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Temperature-induced changes in the bandwidth behaviour of proteins separated with cation-exchange adsorbents[1]

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Abstract

The band-broadening behaviour of several amino acids, peptides and proteins separated under gradient elution conditions has been investigated with the 'tentacle-type' LiChrospher-1000 SO_3^- and the PolySulphoethyl A cation-exchange adsorbents. In particular, the dependencies of the bandwidths of this group of biosolutes on temperature and chromatographic residence time have been examined as part of our ongoing investigations into the influences of secondary equilibrium processes mediated by conformational interconversions of polypeptides or proteins and the ligand structure and flexibility in high-performance anion and cation-exchange chromatographic separations at elevated temperatures. Significantly different band-broadening behaviour was evident with these two adsorbents with solute-, ligand- and temperature-specific effects noted. For several of the proteins examined, bandwidth changes, characteristic of conformational unfolding processes, occurred at higher temperatures with the LiChrospher-1000 SO_3^- adsorbent than with the PolySulphoethyl A adsorbent. However, at lower temperatures, i.e. between 4°C and 25°C, smaller changes in bandwidth behaviour were observed with the PolySulphoethyl A rather than the LiChrospher-1000 SO_3^- adsorbent. In addition, comparative studies with NaCl and $CaCl_2$ as the displacing salt have revealed significantly different band-broadening effects with these two salts when these experiments were carried out at the same temperature with the LiChrospher-1000 SO_3^- adsorbent. The origins of these effects have been discussed in terms of the morphology of these cation-exchange systems and the possible adsorption–desorption mechanisms that apply when proteins interact with these two high-performance ion-exchange chromatographic adsorbents.

Keywords: Band broadening; Temperature effects; Adsorbents; Stationary phases; Proteins; Amino acids; Peptides

1. Introduction

Despite considerable progress over the past decade, detailed knowledge on the mechanism(s) which account for the interaction of proteins with high-performance ion-exchange chromatographic (HPIEC) adsorbents has yet to be fully elucidated at the molecular and/or atomic level. In particular, definitive information is still lacking on the nature of the dynamic interplay which exists between protein structure, surface topography or conformation and the specific chemical and physical structure(s) of the ion-exchange ligands, immobilised onto different types of support materials. One practical consequence of the availability of this information relates to the design and preparation of new types of

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[1] Part CLVI of the series High-performance liquid chromatography of amino acids, peptides and proteins. For Part CLV, see Ref. [50].

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'tailored' ion-exchange adsorbents with the potential to provide enhanced resolution and stability with proteins under conditions of higher separation speed or temperature. The development of the so called 'tentacle-type' ion-exchange adsorbents [1–3] and similar types of polymer-layer adsorbents [4–6] has provided an opportunity to investigate the influence of extended, linear grafted ligand structures on protein retention in HPIEC in comparison with other, more conventional types of 'mono-layer' coverage HPIEC adsorbents. In previous studies, we have investigated as part of this objective the comparative retention behaviour of several proteins separated with 'tentacle-type' strong anion and cation-exchange adsorbents, such as the LiChrospher 1000 TMAE and LiChrospher 1000 SO_3^- adsorbents, and the results compared [7–11] to similar experimental data obtained with other 'monolayer' types of HPIEC adsorbents. These studies have demonstrated that protein solutes interact with 'tentacle-type' adsorbents via a larger number of surface-accessible charge groups than with conventional 'monolayer' type of ion-exchange adsorbents, although the affinities or distribution coefficients of the protein–ligand interaction may be smaller.

In the present investigation, this comparison of the chromatographic behaviour of proteins with 'tentacle-type' adsorbents and other types of polymer-layer adsorbents has been extended through analysis of the bandwidth behaviour (as $4\sigma_t$) of several peptides and proteins separated with the strong cation-exchangers, LiChrospher 1000 SO_3^- and PolySulphoethyl A, under similar elution conditions of increasing temperatures and in the presence of different displacing salts. In addition, this investigation was intended to complement our associated studies [12] detailing the effect of changes in these experimental parameters on the median capacity factor, $\bar{k}$, and the Z_c values of the same group of proteins chromatographed on the same types of adsorbents. Collectively, these studies were designed to represent the initial experimental stage for the development of a quantitative framework for the interpretation of the dependencies of $\bar{k}$, $4\sigma_t$, and Z_c on the residence time, gradient steepness factor, gradient composition and temperature with proteins and polypeptides chromatographed in HPIEC under conditions whereby conformational transitions can/may occur.

2. Materials and methods

The ion-exchange chromatographic adsorbents used were the LiChrospher 1000 SO_3^- adsorbent, obtained from E. Merck (Darmdstadt, Germany) and the PolySulphoethyl A cation-exchange adsorbent, obtained from PolyLC (Columbus, MD, USA). The physical and chemical characteristics of the amino acid, peptide and protein solutes studied in this investigation have been described previously [12]. The mobile phase used was 0.05 *M* sodium acetate at pH 4.0 as solvent A and 1.0 *M* NaCl or 1.0 *M* $CaCl_2$ in 0.05 *M* sodium acetate buffer, pH 4.0, as solvent B. The solutes were eluted with gradient times between 20 and 100 min from 0 to 100% solvent B under different temperature conditions. The bandwidth data for each solute were obtained by measuring the $4\sigma_t$ value (in minutes) at 13.4% of the peak height. Other details related to the selection of the reagents, solutes and experimental conditions are as described previously [12].

3. Results and discussion

3.1. Bandwidth behaviour of control solutes

In this study, the positively charged amino acid derivative dansyl-arginine (dansyl-R, M_r 444.1) and three small peptides, angiotensin III (ANG-III, M_r 931.1), angiotensin II (ANG-II, M_r 1046.2) and angiotensin I (ANG-I, M_r 1296.5), were employed as the control solutes under the different chromatographic conditions. The angiotensin peptides consisted of 7, 8 and 10 amino acid residues and contained 2+, 2+ and 3+ positively charged groups, respectively, at pH 4.0. As previously described [7,12], these control solutes exist as monomeric compounds at the concentrations employed in this study, and exhibit no significant, long-lived secondary or tertiary structures in solution. Consequently, the effective hydrodynamic volumes, surface areas and the bulk diffusive properties of these solutes will remain essentially constant throughout the chromatographic process under the experimental conditions employed.

The origin of the variation in band-broadening behaviour of these control solutes in the gradient

elution mode can be related in the usual manner (if extra-column effects are excluded) to the consequences of the changes in the bulk diffusion, film transfer and surface kinetic effects. Significant changes in band-broadening of these solutes as the temperature of the chromatographic system is increased cannot be ascribed to secondary equilibria associated with conformational effects, because with each of these small solutes the constituent backbone and side-chain functional groups undergo very rapid, time-averaged interconversions between various populations of fully extended structures at the adsorbent surface. In addition, the bulk diffusion coefficient, D_m, is according to the Stokes–Einstein equation [13] linearly dependent on the absolute temperature, T, but inversely dependent on the eluent viscosity, η, whilst the dependency of η on T usually takes the form of log η versus an inverse quadratic T relationship for aqueous solutions of electrolytes [14]. Because of their similar molecular size and mass, changes in the bulk mobile-phase diffusion behaviour of these small solutes will, however, follow a similar and consistently small trend towards smaller values as T is increased with the elution and column systems studied in the present investigations. Any significant differences in the bandwidth behaviour of the control solutes at the same experimental temperature with the two different adsorbents can thus be predominantly related to differences in the physical and chemical characteristics of the stationary phases, i.e. to differences in pore resistance and interaction kinetics due to the different surface chemistries of the adsorbent particles, and also due to changes in the viscosity of the mobile phase as the temperature is increased.

In the case of the protein solutes on the other hand, conformational rearrangements can occur on a much slower time scale. The presence of multiple protein conformers as the temperature is increased with a specified adsorbent will give rise to additional bandspreading or even split peak effects, due to the participation of relatively slow secondary equilibria in solution or at the adsorbent surface and the resulting changes in the surface kinetics and diffusivities of the protein. Similarly, if the temperature is fixed, the appearance of significantly different band-broadening behaviour for proteins of similar molecular mass and hydrodynamic characteristics (i.e. with similar bulk mobile-phase diffusion coefficient, D_m, values) when separated on different adsorbents will be inter alia diagnostic of variations in the surface interaction kinetics. Consequently, comparison of the changes in the bandwidth behaviour of a series of control solutes with the corresponding data for larger polypeptides and proteins following systematic changes in the same set of chromatographic variables, such as adsorbent type, temperature or displacer salt, can be used to provide insight into the effects of the structural hierarchy of proteins on the adsorption–desorption kinetics of the chromatographic interaction as mediated by the surface characteristics of the ion-exchange ligands.

3.2. Influence of temperature on protein bandwidth behaviour with the 'tentacle-type' cation-exchange adsorbent, LiChrospher 1000 SO_3^-

In a previous study [12], temperature was used as an additional parameter to probe the extent by which changes in protein secondary and tertiary structure are reflected in the chromatographic retention behaviour with the LiChrospher 1000 SO_3^- adsorbent. In the present investigation, the influence of temperature on the corresponding experimental bandwidths of the same group of protein solutes have been examined. The dependencies of the bandwidths (as $4\sigma_t$ values) of the control solutes and other proteins on temperature with the LiChrospher 1000 SO_3^- adsorbent using NaCl as the displacing salt are shown in Fig. 1. Representative results for two of the control solutes, ANG-II and ANG-III, demonstrate (Fig. 1a, b) relatively small increases in the magnitude of the bandwidth with increasing gradient time as expected from plate theory. Similar band-broadening behaviour was evident with the other control solutes, dansyl-R, dansyl-K and ANG-I. The results for these control solutes also revealed that between 4°C and 45°C, the experimental bandwidths decreased with increasing temperature and then maintained essentially constant values up to 75°C, again in accord with the predictions of plate theory as adapted to gradient elution RP-HPLC [15,16]. As these low molecular mass solutes have no significant secondary or tertiary structure under the chromatographic conditions employed, the decrease in bandwidth for the control solutes between 4°C and 45°C

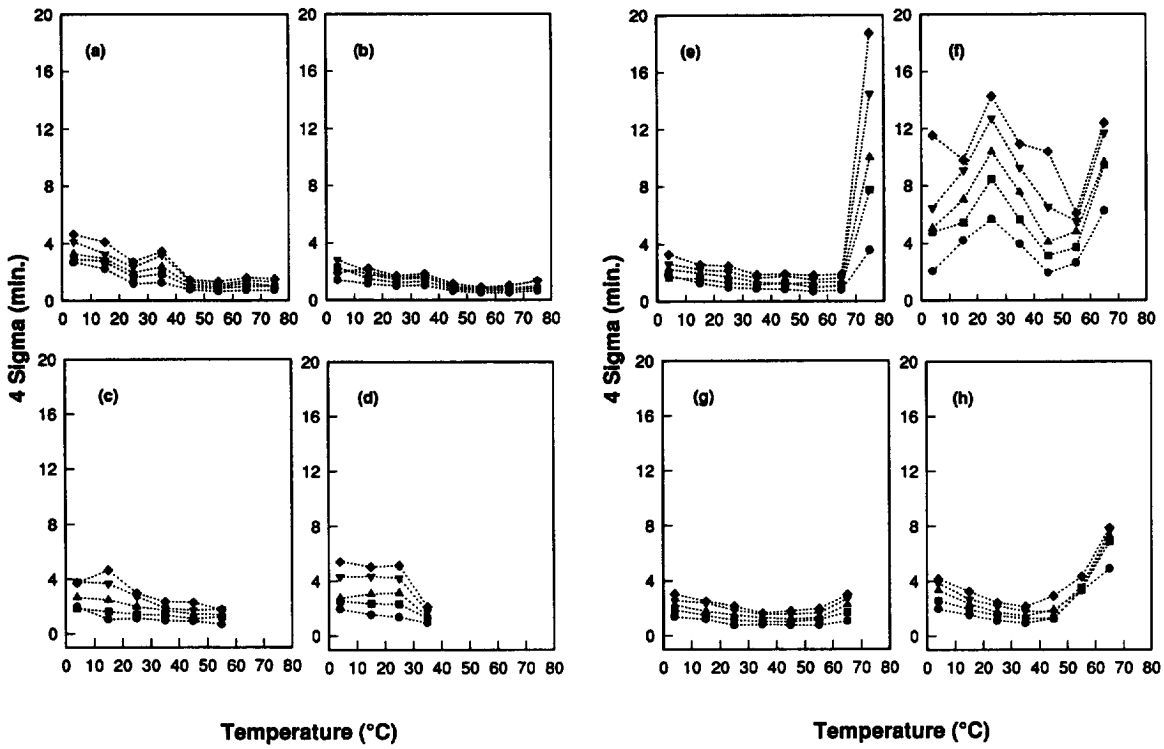

Fig. 1. Dependence of experimental bandwidths ($4\sigma_t$) on temperature for solutes separated on the LiChrospher SO_3^- adsorbent with NaCl as the displacer salt. Solutes: (a) ANG-II; (b) ANG-III; (c) STI; (d) OV; (e) LYS; (f) INS; (g) RIB; (h) CYT. Gradient times used: (●) 20 min, (■) 40 min, (▲) 60 min, (▼)80 min, (◆) 100 min. See Section 2 for other details.

can thus be attributed to the influence of temperature on the mobile-phase viscosity (and hence to changes in the bulk diffusion coefficients, D_ms), variations in the flexibility and solvational status of the immobilised electrostatic ligands which are accessible at the surface of the adsorbent, as well as to the influence of temperature on the ionisation constants (pK_a values) of the charged protein or ligand polyelectrolyte chains [17,18], with all three effects impacting on the interaction kinetics. The cooperative participation of these effects will lead to faster mass transfer kinetics and hence to narrower peak widths.

The dependencies of experimental bandwidths on the temperature for each of the proteins studied with LiChrospher 1000 SO_3^- and NaCl as the displacer salt are plotted in Fig. 1c–h. Comparison of the bandwidth behaviour of the solutes demonstrated that significant differences in the bandwidth dependencies existed between the protein solutes and the low molecular mass control molecules. In particular, examination of Fig. 1f reveals that the magnitude of the experimental bandwidth for bovine insulin (INS, M_r 5700) was much larger than the bandwidth observed for the control molecules and other protein solutes at each temperature and gradient time tested. It is also apparent that the bandwidth for INS fluctuated significantly over the entire temperature range with maxima occurring at 25°C and 65°C. As the pH of the solvent (pH 4.0) is close to the pI value of INS (pI=5.32), aggregation of the molecules may occur due to the low solubility of INS in the aqueous solution. Furthermore, participation of other binding processes mediated by hydrophobic interactions may also contribute to the anomalous bandwidth behaviour of this small protein. Although the Z_c values of the INS with the LiChrospher 1000 SO_3^- adsorbent were the smallest amongst the proteins examined in this study [12], fluctuations in the bandwidth ($4\sigma_t$) values for INS occurred over the same temperature ranges that variations in the Z_c

values occurred [12]. The implication can be drawn from these results that changes in the temperature influence the interaction between INS and the ion-exchange adsorbent in terms of both the retention and bandwidth behaviour as a result of changes in the INS surface structure due to self association and/or unfolding. These results also indicate that it is the nature of the surface interactive properties rather than the molecular size of INS per se that determines the magnitude of the changes in band-broadening under conditions of increasing temperature, i.e. changes in the surface diffusion rate constant rather than the bulk diffusivity represents a dominant contributor to the band-broadening effect.

The magnitude of the experimental bandwidth of bovine ribonuclease (RIB, M_r 12640) with LiChrospher 1000 SO_3^- shown in Fig. 1g gradually decreased with increasing temperature from 4°C to 35°C, similar to the behaviour observed for the control molecules. However, at temperatures between 45°C and 65°C the bandwidth values for RIB increased in a manner which was not apparent for the control solutes. This increased bandwidth may thus indicate disruption of the monomeric, native protein structure due to denaturation or aggregation. The participation of both these effects can be anticipated for this protein due to the disruption of salt bridges and conformational transitions occurring at high temperature during the ion-exchange chromatographic process [19]. At 75°C, the elution of RIB resulted in several smaller split peaks, indicating a high degree of structural disruption of the protein and the appearance of multiple conformers or associated species at this temperature. As the retention time of the RIB peaks increased at 75°C, compared to the retention times for this protein at lower temperatures, this result is consistent with the involvement of hydrophobic interactions becoming a more important contributor to the retention and bandwidth behaviour of this protein at higher temperatures with the LiChrospher 1000 SO_3^- adsorbent.

For horse heart cytochrome *c* (CYT, M_r 12384) the experimental bandwidth (Fig. 1h) obtained with the LiChrospher 1000 SO_3^- adsorbent gradually decreased with increasing temperature from 4°C to 35°C but increased dramatically between 45° and 65°C; CYT was not eluted from the LiChrospher 1000 SO_3^- adsorbent at 75°C. The significant changes in the bandwidth behaviour of CYT above 45°C can be contrasted with those of the control solutes, again indicating that the surface kinetic behaviour of this protein differs from that of the smaller molecules. As the pore size of the LiChrospher 1000 SO_3^- particle is 1000 Å, CYT (as well as all the other protein solutes studied) will have ready access to the interior of the particle. Mass transfer resistance due to film resistance and pore resistance effects are thus not expected to be major contributors to the band-broadening evident with CYT (and the other proteins) as the temperature is increased. However, changes in the surface diffusion properties of the protein due to the participation of additional secondary equilibria in the chromatographic process can again be anticipated to significantly affect the peak width.

Previous studies [20–22] on the influence of temperature on bandwidth behaviour of peptides and proteins in RP-HPLC have demonstrated significant differences in the effect of temperature on the peak width of control small peptides and larger polypeptide molecules which can undergo conformational transitions. In earlier investigations [10–12], hydrophobic interactions were shown to significantly contribute to the interaction of CYT with the 'tentacle-type' LiChrospher 1000 SO_3^- cation-exchange adsorbent at higher temperatures, e.g. 65°C. These changes in the relative contribution of electrostatic and hydrophobic forces thus represent an additional level of complexity in the retention mechanism of proteins with 'tentacle-type' adsorbents (and probably most other types of organic polymeric stationary phases bearing immobilised ion-exchange groups) and will exert a significant impact on the experimental bandwidths at high temperatures. The temperatures at which large changes in bandwidth were noted for CYT also correlated with the large fluctuations in Z_c and log K_c values observed under the same experimental conditions [12]. Collectively, these previous results suggested that both the contact region and the affinity of the protein for the 'tentacle-type' LiChrospher 1000 SO_3^- cation-exchange adsorbent undergo change during the ion-exchange chromatographic process especially at higher temperatures. As a consequence, temperature-induced variations in the protein conformation, state of aggregation and the involvement of hydrophobic

interactions will collectively affect the adsorption–desorption process, leading to the changes in the protein retention and bandwidth behaviour apparent at higher temperatures.

For the protein hen egg white lysozyme (LYS, M_r 14 300) in Fig. 1e, the $4\sigma_t$ versus temperature plots exhibited a small decrease in bandwidth with increasing temperature from 4°C to 65°C which was followed by a large increase in bandwidth at 75°C. This result is indicative that the protein surface structure has been altered and that the ion-exchange kinetics in terms of the surface diffusion contribution to the overall mass transfer process has significantly changed at over the temperature range 45–75°C. The sharp increase in bandwidth compared with the control solutes over the same temperature range suggests that conformational rearrangements induced by the higher temperatures are occurring within the chromatographic time scale. Previous studies with monolayer types of anion-exchange and cation-exchange HPIEC adsorbents with LYS have shown [23] that this protein can undergo hinge motion of its two-domain structure as the initial stages of unfolding, and related phenomena appear to also occur in the RP-HPLC mode [24,25]. This conformational change of the protein was also reflected in the retention behaviour of this protein which resulted in larger Z_c and log K_c values obtained at the same temperature [12]. To establish whether such a dramatic change in bandwidth was caused by chemical degradation of the 'tentacular' surface or, alternatively, the protein, the experiments with LYS were replicated at lower temperatures with the same column and sample after the 75°C experiments were completed. No significant differences from the results originally obtained at the lower temperatures were observed in the peak shape or retention time of LYS with these re-injection experiments.

The bandwidth for soybean trypsin inhibitor (STI, M_r 20 000) in Fig. 1c decreased steadily with increasing temperature up to 55°C. At the higher temperatures of 65° and 75°C STI could not be eluted with 1.0 *M* NaCl from the LiChrospher SO_3^- cation-exchange adsorbent but could be subsequently eluted if the column temperature was again reduced to 25°C. This result suggested that the sharp increase in affinity (log K_c) of STI for the adsorbent at higher temperatures is associated with a conformational change which may be reversed by decreasing the temperature.

In the case of ovalbumin (OV) in the presence of NaCl as the displacing salt, the $4\sigma_t$ values progressively increased up to 45°C (Fig. 1d), after which multiple interconverting bandwidth zones associated with peak-splitting effects became evident. Large deviations in the $4\sigma_t$ values of the eluted peaks of OV were apparent at these higher-temperature conditions. These divergences in the $4\sigma_t$ values for OV and the other polypeptides and proteins from the values predicted on the basis of plate theory and molecular diffusivity arguments for non-interconverting structures [16,25] can be attributed to the formation/presence of interconverting species under the experimental conditions. Whether this behaviour is due to conformational, aggregational or other types of chemical equilibria (i.e. the $\alpha \rightarrow \beta$ rearrangement of aspartic acid residues or deamidation of aspartamide residues [26]) warrants further investigation.

3.3. Influence of $CaCl_2$ on protein bandwidth with the LiChrospher 1000 SO_3^- cation-exchange adsorbent at different temperatures

It is well known that different salts can influence the stability and solubility of proteins in aqueous solution. The effects of salts on protein stability in solution, classified in terms of the Hofmeister series [27], have been extensively discussed in the scientific literature [28–31]. Of the divalent salts, $CaCl_2$ has been reported to have a destabilisation effect on proteins. For example, as the concentration of $CaCl_2$ was increased in a solution containing the fibrous protein collagen, the transition of collagen from a rigid three-chain helically ordered structure to a random coil form progressively took place at lower temperature [30]. This destabilising (salting-in) effect of salts such as $CaCl_2$ on protein structure is believed to be caused: (i) by the direct interaction of the Ca^{2+} cation and the associated Cl^- counterion with various surface-accessible amino acid side-chain groups and polypeptide backbone dipoles within the macromolecule; (ii) by indirect action through effects on the structure and properties of the solvent; or (iii) by a combination of both mechanisms [31].

In more complex systems, such as in an ion-

exchange chromatographic environment, the effects of the displacer salt on protein stability will not simply be evident from a bulk solution effect. In solid–liquid two-phase systems, the salt can specifically affect the mechanism of interaction between the protein and the ionic groups of the adsorbent by perturbing the characteristics of the Donnan layer, or the tertiary structure of the protein once it has adsorbed to the surface. Various studies (e.g. [5–12,32–38]) have demonstrated that the retention properties of proteins in HPIEC are strongly influenced by the nature of the displacer salt. For example, changes in protein retention in anion-exchange chromatography with different sodium, potassium and other M^{1+} and M^{2+} salts have been related to the hydrated ionic radii and have been shown to also be affected by the nature of the co-ions through preferential interactions with the protein solute [5,6,39].

It has also been demonstrated that protein retention in HPIEC and HPHIC is closely related to the water structure stabilising or destabilising effect of the displacer salt in terms of their chaotropic or kosmotropic properties [40–42]. If different displacer salts can affect the conformational status of a protein as it migrates along the column in a time-dependent manner, then these effects will be manifested as significant changes in bandwidth. It has however been observed that significant deviation can arise between the predicted and observed bandwidth values of proteins eluted with monovalent displacer salts in HPIEC when mathematical models based on conventional plate theory are employed to predict the theoretical bandwidth values without taking into account the secondary equilibrium effects associated with protein–salt interactions or protein conformational changes [7–9,37,42]. Because the effect [37–39] of divalent salts, such as $CaCl_2$, on the ion-exchange chromatographic bandwidth behaviour of proteins has not yet been adequately investigated, particularly with the 'tentacle-type' cation-exchange adsorbents, the protein bandwidth behaviour in the presence of $CaCl_2$ as the displacer salt under the same chromatographic conditions as employed with NaCl was examined at different temperatures.

The bandwidth values of various solutes in the presence of the $CaCl_2$ displacer are plotted in Fig. 2 against temperature. The results for the small control molecules such as the angiotensin peptides (Fig. 2a, b) show that the experimental bandwidth increases slightly with increasing gradient time, consistent with the pattern observed for the control solutes when NaCl was used as the displacer salt. However, the magnitude of the bandwidth values for these small molecules (as well as the bandwidth values of the other control solutes, data not shown) was significantly smaller at each individual temperature and gradient time than the values obtained with NaCl as the displacer salt. The origin of this effect remains to be elucidated. The implication which can be drawn from this result however leads to the conclusion that with some proteins peak shapes in zonal elution chromatography under isocratic conditions will be improved when $CaCl_2$ rather than NaCl is used as the displacing salt. Since in the gradient mode the band compression or 'J-effect' [43] will result in comparable reductions in peak-tailing at different k' values for both the NaCl and the $CaCl_2$ elution systems, this finding can only partly be attributed to the higher displacing strength of the Ca^{2+} ion which results in lower median capacity factors for these solutes.

An additional contributor to this effect could have its origin in the extent to which the thickness of the Donnan layer differs between the two salt systems. In the case of the Na^+ and the Ca^{2+} ions, the respective ionic radii are 0.98 Å and 0.99 Å, but with approximately twice the charge density the more solvated Ca^{2+} ion may result in a less diffuse or extended Donnan layer with a different charge potential gradient characteristic than when Na^+ ions are present. This reorganisation of the microenvironment of the 'tentacular' ligand surface will lead to more rapid desorption kinetics for the solute and/or shorter path distances of diffusion through the double layer which the solute must follow as it leaves the ligand surface, thus resulting in lower experimental bandwidths than anticipated on the basis of the NaCl data. The 'tentacular' ligands present in the LiChrospher 1000 SO_3^- cation-exchange adsorbent and related 'tentacle-type' adsorbents have been proposed [1,2] to exist as independently mobile chains made up of repeat units of the cation-exchange ethylsulphonic acid groups, due to the nature of the Ce(IV)-mediated linear graft polymerisation of derivatised methacrylate monomers onto the Glymo-

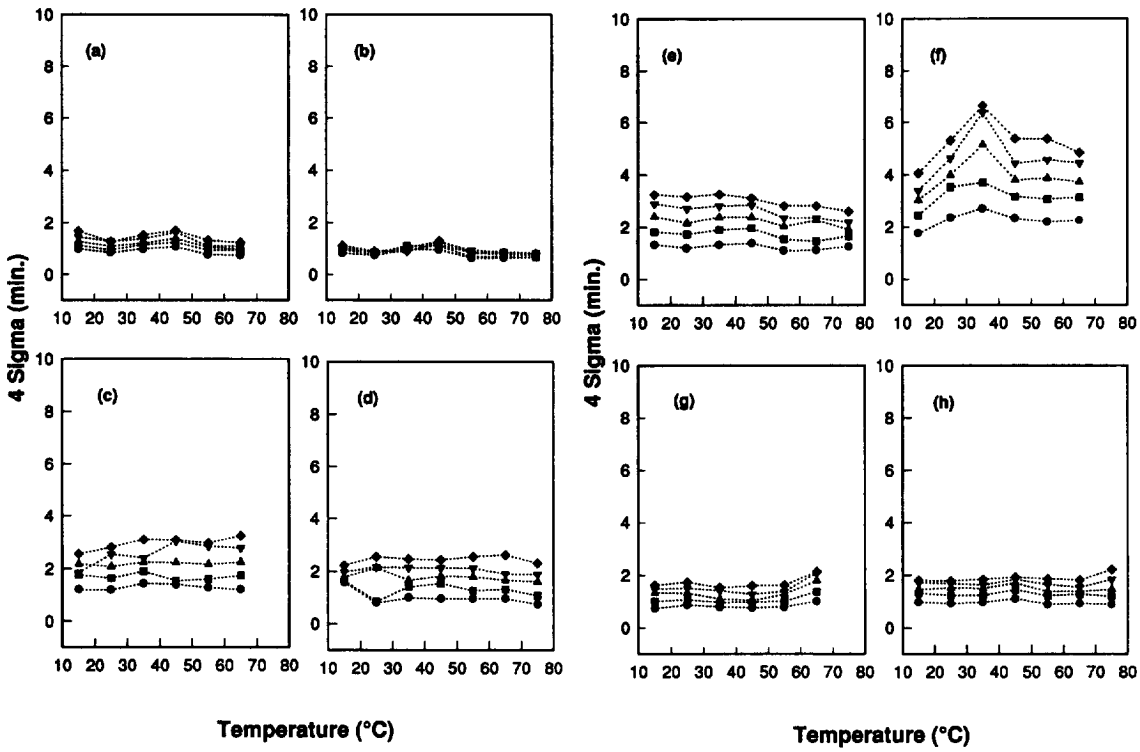

Fig. 2. Dependence of experimental bandwidths ($4\sigma_t$) on temperature for solutes separated on the LiChrospher SO_3^- adsorbent with $CaCl_2$ as the displacer salt. Solutes: (a) ANG-II; (b) ANG-III; (c) STI; (d) OV; (e) LYS; (f) INS; (g) RIB; (h) CYT. Gradient times used: (●) 20 min, (■) 40 min, (▲) 60 min, (▼) 80 min, (◆) 100 min. See Section 2 for other details.

modified LiChrospher 1000. It is highly likely, however, that some cross-linkage may occur between these polyelectrolyte chains due to catenate entanglement of the polymer chains. The observation that different bandwidths are obtained with low molecular mass solutes with the LiChrospher 1000 SO_3^- adsorbent, depending on whether NaCl or $CaCl_2$ is used as the displacing salt, thus suggests that different electrostatic polarisation and double-layer characteristics can be induced in the microenvironment of the ligand surfaces due to the relatively high Na^+ or Ca^{2+} ion concentrations required to maintain the charge electroneutrality of the repetitive SO_3^- groups on these 'tentacular' ligand during the ion-exchange process.

For the larger protein molecules, the dependencies of the bandwidth on temperature in the presence of $CaCl_2$ are plotted in Fig. 2d–h. For CYT, the experimental bandwidth (Fig. 2h) was essentially constant with increasing temperature up to 75°C for the shorter gradient times, but increased slightly at the longer gradient times (60 min to 100 min). A similar trend in the variation of the bandwidths was also evident when NaCl was used as the displacer salt, but the rise in the bandwidth occurred at a significantly lower temperature, e.g. 45°C. As it is unlikely that this change in bandwidth is caused by a change in the bulk diffusion or the film transfer kinetics, then this result suggests that surface kinetics are more rapid when $CaCl_2$ is present in the mobile phase, and that the adsorbed CYT structure is partially stabilised against denaturation as the temperature is increased.

For LYS, a similar dependence of bandwidth on temperature to that seen for CYT was observed (Fig. 2e). The bandwidth decreased slightly with increasing temperature with no increase in bandwidth at higher temperature when $CaCl_2$ was used. In contrast, a very large increase in bandwidth for LYS was observed when NaCl was used as the displacer salt

(Fig. 1e). This result again indicates that $CaCl_2$ may suppress the surface-mediated interconversions such as conformational changes of the protein at temperatures as high as 75°C. It can also be noted that a significant increase in Z_c value was found with NaCl but an essentially similar Z_c value with $CaCl_2$ was observed at these high temperatures. Another example of the apparent stabilising effect of $CaCl_2$ is RIB for which the bandwidth increase commenced at 55°C, which is 10°C higher than the same transition in bandwidth when NaCl was used. The bandwidths of STI and INS showed similar fluctuations for either $CaCl_2$ (Fig. 2c, d) or NaCl (Fig. 1c, d) when the temperature was increased. However, the magnitude of the changes in bandwidth for each protein at each corresponding temperature was generally smaller with $CaCl_2$ than the bandwidth observed when NaCl was used.

It is known that Ca^{2+} is a 'salting in' or destabilising ion [27–31,44–46] for globular proteins in solution. When $CaCl_2$ is used in the bulk mobile phase, a destabilising or unfolding effect is thus anticipated which could be expected to influence the surface structure of the protein and hence cause a change in the value of the contact parameter Z_c and the affinity term log K_c. At the same time, the bandwidth of the solute would be expected to exhibit significantly larger values due to the structural expansion and unfolding of the molecule during the chromatographic process. However, the results of the present study indicate that the bulk 'salting in' effect of $CaCl_2$ is not manifested with this ion-exchange chromatographic system. The smaller increases in the bandwidths of RIB, LYS and CYT at temperatures up to 75°C with $CaCl_2$ as the displacing salt compared to NaCl suggest that $CaCl_2$ has the ability to minimise secondary equilibria events such as denaturation or aggregation which lead to additional band-broadening with various other ion-exchange chromatographic systems.

A further consideration is that the effective ionic strength of the co-ion Cl^- with $CaCl_2$ is twice the value with NaCl. In the cation-exchange chromatographic format used in these investigations, the interaction between the solute, adsorbent and solvent not only involves the negatively charged residues on the protein, the cation of the displacer salt and the positively charged ligands on the adsorbent but the Cl^- co-ions will also selectively interact with the positively charged residues on the protein surface. In addition, the higher displacing strength of $CaCl_2$ will decrease the residence time of the protein in the column which will in turn shorten the time of exposure of the protein to a particular solvent and temperature environment. Consistent with these observations was the finding during the present study that STI and CYT irreversibly bound at higher temperatures to the LiChrospher SO_3^- cation-exchange adsorbent when NaCl was used as the displacing salt but not with $CaCl_2$. These results indicate that with these 'tentacle-type' ion-exchange ligands, $CaCl_2$ may have a moderating effect on the influence of temperature-induced unfolding of the protein structure in the HPIEC environment.

3.4. Comparison of the bandwidth behaviour of proteins on different types of cation-exchange adsorbents under different temperature conditions

Previous studies on the influence of the structure of the ion-exchange ligand on protein retention behaviour [5,36,47,48] have revealed that the density and flexibility of the ligand will impact on the nature of the interaction between the protein solute and the ion-exchange adsorbent and thus affect the magnitude of the Z_c and the log K_c values as well as the adsorption–desorption kinetics. Furthermore, the density and the flexibility of the ligand may contribute to the kinetics of the conformational rearrangement of the protein solute at higher temperatures. As a consequence, the bandwidth behaviours of the same group of amino acids, peptides and proteins were consequently examined with an analogous type of polymeric HPIEC cation exchanger, the Poly-Sulphoethyl A adsorbent, selected for comparative purposes. The physical and chemical properties of these ion-exchange adsorbents have been detailed in previous papers [2,4,6,10–12]. The experimental bandwidth of the solutes obtained under different temperature conditions using NaCl as displacer salt are shown in Fig. 3.

The bandwidth of the control solutes (ANG-II and ANG-III) slightly decreased with increasing temperature from 4°C to 35°C and then remained constant with further increases in temperature. This behaviour is identical to the bandwidth behaviour of

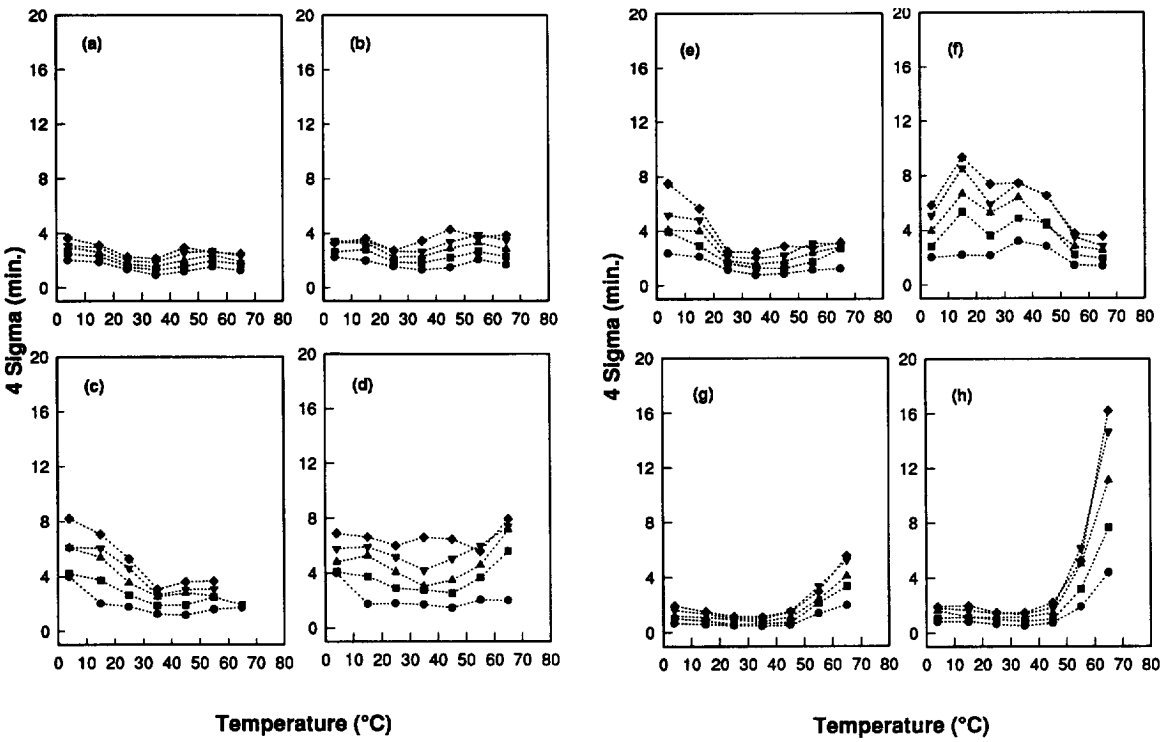

Fig. 3. Dependence of experimental bandwidths ($4\sigma_t$) on temperature for solutes separated on the Polysulphoethyl A adsorbent with NaCl as the displacer salt. Solutes: (a) ANG-II; (b) ANG-III; (c) STI; (d) OV; (e) LYS; (f) INS; (g) RIB; (h) CYT. Gradient times used: (●) 20 min, (■) 40 min, (▲) 60 min, (▼) 80 min, (◆) 100 min. See Section 2 for other details.

these solutes with the LiChrospher 1000 SO_3^- adsorbent. For example, in the bandwidth results for the peptides ANG-II and ANG-III, shown in Fig. 3a, b, small fluctuations in the experimental bandwidths were observed with increasing temperature. However, much larger changes in the bandwidth were observed for the protein molecules, and in particular, three different patterns in the bandwidth plots are evident from comparison of Fig. 3c and e, Fig. 3d and f, and Fig. 3g and h, respectively. For example, the bandwidth of CYT shown in Fig. 3h remained constant for temperatures from 4°C to 35°C and then increased significantly at higher temperatures for all gradient times; CYT was not eluted at 75°C from the PolySulphoethyl A adsorbent. A similar rise in bandwidth at 45°C was also observed with the LiChrospher 1000 SO_3^- adsorbent, suggesting that the overall kinetic properties of CYT are not significantly influenced by the structure of the chromatographic ligand.

Similar results were obtained with RIB (Fig. 3g) which exhibited a small decrease in bandwidth up to 45°C after which there was a significant increase in bandwidth. In contrast, STI and LYS (Fig. 3c, e) exhibited a different bandwidth pattern in which the peak width decreased between 4°C and 35°C with longer gradient times (80 min and 100 min) and remained essentially constant at higher temperatures although a small increase in the bandwidth at temperatures >55°C was observed for LYS. These results are consistent with changes in the diffusional and interactive properties of these proteins having occurred as a result of the so called 'cold' denaturation effect at the lower temperatures. This phenomenon has been documented for a range of proteins [29–31,49] and involves a decrease in the hydrodynamic and interactive properties of the protein due to additional internalisation of hydrophobic amino acid residues at the lower temperatures.

The bandwidth results of all six proteins obtained

Table 1
Experimental bandwidths ($4\sigma_t$) obtained for the various proteins separated on the Lichrospher SO_3^- and the PolySulphoethyl A adsorbent at different gradient times

Protein	Gradient time (min)									
	LiChrospher-SO_3^-					PolySulphoethyl A				
	20	40	60	80	100	20	40	60	80	100
Insulin	5.68	8.47	10.38	12.71	14.27	2.13	3.59	5.27	5.88	7.35
Ribonuclease A	0.74	1.13	1.49	1.84	2.14	0.52	0.58	0.92	1.02	1.16
Cytochrome *c*	1.12	1.45	1.73	2.19	2.43	0.63	0.96	1.11	1.41	1.47
Lysozyme	0.96	1.28	1.73	2.08	2.47	1.14	1.65	1.83	2.13	2.49
Soybean trypsin inhibitor	1.12	1.40	1.98	2.73	2.96	1.76	2.62	3.54	4.59	5.27
Ovalbumin	1.37	2.34	3.15	4.24	5.17	0.68	1.08	1.57	1.99	2.37

with the PolySulphoethyl A adsorbent at 25°C are compared with the results obtained with the LiChrospher 1000 SO_3^- adsorbent at the same temperature in Table 1. At the temperature of 25°C (as well as at 4°C) the bandwidth values for these proteins were greater with the LiChrospher 1000 SO_3^- adsorbent than with the PolySulphoethyl-A adsorbent. However, the bandwidth values of these proteins were smaller with the LiChrospher 1000 SO_3^- adsorbent at higher temperatures (e.g. 55°C). Several important bandwidth differences are thus evident between these comparative data for the two adsorbents. Firstly, the magnitude of the bandwidth for LYS and STI were much smaller on the 'tentacle-type' LiChrospher 1000 SO_3^- adsorbent and there was less fluctuation in the $4\sigma_t$. values. Secondly, the bandwidth behaviour of INS showed an increase in bandwidth at 15°C on the PolySulphoethyl A adsorbent while the increase occurred at 25°C with the LiChrospher 1000 SO_3^- adsorbent. Thirdly, there was a smaller increase in the 4σ values at higher temperatures for RIB and CYT with the LiChrospher 1000 SO_3^- adsorbent compared to the corresponding conditions on the PolySulphoethyl A adsorbent. These results also correlate with the larger degree of fluctuations of Z_c with temperature previously observed for these proteins on the PolySulphoethyl A adsorbent [12].

4. Conclusions

The composition of the surface structure of proteins in terms of the charge density and charge distribution determines the contact region established between a protein and an ion-exchange adsorbent. Changes in the contact area as a consequence of conformational rearrangement, aggregation or other secondary processes will change the affinity of the protein for the adsorbent and in turn will affect the kinetics of the adsorption–desorption process. In the present investigations, these changes have been monitored from variations in the bandwidth behaviour of the proteins and other solutes arising from changes in temperature and residence time. Further evidence has been provided that experimental bandwidth data can be used as an indicator of the dynamic properties of proteins during the chromatographic process. The origins of the differences in bandwidth for the proteins observed with these two different HPIEC adsorbents in the presence of NaCl or $CaCl_2$ as the displacing salt remain fascinating questions worthy of further investigation. In the case of the PolySulfoethyl A adsorbent, the charged poly(2-sulphoethyl aspartamide) chains are attached to the amino-propylated silica support material generating a network of ethylsulphonic acid groups for binding to proteins. With the LiChrospher 1000 SO_3^- adsorbent, on the other hand, the charged ethylsulphonic acid groups are grafted as part of intertwining polymer chains to the surface of a glymo-modified silica support material, potentially providing a different mode of electrostatic interaction between the protein surface and the ligands. In both cases, the surface modification of the silica support material results in a structured coating of relatively short polymer chains of high electrostatic charge potential due to the close proximity of the repeated

monomer ethylsulphonic acid units. The double layer of solvated charged groups and ions so formed at the surface of these adsorbents will result in the generation of a gradient of charge potential extending into the solvent film of mobile phase surrounding the surfaces of the adsorbent which will be very dependent on the extent of flexibility of the ligand chains.

With both the LiChrospher 1000 SO_3^- adsorbent and the PolySulphoethyl A adsorbent changes in temperature will affect the flexibility and hence the accessibility of the immobilised ethylsulphonate ligands to solvent, cations and other ions present in the mobile phase as well as the approaching protein. Because the interaction of a protein with these charged ligands can be considered in terms of the binding of a large, flexible polyelectrolyte chain (the protein) with a second flexible polyelectrolyte chain (the electrostatic ligand) bearing complementary types of charge groups, the protein–ligand interactions will result in a more significant reduction in the effective charge density per unit area of the surface of the adsorbent in the vicinity of this interaction than can occur with low molecular mass solutes. Associated with the resulting reduction in charge repulsion between the individual ligand chains, a decrease in chain solvation and a variation in the gradient of the charge potential and ion concentrations surrounding the ligands can occur.

Since the influence of Ca^{2+} ion compared to Na^+ ion on the $4\sigma_t$ values of proteins chromatographed under the same conditions except for the nature of the displacing salt would suggest that the major contributor to the change in bandwidths for the same protein was associated with a surface diffusion effect, a role for the cation and the associated counterions in modulating the flexibility of the charged ligands can be invoked. Alternatively, the possibility must be considered that the initial binding of the protein is subsequently followed by a series of re-orientations, whereby the protein 'slides' or 'rolls' along the transiently encapsulating charged ligands immediately prior to desorption, thus generating on average a Gaussian affinity distribution of bound species with their associated distribution of kinetic desorption constants. Such a model, characteristic of a multi-layer dissolution mechanism, would be consistent with: (i) the thermodynamic behaviour; (ii) the isothermal adsorption properties whereby the adsorption capacities and the association constants increase with increasing temperature; (iii) the Hill coefficients for the binding which have been found to be less than 1; and (iv) the larger Z_c values observed for protein interactions with these 'tentacular-types' of HPIEC adsorbents [3,7,10–12]. Overall, the results of this investigation have demonstrated that the study of protein bandwidth behaviour at different temperatures represents a sensitive approach to probe the dynamic status of proteins which may undergo conformational transitions and other secondary equilibrium events during the ion-exchange chromatographic process, thus providing valuable insight into the molecular basis of the electrostatic interactions between the protein, solvent and ligand.

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