Effect of protein conformation on experimental bandwidths in reversed-phase high-performance liquid chromatography

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Abstract

The dynamic behaviour of a series of closely related cytochrome c molecules has been investigated in reversed-phase high-performance liquid chromatography. In particular, the influence of temperature and gradient time on the experimental bandwidths of equine, tuna, canine and bovine cytochrome c and equine and tuna apocytochrome c has been determined with a C_{18} and a C_4 sorbent. The observed bandbroadening changes are discussed in comparison to the corresponding retention behaviour of the cytochrome c molecules reported in the preceding paper [1]. The results demonstrate that bandwidth measurements can be used as an indicator of the conformational status of a protein under a particular set of chromatographic conditions.

1. Introduction

The separation of complex mixtures of polypeptides and proteins by reversed-phase high-performance liquid chromatography has improved considerably in recent years as a consequence of a better understanding of the molecular basis of retention and selectivity, together with significant advances in experimental procedures. However, problems often arise as a result of anomalous bandbroadening of eluted peaks of biopolymers, a phenomenon which can hamper the optimisation of the separation conditions. The source of this bandbroadening behaviour can be attributed to a number of secondary equilibrium processes associated with solute aggregation in the bulk mobile phase or at the stationary phase surface, sol–gel equilibria, multisite interaction with the stationary phase surface, specific ion-interaction equilibria involving ionic additives present in the mobile phase, specific pH-dependent equilibria and specific solute-solvation equilibria. While all these interactions can influence the secondary events which give rise to atypical bandbroadening, the dominant factor with proteins is often the presence of multiple conformers due to conformational interconversion [1–5]. Several investigations have characterised the bandbroadening behaviour of peptide solutes and have found that the presence of defined secondary structure strongly affects the experimental peak width [5–11]. While many investigators have reported the occurrence of bandbroadening phenomena during analytical separations of proteins by RP-HPLC, few workers have, however, systematically characterised the dependence of experimental bandwidths on operating conditions.

The kinetic processes which give rise to bandbroadening behaviour of low-molecular-

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mass, rigid organic molecules in gradient elution are influenced by axial dispersion in the bulk mobile phase, dispersion due to slow mass transfer across the solvent–particle boundary and in the intraparticulate spaces and dispersion due to slow mass transfer of the solute at a heterogeneous stationary phase surface [12]. Thus, for small compounds where the molecular surface area and the respective diffusive properties remain essentially constant throughout the chromatographic analysis, the experimental bandwidth can be directly related by the general plate height theory to several diffusion parameters such as the bulk diffusivity D_M, the intraparticulate boundary diffusivity D_P, the surface diffusivity D_S as well as the column residence time which is controlled by the elution time and the flow-rate [12–15]. However, if the solute undergoes conformational rearrangements which are characterised by a rate constant that is comparable to the chromatographic separation time, additional bandspreading and/or distortion of the experimental peak shape will occur leading to asymmetrical or multiple peak formation [1–5,9–11,16–18]. These changes in bandbroadening behaviour will be associated with changes in the hydrodynamic volume and changes in the chromatographic contact region of the solute. Thus, changes in the shape of a symmetrical peak in response to variation in a specific separation parameter such as temperature or column residency time represent kinetic indicators of the dynamic nature of protein backbone flexibility and the conformational transitions of the solute that occur at the solvent–ligand interface. In a previous paper [1], the retention behaviour of a family of cytochrome c molecules was investigated in terms of the influence of temperature on the retention parameters S (related to the chromatographic contact area) and log k_0 (related to the solute affinity for the sorbent). In the present study, bandwidth data associated with the reversed-phase separation of these cytochrome c molecules have been examined. The results are discussed in relation to the kinetics of conformational interconversions that occur during the chromatographic migration of these proteins on both a C_{18} and a C_4 sorbent.

2. Materials and methods

The chromatographic apparatus, chemicals and reagents and chromatographic procedures used in the present study are described elsewhere [1]. In order to examine the dependencies of the experimental bandwidth upon temperature and column residence time for the cytochrome c-related proteins, the experimental bandwidth was measured at 13.4% of the eluted peak height which corresponds to four standard deviations (i.e. 4σ) [9]. The bandwidths were monitored under a range of experimental gradient elution conditions as previously described [1]. These conditions included gradient times between 30 and 120 min at a flow-rate of 1 ml/min at temperatures between 5 and 85°C. All data points were derived from at least duplicate measurements. The 4σ values were determined using a BANDWIDTH macro-software developed within this laboratory for use with a Hewlett-Packard 79995A Chemstation operating software. As loading conditions for the test proteins were approximately $8 \cdot 10^{-5}$ μM, the possibility of solute aggregation in the mobile phase can be excluded [19], and thus the behaviour of 4σ will directly reflect the solute dynamics at the sorbent interface.

3. Results and discussion

3.1. Bandwidth behaviour of control solutes

In order to evaluate the contribution of secondary and tertiary structures to the overall interactive behaviour exhibited by the protein solute, the bandwidth behaviour of two low-molecular-mass solutes, N-acetyl-L-phenylalanine ethyl ester and penta-L-phenylalanine were investigated. The dependence of the experimental bandwidths on temperature for these two molecules chromatographed on the C_{18} sorbent is shown in Fig. 1. The results demonstrated a small increase in the magnitude of the bandwidth with increasing gradient time. This increase
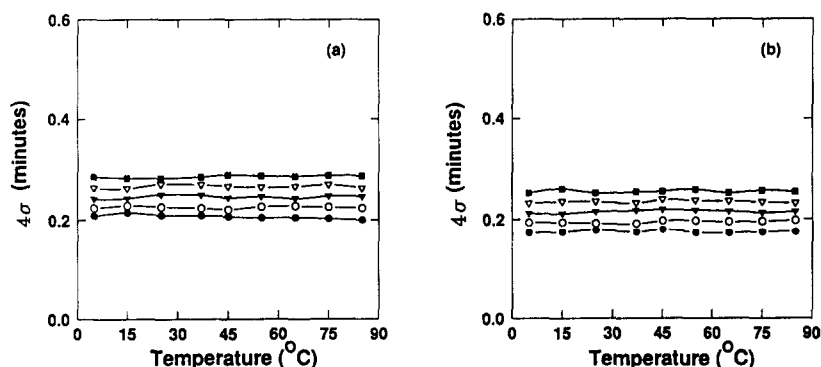
Fig. 1. The dependence of experimental bandwidths, 4σ, on temperature at five different gradient times between 30 and 120 min for (a) penta-L-phenylalanine and (b) N-acetyl-L-phenylalanine ethyl ester chromatographed on the C_{18} sorbent. The gradient times were: ● = 30 min; ○ = 45 min; ▼ = 60 min; ▽ = 90 min; ■ = 120 min.

reflects the axial dispersion of the solute during chromatographic migration which increases proportionately with increased separation time. The results in Fig. 1 also indicate that the bandwidths are essentially independent of operating temperature. These linear dependencies are consistent with either the inability of these solutes to conformationally interconvert or alternatively their ability to interconvert very rapidly with regard to the chromatographic time scale. Almost identical results were obtained for the bandwidth behaviour of these solutes chromatographed on the C_4 sorbent. The mass transport kinetic properties of the control solutes are in accord with many other low-molecular-mass solutes [6,12,13], with the structure and chemical properties of the n-alkyl ligands not significantly affecting the peak shape under these well-controlled conditions. Therefore, the bandwidth values shown in Fig. 1 represent the degree of change in 4σ with increasing temperature which would be expected for low-molecular-mass, non-interconverting solutes. As a consequence, significant deviations in the 4σ values associated with the chromatography of the cytochrome c molecules from that predicted on the basis of molecular diffusivity arguments for a protein of constant shape, can be attributed to the formation of interconverting species.

3.2. Bandwidth behaviour of cytochrome c on the C_{18} sorbent

Equine cytochrome c was chromatographed on the C_{18} sorbent at gradient times between 30 and 120 min and temperatures between 5 and 85°C. The dependence of the experimental bandwidths on gradient time and operating temperature is illustrated in Fig. 2 for both the holo- and the apoprotein. The data for the holo protein (Fig. 2a) reveal that at each temperature, the value of 4σ increased with gradient time, while at each gradient time, 4σ decreased with increasing temperature. However, at certain temperatures, there were large fluctuations in the experimental bandwidths. For example, at 37°C there was a large increase in 4σ at both the 90 and 120 min gradient times. In addition, between 75 and 80°C, a sharp decrease in 4σ was observed. Overall, the results in Fig. 2 are in sharp contrast to the results observed for the control solutes shown in Fig. 1, which exhibited no significant dependency on temperature. Thus, the large changes in the bandwidth behaviour of equine cytochrome c over narrow ranges of experimental conditions are indicative of conformational interconversions which occur particularly near 37°C and between 75 and 80°C at the longer gradient times. Furthermore, the 4σ values ob-
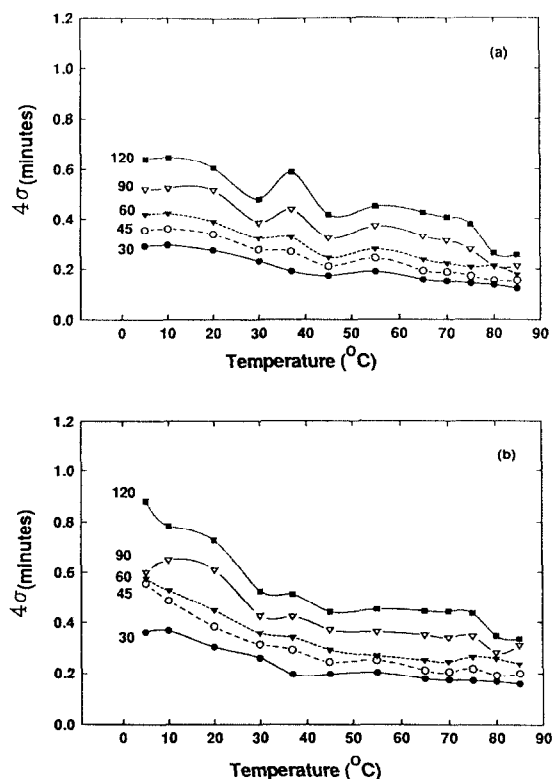
Fig. 2. The dependence of experimental bandwidths, 4σ, on temperature at five different gradient times between 30 and 120 min as indicated for (a) equine cytochrome c and (b) equine apocytochrome c chromatographed on the C_{18} sorbent.

tained for equine cytochrome c were significantly larger at the lower temperatures and longer gradient times than those obtained for the control solutes, as would be expected for larger molecules which occupy a greater hydrodynamic volume and are characterised by a relatively smaller diffusion coefficient. The retention behaviour of the cytochrome c molecules reported previously [1] indicated that the S and log k_O values increased over the same temperature range, consistent with the unfolding of the protein molecule at the higher temperatures. Moreover, the extent of interconversion of the unfolded protein to a refolded molecule would decrease at higher temperatures leading to the presence of predominantly only one form of the protein and thus be consistent with the signifi-

cant decrease in 4σ values. Hence in the low-temperature range, more conformational species will be present, leading to greater perturbation in peak shapes due to these kinetic interconversions. In contrast, at higher temperatures the protein will have less secondary and tertiary structure, ultimately leading to only a single unfolded species migrating in the RP-HPLC system.

The influence of the haem moiety of the cytochrome c molecule was studied by chromatographing equine apocytochrome c under the same experimental conditions. The dependence of the experimental bandwidths on gradient time and temperature for the apoprotein are shown in Fig. 2b. The elution profile of the apoprotein previously showed [1] the presence of two distinct peaks. The bandwidth data plotted in Fig. 2b represents the experimental bandwidths of the early-eluting peak. As was observed for the holo protein, there was an increase in the value of 4σ with increased gradient time and a decrease in 4σ with increased temperature. Thus, the bandbroadening behaviour of equine apocytochrome c was similar to that observed for the holo protein, although the values of 4σ under identical chromatographic conditions for the apoprotein were generally larger. In addition, the variations in 4σ observed at 37°C for the holo protein were not evident for the apoprotein, which suggests that the haem moiety plays a role in the kinetics of structural interconversions which occur during the chromatographic migration. The absence of the haem group would presumably result in a more flexible polypeptide chain with larger hydrodynamic volume which would be manifested as the relatively larger bandwidth values observed at the lower temperatures.

The dependence of experimental bandwidths on gradient time and temperature of tuna cytochrome c and tuna apocytochrome c are shown in Fig. 3. The results for the holo protein in Fig. 3a demonstrate a similar relationship between 4σ, gradient time and temperature to that observed for equine cytochrome c. However, there appears to be a transition in the 4σ values at 37°C which may correspond to a conformational
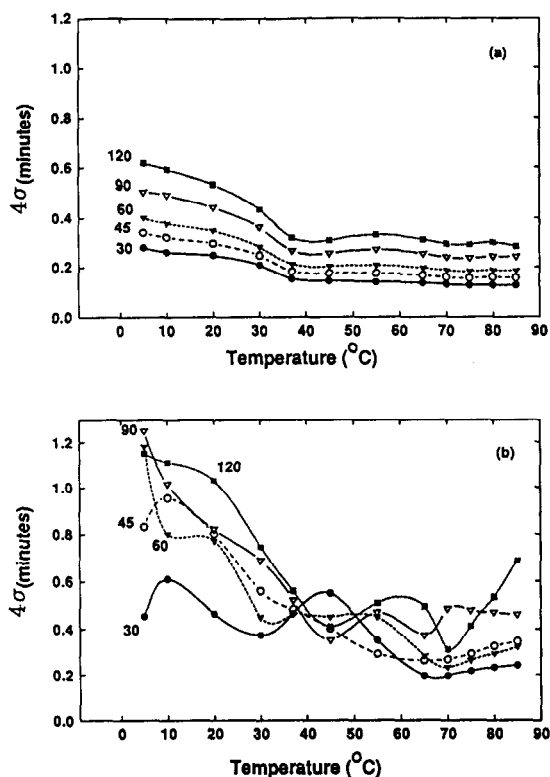
Fig. 3. The dependence of experimental bandwidths, 4σ, on temperature at five different gradient times between 30 and 120 min as indicated for (a) tuna cytochrome c and (b) tuna apocytochrome c chromatographed on the C_{18} sorbent.

apocytochrome c undergoes conformational interconversion at the lower temperatures. The variance in the bandbroadening behaviour observed may also be a direct result of the cold denaturation phenomenon which takes place at these temperatures. In addition, the differences between tuna holo cytochrome c and tuna apocytochrome c again demonstrate the crucial role of the prosthetic haem molecule in the kinetics of the conformational and interactive properties of cytochrome c, and indicate that in the absence of the haem group, the polypeptide backbone readily interconverts between different conformers.

The dependence of the experimental bandwidths on gradient time and temperature for canine and bovine cytochrome c are shown in Fig. 4. Both proteins exhibited similar behaviour

interconversion. Whilst the differences in the amino acid sequence between the equine and the tuna cytochrome c molecule do not result in very dramatic changes in the bandwidth behaviour, differences in the relative stability of the two proteins, as manifested in the kinetics of their interaction with the C_{18} sorbent, are evident. The bandbroadening behaviour of the first eluted peak of tuna apocytochrome c is shown in Fig. 3b and demonstrates significantly different behaviour to either the equine holo or apocytochrome c and the tuna holo cytochrome c. While the trend was observed that the value of 4σ decreased with increasing temperature, these values showed considerable divergence at the different gradient times, with a transition evident around 37°C. Thus, the presence of multiple interconverting peaks demonstrates that tuna
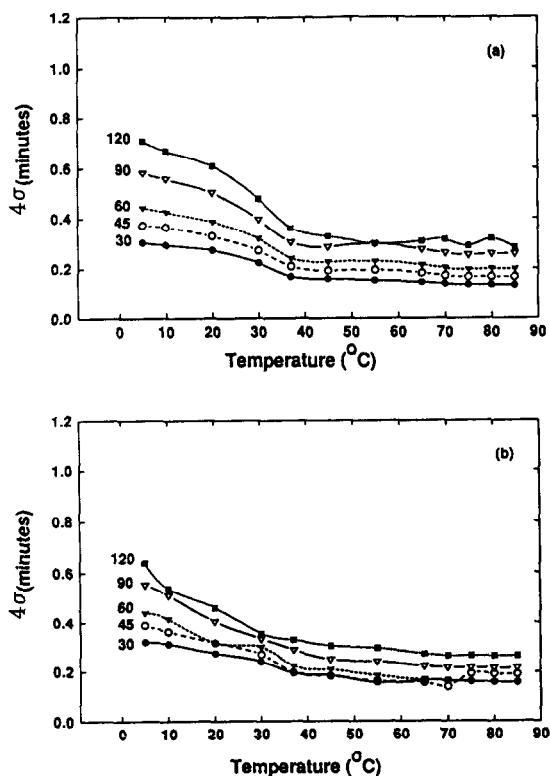

Fig. 4. The dependence of experimental bandwidths, 4σ, on temperature at five different gradient times between 30 and 120 min as indicated for (a) canine cytochrome c and (b) bovine cytochrome c chromatographed on the C_{18} sorbent.

to that observed for equine and tuna cytochrome c with a transition evident around 37°C. Thus between 5 and 37°C the magnitude of 4σ decreased with increasing temperature, while between 37 and 85°C the 4σ value approached a similar magnitude. The small differences in amino acid sequences between the four cytochrome c molecules has been shown to significantly influence the resolution of cytochrome c molecules [1]. Between 5 and 37°C, it can be seen that the magnitudes of the 4σ values were larger for canine cytochrome c while for bovine cytochrome c there were smaller differences in the 4σ value between the five gradient times. Whilst there was an overall similarity in the bandwidth dependencies for the four cytochrome c molecules, subtle differences were evident in the influence of temperature and column residence time on experimental bandwidth. Since the molecular mass and hence the bulk diffusivity of these four proteins will be similar, these differences in 4σ must reflect differences in the kinetics of the conformational interconversion and ligand interaction and can be directly attributed to the amino acid residue substitutions.

3.3. Bandwidth behaviour of cytochrome c on the C_4 sorbent

In order to investigate the effect of ligand structure on the kinetic behaviour of proteins, all cytochrome c molecules were also chromatographed on a C_4 sorbent at temperatures between 5 and 85°C and at gradient times between 30 and 120 min. The dependence of experimental bandwidths on temperature and gradient time are shown in Figs. 5–7. The bandbroadening behaviour for equine cytochrome c shown in Fig. 5a illustrates the generally observed trend that 4σ increased with increasing gradient time and decreased with increasing temperature. Again these results are different to those obtained for the non-interconverting control solutes where no dependency on temperature was observed. At lower temperatures, equine cytochrome c appears to undergo conformational interconversion, a result which was also apparent from the corresponding retention behaviour [1]. At higher
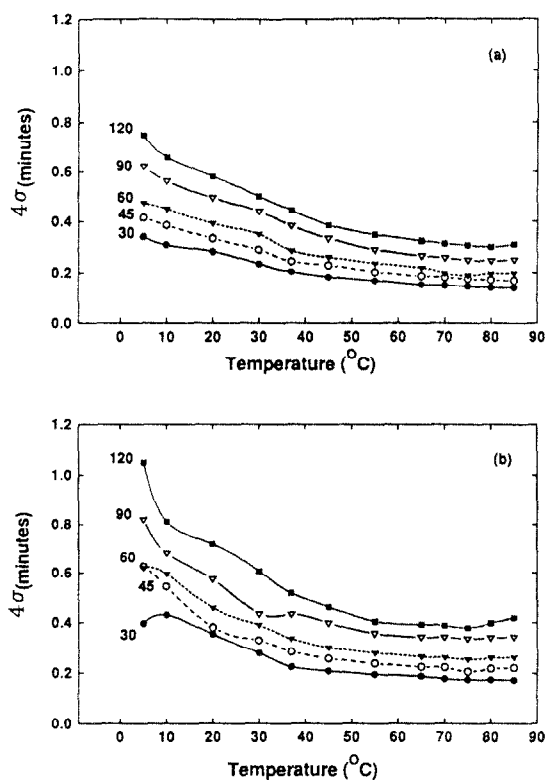

Fig. 5. The dependence of experimental bandwidths, 4σ, on temperature at five different gradient times between 30 and 120 min as indicated for (a) equine cytochrome c and (b) equine apocytochrome c chromatographed on the C_4 sorbent.

temperatures where 4σ approaches a constant value, it is apparent that the rate of conformational interconversion of the protein molecule is too rapid for this effect to be detected as a bandbroadening change. Comparison of the data shown in Fig. 2a with Fig. 5a reveals some significant differences between the bandwidth behaviour of equine cytochrome c on the C_4 and C_{18} sorbents. Firstly, the fluctuations in 4σ at 37°C and 75–80°C observed with the C_{18} sorbent were not apparent with the C_4 sorbent. Secondly, the magnitude of the 4σ values were generally higher with the C_4 sorbent. These differences can be attributed to the differences in the molecular structure of the n-alkyl ligands. Hence, the conformational flexibility, the relative hydrophobicity and the extent of ligand solvation are
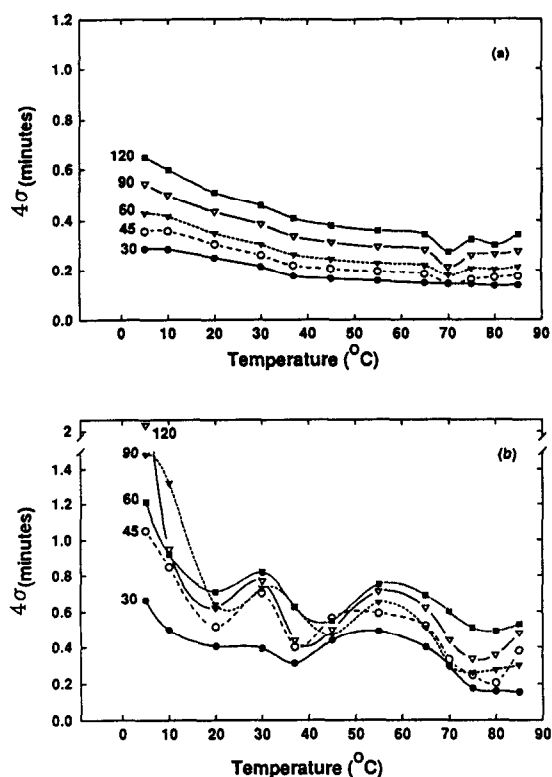
Fig. 6. The dependence of experimental bandwidths, 4σ, on temperature at five different gradient times between 30 and 120 min as indicated for (a) tuna cytochrome c and (b) tuna apocytochrome c chromatographed on the C_4 sorbent.

Fig. 7. The dependence of experimental bandwidths, 4σ, on temperature at five different gradient times between 30 and 120 min as indicated for (a) canine cytochrome c and (b) bovine cytochrome c chromatographed on the C_4 sorbent.

all important factors which influence the adsorption of the protein solute and the potential for the protein solute to undergo conformational interconversion.

The bandbroadening behaviour of equine apocytochrome c is illustrated in Fig. 5b. The elution profile of this molecule revealed the presence of two peaks. The data shown in Fig. 5b represent the experimental bandwidths for the first eluted peak. It is evident from this figure that 4σ exhibited a curvilinear dependency on both gradient time and temperature. In addition, a transition in the bandwidth data occurred between 37 and 45°C while at higher temperatures the 4σ value remained constant. Comparison of these data with the bandwidth behaviour of the holo protein in Fig. 5a reveals that much larger 4σ values were observed for the apoprotein with the
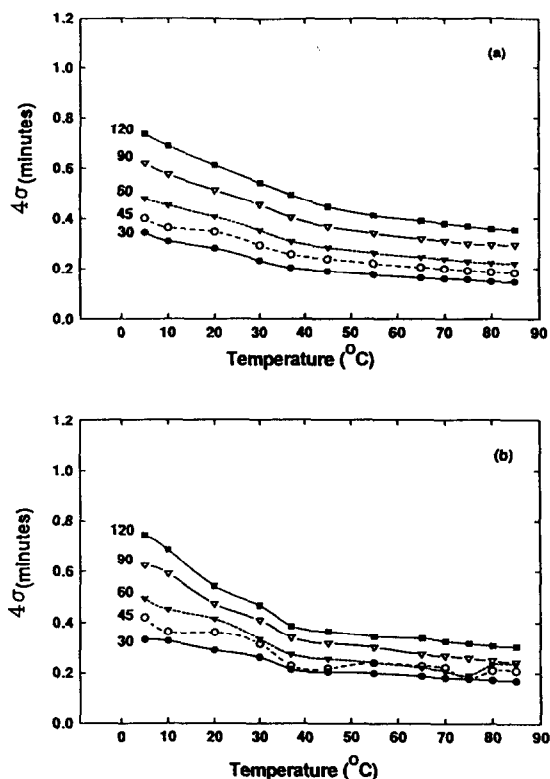
C_4 sorbent between 5 and 45°C, while similar 4σ values were obtained between 45 and 85°C. Thus, the 4σ values at lower temperatures reflect a significant degree of conformational interconversion on the chromatographic time scale, while at higher temperatures the decrease in 4σ values is indicative of an increasingly rapid rate of interconversion. These results once again demonstrate the influence of the haem group in restricting the conformational flexibility of the cytochrome c molecule. Comparison of the bandbroadening behaviour of equine apocytochrome c on both the C_4 and the C_{18} sorbent also reveals that significantly larger 4σ values were observed between 5 and 45°C at gradient times of 90 and 120 min with the C_4 sorbent than with the C_{18} sorbent. It was previously shown [1] that the C_{18} sorbent stabilised the structure of

the second peak compared to the C_4 sorbent and the comparative bandwidths observed at the lower temperatures are again consistent with the presence of a greater degree of conformational interconversion on the C_4 sorbent.

The dependence of experimental bandwidths on gradient time and temperature for tuna cytochrome c and tuna apocytochrome c is illustrated in Fig. 6. As was observed for equine cytochrome c, the values of 4σ for tuna cytochrome c increased with increasing gradient time and decreased with increasing temperature with a transition around 37°C. However, the magnitude of the 4σ values for tuna cytochrome c at the lower temperatures were greater than the corresponding data for equine cytochrome c. This result again demonstrates that the amino acid differences influence the protein's dynamic properties when the molecule undergoes conformational interconversion. The influence of the chromatographic ligand can be seen by comparison of Fig. 3a with Fig. 6a. While the magnitude of the 4σ values are similar with each sorbent, the transition in the bandwidth data around 37°C is much more distinct with the C_{18} sorbent than the transition observed with the C_4 sorbent. Thus, the kinetics of conformational interconversion of tuna cytochrome c appears to be strongly influenced by the nature of the ligand structure. The bandwidth behaviour of tuna apocytochrome c with the C_4 sorbent is shown in Fig. 6b. It can be seen from these data that the changes in the experimental bandwidths were significantly greater than those observed for tuna cytochrome c and that there were large fluctuations over the range of temperatures employed. As the phenomenon of peak splitting was also observed for tuna apocytochrome c on the C_4 sorbent, the changes in experimental bandwidth can therefore be attributed to conformational interconversion of the protein molecule. Thus, while the bandwidth data shown in Fig. 6b represent the data for the first-eluting peak, it is evident from the bandwidth relationships that several closely related structural conformers may be contributing to the peak corresponding to the early-eluting conformer. The conformational interconversion which tuna apocytochrome c un-

dergoes appears to be more extensive as the experimental bandwidths at the lower temperatures are much greater on the C_4 sorbent than on the C_{18} sorbent again demonstrating the stabilising effect of the longer more hydrophobic ligands. These results also further illustrate the influence of the haem group on the structural stability of the cytochrome c molecule.

The dependence of experimental bandwidths on gradient time and temperature for canine and bovine cytochrome c are shown in Fig. 7a and b, respectively. It can be seen that the bandwidth behaviour of these two molecules is similar to that observed for equine cytochrome c with the C_4 sorbent. Thus, 4σ increased with increasing gradient time over the entire temperature range. In addition, between 5 and 45°C, 4σ decreased with increasing temperature, while in the temperature range between 45 and 85°C, 4σ approached a constant value. Comparison with the corresponding data on the C_{18} sorbent in Fig. 4 reveals that the transition in bandwidth values for canine cytochrome c which occurred around 37°C is much less distinct with the C_4 sorbent than with the C_{18} sorbent while the data for bovine cytochrome c on both sorbents was almost identical. Once again, these results demonstrate that the small differences in amino acid sequence between the four cytochrome c molecules gives rise to subtle differences in interactive behaviour in terms of their relative stability and the nature of the conformational transitions which occur under different experimental conditions.

4. Conclusions

The molecular composition of the interactive contact area of the protein solute which presents itself to the chromatographic surface ultimately determines the affinity of the solute for the stationary phase ligands. Changes which occur in the contact region as a consequence of conformational changes will give rise to changes in the retention behaviour of the solute. If the interconversion of a protein solute generates a family of very closely related conformers in

which the chromatographic contact region has also changed, then the small shifts in retention time between these conformers will result in changes in the experimental bandwidths of the protein. The degree to which these conformers can be resolved will depend on the inherent selectivity of the chromatographic system used. Bandwidth measurements of such interconverting systems are therefore a kinetic indicator of the dynamic properties of the protein under a particular set of chromatographic conditions. In the present study, the analysis of protein bandbroadening behaviour has provided information concerning the effects of protein tertiary structure as well as n-alkyl ligand structure on the interactive behaviour of the cytochrome c related proteins in RP-HPLC. Examination of the chromatographic bandwidths has provided insight into the relationship between column residence time and temperature and the conformational dynamics which occur during the chromatographic migration of these protein molecules. Overall, the results have demonstrated that the experimental bandwidth is a very sensitive probe of the dynamic status of a protein solute. In particular, the above experiments demonstrate that both extensive and more subtle changes in protein structure influence the experimental bandwidths of a protein as it undergoes conformational interconversions.

Acknowledgement

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