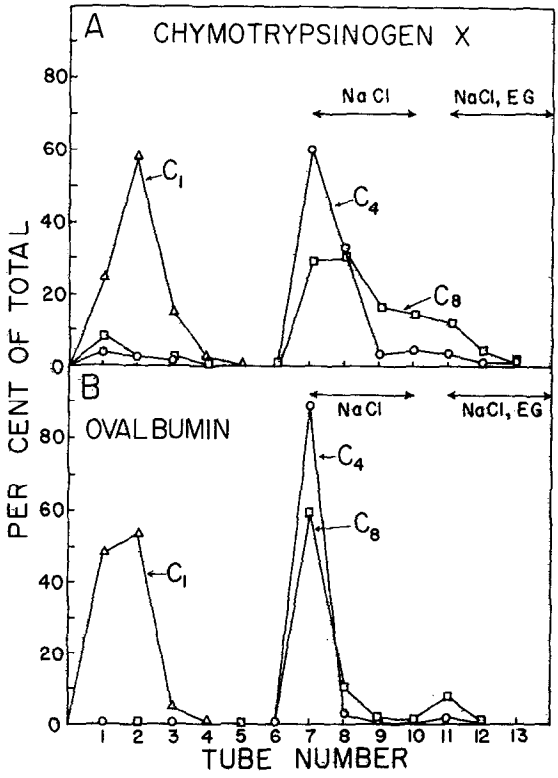

Fig. 2. Binding of chymotrypsinogen X (part A) and of ovalbumin (part B) on the C_1-, C_4- and C_8- adsorbents under the same conditions as described in the legend for Fig. 1.

and C_8.* It should be emphasized that the binding of these negatively charged proteins to the ligands with the longer C-chains is practically "irreversible" in 0.05 M Tris buffer. For rapid elution of these proteins from the adsorbents it is necessary to increase the ionic strength. All of the tested proteins bound by C_4 can be eluted by adding 1 M NaCl to the buffer. With some proteins, e.g., CTg-X and OV, this also holds true for the C_8-adsorbent (Fig. 2). However, reversal of the binding of BSA and of β-LG to C_8 requires further additives. As

* While the present work was being prepared for publication, a paper by Er-el et al. (18) appeared describing studies on protein binding to n-alkyl-Sepharoses in 0.05 M β-glycerophosphate buffer, pH 7. Under their conditions BSA did not bind to the C_4-adsorbent. This could be due to the higher ionic strength of the divalent glycerophosphate as compared to the equimolar but monovalent Tris buffer applied in the present experiments. As shown by the present and also by previous data (10) the ionic conditions are of critical importance in the binding of proteins to this type of adsorbents.

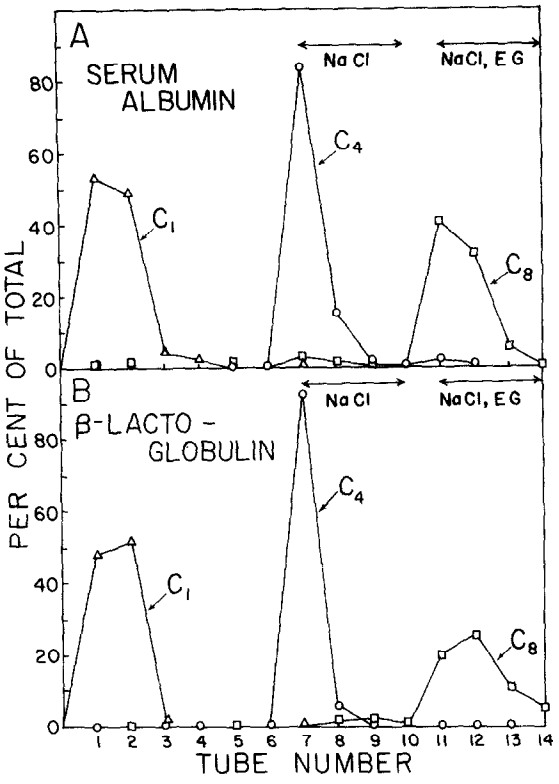

Fig. 3. Binding of bovine serum albumin (part A) and of β-lactoglobulin (part B) on the C_1-, C_4- and C_8-adsorbents under the same conditions as described in the legend for Fig. 1.

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can be seen (Fig. 3) these proteins are released upon the addition of 50 per cent ethylene glycol. The latter decreases the polarity of the medium and tends to weaken hydrophobic bonding.

The fact that only the adsorbents with the larger hydrophobic groups (C_4 and C_8, as opposed to C_1) bind the pertaining proteins indicates that hydrophobic interaction is a primary factor in the binding. This is consistent with the finding that BSA and β-LG, in contrast to OV, bind much stronger to C_8 than to C_4, the former two proteins exhibiting stronger hydrophobic properties than the latter (8). On the other hand, reversal of binding by the addition of salt, as is invariably the case for the C_4-adsorbent, indicates that hydrophobic binding is aided by electrostatic forces. It is known that even highly purified agarose carries negative charges (14). However, positive charges are introduced by the protons taken up by the agarose bound amino groups of the substituting ligand (see ref. 15). This could have a bearing on the finding

that negatively charged proteins in particular are bound by the present adsorbents.* In any event, assuming that the charges are the same on the C_1-, C_4- and C_8-adsorbents and in view of the fact that binding occurs only after substitution by the larger hydrophobic groups, it appears that electrostatic binding is aided by hydrophobic interaction. Furthermore, as seen from the data of Fig. 3, the dissociating effect of salt is enhanced by polarity reducing ethylene glycol. Conversely, the dissociating effect by ethylene glycol was found to be enhanced by salt (see also ref. 10). Thus it appears that the often strong binding of proteins to this type of adsorbents greatly depends on a cooperative and presumably mutually reinforcing effect of (short range) hydrophobic and (long range) electrostatic forces.

DISCUSSION: Although the present type of binding apparently is of a more general and less specific nature than that of an enzyme for its substrate or substrate analogue (see Introduction), this does not necessarily imply that the binding is weak. On the contrary, as shown by the above results, for some proteins and in the case of the more strongly hydrophobic adsorbents, reversal of binding is difficult to achieve.

The possibility exists that protein denaturation occurs due to "detergent-like" action of the hydrophobic ligand. In order to reduce the hydrophobicity of ligands with long (C_{10}) hydrocarbon chains, Yon has introduced additional ionic groups (16). However, the present results suggest that this problem may be circumvented by employing hydrophobic ligands of more moderate size. Also, the effects of relatively simple substituents may be more readily interpretable. On the other hand, the introduction of negative charges instead of or in addition to the present positive charges, might produce useful adsorbents for further studies of this kind.

To provide for a greater variability in the type of ligand, including the introduction of aromatic structures, it might be expedient to attach these ligands to "arms" (see ref. 17) that by themselves are not hydrophobic, e.g., carbohydrates instead of hydrocarbons. The required minimum

* The presence of negative charges on the untreated Sepharose is confirmed by the fact that at extremely low ionic strength (e.g., 0.001) only positively charged proteins are bound (see ref. 10). However, after treatment with CNBr but without the subsequent addition of substituting amine, the opposite is true, i.e., the agarose preferentially binds negatively charged proteins (unpublished data). Thus the possibility cannot be excluded that positive charges are also provided by CNBr-modified, but unsubstituted, sites on the adsorbent.

length of the arm per se may be found to vary from one protein to the next. Another parameter, not yet explored for the present adsorbents, is the effect of pH. Since it is apparent that the overall charge on the protein is a factor, binding would depend on the pH of the medium and the isoelectric point of the protein.

On the basis of these considerations and as already demonstrated with other adsorbents of this type (10), the present substituted agarose preparations present many opportunities for protein separation by column chromatography. Er-el et al. (18) recently have shown that n-alkyl-Sepharoses can be applied to the chromatographic separation and purification of certain enzymes. The present results further emphasize the general applicability of these adsorbents and suggest the use of gradients of polarity reducing agents, of salt and/or of pH, to achieve elution and separation of the proteins.

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