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Influence of temperature on the retention behaviour of proteins in cation-exchange chromatography☆

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Abstract

The chromatographic behaviour of several amino acid derivatives, peptides and proteins has been investigated with the so called "tentacle-type" LiChrospher-100 SO_3^- adsorbent and an analogous poly(2-sulphoethylaspartamide) cation-exchange adsorbent, PolySulphoethyl A. In particular, the dependences of the retention properties of a range of biosolutes on temperature and the chromatographic residence time were evaluated with the objective of gaining further insight into the influence of ligand type and flexibility and the role of solute conformation on the chromatographic behaviour of proteins with these two strong cation-exchange chromatographic adsorbents. The results indicate that significant differences in the chromatographic retention behaviour between proteins and low-molecular-mass solutes occur as a function of temperature and the type of co- and counter ions present in the mobile phase with both adsorbents. Moreover, the dependences of the Z_c and log K_c values on temperature for most of the proteins examined exhibited significant changes in magnitude between 4 and 75°C, whilst no equivalent changes were evident for low-molecular-mass solutes. With the "tentacle-type" LiChrospher-1000 SO_3^- adsorbent at higher temperatures, e.g., at 75°C, most of the proteins could still be eluted although several exhibited very large increases in their retention parameters. With the PolySulphoethyl A adsorbent, on the other hand, none of the proteins examined were eluted at 75°C. The results moreover indicate that hydrophobic interactions play an increasingly important role in protein retention with both types of ion-exchange adsorbents at higher temperatures, but are more dominant with the PolySulphoethyl A ligand. In general, the Z_c values for the proteins with the "tentacle-type" LiChrospher-1000 SO_3^- adsorbent were greater than those obtained with the PolySulphoethyl A adsorbent, suggesting that the "tentacular" ligands present on this strong cation-exchange adsorbent interact with protein molecules through larger contact areas. Collectively, these investigations provide further support for the concept that the adsorption behaviour of proteins with the "tentacle-type" LiChrospher-1000 SO_3^- adsorbent and similar types of "tentacular" ligand systems involves a multilayer dissolution mechanism with the protein interacting with a more diffuse or extended Donnan double layer in the ion-exchange environment, resulting in multi-site binding processes.

Keywords: Temperature effects; Adsorbents; Proteins

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☆ Part CLIII in the series "High-performance liquid chromatography of amino acids, peptides and proteins". For Part CLII, see Ref. [50].

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1. Introduction

The behaviour of proteins in high-performance ion-exchange chromatography (HPIEC) is well known to be influenced by a variety of experimental parameters. In particular, various studies have shown (see, e.g., [1–5]) that the interaction between a protein and an electrostatic adsorbent can be significantly affected by the choice of buffer type, the nature of the co- and counter-ions present in the eluent, the pH conditions used, the flow-rate and the mode of delivery of the mobile phase. Collectively, these studies have also demonstrated that the distribution of the surface charge on the protein, but not the net charge, is an important controlling factor in the electrostatic interaction between a protein solute and an HPIEC adsorbent.

In previous studies [12–14], the relationships between solvent composition and protein structure with several "tentacle-type" anion-exchange adsorbents have been investigated. In the present study, a systematic analysis of the retention behaviour of several proteins and other smaller biosolutes with a "tentacle-type" strong cation exchanger, LiChrospher 1000 SO_3^-, is described. These studies were undertaken with particular reference to the influence of temperature on the characteristics of the electrostatic interactions which occur between proteins and this type of "tentacle-type" cation-exchange adsorbent surface. In addition, the derived chromatographic results were compared with the retention behaviour observed for the same group of solutes under similar temperature conditions using PolySulphoethyl A adsorbent, which also involves a linear grafted polymeric coating, but one based on the incorporation of taurine into cross-linked chains of polysuccinimide immobilized on aminopropylsilica [15].

2. Experimental

2.1. Chemicals and reagents

Bovine erythrocyte carbonic anhydrase, sperm whale myoglobin (type III), hen egg white lysozyme (grade 1), hen egg white ovalbumin, bovine ribonuclease A (type III A), bovine insulin, horse heart cytochrome *c*, soya bean trypsin inhibitor, dansyl-L-arginine, angiotensin-I, angiotensin-II, angiotensin-III, piperazine, Bis-Tris and triethanolamine were purchased from Sigma (St. Louis, MO, USA). Characterization of the proteins followed the procedures described previously [8,12]. Sodium chloride, hydrochloric acid (specific gravity 1.16), sodium acetate and calcium chloride were all AnalaR-grade reagents from Merck Australia (Kilsyth, Australia). Quartz-distilled water was purified with a Milli-Q system (Millipore, Bedford, MA, USA).

2.2. Chromatographic apparatus

All chromatographic studies were carried out with a System Gold chromatographic system from Beckman Instruments (Fullerton, CA, USA), which included a Model 126 programmable solvent module, a Model 166 programmable detector module and a Model 507 autosampler molecule, a System Gold computer system and an Epson LX-400 printer. The LiChrospher 1000 SO_3^- cation-exchange adsorbent was obtained as prepacked columns of dimensions 50 mm × 10 mm I.D. from Merck (Darmstadt, Germany) and the PolySulphoethyl A cation-exchange adsorbent (packed into columns of dimensions 100 mm × 4.6 mm I.D.) was obtained from Poly LC (Columbia, MD, USA). The temperature of the columns was controlled by a column jacket connected to a Model 2209 Multitemp thermostater (Pharmacia, Uppsala, Sweden) with circulating coolant.

2.3. Mobile phase preparation

Solvent A was 0.05 *M* sodium acetate (pH 4.0) and Solvent B was 0.05 *M* sodium acetate containing 1.0 *M* NaCl or 1.0 *M* $CaCl_2$ (pH 4.0). Both eluents were adjusted to the specified pH of 4.0 with 10 *M* HCl using an Orion SA 520 pH meter (Orion, Cambridge, MA, USA). The eluents were then filtered through HAWP 04700 0.45-μm cellulose acetate filters from Millipore

and degassed under vacuum or sparged with helium.

2.4. Sample preparation

The dansylarginine sample was dissolved in buffer A at a concentration of 0.5 mg/ml. The protein and peptide samples were also dissolved in buffer A but at a concentration of 5 mg/ml. These samples were either filtered through 0.22-μm ACRO LC13 filters (Gelman Sciences, Sydney, Australia) or centrifuged with a Eppendorf 5413 centrifuge at 1150 rpm for 10 min and the filtrates/supernatants collected. The samples were stored in a refrigerator at 4°C prior to use.

2.5. Chromatographic procedures

All chromatographic experiments were performed by using a linear salt gradient from 0 to 100% of the solvent B at a constant flow-rate of 1 ml/min. The gradient times chosen for these investigations were 20, 40, 60, 80 and 100 min. Between each gradient elution experiment, solvent A was re-introduced at a flow-rate of 1 ml/min for 20 min as part of the re-equilibration process. The column dead time (t_0) was determined from the breakthrough peak of an unretained salt (e.g., NaCl) by injection of 50 μl of a 1 *M* solution of the salt with 100% solvent A isocratically delivered to the system at a flow-rate of 1 ml/min. The gradient elapse time (t_e) was obtained by pumping solvent B into the chromatographic system with the inlet tube of the column directly connected to the detector. In the elution experiments, various amounts of the samples (10–50 μl) were injected individually or co-injected according to their retention time and peak height of the samples, so as to allow optimal separations to be achieved. All chromatographic data used in the generation of retention plots represented the average of replicated experiments. The chromatographic retention parameters such as the median capacity factor ($\bar{k}$), the median concentration of the displacing salt ($\bar{c}$) and the gradient steepness parameter (b) based on the assumptions of the LSS model [16] were derived using the Pekinese program written in our laboratory for the analysis of ion-exchange chromatographic data [5,11,13]. The retention data obtained from these experiments were subjected to iterative regression analysis of the plot of log $\bar{k}$ versus log $1/\bar{c}$ to determine the slope value (Z_c). The intercept values (log K_c) of these plots were determined by extrapolation of the $\bar{c}$ value to the limiting case of $c \rightarrow 10^{-6}$ mol/l, according to Eq. 1.

3. Results and discussion

3.1. Effect of temperature on protein retention behaviour

Column temperature is an important parameter which is often used to influence the chromatographic behaviour of molecules in order to enhance resolution. In particular, changes in temperature have been commonly used in this manner with reversed-phase high-performance liquid chromatography (RP-HPLC) of small molecules such as amino acids and peptides [17–24]. Variation of the column temperature can also be used to study changes in peptide and protein conformation which occur during the reversed-phase separation process. Such studies also provide insight into the thermodynamics of the surface interaction between the solute and adsorbent. For example, the folding and structural stability of peptides and proteins in RP-HPLC has been extensively investigated through analysis of the retention and band broadening behaviour under different temperature conditions [23,24].

The effect of temperature on the separation of ions and low-molecular-mass polar compounds with ion-exchange adsorbents has also been the subject of extensive investigation for more than 40 years. For example, Kraus and Raridon [25] and Bonner and Overton [26] over 35 years ago documented the effect of temperature on the chemical equilibria of various ions and counterions in ion-exchange processes. Similarly, Partridge and Brimley [27] were able to resolve L-proline, L-valine, L-methionine and a mixture of D- and L-leucine with two synthetic cation

exchangers at 60 and 80°C, whereas these amino acids were not resolved at room temperature. Evaluated column temperatures have also been widely used to achieve the improved resolution and sensitivity of detection as part of amino acid analysis with microparticulate ion-exchange adsorbents [28,29].

In contrast to the above representative examples of studies with low-molecular mass substances and ions, the effect of temperature on the HPIEC behaviour of higher molecular mass biosolutes, in particular proteins, has not been as extensively investigated. Part of the reason for the more limited literature on high-temperature HPIEC of proteins can be attributed to the fact that the equilibrium processes for the ion-exchange separation of proteins are more complex than for the separation of small molecules. In addition, the general concern held by many biologists that elevated temperatures will automatically lead to protein denaturation and precipitation has also been a significant factor. The lower viscosity of the mobile phase and the enhancement of the desorption kinetics at higher temperatures, however, offer the opportunity for improved peak shape and better resolution of closely related protein compounds, particularly in the analytical mode [20].

The use of elevated temperatures for the enhancement of the resolution of proteins in HPIEC has led, however, to some conflicting observations. For example, Vanecek and Regnier [30] reported that the resolution of ovalbumin isoforms from albumin with strong anion-exchange Synchropak SAX adsorbents increased when the temperature was raised from 4 to 25°C. In contrast, a decrease in resolution between β-lactoglobin and carbonic anhydrase [31] was observed with an increase in temperature with Partisil-10 SAX adsorbent. Similar dichotomous results have been observed with various other proteins [9–11,20]. Therefore, the effect of temperature on the ion-exchange chromatographic resolution of proteins cannot yet be readily predicted. However, the available evidence from other areas of chromatographic optimization suggests that variations in column temperature could represent a useful approach to adjusting the chromatographic resolution of proteins and other high-molecular-mass biomacromolecules. In addition, variations in the column temperature and the chromatographic residence time provide a straightforward avenue to investigating the role of protein hierachial structure in HPIEC, particularly since the changes in the secondary and tertiary structures of proteins, which can be induced within the temperature range 0–100°C, occur over a temperature increment where chromatographic separations can also be readily performed. As a consequence, studies on the ion-exchange chromatographic behaviour of proteins over a wide range of temperatures should both provide important information on the role of protein conformation in HPIEC systems and also allow optimization of resolution per se.

Protein retention with ion-exchange adsorbents primarily arises from electrostatic interactions between the protein surface and the charged surface of the adsorbent. A number of theoretical models based on empirical relationships or thermodynamic considerations have been developed to describe protein retention in HPIEC under isocratic and gradient elution conditions (see, e.g., [1,2,10–14,32–34]). Over a limited range of ionic strength conditions, the following empirical equation can be used to describe the gradient elution relationship between the median capacity factor of a protein solute, $\bar{k}$, and the corresponding median salt concentration, $\bar{c}$:

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

where $\log K_c$ is the intercept value of the $\log \bar{k}$ versus $\log 1/\bar{c}$ plot, and is related to the association constant, K_a, for the protein–ligand interaction when $\bar{c} \rightarrow 10^{-6}$ mol/l, and Z_c represents the slope. The magnitude of Z_c and $\log K_c$ at a defined temperature can thus be readily determined by iterative analysis of the plots of $\log \bar{k}$ versus $\log 1/\bar{c}$.

In order to investigate the respective contributions of the ion-exchange ligand structure and the conformational properties of the protein solutes in the retention phenomenon, a basic dansylamino acid derivative and several small

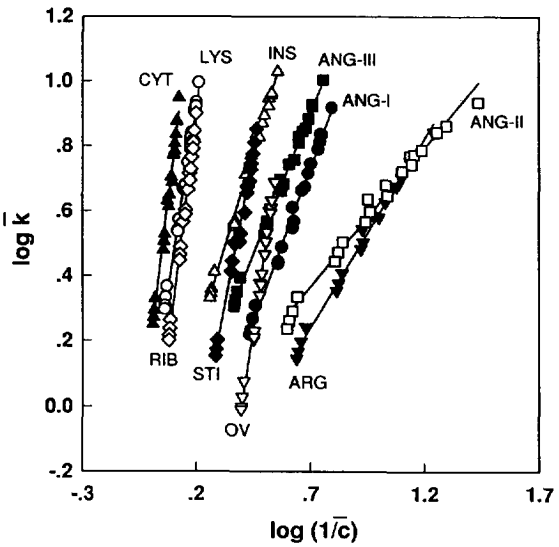

Fig. 1. Plots of log $\bar{k}$ versus log $1/\bar{c}$ with LiChrospher 1000 SO_3^- adsorbent at 25°C with NaCl as the displacer salt. Solutes: ● = ANG I; □ = ANG II; ■ = ANG III; ▼ = ARG; ▲ = CYT; △ = INS; ○ = LYS; ▽ = OV; ◇ = RIB; ◆ = STI. See Experimental for other details.

peptides were investigated as control solutes in this study. These control solutes are low-molecular-mass compounds which exist in solution without any significant secondary structure. Changes in the retention behaviour of these solutes over the temperature range studied will therefore not be related to variations in their conformational structure. In contrast, the conformational behaviour of proteins will be substantially influenced by temperature over the range available in these chromatographic studies. The progress of these temperature induced perturbations of the protein secondary and tertiary structure during the ion-exchange chromatographic separation can thus be monitored from the variations in the magnitude of the Z_c and log K_c values under different temperature conditions.

Fig. 1 shows representative plots of log $\bar{k}$ versus log $1/\bar{c}$ for each solute listed in Table 1 when eluted with 0–1 *M* NaCl as the displacing salt from the LiChrospher 1000 SO_3^- adsorbent at 25°C. The relative positions of the plots for these solutes allowed the experimental data to be considered in terms of three different groups. Clearly, with the availability of additional protein and polypeptide examples, a continuum in the retention dependence behaviour, revealed from the log $\bar{k}$ versus log $1/\bar{c}$ plots as a function of temperature, can be expected for small peptides through to large globular or fibrous proteins. The trends evident with the solutes employed in the present study consequently provide a framework around which the retention dependences of other polypeptides and proteins can be compared. The availability of additional thermodynamic data on the thermal stability of various proteins derived from independent spectroscopic or micro-

Table 1
Physical and chemical properties of the solutes studied

Solute (source)	Abbreviation	p*I*	Molecular mass
ε-Dansylarginine (synthetic)	ARG		444.1
Angiotensin III (synthetic) Arg–Val–Tyr–Ile–His–Pro–Phe	ANG-III		931.1
Angiotensin II (synthetic) Asp–Arg–Val–Tyr–Ile–His–Pro–Phe	ANG-II		1046.2
Angiotensin I (synthetic) Asp–Arg–Val–Tyr–Ile–His–Pro–Phe–His–Leu	ANG-I		1296.5
Insulin (bovine pancreas)	INS	5.32	5700
Ribonuclease A (bovine pancreas)	RIB	9.60	12640
Cytochrome *c* (horse heart)	CYT	11.00	12384
Lysozyme (hen egg white)	LYS	11.00	14300
Soybean trypsin inhibitor (soybean)	STI	4.55	20000
Carbonic anhydrase (bovine erythrocytes)	CA	5.89	30000
Ovalbumin (hen egg white)	OV	4.70	43000

calorimetric measurements would be very beneficial to the interpretation of the trends and variation noted in the parameters derived from the log $\bar{k}$ versus log $1/\bar{c}$ plots in the present investigations and similar recent studies by other investigators. The utilization of such extra-chromatographic approaches has been described elsewhere.

In the context of the present investigations, the first group, corresponding to proteins eluting with longer retention times, included CYT, LYS and RIB. The second group of proteins and peptides, eluting with intermediate retention times, included INS, STI, OV, ANG-I and ANG-III, while the third group, with the earliest elution times, included ANG-II and dansyl-ARG. Closer inspection of the retention data for the first group of solutes reveals that these proteins exhibited sharp increases in their capacity factors over a very narrow range of high salt concentrations. In addition, the p*I* values of this group of proteins (CYT, LYS and RIB) are between 9 and 11, i.e., over 5 units higher than the mobile phase pH value of 4.0. The second group of proteins and peptides exhibited smaller changes in their capacity factors over a wider range of intermediate salt concentrations. The p*I* values of these proteins and peptides were less than 2 units higher than the mobile phase pH value, i.e., p*I* 4.55–5.89. The third group of solutes, including the peptide ANG-II and dansyl-ARG, exhibited the smallest changes in their capacity factors over the lowest salt concentration range. These three elution patterns were evident at all temperatures except 4 and 75°C. It was apparent from these experimental data that all the log $\bar{k}$ versus log $1/\bar{c}$ plots were linear [the correlation coefficients (r^2) ranged between 0.90 and 0.999], indicating that the predominant free-energy changes involved in the interactions between these solutes and this "tentacle-type" absorbent were associated with electrostatic forces.

The retention behaviour of these solutes was further characterized in terms of the effect of changes in temperature on the magnitude of Z_c. Although an experimentally derived constant for a particular protein, adsorbent and chromatographic system, the Z_c values has been used as an empirical measure of the average number of charge groups contributing to the electrostatic characteristics of the portion of the solute surface in contact with the ion-exchange adsorbent. The Z_c values, calculated according to Eq. 1, of the different solutes chromatographed of the LiChrospher 1000 SO_3^- cation exchanger were plotted against temperature (Fig. 2a) to provide an indication of the extent of changes in the electrostatic contact area with increasing temperature. In addition, the corresponding intercept values of the log $\bar{k}$ versus log $1/\bar{c}$ plots, i.e., the log K_c values, were also plotted against temperature as shown in Fig. 2b. Inspection of the data in Fig. 2a and b indicates that the small control solutes exhibited relatively small Z_c and log K_c values compared with the corresponding

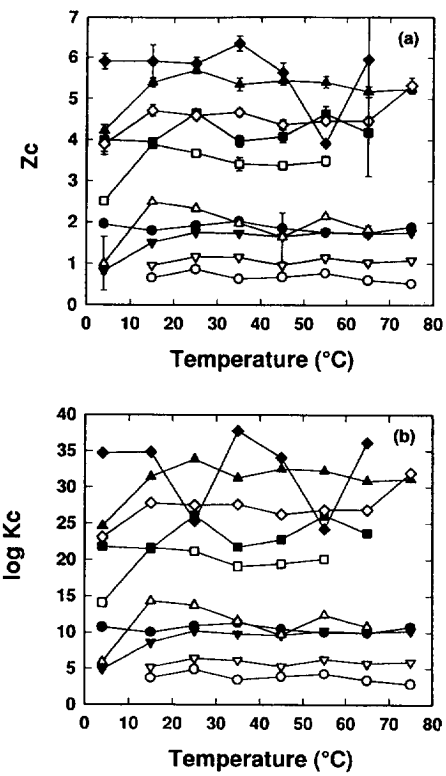

Fig. 2. Plots of (a) Z_c and (b) log K_c versus temperature with LiChrospher 1000 SO_3^- adsorbent at 25°C with NaCl as the displacer salt. Solutes: ● = ANG I; ▽ = ANG II; ▼ = ANG III; ○ = ARG; ◆ = CYT; △ = INS; ◇ = LYS; ■ = OV; ▲ = RIB; □ = STI. See Experimental for other details.

parameters for the larger protein solutes. For example, the control solutes such as dansyl-ARG exhibited a Z_c value of ca. 1 and the angiotensins had Z_c values of ca. 2. The magnitude of these values is consistent with the number of positively charged residues in the amino acid sequences of these solutes (Table 1). In contrast, the majority of the protein solutes, except for INS, had larger Z_c values, typically greater than 3. Dansylarginine and the small peptides contain only one, two or three positive charges and their electrostatic contact areas will be restricted to the fractional contribution of these charge groups. The small contact regions exhibited by this group of solutes are also associated with lower affinity or log K_c values. With the larger polypeptides and proteins, there are a larger number of positively charged residues which are surface accessible and which can interact with the ligand, leading to larger values of Z_c and log K_c However, the maximum value of Z_c is limited by the accessible area of contact and the distribution of the positive charges within this binding region(s) presented by the surface of the protein [1,35].

The nature of the dependence of the Z_c and log K_c values on temperature with LiChrospher 1000 SO_3^- adsorbent shown in Fig. 2a and b also differs between small and large solutes. The Z_c values of small molecules are relatively constant with increasing column temperature, whereas the Z_c values for the large molecules varied over the same temperature range. For example, more significant changes in the Z_c values for CYT, LYS and OV are clearly apparent in Fig. 2a, in contrast to the essentially constant Z_c values for dansyl-ARG and the angiotensin peptides. As noted above, the small control molecules such as dansyl-ARG and the angiotensins exist in solution as fully extended or random coil structures. Since these small molecules do not undergo any secondary or tertiary structural rearrangements, the number of charged groups exposed to the ligands will remain constant and the interaction will be reflected in essentially constant Z_c and log K_c values with increasing temperature. In contrast, the charged amino acid residues that contribute to the electrostatic contact region(s) of the protein surface will depend on the secondary and tertiary structures of the protein. Thus, the larger polypeptide and protein solutes INS, STI, LYS and RIB had relatively small Z_c values at 4°C, but these values increased significantly as the temperature was increased up to 25°C, after which the Z_c and log K_c values for each solute remained essentially constant.

The implication can be drawn from these observations that the retention mechanism with the low-molecular-mass solutes (e.g., dansyl-ARG, ANG-I, -II and -III) remains constant over this temperature interval, but that between 4 and 25°C the mechanism of ligand interaction with the larger polypeptides and proteins changes, presumably because, inter alia, these larger polyelectrolytes undergo conformational changes which result in increases in the magnitude of the electrostatic contact region and the overall binding affinity. Between 25 and 55°C, smaller changes in the Z_c and log K_c values were generally evident for most of the larger solutes, indicating that less significant variations in the secondary or tertiary structure of these solutes may be occurring in this temperature interval. At higher temperatures (e.g., 55–75°C), the degree of variation in the Z_c values for some proteins increased significantly again. For example, the Z_c values for CYT decreased to 3.9 at 55°C but increased again to 5.9 at 65°C, whereas CYT was not eluted at 75°C. Similarly, at 75°C, the Z_c and the log K_c values for LYS increased significantly whereas STI, INS, OV and CYT were also not eluted at this temperature.

When the tertiary structure of a protein changes, as occurs, for example, during unfolding, the contact region between a protein and immobilized ligands on the surface of a support material may increase owing to the distribution of internal salt bridge and exposure of additional charged amino acid residues. This unfolding process may thus allow additional electrostatic interactions between the protein and the adsorbent. Therefore, in a chromatographic environment, increases in the Z_c values of the protein may occur. Concurrently, the unfolded protein surface will exhibit to the chromatographic surface additional accessible hydrophobic amino acid residues. This rearrangement of

the surface structure of the protein at the liquid–solid adsorbent interface may induce or reinforce hydrophobic interactions between the protein and the coulombic adsorbent. The addition of an organic solvent to the mobile phase can be used to verify if any additional hydrophobic interactions are involved at higher temperatures. The elution profiles of CYT with LiChrospher 1000 SO_3^- at 65 and 75°C are shown in Fig. 3 with and without the addition of 20% (v/v) acetonitrile, respectively. It is evident that at 65 and 75°C CYT was not eluted until the organic solvent was added to the mobile phase, indicating the involvement of a significant hydrophobic component in the protein–ligand interaction with this ion-exchange system at these higher temperatures.

Fig. 4 illustrates, as histogram plots, the Z_c

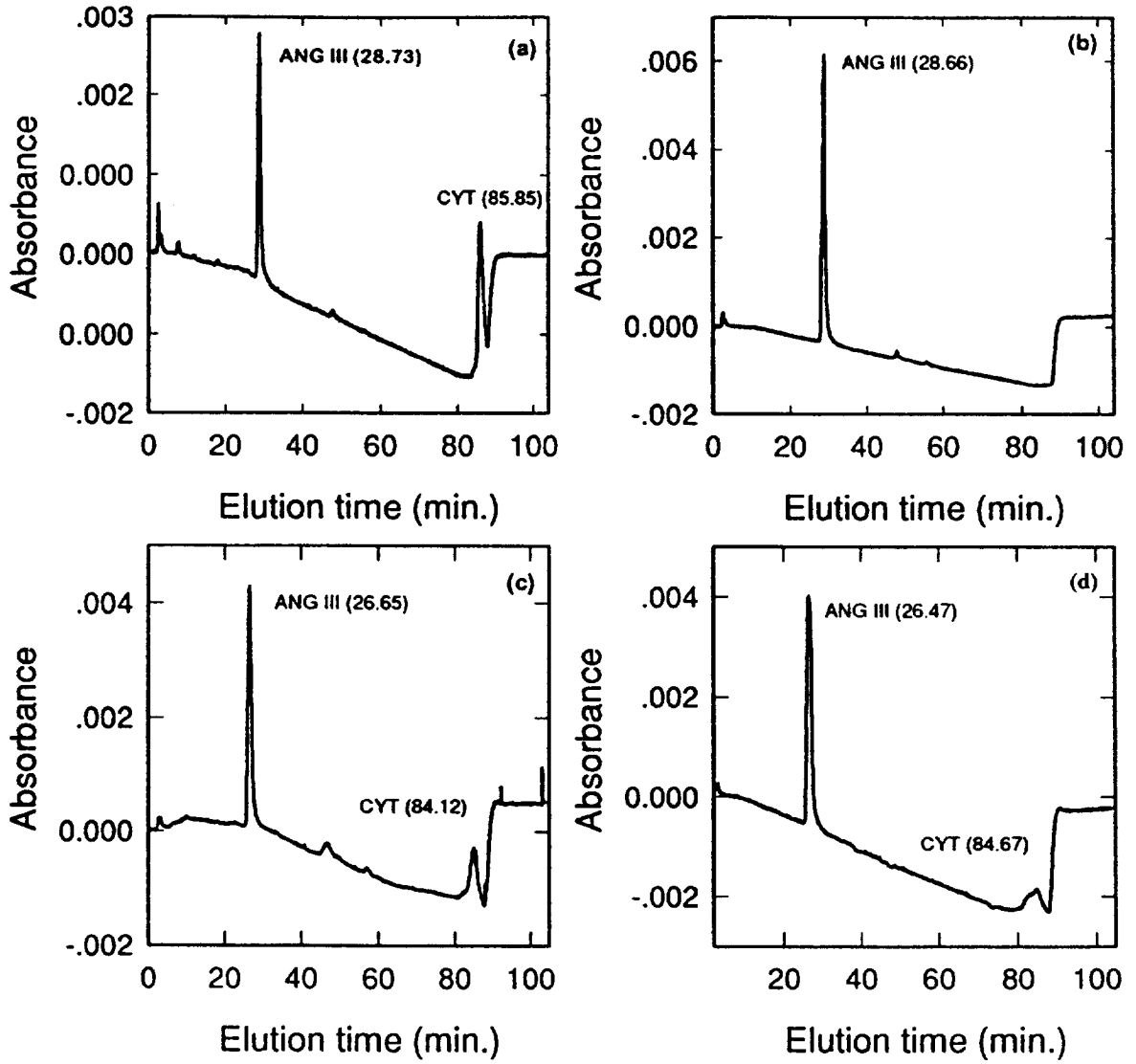

Fig. 3. Chromatograms demonstrating the influence of acetonitrile on the retention behaviour at high temperatures of CYT with LiChrospher 1000 SO_3^- adsorbent and NaCl as the displacer salt. (a) 65°C, no acetonitrile; (b) 75°C, no acetonitrile; (c) 65°C, with 20% acetonitrile in buffer B; (d) 75°C, with 20% acetonitrile in buffer B.

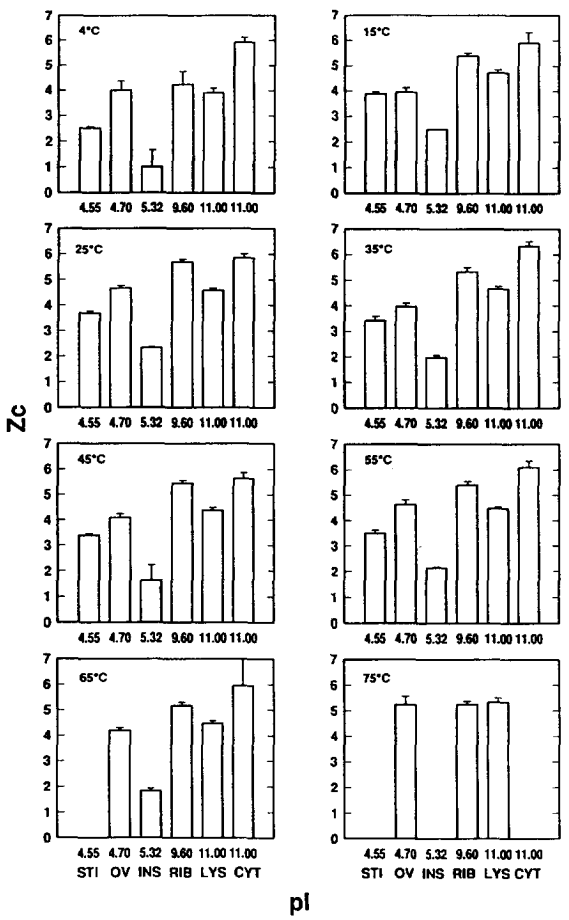

Fig. 4. Histogram representations of the protein Z_c values and pI values at each temperature with LiChrospher 1000 SO_3^- adsorbent and NaCl as the displacer salt. See Experimental for other details.

values of the different proteins compared with their corresponding pI values at each temperature tested. These data reveal that there is no simple additivity relationship between the Z_c value and the pI value. Similarly, as shown in Fig. 5, no simple dependence exists between the Z_c values for each protein and the corresponding molecular mass. Collectively, these results are consistent with the conclusions based on earlier experimental results, and also recent theoretical considerations of protein retention on ion-exchange surfaces [1,2,10,11,33–35], that retention models, such as the net charge hypothesis [36,37], based solely on considerations of the pI value, the net charge, the average surface area and molecular volume or the molecular mass of globular proteins are poor predictors of the retention behaviour of proteins in ion-exchange chromatography. The surface of a protein molecule is characterized by asymmetric distribution of charged amino acid residues, and this property results in regions of varying electrostatic potential. As the temperature increases and the conformation of the protein is disrupted, the electrostatic contact regions will become more extended, which in turn will influence the retention of the protein and the magnitude of the Z_c and log K_c values. Overall, these results reinforce the concept that the contact area between the protein solute and the ion-exchange adsorbent is not directly related to the overall charge of the protein or to its molecular size per se, but rather to the charge anisotropy and its relationship to the distribution of hydrophobic regions present on the protein surface. Analogous conclusions can be reached from the data reported recently in other publications, including the interaction of rat cytochrome b_5 with Mono-Q anion exchanger [38,39] and bovine serum albumin with Sepharose Fast Flow Q [40]. The plurality of this interplay of electrostatic and hydrophobic interactions in protein chromatography with ion-exchange adsorbents has been the subject of various fundamental investigations based on extensions of the Manning counter-ion condensation theory [11,41,42] and thermodynamic considerations [12–14,43].

3.2. Effect of the nature of the displacer salt on the dependence of protein retention behaviour on temperature

It is well known that different salts can influence the ion-exchange chromatographic behaviour of proteins owing to the chaotropic or kosmotropic nature of the cations and anions [5,11,44–47]. In previous studies [12–14], the influence of different monovalent anions on the retention behaviour of several proteins with "tentacle-type" anion-exchange adsorbents was investigated. The results demonstrated that the ionic radius and electronegativity of the mono-

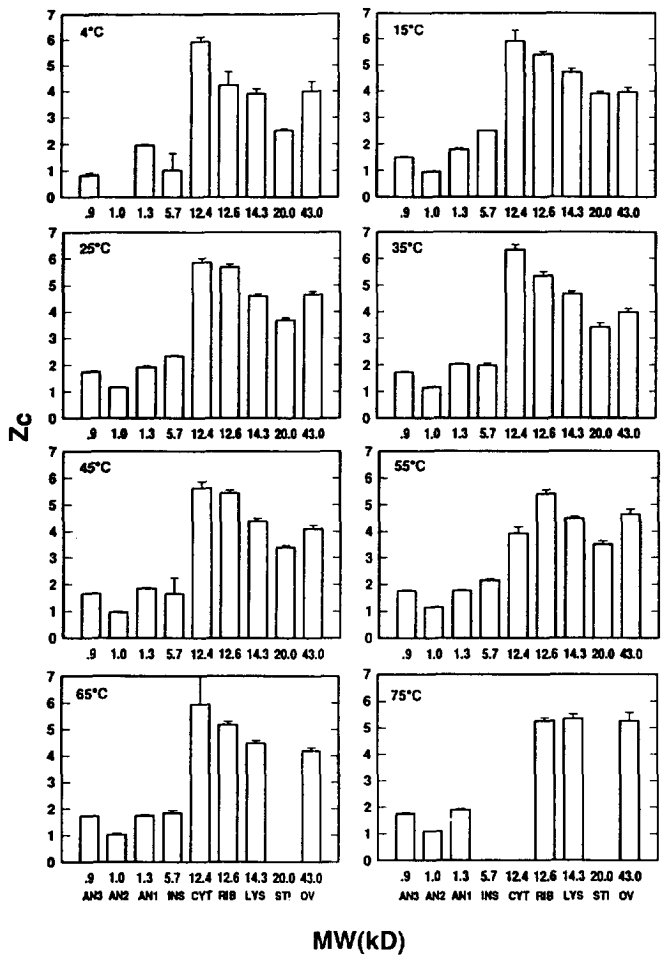

Fig. 5. Histogram representations of the protein Z_c values and the molecular mass values at each temperature with LiChrospher 1000 SO_3^- adsorbent and NaCl as the displacer salt. See Experimental for other details.

valent anion can significantly influence the chromatographic behaviour of proteins with this type of anion-exchange adsorbent. Systematic comparative studies on the effect of monovalent and divalent salts on protein retention behaviour with "tentacle-type" cation-exchange sorbents have not been reported. In the present studies, displacing salt concentrations over the range 0–1 *M* $CaCl_2$ were thus used for the cation-exchange chromatographic separation of the various solutes under similar chromatographic conditions to those described for the 0–1.0 *M* NaCl system.

The Z_c and log K_c values of the various solutes with 0–1.0 *M* $CaCl_2$ as the displacing salt were plotted against temperature (Fig. 6) and compared with the results obtained with 0–1.0 *M* NaCl. These comparative results show that the Z_c values of each solute were greater when NaCl was used as the displacer salt than for $CaCl_2$. Also, some of the proteins were not eluted at high temperatures when NaCl and was used as the displacer salt, but were eluted when $CaCl_2$ was present. For example, with the NaCl system, STI was not eluted at 65 and 75°C whereas OV, INS and CYT were not eluted at 75°C. However, with $CaCl_2$, all the proteins tested were eluted at both 65 and 75°C. Furthermore, the variations in the Z_c and log K_c values of the proteins were

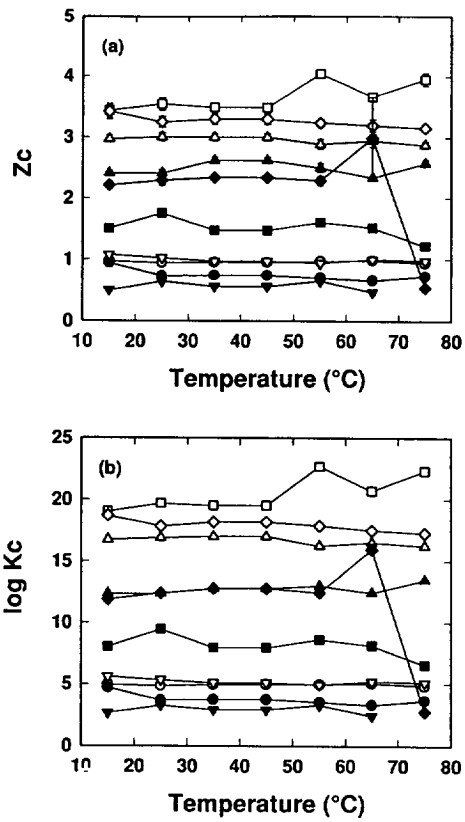

Fig. 6. Plots of (a) Z_c and (b) log K_c versus temperature with LiChrospher 1000 SO_3^- adsorbent at 25°C with $CaCl_2$ as the displacer salt. Solutes: ○ = ANG I; ● = ANG II; ▽ = ANG III; ▼ = ARG; □ = CYT; ■ = INS; △ = LYS; ▲ = OV; ◇ = RIB; ◆ = STI. See Experimental for other details.

observed to be much smaller with the $CaCl_2$ than with the NaCl elution system. The only exceptions were CYT and STI at 65 and 75°C. These observations suggest that both the nature of the cation and the ionic strength of the displacing salt can influence the retention behaviour and thus the magnitude of the Z_c values of protein solutes in cation-exchange HPIEC. Overall, these results confirm that the relative elutropic strength of 1.0 *M* $CaCl_2$ is greater than that of 1.0 *M* NaCl and the elution times of the solutes are reduced accordingly. In particular, the ability of cations to interact with the immobilized ligands and the dipoles of the polypeptide backbone and specific side-chain amino acid groups will modulate the apparent electrostatic charge characteristics of a protein, and these effects will contribute to the change in retention behaviour.

The influence of the valency and activity coefficients of the displacer salt on protein retention behaviour and surface structural characteristics of the protein and electrostatic ligand can be predicted from theoretical treatments of the ion-exchange chromatographic separation of proteins. According to the non-mechanistic stoichiometric model of protein retention behaviour in HPIEC [1,11,13,14], the influence of a divalent cation salt such as $CaCl_2$ on the retention behaviour of a protein in HPIEC can be evaluated in terms of the following relationship:

$$k' = K_a \cdot \frac{A_s}{V_m} \left(\frac{D_{bi}}{a_{ij} b_{ij} D_0 C_i} \right)^z (1 - f) \qquad (2)$$

where K_a, A_s and V_m are equilibrium constant for the interaction of the protein with the ion-exchange adsorbent, the accessible surface area of the adsorbent in m^2/g and the volume of the mobile phase, respectively, and z is the number of charge groups on the protein associated with the chromatographic adsorption/desorption process. The term D_{bi} relates to the initial ligand concentration, D_0 is the displacing ion concentration in mol/l and C_i is the concentration of counter-ions associated with the protein, i.e., the concentration of H^+ ions involved with the protein which are substituted by other cations during the cation-exchange binding events. The relative elutropic strength and activity coefficients of the displacing ions and counter-ions for the ion–protein and the ion–ligand interactions are represented by the terms a_{ij}, and b_{ij}, and the fraction of the adsorbent surface covered by the protein following the adsorption interaction is given by f. If it is assumed that near equilibrium conditions apply and the amount of the protein loaded on to the adsorbent is small, i.e., if only a small percentage of the adsorption capacity, q^*, is involved in the binding and the protein–ligand interaction occurs within a linear region of the adsorption isotherm, then the terms $f \rightarrow 0$ and D_{bi} will remain essentially constant. Under such conditions, the dependence of k' on the con-

centration of the participating ions can be represented by

$$k' = K_a \cdot \frac{A_s}{V_m} \cdot D_{bi}^{z}(a_{ij}b_{ij}D_0C_i)^{-z} \quad (3)$$

For a displacing salt with a divalent cation but a monovalent anion, each cation (e.g., Ca^{2+}) will cause the neutralization of two charge group interactions between the protein and the ligand, with the concentration of the M^{2+} cation, D_0 (in mol/l), required to maintain electroneutrality exactly equal to half of the concentration of the accompanying monovalent (e.g., Cl^-) counter-ions, C_i (in mol/l), associated with the positively charged protein when it is desorbed from the cation exchange adsorbent, and hence

$$k' = K_a \cdot \frac{A_s}{V_m} \cdot D_{bi}^{z}(a_{ij}b_{ij}D_0^{1.5})^{-z} \quad (4)$$

The logarithmic form of Eq. 4 can be expressed, for the case of $CaCl_2$ as the displacing salt, in the following terms:

$$\log k' = \log\left(K_a \cdot \frac{A_s}{V_m} \cdot D_{bi}^{z}\right) + z \log\left(\frac{1}{a_{ij}b_{ij}[Ca^{2+}]^{1.5}}\right) \quad (5)$$

or alternatively

$$\log k' = \log K_i + z \log\left(\frac{1}{a_{ij}b_{ijj}}\right) + 1.5z \log\left(\frac{1}{[Ca^{2+}]}\right) \quad (6)$$

where

$$K_i = K_a \cdot \frac{A_s}{V_m} \cdot D_{bi}^{z} \quad (7)$$

According to Eqs. 2–5, the magnitudes of the slope terms, i.e., the Z_c values, of the isocratically derived log k versus log $1/c$ plots or the gradient-derived log $\bar{k}$ versus log $1/\bar{c}$ plots are predicted to be dependent on the valency of the displacing salt. It can also be seen from Eqs. 2–4 that the relationship between the slope term Z_c and the effective charge term z can be represented by $2z = Z_c$, provided that the valences of the displacing ion and counter-ion are unity, the activity coefficient terms a_{ij} and b_{ij} are independent of the concentrations of the protein, the ligand chains, the displacing ion, the counter-ion and the temperature and the magnitude of the product of a_{ij} and b_{ij} is close to 1. When the valency, n, of the displacing ion is >1, but the counter-ion remains monovalent, then the relationship predicted by Eqs. 1 and 5 follows the dependence $[(n+1)z]/n = Z_c$, again assuming that the value of $a_{ij}b_{ij} \rightarrow 1$ and the other criteria listed above prevail.

The above approach, in principle, allows the effect of the valency and the activity coefficients of different displacer salts to be considered in the evaluation of the Z_c or the corresponding z values. For example, if the retention results for a series of proteins chromatographed with the sample adsorbent, column, flow-rate, elution conditions, buffer composition, pH and temperature, but with NaCl or $CaCl_2$ as the displacing salt, are compared, then the relative differences between these two salt systems can be evaluated in terms of a free energy selectivity parameter, τ^*, such that

$$\tau^* = \log \bar{\alpha} = \log(\bar{k}_{NaCl}/\bar{k}_{CaCl2}) \quad (8)$$

or

$$\tau^* = [\Delta G^\circ_{(CaCl_2)} - \Delta G^\circ_{(NaCl)}]/2.3\ RT = \Delta\Delta G^\circ_{(int,iex)}/2.3\ RT \quad (9)$$

where $\Delta^\circ_{(CaCl_2)}$ and $\Delta G^\circ_{(NaCl)}$ are the Gibbs free energy for the protein–ligand interaction in the presence of $CaCl_2$ and NaCl, respectively, $\Delta\Delta G^\circ_{(int,iex)}$ is the difference in Gibbs free energy for the ion-exchange interaction at the same standard state for these two displacing salt systems, R is the gas constant and T is the absolute temperature.

By utilizing Eqs. 2–7, the following theoretical description for the salt selectivity effect can be obtained for the NaCl (represented below as system 1) and $CaCl_2$ (system 2) elution conditions (and similar expressions would apply for other monovalent or multivalent salt elution conditions), assuming that the chromatographic phase ratio remains constant for the two salt

systems, and the same number of charge groups accessible on the surface of the protein are involved in the interaction with the coulombic ligands, namely

$$\tau^* = \log\left(\frac{K_{a,1}D_{bi,1}^{z}[a_{ij,2}b_{ij,2}D_{0,2}C_{i,2}]^{z}}{K_{a,2}D_{bi,2}^{z}[a_{ij,1}b_{ij,1}D_{0,1}C_{i,1}]^{2}}\right) \quad (10)$$

If the same adsorbent and column are employed, then the initial ligand concentration, D_{bi}, will be identical for the NaCl and the $CaCl_2$ experimental conditions, and Eq. 10 can be simplified to

$$\tau^* = \log\left\{\frac{K_{a,1}(a_{ij,2}b_{ij,2}[Ca^{2+}]^{1.5})^{z}}{K_{a,2}(a_{ij,1}b_{ij,1}[Na^{+}]^{2})^{z}}\right\} \quad (11)$$

As noted above, the τ^* values for a series of proteins, investigated under identical chromatographic conditions, represent the incremental differences in Gibbs free energies manifested by the two salt systems. Hence, from Eqs. 8–11, as the temperature is varied over the same range, linear dependences of the τ^* values on the reciprocal of the temperature are predicted for a series of different proteins, whilst the ratio of the $Z_{c,1}$ and $Z_{c,2}$ values for a particular protein should remain constant, provided that the individual a_{ij} and b_{ij} terms are independent of the temperature and the characteristics (solvated ionic radius, charge density, etc.) of the ions of the displacing salt. On the other hand, if the a_{ij} or b_{ij} terms associated with the protein–ligand interaction are dependent either on the ionic characteristic temperature relationships of the displacing salt or are affected by changes in the conformational or aggregation state of the protein as the temperature is varied, then both salt and temperature specific variations in the $Z_{c,1}/Z_{c,2}$ ratio can be predicted to occur for a series of proteins. If, moreover, at a fixed temperature the product of $a_{ij}b_{ij} \rightarrow 1$ for $CaCl_2$ as the displacing salt, then the relationship between $\bar{k}$ and the experimental salt concentration takes on the more familar form of Eq. 12 (cf., Eq. 6):

$$\log \bar{k} = \log K_c + 1.5z \log(1/[CaCl_2]) \quad (12)$$

According to Eqs. 2–12, when a monovalent salt such as NaCl is used as the displacer salt, the average z value for a protein is predicted to be a half of the slope of the plot of $\log \bar{k}$ versus $\log 1/\bar{c}$. When a divalent salt such as $CaCl_2$ is used as a displacer salt, the z value of a protein is predicted to be two thirds of the slope of the plot of $\log \bar{k}$ versus $\log 1/\bar{c}$. Limited evidence for positive correlations between the retention behaviour and the z values of proteins have been found when di- and trivalent ions have been employed as ionic components of the displacing salt [3,7,40,42,45–47]. If it is assumed that the mechanism of interaction between a protein solute and a charged ligand immobilized on the LiChrospher 1000 SO_3^- adsorbent is not influenced by the nature of the displacer salt, then the extent of change in the Z_c or τ^* value of a protein in response to a change in temperature should be the same for either the 0–1.0 *M* NaCl or the 0–1.0 *M* $CaCl_2$ elution system, since the average orientation of the protein following binding is expected to be initially the same with either displacing salt system. When such criteria apply, then for different proteins the ratios of the slopes of the $\log \bar{k}$ versus $\log 1/\bar{c}$ plots determined at the same temperature with a 0–1 *M* NaCl or a 0–1 *M* $CaCl_2$ elution system are, according to Eqs. 2–11, predicted to be 4:3.

Table 2 lists the ratios of the slopes Z_c of the plots with NaCl and $CaCl_2$ as the displacing salt for each solute tested at each temperature condition with the same "tentacle-type" LiChrospher 1000 SO_3^- adsorbent. The results show that at most temperatures the ratios are significantly different from 1.33 for all the solutes, irrespective of their molecular size or conformational status. In addition, the magnitude of the $Z_{c,1}/Z_{c,2}$ ratio varies differently (and with no linear inverse dependence of τ^* on temperature) for the various solutes as the temperature is increased. These results strongly indicate that the assumptions implicit to the stoichiometric binding model, requiring that the values of z or Z_c are independent of a_{ij} and b_{ij} (and that the product $a_{ij}b_{ij} \rightarrow 1$) over the range of displacing salt concentrations and temperatures employed, are not physically or themodynamically realistic. As a consequence, the results of this investigation

Table 2
Ratios of the Z_c values of the different solutes obtained with 0–1.0 *M* NaCl or 0–1.0 *M* $CaCl_2$ as the displacing salt and LiChrospher 1000 SO_3^- adsorbent

Solute	$Z_{c\,NaCL}/Z_{c\,CaCl_2}$						
	15°C	25°C	35°C	45°C	55°C	65°C	75°C
ARG	1.30	1.35	1.12	1.25	1.19	1.28	N/A[a]
ANG-III	1.40	1.71	1.78	1.60	1.87	1.70	1.78
ANG-II	1.01	1.58	1.55	1.21	1.64	1.56	1.49
ANG-I	1.85	2.03	2.12	1.97	1.81	1.77	2.01
INS	1.67	1.33	1.35b	1.04	1.34	1.20	N/A
RIB	1.58	1.75	1.62	1.60	1.67	1.62	1.66
CYT	1.72	1.65	1.82	1.56	1.51	1.62	N/A
LYS	1.59	1.53	1.56	1.47	1.55	1.52	1.85
STI	1.76	1.60	1.46	1.44	1.52	N/A	N/A
OV	1.64	1.94	1.52	1.65	1.86	1.75	2.03

[a] N/A = not determined owing to a significant change in retention behaviour with either $\bar{k} \rightarrow 0$ or $\bar{k} \rightarrow \infty$.

confirm that the a_{ij} and b_{ij} terms associated with the particular protein-displacing salt system must be taken into account in the interaction between the protein and the ion-exchange adsorbent, both in terms of their effect on the magnitude of the contact area, i.e., the Z_c value, and the overall association constant, K_a, of the protein for the adsorbent during the ion-exchange chromatographic process.

In this context, recent investigations [13,14] on the thermodynamic basis of the adsorption of proteins on LiChrospher 1000 SO_3^- and other HPIEC adsorbents with similar types of "tentacular" ion-exchange ligands take on additional significance. These recent studies and related Hill plot investigations [48] have indicated that the interaction of large, charged biomacromolecules with this class of HPIEC adsorbents essentially follows rectilinear isothermal behaviour involving a multi-layer dissolution mechanism [14], which represents an extension of the ion condensation theory [41]. The thermodynamically favourable interaction [14,43] of a protein with the highly charged polyelectrolyte chains of the ligand will lead to partial neutralization of the regional electrostatic charges on the polymer chains, and result in the formation of more closely packed protein–protein or protein–ligand complexes at the ligand–solvent interface. In essence, the interaction of two different types of flexible polyelectrolyte chains with complementary types of charge characteristics, e.g., the protein and the "tentacular" ligand, will result in a reduction in the effective charge density per unit area of the adsorbent in the microenvironment of the interaction, reinforced by free energy changes associated with a decrease in chain solvation and possibly an entropically driven motion of the "tentacular" ligands to transiently encapsulate the protein. With low-molecular-mass compounds, on the other hand, more dispersed or extended solute–liquid structures can occur because, in these cases, the effective charge density per unit area of the surface of the adsorbent in the microenvironment of the binding of the low-molecular-mass solutes will remain higher, and changes in the solvational and ionic equilibria associated with the Stern double layer will be restricted to considerably smaller regions of the "tentacular" ligand surface.

3.3. Influence of the structure of the ion-exchange adsorbent on the retention behaviour of proteins at different temperatures

In previous studies [3,4,10–13], a number of differences in the retention behaviour of proteins

with anion-exchange adsorbents was observed between the "tentacle-type" LiChrospher 1000 TMAE or Fractogel TMAE adsorbents and the Mono-Q adsorbent, suggesting that differences in ligand morphology and accessibility play an important role in the ion-exchange retention process. In the present study, analogous differences were apparent when the chromatographic behaviour of the same group of amino acids, peptides and proteins were compared using the PolySulphoethyl A and the LiChrospher 1000 SO_3^- cation exchange adsorbents under the same temperature conditions with NaCl as the displacer salt. The PolySulphoethyl A adsorbent is a silica-based ion-exchange adsorbent with the charged ethylsulphonic acid groups attached to the modified polysuccinimide chains at the surface of the aminopropylated silica support material. With the LiChrospher 1000 SO_3^- adsorbent, in contrast, the charged groups are grafted as intertwined polymer chains to the surface of the silica support material, potentially providing a different mode of electrostatic interaction between the protein surface and the ligands.

Fig. 7 shows the dependences of the log $\bar{k}$ versus log $1/\bar{c}$ terms for each solute chromatographed on the cation-exchange adsorbent PolySulphoethyl-A at 25°C. Linear relationships between the log $\bar{k}$ and the log $1/\bar{c}$ values were observed with correlation coefficients ranging from 0.70 to 0.99 for all temperatures. Closer examination of these plots showed a clustered pattern such that the solutes can be similarly divided into three groups as was observed with the "tentacle-type" LiChrospher 1000 SO_3^- adsorbent. At 4°C, the dependence of the capacity factor of the solutes on salt concentration with the PolySulphoethyl A followed the order LYS > CYT > STI ≈ RIB > INS ≈ OV. At 15°C, the order of the dependence changed to LYS > CYT > STI > INS > RIB > OV, with no retention of ANG-II. Between 25 and 35°C, the sequence of the dependence of the capacity factor on the salt concentration changed again to LYS > CYT > INS > RIB > STI > OV. These changes in the retention behaviour of the proteins are again not consistent with the retention being controlled by the net charge of the protein. However,

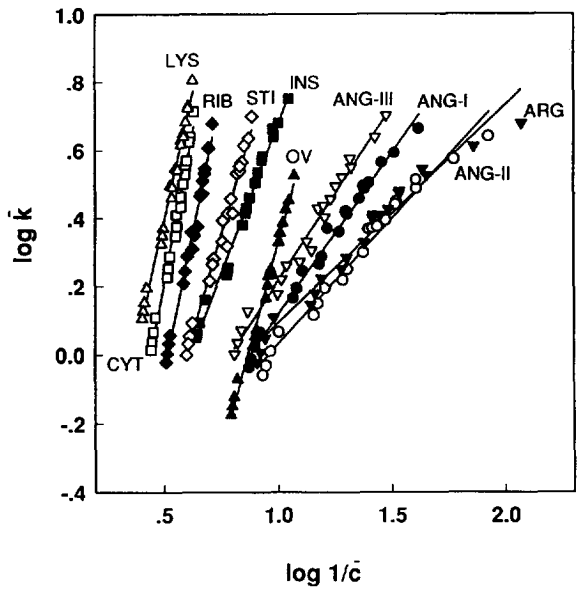

Fig. 7. Plots of log $\bar{k}$ versus log $1/\bar{c}$ with PolySulphoethyl A adsorbent at 25°C with NaCl as the displacer salt. Solutes: ● = ANG I; ○ = ANG II; ▽ = ANG III; ▼ = ARG; □ = CYT; ■ = INS; △ = LYS; ▲ = OV; ◇ = RIB; ◆ = STI. See Experimental for other details.

between 45 and 65°C, the order of the retention dependences of the capacity factors of the different proteins on salt concentration did correlate with the p*I* value of the protein in the order of CYT > LYS > RIB > INS > STI > OV. Comparison of these plots with the corresponding data obtained with the "tentacle-type" LiChrospher 1000 SO_3^- adsorbent reveals significant differences in the dependence of the capacity factors of these proteins on salt concentration. The data obtained with this adsorbent indicated that the retention order of the same group of proteins was unchanged at different temperatures and salt concentrations with the order following the sequence of proteins CYT > LYS > RIB > INS > STI > OV. With the PolySulphoethyl A adsorbent, dansyl-ARG was not retained at any temperature, and none of the protein or peptide solutes were eluted when the temperature was increased to 75°C. In contrast, LYS, RIB, OV, ANG I, ANG II, ANG III and ARG were all eluted from LiChrospher 1000 SO_3^- at 75°C.

Fig. 8 illustrates the dependence of the Z_c and

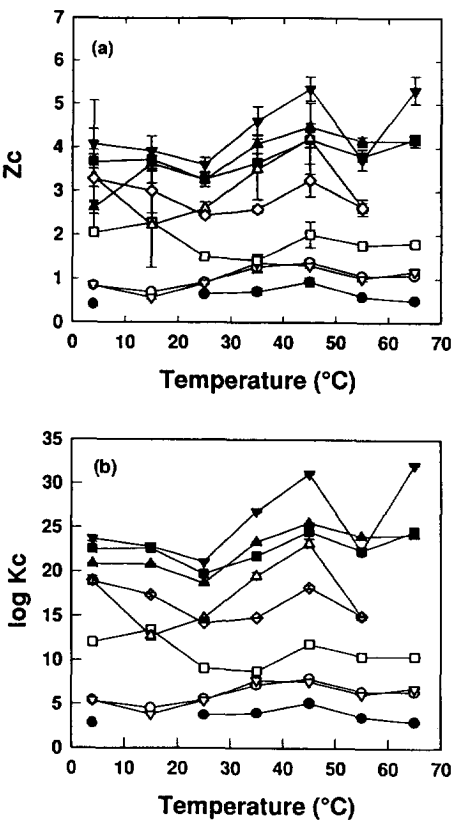

Fig. 8. Plots of (a) Z_c and (b) log K_c versus temperature with PolySulphoethyl A adsorbent with NaCl as the displacer salt. (a) Solutes: ○ = ANG I; ● = ANG II; ▽ = ANG III; ▼ = CYT; □ = INS; ▲ = LYS; △ = OV; ◇ = RIB; ◆ = STI. See Experimental for other details.

log K_c values of the examined solutes on temperature with the PolySulphoethyl A adsorbent. The Z_c and log K_c values for the low-molecular-mass control solutes are small and remain constant with increasing temperature. In contrast, the Z_c and log K_c values for the protein molecules were larger and varied more with increasing temperature. Comparison of these plots with the corresponding data for the "tentacle-type" LiChrospher 1000 SO_3^- adsorbent reveals that the magnitude of the Z_c and log K_c values for all the solutes obtained on the PolySulphoethyl A adsorbent were generally smaller than the values obtained from the "tentacle-type" adsorbent, except for ANG-II, INS and STI at 4°C. However, the degree of variation in the Z_c and log K_c values for the PolySulphoethyl A adsorbent was much greater than the variation observed with the "tentacle-type" LiChrospher 1000 SO_3^- adsorbent. These results indicate that the overall influence of temperature on the protein–adsorbent contact region and the affinity of the protein molecule for the adsorbent is much greater with the PolySulphoethyl A than the LiChrospher 1000 SO_3^- adsorbent. The conclusion which can be drawn from these results is that a larger number of positively charged residues on the protein surface can interact with the LiChrospher 1000 SO_3 "tentacle-type" adsorbent, but that changes in protein conformation are manifested as greater variations in the Z_c and log K_c values with the PolySulphoethyl A adsorbent.

The Z_c values of the solutes obtained with the PolySulphoethyl A adsorbent at different temperatures were also plotted against the isoelectric point of the point (pI) and molecular mass (MW) and the results are shown in Figs. 9 and 10. These results show that no simple relationship exists between the Z_c values obtained for these solutes and the isoelectric point (pI) and molecular weight (MW). These results again demonstrate that the binding behaviour of the contact region established between a protein molecule and the ion-exchange adsorbent is influenced in terms of the contributions from the protein by the number, distribution of charged residues and the conformation, in addition to the structure of the ligands present in the ion-exchange adsorbent.

4. Conclusions

The molecular basis of the interactions of proteins with ion-exchange adsorbents has yet to be fully characterized in terms of the role of the protein three-dimensional structure, the distribution of charged groups on the protein surface and the nature of the ligand structure. The investigations presented in this paper provide further insight into the ion-exchange chromatographic process under conditions where proteins can undergo conformational change. These changes can be detected by analysing the retention pa-

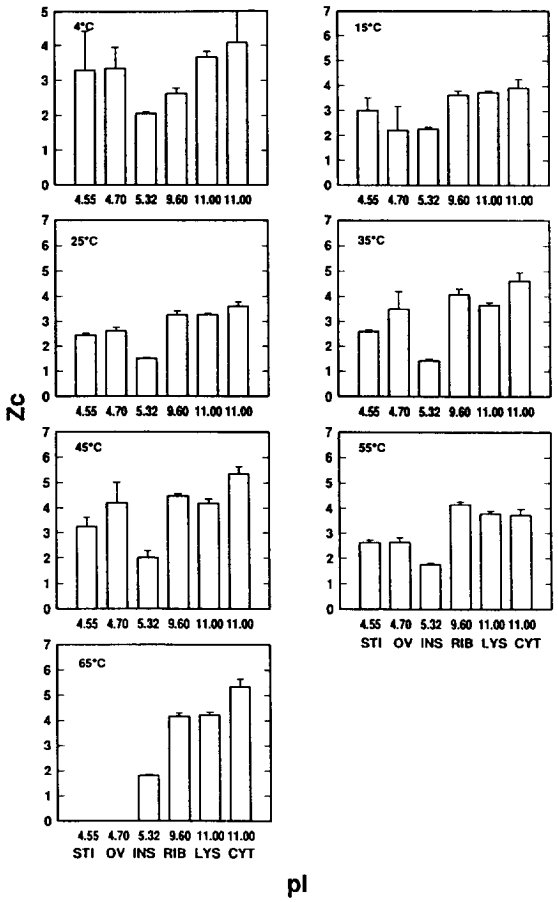

Fig. 9. Histogram representations of the protein Z_c values and pI values at each temperature with PolySulphoethyl A adsorbent and NaCl as the displacer salt. See Experimental for other details.

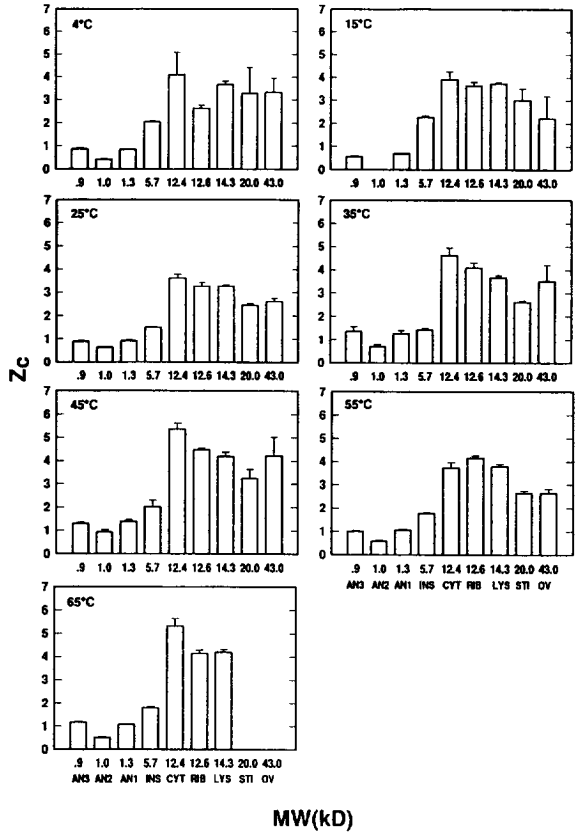

Fig. 10. Histogram representations of the protein Z_c values and molecular masses at each temperature with PolySulphoethyl A adsorbent and NaCl as the displacer salt. See Experimental for other details.

rameters such as the Z_c and log K_c values of the proteins. These studies have established that proteins interact with the "tentacle-type" cation-exchange adsorbent with a higher contact area and affinity than with the more conventional monolayer coverage HPIEC adsorbents, such as PolySulphoethyl A. Overall, these studies have further demonstrated the interplay between the influences of temperature, displacer salt and ion-exchange ligand which modulate protein retention behaviour in HPIEC. In a forthcoming paper [49], the influence of temperature, protein structure, displacer salt and ligand structure on the bandwidth behaviour of proteins will be described for both the LiChrospher 1000 SO_3^- and the PolySulphoethyl A adsorbents in order to elucidate further the mechanism of protein interaction with these two HPIEC adsorbents.

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