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High-performance liquid chromatography of amino acids, peptides and proteins

CXX☆. Evaluation of bandwidth behaviour of proteins chromatographed on tentacle-type anion exchangers

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ABSTRACT

The dynamic behaviour of several proteins chromatographed on the Fractogel-TMAE and LiChrospher-TMAE tentacle-type anion exchangers was investigated through the analysis of experimental bandwidth data. Results were analysed by comparison of experimental data with bandwidths calculated on the basis of the protein assuming a rigid globular structure with a uniform interactive surface using the general plate height theory. The influence of the displacer salts NaCl and KBr and different mobile phase pH conditions (pH 5.5, 6.5, 7.5 and 9.6) on solute bandwidths were investigated. Very similar bandwidth dependencies were observed for all proteins separated on the Fractogel-TMAE sorbent with both displacer salt systems used. However, there was a marked difference in the bandwidth behaviour on the LiChrospher-TMAE sorbent with the two displacer salts. Furthermore, changes in the mobile phase pH resulted in significant differences in the dependence of the bandwidth ratios on both the gradient time and the type of displacer salt.

INTRODUCTION

High-performance ion-exchange liquid chromatography (HPIEC) is now used extensively for the high-resolution analysis and purification of peptides, proteins and polynucleotides [1]. Recently, a new class of ion-exchange sorbents for biochromatography has been introduced, namely the tentacle-type ion exchangers [2,3]. These chromatographic sorbents are characterised by coulombic ligands composed of linear polyelectrolyte chains of average length between 20–50 monomer units graft-polymerised to the hydroxyl groups of a hydrophilic support material. In a previous study [4], we investigated the comparative retention behaviour of proteins separated by strong anion-exchange liquid chromatography on the tentacle sorbents. While some proteins exhibited retention properties which were comparable to that observed with conventional high-performance ion exchangers, other proteins showed significantly different retention properties. In the present study the evaluation of these tentacle-type ion exchangers is continued through analysis of the bandwidth behaviour of several proteins separated by strong anion-exchange chromatography.

EXPERIMENTAL

Chemicals and reagents

Bovine erythrocyte carbonic anhydrase, whale sperm myoglobin (type III), hen egg white lysozyme (grade 1), bovine ribonuclease A (type IIIA), bovine insulin, piperazine, bis-tris, triethanolamine were all purchased from Sigma (St. Louis, MO, USA). The recombinant porcine growth hormone was available in highly purified form from associated studies in this laboratory. Sodium chloride, potassium bromide, hydrochloric acid and hydrobromic acid

☆ For Part CXIX, see ref. 23.

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(AnalaR grade) were obtained from BDH (Port Fairy, Australia).

Quartz distilled water was further purified on a Milli-Q system (Millipore, Bedford, MA, USA). Buffers were adjusted to the respective pH using either hydrochloric acid or hydrobromic acid.

Apparatus

All chromatographic studies were carried out with either a Pharmacia (Uppsala, Sweden) fast protein liquid chromatographic (FPLC) system, as previously described [5,6] or a Beckman System Gold liquid chromatograph. The Fractogel EMD TMAE-650 (Fractogel-TMAE, particle diameter d_p = 25–40 μm, pore size = 650 Å) and the LiChrospher 1000 TMAE (LiChrospher-TMAE, d_p = 5 μm, pore size = 1000 Å) sorbents were obtained as prepacked columns (150 × 10 mm I.D. and 50 × 10 mm I.D., respectively) from E. Merck (Darmstadt, Germany). Samples were injected using glass syringes (Scientific Glass Engineering, Ringwood, Australia), at protein concentrations of 5 mg/ml, typically as 10–100-μl injections. The protein solutions were prepared in mobile phase A and prefiltered through 0.22-μm filters.

Chromatographic procedures

The preparation of eluents and procedures for acquisition of gradient elution data has been described previously [5,6]. Gradient bandwidth parameters ($\sigma_{v,calc}$, $\sigma_{v,exp}/\sigma_{v,calc}$, G, C, D_m and N) were calculated using the Sigma program written in this laboratory in Basic language for IBM AT computers as previously described [6].

RESULTS AND DISCUSSION

Theoretical background

The analysis of the chromatographic performance of proteins separated by interactive modes of chromatography is usually carried out in terms of the evaluation of retention behaviour employing various descriptive, theoretical models. For example, the application of two empirical approaches, namely the stoichiometric displacement model [5,7] and the linear solvent strength (LSS) model [5,8] have considerably increased our understanding of the factors controlling protein retention. While these approaches provide significant information on the physical parameters, *e.g.*, flow-rate, particle size, ligand density, etc., that control the interaction between the stationary phase surface and the protein surface, limited information is derived on the kinetic or mechanistic aspects of the separation. The use of retention parameters derived from the LSS model can be combined with the general plate height theory to provide a method for predicting solute bandwidths so that overall chromatographic resolution can be evaluated and optimized [6,9,10]. A number of studies have adopted this approach for the analysis of retention data derived under reversed-phase [11–15] and ion-exchange conditions [6,10]. It is generally found with small peptides and some proteins that high correlations exist between the experimentally and theoretically derived bandwidths using these procedures [6,10,11]. However significant deviations occur for polypeptides and proteins undergoing secondary equilibria such as conformational rearrangement or aggregation [6,9,15]. In particular, it has been shown in some HPIEC systems that the nature of the displacer salt can exert significant effects on the experimental bandwidths [6]. Previous studies [6,9,10] on protein bandbroadening behaviour in HPIEC have focussed on ion-exchange sorbents derived from mono-layer or cross-linked polymer layer ligand systems. Under conditions of regular retention behaviour, *i.e.*, in the absence of secondary equilibria such as conformational or aggregational effects, excellent correlations between the LSS predictions and experimental peak widths have been documented with these sorbents. The question arises whether sorbents involving the tentacle-type ligand structures also follow similar bandbroadening trends. Recent studies with non-porous sorbents have shown [3] that the adsorption behaviour of tentacle-based HPIEC sorbents is fundamentally different when compared to poly-ethyleneimine (PEI)-based (HPIEC) sorbents. These studies demonstrated that the tentacle-based sorbents are characterised by Hill-type isotherms with pseudo-Gaussian distribution of binding sites, *i.e.*, a type I isothermal surface according to the classification of Gregg and Sing [16].

In the present study the experimental bandwidths of proteins separated on the tentacle-type strong anion-exchangers has been evaluated to assess the utility of the LSS approach with this class of sorbents. According to the LSS concepts [8,10],

under ideal HPIEC conditions of gradient elution, the relationship between peak width, represented by $4\sigma_v$, can be expressed as

$$\sigma_{v,calc} = [(\bar{k}/2 + 1)]GV_mN^{-1/2} \quad (1)$$

where $\bar{k}$ is the median capacity factor of the solute, V_m is the column void volume, N is the plate number and G is the band compression factor which arises from the increase in solvent strength across the solute zone as the gradient developes along the column. The parameter G is given by the expression

$$G^2 = [1 + 2.3b + 1/3(2.3b)^2]/(1 + 2.3b)^2 \quad (2)$$

where b is the gradient steepness parameter. Under normal operating conditions, N can be approximated by

$$N = D_m t_0/Cd_p^2 \quad (3)$$

where d_p is the particle diameter and t_0 is the column deadtime. The diffusion coefficient D_m of the solute in the mobile phase can be expressed in terms of the solute molecular weight, MW, by

$$D_m = 8.34 \cdot 10^{-10}\, T/\eta(MW)^{1/3} \quad (4)$$

where T is the absolute temperature and η is the eluent viscosity. The Knox equation parameter, C, can be estimated from

$$C = \frac{[(1 - x + \bar{k})/(1 + \bar{k})]^2}{15\rho^* a' + 15\rho^* b'\,\bar{k} - 19.2\rho^* x} \quad (5)$$

where x is the interstitial column fraction assumed to be 0.62 for a well-packed column, a' is 1.1 and b' is the surface diffusion parameter which from previous studies is assumed to be equal to 0.72. The restricted diffusion parameter ρ^* was calculated according to the Renkin relationship

$$\rho^* = 1 - 2.104\rho + 2.09\rho^3 - 0.95\rho^5 \quad (6)$$

where ρ is equal to the ratio of the solute diameter to the sorbent pore diameter. The linear logarithmic relationship found between ρ^* and solute molecular weight for the two tentacle-type columns was

$$\log \rho^* = 0.19 - \log MW \quad (7)$$

According to the stoichiometric displacement model, the elution of a protein from a charged surface is accompanied by the adsorption of a stoichiometric amount of displacer counter-ion. As a result, retention properties should be independent of the chemical nature of the displacer co-ion and should only reflect the relative affinity of the counter-ion for the anion-exchange sorbent. In order to utilise these theoretical procedures, the requirement exists that defined values of the particle diameter and pore diameter are available so that the parameters N, C and ρ can be computed according to eqns. 3–6. The use of eqns. 1–7 also assumes that the solute migrates as a unique, conformationally rigid molecule and that the diffusional properties are independent of the nature of the displacing species. However, it is well established that peptides and proteins can undergo conformational and other secondary equilibria in solution and at the surface of chromatographic sorbents [17,18]. If these equilibria do not occur or are extremely rapid compared to the chromatographic separation time, then the ratio between the experimentally observed bandwidth, $\sigma_{v,exp}$, and the bandwidth determined by eqn. 1 should approach unity. However, if these conformational processes occur with similar time scales as the chromatographic retention times, the resulting changes in the diffusional and interactive properties of the solute will lead to a pattern of migration which is more complex than anticipated by conventional plate height theory. In these cases, the ratio, $\sigma_{v,exp}/\sigma_{v,calc}$, will become significantly greater than unity and can be anticipated to show, for example, non-linear dependencies with regard to system residence and gradient steepness.

The influence of NaCl and KBr on protein band-broadening

In the present study, the effect of systematic changes in the displacer salt concentration on the bandwidth behaviour of protein solutes has been investigated under conditions of varied gradient time and constant flow-rate. The proteins listed in Table I were eluted from either the Fractogel-TMAE or the LiChrospher-TMAE strong anion-exchange columns by salt gradients (0–300 mM) varying in time from 20 to 120 min at a constant flow-rate of 1 ml/min. Theoretical bandwidths were calculated according to eqn. 1 and were then compared to the corresponding experimental bandwidths and plotted as a function of the reciprocal of the gradient steepness parameter, b.

Fig. 1 shows plots of the relative $\sigma_{v,exp}/\sigma_{v,calc}$ ratio as a function of $1/b$ for several proteins separated on

TABLE I

PHYSICAL PROPERTIES OF PROTEINS

Protein (source)	Abbreviation	p*I*	MW	D_m (10^{-7} cm^2/s)
Ovalbumin (hen egg white)	OV	4.70	43 000	7.09
Insulin (bovine pancreas)	INS	5.32	5700	13.91
Carbonic anhydrase (bovine erythrocytes)	CA	5.89	30 000	8.00
Growth hormone (porcine recombinant)	GH	6.70	22 100	8.86
Myoglobin (sperm whale muscle)	MYO	7.68	17 500	9.57
Ribonuclease A (bovine pancreas)	RIB	9.60	12 640	10.70
Lysozyme (hen egg white)	LYS	11.00	14 300	10.24

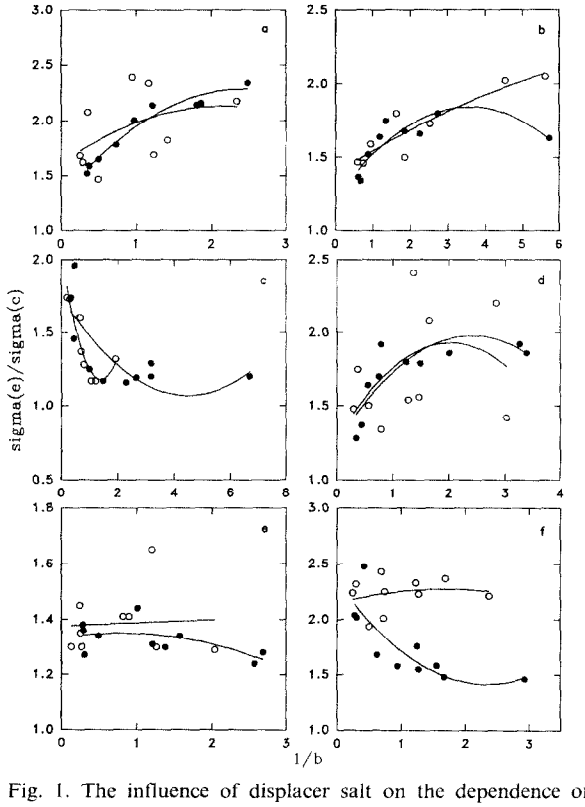

Fig. 1. The influence of displacer salt on the dependence of $\sigma_{v,exp}/\sigma_{v,calc}$ [sigma (e)/sigma (c)] *versus* $1/b$ for several proteins separated on the Fractogel-TMAE sorbent. ○ = NaCl; ● = KBr. Proteins used are (a) OV, (b) INS, (c) CA, (d) GH, (e) MYO and (f) RIB. Data were acquired under conditions of varied gradient time at a flow-rate of 1 ml/min and pH 9.6 as described in the Experimental section and $\sigma_{v,calc}$ was calculated using eqn. 1. All data points were fitted according to a second order regression and fell within 95% confidence limits. See Table I for abbreviations for protein solutes.

the Fractogel-TMAE column with NaCl and KBr as the displacer salt and the mobile phase pH equal to 9.6. It is evident that there is a significant variation in the dependencies of the bandwidth ratio on the residence time with the proteins studied. For OV, INS and GH the bandwidth ratios increase with increasing $1/b$ values for both NaCl and KBr. For CA and MYO the bandwidth ratios approach unity for both displacer salts, while for RIB there is a significant difference in the bandwidth ratio with the two salts. When eluted by steep gradients, protein solutes have reduced column residence times and their elution behaviour is influenced predominantly by mobile phase parameters. The initial decrease in the solute bandwidth ratio at low $1/b$ values seen, for example, with CA with both displacer salts at $1/b < 1$ has been referred to as the "*J*-effect" [8,11]. With $1/b$ values $\ll 1$, this effect results in almost stepwise elution with very pronounced changes in bandwidth dependencies. Moreover, the results of the present study suggest that variations occur in the kinetics of the adsorption process with different proteins on the Fractogel-TMAE tentacle support. In particular, the data indicate that time-dependent exposure of some proteins to the charged TMAE tentacle surfaces and different salt species may strongly influence their interactive and/or diffusional properties, particularly when $1/b$ values are >2.

Fig. 2 shows the corresponding bandwidth plots for the separation of proteins on the LiChrospher-TMAE sorbent with NaCl and KBr as the displacer salts. Comparison of the dependence of the bandwidth ratio on $1/b$ for individual proteins reveals that the influence of the displacer salt on the

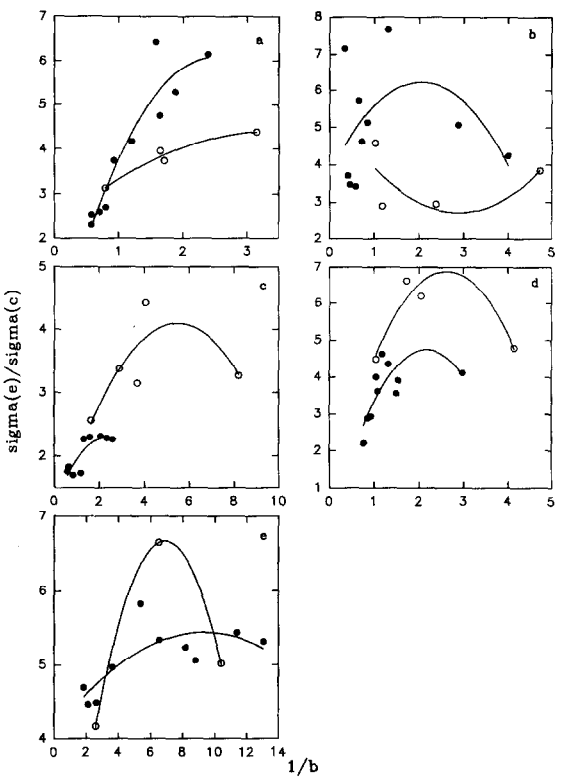

Fig. 2. The influence of displacer salt on the dependence of $\sigma_{v,exp}/\sigma_{v,calc}$ *versus* $1/b$ for several proteins separated on the LiChrospher-TMAE sorbent. ○ = NaCl; ● = KBr. Proteins used are (a) OV, (b) INS, (c) GH, (d) RIB and (e) LYS. See Fig. 1 for other details.

bandwidths is more evident with the LiChrospher-TMAE sorbents than the Fractogel-TMAE sorbent. The bandwidth ratios for OV and INS were larger for KBr than for NaCl. At longer residence times, a number of smaller peaks were resolved from the main OV peak. This phenomenon, which has been observed previously [19], has been attributed to the presence of structural and/or conformational variants of this protein that influence the experimentally observed bandwidth. For GH and RIB larger bandwidth ratios were observed with NaCl compared to KBr. Similar variations in the bandwidth behaviour of proteins separated with different displacer salts on HPIEC sorbents have also been observed, including for example, the conventional Mono-Q strong anion-exchange material [6]. These effects can, in part, be attributed to the kosmotropic or chaotropic properties of various salts which, depending on their concentration, can significantly alter the ion- and ligand-interaction as well as the diffusional properties of proteins in ion-exchange systems. In the case of the tentacle-type sorbents, the specific electrolyte nature of the displacer salt may also influence the dynamic structure of the tentacular surface. While these new phases were designed to specifically interact with a protein in a flexible manner in order to give rise to improved resolution, the interactive process will be extremely sensitive to factors which influence the structure of the ligands as well as the protein solute. Additional evidence for the changes in the dynamic structure and the intercalation of the tentacle polyelectrolyte chains has been provided by the studies on the temperature dependence of protein adsorption with these sorbents [3].

The influence of pH on retention and bandwidth behaviour

Protein retention in HPIEC arises from electrostatic interactions between the protein surface and charged groups immobilized on a stationary phase. According to the net-charge concept, a protein will be retained on an anion-exchange resin when the pH of the eluent is above the p*I* of the protein. However, there are numerous examples [7,9,19–21] where proteins are retained under eluent pH conditions where according to their p*I*, they should be repelled from the stationary phase surface. Nevertheless, the retention of proteins in HPIEC is intimately affected by the pH of the mobile phase. The influence of pH on both the retention and bandwidth properties of the proteins listed in Table I was therefore assessed on the LiChrospher-TMAE sorbent. The retention data were evaluated according to the dependencey of $\log \bar{k}$ on $\log 1/\bar{c}$ according to relationship derived from the LSS model as follows

$$\log \bar{k} = \log K + Z_c \log 1/\bar{c} \quad (8)$$

where $\bar{k}$ is the median capacity factor of the solute and $\bar{c}$ is the corresponding median salt concentration. The value of the parameter Z_c is taken as a measure of the average number of charges located in the contact area established betweeen the protein and the sorbent surface. Tables II and III summarise the results of this evaluation with different mobile phase pHs for both NaCl and KBr respectively. Values of $\log K_c$ were determined by extrapolation

TABLE II

INFLUENCE OF pH ON RETENTION PARAMETERS FOR PROTEINS CHROMATOGRAPHED WITH NaCl

– = Not retained.

Protein	pH 9.6		pH 7.5		pH 6.5		pH 5.5	
	Z_c	log K_c	Z_c	log K_c	Z_c	log K_c	Z_c	log K_c
OV	2.3	11.7	4.4	22.8	2.6	13.4	–	–
INS	1.4	7.2	1.7	8.6	1.4	7.7	–	–
CA	–	–	–	–	–	–	–	–
GH	0.7	3.3	0.7	3.4	–	–	–	–
MYO	–	–	–	–	–	–	–	–
RIB	0.7	2.1	–	–	–	–	–	–
LYS	4.9	24.3	–	–	–	–	–	–

of the log $\bar{k}$ *versus* log $1/\bar{c}$ plots to the limit case of $\bar{c} \rightarrow 10^{-6}$ *M*. Log K_c values also provide an indication of the influence of experimental conditions on the affinities of protein solutes for the sorbent. It is evident from these data that protein retention generally decreases with decreasing pH while none of the test proteins were retained at pH 5.5. This finding with the LiChrospher-TMAE sorbent is in contrast to the results obtained for the isocratic elution of these proteins under similar salt and pH conditions on the Mono-Q sorbent where both OV and CA exhibited significant retention. Additional results which are not anticipated by the net-charge theory were also observed. These included the non-retention of CA and MYO at pH 9.6 as previously noted, and the observation that OV is not retained with mobile phases of pH 5.5 which is still 0.8 pH units above the protein p*I*. These results thus confirm and extend the findings of our previous study [4] on the retention behaviour of proteins on the tentacle-type sorbents which suggested that the density and/or the accessibility of the coulombic binding sites on the silica based material differ from that on the Mono-Q type materials.

Fig. 3 shows the plots of the bandwidth ratio *versus* $1/b$ for OV and INS at pH 7.5 and 6.5 with NaCl and KBr as the displacer salt. For OV, the magnitude of the bandwidth ratio generally increases with decreasing pH with NaCl as the displacing salt, while the opposite effect is observed with KBr. For INS, while similar bandwidth ratio dependencies were observed with both NaCl and KBr at pH 6.5, there were large differences in the relative bandwidth ratio at the higher mobile phase

TABLE III

INFLUENCE OF pH ON RETENTION PARAMETERS FOR PROTEINS CHROMATOGRAPHED WITH KBr

– = Not retained.

Protein	pH 9.6		pH 7.5		pH 6.5		pH 5.5	
	Z_c	log K_c	Z_c	log K_c	Z_c	log K_c	Z_c	log K_c
OV	1.6	7.2	2.5	13.1	2.7	14.0	–	–
INS	1.6	6.5	1.2	6.2	1.1	5.8	–	–
CA	–	–	–	–	–	–	–	–
GH	0.7	2.8	–	–	–	–	–	–
MYO	–	–	–	–	–	–	–	–
RIB	0.4	1.7	–	–	–	–	–	–
LYS	1.9	10.1	–	–	–	–	–	–

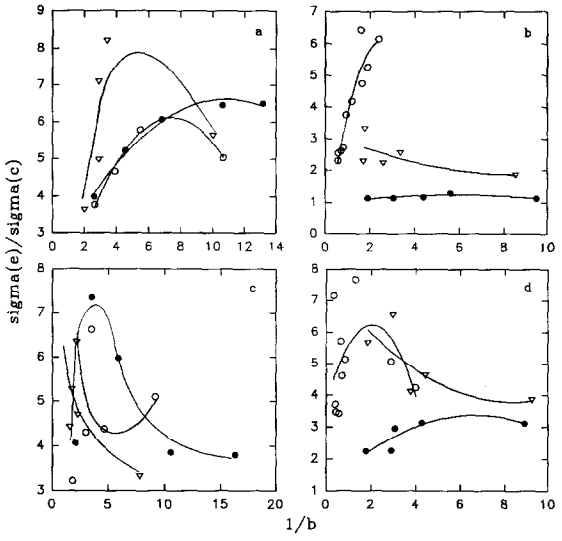

Fig. 3. The influence of mobile phase pH on the dependence of $\sigma_{v,exp}/\sigma_{v,calc}$ *versus* $1/b$ for OV with (a) NaCl and (b) KBr, and INS with (c) NaCl and (d) KBr. ○ = pH 9.6; ● = pH 7.5; ▽ = pH 6.5. See Fig. 1 for other details.

pH values. These results demonstrate that, as the ionisation state of the protein solute is systematically altered through variation of the eluent pH, there is a strong dependence of the kinetic properties of the protein solute on the gradient time and the nature of the displacer salt. Furthermore, the eluent pH can also be anticipated to influence the dynamic structure of the tentacle ligands which will inturn affect the retention and bandwidth behaviour. As a consequence of the nature of the Ce^{4+}-initiated linear graft polymerisation used to prepare the tentacle sorbents, a gradient of tentacles of different monomer lengths may form with porous support materials due to steric restrictions. This effect, coupled with electrostatic repulsion of the positively charged ligands within the pore chambers, could result in the solute experiencing the influence of unmodified sites on the surface of the support matrix. In these circumstances, and particularly with silica based sorbents, the retention and bandwidth behaviour of a protein at different mobile phase pH values would be anticipated not to follow an anion-exchange mode of elution.

CONCLUSIONS

This study has shown that the bandbroadening behaviour of proteins separated on the tentacle-type anion-exchange sorbents is dependent on the nature of the displacer salt, the mobile phase pH and the gradient time. Differences in the bandwidth dependencies for each of the proteins studied also implicate a role for the charge anisotropy of the protein surface structure rather than net charge *per se* in the kinetics of the interactive process. Such effects are consistent with current concepts for the kinetics of molecular docking. Moreover, the experimental data indicate that it cannot be assumed that globular proteins will migrate with these tentacle sorbents as conformationally rigid species with their size and diffusivity predicted solely on the basis of bulk calculations. Rather the results suggest a dynamic interplay between the flexible tentacle ligand and the protein solute under the experimental conditions, a chromatographic behaviour which is somewhat analogous to clathrin-binding phenomena observed with biological membranes [22]. As a result, it can be anticipated that tentacle sorbents of different ligand type should exhibit principally different types of adsorption kinetics depending on the chemical nature of the ligand and the surface properties of the sorbent. This hypothesis is currently being tested with tentacle ligands of different monomer size and chemical composition.

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