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SELECTIVE AROMATIC-HYDROPHOBIC BINDING AND FRACTIONATION OF IMMUNOGLOBULIN BY MEANS OF PHENYL-$(CH_2)_n$-NH-SUBSTITUTED AGAROSES

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. Under specified conditions the major portions of immunoglobulin preparations ("Fraction II", IgG) are selectively bound by agaroses substituted with phenyl-$(CH_2)_n$-NH-groups as compared to agaroses carrying aliphatic n-alkyl-NH-groups of similar hydrophobicity and ligand density. These immunoglobulin preparations, even the most highly purified ones, were found to be inhomogeneous with respect to the aromatic-hydrophobic mode of binding and fractionation through differential adsorption on the basis of the hydrocarbon chain-length of the ligands has been attained.

In a previous report (1) the binding of a number of proteins by n-alkyl-NH-substituted agaroses was compared to the binding of these proteins by agaroses substituted to about the same extent with phenyl-$(CH_2)_n$-NH-groups of closely similar hydrophobicity. It was found that some of the proteins (e.g., serum albumin and β-lactoglobulin) were preferentially bound by the aliphatic adsorbents, whereas others (e.g., chymotrypsinogen and γ-globulin) showed a distinct preference for the aromatic ligands. It was also shown that these preferences tend to be masked by high density and/or by high hydrophobicity of the ligands, especially in the presence of high salt (NaCl) concentrations that may greatly enhance hydrophobic but not necessarily aromatic interaction per se. The present communication is concerned with an investigation of this preferential aromatic effect for the case of γ-globulin, in particular with respect to the apparent inhomogeneity and the possibility of chromatographic fractionation of already highly purified preparations of this type of protein.

EXPERIMENTAL

Materials. The adsorbents were $CH_3(CH_2)_n$- and phenyl$(CH_2)_n$-amino-agaroses, prepared (2) via CNBr-activation of CL-Sepharose 4B (Pharmacia). Before use the adsorbents, all derived from the same batch of activated agarose, were heated for 1 hr at 100° in water (3). The relative extents of the charges introduced by the substitution process, i.e., those on the -NH-

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connector group (4) as well as other charges (e.g., see footnote in ref. 5), were estimated from the maximal amounts of Ponceau S "irreversibly" bound in the absence of salt (6). The dye-binding capacities, in excess of that of the heated unsubstituted CNBr-treated agarose, were taken as a measure of the degrees of substitution of the adsorbents. Earlier data indicated that these Ponceau-binding capacities of n-alkylamino-agaroses are proportional to the protein binding capacities in the case of electrostatic binding by a series of different adsorbents at low salt concentrations (7) as well as in the case hydrophobic binding by various preparations of the same adsorbent at high salt (NaCl) concentrations (8). The adsorbents were stored at pH 3-4 and 5°, i.e., under stabilizing conditions (e.g., see refs 9,10).

The preparations of γ-globulin were Fraction II (USB), Fraction II, grade A (CALBIOCHEM) and bovine IgG (MILES).

Methods. A solution of 2-4 mg of the protein in 0.01 M Na-phosphate, pH 6.8, and containing a specified amount of NaCl was applied at room temperature to the first of a series of 1 ml columns of the adsorbents in disposable Pasteur pipets, arranged in sequence of increasing hydrophobicity, the hydrophobicity of a phenyl-group being equal to 3-4 straight-chain methylene groups (11,12). The columns were interconnected by means of TEFLON tubing and OMNIFIT connectors (BIOLAB, Dover N.J.) and the protein washed into the gradient with 70-130 ml of the ambient buffer-salt solution by means of a peristaltic pump. Use of the hydrophobicity gradient (13) is to ensure that the binding of a particular protein species occurs on a column that provides not more than the minimum affinity needed and consequently to raise the likelyhood that the binding is quantitatively reversed by means of a relatively mild (non-denaturing) eluant (see also ref. 14). For this purpose the columns, after completion of the run, were disconnected and separately eluted with 50 per cent ethylene glycol (15). The protein contents of the eluates were determined spectrophometricaly.

RESULTS

Aromatic-hydrophobic binding versus hydrophobic binding per se. Fig. 1 shows that in the presence of 4 M NaCl the major portion of the protein in "Fraction II" is strongly bound by non-aromatic short-chain n-alkyl-adsorbents, the maximum of binding occurring with the propylamino-agarose. However, the extent of binding rapidly decreases with decrease of the salt concentration, which is indicative of hydrophobic binding (e.g., see ref. 16). Upon lowering of the salt concentration to 0.5 M NaCl and under the applied conditions, nearly all of the protein passes through the entire series of non-aromatic hydrophobic columns, including the C_7*- and C_8-adsorbents.

In contrast to the non-aromatic n-alkyl-adsorbents, which in 0.5 M NaCl bind little or none of the protein, the data of Fig. 2 indicate that at the same low salt concentration, a considerable fraction of the protein is held by

*Abbreviations: C_1-C_8, agaroses substituted with n-alkylamines with hydrocarbon chain lengths of 1-8; C_4-phe, 4-phenyl-n-butyl-NH-agarose; C_2-phe, 2-phenyl-ethyl-NH-agarose; γ-G, γ-globulin (immunoglobulin).

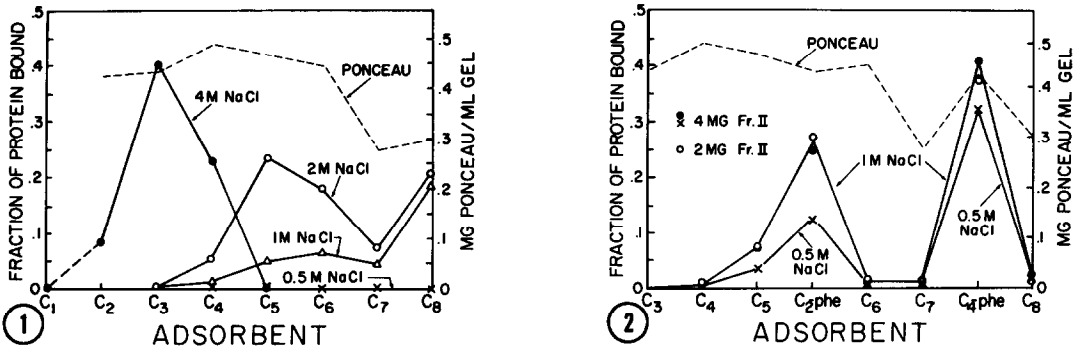


Fig. 1. Effect of 4 M, 2 M, 1 M, or 0.5 M NaCl, on the extent of non-aromatic hydrophobic binding of γ-globulin (Fr. II) on a hydrophobicity gradient of n-alkylamino-agaroses after application of 70, 100, 78 or 100 ml of the ambient salt solutions respectively. Dashed curves indicate the Ponceau-binding capacities of the adsorbents (see EXPERIMENTAL).

Fig. 2. Effect of salt (NaCl) concentration and of the amount of protein on the extent of aromatic-hydrophobic binding of γ-globulin (Fr. II) by the hydrophobicity gradient of Fig. 1 and under the same conditions, but with aromatic (phenyl-n-alkyl) columns interposed as indicated (see text). The extent of wash-in was ≈80 ml for all three runs.

the C_2-phe column and an even larger fraction by the more hydrophobic C_4-phe-column. However, it is also noted that the total recovery of the protein is much lower in 0.5 M NaCl than when the ambient salt concentration is 1.0 M. As can be seen, this is due primarily to the lower extent of binding of the first, apparently most hydrophobic, fraction bound by the C_2-phe-column. For this reason and also because higher salt concentrations may result in extensive non-aromatic, purely hydrophobic, binding (see Fig 1), subsequent experiments were carried out in the presence of 1 M NaCl. Although at this salt concentration considerable binding occurs by the C_5-adsorbent (see also below), binding further up the gradient is almost exclusively by the aromatic adsorbents. This is emphasized by the fact that a large fraction of the protein is bound by the C_2-phe and that the remainder passes through the (more hydrophobic) C_6- and C_7- adsorbents, and is not bound until it reaches the C_4-phe column.

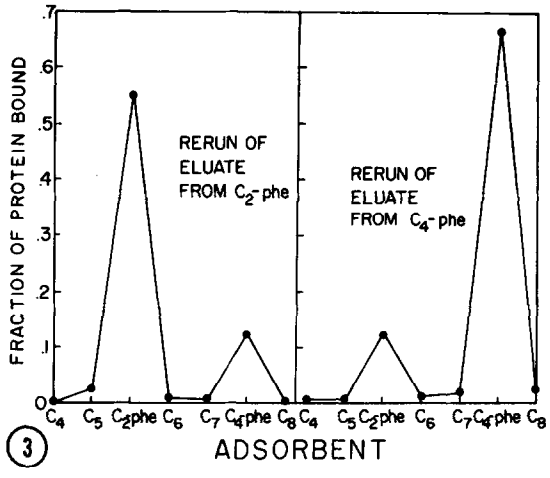
The data of Fig. 2 also show that in 1 M NaCl the results are largely independent of the amount of γ-globulin employed (either 4 or 2 mg), indicating that the binding of protein by C_4-phe was not due to overloading of the

C_2-phe-column and "spilling over" of protein onto the more hydrophobic column.

Effect of ligand density of adsorbent. The relative extents of protein binding by the adsorbents likely are affected by differences in their ligand contents. Normalization of the data with respect to this factor strictly applies only to the case of saturation of the adsorbents with the protein (e.g., see ref. 17). As seen from the absence of saturation revealed by the data of Fig. 2, this does not seem generally to be the case with the amounts of protein applied here. However, if "normalization" of the data of Figs. 1 and 2 nonetheless is carried out on the basis of the Ponceau-binding capacities of the adsorbents - and which under sub-saturating conditions would tend to exaggerate the relative effect of the ligand concentration - it is found that, qualitatively, this factor does not affect the conclusions drawn from the data. It should also be noted that all of the results presented in this report were obtained with the aid of the same set of adsorbents.

Fractionation of γ-globulin through differential aromatic-hydrophobic adsorption. Comparison of various preparations of the protein. The data of Fig. 3 show that when the two fractions recovered from the C_2-phe and C_4-phe columns (Fig. 2), are dialyzed and separately rechromatographed on the same series of adsorbents, the bulk of each of these fractions again is found on the adsorbent by which it was originally held. The fact that one of the protein fractions is held by a less hydrophobic adsorbent than the other would suggest that the first one is the more hydrophobic of the two, which would be in accord with the finding that the binding of this fraction is more dependent on the presence of salt than the fraction predominantly bound by the C_4-phe adsorbent (see Fig. 2). On the other hand, the possibility can not be excluded that the protein species bound by the C_4-phe-adsorbent require a longer "spacer" between the matrix and the aromatic group.

Fig. 4 shows a comparison of the distribution of different preparations of immunoglobulin washed into the series of interconnected columns with 130 ml of the medium. It can be seen that part of all of these preparations is found



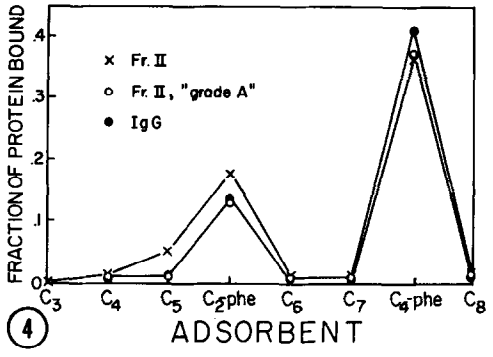


Fig. 3. Separate reruns of the dialyzed fractions eluted from the C_2-phe- and C_4-phe-columns of Fig. 2 (1 M NaCl) on the same series of adsorbents and under the same conditions.

Fig. 4. Comparison of the chromatographic behavior of various immunoglobulin preparations on the same series of adsorbents as for Figs. 2 and 3. Four mg of protein dissolved in 1 M NaCl was washed into the gradient with the aid of 130 ml of the ambient medium.

on the C_2-phe- and part on the C_4-phe-adsorbent, whereas little or none is held by the interposed aliphatic C_6-and C_7-columns. However, a considerable part of the presumably least pure Fraction II preparation is found on the C_5-adsorbent. It is of interest that little or none of this, apparently hydrophobic but non-aromatic, binding occurs with IgG and with Fraction II, grade A. The additional binding by the C_2-phe, observed only in the case of the least pure preparation, also could be caused by purely hydrophobic interaction. In any event, it appears that, at least in 1 M NaCl, "pure" immunoglobulin is almost exclusively bound by the aromatic adsorbents. This is further confirmed by the finding that the reruns of the adsorbates of C_2-phe and C_4-phe (Fig. 3), in contrast to the original preparation, also show little or no binding by the C_5-adsorbent. The separability into fractions of different adsorptive properties and the persistence of the distribution of the protein over both of the employed aromatic adsorbents, even for the case of IgG (Fig. 4), indicates an inherent inhomogeneity of immunoglobulin with respect to aromatic binding sites (see DISCUSSION).

DISCUSSION

Preferential binding of γ-globulin as compared to a much lower affinity of serum albumin for agarose substituted with ligands carrying aromatic groups, at least in the case of phenylalanine, had been noted previously (13, 18) and was tacitly ascribed to differences in the hydrophobicities of these proteins. The first indication of a propensity of γ-G for aromatic binding per se was not noted until later (1). The present results show that the relative extents of hydrophobic and aromatic binding, which are assumed to be synergistic (1), greatly depends on and can be manipulated by the salt (NaCl) concentration. The results also indicate that the selective aromatic binding of γ-globulin can be applied to its purification (see Fig. 4). However, perhaps more important is the observation of the persistent inhomogeneity of binding, even of the IgG preparation (Fig. 4). This would be in accord with the finding that the hapten binding sites of γ-G tend to be aromatic (see ref. 19). Since these binding areas are on the variable portion of the molecule, the interaction of such sites with aromatic adsorbents would be inherently inhomogeneous. On the other hand, on this basis nearly identical results with different preparations of the protein (Fr. II, grade A and IgG, see Fig. 4) presumably originating from different sources, could not be expected.

The present results do not imply that only two major fractions with different aromatic-hydrophobic binding properties are involved. It may be assumed that part of the fraction that passes through the C_2-phe-column (Fig. 2) and subsequently is bound by C_4-phe, might have been held by a C_3-phe-column. Furthermore, the fraction (≈30 percent) that in the presence of 0.5 M NaCl passes through the entire series of adsorbents may possibly be held by adsorbents with phenyl$(CH_2)_n$- ligands with hydrocarbon chain lengths>C_4. It will also be of interest to study the interactions of immunoglobulins with adsorbents carrying aromatic groups other than phenyl. Variation of the aromatic moiety per se can be expected to provide many opportunities for fractionation in addition to those based on the C-chain length of the non-

aromatic purely hydrophobic moiety of the ligands employed in the present investigation.

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