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Journal of Chromatography A, 828 (1998) 357–364

JOURNAL OF CHROMATOGRAPHY A

Protein–protein interactions on weak-cation-exchange sorbent surfaces during chromatographic separations

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Abstract

This paper examines the nature of chromatographic separations on a weak cation-exchange material in which immobilized protein coats 24% or less of the sorbent surface. It was found that columns on which proteins were immobilized still behaved as a cation-exchange chromatography sorbents, but their selectivity was different from the parent weak cation-exchange column. This was interpreted to mean that in addition to the normal electrostatic interactions expected in ion- exchange chromatography, protein analytes interact with immobilized protein on the sorbent surface. Anionic proteins were not adsorbed, indicating that immobilized proteins were acting synergistically with ionic stationary phase groups to enhance retention. It is concluded that these protein–protein interactions occur after proteins are captured by the primary interaction mechanism of the column, in this case, electrostatic interaction. Protein–protein interaction is a secondary, lateral process. These lateral interactions were observed between 4% and 24% surface saturation. The significance of this observation is that in preparative chromatography and the case of ''fouled'' columns, strongly adsorbed proteins could alter the elution characteristics of sample proteins being target for analysis or purification.

Keywords: Ion exchangers; Protein–protein interactions; Stationary phases, LC; Proteins

1. Introduction

A unique property of proteins is that a small group of amino acids at their surface, sometimes even a single residue, can dominate chromatographic behavior in one separation mode but have little impact on another [1–4]. This is because proteins are three-dimensional structures with great diversity in the distribution of surface functional groups. This surface diversity makes a critical contribution to their behavior as catalysts, receptors and molecular transporters in addition to their chromatographic properties. Based on four decades of accumulated literature, a number of conclusions and predictions relating to the chromatographic behavior of proteins have been made [5]. One is that it is sterically impossible for all of the amino acid residues in a protein to simultaneously contact the surface of a chromatographic sorbent. This is a critical issue as will be seen below. Second, small groups of residues at or near the exterior surface are able to dominate chromatographic behavior because they are the most likely to be in the protein–sorbent interface. This is why heterogeneity in the amino acid distribution at, or near protein surfaces is so important in achieving selectivity in liquid chromatographic separations[1]. Fourth, the region of the external surface of a protein

[1]The ''foot print'' hypothesis of chromatographic adsorption [1] is in fact, based on the conclusion that a small group of amino acids with a unique spatial distribution at the surface of a protein can play a major role in determining chromatographic behavior.

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0021-9673/98/$ – see front matter
PII: S0021-9673(98)00641-4

that determines chromatographic behavior may vary between chromatographic modes; i.e., different parts of the surface may be involved in ion-exchange, hydrophobic interaction, and the various forms of chromatography. Fifth, structural changes in a protein that alter amino acid composition in the solute–sorbent contact region will alter chromatographic behavior. And finally, it has been found that chromatographic matrices can alter the structure of an adsorbed protein and change its chromatographic behavior.

Contributions of chromatographic sorbents themselves to the separation process are recognized by the designation of charged column packings ''ion-exchange chromatography'' sorbents [6–17], weakly hydrophobic supports as ''hydrophobic interaction chromatography'' materials [18,19], metal chelating phases as ''immobilized metal affinity'' sorbents, and immunosorbents as ''bioaffinity chromatography'' columns. ''Reversed-phase'', ''normal-phase'' and ''size exclusion'' [6,20,21] are still other cases. Having thus designated the various chromatographic separation modes and used these names widely in writings, few chromatographers actually believe chromatography occurs by a single, exclusive process as these names imply. It is also widely appreciated that even chromatographic sorbents with the same stationary phase can have very different selectivity [22–24]. Differences in ligand density, the coupling group through which the stationary phase is attached to the support, the general degree of matrix hydrophilicity, and other functional groups in the matrix which interact directly with the protein are additional variables that determine selectivity.

Surfaces that do not interact strongly with proteins are also known. The broad statement can be made that proteins do not generally interact strongly with each other non-specifically and therefore do not make good stationary phases. For example, serum albumin and immunoglobulin G are not suitable chromatographic stationary phases for general protein separations. The reason is obvious, if proteins generally interacted strongly with each other, protein solutions would gel. The requisite molecular diffusion and convective transport necessary in higher life forms would be impossible in protein gels. Exceptions are specific receptor–ligand interactions such as antigen and antibody interactions, hormones and their receptors, enzymes and their pseudo-substrates, lectin–oligosaccharide pairs and transport proteins.

Chromatographic behavior in the preparative mode is frequently load dependent and non-linear at high protein loading. This is widely attributed to the lack of available sorbent surface and adsorption of multiple layers of the specific protein being isolated through intermolecular interactions [25–29]. This explanation is perhaps too simple. This would require a molecular recognition process in which a protein discriminates between one of it's own species and other very similar proteins at chromatographic sorbent surfaces. The question to be examined here is whether inter-species interactions are preferred and the possibility that other types of intermolecular interactions occur on chromatographic sorbents.

2. Experimental

2.1. Materials

LiChrospher 1000 Diol Silica (10 μm, 1000 Å) was purchased from E. Merck (Darmstadt, Germany). Cesium (Ce) (IV) sulfate, acrylic acid, 1,3-dicyclohexyl carbodiimide (DCC), ethanolamine (EA), were obtained from Aldrich (Milwaukee, WI, USA). Albumin (HSA) (human serum), trypsinogen (TRYN) (bovine pancreas), α-chymotrypsinogen A (CHYM) (bovine pancreas), cytochrome *c* (BCYT C) (bovine heart), cytochrome *c* (HCYT C) (horse heart), lysozyme (LYS) (chicken egg white), ribonuclease A (RNase A) (bovine pancreas), and *N*-hydroxysuccinimide (NHS) were purchased from Sigma (St. Louis, MO, USA). Sodium chloride, sodium phosphate monobasic, sodium phosphate dibasic heptahydrate and tetrahydrofuran (THF) were procured from Mallinckcrodt (Phillipsburg, NJ, USA).

2.2. Instrumentation

Sorbents were slurry packed into 50×4.6 mm I.D. stainless steel columns using a high-pressure packing pump (Shandon Southern Instrument, Sewickley, PA, USA). Chromatographic evaluations were performed on BioCAD liquid chromatograph (PerSeptive Bio-

systems, Framingham, MA, USA). Absorbance was monitored at 280 nm.

2.3. Synthesis of polyacrylate stationary phase

A method developed by Mino and Kaizerman [30] and modified by Ratnayake and Regnier [31] was used to synthesize the polyacrylate bonded phase. One gram of diol silica was transferred into a 100-ml three-necked round bottom flask, and 50 ml of deionized water was added. Following shaking, 3 ml of acrylic acid was added and the suspension purged with nitrogen while shaking. Polymerization was initiated by the addition of Ce(IV) sulfate (0.5 g) at 50–55°C for 7 h under nitrogen. The suspension was then cooled to room temperature and filtered in a sintered-glass funnel, washed with 0.5 *M* H_2SO_4 until it was white and then washed with water to neutrality. Finally the sorbent was rinsed with THF and dried under vacuum.

2.4. Protein immobilization

Polyacrylate grafted silica (0.1 g) was transferred into a 20 ml polypropylene tube, followed by the addition of 3 ml of 30 m*M* NHS. The mixture was shaken for 30 min after which 3 ml of 30 m*M* DCC in dioxane was added and shaking was continued for 2 h. The sorbent was isolated by centrifugation and the supernatant discarded. The silica was then washed with dioxane and water to remove excess DCC and NHS.

Two ml of either a 3.125 mg/ml solution of HSA or 0.625 mg/ml of another protein such as CHYM, BCYT C, LYS or RNase A, respectively was added in 0.1 *M* $NaHCO_3$ (pH 7.5) to the activated polyacrylate sorbent and the suspension continuously agitated for 24 h at room temperature. The sorbent was then isolated by centrifugation, 1.25 ml of 20% ethanolamine in 0.1 *M* $NaHCO_3$ (pH 7.5) was added to the particles to block unreacted activated site and the suspension agitated for 1 h. The particles were again isolated by centrifugation and sequentially washed with the following solutions; 3 ml of 0.01 *M* phosphate (pH 6.0), 3 ml of 1 *M* NaCl in 0.01 *M* phosphate (pH 6.0), 3 ml of 10% ethylene glycol, and 3×3 ml water washes. The sorbent was reisolated between washing steps by centrifugation.

2.5. Chromatographic evaluations

Protein-immobilized silica was packed into 50× 4.6 mm I.D. stainless steel columns. The ion-exchange columns were eluted with a linear 15 min gradient ranging from 0.01 *M* phosphate (pH 6.0) to 1.0 *M* NaCl in 0.01 *M* phosphate (pH 6.0) at a flow-rate of 1 ml/min.

Protein samples were prepared in 0.01 *M* phosphate (pH 6.0). Columns were evaluated with a mixture of TRYN, CHYM, BCYT C, HCYT C, LYS (4 mg/ml each) and RNase A (10 mg/ml). *Z* numbers and *I* values were determined by retention times of proteins under isocratic elution conditions at different concentrations of NaCl in 0.01 *M* phosphate (pH 6.0).

3. Results and discussion

3.1. The model system

The surface environment under which non-linearity occurs in preparative chromatography has been created dynamically through heavy sample loading in past studies [28]. Unfortunately, surfaces formed dynamically are very hard to define and study. The rationale used in these studies was that immobilized proteins could be used to partially saturate a sorbent surface and provide a mimic of a heavily loaded column. This strategy has several advantages. One is that the composition and concentration of protein on a sorbent surface can be permanently defined. Another is that protein composition on the sorbent surface is independent of sample load. Still another is that specific interactions between proteins can be examined. For example, it is possible to address the question of whether a protein is more likely to interact with its own species than another. The limitation of this approach is that translation is severely restricted in covalently bound as opposed to adsorbed proteins. For this reason, immobilized proteins probably do not exactly mimic adsorbed proteins in chromatographic systems.

3.2. Sorbent synthesis

The weak cation-exchange sorbent, PAA-6, was

prepared by a Ce(IV) initiated polymerization of acrylic acid on the surface of a diol-bonded phase support [31,32]. This polyacrylate bonded phase is known to be fimbriated (tentacular) and of high loading capacity [33]. The PAA-6 sorbent was used to immobilize all the proteins used in this study.

Recognizing that carboxyl groups in weak cation-exchange chromatography sorbents are frequently used in protein immobilization, mimics of a heavily loaded preparative column were created by covalently bonding proteins to NHS-activated polyacrylic acid cation-exchange materials through amide bond formation. Unreacted NHS groups were blocked by ethylene amine. The degree of surface loading was controlled both by reaction time and protein concentration. Proteins immobilized on the PAA-6 sorbent and their designation are listed in Table 1.

3.3. Evaluation of protein–surface interactions

The interaction of proteins with sorbents was evaluated in two ways. One was to determine the chromatographic behavior of a series of proteins in the ion-exchange mode and evaluate changes in selectivity on immobilized protein columns relative to the underivatized PAA-6 weak cation-exchange sorbent. The other was to examine the ion-exchange equilibrium involved in adsorption.

Clearly, the immobilized protein columns still behave as weak cation-exchange columns, albeit with significant differences in retention and selectivity (Fig. 1). One notable difference is that the peaks are broader with the immobilized protein columns. It is not clear whether this arises from restricted diffusion in the sorbent pores, diminished desorption kinetics at the surface of the ion exchanger, or from some other phenomenon. A second is the changes in retention time (Table 2) and peak reversal seen with lysozyme and ribonuclease A on all the immobilized protein columns (Fig. 1 and Table 2). Retention generally increased or remained the same in all cases except with ribonuclease A and cytochrome *c*. Decreases in ribonuclease A retention were seen with all the immobilized proteins except human serum albumin. Although smaller, significant decreases in cytochrome *c* retention were also seen in all cases except with human serum albumin. A third difference relative to the parent PAA-6 sorbent were the substantial changes in selectivity seen with the immobilized proteins.

Efforts to understand the nature of these changes were directed at an examination of the ion-exchange equilibrium. Ion-exchange adsorption may be described by the equilibrium

$$K_f = [P_b][D_0]^Z / [P_0][D_b]^Z \quad (1)$$

where K_f represents a formation constant; $[P_0]$ is the protein concentration in the mobile phase; $[P_b]$ is the protein concentration adsorbed onto the stationary phase; $[D_0]$ is the concentration of desorbing agent in the mobile phase; $[D_b]$ is the concentration of desorbing agent at the surface of the stationary phase; and Z is the number of molecules of salt displaced from the sorbent by a molecule of protein when it is adsorbed. This equilibrium can be related to the chromatographic capacity factor (k') by the equation

$$\log k' = Z \log 1/[D_0] + \log I \quad (2)$$

where $I = K_f \Phi [D_b]^Z$, a group of constants [6,7]. The term Φ is chromatographic phase ratio. Z number and the I value for a protein are obtained by plotting $\log k'$ vs. $\log 1/[D_0]$. The number of charged groups

Table 1
Description of columns used in the study

Column abbreviation	Description
PAA-6	Cation-exchange sorbent made by polymerizing 6% (v/v) acrylic acid on diol silica (10 μm, 1000 Å)
PAA-HSA	6.25%[a] g/g HSA immobilized on PAA-6 sorbent
PAA-CHYM	1.25%[a] g/g CHYM immobilized on PAA-6 sorbent
PAA-BCYT C	1.25%[a] g/g BCYT C immobilized on PAA-6 sorbent
PAA-LYS	1.25%[a] g/g LYS immobilized on PAA-6 sorbent
PAA-RNase A	1.25%[a] g/g RNase A immobilized on PAA-6 sorbent

[a] Based on the amount of proteins added in sorbent.

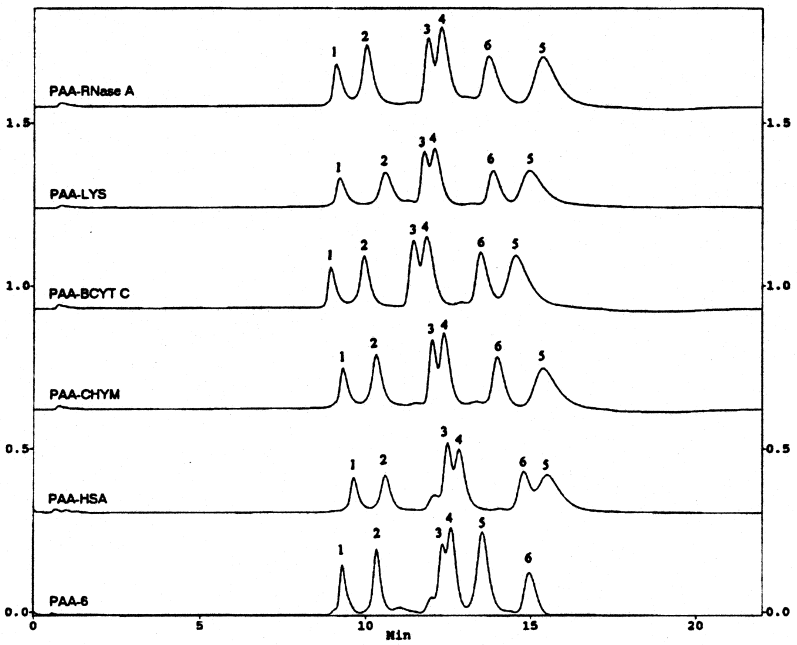

Fig. 1. Retention of (1) trypsinogen, (2) α-chymotrypsinogen A, (3) bovine cytochrome *c*, (4) horse cytochrome *c*, (5) lysozyme, (6) ribonulease A by columns of PAA-6, PAA-HSA, PAA-CHYM, PAA-BCYT C, PAA-LYS and PAA-RNase A. Columns: 50×4.6 mm I.D.; mobile phase: (A) 10 m*M* phosphate buffer, pH 6.0; (B) 1.0 *M* NaCl in A; flow-rate: 1.0 ml/min; gradient: 0–100% B in 15 min; detection: UV at 280 nm.

in a protein involved in its adsorption are thought to be represented by *Z*. Changes in *Z* number would suggest that the number of groups involved in electrostatic interactions at the sorbent surface had changed. On the other hand, changes in the *I* value would most likely be associated with changes in the equilibrium constant, i.e., the strength of the interaction with the surface. Although more sophisticated models of ion-exchange retention exist, they are more difficult to apply experimentally [10–17] and were not used in this study. It should be understood that neither the model given above, nor any other known model has been tested to the extent that they have been shown to totally reflect reality with native proteins. There is still a substantial element of speculation in all analyses of protein retention data by theoretical models. The data presented below is no exception.

It is seen in Table 3 that the substantial changes in relative retention and selectivity are the result of a combination of changes in *Z* numbers and *I* values with the immobilized protein columns. *Z* numbers

Table 2
Retention time of proteins on polyacrylate and immobilized protein polyacrylate columns

Column	TRYN	CHYM	BCYT C	LYS	RNase A
PAA-6	9.27	10.32	12.30	13.53	14.95
PAA-HSA	9.64 (+0.37)[a]	10.57 (+0.25)	12.48 (+0.18)	15.51 (+1.98)	14.77 (−0.18)
PAA-CHYM	9.30 (+0.03)	10.32 (+0.00)	11.99 (−0.31)	15.36 (+1.83)	13.97 (−0.98)
PAA-BCYT C	8.93 (−0.34)	9.95 (−0.37)	11.43 (−0.87)	14.52 (+0.99)	13.47 (−1.48)
PAA-LYS	9.21 (−0.06)	10.57 (+0.25)	11.74 (−0.56)	14.95 (+1.42)	13.84 (−1.11)
PAA-RNase A	9.08 (−0.19)	10.01 (−0.31)	11.86 (−0.44)	15.36 (+1.83)	13.72 (−1.23)

[a] The degree of change in a value relative to PAA-6.

Table 3
Retention characteristics and affinity properties of proteins on polyacrylate and immobilized protein polyacrylate sorbents

Column	TRYN	CHYM	BCYT C	LYS	RNase A
	Z number				
PAA-6	2.45	3.34	4.20	4.53	5.32
PAA-HSA	3.15 (+0.70)[a]	3.73 (+0.39)	4.26 (+0.06)	5.03 (+0.50)	5.18 (−0.14)
PAA-CHYM	2.16 (−0.29)	3.15 (−0.19)	4.13 (−0.07)	5.42 (+0.89)	5.36 (+0.04)
PAA-BCYT C	2.36 (−0.09)	3.37 (+0.03)	3.96 (−0.24)	5.35 (+0.82)	5.26 (−0.06)
PAA-LYS	3.65 (+1.20)	3.60 (+0.26)	4.15 (−0.05)	5.13 (+0.60)	5.32 (0.00)
PAA-RNase A	2.18 (−0.27)	3.12 (−0.22)	4.22 (+0.02)	5.55 (+1.02)	5.32 (0.00)
	I ($\cdot 10^{-2}$) value				
PAA-6	1.05	1.07	1.95	1.78	3.55
PAA-HSA	2.00 (+0.95)	0.36 (−0.71)	1.29 (−0.66)	2.69 (+0.91)	2.09 (−1.46)
PAA-CHYM	0.55 (−0.50)	1.00 (−0.07)	1.41 (−0.54)	2.51 (+0.73)	1.48 (−2.07)
PAA-BCYT C	0.76 (−0.29)	0.62 (+0.45)	1.38 (−0.57)	1.62 (−0.16)	1.35 (−2.20)
PAA-LYS	0.08 (−0.97)	0.69 (−0.38)	1.20 (−0.75)	2.95 (+1.17)	1.41 (−2.14)
PAA-RNase A	1.02 (−0.03)	0.83 (−0.24)	1.05 (−0.90)	1.66 (−0.12)	1.10 (−2.45)

[a] The degree of change in a value relative to that observed with PAA-6.

and *I* values generally increased or remained unchanged relative to PAA-6 in all cases except the *I* values for cytochrome *c*. In contrast, the *Z* numbers and *I* values for α-chymotrypsinogen A stay the same on the immobilized protein sorbent surfaces compared to the control, i.e., PAA-6. Some variations are due to experimental errors. *Z* number of ribonuclease A also stays same on protein-immobilized sorbent surfaces compared to the control, i.e., PAA-6, but *I* value decreases on protein-immobilized sorbent surfaces. Both *Z* number and *I* value of lysozyme, cytochrome *c* and trypsinogen are varied on protein-immobilized sorbents. Interestingly, the changes of *Z* numbers and *I* values are different with different analyte proteins. For lysozyme, *Z* number increases on all protein-immobilized sorbent surfaces, and *I* value increases on HSA-, CHYM- and LYS-immobilized sorbent surfaces. For bovine cytochrome *c*, both *Z* number and *I* value decreases on BCYT C-immobilized sorbent surface. For trypsinogen, *Z* number and *I* value increase on HSA-immobilized sorbent surface, *Z* number increases but *I* value decreases on LYS-immobilized sorbent surface.

Based on the results in Table 3 the following explanations and rationalizations of the data are possible. The increase in retention time of trypsinogen on the human serum albumin column (PAA-HSA) would appear to be the result of an increase in the number of charges involved (+0.70 in *Z*) and the strength of the interaction with the sorbent (+0.95 in *I*). Increasing numbers of interactions can either be due to an additional electrostatic interaction with HSA or a change in the orientation of trypsinogen relative to the sorbent surface. Although there was little change in the retention time of trypsinogen on the PAA-LYS column, there were large, compensating changes in the retention mechanism. The number of charges increased (+1.20 in *Z*) while the strength of the interaction decreased (−0.97 in *I*). This could occur in any of several ways. One would be that the orientation of adsorption was changed sufficiently to diminish hydrophobic binding while simultaneously enabling greater electrostatic binding. This could happen by reorienting the molecule in such a way that a hydrophobic site on trypsinogen is no longer in the protein–sorbent interface. Another would be that the distance of charged groups from the sorbent increases, perhaps lysozyme alters the orientation of trypsinogen

to the sorbent in such a way that more charges are involved in adsorption but they are further from the surface.

Changes in the retention time, *Z* number, and *I* values of α-chymotrypsinogen A (CHYM) and bovine cytochrome *c* (BCYT C) with the immobilized protein sorbents were relatively modest in all cases. In the case of CHYM the changes do not follow a trend and are too small to merit discussion. BCYT C is different. Although small, most of the changes in retention time, *Z* number, and *I* value are negative. This would appear to be a clear case of the immobilized proteins altering the orientation of a protein analyte on the sorbent surface. The data do not suggest how this occurs. In view of the fact that there are large areas of unencumbered surface in these sorbents and the amount of analyte is small, there are no spatial restrictions on adsorption. If it is true that there is an alteration of orientation with the immobilized protein sorbents, it must be because of lateral interactions of the analyte with immobilized proteins.

The retention time of lysozyme with the immobilized protein sorbents increases in all the cases examined. Clearly this is due to an increased electrostatic interaction; increases in *Z* number ranged from +0.50 to +1.02. *I* values also increased from +0.73 (PAA-CHYM) to +0.91 (PAA-HSA) and +1.17 (PAA-LYS) except with PAA-BYCT C (−0.16) and PAA-RNase A (− 0.12). It is not apparent whether the increased interaction is with the weak cation-exchange stationary phase or immobilized protein.

The behavior of ribonuclease A on immobilized protein columns was the most interesting of the proteins studied. In all cases, its retention time was decreased by large negative values of *I* ranging from −1.46 to −2.45. In contrast, *Z* values were unchanged. These results are interpreted to mean that the decreases in retention were the result of increases in electrostatic repulsion. This would seem logical in that the immobilized ribonuclease A, lysozyme, bovine cytochrome *c* and α-chymotrypsinogen A are all positively charged. It is to be expected that ribonuclease A would be electrostatically repelled from these immobilized protein sorbents. The puzzling thing is that lysozyme has an even greater net positive charge than ribonuclease A but it was not electrostatically repelled. Protein structure must also play a role in the lateral interaction of proteins at surfaces.

4. Conclusions

The data presented in this paper allow several conclusions to be made about protein–protein interactions at surfaces in preparative chromatography. One is that there is strong evidence for lateral interactions between proteins on surfaces and that these proteins do not have to be of the same species. This is important because it means that the chromatographic behavior of a protein will be influenced increasingly by other components in the mixture as sample loading increases. Proteins that will have the greatest impact will be those that are either strongly retained or irreversibly adsorbed.

A second conclusion is that preferential interactions of protein analytes with their own species at surfaces plays a small role in chromatographic behavior in cation-exchange chromatography. Interactions of a protein analytes were generally as strong or stronger with other proteins. This is very important because it shows that formation of homodimers or multiple layers of a protein species at a surface is not inherently preferred over lateral association with other proteins. It also brings into question the possibility that proteins ever form multiple layers at surfaces except at extremely high concentrations.

A third conclusion is that in cation-exchange chromatography proteins must first be adsorbed electrostatically before lateral interactions occur. Although not examined in this study, this conclusion probably applies to other retention mechanisms as well. In all cases, only those proteins captured by the underivatized weak cation-exchange column were also captured by the immobilized protein columns. This is extremely significant in that it indicates that protein– protein association alone at sorbent surfaces must play a minor, if any role in preparative chromatography. The only time protein–protein association might play a role would be at extremely high concentration, i.e., in solutions of 10–100 mg/ml of protein. The role of the sorbent in protein–protein

association is apparently to cause protein concentration at the surface to reach the high levels necessary for intermolecular association. The only way electrostatically adsorbed proteins can interact is through lateral diffusion. The nature of protein–protein association at surfaces will likely differ from that in solution. There is a high probability that regiospecific adsorption of proteins to the sorbent surface and restrictions in translational motion will not allow the full range of interactions possible in solution. Only when the sorbent surface is completely saturated and protein concentration in solution approaches that at the sorbent surface will protein–protein association without primary capture become a mechanism of adsorption. Clearly the probability of lateral protein–protein interaction at surfaces is much higher than in solution.

A fourth conclusion is that lateral interactions between proteins at surfaces can alter selectivity. This is most likely due to a combination of phenomena ranging from differential intermolecular association between proteins on the surface to alterations in orientation of binding.

It is apparent from the forgoing discussion that the chromatographic behavior of a protein on a heavily loaded preparative chromatography columns will depend both on specific interactions with the stationary phase and with other proteins in the mixture being separated.

Acknowledgements

The authors gratefully acknowledge financial support from National Institute of Health grant number 25431.

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