Journal of Chromatography, 616 (1993) 189–195
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam

CHROMBIO. 6848

Quantitation of adsorption capacity of immunoglobulin G on histidine–aminohexyl Sepharose and determination of affinity constant

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(First received September 15th, 1992; revised manuscript received March 12th, 1993)

ABSTRACT

Histidine, a pseudobiospecific ligand, had been utilized to purify several proteins such as chymosin, acidic protease, carboxypeptidase Y and immunoglobulin G (IgG). A detailed study was undertaken to purify IgG on histidine coupled to aminohexyl Sepharose [A. El-Kak and M. A. Vijayalakshmi, *J. Chromatogr.*, 510 (1991) 29]. To better understand the force of interaction between IgG and histidine coupled to aminohexyl Sepharose, the equilibrium dissociation constant (K_D) was determined by standard techniques such as frontal and zonal elution. The maximum capacity (Q_X) and K_D were determined to be 11.6 mg IgG per ml gel and $2.4 \cdot 10^{-6}$ *M*, respectively, by frontal analysis. Using zonal elution with histidine as a competing soluble free ligand in the equilibrating buffer, the values K_D between IgG and soluble free histidine and between IgG and immobilized histidine were determined to be 0.351 *M* and $2.4 \cdot 10^{-5}$ *M*, respectively. The zonal elution value is approximately ten times higher than that estimated by frontal analysis. It was verified again by equilibrium binding analysis. Using this technique we determined K_D and Q_X to be $4.6 \cdot 10^{-6}$ *M* and 9 mg/ml, respectively, which are very close to the frontal analysis results.

INTRODUCTION

The success of a chromatographic separation depends upon the biospecific interaction between the protein of interest and the immobilized ligand. All other components of a mixture should have little or no affinity for the ligand. In order to better understand the interaction of the ligand with the protein it is necessary to know the strength of their affinity in the form of the equilibrium constant, K_a [1]. Several chromatographic approaches are available to quantify the affinity constants. Two experimental approaches in this field predominate, namely frontal and zonal elution analyses [2,3]. Batch-type equilibrium binding analysis constitutes another non-chromatographic method [4,5].

Several proteins and peptides have been purified using histidine ligand affinity chromatography on both analytical and preparative scales. These include acidic protease [6], chymosin [7], carboxypeptidase Y [8] and immunoglobulin G (IgG) polyclonal [9] and monoclonal [10]. Melin and Vijayalakshmi [11] found the equilibrium dissociation constant (K_D) to be $2.82 \cdot 10^{-2}$ *M* between histidine and IgG by zonal elution analysis when histidine was coupled directly to Sepharose by epichlorohydrin activation.

In this study we have determined the maximum adsorption capacity, Q_x, and K_D for histidine coupled to aminohexyl Sepharose (H-AH-Sepharose) gel by frontal and zonal analyses and

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0378-4347/93/$06.00

compared them with the values obtained by batch-type equilibrium binding analysis.

EXPERIMENTAL

Materials

All buffers were prepared with deionized water purified by a Milli-Q system (Millipore, France) and the reagents were of analytical grade. Tris-(hydroxymethyl)aminomethane was purchased from Merck (Darmstadt, Germany), Blue Dextran 2000 from Pharmacia Fine Chemicals (Lot No. C064) and sodium chloride from Sigma (St. Louis, MO, USA). Pure IgG from human placenta (Lot No. 91G5010, 157 g/l) containing glycine (20 g/l), sodium chloride (1 g/l) and traces of IgA (<3%) was a kind gift from Dr. Grand George of Institut Merieux, France. All mobile phases were degassed under vacuum. Histidine coupled to aminohexyl Sepharose (H-AH-Sepharose) was prepared in our laboratory as described in ref. 10.

Chromatographic conditions for IgG were as follows: adsorption in the presence of 25 m*M* Tris–HCl buffer, pH 7.4, and elution with the same buffer containing 0.2 *M* sodium chloride [10].

Determination of IgG concentration:

The concentration of IgG was determined by measuring the absorbance (*A*) of the solution at 280 nm (assuming $A^{1\%}_{280\ nm} = 14$ for IgG), the molecular masss of IgG being approximately 150 000.

Frontal analysis

For this study, a column of 1.3 cm × 1 cm I.D. was filled with H-AH-Sepharose gel and a preparation of human placental IgG, containing 157 mg/ml IgG (*ca.* 95% IgG), was used. The experiment was carried out as described in ref. 12. To the column, equilibrated with 25 m*M* Tris–HCl, pH 7.4, a protein solution approximately diluted in the same buffer was fed continuously until the absorbance of the effluent at 280 nm attained a plateau. Then the column was washed with the equilibrating buffer until the absorbance of the effluent at 280 nm reached zero. The capacity of the gel at this particular concentration of the protein was determined by eluting with the equilibrating buffer containing 0.2 and 1 *M* sodium chloride. The experiment was repeated at different concentrations upto 25 mg/ml IgG. By doing so the capacity of the column, Q_a, obtained at different concentrations (C) could be utilized to determine the equilibrium relationship of the protein between the liquid and the solid phase (gel).

The data obtained were fitted to the Langmuir model as shown below:

$$Q_a = \frac{Q_x C}{K_D + C} \quad (1)$$

where C is the concentration of the protein solution (mg IgG per ml), K_D is the equilibrium dissociation constant (ml/mg or M^{-1}), Q_a is the equilibrium ligand-bound IgG concentration (mg IgG per ml gel) and Q_x is the maximum adsorption capacity of the gel (mg IgG per ml gel)

Equilibrium binding analysis

The experimental method is similar to that mentioned in refs. 4 and 5. The experiment was carried out at room temperature (20–22°C). H-AH-Sepharose equilibrated with 25 m*M* Tris–HCl buffer, pH 7.4, was allowed to settle in a 10-ml graduated cylinder to a constant final bed volume (at least 30 min). The gel was then homogeneously suspended in an equal volume of the equilibrating buffer and 200-μl aliquots of the suspension were added to a series of Eppendorf tubes, in duplicate, containing 100 μl of IgG of concentrations ranging from 0 to 12.5 mg/ml in the equilibrating buffer.

After incubation for 30 min with intermittent shaking, the tubes were centrifuged (30 s at 1200 rpm, 186 *g*) to pellet the gel and 100 μl of the clear supernatant were withdrawn from each tube to determine the unbound protein concentration (C). The ligand-bound protein concentration, Q_a, was determined from the difference between the initial and equilibrium protein concentration, C. These data were plotted according to the transformed Langmuir equation to determine K_D and

Q_x as shown below:

$$\frac{1}{Q_a} = \frac{K_D}{Q_x}\ \frac{1}{C} + \frac{1}{Q_x} \quad (2)$$

$$\frac{C}{Q_a} = \frac{1}{Q_x} C + \frac{K_D}{Q_x} \quad (3)$$

$$\frac{Q_a}{C} = -\frac{1}{K_D} Q_a + \frac{Q_x}{K_D} \quad (4)$$

$$\ln \frac{Q_a}{L} = \ln K_a + n \ln C \quad (5)$$

where $L = (Q_x - Q_a)$ is the equilibrium ligand concentration and n is the protein interaction cooperativity.

Zonal elution

For this study, a column of 2.3 cm × 1 cm I.D. filled with H-AH-Sepharose gel was used. From the previously published work [10] it is known that H-AH-Sepharose adsorbs human placental IgG_1 in the presence of 25 m*M* Tris–HCl buffer, pH 7.4, and IgG_1 is eluted with the same buffer in the presence of 0.2 *M* sodium chloride. Therefore, for this study only IgG_1 was used.

Determination of the void volume (V_m)

Blue Dextran 2000 was chosen as a tracer to determine void volume because its molecular size is greater than the pore size of the gel. The tracer is eluted in the void volume. It is known from a previous report [11] that Blue Dextran 2000 does not interact with the gel in the presence of 0.05 *M* Tris–HCl buffer, pH 9.0. After the column has been equilibrated with 0.05 *M* Tris–HCl buffer, pH 9.0, a 1-ml sample of a solution containing 5 μg of Blue Dextran 2000 was injected and the elution volume was measured at a flow-rate of 27.6 ml/h. The absorbance of the effluent was continuously monitored at 254 nm.

Determination of the unretarded volume (V_0)

It is known from a previous report [10] that IgG_1 does not interact with histidine when the equilibrating buffer contains 0.2 *M* sodium chloride. Once the column has been equilibrated with 25 m*M* Tris–HCl buffer, pH 7.4, containing 1.0 *M* sodium chloride, a 850-μg sample of purified IgG_1 was injected at a flow-rate of 27.6 ml/h and the elution volume was measured to determine the void volume. In our case we used 1.0 *M* sodium chloride in the equilibrating buffer instead of 0.2 *M* sodium chloride to avoid ionic interactions.

Determination of the elution volume of IgG_1 in the presence of free ligand (V)

To an H-AH-Sepharose column previously equilibrated with 25 m*M* Tris–HCl, pH 7.4, 850 μg of IgG_1 were injected and the elution volume was measured. The flow-rate was maintained at 27.6 ml/h. Different concentrations of the free ligand ranging from 0 to 200 m*M* were used and the elution volume was measured for each free ligand concentration. From the gradient and intercept of the plot $1/(V - V_0)$ *versus* L, K_L and $K_{\bar{L}}$ could be calculated [13].

$$\frac{1}{V - V_0} = \frac{K_{\bar{L}}}{(V_0 - V_m)\,\bar{L}} + \frac{K_{\bar{L}}\,L}{K_L(V_0 - V_m)\bar{L}} \quad (6)$$

where K_L is the dissociation constant for IgG and soluble free ligand (histidine) and $K_{\bar{L}}$ is the dissociation constant for IgG and immobilized ligand (histidine). K_L could be calculated from the ratio of intercept to slope and $K_{\bar{L}}$ could be calculated from the intercept.

RESULTS

The interaction of IgG and histidine coupled

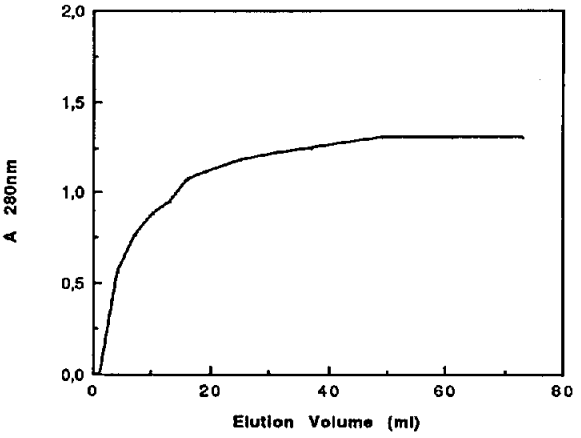

Fig 1. Frontal analysis of immunoglobulin G (1 mg/ml) on H-AH-Sepharose. For experimental details, see text. Linear velocity, 35.1 cm/h; temperature, 4°C.

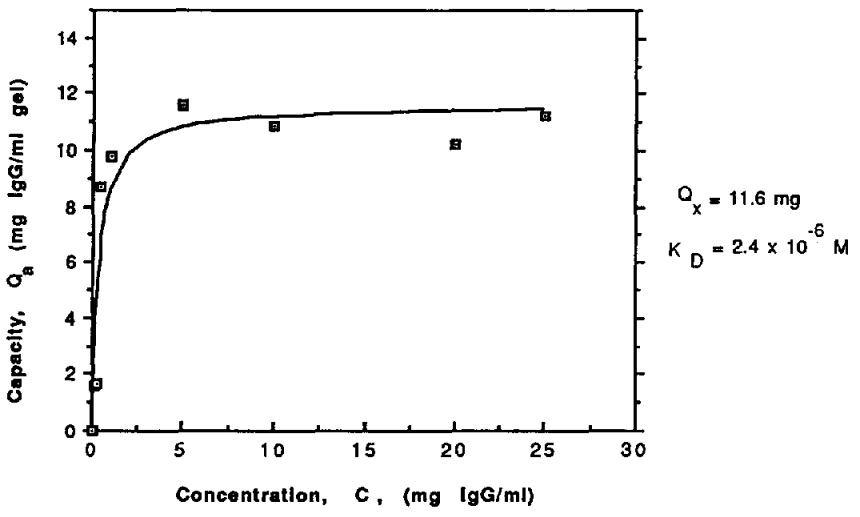

Fig 2. Adsorption isotherm of human IgG on H-AH-Sepharose.

to aminohexyl Sepharose was evaluated by frontal affinity chromatography. Fig. 1 shows the frontal analysis profile for IgG at a concentration of 1 mg/ml. This profile is representative of the concentrations we used.

Data were analysed by eqn. 1. Fig. 2 shows that the histidine–IgG complex has an apparent equilibrium dissociation constant (K_D) of 2.4 · 10^{-6} M with a maximum binding capacity of approximately 11 mg IgG per ml gel. Data as analysed according to eqn. 1 seem to produce a reasonable fit.

The same parameters (K_D and Q_x) were determined for histidine–IgG interaction using a non-chromatographic equilibrium binding approach (Scatchard analysis). Fig. 3 illustrates the results of equilibrium binding analysis plotted according to eqns. 2–5 and shows the estimated K_D and Q_x to be 4.6 · 10^{-6} M and 9 mg IgG per ml gel. The difference in the binding capacity and the affinity constant by these two methods is not large.

In the zonal elution experiment ($V_0 - V_m$) was found to be 0.37 ml. From Fig. 4 it is readily seen that retardation of IgG increased as the concentration of free histidine, L, in the 25 mM Tris–HCl buffer, pH 7.4, decreased. The binding constants measured by this method are K_L = 2.4 · 10^{-5} M and K_L = 3.5 · 10^{-1} M. The results obtained by three different methods are summarized in Table I.

DISCUSSION

The shape of the elution front in Fig. 1 is sharp for H-AH-Sepharose, indicating an even distribution of the protein zone along the entire length of the column. This is apparently due to the uniformity in packing of the column.

Fig. 2 shows the adsorption isotherm. It follows a typical Langmuir-type isotherm and is reasonably well fitted to the simple Langmuir isotherm equation. The frontal chromatography is, however, labour-intensive and requires significantly more protein. Unarska *et al.* [14] have determined 13 mg/ml for γ-globulin as a maximum binding capacity on protein A-bound Sepharose. In our case we obtained 11 mg/ml, which is a similar value.

Equilibrium binding analysis enables easy and fast determination of K_D and Q_x. The Scatchard plot is sensitive to heterogeneous and cooperative binding effects [4]. The Hill plot (Fig. 3d) requires prior knowledge of the total binding site capacity to calculate the equilibrium concentration of free binding sites remaining at each concentration of interacting solute. In contrast, the Scatchard plot allows the total number of binding sites to be determined by extrapolation without the need to reach saturation experimentally (Fig. 3d).

Non-homogeneous interaction of protein has been demonstrated by Hutchens and co-workers

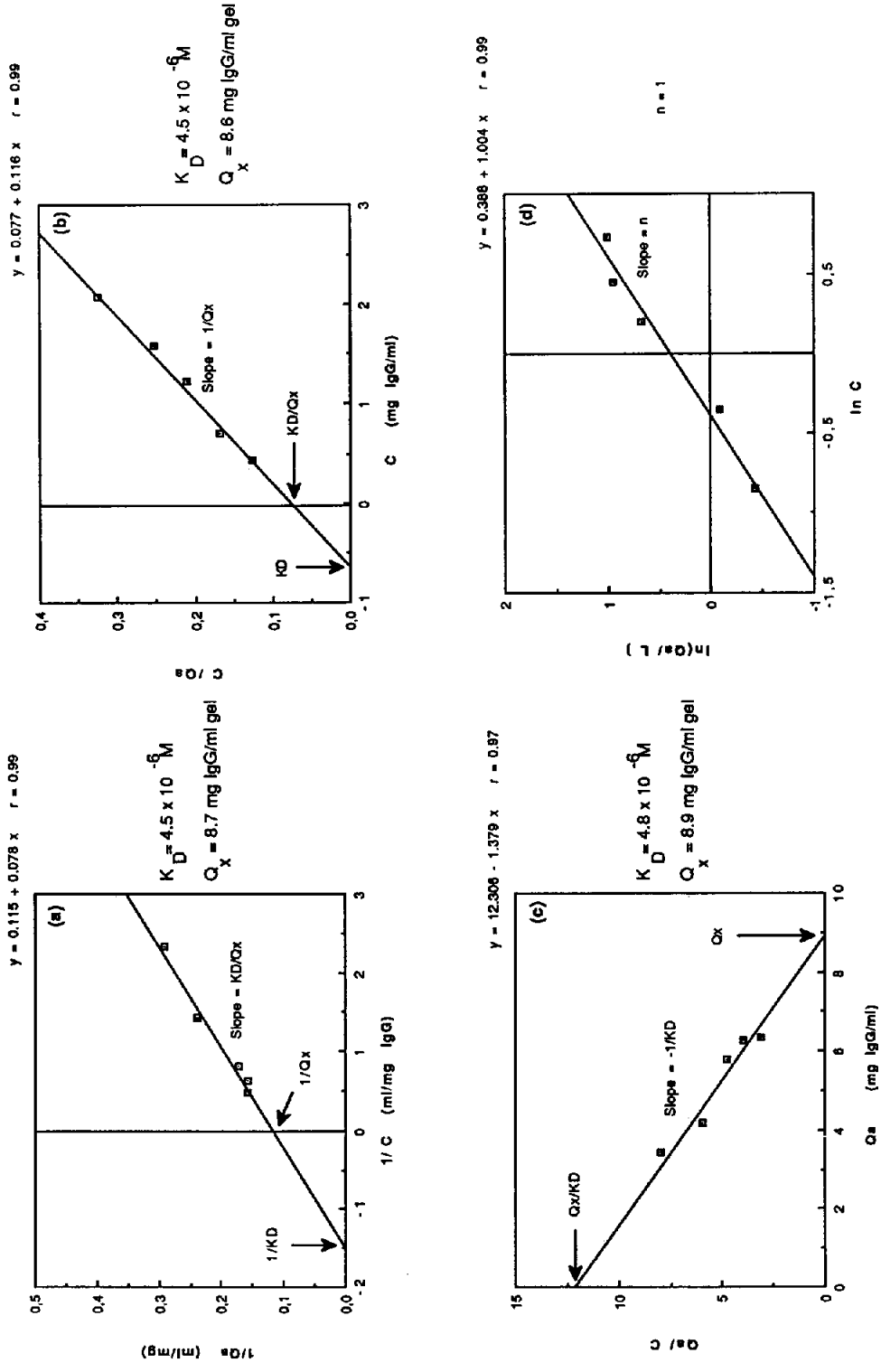

Fig. 3. Graphical representation of eqns. 2–4 for the determination of affinity constant (K_D) and binding capacity (Q_x) shown in (a)–(c). C and Q_a were determined as described in the Experimental section. The value of L utilized in eqn. 5 was calculated as $Q_x - Q_a$ using the average value of Q_x determined in the plots (a–c). (d) Determination of n (Hill plot).

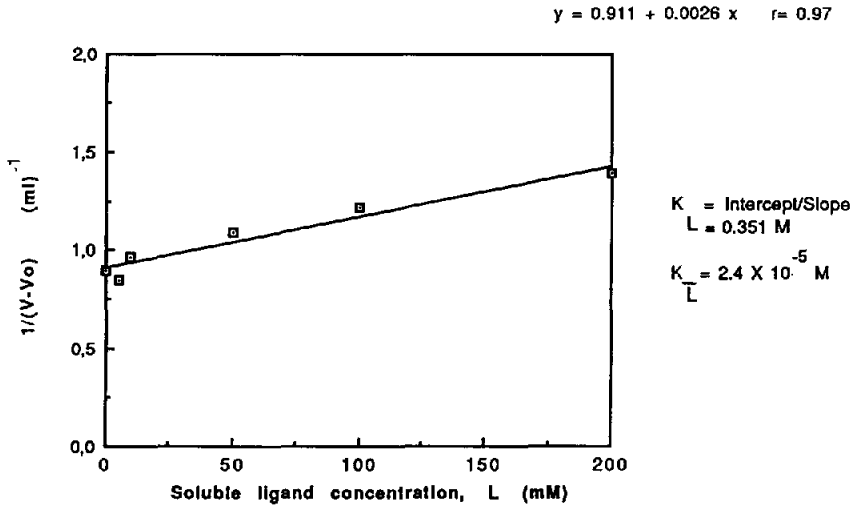

Fig 4. Elution data plotted as $1/(V - V_0)$ *versus* L. Dissociation constants, K_L and $K_{\bar{L}}$, were calculated from the linear plot using eqn. 6.

[4,5] using a commercially available ovalbumin. The elution front showed a marked difference by frontal analysis for the same protein without and after additional purification. This was verified by the Scatchard plot [4]. This type of divergence was not observed in our case. Therefore it could be concluded that the IgG sample that we used is pure and interaction with histidine is homogeneous. The value obtained for $n = 1$ from Fig. 3d proves that there is no cooperativity in protein interaction. This static (non-chromatographic) approach eliminates many variables and assumptions often required for the dynamic events associated with the chromatographic approaches. Results obtained from the equilibrium binding analyses agree well with those of the frontal analyses.

The linear relation between $1/(V - V_0)$ and L (Fig. 4) according to eqn. 6 indicates clearly that the interaction between IgG and the immobilized histidine is monovalent based on zonal elution chromatography [3]. In fact, a similar monovalent interaction between IgG and immobilized histidine was observed previously using a Sepharose 4B coupled to histidine without the aminohexyl (AH) spacer arm [11].

The immobilized histidine shows a much higher affinity than the soluble ligand ($K_L \gg K_{\bar{L}}$). This can be at least partially explained by the contribution from the AH group used as the spacer. Hence, the use of free histidine as the competing soluble ligand in the zonal elution mode is questionable. We are in fact pursuing this study by using a few alkyl derivatives of histidine as competing soluble ligands.

TABLE I

SUMMARY OF RESULTS DETERMINED BY DIFFERENT METHODS

No.	Method	Dissociation constant K_D (M)	Capacity (mg IgG per ml gel)
1	Frontal analysis	$2.4 \cdot 10^{-6}$	11.6
2	Equilbrium binding analysis	$4.6 \cdot 10^{-6}$	8.7
3	Zonal elution	$2.4 \cdot 10^{-5}$	–

ACKNOWLEDGEMENTS

The kind gift of IgG sample of human placenta from Dr. Grand George of Institut Merieux, France, is gratefully acknowledged. We gratefully acknowledge stimulating and fruitful discussions with Professor E. Sulkowski (Buffalo, NY, USA).

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