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Competitive ion-exchange adsorption of proteins: Competitive isotherms with controlled competitor concentration

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Abstract

The competitive adsorption processes inevitably present in chromatographic separations of complex mixtures have not been extensively studied. This is partly due to the difficulty of measuring true competitive isotherms, in which all system parameters (including competitor concentrations) are held constant. We report a novel approach to determining competitive protein adsorption isotherms in which the competitor concentration is held constant across the entire isotherm. By using the heme prosthetic group in cytochrome b_5 as a quantitative spectrophotometric label, competitive isotherms between cytochrome b_5 and α-lactalbumin can be constructed. Similarly, manganese-substituted protoporphyrin IX heme replacement allows the non-perturbing labeling of individual cytochrome b_5 conservative surface charge mutants by replacement of a single atom in the interior of the protein. This labeling allows the study of competition between cytochrome b_5 charge mutants of identical size and shape, which differ only in charge arrangement. Using these techniques, the effect of competing species on equilibrium behavior and the apparent heterogeneity of anion-exchange adsorbents in the presence of competitors can be quantitatively studied by fitting the data to two popular single-component binding models, the Temkin and the Langmuir–Freundlich (L–F) isotherms.

Keywords: Protein adsorption; Competitive adsorption; Ion-exchange; Competitive binding; Competitive isotherms; Anion-exchange; Temkin isotherm; Langmuir–Freundlich isotherm

1. Introduction

All practical applications of chromatography involve the separation of complex mixtures. The competitive adsorption processes involved have not been extensively studied, however, and knowledge of these phenomena would be invaluable in developing improved adsorbents and methods. In addition, the effectiveness of any chromatographic model is strongly dependent not only on the accurate description of the relatively well-studied transport effects, but also on the accurate description of multi-component adsorption equilibria [1]. There are many factors which influence protein adsorption, the main contributors being affinity of the protein for the adsorbent, the energetic heterogeneity of the adsorbent, uneven ligand distribution on the protein surface [2–4], competition from other species (other solutes, counterions, etc.) and interactions between solutes, both adsorbed and in solution. The ubiquitous Langmuir isotherm [5] is often used to describe adsorption to a variety of surfaces [6–10]; however this isotherm model is unable to capture all the important aspects of many protein adsorption interactions including ion exchange [11–16]. This work seeks to characterize competitive adsorption equilibrium behavior in anion-exchange chromatography exclusive of kinetic effects using true competitive isotherms on non-porous ion-exchange media. The goal is to characterize the most fundamental processes of competitive protein adsorption, particularly how the presence of competing proteins affects the affinity and apparent heterogeneity of the adsorbent surface. Another interesting question to be addressed is whether or not the reduction of apparent heterogeneity in a chromatographic system is due to surface crowding effects at higher loadings, or if the presence of competitor on the surface of the adsorbent is af-

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fecting the true energetic heterogeneity of the adsorbent surface.

Competitive and single-component protein adsorption have been characterized by a variety of experimental methods [1,16–21]. These methods involve determination of single and multicomponent binding equilibria in ion-exchange systems including batch adsorption [1,16,21,22], determination from chromatographic peak shapes [23], frontal chromatography [24], and breakthrough curves [20]. These studies, however, focus mainly on experiments involving the use of porous adsorbent particles. Even in batch adsorption, transport phenomena in pores can alter the apparent equilibrium behavior of a multicomponent system due to pore blockage and accessibility effects. Several authors have reported single and multicomponent batch protein adsorption data [1,16,19,25]. In the case of multicomponent (binary) systems, interpretation of the competitive isotherms obtained is complicated by the simultaneously varying concentrations of each of the two proteins. These data, therefore, do not constitute a set of individual competitive isotherms, but formally are a set of data points each lying on a different isotherm [16,19]. With such a data set, it is difficult to identify what quantitative effects are due to the presence of the competitor, and what effects are due to the varying liquid phase concentration of the competitor.

We have undertaken to address these issues with the use of a non-porous adsorbent (Amersham Mini Q beads) along with novel methods of isotherm measurement and competitor labeling. Mini Q beads are small (3 μm) non-porous hydrophilic polyether anion-exchange particles with a strong quaternary ammonium functionality. The non-porosity and small size of these particles allows rapid equilibration, and their low capacity reduces the amount of material required for determination of complete isotherms. We have also utilized a non-perturbing labeling method which relies on the replacement of the naturally occurring heme in cytochrome b_5 with a Mn(III)-substituted protoporphyrin. This labeling provides two unique chromophores which allows simultaneous quantitation of cytochrome b_5 mutant mixtures spectrophotometrically.

2. Materials and methods

2.1. Model proteins

The proteins used in this study were bovine α-lactalbumin (Calcium depleted, Sigma, product L 6010), recombinant tryptic fragment of wild-type (wt) cytochrome b_5 and two charge mutants of cytochrome b_5. Cytochrome b_5 is a 13.6 kDa recombinant form of the tryptic core of wild-type hepatic microsomal rat cytochrome b_5, with an isoelectric point (p*I*) of 5.3. In addition to the wild-type cytochrome b_5, two conservative site-directed surface charge mutants of cytochrome b_5, E11Q and E44Q (glutamic acid to glutamine at positions 11 and 44, respectively), were used. The mutation E44Q disrupts a charge cluster on the surface of cytochrome b_5, whereas the E11Q mutation does not disrupt the cluster. The effect of these mutations is to create two nearly identical proteins (i.e. same total charge, size, shape and stability) differing only in affinity for the ion-exchange adsorbent [26,27]. α-Lactalbumin is a 14.2 kDa protein with a p*I* of 5.0 which was chosen because it is readily available, has been well characterized in the literature and has often been used in adsorption experiments [20].

2.2. Protein purification

The cytochrome b_5 wild-type, E11Q, and E44Q forms were purified from cultures of *E. coli* strain TB1 harboring the corresponding plasmids obtained from the laboratory of Prof. Steven Sligar, UIUC [22]. Flasks containing 1 L of LB medium were inoculated and cultured at 37 °C with moderate agitation (80 rpm; reduced oxygen tension promotes heme incorporation) for 24 h and then harvested by centrifugation at $1000 \times g$ in a Beckman J2–20 centrifuge. The resulting cell pellets were frozen at −20 °C for at least 24 h or until needed. For cell lysis, the cell paste (typically 50–100 g) was suspended in 200 ml of lysis buffer (20 mM Tris, 0.1% Triton X-100, 1 mM PMSF, and 15 mM spermidine to clear nucleic acids (Sigma; DeWalt et al. [28]). The suspension was then lysed at 4 °C using a French press (Aminco) with a back pressure of $10{,}000 \pm 1000$ psi. The resulting lysate was centrifuged at $14{,}000 \times g$ in a Beckman J2–21 centrifuge to remove cell debris and precipitated nucleic acids [28], followed by microfiltration through a 0.2 μm membrane filter. The clarified lysate (∼200 ml) was loaded on a 4.5 cm diameter Amicon column packed with Q Sepharose Fast Flow (Amersham) with a bed height of 5 cm, washed with 3 column volumes of 20 mM Tris, pH 8.0 at 10 ml/min and the product eluted with 0 to 400 mM NaCl over 5 column volumes at 5 ml/min. The resulting fractions containing cytochrome b_5 (identified by absorbance at 412 nM) were pooled and concentrated in an Amicon Centri-prep YM-10 concentrator to a concentration of 10 mg/ml. The concentrated samples were loaded onto a pre-packed HiPrep 26/60 S-100 Sephacryl size-exclusion chromatography (SEC) column (Amersham) using a Superloop (Amersham) and repeated injections of 3 ml at a flowrate of 1.5 ml/min. The resulting fractions of cytochrome b_5 were essentially pure as judged by sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE), however a subsequent anion-exchange step was added to remove trace contaminants as well as to concentrate the product. The pooled fractions from the SEC column were loaded directly onto a 3.2 cm diameter Amicon column containing Q Sepharose HP at a bed height of 5 cm. A gradient (5 column volumes) from 0 to 400 mM NaCl was run and the resulting cytochrome b_5 fractions (determined by absorbance at 412 nm), were pooled. The resulting product was desalted by dialysis against 10 mM Tris–HCl, pH 8.0 and the product was sterile filtered (0.22 μm) and

aliquoted into 2 ml cryotubes and stored at −80 °C until needed.

2.3. Manganese protoporphyrin labeling

Cytochrome b_5 was labeled by replacing a single, buried atom by reconstitution with manganese-substituted protoporphyrin IX (manganese protoporphyrin IX; Porphyrin Products) of cytochrome b_5 apoprotein prepared by a modification of a procedure originally developed by Teale [29,30]. The protein to be reconstituted was first diluted with 100 mM KPO_4, pH 7.0 to a maximum concentration of 0.5 mg/ml of protein. The cytochrome b_5 (kept on ice throughout the procedure) was then stripped of its native heme by addition of cold 2 M HCl until a pH of 2.0 was reached and extraction using one volume of methyl ethyl ketone (MEK), retaining the apoprotein-containing aqueous phase. The aqueous phase was then dialyzed against 4 L of 10 mM Tris–HCl, 1 mM EDTA, pH 8.0 to extract any residual MEK and to return the pH to neutrality.

The apoprotein (wild type, E11Q or E44Q) was reconstituted with the manganese protoporphyrin according to the method of Martinis et al. [31]. A 1 mg/ml solution of manganese protoporphyrin was prepared by dissolving 10 mg manganese protoporphyrin IX in 1 ml of 100 mM NaOH, followed by addition of 9 ml of deionized water. The resulting solution was slowly (over 30 min) added to the apoprotein in a molar ratio of 2:1 manganese protoporphyrin to apoprotein. The mixture was then kept at 4 °C for 24 h to allow the protoporphyrin to incorporate. The resulting solution was placed on a gravity flow LC column filled with S-100 Sephacryl (Amersham) in order to remove any residual manganese protoporphyrin IX. The resulting product was dialyzed against 10 mM Tris–HCl, pH 8.0 and concentrated in an Amicon concentrator. The absorbance of the product was measured at 368 and 469 nm to ensure no residual protoporphyrin remained. Correctly reconstituted Mn(III)-cytochrome b_5 had an A_{368}/A_{469} ratio of 1.1. Any remaining unincorporated manganese protoporphyrin gave a ratio greater than 1.1. As a final concentrating step, the reconstituted Mn(III)-cytochrome b_5 was loaded onto a 2.5 cm Amicon column containing Q Sepharose HP, and eluted with a step to 400 mM NaCl. The product was dialyzed against 10 mM Tris–HCl, pH 8.0, sterile filtered and stored at −80 °C until used.

2.4. Quantitative detection of model proteins

We utilized a spectrophotometric method for the quantitation of each component of the binary protein mixtures used in this study. Native cytochrome b_5 contains a heme prosthetic group which provides a unique spectrophotometric label, allowing convenient quantitation using absorbance at 412 nm. By simultaneous detection at 280 and 412 nm, the amounts of cytochrome b_5 and another protein, in this case α-lactalbumin, can readily be determined. For a path length of 1 cm, the concentration of cytochrome b_5 and α-lactalbumin can be given respectively as:

$$C_{b_5} = \frac{A_{412}}{\varepsilon_{b_5}^{412}} \tag{1}$$

and

$$C_{ALN} = \frac{1}{\varepsilon_{ALN}^{280}}[A_{280} - A_{412} \cdot R_Z^{-1}] \tag{2}$$

where R_Z is defined as

$$R_Z = \frac{\varepsilon_{b_5}^{412}}{\varepsilon_{b_5}^{280}}. \tag{3}$$

Replacement of the naturally-occurring iron heme with a manganese-substituted protoporphyrin IX allows simultaneous determination of the concentrations of the two test proteins using the 412 nm spectral maximum of the natural [Fe(III)] heme and the 469 nm maximum of the manganese-substituted protoporphyrin IX cytochrome b_5. Fig. 1 shows a comparison of the two distinct spectra of the Fe(III) and Mn(III)-cytochrome b_5.

Resonance Raman spectroscopy was employed to assure the stability of the manganese-substituted cytochrome b_5. Frequency shifts for the Mn(III)-cytochrome b_5 species indicate that the protoporphyrin group remains six-coordinate (the same as the wild type heme species), although the results indicate some weakening of the metal-to-histidine ligands [30]. In order to verify that this slight weakening did not perturb the binding characteristics of the Mn(III)-cytochrome b_5, isocratic HPLC experiments were carried out to ensure the behavior of the native heme cytochrome b_5 was similar to the manganese substituted form. The isocratic analyses were carried out at 125, 150, 175, and 200 mM NaCl on a Mono-Q HR 5/5 column at a flowrate of 1 ml/min. The retention times for the wild type cytochromes matched those found for the manganese substituted cytochromes, with an average difference of less than 5%. Other substituted protoporphyrins were also prepared and tested, including zinc(II) protoporphyrin IX, cobalt(II) protoporphyrin IX, and tin(IV) protoporphyrin IX. None of these were successful, however: cytochromes substituted with these protoporphyrins were unstable on Mono Q HPLC, indicating the coordination between the metal and the axial histidines was too weak to keep the protein in the native conformation while bound to an ion-exchange matrix.

Batch binding isotherms with Mini Q beads were also used to confirm the stability of the manganese-substituted cytochrome b_5 species on this adsorbent. Single-component isotherms at 100 mM NaCl showed no significant deviations in binding behavior, leading to the conclusion that the manganese version of the cytochromes b_5 is stable while bound to the ion-exchanger Mini Q (data not shown). This was further confirmed by closure of mass balances on the protein during isotherm determination experiments.

Because each chromophore contributes a small amount to each absorbance (e.g. the Mn(III)-cytochrome b_5 contributes

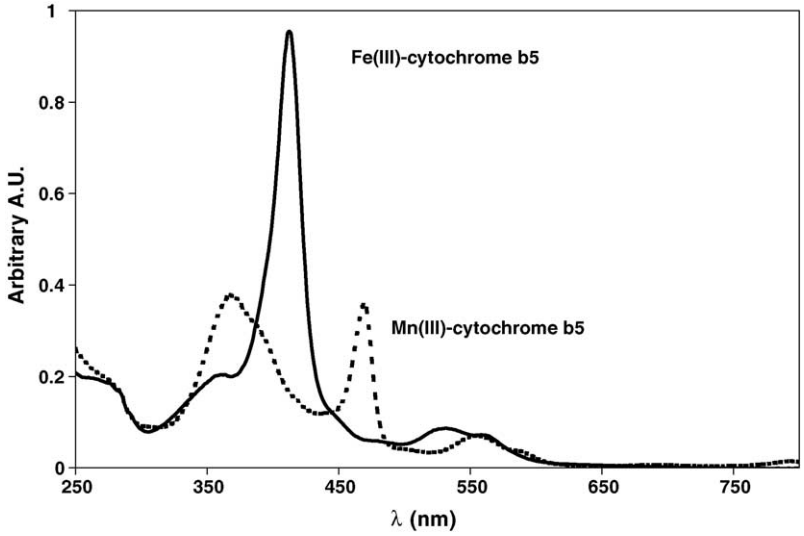

Fig. 1. Absorbance spectra of Fe(III)-cytocrhrome b_5 and Mn(III)-cytochrome b_5 superimposed. The Fe(III)-cytochrome b_5 spectrum (solid line) exhibits a maximum at 412 nm. The Mn(III)-cytochrome b_5 spectrum (dotted line) exhibits maxima at 368 and 469 nm.

a small amount to A_{412}), a relation was developed to deconvolute the raw absorbance data from the mixtures.

For the concentration of the natural [Fe(III)] heme species:

$$C_{\mathrm{Feb_5}} = \varepsilon^{412}_{\mathrm{Feb_5}} \frac{A_{412} - A_{469}M}{1 - MF^{-1}} \tag{5}$$

where

$$M = \frac{\varepsilon^{412}_{\mathrm{Mnb_5}}}{\varepsilon^{469}_{\mathrm{Mnb_5}}} \tag{6}$$

and

$$F = \frac{\varepsilon^{412}_{\mathrm{Feb_5}}}{\varepsilon^{469}_{\mathrm{Feb_5}}}. \tag{7}$$

Similarly, for the Mn(III)-cytochrome b_5, the concentration is given as

$$C_{\mathrm{Mnb_5}} = \varepsilon^{496}_{\mathrm{Mbb_5}} \frac{A_{469} - A_{412}F^{-1}}{1 - MF^{-1}}. \tag{8}$$

By measuring the absorbance of samples at two wavelengths, 412 and 469 nm, we are able to quickly determine the individual concentrations of the two labeled proteins E11Q and E44Q.

2.5. Competitive isotherms

A complete binary isotherm would in theory consist of a two-dimensional surface, in which the two protein liquid-phase concentrations would be on e.g., the *xy*-plane and the *z*-axis would represent the amount of one of the proteins bound to the stationary phase. The most valuable practical isotherm data, and the goal of this work, is an experimental isotherm taken parallel to the *x* or *y*-axis such that the concentration of one of the proteins (here called the competitor) remains constant in one phase. In this work, we have chosen to control the concentration of the competitor in the liquid phase [16]. Controlling competitor concentration in either phase is difficult (and has not previously been reported), because when a second protein is added to the system, some of the other protein is inevitably displaced into the liquid phase. In order to construct controlled isotherms, we used a relatively large liquid phase volume and a low capacity non-porous adsorbent to avoid the problem of a large amount of protein being displaced by a competitor. Perhaps most importantly, we added the second protein in a solution matching the equilibrium concentration of the first one added, to minimize perturbation of the system.

Each competitive isotherm consisted of nine points measured in duplicate or triplicate. One protein was chosen as the competitor and the other as the variable supernatant protein, called the "test protein". Each microcentrifuge tube (1.8 ml Axygen) with 10 μl of Mini Q beads was pre-equilibrated in 50 or 100 mM NaCl in 10 mM Tris–HCl at pH 8.0. Following this, the beads were pre-equilibrated with 400 μl of the competitor protein solution for 1 h, a time found in control experiments to be sufficient for equilibration. The tubes were centrifuged at 10,000 × *g* and the supernatant decanted for analysis by spectrophotometry. The absorbance of the supernatant was measured for three tubes (variation was routinely less than 5%), and the average value used to calculate the supernatant competitor concentration. Binary mixtures were then prepared with an additional protein, in which the competitor concentration was taken as the average equilibrium supernatant concentration (to keep competitor constant) and the other protein was varied between 0 and ca. 1 mg/ml. The binary mixtures were mixed with the adsorbent and allowed to equilibrate for 1 h. The samples were then centrifuged, the supernatant decanted for measurement of the equilibrium concentrations, and the adsorbent washed with 1.8 ml of 10 mM

Tris, pH 8.0 to ensure that no supernatant protein remained in the interstices of the beads. The bound proteins were then eluted from the adsorbent with 300 μl of 10 mM Tris, 1 M NaCl for 1 h and the resulting eluate was then decanted for analysis of the adsorbed proteins.

2.6. Data analysis

Adsorption data were analyzed using two isotherm models originally derived for single component adsorption, the Temkin and Langmuir–Freundlich (L–F) models. The Temkin model is represented as:

$$q = q_T \ln[1 + K_T C] \tag{9}$$

where q is the amount of solute bound to the adsorbent and C is the concentration of solute in the liquid (supernatant) phase. K_T is the maximum affinity and q_T is the differential surface capacity per unit of binding energy [15] and is inversely proportional to the heterogeneity and the extent of adsorbate–adsorbate interactions [15,32].

The Langmuir–Freundlich model is given as:

$$q = \frac{C^{n_H}}{K_d^{n_H} + C^{n_H}} \tag{10}$$

where n_H is the heterogeneity parameter and K_d is the average dissociation constant [21]. This isotherm reduces to the classic Langmuir isotherm when n_H equals unity, corresponding to a completely homogeneous surface. By constructing isotherms in which one competitor concentration remains constant, we can analyze our data using these one-component models to measure how the affinity constants and other parameters are influenced by the presence of competitors. Data fitting was performed using TableCurve 2D v5.01software, and standard error and goodness of fit parameters were obtained from the software to gauge the validity of the fit to the data. TableCurve 2D uses the Levenburg–Marquardt algorithm for fitting user-defined non-linear equations. In order to avoid the pitfalls of local minima in the non-linear least squares solution, TableCurve is equipped with a graphical interface which allows the manual adjustment of initial guess vectors to ensure that a true global minimum is found.

2.7. Affinity and apparent heterogeneity

One of the major goals of this work is to quantitatively describe the changes in protein adsorption produced by the presence of a competing protein species. The Langmuir–Freundlich and Temkin models have their own interpretations of the characteristics of primary interest, affinity and apparent heterogeneity. The Langmuir–Freundlich model assumes a symmetric quasi-Gaussian distribution of adsorbent site affinities. The average value of the affinity distribution is K_d and the width of the distribution is related to the heterogeneity parameter n_H as shown by Sips [33]. As n_H approaches 0 (large negative cooperativity), the width of the distribution becomes infinite. As n_H approaches 1 (a completely homogeneous binding surface, equivalent to the classic Langmuir model), the distribution becomes a Dirac delta function with value K_d. The dissociation constant K_d is inversely proportional to affinity, therefore an increase in the K_d implies a decrease in affinity.

The Temkin model has a uniform underlying affinity distribution which is described as a step function ranging from 0 to a maximum affinity of K_T. Because K_T gives the maximum affinity, it also determines the width of the distribution. The Temkin model also contains a parameter which, in terms of statistical–mechanical aspects, can describe both surface heterogeneity and lateral adsorbate–adsorbate interactions [15,32]. This parameter, q_T, is inversely proportional to the surface heterogeneity as well as the extent of unfavorable lateral interactions (i.e. q_T decreases with increasing heterogeneity and with increasing unfavorable lateral interactions). The parameter q_T is inversely proportional to the width of the distribution, meaning that a lower value of q_T indicates a wider distribution, or a more heterogeneous surface. At the same time, q_T is also inversely proportional to the amount of unfavorable lateral interactions between adsorbed species [32]. In essence, a system of binding isotherms which shows an increase in q_T and a decrease in K_T shows that the system is undergoing a decrease in energetic heterogeneity only (for example, as the amount of competitor in the system increases). A system which shows only an increase in q_T indicates a decrease in the number of unfavorable interactions between bound proteins on the surface of the adsorbent. A detailed description and derivation of this analysis can be found in Johnson et al. [34]. The parameters of both the Langmuir–Freundlich and Temkin models were used to quantitatively analyze the effects of competing proteins in binary systems.

3. Results and discussion

3.1. Cytochrome b_5/α-lactalbumin binary system

Competitive isotherms were constructed using varying amounts of α-lactalbumin as the competitor. Fig. 2 shows an example of such a competitive isotherm. Of note is the constant concentration of α-lactalbumin in the supernatant over the entire range of the isotherm. Because the competitor concentration does not vary in the supernatant, the only independent variable is the amount of cytochrome b_5 in the supernatant. The dependent variables are thus the amounts of cytochrome b_5 and α-lactalbumin bound to the adsorbent. Constant parameters in the system are ionic strength, pH, temperature, and competitor concentration in the supernatant. Fig. 3 shows a series of isotherms, each with a different α-lactalbumin competitor concentration. An interesting qualitative feature of this system is the strong initial sensitivity of the cytochrome b_5 to small amounts of α-lactalbumin, in contrast to the lower sensitivity at higher

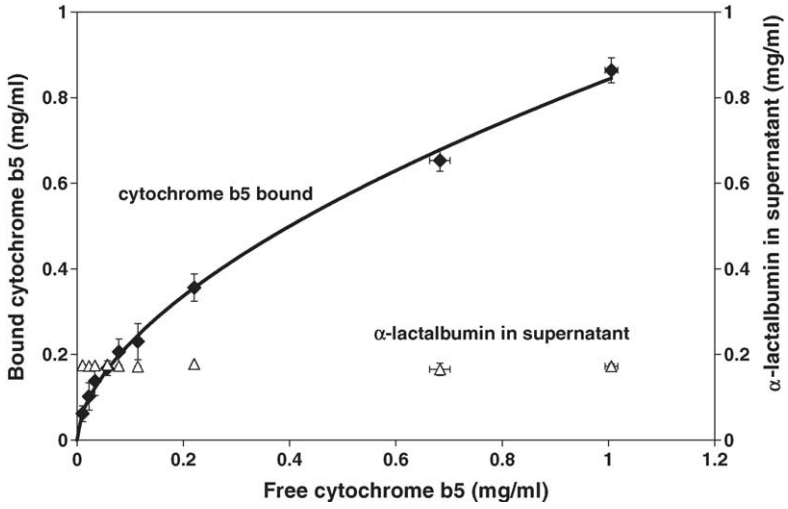

Fig. 2. True competitive isotherm on Mini Q: cytochrome b_5 ($Cytb_5$) wild-type with 0.170 ± 0.003 mg/ml α-lactalbumin (ALN), 10 mM Tris, 50 mM NaCl, pH 8. Supernatant concentration of $Cytb_5$ is varied from 0 to 1 mg/ml. The supernatant concentration of ALN is kept constant at 0.17 mg/ml (secondary *y*-axis, △). Amount of $Cytb_5$ bound (mg/ml adsorbent, ♦) is shown on primary *y*-axis.

competitor concentrations. This may indicate the presence of a subset of "special" sites for which the cytochrome b_5 competes much more effectively, even at higher concentrations of α-lactalbumin. The initial sensitivity of the cytochrome b_5 to the α-lactalbumin may also be partially explained by a kinetically trapped binding of the proteins to the adsorbent. Order-of-addition competitive isotherms show [30] that when α-lactalbumin is added to the adsorbent first, the amount of cytochrome b_5 that can bind is slightly reduced.

A more quantitative analysis of the binary systems can be obtained by fitting the bound cytochrome b_5 data to single-component Langmuir–Freundlich and Temkin isotherms. Table 1 shows fitted Langmuir–Freundlich parameters (reciprocal of affinity K_d, and heterogeneity, n_H) of the isotherms plotted in Fig. 3. It is evident that the presence of α-lactalbumin significantly reduces the adsorbent affinity of cytochrome b_5, as reflected in the immediate increase of the dissociation constant (K_d). The heterogeneity parameter (n_H), in contrast, initially remains relatively unchanged. At higher loadings of α-lactalbumin the cytochrome b_5 adsorption heterogeneity parameters increase, indicating a decrease in apparent heterogeneity. It is apparent (at least within the interpretation of the L–F model) that the affinity is more sensitive to low competitor loadings than is the heterogeneity parameter. The results indicate that, at least for this system, the effect of the competitor is to reduce affinity (more at low competitor loadings) and lower heterogeneity (at higher competitor loadings). This can be interpreted as either an apparent affinity change due to increasing adsorbate–adsorbate interactions at higher loadings, or as reflecting the pres-

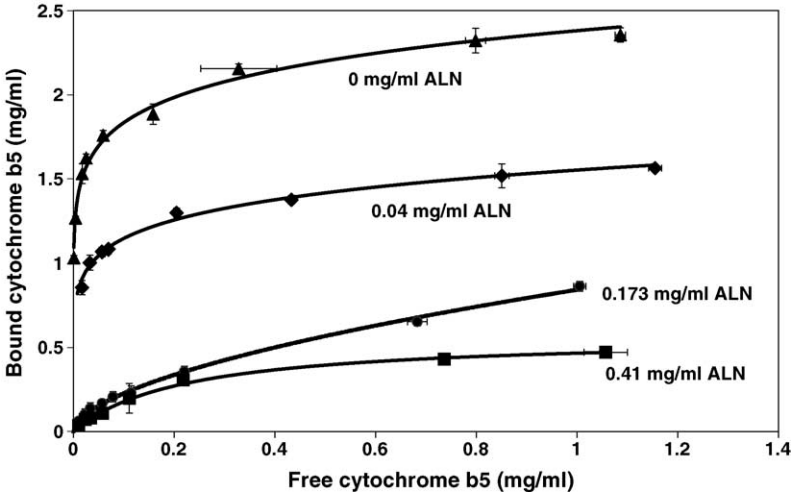

Fig. 3. Competitive cytochrome b_5 adsorption isotherms with α-lactalbumin as the competitor, 50 mM NaCl. Each curve shows the amount of cytochrome b_5 bound (mg/ml adsorbent) in the presence of a different constant supernatant concentration of α-lactalbumin competitor. Data fits are obtained from the single component Langmuir–Freundlich model.

Table 1
Langmuir–Freundlich and Temkin parameters for cytochrome b_5/α-lactalbumin system

Competitor (α-lactalbumin) (mg/ml)	Langmuir–Freundlich parameters		Temkin parameters	
	K_d (mg/ml)	n_H	K_T (mg/ml)$^{-1}$	q_T (mg/ml)
0	0.014 ± 0.006	0.26 ± 0.03	210000 ± 91000	0.19 ± 0.01
0.04	0.761 ± 0.01	0.23 ± 0.02	13000 ± 5000	0.16 ± 0.01
0.10	6.917 ± 0.76	0.24 ± 0.02	6000 ± 3000	0.14 ± 0.01
0.20	6.916 ± 1.33	0.59 ± 0.07	40 ± 10	0.15 ± 0.02
0.40	3.253 ± 2.21	0.81 ± 0.09	6 ± 4	0.36 ± 0.18

The maximum saturation parameter (for L–F) is fixed at 3 mg/ml. Conditions were 10 mM Tris, 50 mM NaCl, pH 8.0.

ence of heterogeneous sites for which the proteins compete [22].

The data were also fitted to the Temkin isotherm. It is apparent that the Temkin affinity constant, K_T, decreases with increasing competitor concentration (Table 1). The parameter q_T, however, does not vary in a statistically meaningful way. It is important to note that due to the nature of the Temkin isotherm, the affinity term reflects the width of the affinity distribution, and thus the energetic heterogeneity of the adsorbent. Therefore, due to the nature of the Temkin isotherm, a reduction in the Temkin affinity constant must imply a reduction in heterogeneity, or vice versa.

3.2. Cytochrome b_5 E11Q/E44Q binary system

By constructing competitive isotherms between cytochrome b_5 conservative charge mutants E11Q and E44Q, we were able to observe how a competitor differing only in affinity (and not size, shape, or diffusivity) can affect competitive behavior. Fig. 4 shows the differing single-component behavior of the two mutants, in which the weaker binding of the E44Q is due to the disruption of a charge cluster on the surface of the protein. By competing two proteins with identical characteristics that differ only in affinity, we were able to probe the effect of affinity alone on binary competitive behavior. Fig. 5 shows the result of competitive isotherms in which the mutant E44Q (referred to as the "weak binder") is taken as the competitor and E11Q (referred to as the "strong binder") is varied in the supernatant as the test protein. As can be seen from the adsorption data, there is an apparent decrease in the amount of protein bound in the presence of the competitor. It is qualitatively apparent from these isotherms that the effect of the weak binder (E44Q) competitor is to reduce the amount of the stronger (E11Q) that is bound, as is expected.

A more quantitative analysis can be made by fitting the adsorption data to the Langmuir–Freundlich and Temkin isotherms (Table 2). As the amount of competitor is increased from 0 to 0.3 mg/ml, the value of the test protein dissociation constant increases. This demonstrates that the presence of the weaker competitor can suppress the adsorption of the strong binder on the ion-exchange adsorbent. An interesting feature is the high sensitivity of the disassociation constants to small amounts of the competitor, while there is an insignificant effect on the heterogeneity parameter (n_H).

The data for this system were also analyzed using the Temkin model. As before, the affinity constant shows a decreasing affinity (a lowering of the Temkin affinity con-

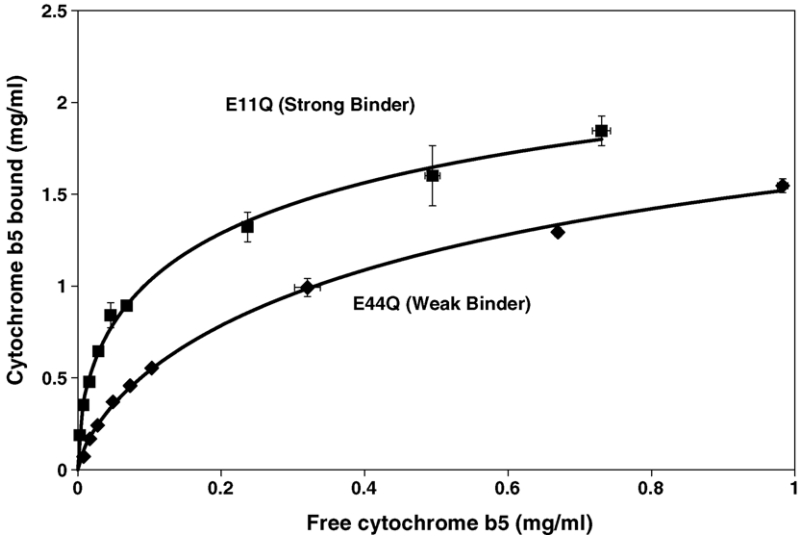

Fig. 4. Single component isotherms for adsorption of cytochrome b_5 E11Q and E44Q mutants on Mini Q, 10 mM Tris, 100 mM NaCl, pH 8.0. Both proteins have the same overall charge, but they have different binding affinities. Curve fits obtained using the Langmuir–Freundlich model. The E11Q is referred to as the "Strong Binder" due to its higher affinity for the adsorbent. E44Q is similarly referred to as the "Weak Binder".

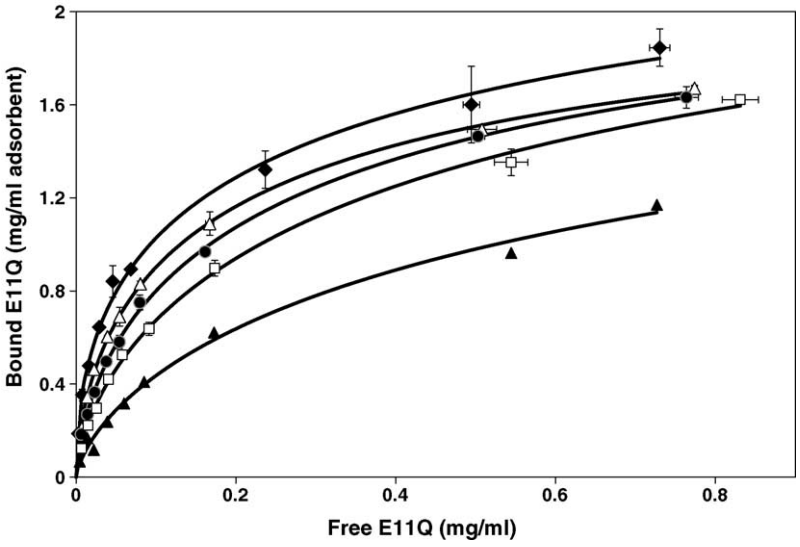

Fig. 5. True competitive isotherms on Mini Q with cytochrome b_5 mutants in which the weak binder (E44Q) is the competitor. The concentration of the strong binder (E11Q) is varied in the supernatant from 0 to 1 mg/ml, and the competitor is held constant (within 5%) for each curve. The amount of the E11Q bound (mg/ml adsorbent) to the adsorbent is shown as a function of supernatant concentration. Curve fits obtained using the Langmuir–Freundlich model. (♦) Pure E11Q, (△) E11Q with 30 μg/ml E44Q, (●) E11Q with 60 μg/ml E44Q, (□) E11Q with 160 μg/ml E44Q, (▲) E11Q with 300 μg/ml E44Q.

stant) of the strong binder due to the presence of the weaker binder. Table 2 shows the resulting parameters. The q_T parameters do not vary significantly over the range examined.

3.3. Cytochrome b_5 E44Q/E11Q binary system

Competitive isotherms were constructed as above, except this time the competing protein was the strong binder E11Q and the weak binder E44Q was varied in the supernatant. Qualitatively, the data are similar to Fig. 5 and the figure is omitted for brevity. Once again, the Langmuir–Freundlich dissociation constant (K_d) of the test protein is increased (affinity is decreased) with increasing competitor concentration as expected (Table 3). The presence of the strong binder on the weaker E44Q has a more pronounced effect on the heterogeneity than the system above. The heterogeneity parameter of 0.95 for the 30 μg/ml of strong competitor implies that the weak binder is seeing an effectively homogeneous surface. In fact, both the 15 and 30 μg/ml data fit very well to the classical Langmuir isotherm, indicating an apparently homogeneous surface in the presence of small amounts of the strong binder E11Q. What this implies is the ability of the strong binding E11Q to reduce the surface heterogene-

Table 2
Langmuir–Freundlich and Temkin parameters for E11Q/E44Q system at 100 mM NaCl

Competitor (E44Q) (μg/ml)	Langmuir–Freundlich parameters		Temkin parameters	
	K_d (mg/ml)	n_H	K_T (mg/ml)$^{-1}$	q_T (mg/ml)
0	0.08 ± 0.002	0.70 ± 0.04	257 ± 66	0.31 ± 0.03
30	0.13 ± 0.03	0.71 ± 0.04	121 ± 9	0.35 ± 0.01
60	0.20 ± 0.03	0.81 ± 0.03	74 ± 19	0.38 ± 0.05
160	0.34 ± 0.03	0.80 ± 0.03	57 ± 8	0.35 ± 0.03
300	0.76 ± 0.23	0.68 ± 0.11	36 ± 28	0.28 ± 0.14

The maximum saturation parameter (for L–F) is fixed at 2 mg/ml. Conditions were 10 mM Tris, 100 mM NaCl, pH 8.0.

Table 3
Langmuir–Freundlich and Temkin parameters for E44Q/E11Q system at 100 mM NaCl

Competitor (E11Q) (μg/ml)	Langmuir–Freundlich parameters		Temkin parameters	
	K_d (mg/ml)	n_H	K_T (mg/ml)$^{-1}$	q_T (mg/ml)
0	0.32 ± 0.06	0.81 ± 0.06	31 ± 5	0.38 ± 0.03
5	0.39 ± 0.02	0.80 ± 0.01	31 ± 3	0.35 ± 0.02
15	0.48 ± 0.07	0.85 ± 0.04	23 ± 2	0.33 ± 0.02
30	0.79 ± 0.06	0.95 ± 0.03	7 ± 0.3	0.43 ± 0.02

The maximum saturation parameter (for L–F) is fixed at 2 mg/ml. Conditions were 10 mM Tris, 100 mM NaCl, pH 8.0.

Table 4
Langmuir–Freundlich parameters for the E44Q/E11Q system at 50 mM NaCl

Competitor (E11Q) (μg/ml)	Langmuir–Freundlich parameters		Temkin parameters	
	K_d (mg/ml)	n_H	K_T (mg/ml)$^{-1}$	q_T (mg/ml)
0	0.014 ± 0.003	0.42 ± 0.02	11500 ± 2030	0.19 ± 0.01
20	0.16 ± 0.01	0.79 ± 0.01	52 ± 3	0.71 ± 0.02
70	0.15 ± 0.01	0.91 ± 0.02	31 ± 3	0.84 ± 0.04
200	0.23 ± 0.03	1.02 ± 0.06	17 ± 2	0.95 ± 0.06

q_{max} was held at 3 mg/ml.

ity that the weak binding E44Q "sees" on the surface of the adsorbent.

In terms of the Temkin model, the data shows a very small effect on the q_T parameter over the range of competitor concentrations studied while affinity is reduced approximately four-fold over the course of the competitor range (Table 3).

Interestingly, the effect of the competitor on the heterogeneity parameter is much more pronounced at lower ionic strength. The effect of the stronger binder (E11Q, held constant as competitor) on the binding of the weaker E44Q was determined at a reduced ionic strength (50 mM versus 100 mM NaCl). It is apparent that at reduced ionic strength, the affinity and heterogeneity parameters (Table 4) are very sensitive to small amounts of the strong competitor (E11Q), but less sensitive at higher concentrations. The affinity values are constant within experimental error between 20 and 70 μg/ml competitor, suggesting that a change in apparent heterogeneity (whether due to energetic heterogeneity or lateral interactions) is the main factor affecting the binding at these conditions.

An analysis using the Temkin model leads to similar conclusions. There is a strong sensitivity of both the Temkin affinity constant and Temkin parameter q_T to the initial small amount of the strong competitor (E11Q). The inverse covariation of the K_T and q_T parameters may indicate that the effect of the strong competitor on adsorption of the weak binder is to reduce the energetic heterogeneity of the adsorbent surface. An increase only in unfavorable interactions would have the effect of decreasing q_T, while keeping K_T constant [34]. These results suggest that at lower ionic strengths, apparent heterogeneity is more affected by competitors than at higher ionic strengths, and is not solely due to lateral interactions of bound molecules.

3.4. Adsorbent heterogeneity effects versus lateral interaction effects

In the above, there is some uncertainty whether the changes in the Langmuir–Freundlich heterogeneity parameter are due to changes in adsorbent heterogeneity or to unfavorable lateral interactions among adsorbed species as surface coverage increases. In the two systems E11Q/E44Q (300 μg/ml E44Q competitor) and E44Q/E11Q (30 μg/ml E11Q competitor), the amount of competitor bound to the adsorbent in both cases varies from about 0.6 mg/ml adsorbent to 0.2 mg/ml adsorbent (see Figs. 6 and 7). If one looks at the Langmuir–Freundlich heterogeneity parameters, however, the system with the strong E11Q as the competitor shows almost complete homogeneity (n_H = 0.95) while the system with the weak E44Q as the competitor shows more hetero-

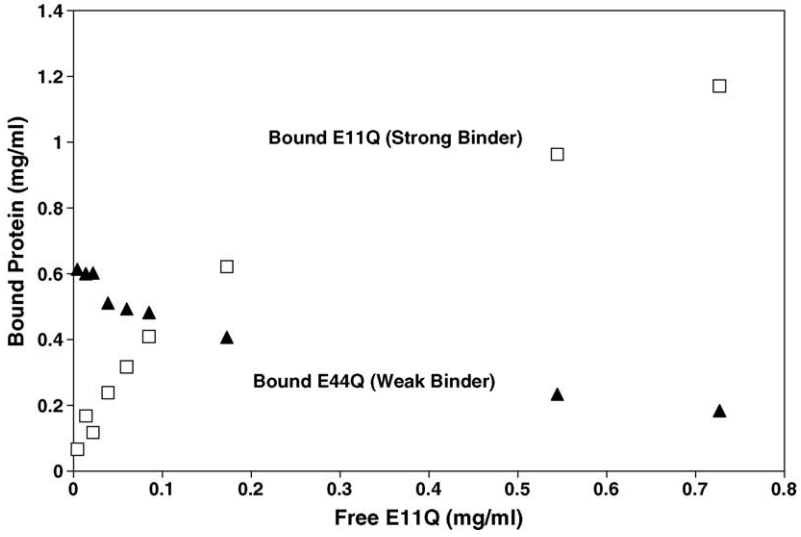

Fig. 6. Competitive Isotherm E11Q/E44Q (300 μg/ml). These data are a binary isotherm in which the weak binder (E44Q) is present at 300 mg/ml in the supernatant. Strong binder (□, E11Q) is varied in the supernatant, weak binder (E44Q) held constant at 300 ± 10 μg/ml. Amounts of bound (mg/ml adsorbent) E11Q and bound E44Q (▲, competitor) are shown as a function of E11Q in the supernatant.

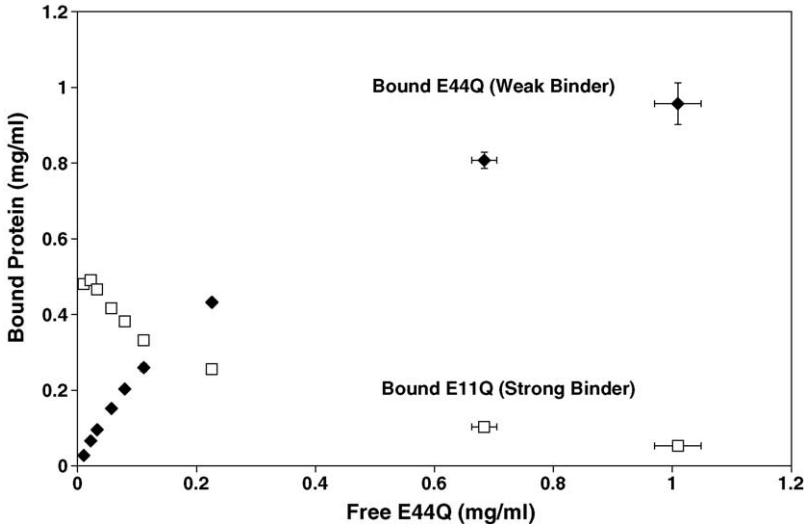

Fig. 7. Competitive isotherm E44Q/E11Q (30 μg/ml) at 100 mM NaCl. This data is the same as the 30 μg/ml curve in Fig. 5; however the bound competitor is also shown. Weak binder (E44Q) is varied in the supernatant, strong binder (E11Q) held constant in supernatant at 30 ± 3 μg/ml. Amounts of bound (mg/ml adsorbent) E44Q (♦) and E11Q (□, the competitor) are shown as a function of E44Q in the supernatant.

geneity (n_H = 0.68). If the Langmuir–Freundlich heterogeneity parameter was solely influenced by lateral interactions, then one would expect these values to be closer when the same amount of competing adsorbate (of the same size, shape and total charge) is bound to the surface. Also, the value of the heterogeneity parameter for the E11Q/E44Q (300 μg/ml E44Q competitor) system is essentially the same as for the pure component E11Q isotherm. What this suggests is that the weak binder is unable to influence the apparent heterogeneity the E11Q "sees" on the surface, while the strong binder is more able to influence what the weaker E44Q "sees". While this evidence is highly suggestive, in many cases it will remain difficult to separate true energetic heterogeneity effects from lateral surface interactions of the adsorbed molecules. This may not be a barrier to practical applications, however, if the overall effects of both heterogeneity and lateral interactions can be "lumped" with an apparent heterogeneity parameter such as the Langmuir–Freundlich heterogeneity parameter.

3.5. Summary

It is apparent that the presence of a competitor protein reduces the affinity of the test protein, which is expected. In some cases however, an unexpected initial extreme sensitivity to small amounts of competitor was observed. For example, in the cytochrome b_5/α-lactalbumin system, the affinity of the cytochrome b_5 is most affected by small amounts of α-lactalbumin. The effect of competitor on the apparent heterogeneity is not as straightforward. For the α-lactalbumin/cytochrome b_5 system, the apparent heterogeneity is only affected at higher loadings of α-lactalbumin competitor, while affinity is most affected at lower loadings. This behavior was not seen with the cytochrome b_5 mutant systems. The heterogeneity of these systems was only sensitive to competitor at lower ionic strength (and greater Debye length of electrostatic interactions), while at higher ionic strength heterogeneity was only affected by competitors at the higher loadings of the strong competitor (E11Q).

4. Conclusions and extensions

For the first time, a set of truly competitive protein adsorption isotherms is reported. Also, the effects of competing protein species on the adsorption of other proteins were quantitatively described in terms of heterogeneity and affinity parameters of two popular binding models, the Temkin and the Langmuir–Freundlich models. These competitive isotherms can be a valuable tool in elucidating the intricacies of competitive adsorption, and the quantitative data obtained could aid the development of more realistic models of the binding of proteins on ion-exchange matrices.

Using the experimental protocols presented in this paper, we have been able to directly show how the effects of a competing protein can quantitatively affect equilibrium parameters such as affinity and apparent heterogeneity in a true-equilibrium binary system. In the α-lactalbumin/cytochrome b_5 system, we observed an unexpected sensitive initial effect of the presence of competing α-lactalbumin on the affinity of the cytochrome b_5 to the adsorbent while at higher concentrations the affinity was not greatly affected. In a real chromatographic application, such an empirical knowledge of how a protein's affinity is affected by competitors would be useful in predicting the chromatographic behavior in both low concentration competitor regimes and high concentration competitor regimes, such as during the removal of a trace contaminant from a product stream or during high-load displacement chromatography.

We have also shown that the presence of a competitor can alter the apparent heterogeneity of an adsorbent. For the case of the E11Q and E44Q cytochrome b_5 systems, the weaker binding E44Q was unable to influence the observed heterogeneity that the stronger binding E11Q was "seeing" on the surface of the adsorbent, whereas the stronger binding E11Q was able to reduce the amount of apparent heterogeneity of the surface experienced by the weaker binding E44Q. The data also show that the observed heterogeneity effects are likely due to true energetic heterogeneity, as opposed to lateral interactions at higher protein loadings. Such behavior in terms of an affinity distribution interpretation has interesting implications for real chromatographic systems. The preference of one protein for a certain subset of sites on an adsorbent (indicated by a reduction in apparent heterogeneity by one protein but not by the other) immediately suggests the deliberate construction of types of adsorbents that contain sites which would be preferred by a certain protein. This could conceivably allow for the manipulation of the adsorbent surface to tailor specific selectivities for a particular protein in a mixture. We are beginning to design deliberately clustered adsorbents to this end. The use of the competitive isotherms described herein will be a valuable tool to study how the design of these different adsorbents affects selectivities among different proteins.

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