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

XCVII[a]. The influence of the gradient elution mode and displacer salt type on the retention properties of closely related protein variants separated by high-performance anion-exchange chromatography

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SUMMARY

The influence of different elution modes, gradient times and flow-rates on the relative retention of closely related variants of carbonic anhydrase and ovalbumin has been investigated using high-performance ion-exchange chromatography. Three isoform species of carbonic anhydrase and four isoforms related to ovalbumin eluted by anion-exchange chromatography were characterised by isoelectric focusing and sodium dodecylsulphate–polyacrylamide electrophoresis. Gradient retention data were collected using several different alkali metal halides as the displacer salt, in order to systematically evaluate the effect on selectivity of different anions and cations in the series F^-, Cl^- and Br^-, and Li^+, Na^+ and K^+. While the selectivity between the different ovalbumin isoform species remained essentially constant with each displacer salt, solute Z_c-values [*J. Chromatogr.*, 458 (1988) 27] varied with the type of salt. In contrast, non-parallel retention plots were obtained for the carbonic anhydrase isoforms with the Z_c values different for each isoform. Furthermore, significant differences in chromatographic behaviour for these proteins were observed between experiments carried out under gradient elution conditions with either varied gradient time and constant flow-rates or fixed gradient time and varied flow-rates. These results are discussed in terms of the influence of column residence time and protein-salt interactions of the solute's interactive ionotope and the concomitant effects these structural perturbations may have on chromatographic behaviour.

INTRODUCTION

During the past decade, high-performance liquid chromatography (HPLC) has developed into a powerful technique for the analysis of complex mixtures of biological

[a] For Part XCVI, see ref. 17.

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macromolecules[1–3]. The advent of protein engineering, cell culture technologies and new methods for rapid peptide synthesis has necessitated the development of very sensitive and reliable separation strategies for the purification of target peptides and proteins from crude feedstock solutions contaminated with other structural or charged variant species. Production of polypeptides and proteins by recombinant DNA or chemical synthesis methods often result in a higher incidence rate of variant species than normally found in natural systems[3], arising from expression errors, post-translational processing or incomplete peptide syntheses. In the fields of medicine and veterinary science, the removal of these variants is paramount if the target protein or peptide is intended for therapeutic use.

Removal of contaminating protein variants using the various modes of adsorption chromatography available today will only be successful if subtle differences exist between the stationary phase binding site on the contaminants and on the target protein. The further development of strategies for the separation of very closely related protein solutes requires an understanding of the relationship between chromatographic behaviour and solute structure. At the present time, the extent to which various chromatographic systems can probe and detect changes in the microstructure of protein binding sites has not been fully elucidated. In this study the influence of experimental conditions on the chromatographic behaviour of the closely related structural variants of bovine carbonic anhydrase and ovalbumin has been investigated. Retention data were used to obtain protein Z_c values[1,2], as a measure of the average number of electrostatic interactions occurring between the solute and stationary phase surfaces. Perturbations in the microstructure of the protein binding site were monitored by changes in Z_c with systematic changes in the displacer salt type and the buffer concentration.

MATERIALS AND METHODS

Chromatographic procedures

All chromatographic procedures were performed with a Pharmacia (Uppsala, Sweden) fast protein liquid chromatography (FPLC) system. The source of chemicals and reagents, the purity of the proteins and methods used for the numerical analysis of chromatographic retention and bandwidth data have been described in detail elsewhere[1,4].

Isoelectric focusing and sodium dodecylsulphate electrophoresis

Acrylamide and sodium dodecylsulphate (SDS) were of electrophoresis grade from BDH (Clayton, Australia). Ampholines (pH ranges: 3.5–10, 5–7, 4–6 and 9–11) and standard proteins of known isoelectric points (p*I*) were obtained in kit form from Pharmacia. All other chemicals were of analytical grade or better. Isoelectric focusing (IEF) was carried out using a Bio-Rad (Richmond, CA, U.S.A.) Bio-phoresis horizontal electrophoresis cell powered by a LKB (Bromma, Sweden) 2297 Macrodrive 5 constant-power supply. The horizontal bed was cooled to 4°C. Gel dimensions were 230 mm × 115 mm × 1 mm. SDS-polyacrylamide gel electrophoresis (PAGE) was performed using slab gels (200 mm × 170 mm × 1 mm) and an "in house" built vertical gel apparatus. Protein samples collected from Mono-Q column experiments were desalted in the following manner. A 200-μl aliquot of sample was diluted up to

2 ml total volume by addition of the desalting buffer (20 m*M* Tris · HCl, 0.02% Brij-35, pH 7.4). Protein samples were then concentrated and desalted in Centricon-10 microconcentrators (Amicon, Danvers, MA, U.S.A.) by centrifuging at 2800 *g* for 60 min using a Sorvall RC-5B centrifuge fitted with a SS-34 head (Dupont, Wilmington, DE, U.S.A.) or until approximately 200 μl of sample remained. The remaining sample was then dialysed for 15 h in a solution of 0.02% Brij-35 using Spectrapore (Spectrum Medical Industries, Los Angeles, CA, U.S.A.; 600–8000 molecular weight cut off) dialysis tubing.

RESULTS AND DISCUSSION

The separation and characterisation of isoforms for carbonic anhydrase and ovalbumin

Chromatographic elution profiles for many commercially available proteins, as well as proteins isolated in the research laboratory, often show multiple peaks corresponding to the existence of several structurally related species. If these proteins are isoforms of the mature, expressed form of the protein, *e.g.* if they are functionally related proteins of very similar sequence and composition arising from minor genomic or post-translational modifications in overall charge and/or hydrophobicity, then such isoform mixtures can conveniently be used to examine the chromatographic behaviour of related protein variants in terms of structure–retention dependencies. Typical of such chromatographic behaviour are the high-performance ion-exchange chromatography (HPIEC) elution profiles for bovine carbonic anhydrase and ovalbumin shown in Fig. 1, which reveal the presence of multiple peaks for each protein. The bovine carbonic anhydrase sample used in this study is known from other studies to contain at least two charged variant species which gave rise to peaks 1 to 3. These species can be readily distinguished on the basis of their p*I* values and are referred to as carbonic anhydrase II, p*I* 5.4 ($CA\text{-}II_{5.4}$) and carbonic anhydrase II, p*I* 5.9 ($CA\text{-}II_{5.9}$). Furthermore, comparative studies[5] on the $CA\text{-}II_{5.4}$ and $CA\text{-}II_{5.9}$ isoforms have shown that they are genetic variants where, in the latter case, an Arg residue is replaced in the primary sequence structure by a Gln residue at position 56.

Chromatographic analysis of high-quality commercial preparations of ovalbumin by other workers[6] has indicated that the preparations are homogeneous in

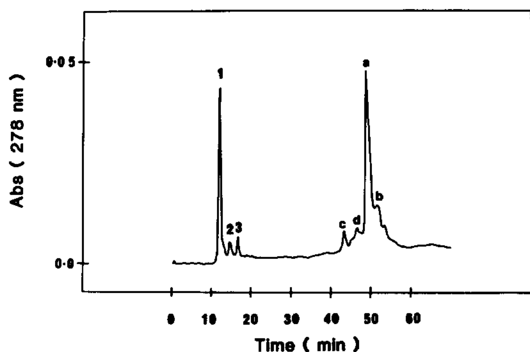

Fig. 1. Chromatogram demonstrating the presence of three bovine carbonic anhydrase isoforms (peaks 1–3) and four ovalbumin isoforms (peaks a–d), eluted with lithium chloride as the displacer salt and gradient time of 85.7 min at a flow-rate of 1 ml/min.

terms of molecular weight as determined by gel filtration. However, several chromatographic zones are also observed by ion-exchange techniques which suggests that proteins associated with these peaks could be charge or structural isoforms of ovalbumin. In order to further confirm the molecular characteristics of isoforms for carbonic anhydrase and ovalbumin with the preparations used in the present studies, samples recovered from the chromatographic studies (Fig. 1) were characterised using IEF and SDS-PAGE.

A comparison of the SDS-PAGE and IEF behaviour for the bovine carbonic anhydrase sample in peaks 1 to 3 to that for highly purified samples of CA-II$_{5.4}$ and

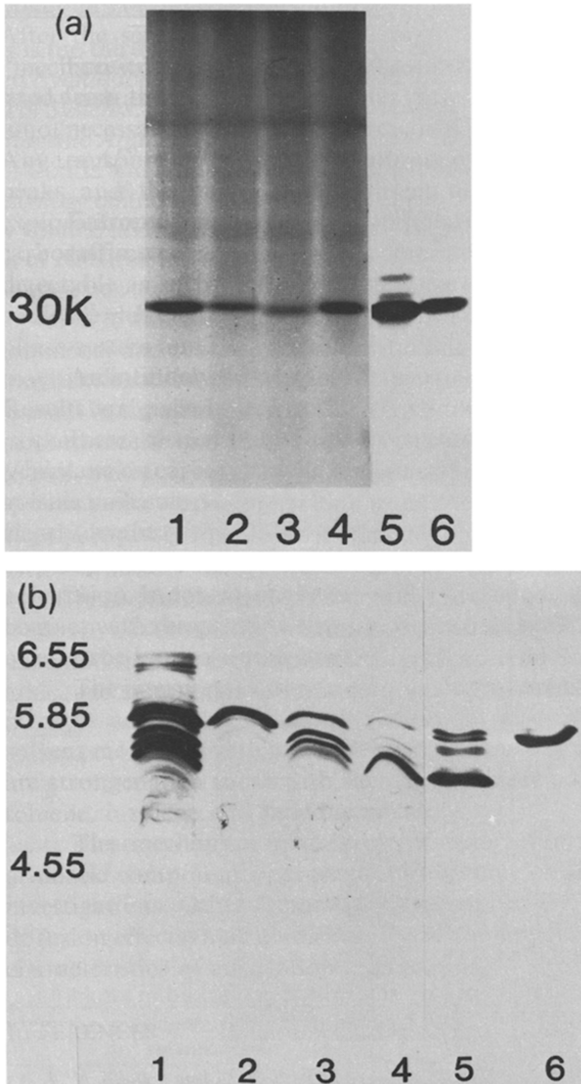

Fig. 2. Electrophoretic analysis of bovine carbonic anhydrase by (a) SDS-PAGE and (b) IEF. Samples: 1 = commercial sample; 2 = CA-1; 3 = CA-2; 4 = CA-3; 5 = CA-II$_{5.4}$; 6 = CA-II$_{5.9}$. See Materials and Methods for other details.

CA-$II_{5.9}$ is shown in Fig. 2a and b. SDS-PAGE shows three bands of identical molecular weight. However, IEF indicates the existence of the various charged isoforms. Peak 1 (CA-1), the major species corresponds to CA-$II_{5.9}$, while peak 3 (CA-3) can be attributed to CA-$II_{5.4}$. Two additional protein species with isoelectric points at p*I* 5.60 and 5.70 were found in fractions taken from peak 2 and were identified as CA-$2_{5.6}$ and CA-$2_{5.7}$. The structural origin of these latter two protein isoforms is currently under investigation.

Fig. 3a and b shows the SDS-PAGE and IEF gels for all the ovalbumin fractions collected after ion-exchange chromatography and an unchromatographed ovalbumin

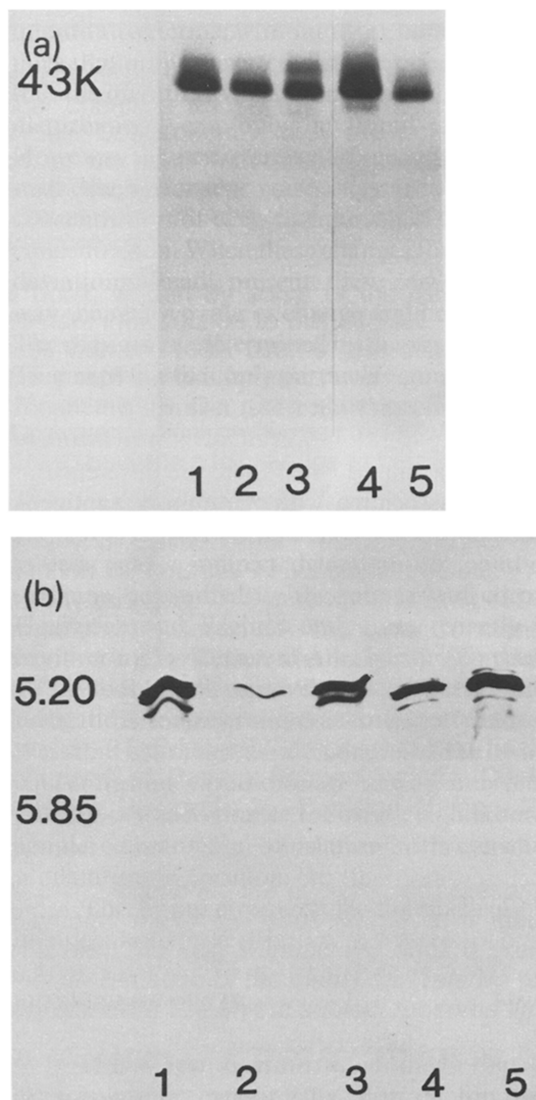

Fig. 3. Electrophoretic analysis of ovalbumin by (a) SDS-PAGE and (b) IEF. Samples: 1 = Commercial sample; 2 = Ov-c; 3 = Ov-d; 4 = Ov-a; 5 = Ov-b. K = Kilodaltons.

sample. The ovalbumin preparation was resolved into the four chromatographic peaks shown in Fig. 1, with the isoforms termed Ov-c, Ov-d, Ov-a and Ov-b, respectively, where Ov-a represents the major species of ovalbumin. The SDS-PAGE and analytical IEF results (Fig. 3) indicate that the ovalbumin species corresponding to each of the four chromatographic peaks have similar molecular weights and charge distributions. The protein species contributing to the HPIEC profile of ovalbumin therefore do not represent nett charge variants of the dominant form. Structural heterogeneity with ovalbumin is known to arise from the type or number of neutral sugars attached to the peptide chain[7,8]. In addition, the formation of different conformational species, with different interactive properties under the chromatographic conditions used, would also give rise to a complex, multi-peak elution profile. These structural variations are not detected by methods such as IEF or chromatofocusing which separate according to nett charge or SDS-PAGE unless substantial differences in molecular mass existed between the isoforms. The existence of similar structural variants as revealed by HPIEC has been documented with other glycoproteins and is believed to be responsible for the complex elution profiles observed for electrophoretically pure samples of, for example, thyroid stimulating hormone[9].

The effect of displacer salt type and varied gradient (VG) times on isoform $Z_{c,VG}$ values

Protein isoforms will only be separated by a particular mode of adsorption chromatography if the binding surfaces of each protein species involved in the retention process differ from each other. Furthermore, the number of functional groups at the surface of the protein which interact with the sorbent will be dependent upon the mode of elution. For example, resolution of variant proteins by immunoaffinity chromatography will only be achieved when there are differences in highly specific areas on the protein surface associated with the epitope (antigenic determinant) and these differences directly contribute to the binding process with the immobilised antibody. For other modes of adsorption chromatography such as reversed-phase, hydrophobic-interaction or ion-exchange systems, the binding site on the protein may not be as structurally specific and could occur over a much larger region of the surface area of the solute molecule or alternatively involve multisite regions of interaction. Therefore, structural changes over a far greater portion of the protein that may be invisible to immunoaffinity methods, can be detected by these chemical ligands in adsorptive chromatographic methods.

The ability of the chromatographic systems with immobilised chemical ligands to detect and anticipate small compositional or structural changes in the binding regions of proteins has not been comprehensively investigated in a systematic manner in terms of structure–retention dependencies, although the application literature on protein purification abounds with anecdotal examples of the resolution of charge and structural variations of the same protein. However, recent studies[10–14] on the HPIEC and hydrophobic-interaction chromatographic properties of lysozyme indicate that the correlation of the three-dimensional protein structure and chromatographic properties can be used to provide significant insight into the structural factors including subtle changes in the topographic features of proteins which control or influence the selectivity of chromatographic systems. To further investigate the potential of HPIEC as a functional molecular probe for determining subtle charge variations in protein structure, the binding behaviour of the variants of bovine

carbonic anhydrase and ovalbumin was investigated as a function of the mobile phase composition, the gradient time, t_G and eluent flow-rate. The chromatographic data were analysed in terms of the Chained Pek-n-ese programme[2], which integrates relative retention and peak width in the gradient mode ($\bar{k}$, $\bar{\sigma}_v$) with the chromatographic variables, t_G, flow-rate (F), Δc, etc. The HPIEC data were then displayed as plots of log $\bar{k}$ *versus* log $1/\bar{c}$ where $\bar{k}$ and $\bar{c}$ are the median capacity factor and the concentration of the displacing salt respectively.

Fig. 4a–h shows the retention plots of log $\bar{k}$ *versus* log $1/\bar{c}$ for the isoforms of bovine carbonic anhydrase and ovalbumin eluted with a variety of alkali metal halide salts. Retention data were obtained by varying the gradient time between 8.6 and 171.1 min at a constant flow-rate of 1 ml/min with the buffer pH 9.6. The retention maps illustrate that mobile phase composition can be used to optimise the selectivity of the HPIEC separation of these isoform species. For example, Fig. 4d and g shows that the selectivity between the various bovine carbonic anhydrase variants was maintained while their elution volumes were reduced with a change in salt type from lithium chloride to potassium bromide. Other salts such as sodium chloride or sodium bromide (Fig. 4b and c) were found to decrease the selectivity between the isoforms as the gradient time was reduced. Evidently, these latter sodium salts increase the resolution of the separate isoforms of carbonic anhydrase when longer gradient times are used.

Tables I and II show the $Z_{c,VG}$ values for varied gradient time (± 1 S.D.) and coefficients of determination (r^2) in parentheses obtained from the retention data in Fig. 4a–h for bovine carbonic anhydrase and ovalbumin respectively. The $Z_{c,VG}$ values were obtained by regression analysis of linear regions of plots for log $\bar{k}$ *versus* log $1/\bar{c}$. According to the stoichiometric displacement model[15], Z_c values are a quantitative measure of the number of electrostatic interactions occurring between the solute and stationary phase medium. If the salt species in the eluent has no influence on the distribution or orientation of interactive charges on the protein surface, experimental $Z_{c,VG}$ values will be independent of the displacer ions used. However, Tables I and II show that protein $Z_{c,VG}$ values are not constant over the range of the elution conditions employed and vary significantly with the type of displacer salt used. Furthermore, the effect of individual chaotropic (water structure breaking) and kosmotropic (water structure making) displacer anions and cations on the $Z_{c,VG}$ values of both the bovine carbonic anhydrase and ovalbumin isoforms follow similar trends in the magnitude of $Z_{c,VG}$ as previously observed for other proteins[12]. These results thus provide additional support for the following generalisations pertinent to the selection of displacer salts in HPIEC, namely: (1) $Z_{c,VG}$ values increase as both the anion and cation of the displacer salt becomes more chaotropic in nature. (2) $Z_{c,VG}$ values increase as both the anion and cation became more kosmotropic in nature. (3) $Z_{c,VG}$ values decrease when the displacer salt contains a combination of a chaotropic and kosmotropic ion.

The results in Tables I and II can be further used to evaluate the effect of the salt type on the ionotopic microstructure (coulombic binding site) of related variant species. Significant variations in the $Z_{c,VG}$ value indicate a change in the number of electrostatic interactions occurring between the protein and stationary phase. Under these circumstances, the most likely cause of changes in $Z_{c,VG}$ values are through salt-induced perturbations in the ionotopic structure. For example, the $Z_{c,VG}$ values for Ov-a, Ov-c and Ov-d varied with changes in the displacer salt which suggests that

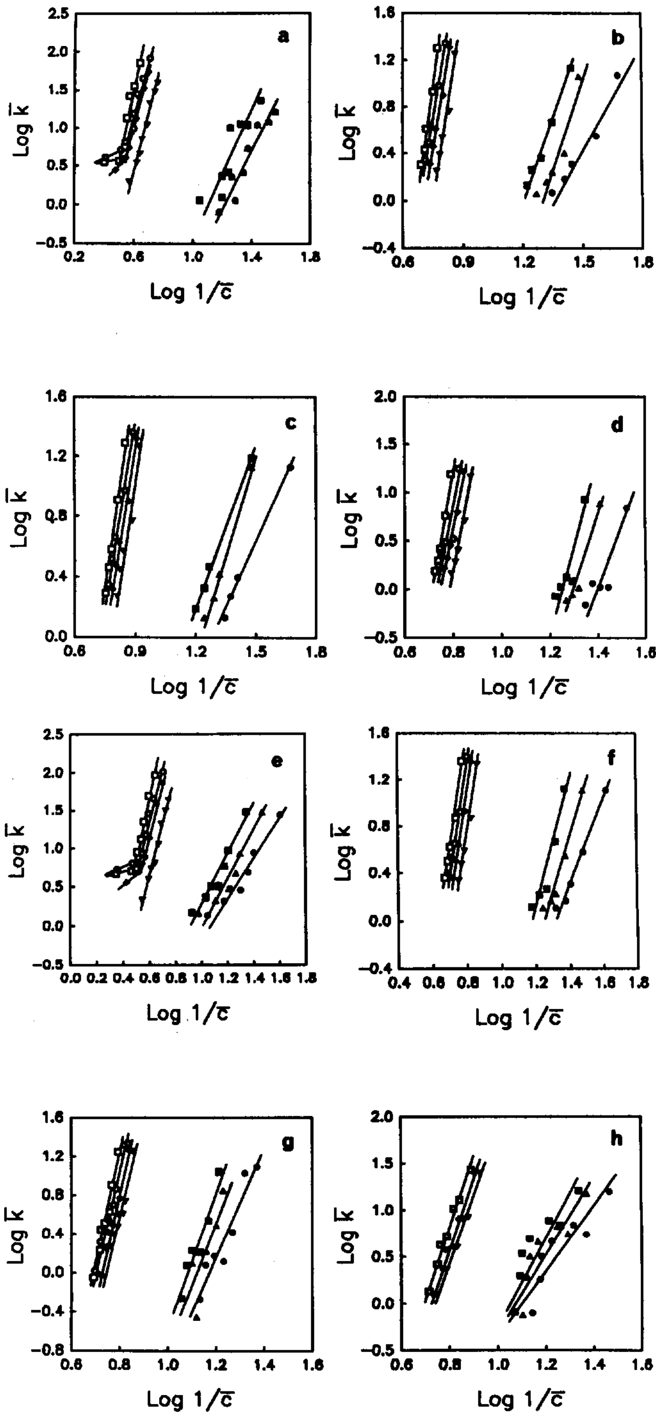

Fig. 4. Plots of log $\bar{k}$ *versus* log $1/\bar{c}$ for bovine carbonic anhydrase isoforms 1, 2 and 3 and ovalbumin isoforms a, b, c and d. The plots were derived from gradient elution data at pH 9.6, a flow-rate of 1 ml/min with gradient times between 8.6 and 171.1 min for (a) NaF, (b) NaCl, (c) NaBr, (d) LiCl, (e) KF, (f) KCl, (g) KBr and (h) LiBr. Isoforms: ● = CA-1; ▲ = CA-2; ■ = CA-3; ▼ = Ov-c; ◆ = Ov-d; ○ = Ov-a; □ = Ov-b.

TABLE I

Z_c VALUES FOR CARBONIC ANHYDRASE VARIANTS OBTAINED BY LINEAR REGRESSION OF VARIED GRADIENT TIME EXPERIMENTS

Coefficients of determination are given in parentheses.

Salt	*Protein*		
	CA-1	*CA-2*	*CA-3*
LiF	Not done	Not done	Not done
LiCl	5.60 ± 2.12 (0.78)	7.44 ± 1.24 (0.95)	7.62 ± 1.98 (0.83)
LiBr	3.14 ± 0.71 (0.80)	3.67 ± 0.87 (0.78)	3.81 ± 0.86 (0.80)
NaF	3.55 ± 0.45 (0.91)	3.55 ± 0.45 (0.92)	3.52 ± 0.69 (0.81)
NaCl	3.07 ± 0.21 (0.99)	5.40 ± 1.18 (0.91)	4.46 ± 0.28 (0.99)
NaBr	2.92 ± 0.17 (0.99)	4.31 ± 0.22 (1.00)	3.55 ± 0.13 (1.00)
KF	2.37 ± 0.25 (0.95)	2.64 ± 0.36 (0.92)	3.15 ± 0.36 (0.94)
KCl	3.84 ± 0.05 (1.00)	5.10 ± 0.28 (0.99)	5.09 ± 0.92 (0.91)
KBr	5.60 ± 0.75 (0.92)	7.11 ± 2.43 (0.68)	7.33 ± 1.02 (0.92)
LiF	Not done	Not done	Not done
NaF	3.55 ± 0.45 (0.80)	3.55 ± 0.45 (0.92)	3.52 ± 0.69 (0.81)
KF	2.37 ± 0.25 (0.95)	2.64 ± 0.36 (0.92)	3.15 ± 0.36 (0.94)
LiCl	5.60 ± 2.12 (0.78)	7.44 ± 1.24 (0.95)	7.62 ± 1.98 (0.80)
NaCl	3.07 ± 0.21 (0.99)	5.40 ± 1.18 (0.91)	4.46 ± 0.28 (0.99)
KCl	3.84 ± 0.05 (1.09)	5.10 ± 0.28 (0.99)	5.09 ± 0.92 (0.91)
LiBr	3.14 ± 0.71 (0.80)	3.67 ± 0.87 (0.78)	3.81 ± 0.86 (0.80)
NaBr	2.92 ± 0.17 (0.99)	4.31 ± 0.22 (1.00)	3.55 ± 0.13 (1.00)
KBr	5.60 ± 0.75 (0.92)	7.11 ± 2.43 (0.68)	7.33 ± 1.02 (0.92)

the size of the ionotopic region is strongly influenced by the nature of the eluting salt. The $Z_{c,VG}$ values for the three variants, however, did not differ significantly within a particular salt system. For example, elution with potassium chloride and potassium fluoride gave the following results: *i.e.*, for KCl: $Z_{c,VG}$ (Ov-a) = 8.40 ± 0.76, $Z_{c,VG}$ (Ov-c) = 8.50 ± 0.71, $Z_{c,VG}$ (Ov-d) = 8.70 ± 0.55 and with KF, $Z_{c,VG}$ (Ov-a) = 6.37 ± 0.57, $Z_{c,VG}$ (Ov-c) = 7.05 ± 0.48, $Z_{c,VG}$ (Ov-d) = 6.70 ± 0.57. This observation further indicates that the interactive behaviour of the ionotopes of the ovalbumin variants is influenced in a similar manner on exposure to various salts. The essentially constant $Z_{c,VG}$ values for Ov-a, Ov-c and Ov-d indicate the existence of ionotopes with equivalent charge densities, and for proteins with closely related structures these ionotopes are likely to be located in identical areas of the three-dimensional structure of the protein. Parallel, yet non-superimposable, plots of log $\bar{k}$ *versus* log $1/\bar{c}$ for these protein variants further clearly indicate that these ionotopes differ, however, in their affinity for the support surface. The variation in affinities for the stationary phase can be attributed to unique structural differences within each glycoprotein isoform which may be a result of variations in conformation and/or compositional heterogeneity of the carbohydrate moiety. As such, the chromatographic data derived from the interaction of an immobilised chemical ligand, *e.g.* a quaternary ammonium group, with a protein as evidenced in HPIEC (or other

TABLE II

Z_c VALUES FOR OVALBUMIN VARIANTS OBTAINED BY LINEAR REGRESSION OF VARIED GRADIENT TIME EXPERIMENTS

Coefficients of determination are given in parentheses.

Salt	*Protein*			
	Ov-a	*Ov-b*	*Ov-c*	*Ov-d*
LiF	Not done	Not done	Not done	Not done
LiCl	9.92 ± 2.90 (0.80)	15.34 ± 1.01 (0.99)	12.41 ± 1.08 (0.98)	12.26 ± 0.87 (0.99)
LiBr	7.30 ± 0.46 (0.98)	7.30 ± 0.46 (0.98)	6.91 ± 0.54 (0.97)	7.17 ± 0.57 (0.97)
NaF	6.56 ± 0.71 (0.95)	11.21 ± 1.78 (0.91)	7.72 ± 0.45 (0.98)	7.55 ± 0.41 (0.99)
NaCl	9.40 ± 0.50 (0.99)	11.71 ± 0.93 (0.98)	10.31 ± 1.05 (0.97)	9.74 ± 0.55 (0.99)
NaBr	7.83 ± 0.48 (0.99)	10.01 ± 0.23 (1.00)	8.23 ± 1.02 (0.96)	7.95 ± 0.52 (0.99)
KF	6.37 ± 0.57 (0.97)	7.69 ± 0.64 (0.97)	7.05 ± 0.48 (0.98)	6.70 ± 0.40 (0.99)
KCl	8.40 ± 0.76 (0.98)	9.48 ± 0.74 (0.98)	8.50 ± 0.71 (0.98)	8.69 ± 0.55 (0.99)
KBr	9.48 ± 0.69 (0.97)	11.94 ± 1.05 (0.96)	9.81 ± 1.06 (0.95)	9.94 ± 1.00 (0.95)
LiF	Not done	Not done	Not done	Not done
NaF	6.56 ± 0.71 (0.95)	11.21 ± 1.78 (0.91)	7.72 ± 0.45 (0.98)	7.55 ± 0.41 (0.99)
KF	6.37 ± 0.57 (0.97)	7.69 ± 0.64 (0.97)	7.05 ± 0.48 (0.98)	6.70 ± 0.40 (0.99)
LiCl	9.92 ± 2.90 (0.80)	15.34 ± 1.01 (0.99)	12.41 ± 1.08 (0.98)	12.26 ± 0.87 (0.99)
NaCl	9.40 ± 0.50 (0.99)	11.71 ± 0.93 (0.98)	10.31 ± 1.05 (0.97)	9.74 ± 0.55 (0.99)
KCl	8.40 ± 0.76 (0.98)	9.48 ± 0.74 (0.98)	8.50 ± 0.71 (0.98)	8.69 ± 0.55 (0.99)
LiBr	7.30 ± 0.46 (0.98)	7.30 ± 0.46 (0.98)	6.91 ± 0.54 (0.97)	7.17 ± 0.57 (0.97)
NaBr	7.82 ± 0.48 (0.99)	10.01 ± 0.23 (1.00)	8.23 ± 1.02 (0.96)	7.95 ± 0.52 (0.99)
KBr	9.48 ± 0.69 (0.97)	11.94 ± 1.05 (0.96)	9.81 ± 1.06 (0.95)	9.94 ± 1.00 (0.95)

adsorptive modes) have formal similarities to the competitive binding observed with biological affinate–ligand interactions in solution or as solid phase assays.

The electrophoretic data shown in Fig. 3 suggest that Ov-b has similar molecular size and charge distribution to Ov-a, Ov-c and Ov-d. However, the $Z_{c,VG}$ values (Table II) indicate that the HPIEC binding site for Ov-b differs significantly from those of the other isoforms under the studied elution conditions. This variant generally exhibited larger $Z_{c,VG}$ values than those observed for the other ovalbumin isoforms. The larger $Z_{c,VG}$ values suggest that an increased number of electrostatic interactions were involved in the sorption of Ov-b to the stationary phase surface. Again, variations in conformation or in the composition of the carbohydrate moiety of Ov-b, undetectable by IEF methods, are likely causes for the increase in $Z_{c,VG}$.

For the bovine carbonic anhydrase isoforms denoted CA-1, CA-2 and CA-3, electrophoretic data indicate that they are charged variants. Inspection of the data in Table I shows their $Z_{c,VG}$ values varied with the type of displacer salt. Furthermore, the $Z_{c,VG}$ values for both CA-2 and CA-3 were found to be similar in most salt systems. These results indicate that the interactive ionotopes on CA-2 and CA-3 were responding in a similar manner to changes in the type of displacer salt and that these two isoforms interact with the sorbent through areas of similar charge density and topography. However, the parallel, but non-superimposable, retention plots of log

$\bar{k}$ *versus* log $1/\bar{c}$ shown for CA-2 and CA-3 in Fig. 4a–h, indicate that the ionotopes of these isoforms differ in their relative affinity for the stationary phase. The $Z_{c,VG}$ values for major isoform species CA-1 were found to be similar to those for CA-2 and CA-3 in four of the displacer salts investigated, *i.e.*, LiCl, LiBr, NaF and KBr. This observation reveals that the interactive ionotope for CA-1 has similar charge features to those present on CA-2 and CA-3. The $Z_{c,VG}$ values for CA-1, however, were typically smaller than those for CA-2 and CA-3 when NaCl, NaBr, KF, or KCl were used as displacer salts. The relative changes in $Z_{c,VG}$ between these charge variants appears to result from specific salt effects on the protein hierarchical structure influencing the electrostatic potential of the ionotopes.

The effect of salt type and varied flow (VF) gradient elution conditions on isoform $Z_{c,VF}$ values

The plots of log $\bar{k}$ *versus* log $1/\bar{c}$, (Fig. 5a–h) show the effect of salt type and flow-rate on the gradient retention behaviour for the various isoforms of carbonic anhydrase and ovalbumin. Flow-rates were varied between 0.1 and 2.0 ml/min, while the gradient time, t_G, was held constant at 17.1 min. At lower flow-rates many of the retention plots for the related isoforms were found to converge as selectivity, α, between the different species approached unity. Hence, the purification of protein isoforms is best accomplished at high flow-rates *e.g.* > 1 ml/min if a small gradient time, t_G is used in conjunction with the other chromatographic conditions of this study.

Tables III and IV shows the $Z_{c,VF}$ values ($\pm$ 1 S.D.) and the coefficients of determination (r^2) obtained from the regression analyses of the varied flow retention data shown in Fig. 5a–h for carbonic anhydrase and ovalbumin respectively. As is evident from Table III, the $Z_{c,VF}$ values for the carbonic anhydrase isoforms, CA-2 and CA-3 differed from the $Z_{c,VF}$ value for the major isoform CA-1. Furthermore, $Z_{c,VF}$ for CA-2 and CA-3 were similar only for the NaBr, KF or KBr salt systems. This result differs to that found under conditions of fixed flow-rate and varied gradient time where equivalent $Z_{c,VG}$ values for all three isoforms were obtained for the majority of displacer salts investigated. These data reveal that under conditions of varied flow, the ionotopes of CA-2 and CA-3 exhibit similar charge densities and topographic locations when eluted with either NaBr, KF or KBr. The remaining five salts in the series have therefore changed relative charge densities of the binding domains of these isoforms through specific ion-binding effects or have altered the displacement mechanism of these charged variants.

The trends in the relative magnitudes of the $Z_{c,VF}$ values (Table IV) for the ovalbumin variants were found to differ from the patterns observed with the $Z_{c,VG}$ values (Table II). For example, the isoforms Ov-a, Ov-c and Ov-d were observed under varied gradient time conditions (Table II) to have similar $Z_{c,VG}$ values when eluted with each type of displacer salt. Inspection of the data for varied flow-rate conditions (Table IV) shows that equivalent $Z_{c,VF}$ values were only obtained for the Ov-a, Ov-c and Ov-d proteins when eluted with LiCl, NaF, KCl or KBr. These results suggest that the isoforms Ov-a, Ov-c and Ov-d appear to interact through ionotopes of similar charge density with differing affinities for the sorbent. However, their $Z_{c,VF}$ values for Ov-a, Ov-c and Ov-d became significantly different in the presence of LiBr, NaCl, NaBr or KF, presumably as a result of specific salt effects which perturb the interactive ionotopes of these three protein variants. Alternatively, these three ovalbumin

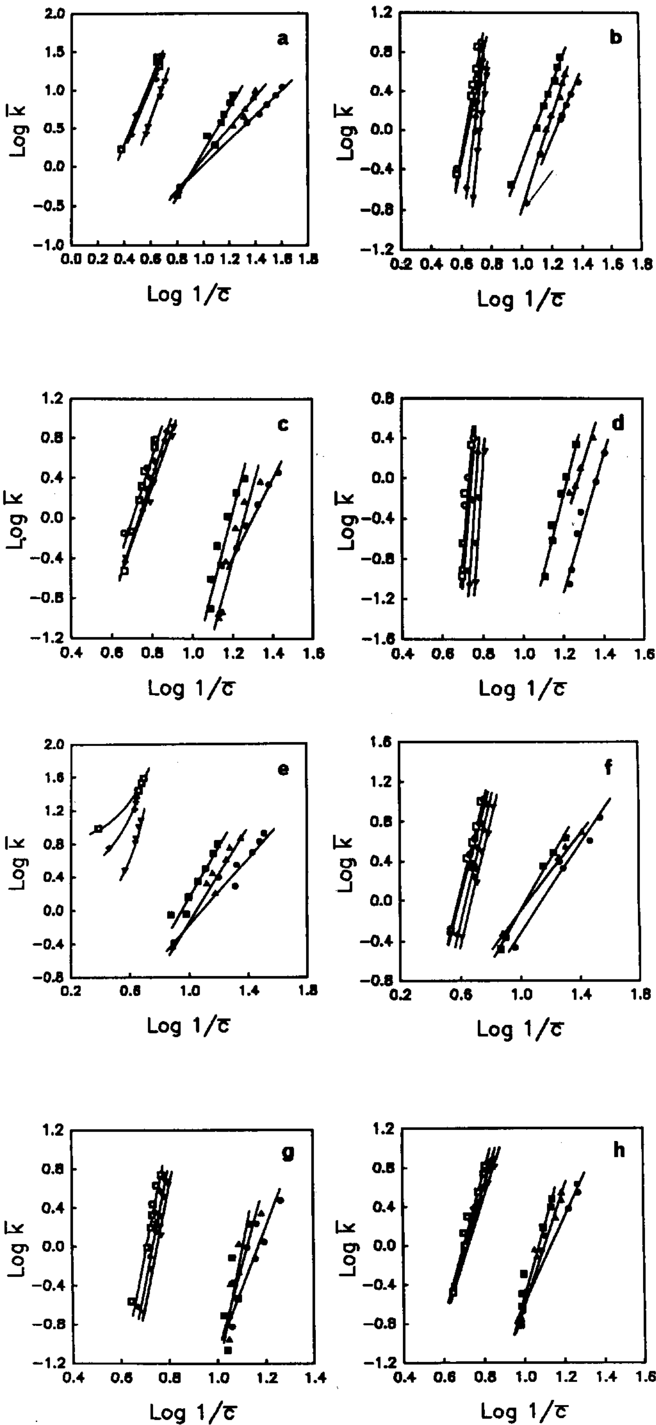

Fig. 5. Plots of log $\overline{k}$ *versus* log $1/\overline{c}$ for bovine carbonic anhydrase isoforms 1, 2 and 3 and ovalbumin isoforms a, b, c and d. The plots were derived from gradient elution data at pH 9.6, gradient time of 17.1 min and flow-rate varying between 0.2 and 2.0 ml/min for (a) NaF, (b) NaCl, (c) NaBr, (d) LiCl, (e) KF, (f) KCl, (g) KBr and (h) LiBr. See legend to Fig. 4 for other details.

TABLE III

Z_c VALUES FOR CARBONIC ANHYDRASE VARIANTS OBTAINED BY LINEAR REGRESSION OF VARIED FLOW-RATE EXPERIMENTS

Coefficients of determination are given in parentheses.

Salt	*Protein*		
	CA-1	*CA-2*	*CA-3*
LiF	Not done	Not done	Not done
LiCl	6.76 ± 0.80 (0.95)	4.74 ± 0.49 (0.98)	8.10 ± 0.73 (0.97)
LiBr	4.16 ± 0.40 (0.96)	5.56 ± 0.57 (0.95)	6.77 ± 0.75 (0.94)
NaF	1.57 ± 0.07 (0.99)	2.12 ± 0.10 (0.99)	2.93 ± 0.29 (0.95)
NaCl	3.31 ± 0.16 (0.99)	4.76 ± 0.22 (0.99)	3.83 ± 0.18 (0.99)
NaBr	4.53 ± 0.53 (0.95)	6.87 ± 0.99 (0.91)	6.90 ± 0.97 (0.91)
KF	2.01 ± 0.22 (0.95)	2.81 ± 0.30 (0.95)	2.90 ± 0.35 (0.93)
KCl	2.17 ± 0.24 (0.97)	2.01 ± 0.07 (1.00)	2.60 ± 0.13 (0.99)
KBr	5.95 ± 1.19 (0.83)	7.82 ± 2.23 (0.71)	9.53 ± 3.22 (0.64)
LiF	Not done	Not done	Not done
NaF	1.57 ± 0.07 (0.07)	2.12 ± 0.10 (0.99)	2.93 ± 0.29 (0.95)
KF	2.01 ± 0.22 (0.95)	2.81 ± 0.30 (0.95)	2.90 ± 0.35 (0.99)
LiCl	6.76 ± 0.80 (0.95)	4.74 ± 0.49 (0.98)	8.10 ± 0.73 (0.97)
NaCl	2.94 ± 0.13 (0.99)	4.76 ± 0.22 (0.99)	3.83 ± 0.18 (0.99)
KCl	2.17 ± 0.24 (0.97)	2.01 ± 0.07 (1.00)	2.60 ± 0.13 (0.99)
LiBr	4.16 ± 0.40 (0.96)	5.56 ± 0.57 (0.95)	6.77 ± 0.75 (0.94)
NaBr	3.59 ± 0.18 (0.99)	6.87 ± 0.99 (0.91)	6.90 ± 0.97 (0.91)
KBr	5.95 ± 1.19 (0.83)	7.82 ± 2.23 (0.71)	9.53 ± 3.22 (0.64)

isoforms may interact through identical ionotopes but are eluted via different displacement mechanisms.

The relationship between Z_c values for Ov-a and Ov-b eluted under varied flow-rate experiments was also different to that found under conditions of varied gradient time. Similar $Z_{c,VF}$ values were obtained for both variants in the different salt systems. Furthermore, the use of LiBr, NaF, KF or KBr as displacer salts resulted in both isoforms eluting as a single peak under conditions of varied flow-rate. The ramifications of these observations are that if a matrix of displacing salts is used, then selectivity optimisation of the IEC of proteins can be rationally achieved under conditions of varied gradient time or flow-rate.

These results also indicate that subtly different modes of gradient elution can be employed to influence the electrostatic interactive surface of proteins, presumably as a consequence of the contact or residency requirements once the protein has been adsorbed. Previous studies[12,13] have shown that both the type of salt and the column residence time can influence the Z_c value of proteins in HPIEC. This observation suggests that changes in the physicochemical basis of the sorption and desorption process can be induced by experimental factors which are commonly used to manipulate the elution time. Column residence time is one factor which differentiates the sorption conditions in varied gradient time and varied flow-rate experiments. As

TABLE IV

Z_c VALUES FOR OVALBUMIN VARIANTS OBTAINED BY LINEAR REGRESSION OF VARIED FLOW-RATE EXPERIMENTS

Coefficients of determination are given in parentheses.

Salt	*Protein*			
	Ov-a	*Ov-b*	*Ov-c*	*Ov-d*
LiF	Not done	Not done	Not done	Not done
LiCl	20.40 ± 3.57 (0.92)	24.73 ± 6.59 (0.88)	21.56 ± 6.75 (0.84)	24.28 ± 6.61 (0.87)
LiBr	7.45 ± 0.68 (0.96)	7.45 ± 0.68 (0.96)	5.95 ± 0.32 (0.99)	6.49 ± 0.42 (0.98)
NaF	4.09 ± 0.36 (0.99)	4.09 ± 0.36 (0.99)	4.81 ± 0.27 (0.99)	3.98 ± 0.41 (0.97)
NaCl	7.42 ± 0.79 (0.95)	8.09 ± 1.40 (0.89)	12.42 ± 0.56 (0.99)	10.97 ± 0.56 (0.99)
NaBr	8.05 ± 0.36 (0.99)	8.22 ± 0.47 (0.98)	5.48 ± 0.32 (0.98)	6.29 ± 0.34 (0.99)
KF	4.43 ± 0.21 (1.00)	4.43 ± 0.21 (1.00)	6.70 ± 0.69 (0.98)	9.09 ± 0.91 (0.99)
KCl	5.65 ± 0.63 (0.95)	5.97 ± 0.88 (0.92)	5.98 ± 0.44 (0.98)	6.04 ± 0.51 (0.97)
KBr	10.92 ± 0.88 (0.97)	10.92 ± 0.88 (0.97)	12.49 ± 1.17 (0.96)	11.56 ± 1.08 (0.96)
LiF	Not done	Not done	Not done	Not done
NaF	4.09 ± 0.36 (0.99)	4.09 ± 0.36 (0.99)	4.81 ± 0.27 (0.99)	3.98 ± 0.41 (0.97)
KF	4.43 ± 0.21 (1.00)	4.43 ± 0.21 (1.00)	6.70 ± 0.69 (0.98)	9.09 ± 0.91 (0.99)
LiCl	20.40 ± 3.57 (0.92)	24.73 ± 6.59 (0.88)	21.56 ± 6.75 (0.84)	24.28 ± 6.61 (0.87)
NaCl	7.42 ± 0.79 (0.95)	8.09 ± 1.40 (0.89)	12.42 ± 0.56 (0.99)	10.97 ± 0.56 (0.99)
KCl	5.65 ± 0.63 (0.95)	5.97 ± 0.88 (0.92)	5.98 ± 0.44 (0.98)	6.04 ± 0.51 (0.97)
LiBr	7.45 ± 0.45 (0.96)	7.45 ± 0.68 (0.96)	5.95 ± 0.32 (0.99)	6.49 ± 0.42 (0.98)
NaBr	8.05 ± 0.36 (0.99)	8.22 ± 0.47 (0.98)	5.48 ± 0.32 (0.98)	6.29 ± 0.34 (0.99)
KBr	10.92 ± 0.88 (0.97)	10.92 ± 0.88 (0.97)	12.49 ± 1.17 (0.96)	11.56 ± 1.08 (0.96)

the gradient time is increased protein solutes will progressively experience longer column residence times under chromatographic conditions of fixed flow-rate as well as being exposed, with long gradients, to very low rates of change in the displacer salt concentration. Under conditions of relatively long gradient time and very shallow gradients approaching isocratic conditions, there is sufficient time for the solute molecules to orientate themselves at the solute–stationary phase surface. This continuous "docking/re-docking" process will thus be very responsive to secondary equilibrium reactions that occur during or following sorption. Such dynamic interactive processes will have several consequences. Firstly, the equilibrium association constant may increase with time. Secondly, the Z_c value is anticipated to be influenced by the choice of t_G much more than by the choice of flow-rate condition. Thirdly, the increase in Z_c values or equilibrium binding constants observed as a protein unfolds with coulombic sorbents would also be consistent with these processes. Similar mechanisms may also contribute to the changes in the adsorption capacity observed with bath (batch) adsorption/desorption of proteins with preparative IEC and biomimetic affinity sorbents[16]. Under adsorption/desorption conditions where the three-dimensional shape of the protein, and more importantly the shape of the ionotope, is constant the magnitude of the Z_c values obtained for solutes eluted in this manner will reflect the maximum number of charged amino acid residue side

chains that contribute to the binding site on the molecule. Conversely, when the three-dimensional geometry of the protein's ionotope changes due to conformational transitions or salt-bridge perturbations then the magnitude of the Z_c values will vary depending on the salt type or column residence time. On this basis, related isoforms which are dynamically equivalent at the sorbent surface are unlikely to exhibit Z_c values which vary with a particular salt system. Similarly, when the protein isoforms are not dynamically equivalent, as may be revealed, for example, through differences in Z_c values obtained under constant and varied flow-rate conditions but with different salt systems, then the chromatographic ligand is effectively probing an ionotope of different molecular dimensions and/or charge density.

The effect of piperazine buffer concentration and varied gradient time on isoform Z_c values

Recent studies[1] have shown that changes in the eluent buffer concentration can also significantly influence solute retention in gradient HPIEC systems. Because of its buffer capacity over a wide pH range, piperazine has found numerous applications in IEC separation of proteins, particularly with eluents of basic pH value. In associated studies[1], piperazine concentrations of 40 m*M* or greater were found to significantly change the interactive properties of the CA-$II_{5.9}$ isoform in anion HPIEC under gradient elution conditions. To further investigate which factors influence protein binding sites in HPIEC, the effect of piperazine concentration on the retention of structural variants of the other bovine carbonic anhydrase isoforms and ovalbumin was investigated. The $Z_{c,VG}$ values corresponding to different piperazine conditions were obtained from regression analysis of plots for log $\bar{k}$ *versus* log $1/\bar{c}$ for these proteins eluted under gradient conditions. Solute species were eluted from a Mono-Q strong-anion-exchange column using a sodium chloride gradient increasing linearly between 0 and 300 m*M*. Piperazine concentrations of 0, 5, 10, 20, 30, 40 and 80 m*M* were selected for use in this study. At each buffer concentration, solute retention data were obtained for four different gradient times, *i.e.*, t_G = 17.1, 34.3, 60 and 100 min. The Z_c values obtained for each isoform are shown in Tables V and VI and are plotted against the piperazine concentration in Fig. 6a and b.

TABLE V

Z_c VALUES FOR CARBONIC ANHYDRASE VARIANTS OBTAINED BY LINEAR REGRESSION OF VARIED BUFFER EXPERIMENTS

Coefficients of determination are given in parentheses.

Piperazine (mM)	*Protein*		
	CA-1	*CA-2*	*CA-3*
80	1.00 ± 0.11 (0.98)	1.28 ± 0.07 (0.99)	1.54 ± 0.06 (1.00)
40	1.96 ± 0.51 (0.88)	2.49 ± 1.02 (0.75)	2.56 ± 0.61 (0.90)
30	4.20 ± 0.53 (0.97)	5.36 ± 0.07 (1.00)	3.77 ± 0.96 (0.89)
20	4.09 ± 0.28 (0.99)	4.56 ± 0.65 (0.96)	5.21 ± 0.30 (0.99)
10	4.09 ± 0.40 (0.98)	5.71 ± 0.34 (0.99)	5.58 ± 0.16 (1.00)
5	4.36 ± 0.81 (0.94)	6.66 ± 0.61 (0.99)	6.24 ± 0.66 (0.98)
0	4.04 ± 1.51 (0.78)	6.00 ± 2.07 (0.89)	6.47 ± 1.57 (0.94)

TABLE VI

Z_c VALUES FOR OVALBUMIN VARIANTS OBTAINED BY LINEAR REGRESSION OF VARIED BUFFER EXPERIMENTS

Coefficients of determination are given in parentheses.

Piperazine (mM)	*Protein*			
	Ov-a	*Ov-b*	*Ov-c*	*Ov-d*
80	6.63 ± 0.57 (0.99)	8.40 ± 0.19 (1.00)	6.77 ± 0.27 (1.00)	6.58 ± 0.21 (1.00)
40	6.31 ± 0.80 (0.97)	7.12 ± 0.99 (0.96)	6.14 ± 0.71 (0.97)	6.27 ± 0.76 (0.97)
30	9.42 ± 0.57 (0.99)	12.08 ± 0.64 (0.99)	9.71 ± 0.48 (1.00)	9.53 ± 0.19 (1.00)
20	9.00 ± 0.42 (1.00)	10.56 ± 0.50 (1.00)	9.04 ± 0.29 (1.00)	9.20 ± 0.53 (0.99)
10	9.29 ± 0.32 (1.00)	10.52 ± 0.29 (1.00)	9.42 ± 0.33 (1.00)	9.51 ± 0.29 (1.00)
5	9.73 ± 0.06 (1.00)	11.70 ± 0.77 (0.99)	9.76 ± 0.43 (1.00)	10.14 ± 0.27 (1.00)
0	12.20 ± 4.64 (0.87)	13.97 ± 2.66 (0.97)	40.33 ± 18.42 (0.83)	23.27 ± 4.38 (0.97)

Examination of the data shown in Table V and Fig. 6a reveals that the effects of the piperazine concentration on the Z_c values for CA-1 differed to those for CA-2 and CA-3. Between 0 and 30 mM piperazine, the Z_c values for CA-1 remained constant and independent of the buffer concentration. At piperazine concentrations > 30 mM the amount of positively charged piperazine buffer complexing onto the surface of the protein and sorbent was sufficient to induce a change in the protein–sorbent interactive properties, causing significant decreases in $Z_{c,VG}$. In contrast CA-2 and CA-3 had similar $Z_{c,VG}$ values at each buffer concentration which progressively decreased over

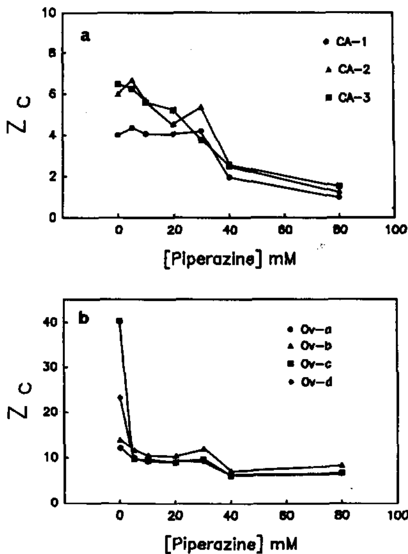

Fig. 6. Plots of Z_c *versus* the concentration of piperazine buffer under conditions of gradient elution with gradient times between 17.1 and 100 min and flow-rate of 1 ml/min for (a) carbonic anhydrase isoforms 1, 2 and 3 and (b) ovalbumin isoforms a, b, c and d.

the range 0 to 80 m*M* piperazine. Overall, these results indicate that the ionotopes of CA-2 and CA-3, although similar in charge density, were susceptible to changes in piperazine concentration. In the absence of buffer, the $Z_{c,VG}$ values for each of the carbonic anhydrase variants represent the maximum number of electrostatic interactions that can occur between each charge variant and the stationary phase in an aqueous environment at high pH.

Inspection of the data in Table VI and Fig. 6b reveals that the $Z_{c,VG}$ values for Ov-a, Ov-c and Ov-d did not vary significantly between concentrations of 10 to 80 m*M* piperazine. The complexation of both uni- and bivalent positively charged piperazine ions onto the surface of these proteins, above a critical buffer concentration of *ca.* 10 m*M*, appears to generate a coulombic binding surface on these isoforms of similar size. As the buffer concentration approaches zero, *i.e.*, below 10 m*M*, the $Z_{c,VG}$ values for the two isoforms Ov-c and Ov-d increase significantly. In the absence of buffer, more negatively charged sites on Ov-a, Ov-c and Ov-d become accessible for interaction with the stationary phase surface. The $Z_{c,VG}$ values obtained at 0 m*M* piperazine thus represent an estimate of the maximum number of electrostatic interactions that can occur between the solute and stationary phase in an aqueous environment at pH 9.6. The decrease in $Z_{c,VG}$ values observed with increasing buffer concentration over the range 0–10 m*M* is consistent with the stoichiometric displacement model[14,15]. These dramatic changes in the magnitude of $Z_{c,VG}$ provide further support for the conclusion that over the range 0–10 m*M* piperazine Ov-a and Ov-b have similar, if not identical, ionotopic areas whilst in comparison, significantly different ionotopic areas are manifested by Ov-c and Ov-d at 0 m*M* piperazine. Such large differences seen in the Z_c values of protein isoforms as the buffer concentration is decreased may be a result of conformational changes. The protein isoform that is least conformationally stable under conditions of very low ionic strength will unfold to the greatest extent, and additionally accessible amino acid residues will contribute to further electrostatic interactions. This behaviour will be manifested as a large increase in Z_c at a particular buffer concentration. Such behaviour has already been proposed for subtilisin variants[13], lysozymes[11,14,18] and myoglobins[18]. If a similar phenomenon is occurring with the ovalbumin isoforms then on the basis of the changes in Z_c values observed for the above elution conditions, the relative conformational stability of the three isoforms in the absence of piperazine buffer follows the order Ov-a > Ov-d > Ov-c.

CONCLUSION

In this study the ability of HPIEC systems to probe subtle changes in the tertiary structure of several protein variants has been investigated. The use of structural variants of proteins permits the existence of different ionotopes on protein surfaces to be clearly delineated. These results further contribute towards an understanding of the factors which influence the electrostatic interactions occurring between the charged surfaces of the protein and the sorbent. This information can also be used to selectively manipulate the interactive properties of proteins for the purpose of improving resolution in chromatographic separations. For example, changes in the piperazine buffer concentration were found to influence the interactive behaviour of protein variants. Manipulation of the buffer concentration provides one additional avenue to regulate protein binding behaviour during the optimisation of chromatographic

separations where very high resolution is required *e.g.* with protein variants. These studies also clearly demonstrate that specific manipulation of displacing salt conditions in terms of salt type or rate of change of salt concentration can significantly enhance the selectivity of closely-related protein molecules.

The results presented here have important implications in studies on protein microheterogeneity and demonstrate the utility of HPIEC to complement other techniques such as IEF and chromatofocusing for the characterisation of microheterogeneous proteins derived from natural or recombinant DNA sources. Associated investigations underway in this laboratory will further characterise the influence of changes in the ionotopic structure of proteins and peptides as the biosolute approaches the stationary phase surface, through correlation of the chromatographic behaviour of several proteins with their three-dimensional structures determined by X-ray crystallographic and nuclear overhauser NMR spectroscopic procedures.

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