Journal of Chromatography, 597 (1992) 57–66
Elsevier Science Publishers B.V., Amsterdam

CHROM. 23 828

Study of high-affinity interactions by quantitative affinity chromatography

Analytical expressions in terms of total ligand concentration

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ABSTRACT

Analytical expressions are derived for the description of ligand-facilitated and ligand-retarded desorption of partitioning solute in terms of total ligand concentration in quantitative affinity chromatography. Their application is then illustrated by consideration of results from recycling partition equilibrium studies of the heparin-facilitated desorption of thrombin from heparin-Sepharose, and of the competition between methyl-α-D-mannoside and *p*-nitrophenyl-α-D-mannoside for concanavalin A immobilized on CPG-170. Finally, published frontal affinity chromatographic data for the NADH-dependent elution of rabbit muscle lactate dehydrogenase from oxamate-Sepharose are reanalysed using these equations to demonstrate the characterization of a system reflecting the binding of a solute–ligand complex to an affinity matrix. This investigation extends the scope of quantitative affinity chromatography to include not only the study of solute–ligand interactions governed by larger binding constants but also the characterization of interactions in which the partitioning solute and ligand are both macromolecular, and eliminates the need for prior dialysis to establish the free ligand concentration required for application of earlier analytical expressions.

INTRODUCTION

Analytical expressions for the evaluation of binding constants by quantitative affinity chromatography have traditionally been derived in terms of the free concentration of ligand effecting changes in the distribution of partitioning solute between matrix-bound and liquid-phase states [1–4]. Consequently, application of the technique has been confined mainly to studies of relatively weak interactions for which the total ligand concentration approximates the required free concentration [1,3], or to studies of high-affinity interactions for which the concentrations of free ligand were established by prior equilibrium dialysis or its gel chromatographic counterpart [5–7]. The recent realization [8,9] that affinity chromatographic behaviour reflecting competition between ligand and matrix sites for partitioning solute is also amenable to quantitative interpretation in terms of total ligand concentration therefore represents a major theoretical breakthrough. By eliminating the need for prior dialysis to establish the free ligand concentration, this development extends the scope of quantitative affinity chromatography to include the characterization of interactions in which the partitioning solute and ligand are both macro-

0021-9673/92/$05.00

molecular. It also confers greater versatility on the recycling partition technique of quantitative affinity chromatography [3,5,8], in which successive additions of solute and then of ligand allow evaluation of the ligand-binding constant from a single experiment [8].

The initial aim of this investigation is to derive quantitative expressions in terms of total ligand concentration for the various affinity chromatographic situations involving an interplay of two equilibria. Use of these expressions is then illustrated with three experimental systems: a recycling partition equilibrium study with heparin-Sepharose as affinity matrix to evaluate solute–matrix and solute–ligand association constants for the thrombin–heparin interaction; a recycling partition study to determine solute–matrix and ligand–matrix association constants from methyl-α-D-mannoside-effected displacement of *p*-nitrophenyl-α-D-mannoside from concanavalin A immobilized on CPG-170 [10,11]; and a frontal affinity chromatographic study [12] of the retarding effect of NADH on the elution of lactate dehydrogenase from oxamate–Sepharose to evaluate intrinsic association constants for the binding of NADH to the enzyme and for interaction of this binary complex with the immobilized oxamate.

THEORY

In affinity chromatography the effect of including a ligand, S, in the mobile phase is usually either to facilitate desorption of the solute, A, because of interactions that are competitive, or to retard desorption of the solute through the formation of complexes that enhance the interaction with matrix, X. For systems with only two types of interactions there are two competitive situations, one in which the interactions of ligand and matrix with solute are mutually exclusive, and the other in which the competition is between solute and ligand for matrix sites. On the other hand, solute-retarded desorption occurs when the formation of a solute–ligand complex is a prerequisite for interaction with matrix sites, and also when the adsorption of solute reflects interaction with a liganded matrix site. These four situations are considered in turn.

Competition between matrix and ligand for solute sites

Consider the situation in which an *f*-valent solute, A, can react either with matrix sites, X, or with ligand, S, both of which are univalent. For such a system the distribution of partitioning solute is related to the intrinsic association constants [13] for the solute–matrix (k_{AX}) and solute–ligand (k_{AS}) interactions by the expression [3]

$$k_{AX} = \frac{\{(1 - ([\bar{A}]/[\bar{\bar{A}}])^{1/f}\}(1 + k_{AS}[S])}{([\bar{A}]/[\bar{\bar{A}}])^{1/f}[[\bar{\bar{X}}] - f[\bar{\bar{A}}]\{1 - ([\bar{A}]/[\bar{\bar{A}}])^{1/f}\}]} \quad (1)$$

where $[\bar{\bar{A}}]$ and $[\bar{\bar{X}}]$ denote the total concentrations of solute and matrix sites, respectively. $[\bar{A}]$ refers to the total concentration of partitioning solute (free and complexed) in the liquid phase of a mixture with free ligand concentration [S]. It should be noted that $\{1 - ([\bar{A}]/[\bar{\bar{A}}])^{1/f}\}/([\bar{A}]/[\bar{\bar{A}}])^{1/f} = ([\bar{\bar{A}}]/[\bar{A}])^{1/f} - 1 = ([\bar{\bar{A}}]^{1/f} - [\bar{A}]^{1/f})/[\bar{A}]^{1/f}$ [6,14]. On making the substitution

$$\bar{k}_{AX} = k_{AX}/(1 + k_{AS}[S]) \quad (2)$$

where $\bar{k}_{AX}$ is the constitutive association constant (or apparent value of k_{AX} when measured in the presence of ligand), eqn. 1 may be arranged as

$$([\bar{\bar{A}}]^{1/f} - [\bar{A}]^{1/f})/[\bar{A}]^{1/f} = \bar{k}_{AX}[\bar{\bar{X}}] - f\bar{k}_{AX}[\bar{A}]^{(f-1)/f}\{[\bar{\bar{A}}]^{1/f} - [\bar{A}]^{1/f}\} \quad (3a)$$

which is readily applied to results of partition equilibrium studies ($[\bar{A}]$ as a function of $[\bar{\bar{A}}]$). A series of partition equilibrium experiments with a range of solute concentrations in the absence of ligand thus allows k_{AX} ($\bar{k}_{AX}$ when $[S] = 0$) to be evaluated from the slope ($-fk_{AX}$) of the linear plot of $([\bar{\bar{A}}]^{1/f} - [\bar{A}])^{1/f})/[\bar{A}]^{1/f}$ *versus* $[\bar{A}]^{(f-1)/f}([\bar{\bar{A}}]^{1/f} - [\bar{A}]^{1/f})$, which is the multivalent counterpart [15] of the Scatchard plot for a univalent solute [16].

The unavailability of values for $[\bar{\bar{A}}]$ in frontal affinity chromatography is offset by incorporating the mass conservation requirement [5] that $\bar{V}_A[\bar{A}] = V_A^*[\bar{\bar{A}}]$, where $\bar{V}_A$ is the measured elution volume on a column for which V_A^* is the volume accessible to A (the elution volume in the absence of any interaction with the matrix). By this means it is possible to eliminate the terms in $[\bar{\bar{A}}]$ and hence to obtain a relationship solely in terms of elution volumes and $[\bar{A}]$, the applied concentration of solute in a frontal chromatographic experiment. From this chromatographic counterpart of eqn. 3a, namely,

$$(\bar{V}_A/V_A^*)^{1/f} - 1 = \bar{k}_{AX}[\bar{\bar{X}}] - \bar{k}_{AX}(\bar{V}_A/V_A^*)^{(f-1)/f}[\bar{A}]\{(\bar{V}_A/V_A^*)^{1/f} - 1\} \quad (3b)$$

the equivalent of the multivalent Scatchard plot is the dependence of $(\bar{V}_A/V_A^*)^{1/f} - 1$ on $(\bar{V}_A/V_A^*)^{(f-1)/f}[\bar{A}]\{(\bar{V}_A/V_A^*)^{1/f} - 1\}$.

The total ligand concentration, $[\bar{S}]$, is introduced into the analysis by writing the binding function, r (moles of ligand bound/total moles of solute), for the interaction of ligand with f equivalent and independent sites on the partitioning solute [13], namely,

$$r = ([\bar{S}] - [S])/[\bar{A}] = fk_{AS}[S]/(1 + k_{AS}[S]) \quad (4)$$

Clearly, eqn. 4 can be rearranged such that the concentration of free ligand, [S], is expressed as

$$[S] = [\bar{S}] - fk_{AS}[\bar{A}][S]/(1 + k_{AS}[S]) \quad (5)$$

which may be combined with eqn. 2 to give [8]

$$R = 1 + k_{AS}\{[\bar{S}] - (R - 1)f[\bar{A}]/R\} \quad (6)$$

where $R = k_{AX}/\bar{k}_{AX}$ has been substituted for the experimentally measurable ratio of solute–matrix association constants in the absence and presence of ligand. By measuring R at a series of total ligand concentrations, the value of k_{AS}, the solute–ligand intrinsic binding constant, may be determined from the slope of the dependence of R, or $(R - 1)$ [8], on $[\bar{S}] - (R - 1)f[\bar{A}]/R$.

Competition between solute and ligand for matrix sites

We now consider the situation in which ligand-facilitated desorption reflects competition between the solute and a univalent ligand for matrix sites, the latter interaction being governed by the binding constant k_{XS}. For this system the distribution of partitioning solute continues to be described by eqn. 3, but with the constitutive association constant ($\bar{k}_{AX}$) given by [4]

$$\bar{k}_{AX} = k_{AX}/(1 + k_{XS}[S]) \quad (7)$$

In frontal affinity chromatographic studies of this competitive situation with k_{AX} and k_{XS} as the operative equilibrium constants, the total concentrations of ligand and partitioning solute in the liquid phase are also the free concentrations, and hence eqn. 7 provides the relationship for analysing such data. In partition equilibrium studies the measured concentration of partitioning solute in the liquid phase, $[\bar{A}]$, continues to be the free concentration, but only the total ligand concentration in the biphasic system, $[\bar{\bar{S}}]$, is known. The free concentration, [S], is introduced by noting that

$$[\bar{\bar{S}}] = [S](1 + k_{XS}[X]) \quad (8)$$

from which [X], the effective concentration of free matrix sites, may be eliminated on the basis that the intrinsic binding constant for the interaction of an f-valent partitioning solute with univalent matrix sites is defined as

$$k_{AX} = ([\bar{\bar{A}}]^{1/f} - [\bar{A}]^{1/f})/([X][\bar{A}]^{1/f}) \quad (9)$$

Combination of eqns. 7–9 then yields the relationship

$$(R - 1)k_{AX}[\bar{A}]^{1/f} = k_{XS}\{k_{AX}[\bar{A}]^{1/f}[\bar{\bar{S}}] - (R - 1)([\bar{\bar{A}}]^{1/f} - [\bar{A}]^{1/f})\} \quad (10)$$

where, as before, $R = k_{AX}/\bar{k}_{AX}$. The value of k_{XS} may therefore be obtained from the slope of the dependence of $(R - 1)k_{AX}[\bar{A}]^{1/f}$ on $k_{AX}[\bar{A}]^{1/f}[\bar{\bar{S}}] - (R - 1)([\bar{\bar{A}}]^{1/f} - [\bar{A}]^{1/f})$. As required, substitution of a value of unity for f in eqn. 10 yields the expression derived previously [8] for this type of ligand-facilitated desorption of a univalent partitioning solute.

Requirement of a solute–ligand complex for interaction with matrix

We now turn to systems exhibiting ligand-retarded desorption of the partitioning solute as the result of ternary complex formation between ligand, solute and matrix. Consider, first, the situation which entails the reaction of soluble solute–ligand complexes with matrix sites, k_{AS} and k_{ASX} being the relevant intrinsic association constants. The absence of a solute–matrix interaction when ligand is not present means that there is no information, apart from V_A^*, to be gained from partition studies of A alone. Both intrinsic association constants must therefore be evaluated from affinity chromatographic experiments in the presence of ligand. In that regard, k_{ASX} can be determined either by applying eqn. 3 to results obtained with a concentration of ligand sufficient to saturate all sites on the solute, or by extrapolating $\bar{k}_{AX}$ values obtained by this means with lower ligand concentrations to the limit of infinite ligand concentration ($1/[S] \rightarrow 0$).

At low ligand concentrations the smaller magnitude of $\bar{k}_{AX}$ reflects the existence of unliganded A

sites with no affinity for matrix. On the grounds that the fractional saturation of A sites with ligand is $k_{AS}[S]/(1 + k_{AS}[S])$ [13], the relationship between k_{AX} and k_{ASX} becomes [4]

$$\bar{k}_{AX} = k_{ASX}k_{AS}[S]/(1 + k_{AS}[S]) \quad (11)$$

Thus, provided that k_{ASX} is first determined by one of the methods suggested above, it is possible to define a ratio of solute–matrix association constants, $Q = k_{ASX}/\bar{k}_{AX}$, which allows eqn. 11 to be rewritten as

$$1/(Q - 1) = k_{AS}[S] \quad (12)$$

The relationship between the free ligand concentration, [S], and $[\bar{S}]$, the total ligand concentration in the liquid phase, is obtained by combining eqns. 5 and 11 to give

$$[S] = [\bar{S}] - f\bar{k}_{AX}[\bar{A}]/k_{ASX} \quad (13)$$

whereupon eqn. 12 becomes

$$1/(Q - 1) = k_{AS}([\bar{S}] - f[\bar{A}]/Q) \quad (14)$$

Characterization of the system is, in principle, completed by evaluating k_{AS} as the slope of a plot of $1/(Q - 1)$ *versus* $([\bar{S}] - f[\bar{A}]/Q)$.

Although eqn. 14 may be applied to frontal affinity chromatographic data, where $[\bar{S}]$ and $[\bar{A}]$ are defined by the composition of the applied solution, it requires further modification for use with partition equilibrium studies, where the only available ligand concentration is again $[\bar{\bar{S}}]$. The difference between $[\bar{\bar{S}}]$ and $[\bar{S}]$ reflects the concentration of ligand associated with matrix in the form of ASX complexes, and clearly this difference must also equal the concentration of A sites involved in such complexes.

Evaluation of the constitutive association constant for the solute matrix interaction via eqn. 3 is based on the definition of this intrinsic constant as [3,6]

$$\bar{k}_{AX} = \frac{\{1 - ([\bar{A}]/[\bar{\bar{A}}])^{1/f}\}f[\bar{\bar{A}}]}{[X]([\bar{A}]/[\bar{\bar{A}}])^{1/f}f[\bar{\bar{A}}]} \quad (15)$$

On the grounds that the numerator of eqn. 15 corresponds to the concentration of A sites complexed with matrix [3], it may be identified with the difference between $[\bar{\bar{S}}]$ and $[\bar{S}]$:

$$\{1 - ([\bar{A}]/[\bar{\bar{A}}])^{1/f}\}f[\bar{\bar{A}}] = [\bar{\bar{S}}] - [\bar{S}] \quad (16)$$

Substitution of the consequent expression for $[\bar{\bar{S}}]$ into eqn. 14 then gives

$$1/(Q - 1) = k_{AS}\{[\bar{\bar{S}}] - f[\bar{A}]^{(f-1)/f}([\bar{A}]^{1/f} - [\bar{A}]^{1/f}) - f[\bar{A}]/Q\} \quad (17)$$

which provides the means of evaluating k_{AS} from partition studies.

Requirement of a matrix–ligand complex for interaction with solute

To conclude this section we treat the other interplay of two equilibria that leads to ligand-retarded desorption, namely the interaction of the solute with a matrix–ligand complex, XS. Theoretical consideration of this alternative pathway of ternary complex formation between solute, matrix and ligand leads to the following dependence of k_{AX} on the free ligand concentration [4]:

$$\bar{k}_{AX} = k_{XSA}k_{XS}[S]/(1 + k_{XS}[S]) \quad (18)$$

where k_{XSA} is the intrinsic association constant for reaction of the solute with the matrix–ligand complex, the formation of which is governed by k_{XS}. Because this expression is formally identical with eqn. 11, it follows that k_{XSA} and k_{XS} may be evaluated from ($\bar{k}_{AX}$, [S]) data by the same procedures as those described for systems in which ternary complex formation is governed by k_{ASX} and k_{AS}.

For situations in which solute adsorption reflects the binding of solute to liganded matrix sites, the absence of a solute–ligand interaction in the liquid phase ensures that the analogue of eqn. 12, *viz.*,

$$1/(Q - 1) = k_{XS}[S] \quad (19)$$

with $Q = k_{XSA}/\bar{k}_{AX}$, may be applied to frontal affinity chromatographic results because of the identity of [S] and $[\bar{S}]$. However, interpretation of partition equilibrium studies requires an expression in terms of $[\bar{\bar{S}}]$, which is obtained by taking into account the following factors. First, the concentration of ligand present as ternary complex again equals the concentration of bound solute, and is therefore given (eqn. 16) by $\{1 - ([\bar{A}]/[\bar{\bar{A}}])^{1/f}\}f[\bar{\bar{A}}]$. Secondly, by analogy with eqn. 15, the intrinsic association constant for ternary complex formation, k_{XSA}, may be written as

$$k_{XSA} = \frac{\{1 - ([\bar{A}]/[\bar{\bar{A}}])^{1/f}\}f[\bar{\bar{A}}]}{[XS]([\bar{A}]/[\bar{\bar{A}}])^{1/f}f[\bar{\bar{A}}]} \quad (20)$$

which allows the concentration of the matrix–ligand complex, [XS], to be expressed as

$$[XS] = ([\bar{A}]^{1/f} - [\bar{A}]^{1/f})/(k_{XSA}[\bar{A}]^{1/f}) \quad (21)$$

On the grounds that $[S] = [\bar{\bar{S}}] - ([\bar{\bar{S}}] - [\bar{S}]) - [XS]$, combination of eqns. 16 and 21 with eqn. 19 leads to the conclusion that

$$1/(Q-1) = k_{XS}\{[\bar{\bar{S}}] - ([\bar{A}]^{1/f} - [\bar{A}]^{1/f})[f[\bar{\bar{A}}]^{(f-1)/f} + 1/(k_{XSA}[\bar{A}]^{1/f})]\} \quad (22)$$

which provides the means of evaluating k_{XS} from partition equilibrium studies of systems within this category.

EXPERIMENTAL

Materials

Human α-thrombin was kindly provided by Dr. J. Fenton, New York State Department of Public Health, Albany, NY, USA. Heparin (fraction 9, M_r 5600) was purified by gel chromatography of heparin from pig intestinal mucosa by the method of Laurent *et al.* [17]. Concanavalin A, controlled-pore glass beads (glyceryl-CPG 170), α-D-methylmannoside and *p*-nitrophenyl-α-D-mannoside were obtained from Sigma (St. Louis, MO, USA). The tresylation procedure described by Nilsson and Larsson [18] was used to immobilize concanavalin A on glyceryl-CPG 170 beads. Heparin-Sepharose was purchased from Pharmacia (Piscataway, NJ, USA).

Thrombin concentrations were determined spectrophotometrically on the basis of a molecular weight of 36 500 and an absorption coefficient ($A^{1\%}_{1\,cm}$) of 17.4 at 280 nm [19]. A molar absorption coefficient of 10 000 at 305 nm was used to determine concentrations of *p*-nitrophenylmannoside [20].

Affinity chromatographic studies of ligand binding

The binding of heparin to thrombin at 25°C was studied by affinity chromatography on heparin-Sepharose in accordance with the recycling partition equilibrium technique used previously [3,5,8]. An aliquot (10 μl) of thrombin (0.2 m*M*) in 0.05 *M* 4-(2-hydroxyethyl)-1-piperazineethanesulphonic acid (HEPES)–0.125 *M* NaCl (pH 7.4) was added to a slurry of heparin-Sepharose (10 mg) in the same buffer [$(V^*_A)_0$ = 6.0 ml] and the absorbance of the recycling liquid phase was measured continuously at 280 nm with a Pharmacia UV-M monitor. After attainment of partition equilibrium (*ca.* 30 min), the process was repeated several times to obtain sufficient data for characterizing the thrombin–matrix interaction in terms of intrinsic affinity constant (k_{AX}) and effective matrix-site concentration ($[\bar{\bar{X}}]_0$). At that stage desorption of thrombin was effected by successive additions of aliquots (10 μl) of heparin (0.62 m*M*) to generate data for the evaluation of k_{AS} via eqn. 6. A detailed description of these procedures appears elsewhere [8].

In similar recycling partition equilibrium studies of the binding of methylmannoside to immobilized concanavalin A by competitive displacement of *p*-nitrophenylmannoside, aliquots (40 μl) of the latter (2.8 m*M*) were first added to a slurry (*ca.* 4 ml) of concanavalin A–CPG 170 equilibrated at 20°C with acetate–chloride buffer (pH 5.5, I = 0.50) [14]. The concentration of *p*-nitrophenylmannoside in the liquid phase, [Ā], was monitored continuously at 305 nm with an ISCO V^4 absorbance detector. Aliquots (100 μl) of methylmannoside (10 m*M*) were then added to generate data for the evaluation of k_{XS} via eqn. 10. Finally, solid methylmannoside (0.1 g) was added to displace all *p*-nitrophenylmannoside from the matrix and hence allow retrospective evaluation of V^*_A at each stage of the experiment; the volume occupied by the solid methylmannoside was calculated on the basis of a partial specific volume of 0.60 ml/g.

RESULTS AND DISCUSSION

By demonstrating that affinity chromatographic behaviour reflecting all four possible interplays of two chemical equilibria is amenable to quantitative description in terms of total ligand concentration, this investigation serves to indicate the general significance of the initial realization [8] that competition between ligand and matrix sites for solute could be described in such terms, and has thereby achieved its first goal. We now wish to illustrate the application of the appropriate quantitative expressions to experimental systems complying with three of the four different classes of affinity chromatographic behaviour. In that regard the fourth category of system, *viz.*, ligand-retarded desorption through interaction of the solute with a matrix–ligand complex, is unlikely to be encountered in an affinity system chosen by the experimenter.

Heparin-facilitated desorption of thrombin from heparin-Sepharose

This situation involving competition between ligand and matrix sites for solute has, of course, already been illustrated by the earlier characterization of the interaction between high-affinity heparin and antithrombin III [8]. However, the substitution of thrombin for antithrombin introduces the problem of ligand multivalency, because the interaction of thrombin with heparin is not confined to the specific pentasaccharide sequence associated with the binding of antithrombin to heparin. Indeed, the binding of thrombin may well reflect non-specific interaction with hexasaccharidic (3-disaccharidic) segments of the heparin chain [21]. Inasmuch as the total concentration of matrix sites, $[\bar{\bar{X}}]$, is an effective thermodynamic parameter based on the concept of uniform distribution of matrix sites throughout the volume accessible to solute [1], it is immaterial whether the adsorption of thrombin to heparin-Sepharose entails specific or non-specific matrix sites. In fact, advantage has already been taken of this situation in the use of Sephadex as an affinity matrix to quantify the interactions of concanavalin A with methylglucoside [22] and ovalbumin [14].

Characterization of the thrombin–matrix interaction is summarized in Fig. 1a, about which the following points merit comment. (i) Because of the small amount of heparin-Sepharose (10 mg) in the slurry (6 ml), $(V_A^*)_0$ was taken as the volume of buffer present, a quantity established by reweighing the previously tared partition cell. (ii) As before [8], the progressive decrease in $[\bar{\bar{X}}]$ with increase in V_A^* resulting from each successive addition of solute has been taken into account by expressing $[\bar{\bar{X}}]$ in terms of its initial value, $[\bar{\bar{X}}]_0$, as $\{(V_A^*)_0/V_A^*\}[\bar{\bar{X}}]_0$, which leads to the manner of presentation adopted in Fig. 1a for the plot of results in accordance with eqn. 3a for a univalent partitioning solute. (iii) On the basis of the combined results for three separate recycling partition experiments shown in Fig. 1a, $k_{AX} = 1.7\ (\pm 0.3) \cdot 10^6$ l/mol and $[\bar{\bar{X}}]_0 = 3.2\ (\pm 0.4)\ \mu M$.

Consideration of results reflecting the displacement of thrombin from heparin-Sepharose by soluble heparin clearly has to await a decision about the magnitude of $[\bar{\bar{S}}]$ to be used in eqn. 6. In the theory section $[\bar{\bar{S}}]$ was identified as the total molar concentration of competing ligand, but that description was based on assumed univalency of the ligand. Although multivalency of both solute and ligand

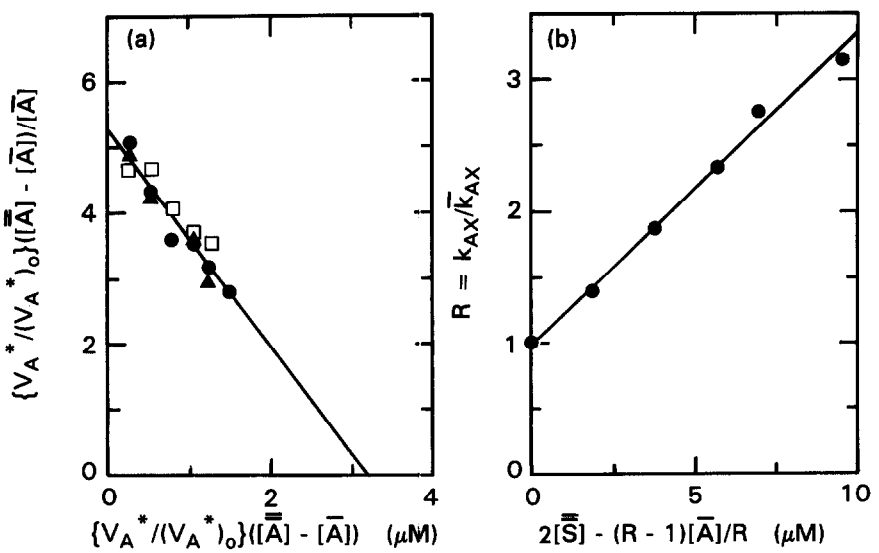

Fig. 1. Characterization of the interaction between heparin and thrombin by recycling partition equilibrium studies with heparin–Sepharose as affinity matrix. (a) Evaluation of the thrombin–matrix affinity constant (k_{AX}) and the effective concentration of matrix sites ($[\bar{\bar{X}}]_0$) by Scatchard analysis of three separate sets of data. (b) Dependence of the constitutive solute–matrix association constant ($\bar{k}_{AX}$) on total ligand concentration, the results being plotted in accordance with the form suggested by eqn. 6 for the determination of k_{AS}, the binding constant for the interaction of thrombin with the bivalent heparin.

poses an intractable problem in quantitative affinity chromatography, the partition of a univalent solute can be handled provided that a valence, g, can be assigned to S [14]: $[\bar{S}]$ is then replaced by $g[\bar{S}]$, the total concentration of equivalent and independent sites for thrombin on the heparin chain. In a recent investigation of the thrombin–heparin interaction [21], the results for a similar heparin sample (M_r = 5600) were amenable to interpretation either in terms of a site-specific model with two binding sites for thrombin on this heparin chain comprising nine disaccharide repeating units, or an alternative model involving non-specific interaction with any hexasaccharidic repeat segment. For the latter model there are seven possible ways in which the first thrombin molecule can bind [21], but in only one case is it possible for three thrombin molecules to be attached to the one heparin chain. On statistical grounds the average number of sites is therefore $\{(1 \cdot 3) + (6 \cdot 2)\}/7$, *i.e.*, 2.1. We therefore consider that the heparin is effectively bivalent (g = 2), irrespective of whether the binding of thrombin is considered in terms of site-specific or non-specific models. From the consequent plot of the experimental results in accordance with eqn. 6 (Fig. 1b), a value (± 2 S.E.M.) of 2.4 (± 0.3) $\cdot 10^5$ l/mol is obtained for k_{AS}. This result is in reasonable agreement with the earlier fluorescence study (Table II in ref. 21), which yielded intrinsic association constants of 5.6 (± 0.9) $\cdot 10^5$ and 1.6 (± 0.08) $\cdot 10^5$ l/mol for the specific and non-specific models, respectively, of heparin with M_r = 5600.

Competition between carbohydrates for immobilized concanavalin A

The displacement of *p*-nitrophenylmannoside from immobilized concanavalin A by methylmannoside [10,11] is used to illustrate the situation in which desorption reflects competition between solute and ligand for matrix sites. Quantitative expressions for this and the previous competitive case are formally identical when written in terms of free ligand concentration (*cf.*, eqns. 2 and 7), but the similarity does not extend to descriptions in terms of total ligand concentration (*cf.*, eqns. 6 and 10). Because eqns. 6 and 10 differ so markedly in form, a decision about the nature of the competitive ligand interaction is clearly a prerequisite for evaluation of its magnitude from partition (chromatographic) studies in which only the total ligand concentration is available. In that regard there should be little cause for confusion in affinity chromatographic

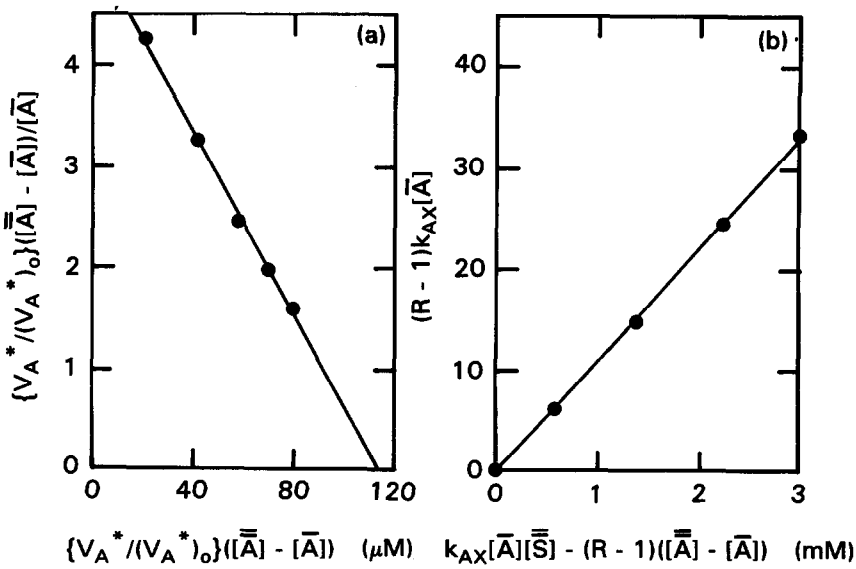

Fig. 2. Quantitative affinity chromatographic characterization of the competitive interactions of *p*-nitrophenylmannoside (A) and methylmannoside (S) for concanavalin A immobilized on CPG 170. (a) Evaluation of k_{AX} and $[\bar{X}]_0$ by Scatchard analysis. (b) Evaluation of k_{XS} from the dependence of the constitutive solute–matrix association constant ($\bar{k}_{AX}$) on total ligand concentration (eqn. 10).

experiments where the chemical properties of the immobilized reactant dictated its selection as affinity matrix.

Results of a recycling partition study of the interaction between solute (*p*-nitrophenylmannoside) and immobilized concanavalin A are presented in Fig. 2a, from which values of 4.5 (± 0.6) $\cdot 10^4$ l/mol and 114 (± 8) μM are obtained for k_{AX} and $[\bar{\bar{X}}]_0$, respectively. We note that k_{AX} is of the same order of magnitude as, but larger than, the association constant of $2.6 \cdot 10^4$ l/mol reported by Anderson and Walters [11] for the interaction between *p*-nitrophenylmannoside and immobilized concanavalin A under fairly similar conditions. However, the concanavalin A matrix used in that study exhibited heterogeneity [11], as did the affinity matrix used by Muller and Carr [10], who reported an even lower average value, $1.6 \cdot 10^4$ l/mol, but also presented evidence (Fig. 6 in ref. 10) for sites with binding constants as high as $7.6 \cdot 10^4$ l/mol. The fact that results obtained with the present concanavalin A affinity matrix are described adequately by the concept of a single class of matrix sites with higher affinity for solute (k_{AX}) than the average value exhibited by either of its predecessors [10,11] may reflect the decision to immobilize the concanavalin A as a fully saturated lectin–methylmannoside complex. This course of action may have afforded at least partial protection of amino groups in the immediate vicinity of the carbohydrate-binding site against involvement in the random covalent coupling of lectin to matrix.

Although a reaction characterized by an equilibrium constant of 10^4 l/mol can hardly be described as a high-affinity interaction, the large effective concentration of matrix sites in the present study ($[\bar{\bar{X}}]_0 = 114\ \mu M$) bestows upon the system the problems associated with the characterization of stronger interactions. The competition between methylmannoside and *p*-nitrophenylmannoside for immobilized concanavalin A sites is summarized in Fig. 2b, which plots results from the second half of the recycling experiment in accordance with eqn. 10: a value of 1.1 (± 0.1) $\cdot 10^4$ l/mol for k_{XS} is obtained from the slope. In keeping with the result for the binding of *p*-nitrophenylmannoside to the present affinity matrix, this association constant for the interaction of methylmannoside with the immobilized concanavalin A is slightly larger than the corresponding values of 7600 [10] and 7400–8500 l/mol [11] obtained in the earlier affinity chromatographic studies of this system.

NADH-retarded desorption of lactate dehydrogenase from oxamate-Sepharose

Consideration is now given to an example of the more likely affinity chromatographic situation involving ligand-retarded desorption, namely that in which solute adsorption reflects ternary complex formation between matrix sites and a binary solute–ligand complex. For this purpose we return to a frontal affinity chromatographic study of the NADH-retarded elution of rabbit muscle lactate dehydrogenase (0.9 μM) from oxamate-Sepharose [12].

Because only a single concentration of enzyme was used, the following analysis necessarily entails an assumption that the concentration of matrix sites greatly exceeds that of partitioning solute. With that proviso, the expression for $\bar{k}_{AX}$ (eqn. 3b) becomes [6,14]

$$(\bar{V}_A/V_A^*)^{1/f} - 1 = \bar{k}_{AX}[\bar{\bar{X}}] \qquad (23)$$

Its combination with eqn. 11 then leads to the conclusion that the affinity chromatographic behaviour of such a system should be described by

$$(\bar{V}_A/V_A^*)^{1/f} - 1 = k_{ASX}k_{AS}[\bar{\bar{X}}]\,[S]/(1 + k_{AS}[S]) \qquad (24)$$

However, although a value of 4 for f is indicated by the tetrameric subunit structure of lactate dehydrogenase, the results of the frontal affinity chromatographic study with $[\bar{A}] = 0.9\ \mu M$ [12] signified conformity to eqn. 24 with $f = 1$. This situation occurs when steric factors preclude multiple attachment of solute to matrix even though f is not unity [1,12,23]. Under those conditions eqn. 24 becomes

$$(\bar{V}_A/V_A^*) - 1 = fk_{ASX}k_{AS}[\bar{\bar{X}}]\,[S]/(1 + k_{AS}[S]) \qquad (25)$$

whereupon the double-reciprocal linear transform of this expression has an ordinate intercept of $1/(fk_{ASX}[\bar{\bar{X}}])$. Such a plot with $[\bar{S}]$ substituted for [S] is shown in Fig. 3a.

A second feature of those results for the affinity chromatographic behaviour of NADH–lactate dehydrogenase mixtures on oxamate-Sepharose [12] is the fact that the total enzyme concentration was too large for the approximation to be made that $[S] \approx [\bar{S}]$. Consequently, Fig. 3a may only be used to

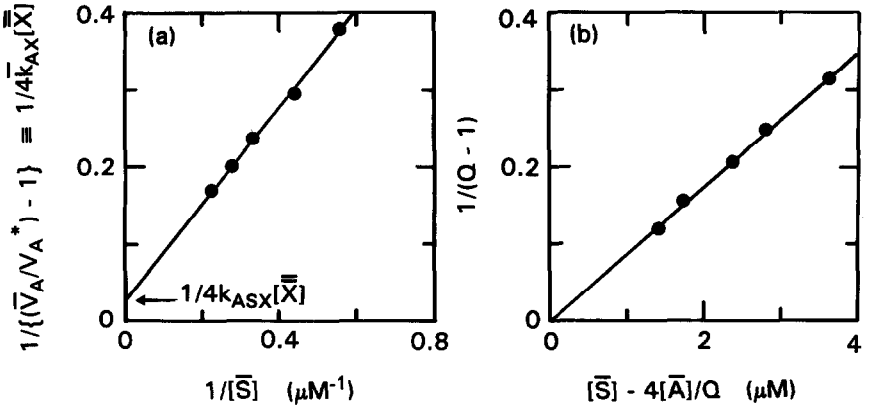

Fig. 3. Evaluation of the intrinsic binding constant (k_{AS}) for the interaction of NADH with lactate dehydrogenase by frontal affinity chromatography of enzyme–NADH mixtures on an oxamate-Sepharose column. (a) Analysis of results, inferred from Fig. 1 of ref. 12, on the basis of the double-reciprocal linear transform of eqn. 25 with the free ligand concentration ([S]) taken as [S]. (b) Consequent evaluation of k_{AS} via eqn. 14.

establish the magnitude of $1/4k_{ASX}[\bar{\bar{X}}]$. This parameter is then used in conjunction with the experimental points to define the ratio of solute–matrix affinity constants, $Q = 4k_{ASX}[\bar{\bar{X}}]/(4\bar{k}_{AX}[\bar{\bar{X}}]) = 1/\{(\bar{V}_A/V_A^*)^{1/f} - 1\}$, for each [S̄]. A plot of the Q values in accordance with eqn. 14 exhibits the predicted linear dependence (Fig. 3b), the slope of which yields an intrinsic association constant (k_{AS}) of $9 \cdot 10^4$ l/mol for the enzyme–NADH interaction. This value of k_{AS} was also obtained previously [12] by the more tedious procedure of evaluating [S] by iterative solution of the quadratic equation that relates [S], [Ā], k_{AS} and [S̄], using the approximate value of k_{AS} from Fig. 3a as an initial estimate of the solute–ligand affinity constant.

CONCLUSIONS

By providing analytical expressions in terms of total ligand concentration for all four affinity chromatographic situations involving an interplay of two chemical equilibria, this investigation has reinforced the importance of the earlier finding [8] that ligand-facilitated elution of partitioning solute could be described in such terms. Elimination of the earlier requirement of a value for free ligand concentration means that high-affinity interactions may be characterized far more readily, and further, the previous restriction that the ligand be small is also removed. This study, which not only outlines the theoretical developments but also illustrates the application of the analytical expressions, has enhanced even further the versatility of quantitative affinity chromatography as a means of characterizing interactions between dissimilar molecules, large or small.

ACKNOWLEDGEMENTS

The support of this study by the Australian Research Council (D.J.W.), an Established Investigatorship from the American Red Cross (C.M.J.) and the Southeastern Michigan Regional Blood Services is gratefully acknowledged.

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