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HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY OF AMINO ACIDS, PEPTIDES AND PROTEINS

LXXXVIII*. CALCULATION OF THE AVERAGE DISTANCE BETWEEN PROTEIN SOLUTES AND THE STATIONARY PHASE DURING ISOCRATIC ANION-EXCHANGE CHROMATOGRAPHY

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SUMMARY

This investigation deals with protein retention behaviour in high-performance anion-exchange chromatography in terms of the average distance of approach between the protein solute and the positively charged anion-exchange stationary-phase surface. The theoretical treatment is based on a modified Debye-Hückel theory for spherical impenetrable ions, where the electrostatic potential energy has been related to the chromatographic capacity factor, k'. Results are presented for three globular proteins, eluted isocratically from a Mono-Q strong anion-exchange resin with sodium choride as the displacer salt by a mobile phase with pH in the range 5.50–9.60. Analysis of experimental retention data indicates that topographically predefined, charged regions on the protein surface, called ionotopes, control the orientation and approach distance of the protein solute.

INTRODUCTION

High-performance ion-exchange chromatography (HPIEC) has emerged in recent years as an important method for the analysis and purification of peptides, proteins and polynucleotides. However, a quantitative model describing the fundamental mechanistic processes that control the ion-exchange separation of proteins on microparticulate supports coated with charged ligands is not yet available. Protein retention at ion-exchange surfaces arises from electrostatic interactions between the zwitter-ionic protein surface and the charged stationary-phase surface. Previously, a number of non-mechanistic models have been utilised to assess the retention and bandwidth behaviour of proteins derived from experimental studies with HPIEC[1–5]. These studies have typically evaluated variation in protein selectivity or band-broadening as a function of the ionic strength, mobile-phase pH or the nature of the

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displacer ion through changes in the electrostatic surface potential of the polyelectrolyte surface. Variation of the chromatographic experimental parameters alters the affinity of the solute for the stationary phase through changes in the overall electrostatic surface charge ratio by protonation/deprotonation or through specific electrostatic interactions of the displacer co-ions and counter-ions with surface charge groups on the protein solute or the coulombic ligand. While it is assumed that these interactions will take place at the surface of a conformationally intact protein solute, changes in the three dimensional structure of the protein, which may result from time-dependent exposure to certain chemical environments both in the mobile phase and at the stationary-phase surface during the ion-exchange process, will also clearly have significant effects on solute retention characteristics. The affinity of the solute for the stationary phase is reflected in the retention behaviour of the solute, which will thus be dependent on the relative charge distribution at the surface of both the protein and the stationary phase. Changes in these affinity dependencies will, through the contribution of mutually attractive and repulsive forces and steric bulk, influence the distance to which the protein is able to approach the stationary phase. A method has therefore been investigated for calculating the average distance of approach between a solute molecule and the ionic groups attached to the support surface during anion exchange chromatography. This procedure, which is based on a modified Debye-Hückel theory for spherical, impenetrable ions, allows the evaluation of chromatographic retention data in terms of specific geometric and thermodynamic parameters, and thus provides further mechanistic insight into the behaviour of proteins at charged surfaces in the presence of solvated ions.

EXPERIMENTAL

Apparatus

All chromatographic experiments were performed with a Pharmacia (Uppsala, Sweden) Fast Protein Liquid Chromatography (FPLC) system consisting of two P-500 syringe pumps, a V-7 injector and a 278-nm fixed-wavelength single-path UV monitor, coupled to a two channel REC 482 pen recorder and a Perkin-Elmer (Norwalk, CT, U.S.A.) LCI-100 integrator. Isocratic elution was controlled with a Pharmacia GP-250 solvent programmer. Thermostatted, prepacked Mono-Q strong anion-exchange columns (HR 5/5, 50 × 5 mm I.D.) were used throughout. The pH measurements were performed with an Orion Research Products (Cambridge, MA, U.S.A.) SA 520 meter, equipped with a combination glass electrode. All chromatographic separations were carried out at ambient temperatures of 20°C.

Chemicals and reagents

Bovine erythrocyte carbonic anhydrase, sperm whale skeletal muscle myoglobin (type iii), hen egg ovalbumin (grade v), human serum albumin, Bis-Tris, triethanolamine and piperazine were purchased from Sigma (St. Louis, MO, U.S.A.) and characterised by procedures previously established in this laboratory. Sodium chloride (AnalaR grade) was obtained from BDH (Port Fairy, Australia). Quartz-distilled water was further purified on a Milli-Q system (Millipore, Bedford, MA, U.S.A.), hydrochloric acid (sp. gr. 1.16, AnalaR grade) was purchased from BDH.

Chromatographic procedures

Eluent A was a 0.02 *M* solution of a suitable buffering ion. The buffers used were piperazine (pH 5.50 and pH 9.60), Bis-Tris (pH 6.50) and triethanolamine (pH 7.50). In all cases, eluent B was 0.02 *M* with respect to the appropriate buffer and contained 0.3 *M* sodium chloride as the displacer salt. The pH of both eluents was adjusted to the required pH by addition of hydrochloric acid. Eluents A and B were filtered (0.45-μm cellulose acetate HAWP 04700 (Millipore) and degassed under vaccum. Protein solutions were prepared by dissolving the protein in eluent A at a concentration of 5 mg/ml unless otherwise specified. Protein solutions were filtered before use through 0.22-μm ACRO LC13 filters (Gelman Sciences, Sydney, Australia). Protein sample sizes ranged from 5 to 1000 μg with injection volumes between 10 and 200 μl. At each pH, samples were eluted isocratically, starting at 100% eluent B and repeated, at decreasing concentrations of B until 5% eluent B was reached or protein elution volumes were greater than 30 column volumes.

At each mobile phase pH, log k' and log $1/c$ data were collected for each protein and subjected to an iterative regression analysis to determine the y-intercept (log K), slope (Z_c) and correlation coefficient (r^2) from the plot log k' *versus* log $1/c$. The PROTDIST program, developed in this laboratory for use on IBM XT or AT-compatible personal computers, was used for data analysis.

RESULTS AND DISCUSSION

Theoretical concepts

The mass distribution of a protein solute P with charge $\pm a$, in HPIEC under near equilibrium conditions can be described by the stoichiometric equation

$$(P^{\pm a})_m + Z_t(D^{\pm b})_s \rightleftarrows (P^{\pm a})_s + Z_t(D^{\pm b})_m \quad (1)$$

where $D^{\pm b}$ is the displacer counter-ion and the subscripts m and s represent the mobile-phase and stationary-phase component, respectively. For large polyelectrolyte molecules, such as peptides and proteins, Z_t represents the ratio of the effective surface charge to that on the counter-ion. If conditions of near-equilibrium apply for the chromatographic system, the above distribution model can be rewritten for a monovalent co-/counter-ion solvent system in terms of the dependency of the capacity factor, k', on the concentration of the displacing ion, c, such that for a generalised adsorption process

$$\log k' = \alpha + \beta[\log(1/c)] + \gamma[\log(1/c)]^2 + \delta[\log(1/c)]^3 + \ldots \quad (2)$$

In linear elution chromatography, eqn. 2 is often represented as a non-mechanistic linear expression, which describes the relationship between solute retention and the displacer ion concentration as follows

$$\log k' = \log K + Z_c \log (1/c) \quad (3)$$

where k' is the solute capacity factor, c is the concentration of the displacer ion and K is the overall distribution constant. Values of both log K and Z_c can be obtained by

evaluating the intercept and slope, respectively, of plots of log k' *versus* log $1/c$ by regression analysis. The chromatographically determined Z_c value, derived from eqn. 3, has frequently been taken to represent the number of charged interactive binding sites established between the protein solute and the stationary phase suport such that $Z_c \leq Z_t$. In anion-exchange systems, Z_c values have been found to increase with pH[3,4] and reach a maximum value at a pH which is solute-dependent. The magnitude of Z_c, the shape of the Z_c *versus* pH plots, and the relationships between Z_c and pH or ionic condition, clearly are dependent on the number of exposed charged groups and their distribution on the surface of the protein. In this context, the participation of intrinsic ion bridges between complementary charge centres within the interior or periphery of the protein or extrinsic ion bridges between solvated ions and complementary charge groups at the protein surface would be expected to have a fundamental influence on Z_c values. For example, in anion-exchange systems, operated under normal pH conditions, the non-uniform charge distribution at the surface of the protein will result in localised areas of high electrostatic potential. These regions arise as a consequence of either continuous (*i.e.* sequentially linked amino acids) or discontinuous (*i.e.* topographic through space interactions) clustering of anionic amino acids. This unique interactive patch (or patches) on the surface of the protein can thus be represented as a contour surface or ionotope. Since these regions represent the sites of highest interactive potential which orient or direct the approach of the protein to the charged surface of the stationary phase, they thus act as "docking" sites of defineable surface contact area, A_{cont}, and charge anisotropy, Z_{anis}. It is thus not surprising that many different proteins are capable of being bound to and eluted from a particular ion exchanger with the same apparent k' and even the same band dispersion. What these observations reveal is the similarity and even constancy of the shape and surface characteristics of the interactive sites of a particular set of proteins, manifested through their ionotopes, a concept which is formally analogous to the mimotope strategy recently developed for immunoaffinity chromatography[6]. The proximity and orientation with which a particular protein solute is able to approach the stationary-phase ligands will thus depend on a number of factors. These include surface charge asymmetry and the steric bulk of the protein as well as the stationary phase charge and ionic concentration gradient at the liquid–solid interface, *i.e.* the thickness of the double layer between the solid phase and the bulk solution. As chromatographic retention data is a measure of the free energy of interaction between the solute and stationary phase, the influence of experimental conditions on the approach distance and orientation of a protein solute at the charged surface can be assessed through calculation of the electrostatic potential of the protein which, in turn, allows an average approach distance to be determined. Insight into the mechanistic behaviour of proteins at an ion-exchange surface can be obtained from a number of experimental methods including (i) evaluation of the kinetics of structure–retention behaviour, (ii) analysis of isothermal behaviour and adsorption–desorption kinetics, and (iii) physicochemical treatments which provide quantitative information on protein volume or area occupancy at the surface or data on the minimum approach distance. Studies[7] on the adsortion of polyelectrolytes at charged surfaces have established that the struture of adsorbed solute layers is governed by a subtle balance between enthalpic and entropic factors. For homopolymers, such as polylysine with a repeating segmental unit, these factors include the segmental adsorption energy, the chain-con-

formation entropy, the entropy of mixing segments in a defined solvent and the interaction between segments and solvent components. Theoretical treatments have yet to be generalised to describe the behaviour at coulombic interfaces of charge asymetric, compositionally different biopolymers, which do not behave like statistical coils and which have unique tertiary or quaternary hierachial structures such as those found for globular proteins. In these more complex cases, free energy changes associated with the adsorption properties of proteins are largely determined by the extent to which their bulk conformation is retained during adsorption. If the tertiary structure is unaffected, the adsorption behaviour can be accommodated relatively simply in energetic terms and can be represented as a well-controlled interaction. For proteins which unfold upon adsorption, entropic contributions become important and sometimes dominant. Under these circumstances the orientational frequency and preference of a protein, as it approaches a coulombic surface, can be represented by a kinetically and thermodynamically favoured charge vector. This charge vector will thus correspond to the charged surface of a hypothetical ionotope of predefined interactive surface occupancy and adsorption/desorption kinetics. If it is assumed that the retention process is governed by electrostatic interactions, then Debye-Hückel theory for spherical impenetrable ions can be adapted to evaluate the behaviour of this ionised protein at an HPIEC packing surface. However, it must be recognised that, although this treatment assumes that long range Van der Waals and Lifshitz forces or hydrogen bonding effects play a minor role in the retention process, participation of these phenomena at different elution conditions will lead to second order relationships between log k' and log $1/c$ which are characteristic of changes in the surface hydrophobicity or water structure. As a consequence, non-ideality in retention behaviour analysed according to the assumptions of the Debye-Hückel theory provides insight into the influence of matrix–solute hydrophobic effects or solvation effects. These combined secondary effects can then be analysed in terms of solvophobic and/or solvation theory.

Fig. 1 shows a simplified graphic representation of the Debye-Hückel theory for spherical impenetrable ions[8], as adapted for a macro-ionic ionotope at an ion-exchange packing surface. This model assumes that the protein solute, or more accurately, the topographic region on the protein surface which interacts with the

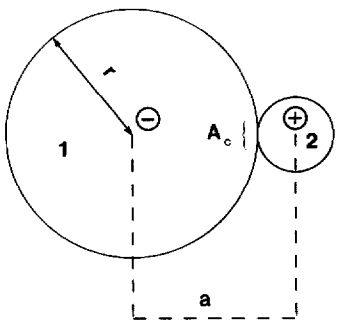

Fig. 1. Theoretical model used in the derivation of eqn. 9 to calculate the solute approach distance, a, consisting of the anionic macro-ion (1) of radius r, and the cationic stationary-phase ligand (2), which interact through the coulombic interaction area (A_c).

HPIEC ligands to create the apparent coulombic contact area, A_{cont}, of the ionotope, can be represented by a charged surface with effective radius r, and is located at the fixed centre of a co-ordinate system. Both the protein ionotope and those ions present at the solute–stationary phase interface are found in a solvent with a dielectric constant, D. The charge vector, q, associated with the ligand–solute interaction established between the stationary phase and the protein is assumed to be either evenly distributed over the macro-ionic ionotope surface or spherically symmetrical in its location. All other interacting ions found at the electrostatic binding site, which include charge support groups, buffer ions and the displacer salt, are assumed to be univalently charged and mobile relative to the central macro-ion. The probability of protein–protein interaction is assumed to be infinitesimally small. The distance, a, in Fig. 1 thus represents the average distance of closest approach between the vectorial centre of the ionotopic surface and the point charge of a univalent ligand ion at the solute–stationary phase interface.

The electrical work in placing charges on the central ion is the product of potential and charge. The work involved in adding a charge, dq, to a point with electrical potential, ψ, is

$$dW_e = \psi dq \tag{4}$$

Hence, the electrostatic free energy, W_e, can be defined in terms of a hypothetical discharge state of the protein ion such that

$$W_e = \int_0^q \psi dq \tag{5}$$

The potential at the surface where the charge is located may then be represented as

$$\psi = \left(\frac{q}{Dr}\right)\left(1 - \frac{\kappa r}{1+\kappa a}\right) \tag{6}$$

Substituting eqn. 6 into eqn. 5 and assuming that Zq is the final effective charge at the point of interest *i.e.*, the macro-ion surface

$$W_e = \int_0^{Zq} \left(\frac{q}{Dr}\right)\left(1 - \frac{\kappa r}{1+\kappa a}\right) \tag{7}$$

Integration of eqn. 7 gives

$$W_e = \left(\frac{Z^2q^2}{2Dr}\right)\left(1 - \frac{\kappa r}{1+\kappa a}\right) \tag{8}$$

By solving eqn. 8 for the a term we obtain the value for the average distance between

the protein ion and the charged ligands at the solute–stationary phase interface, *e.g.*

$$a = \left(\frac{Z^2q^2}{Z^2q^2 - 2DrW_e}\right) - \frac{1}{\kappa} \tag{9}$$

where Z is the magnitude of charge, q is the sign of the charge and r is the protein radius, which is calculated from molecular weight (MW) using the relationship

$$r = (0.39\ \mathrm{MW})^{1/3} \tag{10}$$

The quantity κ^{-1} has the dimensions of length and is referred to as the Debye length. It is an approximate measure of the thickness of the ionic atmosphere or the distance over which the electrostatic field of an ion extends with appreciable strength. The κ term can be calculated by using the following relationship

$$\kappa^2 = \left(\frac{1000\ Ne^2}{\varepsilon_0\ DkT}\right) \sum c_i Z_i \tag{11}$$

where N is Avogadro's number, e is the protonic charge, ε_0 is the permitivity in vacuum, k is the Boltzman constant and T is the temperture (K). The term $\Sigma c_i Z_i$ represents the summation of all charged species of concentration, c, and charge, Z.

If it is assumed that solute elution is carried out under conditions which maximise electrostatic interactions, then the electrostatic free energy term, ΔG_e, for ion-exchange solute retention can be considered to be equivalent to W_e. Thus, in the absence of other secondary retention phenomena the a term from eqn. 9 may be calculated by the following substitution

$$W_e = \Delta G_e = -RT \ln K_d \tag{12}$$

The equilibrium constant, K_d, for the ion-exchange process can be expanded to give

$$\Delta G_e = -RT (\ln k' - \ln \varphi) \tag{13}$$

where R is the gas constant, k' is the chromatographic capacity factor and φ is the dimensionless phase ratio, equal to the ratio of the volume of the stationary phase (V_s) to the volume of the mobile phase (V_m).

The theoretical dependence of electrostatic energy on the distance between two charged bodies is shown in Fig. 2. The right side illustrates the increasing electrostatic attractive energy of a system were two species of opposite charge are brought progressively closer together until a minimum separation distance is reached. This would be analogous to the adsorption phase of HPIEC, where the experimental conditions, such as the concentration of displacer salt and mobile phase pH, will determine the average distance between the charged protein solute and stationary-phase ligands. Conversely, the energy associated with the repulsion between two molecules of similar charge is illustrated on the left side of Fig. 2, where a decrease in the separation distance results in an increase in the repulsive energy. Solute retention in

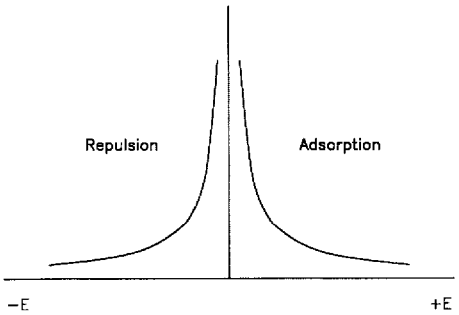

Fig. 2. Plot of the theoretical dependence of distance between two oppositely or similarly charged particles on the electrostatic energy of attraction or repulsion.

HPIEC arises from attractive electrostatic interactions, and hence the degree of repulsion depicted in Fig. 2 cannot be usually assessed from ion-exchange chromatographic data. However, these conditions will occur when proteins are chromatographed in anion-exchange systems at pH levels well below their known p*I*, *i.e.* when the surface of the protein assumes an overall positive charge, resulting in repulsion between the solute and the stationary phase. However, it is noteworthy that several examples have been reported[2,3,9] where proteins exhibit significant retention at a pH equal to or below their p*I*. The analysis of retention data in terms of the dependence of approach distance on mobile phase pH therefore provides the basis for assessing the ability of ionotopic regions at the protein surface to interact with the stationary phase, and gives greater insight into the factors that control the orientation and interaction of these topographic regions of proteins with charged surfaces in general.

Influence of pH on solute approach distance

Isocratic retention data for four globular proteins, listed in Table I, were obtained on a Mono-Q strong anion-exchange column. The proteins varied in molecular weight from 17 500 to 69 000 with p*I* values in the range 4.70–8.18. All proteins were chromatographed at a constant buffer concentration (20 m*M*) and various pH values, the sodium chloride concentration varying between 0 and 300 m*M*,

TABLE I
PHYSICAL PARAMETERS OF PROTEINS

Protein	*pI*	*MW*	*Z*			
			pH			
			5.50	*6.50*	*7.50*	*9.60*
Ovalbumin (eggwhite)	4.70	43 500	3.1	6.2	5.9	7.7
Albumin (human serum)	5.85	69 000	1.8	4.8	4.2	11.1
Carbonic anhydrase (bovine erythrocytes)	5.89	30 000	0.2	0.4	0.6	4.4
Myoglobin (sperm whale muscle)	7.68, 8.18	17 500	0.0	0.1	0.2	2.8

and at a flow-rate of 1 ml/min. Z_c values were calculated by regression analysis of plots of log k' *versus* log $1/c$ at each buffer pH. The Z_c values for each solute, which is defined as the number of charged interactions occuring between the protein solute and stationary phase, were then substituted for Zq in eqn. 9.

Figs. 3–6 show plots of the approach distance, a, as a function of the chromatographic capacity factor, k', for each protein as the mobile phase pH approaches and/or passes through their pI values. For ovalbumin and human serum albumin (Figs. 3 and 4) there is an inverse relationship between the approach distance and k'. Thus, as k' is systematically increased, a minimum a value is reached where the protein ion is thermodynamically restricted from moving any closer to the packing surface, regardless of further decreases in displacer ion concentration (*i.e.* further increases in k' values). Furthermore, the minimum a value decreases with decreasing solvent pH. This indicates that the protein ion orients itself progressively closer to the sorbent surface as the overall anionic charge on the solute surface diminishes and as the dominant interactive charge groups become more spatially isolated.

The net-charge concept has been extensively used to predict solute retention on HPIEC packings as a function of solvent pH. For an anion-exchange column, solute retention is predicted according to this concept to decrease with a decrease in solvent pH. When the solvent pH is equivalent to or below the solute pI, the solute is no longer retained on the anion-exchange column due to repulsive forces dominating the interaction between the sorbent surface and the solute molecule. However, it has been demonstrated[2,3,9] that considerable retention can occur at protein isoelectric points, a behaviour which is consistent with the intramolecular charge asymmetry on the surface accessible regions of the protein. The influence on the minimum approach distance of residual charged groups which exist at a pH close to the pI of the protein is clearly evident in Figs. 4 and 5. Human serum albumin and carbonic anhydrase have almost identical pI values of 5.85 and 5.89. On the basis of the net charge concept, similar dependencies of the a value on k' would be anticipated for these two proteins. Comparison of Figs. 4 and 5 show that human serum albumin exhibits significant retention over the entire pH range, while there is little electrostatic interaction between carbonic anhydrase and the stationary phase at pH 5.50 and 6.50. In these cases, as the solvent pH approaches and passes through the solute pI, the solute becomes

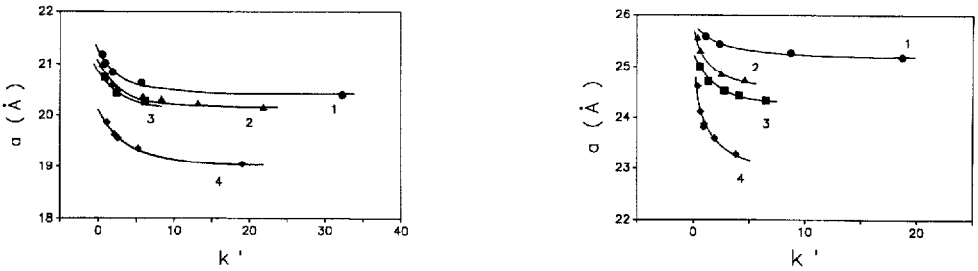

Fig. 3. Plots of approach distance, a, *versus* capacity factor, k', for ovalbumin. Data points were derived from measurements made with varying sodium chloride concentrations and solvent pH values of (1) 9.60, (2) 7.50, (3) 6.50 and (4) 5.50. See Experimental for other details.

Fig. 4. Plots of a *versus* k' for human serum albumin. See legend to Fig. 3 for other details.

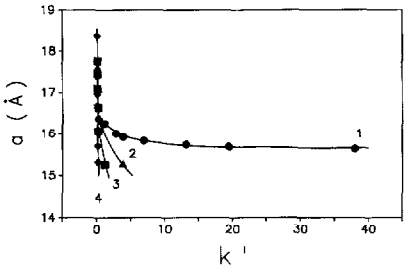

Fig. 5. Plots of *a versus k'* for carbonic anhydrase. See legend to Fig. 3 for other details.

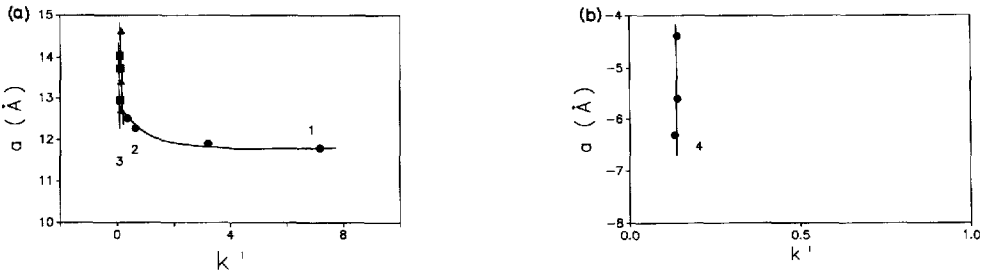

Fig. 6. (a) Plots of *a versus k'* for myoglobin with solvent pH values equal to (1) 9.60, (2) 7.50 and (3) 6.50. See legend to Fig. 3 for other details. (b) Plots of *a versus k'* for myoglobin with solvent pH equal to 5.50.

increasingly positive and this results in lower k' values for carbonic anhydrase. However, it is clearly apparent that the interactive sites or ionotopes for carbonic anhydrase and human serum albumin are significantly different, resulting in the case of human serum albumin in a closer approach to the stationary-phase ligands. Myoglobin, with a p*I* equal to 7.68, is poorly retained below pH 9.60. Indeed, as k' approaches zero, the first term in eqn. 9, which incorporates the interactive energy term, approaches a constant value. Under these conditions eqn. 9 reduces to a dependence of the a value on κ, and this can lead to experimental a values corresponding to solute repulsion from the ligand surface, as seen for myoglobin in Fig. 6b. Similar influences of pH on solute retention also have important application in the use of chromatofocusing for the separation and purification of protein samples. The choice of buffer species and pH gradient range in the optimisation of experimental conditions will significantly influence the interactive properties of protein solutes, and judicious manipulation of solvent pH would clearly enhance the separation of closely related proteins through selective changes of the surface charge.

The difference between the minima of plots for a versus k' for different proteins, *i.e.* the Δa *versus* k' function, provides an indication of the types of molecular changes that occur at the protein–stationary phase interface as a result of a decrease in buffer pH. In Figs. 3 and 4 the distance change between asymptotes at pH 9.60, 7.50 and 6.50 varies up to 1 Å. These differences may reflect changes in double-layer thickness, particularly since different buffer ions were used at pH 9.60, 7.50 and 6.50. Piperazine buffer was used at both pH 9.60 and 5.50 and the distances between the two curve minima for these pH values were 1.4 Å and 2.0 Å for ovalbumin and human serum albumin, respectively. Such distances are similar to those calculated for nearest-neighbour hydrogen-bonding effects for O–H distances (1.86 Å) in water[10], indicating

a difference in the thickness of the layer of solvating water molecules present at the interactive surface of either the central macro-ion or the stationary phase. Changes in the degree of protein solvation have significant implications in the conformational stability of protein solutes during HPIEC. Furthermore, substantial repulsive or hydration forces occur when solvated surfaces, such as lipid bilayers[11,13], are brought within close proximity (< 30 Å) of each other. The origin of these hydration forces has been studied[14,15] for mica surfaces, separated by distances of less than 30 Å, in a variety of electrolyte solutions. With mica, competitive adsorption between metal ions, such as Na^+ (which on adsorption strongly orients water dipoles), and H^+ ions was found to produce pH-dependent switching mechanism. The phenomenon of metal/hydrogen ion exchange at various surfaces and its correlation with short-range hydration forces has been advanced to account for thylakoid membrane stacking and unstacking in chloroplasts[16]. In ion-exchange chromatography with sodium chloride or other sodium salts as displacers a sodium/hydrogen ion switching mechanism may also be active at the solute–stationary phase interface, whereby the lowering of buffer pH from 9.60 to 5.50 causes the sorbed sodium ions at the protein surface to be replaced by hydrogen ions. This results in a decrease of the hydration forces, reflected by the observed changes in the *a* value between asymptotes at pH 9.60 and 5.50 for ovalbumin and human serum albumin in Figs. 3 and 4.

CONCLUSION

Estimation of the distance within which a protein solute can approach the charged stationary-phase surface in HPIEC provides the basis for a more detailed analysis of chromatographic retention data in electrostatic-interaction systems. The present study indicates that small changes in the charge of the interactive or ionotopic surface of the solute can strongly influence the isocratic retention behaviour of proteins through changes in the electrostatic potential. While these results would be anticipated from qualitative consideration of the amphoteric nature of protein surface structure, the current study represents a detailed quantitative approach to the analysis of these molecuar events. Identification of the ionotopic surface of proteins would clearly allow the effects of pH on the selectivity of closely eluted proteins to be more effectively exploited in ion-exchange, hydrophobic-interaction and chromatofocusing systems. The battery of experimental parameters for selectivity optimisation also includes the nature of the displacing salt and the use of gradient elution. Future papers will present the results of investigations on the influence of these parameters on the orientation of protein solutes at charged surfaces in HPIEC.

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