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FRONTAL AFFINITY CHROMATOGRAPHY: THEORY FOR ITS APPLICATION TO STUDIES ON SPECIFIC INTERACTIONS OF BIOMOLECULES

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SUMMARY

Affinity chromatography is very useful in the investigation and characterization of specific interaction between biomolecules. Frontal analysis in affinity chromatography is advantageous from both theoretical and experimental viewpoints. The theory is very simple because we can describe this system by means of a simple equilibrium problem. Chromatographic data can be related easily to the amount of interacting molecules and the equilibrium constant. Useful equations analogous to those of enzyme kinetics can also be derived easily. Thus, frontal affinity chromatography provides information almost identical to that obtainable by enzyme kinetic studies. In addition, this method is more general because it does not depend on enzymatic activity. Experiment is very easy and does not require any special equipment. It is a powerful tool, especially for complicated systems where it has been difficult to find an appropriate method.

INTRODUCTION

Since affinity chromatography is based on specific interactions between biological molecules, it should be useful in the investigation and characterization of such interactions. However, affinity chromatography is not familiar to many researchers as an analytical tool, and they may not realize that it is treatable quantitatively. In the opinion of the author, affinity chromatography is very straightforward and suitable for quantitative treatment, because it is a combination of a specific interaction and a powerful separation technique. Studies from such points of view have appeared [1—15].

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Frontal chromatography has not been widely used for such a purpose. Only a few reports have referred to the application of frontal chromatography for limited cases [3, 5, 7, 12]. We found that frontal chromatography has many analytical advantages in comparison to ordinary chromatography and have studied extensively its application to various interacting systems [7, 16—21]. This method was able to provide high-quality information comparable to that obtainable by enzyme kinetic analysis, by means of very simple experiments. In this paper, the theory required for the application of frontal affinity chromatography to specifically interacting biomolecules is systematically described.

GENERAL CONSIDERATIONS

For analysis of frontal chromatography, a solution of one of the interacting substances, A, is applied continuously at constant concentration, $[A]_o$, to a column on which its counterpart, B, is immobilized. The adsorbent should not be too strong. The resultant elution profile is composed of an elution front and a plateau (Fig. 1). The elution volume of the front, V, is measured. The V value is approximately equal to the volume of effluent corresponding to $[A]_o/2$. However, if the elution front is not symmetrical with regard to the midpoint, V should be calculated as follows. If fractions of constant volume are collected, V can be determined by using the following equation:

$$V = na + a \frac{\sum_{i=1}^{n} [A]_i}{[A]_o} \tag{1}$$

where a is the volume of one fraction, n is the tube number of a certain fraction at the plateau, and $[A]_i$ is the concentration of fraction i. This value corresponds to the hypothetical elution front if the boundary of A is not disturbed at all during passage through the column. Although V includes the volume of the tubing from the outlet of the column to the fraction collector, this can be neglected because we always consider values relative to V_o, which is the elution volume in the case without specific interaction. Thus, in frontal chromatography, the elution volume can be determined accurately regardless

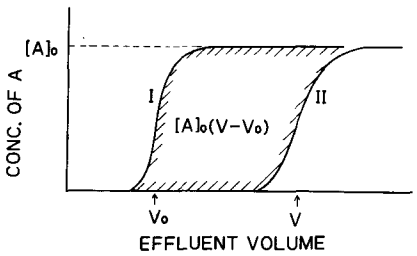

Fig. 1. Elution profiles in frontal affinity chromatography. Curve II is the elution pattern of the ligand, curve I is that under the conditions where specific adsorption is completely suppressed.

of the volume of one fraction. In ordinary chromatography, it is difficult to determine the precise peak position if the peaks are broad or asymmetrical. Although the shape of the actual elution front is not always symmetrical due to microscopic non-ideal effects, it is possible to derive useful information on analysis; this point is not discussed in the present theory. A suitable affinity adsorbent must be prepared, but this is not a serious problem. A rather weak adsorbent, which is not suitable for preparative purposes because of leakage of the target substance, will be effective.

Besides the technical and operational advantages mentioned above, theoretical treatment of frontal affinity chromatography is very easy because the concentration of free A, [A], is always constant and equal to the initial concentration, $[A]_0$, once dynamic equilibrium is reached in the column. This is because, even if a portion of A is trapped, A is continuously supplied and the initial concentration is maintained (Fig. 2). Furthermore, the elution front is retarded to an extent corresponding to the amount of adsorbed A, which greatly simplifies derivation of the equations.

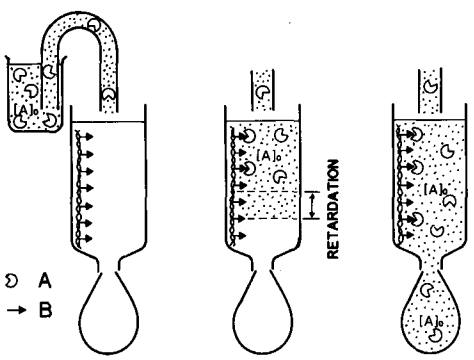

Fig. 2. Schematic presentation of frontal affinity chromatography.

For development of the analytical procedure, we assume the simplest model. We only consider the equilibrium state, i.e. merely consider the extent of retardation and not the shape of the elution front. Thus, the dynamic aspect of chromatography is neglected. The column is thought of as a cell for equilibrium dialysis. The chromatographic pattern is used only to determine the accurate amount of adsorbed target substance. Immobilized ligand is assumed to be distributed uniformly in the column and the volume of stationary phase is neglected because Sepharose 4B is used as a support matrix. The bed of wet gel contains <2% agarose and the pore size is large enough for almost all protein molecules. Consideration of the diffusion of A or turbulence of flow is unnecessary. Once the plateau is reached, the column is in a state of dynamic equilibrium, and the situation is reduced to a simple equilibrium problem.

For application to more complicated systems, such as analysis of ternary complexes, equations are often derived under the conditions where the concentration of one of the interacting molecules can be neglected, as in enzyme kinetic analysis.

BASIC EQUATIONS

First, we define four interacting substances: A, a ligand that interacts with an affinity adsorbent; B, an immobilized ligand on the affinity adsorbent; I, a counter-ligand that competes with A and inhibits the binding of A to B (i.e. a competitive inhibitor of enzymes); E, an effector that affects the binding of A to B. For these substances, the following three dissociation constants are defined:

$$K_d = \frac{[A][B]}{[AB]} \tag{2}$$

$$K_i = \frac{[A][I]}{[AI]} \tag{3}$$

$$K_e = \frac{[A][E]}{[AE]} \tag{4}$$

$[X]$ and $[X]_o$ represent concentration and initial concentration of any substance X, respectively.

The essential point of the present consideration is how to relate these dissociation constants to the data obtained from chromatographic experiments. Parameters for chromatography are defined as follows: $[B]_o$, amount of immobilized ligand per unit volume (expressed as concentration) of affinity adsorbent; v, bed volume of the column; B_t, total amount of the immobilized ligand, i.e. $v[B]_o$; V, elution volume of A; V_o, elution volume of A under the conditions where the specific interaction of A and B is completely suppressed; V_i, V in the presence of I; V_e, V in the presence of E.

The larger the amount of A bound to B, the larger will be the value of $(V - V_o)$. It is clear from Fig. 1 that $[A]_o(V - V_o)$ is equal to "specifically" adsorbed A.

The dissociation constant can be related to the parameters of chromatography as follows [7, 16, 17]:

$$K_d = \frac{[A][B]}{[AB]} = \frac{[A]_o\{[B]_o - [A]_o(V - V_o)/v\}}{[A]_o(V - V_o)/v} = \frac{B_t}{V - V_o} - [A]_o \tag{5}$$

If chromatography is carried out in the presence of I or E, the apparent dissociation constant, $K_{d(app)}$ can be calculated by replacing V with V_i or V_e.

ANALYSIS OF THE INTERACTION BETWEEN ADSORBENT (B) AND LIGAND (A)

Dependence of the amount of adsorbed A on $[A]_o$

Eqn. 5 can be rearranged to various useful forms. First, the following equation can be derived [18]:

$$\frac{1}{[A]_o(V - V_o)} = \frac{K_d}{B_t} \cdot \frac{1}{[A]_o} + \frac{1}{B_t} \tag{6}$$

When V values are obtained for various $[A]_o$ values, the reciprocal of

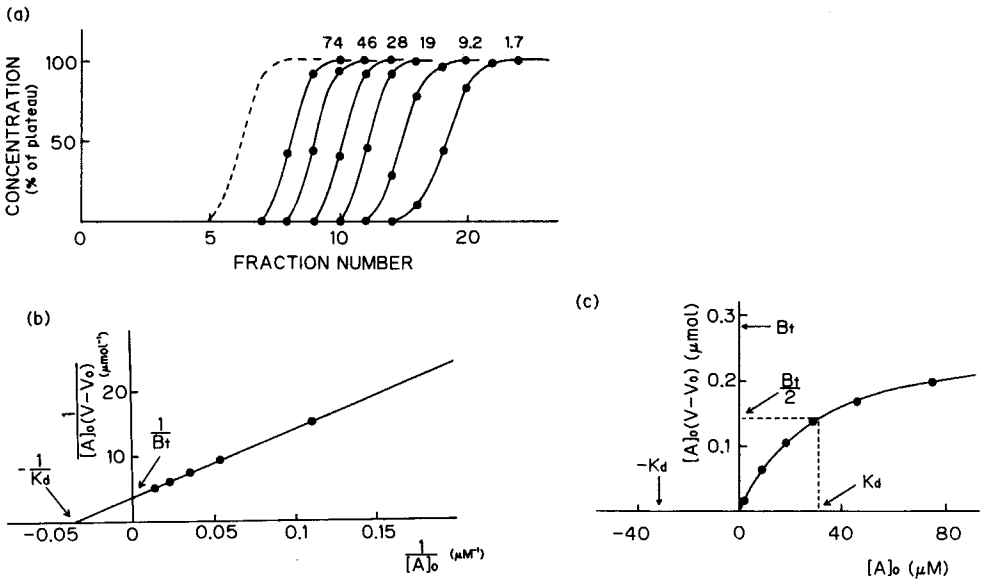

Fig. 3. (a) Elution profiles in frontal affinity chromatography for various concentrations (indicated in μM in the figure) of guanosine on a carboxymethylated RNase T_1 Sepharose column. The broken line represents the elution profile of adenosine (for the determination of V_o). Bed volume was 4.3 ml and total amount of immobilized protein was 500 nmol. 0.05 M Acetate—sodium hydroxide buffer (pH 5.5) was used. Flow-rate was 5.0 ml/h and temperature was 4°C. Fractions of 1.06 ml were collected and A_{280} values were measured. The V value for each elution profile was calculated by using eqn. 1. (b) Analysis of the above data by the $1/[A]_o(V - V_o)$ versus $1/[A]_o$ plot (see eqn. 6). Dependency of the amount of adsorbed A on the concentration of A. This plot is analogous to the Lineweaver—Burk plot. The data point for $[A]_o = 1.7$ μM was omitted even though it fitted on the straight line, because it was too far from the other points. The B_t value calculated from the intercept on the ordinate ($1/B_t$) was 290 nmol. This indicates that ca. 60% of the immobilized protein retained binding ability. The K_d value calculated from the intercept on the abscissa ($-1/K_d$) was 29 μM. This value was reasonable compared to the reported dissociation constant for free carboxymethylated RNase T_1 [22]. (c) Analysis of the above data by the $[A]_o(V - V_o)$ vs. $[A]_o$ plot. This plot is analogous to the Michaelis—Menten plot. Values of B_t and K_d can be calculated from the coordinates of two asymptotes of the hyperbola. Essentially the same values as shown above were obtained.

$[A]_o(V - V_o)$, i.e. adsorbed A, is a linear function of the reciprocal of $[A]_o$. The intercept on the abscissa corresponds to $-1/K_d$, and that on the ordinate corresponds to $1/B_t$. Thus, K_d can be determined even when B_t is unknown. This equation resembles the Lineweaver—Burk equation, which is frequently used for enzyme kinetics. It is apparent that $[A]_o(V - V_o)$ and K_d correspond to substrate concentration and the Michaelis constant, respectively. Thus, affinity chromatography can be discussed at the same level as enzyme kinetics. This is reasonable because both systems are based on the Langmuir binding isotherm. Fig. 3 shows an example of such analysis. Interaction of chemically modified ribonuclease (RNase) T_1 (catalytic activity was lost but specific binding ability was retained) and a substrate analogue, guanosine, was studied. In Fig. 3a, a set of elution profiles for various initial concentrations of guanosine on a column of immobilized carboxymethylated RNase T_1 is given. Fig. 3b shows a plot according to eqn. 6.

From eqn. 6, the meaning of B_t becomes clearer; it is the limit of the amount of specifically adsorbed A when $[A]_o$ approaches infinity. This value also represents the amount of B that retains binding ability for A (which is often smaller than the total amount of immobilized B). Once B_t has been determined, K_d can be determined by a single experiment using one $[A]_o$.

Eqn. 5 can be rearranged to the following form [17]:

$$[A]_o(V - V_o) = \frac{B_t[A]_o}{[A]_o + K_d} = \frac{-B_t K_d}{[A]_o + K_d} + B_t \tag{7}$$

This equation is analogous to the Michaelis—Menten equation. Thus, a plot of $[A]_o(V - V_o)$ versus $[A]_o$ gives a hyperbola. The asymptote parallel to the abscissa corresponds to B_t and the other asymptote parallel to the ordinate corresponds to $-K_d$ (Fig. 3c). The $[A]_o$ value corresponding to $B_t/2$ is equal to K_d. Eqns. 6 and 7 give essentially the same information. From these considerations, it is apparent that affinity chromatography can be treated in the same way as enzyme kinetics.

Dependence of elution volume on $[A]_o$

Next, we consider problems concerning the chromatography. From eqn. 5, eqn. 8 can be derived [18]:

$$V = V_o + \frac{B_t}{[A]_o + K_d} \tag{8}$$

This equation is useful to describe the characteristics of frontal affinity chromatography. The plot of V versus $[A]_o$ is also a hyperbola and the two asymptotes correspond to $-K_d$ and V_o (Fig. 4). The smaller the $[A]_o$ value, the larger is V. If $[A]_o \ll K_d$, i.e. if $[A]_o$ is negligible compared to K_d, V approaches the maximum value, V_m:

$$V_m = V_o + \frac{B_t}{K_d} \tag{9}$$

V_m is apparently independent of $[A]_o$. (This corresponds to eqn. 10 of ref. 9, though the latter was derived for ordinary chromatography.)

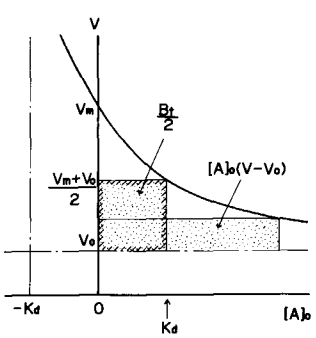

Fig. 4. The V versus $[A]_o$ plot (see eqn. 8). Dependency of the elution volume of A on the concentration of A. Both elution volume and adsorbed amount can be predicted for a given $[A]_o$, provided that the values of K_d and B_t are known.

If $[A]_o$ increases, V becomes smaller. However, V cannot be smaller than V_o. Thus, V_o is the limit of V when $[A]_o$ approaches infinity, i.e. the immobilized ligand is saturated. At a certain concentration of $[A]_o$, the amount of adsorbed A corresponds to the area of the dotted rectangle in Fig. 4. It is easily seen that when $[A]_o = K_d$, adsorbed A becomes equal to $B_t/2$, i.e. half of the maximum capacity, and V becomes $(V_m + V_o)/2$. Thus, the elution volume varies from V_m to V_o depending on $[A]_o$, and the amount of adsorbed A varies from $[A]_o(V_m - V_o)$ to B_t.

The reason why V becomes equal to V_m (which is independent of $[A]_o$) is that if $[A]_o$ is very small, $[A]_o(V - V_o)$ is approximately proportional to $[A]_o$. Thus, when $[A]_o$ doubles, the amount of adsorbed A also doubles and does not result in any change in V. This is analogous to enzyme kinetics in which velocity is approximately proportional to substrate concentration if the latter is negligible in comparison to K_m.

Eqn. 8 is helpful for designing affinity adsorbents because we can predict the chromatographic result. This equation can be directly applied to the case in which we intend to concentrate a certain substance from a dilute solution. Assume that we wish to prepare an affinity adsorbent to concentrate an enzyme (mol. wt. 50 000) and succeed in immobilizing a competitive inhibitor ($K_i = 10^{-7}$ M) at $[B]_o = 10^{-4}$ M. If we apply a dilute enzyme solution (e.g. $[A]_o = 10^{-7}$ M, 5 μg/ml) continuously to a column (bed volume v = 10 ml), V will be ca. 5000 ml. This means that we can apply as much as ca. 4000 ml to the column before leakage of the enzyme will occur (binding strength is assumed here to be unchanged after immobilization, i.e. $K_i \approx K_d$). The amount of adsorbed enzyme, $[A]_o(V - V_o)$, will be 25 mg, which corresponds to half of the capacity of the column. If $[A]_o = 10^{-6}$ M (50 μg/ml), V is ca. 900 ml and the amount of adsorbed enzyme at that time will be ca. 45 mg. If $[A]_o = 10^{-5}$ M (500 μg/ml), the enzyme will begin to leak at ca. 100 ml. The retained enzyme then is ca. 50 mg, which corresponds to the maximum capacity of the adsorbent.

Consider also the case of a weak affinity adsorbent. Suppose we have a column of the same size containing a competitive inhibitor of $K_i = 10^{-4}$ M at $[B]_o = 10^{-4}$ M. Even if $[A]_o = 10^{-6}$ M, the enzyme will begin to leak at ca. < 20 ml. Only 0.5 mg of the enzyme will be retained.

This method is not directly applicable to ordinary chromatography because [A] is subject to change during passage through the column. However, in limited cases, i.e. when [A] is negligible in comparison to K_d, the system can be treated similarly. In this case, the value $[B]_o/K_d$ can be considered as the ratio of elution volume to bed volume. Thus, for example, if we prepare an affinity adsorbent for which $[B]_o$ is ten times K_d, A will appear at a volume of ten times the bed volume.

Simplified system in which $[A]_o$ can be neglected

Equation 9 can be rearranged as follows [7, 16, 17]:

$$K_d = \frac{B_t}{V_m - V_o} \tag{10}$$

This means that we can determine the K_d value without considering the term

$[A]_o$. In other words, [B] can be considered as $[B]_o$ because [AB] is negligibly small compared to $[B]_o$. This relation is very useful because we can determine K_d even if the exact concentration of A is unknown (e.g. unpurified protein, material of unknown molecular weight, etc.), provided that the elution profile can be obtained by appropriate procedures (e.g. measurement of enzymatic activity, immunochemical method, etc.).

This relation can also be used to compare the K_d values of a system under various conditions. The following equation is useful [7, 16, 17]:

$$\frac{K_{d(I)}}{K_{d(II)}} = \frac{V_{m(II)} - V_o}{V_{m(I)} - V_o} \tag{11}$$

where subscripts I and II represent different conditions. K_d is inversely proportional to the extent of retardation.

Presence of two ligands, A_1 and A_2

Consider the case in which two specific ligands, A_1 and A_2, are applied to the immobilized B column. For each interaction, K_d is defined as follows:

$$K_{d1} = \frac{[A_1][B]}{[A_1B]} \tag{12}$$

$$K_{d2} = \frac{[A_2][B]}{[A_2B]} \tag{13}$$

If $K_{d1} > K_{d2}$, A_1 moves faster than A_2. However, adsorbed A_1 will be replaced later by A_2. Fig. 5 illustrates this situation. A similar illustration can be found in ref. 5. $[A_1]_o'$, which is the height of the first step, is higher than $[A_1]_o$. After dynamic equilibrium has been attained, $[A_1]$ becomes $[A_1]_o$, and $[A_2]$ becomes $[A_2]_o$. The amounts of adsorbed A_1 and A_2 are

$$[A_1B]v = (V_2 - V_o)[A_1]_o - (V_2 - V_1)[A_1]_o' \tag{14}$$

$$[A_2B]v = (V_2 - V_o)[A_2]_o \tag{15}$$

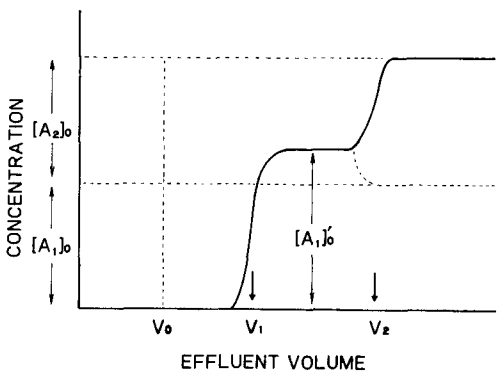

Fig. 5. Elution profile observed when a mixture of two specific ligands, A_1 and A_2, are applied. The binding of A_2 is stronger than that of A_1.

Thus, each K_d can be expressed as follows:

$$K_{d1} = \frac{[A_1][B]}{[A_1B]} = \frac{[A_1]_o\{[B]_o - [A_1B] - [A_2B]\}v}{(V_2 - V_o)[A_1]_o - (V_2 - V_1)[A_1]_o} \quad (16)$$

$$= \frac{[A_1]_o\{B_t - (V_2 - V_o)[A_1]_o + (V_2 - V_1)[A_1]_o - (V_2 - V_o)[A_2]_o\}}{\{(V_2 - V_o)[A_1]_o - (V_2 - V_1)[A_1]_o\}}$$

$$K_{d2} = \frac{[A_2][B]}{[A_2B]} = \frac{[A_2]_o\{[B]_o - [A_1B] - [A_2B]\}v}{(V_2 - V_o)[A_2]_o}$$

$$= \frac{\{B_t - (V_2 - V_o)[A_1]_o + (V_2 - V_1)[A_1]_o - (V_2 - V_o)[A_2]_o\}}{(V_2 - V_o)} \quad (17)$$

These equations are too complicated for practical use. However, if $[A_1]_o \ll K_{d1}$, and $[A_2]_o \ll K_{d2}$, [B] can be considered approximately equal to $[B]_o$. In such a case, each K_d can be determined independently because it can be considered that chromatography is carried out by using two columns.

ANALYSIS OF THE INTERACTION BETWEEN THE LIGAND AND OTHER SOLUBLE MOLECULES THAT AFFECT THE BINDING OF THE LIGAND TO THE AFFINITY ADSORBENT

Effect of counter-ligand (I)

Now, we will consider a more complicated situation where a substance that affects the interaction between A and B is present. Analysis of such systems gives information on the interaction between A and these molecules. To simplify, we consider only limited systems where $[A]_o$ can be neglected, i.e. $[A]_o \ll K_d$ [7]. In the presence of a counter-ligand (I), elution of A is accelerated and the elution volume is decreased to V_i. From the operational point of view, the column is first equilibrated with I (to $[I]_o$), and a solution of A dissolved in the same buffer is applied. To simplify, $[I]_o$ is also assumed to be extremely large in comparison to $[A]_o$. Under these conditions, the amount of adsorbed A decreases from $[A]_o(V_m - V_o)$ to $[A]_o(V_i - V_o)$, and thus:

$$\frac{V_i - V_o}{V_m - V_o} = \frac{1}{1 + [I]/K_i} = \frac{1}{1 + [I]_o/K_i} \quad (18)$$

K_i can be calculated by using the following equation:

$$K_i = \frac{V_i - V_o}{V_m - V_i}[I]_o \quad (19)$$

If $[I]_o = K_i$, V_i will be intermediate between V_o and V_m. For a more accurate determination of K_i, V_i values in the presence of various concentrations of I are measured and analysed by means of the following equation:

$$V_i = V_o + K_i \frac{V_m - V_i}{[I]_o} \quad (20)$$

A plot of V_i versus $(V_m - V_i)/[I]_o$ gives a straight line (Fig. 6). The intercept

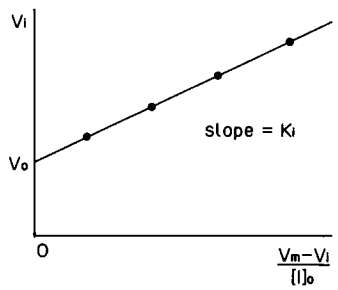

Fig. 6. Analysis of the effect of a soluble counter-ligand (I). The V_i versus $(V_m - V_i)/[I]_o$ plot (see eqn. 20). The dissociation constant of I (K_i) can be calculated from the slope. This method resembles the analysis of competitive inhibition in enzyme kinetics.

on the ordinate corresponds to V_o and the slope to K_i. It is apparent that V_o is the limiting value of V when $[I]_o$ approaches infinity and A becomes saturated with I. This procedure is essentially similar to those reported by Dunn and Chaiken (eqn. 17 of ref. 2) and Nichol et al. (eqn. 23 of ref. 3), though the former authors did not use frontal chromatography.

This method is analogous to the analysis of competitive inhibition in enzyme kinetics. The interaction between A and I is measured indirectly in terms of the decrease in the elution volume, instead of the decrease in velocity in the case of enzyme kinetics. One of the advantages of the indirect method is that a wide range of K_i values can be determined without changing the concentration of A. It is not necessary to use a high concentration of A even in the case of a very weak interaction. Moreover, this indirect specific method is not susceptible to non-specific interaction, which often interferes with direct methods. The most important feature of this method is that it is applicable not only to enzyme—inhibitor systems but also to all systems involving a specific interaction (antigen—antibody, lectin—saccharide, etc.), because it does not depend on enzymatic activity. As regards the application to enzyme systems, it can be carried out under conditions where the enzyme is no longer active, e.g. at extreme pH or very low temperature.

Presence of two competitive inhibitors

It is possible to extend the treatment to a situation where two competitive inhibitors, I_1 and I_2, are present [17]. $[A]_o$ should always be negligible compared to the concentrations of both inhibitors, $[I_1]_o$ and $[I_2]_o$. If we define the elution volume of the enzyme as $V_{(1+2)}$, the following equation is obtained:

$$\frac{K_{d(app)}}{K_d} = 1 + \frac{[I_1]_o}{K_{i(1)}} + \frac{[I_2]_o}{K_{i(2)}} = \frac{V_m - V_o}{V_{(1+2)} - V_o} \tag{21}$$

where $K_{i(1)}$ and $K_{i(2)}$ are the dissociation constants for AI_1 and AI_2, respectively. If only I_1 is present, we have essentially the same equation as eqn. 20, thus, eqn. 22 is derived:

$$\frac{V_1 - V_o}{V_{(1+2)} - V_o} = 1 + \frac{[I_2]_o}{K_{i(2)}(1 + [I_1]_o/K_{i(1)})} \tag{22}$$

This is rearranged to

$$V_{(1+2)} = V_o + K_{i(2)} \frac{V_1 - V_{(1+2)}}{[I_2]_o} \left(1 + \frac{[I_1]_o}{K_{i(1)}}\right) \tag{23}$$

This equation resembles eqn. 20. Under fixed $[I_1]_o$, a plot of $V_{(1+2)}$ for various $[I_2]_o$ against $(V_1 - V_{(1+2)})/[I_2]_o$ gives V_o from the intercept on the ordinate and $K_{i(2)}\{1 + [I_1]_o/K_{i(1)}\}$ from the slope. If $K_{i(1)}$ is known, $K_{i(2)}$ can be calculated. This procedure resembles the analysis of competitive inhibition by means of the Lineweaver—Burk plot.

Presence of an effector

Consider an effector that binds to A (not to the binding site for B) and affects the interaction between A and B. The reaction is illustrated as follows:

$$\begin{array}{ccc} A + B & \xleftrightarrow{K_d} & AB \\ + & & + \\ E & & E \\ K_e \updownarrow & & \updownarrow K_{e'} \\ EA + B & \xleftrightarrow{K_{d'}} & EAB \end{array}$$

The following dissociation constants are defined:

$$K_{d'} = \frac{[EA][B]}{[EAB]} \tag{24}$$

$$K_{e'} = \frac{[AB][E]}{[EAB]} \tag{25}$$

If $K_{d'} < K_d$, E strengthens the interaction between A and B. If $K_{d'} > K_d$, E non-specifically inhibits the interaction. The apparent dissociation constant $K_{d(app)}$ derived from the elution volume, V_e, is

$$K_{d(app)} = \frac{([A] + [EA])[B]}{[AB] + [EAB]} \tag{26}$$

From eqn. 26, we obtain

$$\frac{V_e - V_o}{V_m - V_o} = \frac{K_d}{K_{d(app)}} = \frac{[A][B]}{[AB]} \cdot \frac{[EAB] + [AB]}{\{[A] + [EA]\} \cdot [B]} = \frac{1 + [EAB]/[AB]}{1 + [EA]/[A]} = \frac{1 + [E]/K_{e'}}{1 + [E]/K_e} = \frac{1 + [E]_o/K_{e'}}{1 + [E]_o/aK_{e'}} \tag{27}$$

where $a = K_e/K_{e'} = K_d/K_{d'}$.
This equation is reported for the first time.

EXTENSION TO LIGANDS HAVING MULTIPLE BINDING SITES

We have considered ligand molecules having only one binding site. Now, we extend the application to a multivalent system [20]. First, we will consider

the simplest situation. We assume a protein having two identical and independent binding sites, which binds to an immobilized ligand with only one site at a time; this situation is illustrated below (second binding is not considered):

$$AB \xleftarrow[K_d]{B} A \xrightarrow[K_d]{B} BA$$

AB and BA represent complexes in which A binds to B at different sites.

$$[BA] = [AB] = \frac{[BA] + [AB]}{2} = \frac{[AB]_t}{2} \tag{28}$$

where $[AB]_t$ is the total concentration of the AB complex. The intrinsic dissociation constant, K_d, is expressed as follows:

$$K_d = \frac{[A][B]}{[BA]} = \frac{[A]_o\{[B]_o - [BA] - [AB]\}}{[BA]}$$

$$= \frac{[A]_o\{[B]_o - 2[BA]\}}{[BA]} = \frac{[A]_o[B]_o}{[BA]} - 2[A]_o$$

$$= 2\left\{\frac{B_t}{V - V_o} - [A]_o\right\} \tag{29}$$

This equation means that we shall have an apparent K_d of half the intrinsic K_d if we do not know the valency of the protein. In other words, the apparent K_d is smaller than the true K_d in the case of a multivalent system.

Eqn. 29 can be rearranged to

$$V = V_o + \frac{B_t}{[A]_o + K_d/2} \tag{30}$$

Fig. 7 shows the V versus $[A]_o$ plot. The solid line represents the case of divalent protein and the broken line the case of monovalent protein. If $[A]_o \ll$

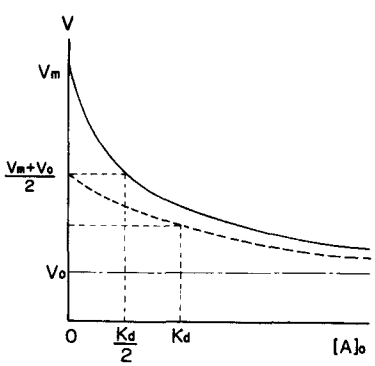

Fig. 7. Comparison of V versus $[A]_o$ plots for monovalent and divalent systems. The solid line represents the case of divalent protein and the broken line represents the case of monovalent protein. If the ligand concentrations are small compared to K_d, V for the divalent ligand will be approximately twice that for the monovalent ligand.

K_d:

$$V_m = V_o + 2\frac{B_t}{K_d} \tag{31}$$

thus, the extent of retardation, $(V_m - V_o)$, is doubled.

The relationship between free A and adsorbed A in a dynamic equilibrium state is as follows. If we define $[A]_t$ as the total concentration of A in the column:

$$[A]_t = [A] + [BA] + [AB] = [A]\left\{1 + \frac{2[B]}{K_d}\right\} \tag{32}$$

Thus,

$$\frac{[BA] + [AB]}{[A]} = \frac{1 + \frac{2[B]}{K_d}}{1 + \frac{K_d}{2[B]}} = \frac{2[B]}{K_d} \tag{33}$$

In the case of monovalent A, this value was $[B]/K_d$.

If $[A]_o \ll K_d$:

$$V_m - V_o = \frac{B_t}{K_{d(app)}} \tag{34}$$

Then, the relation between $K_{d(app)}$ and the intrinsic dissociation constant (K_d) can be expressed as follows:

$$K_{d(app)} = \frac{[A][B]}{[AB] + [BA]} = \frac{1}{2}K_d \tag{35}$$

From eqns. 33 and 34, we obtain:

$$K_d = \frac{2B_t}{V_m - V_o} \tag{36}$$

For a multivalent protein that has n identical and independent binding sites, we can similarly derive the following equations:

$$K_d = n\left\{\frac{B_t}{V - V_o} - [A]_o\right\} \tag{37}$$

$$V = V_o + \frac{B_t}{[A]_o + \frac{K_d}{n}} \tag{38}$$

$$V_m = V_o + n\frac{B_t}{K_d} \tag{39}$$

$$K_d = \frac{nB_t}{V_m - V_o} \tag{40}$$

Thus, the extent of retardation will be n times that of monovalent protein, and the intrinsic K_d is n times the apparent K_d.

Effect of counter-ligand

Analysis of the interaction between A and the immobilized ligand, B, is rather simple, because once A binds to B, further binding need not be considered. The situation seems to be more complicated if a counter-ligand that moves freely in the solution is added. However, under limited conditions, i.e. $[A]_o \ll K_d$ and $[A]_o \ll [I]_o$, the equation can be extensively simplified and we can obtain eqns. 18—20. Thus, we can calculate K_i of the counter-ligand by means of the same equation regardless of the valency of the protein. For details of the derivation of equations, see ref. 20. Analysis of multivalent systems has been described in refs. 11 and 12, though they were not carried out by frontal chromatography.

CONCLUSION

Although the theory of frontal affinity chromatography presented here is based on the simplest model, and an almost ideal state is assumed, it has proved to be extremely useful as a general procedure to investigate the interaction of proteins and specific ligands. Equilibrium constants obtained by experiments were consistent with those obtained by other methods for soluble systems such as enzyme kinetics and equilibrium dialysis. We first applied it to studies on the characteristics of the active sites of enzymes. The interaction of trypsin with various affinity adsorbents was analysed and the utility of this procedure was demonstrated [16]. The method was further developed to analyse the interaction of trypsin and counter-ligands (competitive inhibitors, substrate analogues, etc.) [17]. The characteristics of immobilized trypsin were also studied [18]. These experiments established that frontal chromatography can provide information almost identical to that obtained by enzyme kinetic studies. From several viewpoints, it is superior to enzyme kinetics because it is applicable even when the enzyme is no longer active. This was demonstrated in the case of analysis of the binding properties of anhydrotrypsin and anhydrochymotrypsin (enzymatically inactive, but retaining specific binding ability) [19]. As regards studies on other enzymes, binding of substrate analogues to ribonuclease T_1 was also analysed [23].

The great utility of this method was demonstrated for the quantitative investigation of binding specificity of a non-enzyme saccharide-binding protein, concanavalin A. Mono- and disaccharides were used as counter-ligands in the frontal chromatography of concanavalin A on immobilized glucose columns and accurate K_d values were determined [20]. In this experiment, frontal affinity chromatography was also proved to be applicable to multivalent systems. On the other hand, the bindings of ten asparagine-linked oligosaccharides derived from ovalbumin were analysed by using an immobilized concanavalin A column, and the carbohydrate structural characteristics important for the binding of concanavalin A were clarified [21]. Frontal affinity chromatography is thus a powerful tool, especially for complicated systems such as lectins and complex carbohydrates.

This method is complementary to other methods, e.g. kinetics, difference spectra, equilibrium dialysis, gel filtration, etc., and is advantageous from several points of view. For example, the interaction can be measured without altering the equilibrium. The introduction of reporter groups, such as fluorescent or chromogenic probes, is not necessary, nor is specially devised equipment required. Though the experimental procedure is simple, the data obtained are highly reproducible and accurate. The theoretical basis is very simple and the physical significance of K_d and B_t is very clear. This method should prove to be a very efficient tool for research into various biospecific interactions.

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