ELSEVIER

Journal of Chromatography A, 676 (1994) 17–31

JOURNAL OF CHROMATOGRAPHY A

A comparative study of the retention behaviour and stability of cytochrome *c* in reversed-phase high-performance liquid chromatography

K.L. Richards, M.I. Aguilar *, M.T.W. Hearn

Department of Biochemistry and Centre for Bioprocess Technology, Monash University, Wellington Road, Clayton 3168, Australia

Abstract

The retention behaviour of equine, tuna, canine and bovine cytochrome *c* and the corresponding equine and tuna apocytochrome *c* has been investigated using reversed-phase gradient elution chromatographic procedures. A range of operating temperatures between 5 and 85°C were utilised to monitor the influence of protein unfolding on the corresponding retention parameters. Chromatographic measurements were obtained with both an *n*-octadecyl (C_{18}) and *n*-butyl (C_4) sorbent in order to investigate the role of ligand structure on protein retention. The results demonstrated significant differences in the S and log k_0 values between all cytochrome *c* proteins despite the very high degree of sequence homology. These observations suggest that specific amino acid substitutions are directly involved in the chromatographic contact region of the cytochrome *c* molecules. In addition, the prosthetic haem moiety was also shown to provide a significant structural role in terms of relative protein stability under reversed-phase chromatographic conditions. Overall, the results of the present study further demonstrate the utility of interactive modes of chromatography to provide information on physicochemical aspects of protein–surface interactions.

1. Introduction

The success of reversed-phase high-performance liquid chromatography (RP-HPLC) in the analysis and purification of low-molecular-mass compounds and small peptides is increasingly finding parallels for the analysis of proteins. However, the potential loss of biological activity, the formation of multiple peaks of otherwise pure samples and lower levels of recovery arising from protein denaturation or conformational changes remain the main factors which contribute to the less common use of preparative RP-HPLC compared to ion-exchange and affinity chromatographic methods for protein purification. A number of experimental parameters have been established in order to characterise the interactive behaviour of peptides and proteins in RP-HPLC. Experimentally derived Z or S values, which are empirical terms describing the magnitude of the chromatographic contact region and which are calculated from the slope of isocratic or gradient retention plots, *i.e.* from plots of the logarithmic capacity factor log k, *versus* the reciprocal of the molar concentration $1/[c]$, or the molar fraction, ψ, of the organic modifier have been correlated with various physicochemical parameters of peptides and pro-

* Corresponding author.

0021-9673/94/$07.00
SSDI 0021-9673(94)00092-N

teins [1–6]. In addition, the influence of column temperature, solvent strength and the nature of the *n*-alkyl ligand on peptide retention behaviour has also been studied [8,9]. Investigations have also described the characterisation of protein folding and stability in reversed-phase chromatographic systems [9–19]. In particular, it has been established that the Z/S value of a protein often increases with the degree of denaturation as a result of the formation of (a) larger chromatographic contact region(s) in the unfolded species [7,16]. These studies have therefore established that conformational intermediates with different folded states can give rise to different retention behaviour.

In the present study, the effect of gradient time, operating temperature and type of immobilised *n*-alkyl ligand on the retention behaviour of various closely related cytochrome c species [*i.e.* equine (horse heart), tuna (tuna heart), canine (dog heart) and bovine (cattle heart)] and the corresponding equine and tuna apocytochrome *c* have been examined. In order to validate the relationship between protein conformation and the corresponding retention parameters in RP-HPLC, the retention behaviour of this series of cytochrome *c*-related proteins was monitored under chromatographic conditions chosen to manipulate secondary and tertiary structure.

2. Materials and methods

2.1. Chemicals and reagents

Acetonitrile (HPLC grade) was obtained from Mallinckrodt (Paris, KY, USA) and trifluoroacetic acid (TFA) was purchased from Pierce (Rockford, IL, USA). Water was quartz-distilled and deionised in a Milli-Q system (Millipore, Bedford, MA, USA). Equine (horse heart), tuna (tuna heart), canine (dog heart) and bovine (cattle heart) cytochrome *c* were obtained from Sigma (St. Louis, MO, USA). All other reagents were analytical reagents or the best available grade. All mobile phases were filtered prior to use with a 0.2-μm Millipore Durapore filter.

2.2. Apparatus

All chromatographic measurements of the retention behaviour were performed on a Hewlett-Packard (Waldbronn, Germany) Model HP 1090M liquid chromatograph with a binary-drive solvent-delivery system, equipped with a HP 79847A autosampler and a HP 79846A autoinjector. Temperature was controlled by a HPW90A heated column compartment or for lower temperatures by immersion of the column in a column water jacket coupled to a recirculating cooler (FTS Systems, NY, USA). All measurements were routinely monitored at 215 and 400 nm utilising a HP 79880A diode array detector. Chromatographic analysis and peak integration were carried out by a HP 9153C professional computer with the HP79995A "Chemstation" operating software. Further peak analysis was performed using the "Bandwidth" software developed within this laboratory and compatible with the "Chemstation" software package.

Reversed-phase chromatography was carried out with Bakerbond Widepore *n*-octadecyl silica (C_{18}) and *n*-butyl silica (C_4) stationary phases (J.T. Baker, Phillipsburg, NJ, USA) with nominal particle diameters of 5 μm and an average pore size of 30 nm, packed into 250 × 4.6 mm I.D. stainless-steel cartridges. All pH measurements were performed with an Orion Model SA520 pH meter (Orion, Cambridge, MA, USA).

2.3. Chemical preparation of apocytochrome c

Apocytochrome *c* was prepared by modification of the silver sulphate method [20]. To individual solutions of either equine or tuna cytochrome *c* [1 mg/ml, dissolved in 33% (v/v) acetic acid] was added a 60 molar excess of Ag_2SO_4. The reaction mixture was shielded from light and incubated at 37°C for a period of 48 h. Silver was precipitated by acidification with 1 *M* H_2SO_4 and the supernatant transferred to a fresh reaction vessel. The protein was precipitated from the supernatant by the addition of acetone (3.0 ml) and the solution chilled in ice for

approximately 30 min. The acetone was decanted and the protein pellet was dried under a stream of nitrogen. The protein was redissolved in 0.09% TFA in acetonitrile–water (50:50) and stored at 4°C.

2.4. Chromatographic procedures

Bulk solvents were filtered and degassed by sparging with helium. Linear gradient elution was performed using 0.1% TFA in Milli-Q water and 0.09% TFA in acetonitrile–water (50:50) over gradient times of 30, 45, 60, 90 and 120 min with a flow-rate of 1 ml/min at temperatures of 5, 10, 20, 30, 37, 45, 55, 65, 70, 75, 80 and 85°C. All data points were derived from at least duplicate measurements with retention times between replicates typically varying by less than 1%. The concentrations of protein solutions were 1.0 mg/ml and injection sizes ranged between 2–10 μl. The column dead volume was taken as the retention time of the non-interactive solute, sodium nitrate.

2.5. Computational procedures

The various chromatographic parameters log $\bar{k}$, $\bar{\psi}$, S and log k_0 were calculated using the Pek-n-ese program [3] written in Pascal for IBM-compatible computers. Statistical analysis involved ANOVA linear regression analysis was carried out utilising Sigma Plot (version 5.0) graphics software for IBM-compatible computers.

2.6. Computer analysis of protein structures

The surface distribution of amino acid residues of different cytochrome *c*'s were visualised using a Silicon Graphics Model 4D IRIS workstation using the INSIGHT II operating software from Biosym Technologies (San Diego, CA, USA). The amino acid sequences of each cytochrome *c* molecule were obtained from the PSD-Kyoto protein sequence data base. The atomic coordinates for tuna cytochrome *c* were obtained from the Brookhaven data bank. The tertiary structure of equine, bovine and canine cytochrome *c* were predicted from the X-ray crystallographic structure of tuna cytochrome *c* by substitution of the appropriate amino acid residues in the three-dimensional structure followed by refinement of the structure using molecular dynamics and energy minimisation [21].

3. Results and discussion

3.1. Retention behaviour of cytochrome c on the C_{18} sorbent

Linear solvent strength (LSS) concepts have been widely used in RP-HPLC to derive chromatographic parameters related to the interaction of the solute molecule with the sorbent [1,3–7]. Thus, under regular reversed-phase gradient elution conditions, linear relationships are frequently observed between the logarithm of the median capacity factor, log $\bar{k}$, and the median organic mole fraction, $\bar{\psi}$, with the experimental data readily analysed according to the following empirical expression

$$\log \bar{k} = \log k_0 - S\bar{\psi} \quad (1)$$

The S and log k_0 values can be determined by linear regression analysis of log $\bar{k}$ *versus* $\bar{\psi}$ experimental data according to Eq. 1. Previously, the S and log k_0 values have been related, via the solvophobic theory, to the magnitude of the chromatographic contact area established by the protein solute and the affinity which the solute has for the chromatographic ligands [22]. In the present investigation, gradient elution data were obtained for a series of cytochrome *c* molecules as part of our on-going studies on the mechanism of interaction of proteins in RP-HPLC. In particular, the S and log k_0 values were determined for equine, tuna, canine and bovine cytochrome *c* and the chemically modified proteins, equine apocytochrome *c* and tuna apocytochrome *c*. The amino acid residue differences between these different proteins are listed in Tables 1 and 2. A range of operating temperatures were also utilised to monitor the influence of protein unfolding on the corresponding retention param-

Table 1
Differences in amino acid sequences in four cytochrome *c* species

Species	Sequence position number																			
	4	9	22	28	33	44	46	47	54	58	60	61	62	88	89	92	95	100	103	104
Equine	Glu	Ile	Lys	Thr	His	Pro	Phe	Thr	Asn	Thr	Lys	Glu	Glu	Lys	Thr	Glu	Ile	Lys	Asn	Glu
Tuna	*Ala*	*Thr*	*Asn*	*Val*	*Trp*	*Glu*	*Tyr*	*Ser*	*Ser*	*Val*	*Asn*	*Asn*	*Asp*	Lys	*Gly*	*Gln*	*Val*	*Ser*	*Ser*	–
Canine	Glu	Ile	Lys	Thr	His	Pro	Phe	*Ser*	Asn	Thr	*Gly*	Glu	Glu	*Thr*	*Gly*	*Ala*	Ile	Lys	Asn	Glu
Bovine	Glu	Ile	Lys	Thr	His	Pro	Phe	*Ser*	Asn	Thr	*Gly*	Glu	Glu	Lys	*Gly*	Glu	Ile	Lys	Asn	Glu

Italicised amino acids represent the residues which differ from the amino acid sequence of equine cytochrome *c*.

eters. All cytochrome *c* molecules were chromatographed on the *n*-octadecyl (C_{18}) sorbent at five different gradient times ranging between 30 and 120 min at a flow-rate of 1 ml/min at twelve different temperatures ranging between 5 and 85°C. Fig. 1 shows the chromatographic separation of the four cytochrome *c* molecules with a 120-min gradient time at 25°C. It is clear from this chromatogram that significant selectivity differences exist between these closely related proteins. As discussed below and listed in Table 1, there are only three amino acid differences between the equine and bovine cytochrome *c* and five amino acid differences between the equine and canine cytochrome *c*. The high degree of resolution between these protein molecules indicates that these specific amino acid differences play a significant role in the interactive behaviour of these proteins.

In order to further characterise the retention behaviour of these protein molecules, the retention data obtained for each cytochrome *c* related protein were analysed according to the LSS model to derive values for log $\bar{k}$ and $\bar{\psi}$. The log $\bar{k}$ *versus* $\bar{\psi}$ retention plot for equine holocytochrome *c* over the range of temperatures is shown in Fig. 2. Iterative linear regression analysis of the log $\bar{k}$ *versus* $\bar{\psi}$ experimental data yielded the values of the retention parameters *S* and log k_0 at different temperatures. Fig. 3 shows the change in *S* and log k_0 values of the four cytochrome *c* molecules with increasing temperature for each protein chromatographed on the *n*-octadecyl sorbent. Between the temperatures of 5 and 10°C, with the exception of bovine cytochrome *c*, there was a small but significant decrease in the values of both the *S* and log k_0 values. As the temperature increased between 10 and 80°C, a continuous increase in both the *S* and log k_0 values was observed for all proteins. Except for equine cytochrome *c* which showed a continual increase up to 85°C, between

Table 2
Specific differences in the amino acid composition of four cytochrome *c* species

Species	Compared to species	No. of amino acid residues difference
Equine	Tuna	19
Equine	Bovine	3
Equine	Canine	5
Tuna	Bovine	17
Tuna	Canine	18
Bovine	Canine	2

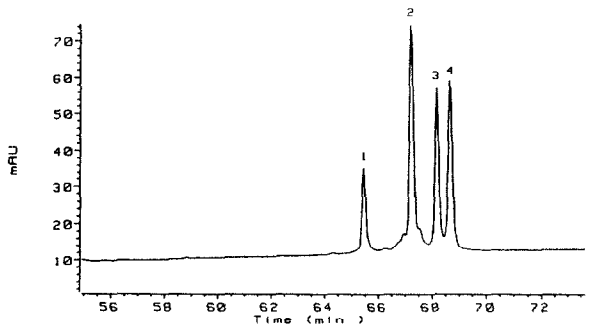

Fig. 1. RP-HPLC chromatogram showing the elution profile of a mixture of (1) bovine, (2) equine, (3) tuna and (4) canine cytochrome *c*. The profile was obtained with a gradient time of 120 min at 25°C. For other chromatographic conditions see the Materials and methods section.

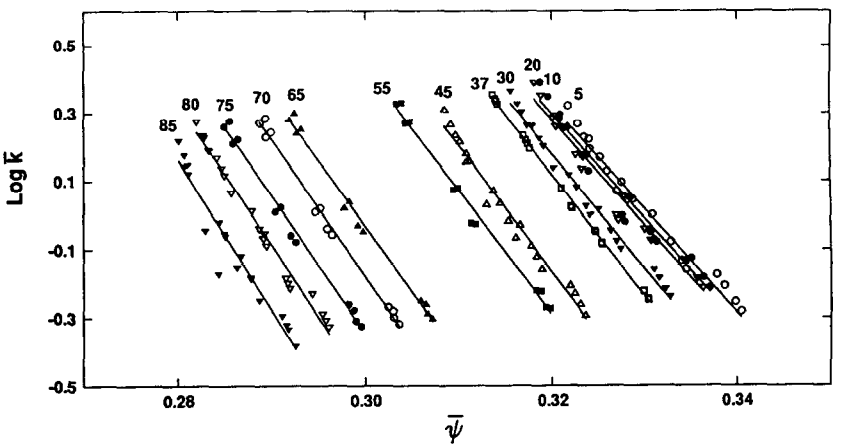

Fig. 2. Plots of log $\bar{k}$ *versus* $\bar{\psi}$ for equine cytochrome *c* chromatographed on a C_{18} sorbent at 12 different temperatures between 5 and 85°C as indicated. For other chromatographic conditions see the Materials and methods section.

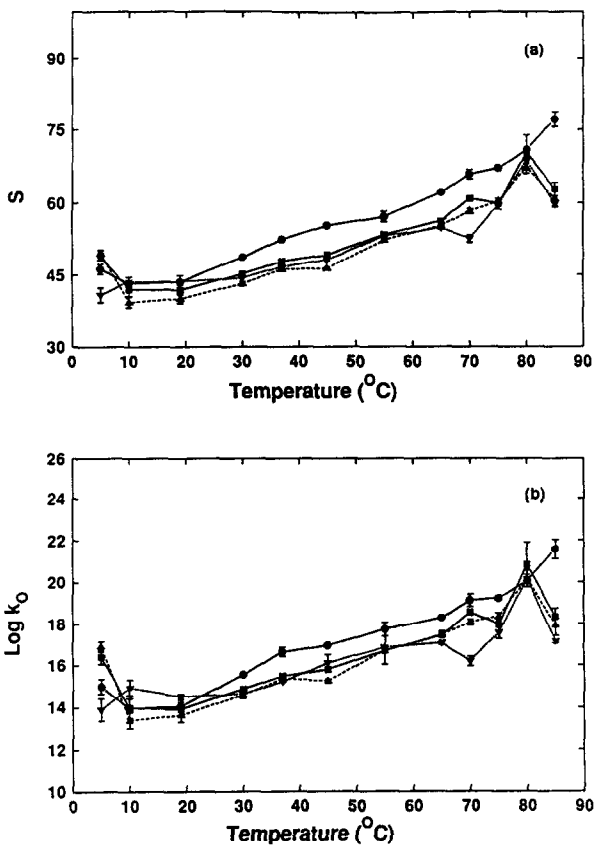

Fig. 3. Dependence of (a) *S* and (b) log k_0 on temperature for each of the holo-cytochrome *c* molecules separated on the C_{18} sorbent. ● = Equine; ■ = tuna; ▲ = canine; ▼ = bovine.

80 and 85°C, both the *S* and log k_0 values for tuna, canine and bovine cytochrome *c* all showed a decrease. If it is recalled that the *S* and log k_0 values represent a measure of the size of the interactive binding region on the surface of the cytochrome *c* protein and its affinity for the stationary phase ligands respectively, then changes in these retention parameters can be related to changes in the structure of the protein which are occurring as a response to increases in the operating temperature. Furthermore, the changes in the *S* value with increasing temperature can be related to the extent of unfolding of the protein structure. The appearance of these retention plots in Fig. 3 can be contrasted with the corresponding data observed for the low-molecular-mass control solutes N-acetyl-L-phenylalanine ethyl ester and penta-L-phenylalanine on the same *n*-octadecyl silica which are shown in Fig. 4. These small solutes have no significant secondary structure and exhibit little change in the magnitude of the *S* value over the entire range of temperatures employed. The corresponding affinity or log k_0 term for these two control solutes demonstrated only a small decrease in value with increased operating temperature, as would also be expected for a conformationally rigid solute. Moreover, as the *S* and log k_0 values obtained for these control

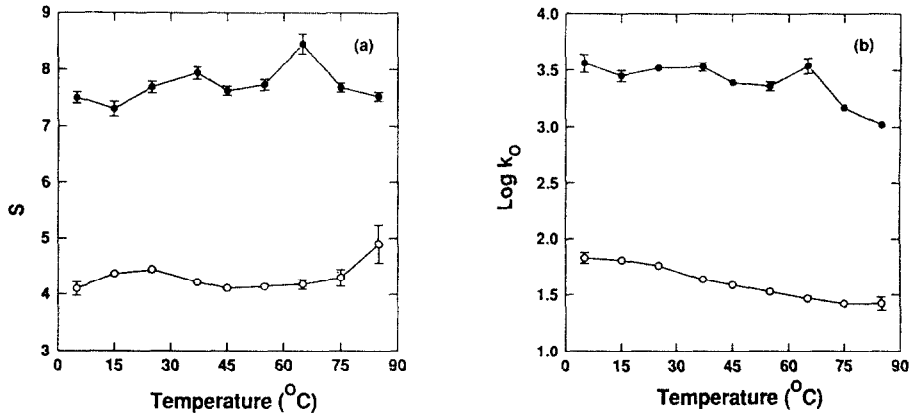

Fig. 4. Dependence of (a) S and (b) log k_0 on temperature for penta-L-phenylalanine (●) and N-acetyl-L-phenylalanine ethyl ester (○) separated on the C_{18} sorbent.

solutes reflected very small changes over the temperatures employed, these results provide evidence that the relative stability and phase characteristics of the sorbent ligands were unchanged with increases in the operating temperature. The changes in the retention data for the cytochrome *c* molecules can thus be related to changes in conformation of these proteins with increases in the operating temperature rather than specific changes in the interactive characteristics of the ligands.

In the low-temperature range 5–10°C, decreases were observed in the experimental S values for three (equine, tuna and canine) cytochrome *c*'s, suggesting a reduction in the contact area of the interactive binding site. The affinity of this interaction was also reduced at 10°C relative to 5°C. At low temperatures, a reduction in the size of the interactive binding region may occur as result of refolding. The experimental data thus suggest that at 5°C the holo-cytochrome *c* molecules may be in a less compact state, compared to that adopted at 10°C. Other studies on the effect of temperature on the three-dimensional structure of equine cytochrome *c* have demonstrated that the cytochrome *c* molecule loses activity at low pH and low temperature arising from the formation of a molten globule state which retains the secondary structural features of the native state, but is characterised by weakened tertiary hydrogen bonding [23–25]. This so-called cold denaturation effect will result in a more expanded tertiary structure with the internalised non-polar amino acid residues still buried and not accessible to the solvent (or other probing ligands). As a consequence of the slightly expanded surface characteristics of this molten globule form, distortion of the geometry of the surface-accessible interacting amino acid side chains will result in a greater contact area (*i.e.* increased S value) and increased free energy change upon binding (*i.e.* higher log k_0) as evident in the increased S and log k_0 values observed at 5°C.

As the temperatures increased over the range 10–80°C, there was a gradual, nearly linear dependence on temperature of the change in the interactive area of cytochrome *c*, as determined by the S values from approximately 14 to 20, which also coincided with an increase in the log k_0 values. The dependence of these parameters on temperature indicates that the protein three-dimensional structure is unfolding, by a process which involves the exposure of previously buried hydrophobic amino acids. Between 80 and 85°C however, while the S and log k_0 values for equine cytochrome *c* continued to increase, these parameters showed a significant decrease for the

tuna, bovine and canine molecules. In addition, between 20 and 85°C, the S and log k_0 values for the equine molecule were consistently higher than the corresponding values for the tuna, bovine and canine molecules. These results demonstrate that the differences in amino acid composition lead to subtle differences in the interactive behaviour of these protein molecules. Whilst there is essentially no difference in the tertiary structures of these proteins in the crystalline state and in bulk solution [26], the differences in retention behaviour suggests that there may be structural differences when placed within the chromatographic environment. What then is the origin of these changes in selectivity, S and log k_0 values at different temperatures observed for these proteins? Table 1 lists the amino acid differences between the different cytochrome c species. For closely related proteins which exhibit significantly different selectivity and band-broadening dependencies on chromatographic variables such as gradient time and temperature, these changes in retention behaviour will have their origin in the amino acid substitutions either by being directly located in the same chromatographic binding region or by causing a change in the tertiary structure, which can affect the shape/composition of the contact region. Of the cytochrome c's examined, the bovine species is the most closely related to the equine species with only three amino acid residue differences. Furthermore, alignment of these amino acid residue replacements at positions 47, 60 and 89 with the equine crystal structure indicates that each amino acid residue difference occupies a solvent exposed position on the surface of the three dimensional structure [27]. These three amino acid residue differences are moreover located in three distinct regions on the surface of the cytochrome c protein. Position 47 ($Thr_{equine} \rightarrow Ser_{bovine}$) is located in the omega (Ω) loop at the bottom of the haem group. Position 60 is located at the N-terminus of the third α-helix where the positively charged lysine residue in the equine protein has been replaced with the non-charged glycine residue in the bovine protein. The remaining amino acid residue difference at position 89 ($Thr_{equine} \rightarrow Gly_{bovine}$) is located on the hydrophilic face of the C-terminal helix. As a consequence of the folding of these proteins, residues 47 and 89 are in close proximity to the haem pocket whilst residue 60 is located on the opposite face of the molecule. Similarly, alignment of the two additional amino acid substitutions which occur between canine and equine cytochrome c at positions 88 ($Lys_{equine} \rightarrow Thr_{canine}$) and 92 ($Glu_{equine} \rightarrow Ala_{canine}$) reveal that these substitutions form a cluster with residue 89, again in close proximity to the haem crevice. Moreover, two of the three amino acid residues in this region in the equine protein are charged (Lys^{88}) or very polar (Glu^{92}), whilst the substituted amino acid residues in the canine protein (Thr^{88} and Ala^{92}) are neutral or non-polar at pH 2. In contrast, there are 19 amino acid residue differences between equine and tuna cytochrome c, 17 of which occupy surface positions within the native crystal structure [27,28] and two occupy internal positions. The change in nett charge accompanying the amino acid substitutions for the tuna species compared to the equine species may account for the differences in the magnitude of the S and log k_0 parameters at each of the temperatures studied. In particular, several amino acid residues which are positively charged in the equine protein are substituted with neutral amino acid residues in the tuna protein, resulting in a relative nett loss of five positive charges for the tuna cytochrome c molecule. Taking into consideration the electrostatic charge differences on the surface of each of the proteins, and the difference in retention behaviour observed when the chromatographic variables were held constant, then clearly these amino acid differences are responsible for the observed differences in selectivity and in the S and log k_0 values. Overall, the experimental data suggest that the amino acid residues flanking the haem crevice are predominant contributors to the S and log k_0 values (*i.e.* the chromatographic contact region) of cytochrome c. Detailed analysis of the hydrophobic surface free energy of each cytochrome c molecule is currently being

investigated with specific reference to the role of the non-polar haem cleft in the protein–ligand interaction.

3.2. The retention behaviour of apocytochrome c on the C_{18} sorbent

In order to establish the role of the haem moiety in the interaction of the holo-protein with the *n*-octadecyl ligands, additional experiments were carried out with the corresponding equine and tuna apocytochrome *c* proteins using the same methods of chromatographic analysis. Injection of both the homogeneous apoproteins at temperatures of 5 and 10°C yielded two distinct, resolvable peak zones. The chromatograms for equine apocytochrome *c* at five different gradient times at 5°C are shown in Fig. 5. As is evident from these data, the appearance of the two peaks was dependent upon operating temperature and column residence time. In general, the degree of peak splitting was greatest at lower temperatures and shorter gradient times. For equine apocytochrome *c* at 5°C, the second peak had almost disappeared when a 120-min gradient time was employed, whilst at 10°C, the second (later eluting) peak was absent when either the 90- and 120-min gradient times were employed. At 20°C, the second peak was not evident for any of the gradient times. Furthermore, the relative proportions of these two peaks changed with increasing gradient times, such that the integrated area of the later-eluting peak (peak 2, Fig. 5) diminished as the area of the early-eluting peak increased (peak 1, Fig. 5). For tuna apocytochrome *c* at 5°C, this peak splitting effect was also observed at all gradient times, whilst the appearance of the second peak was observed at 30, 45 and 60 min at 10°C. A similar phenomenon has been reported in the literature for other homogeneous proteins, where multiple peak formation in the RP-HPLC elution profile has been observed with variations in column residency times and temperature [16–19,29,30]. Furthermore, these investigations have shown that the conformers correspond to both biologically active and denatured structures, with the denatured structure typically eluting later with RP-HPLC systems. The results of the present study with apocytochrome *c* suggest that an additional pathway of conformational change, *i.e. cold* denaturation, can occur as a consequence of reversible conformational changes under gradient elution conditions. Detection of this phenomenon of conformational interconversion is the result of relatively slow kinetic processes which occur within the chromatographic time scale. Assuming that this interconversion can be described by a first-order rate process as outlined in the following scheme

$$\text{Unfolded}_{\text{cold}} \overset{k_1}{\rightleftharpoons} \text{Native} \overset{k_2}{\rightleftharpoons} \text{Denatured}_{\text{hot}}$$

the apparent first-order rate constants for the unfolding process associated with the cold denaturation, k_1, can be determined. This analysis was carried out using the dependence of the natural logarithm of the relative peak area (A) of the second conformer (*i.e.* log $(A_2/[A_1 + A_2])$ on the residency time of the second conformer, as shown in Fig. 6. The rate constants determined from the slopes of these plots are listed in Table 3. On the C_{18} sorbent, the rate 1 constants were greater for equine than for tuna apocytochrome *c*, while the rate of disappearance of the second peak was greater at 10°C than at 5°C.

The dependence of the S and log k_0 values on temperature for the two apocytochrome *c* molecules are shown in Fig. 7. It is generally observed in RP-HPLC that polypeptides elute later in an

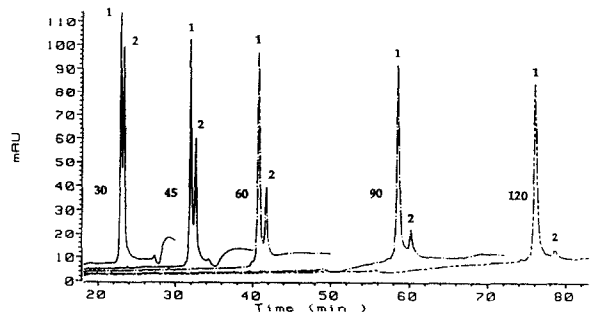

Fig. 5. RP-HPLC chromatogram showing the elution profiles obtained for equine apocytochrome *c* chromatographed on the C_{18} sorbent at 5°C at gradient times of 30, 45, 60, 90 and 120 min, as indicated. The first-eluted conformer is denoted as 1 and the second-eluted conformer is denoted as 2.

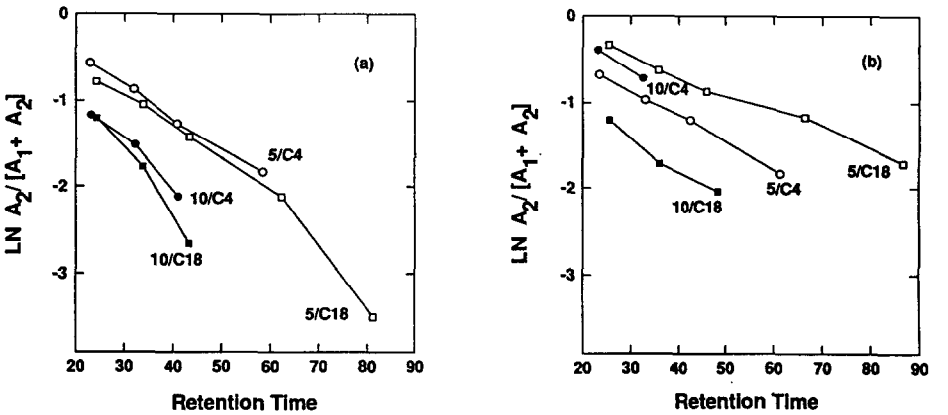

Fig. 6. The rates of structural conversion of the second apocytochrome *c* conformer to the first apocytochrome *c* conformer. Time scales in min. (a) Equine apocytochrome *c*; (b) tuna apocytochrome *c*. 5/C4, 5/C18 corresponds to data derived at 5°C with the C_4 and C_{18} sorbent, respectively; 10/C4, 10/C18 corresponds to data derived at 10°C with the C_4 and C_{18} sorbent, respectively.

unfolded form than in a folded conformation, due to the greater exposure of the inner core hydrophobic amino acid residues [19,30]. In the case of both the equine and tuna apocytochrome *c* two peaks were resolved at 5 and 10°C, with the *S* and log k_0 values of the second peak indicating a larger interactive contact area for this species than for the early-eluting species. However, it is evident that the second peak converts to the first peak at 5°C and to a lesser extent at 10°C, indicating that this type of conformational change was reversible. Between the temperatures of 10 and 85°C, where only one peak was observed, an increase in both these retention parameters was found for the apocytochrome *c* protein molecule. Thus, the temperature-induced increase in both retention pa-

Table 3
First-order rate constants for the cold denaturation of apocytochrome *c*

Temperature (°C)	Column	Rate constant ($mAU\,s^{-1} \times 10^4$)	
		Equine	Tuna
5	C_{18}	8.3	3.3
10	C_{18}	13.3	6.7
5	C_4	6.7	5.0
10	C_4	8.3	6.7

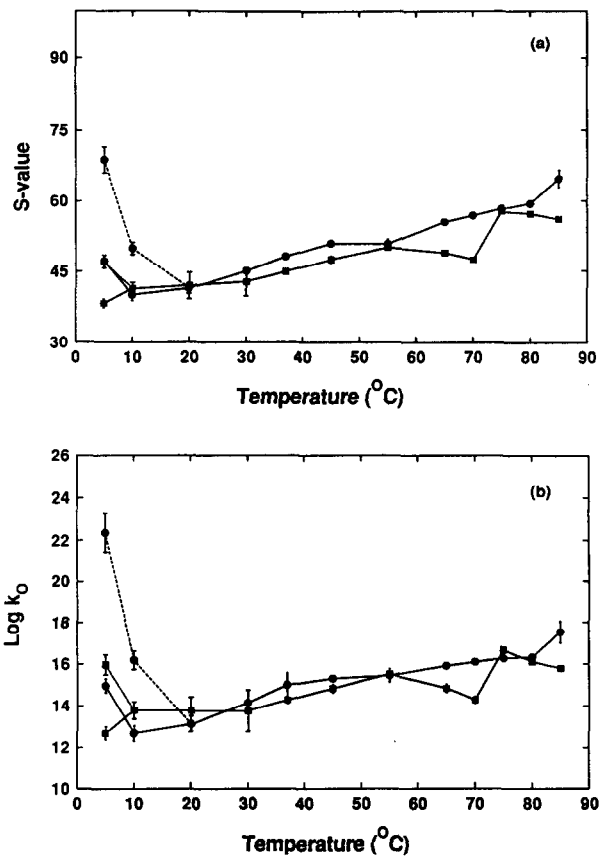

Fig. 7. Dependence of (a) *S* and (b) log k_0 on temperature for equine (●) and tuna (■) apocytochrome *c* separated on the C_{18} sorbent. The data points connected by broken lines represent the *S* and log k_0 values for the second conformer.

rameters is consistent with changes in the protein secondary and tertiary structure. These results suggest that at low temperatures in the presence of both the stationary and mobile phases, apocytochrome *c* adopts a folded conformation, which is subject to denaturation at higher temperatures. Relevant independent studies involving circular dichroism [31–33], Fourier-transform infrared spectroscopy [34] and nuclear magnetic resonance (NMR) spectroscopy [35] measurements of the conformational properties of apocytochrome *c* when associating with lipid micelles or phospholipid vesicles have unequivocally shown that the water–lipid interface induces a highly dynamic folded state in the molecule, involving the stabilisation of secondary structural units. Moreover, these results collectively suggest that the lipid-induced folding of the apoprotein may represent a folded intermediate in the cytochrome *c* folding pathway [35]. These results are vastly different to the bulk solution structure for the apoprotein in the freely solvated monomeric state in physiological buffers, which show an absence of any stabilised secondary structure, with the exception of the N- and C-terminal regions of the polypeptide chain [33]. Thus, these experimental results suggest that apocytochrome *c* can adopt a significant amount of secondary and tertiary structure in the presence of the hydrocarbonaceous adsorbent.

The S and log k_0 values for both the equine holo- and apocytochrome *c* proteins have been replotted for comparison in Fig. 8. It is evident from these plots that the equine holo-protein exhibited higher S and log k_0 values than the apoprotein. A similar relationship between tuna holo- and tuna apocytochrome *c* was also apparent. Furthermore, it is evident from these retention data that although the general unfolding trajectories of these two proteins are similar, they are not identical, with incremental increases in the ΔS and $\Delta\log k_0$ values occurring with temperature. Since the only compositional difference between the apoprotein, when compared to the holo-protein, is the presence/absence of the haem moiety, these results demonstrate that the haem moiety also plays a role in determining the S and log k_0 values associated with the interactive structure of cytochrome *c*, an effect which becomes more pronounced with increasing temperature. For example, the haem molecule may contribute directly to the binding domain or its removal may cause changes in the three-dimensional structure which then change the properties of the binding domain and in this way change the experimental S and log k_0 values. The haem moiety is located within a hydrophobic crevice in the overall globular structure of the protein, with one hydrophobic exposed edge [28]. It is possible that the protein orients itself with the crevice aligned with the hydrophobic ligands. As the crevice itself is lined with hydrophobic residues, this orientation would facilitate hydrophobic

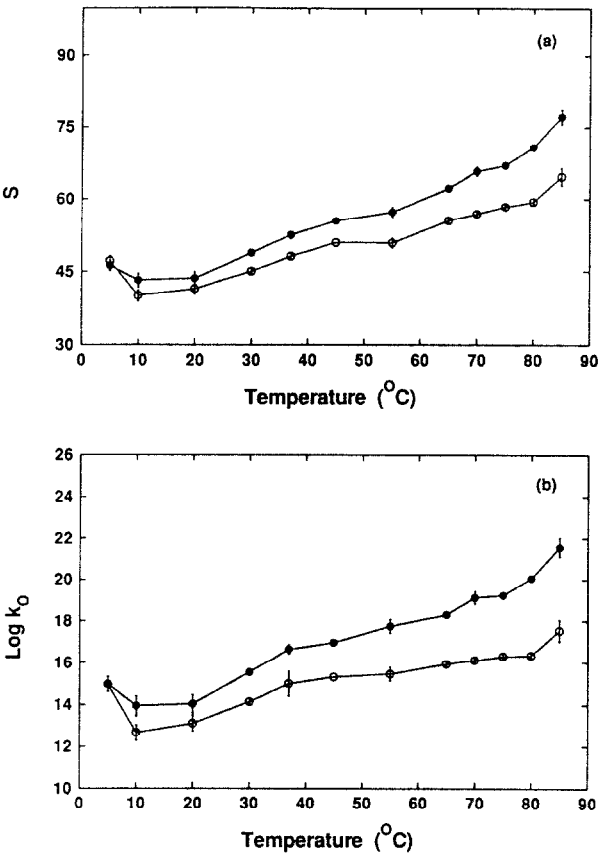

Fig. 8. Dependence of (a) S and (b) log k_0 on temperature for equine holo- (●) and apo- (○) cytochrome *c* separated on the C_{18} sorbent.

interaction, especially as the protein unfolds and the hydrophobic contact area is increased.

3.3. Retention behaviour of cytochrome c on the C_4 sorbent

The chemical properties of the *n*-octadecyl and *n*-butyl ligand dictate that both types of *n*-alkyl silica sorbents are likely to interact with the surface topography of the biosolute in a subtly different manner. This view is supported by various literature studies which have demonstrated that different *n*-alkyl chain lengths (in particular, *n*-octadecyl *versus* *n*-butyl) probe different regions on the surface of a biosolute as a consequence of differences in ligand structure [8,11]. In addition, molecular dynamics studies on the mobility of the *n*-alkyl ligands when immobilised onto a silica surface have revealed significant conformational differences in the structure of *n*-octadecyl and *n*-butyl ligands [36], in agreement with results from NMR spectroscopic techniques [37,38]. The different flexibilities of the C_{18} and the C_4 ligands in conjunction with their different chemical properties when presented for interaction with biosolutes were thus anticipated to be revealed as significant changes in chromatographic retention behaviour of the different cytochrome *c*-related proteins.

The four cytochrome *c* molecules were chromatographed at five different gradient times ranging between 30 and 120 min at a flow-rate of 1 ml/min at twelve different temperatures ranging between 5 and 85°C. Fig. 9 shows the change in the *S* and log k_0 values of the cytochrome *c*'s when subjected to increases in temperature. These thermal denaturation curves illustrate that the retention behaviour of the cytochrome *c* molecules in the presence of the C_4 ligands is much more complex than found with the C_{18} ligands. Furthermore, the dependence of *S* and log k_0 on temperature for the control solutes N-acetyl-L-phenylalanine ethyl ester and penta-L-phenylalanine on the C_4 sorbent were similar to that observed with the C_{18} sorbent (data not shown) again demonstrating that the changes in

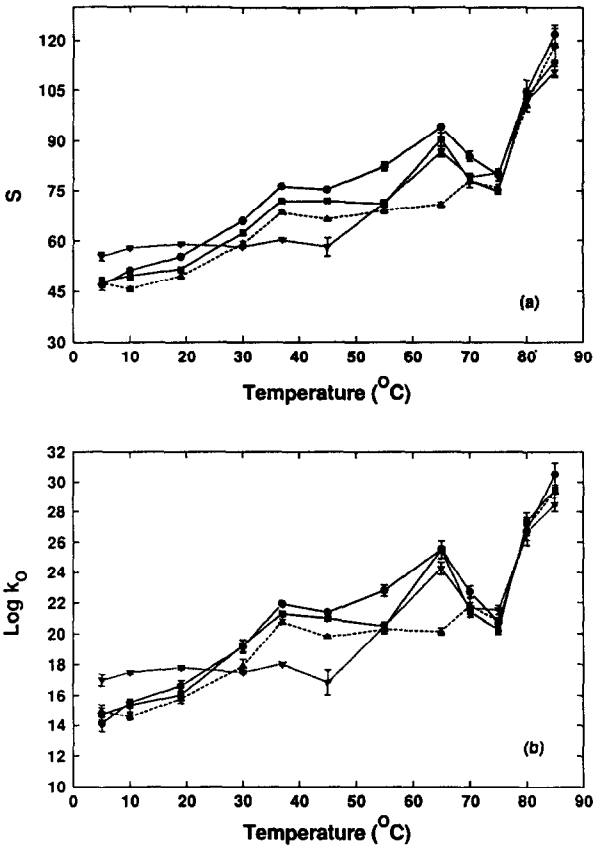

Fig. 9. Dependence of (a) *S* and (b) log k_0 on temperature for each of the holo-cytochrome *c* molecules separated on the C_4 sorbent. ● = Equine; ■ = tuna; ▲ = canine; ▼ = bovine.

S and log k_0 values in Fig. 9 can be related to changes in protein conformation. Initially, between 5 and 37°C there was a steady increase in the magnitude of both the *S* and log k_0 retention parameters. At 5°C, the experimental results suggest that the protein molecules are likely to exist as folded structures, whilst the structures characterised at 37°C are significantly unfolded. Between 37 and 65°C, the *S* and log k_0 values increased further, although the relative changes were less pronounced. Between 65 and 75°C a reproducible decrease in the magnitude of both the retention parameters was observed. In the very high temperature range of 80–85°C both the *S* and log k_0 parameters increased significantly in

value. The overall conformation of the holo-cytochrome *c* molecules may correspond to the fully unfolded form at 85°C. Heat perturbation of the protein structure results in the unfolding of the cytochrome *c* domains, subdomains and specific secondary structures. At some point in the unfolding pathway, the haem moiety will become exposed to the immobilised hydrophobic ligands which will increase the attraction of the protein moiety for the stationary phase. Overall, for the holo-proteins, the experimental results indicate that with the C_4 ligands there are distinct phases during the denaturation of the three-dimensional protein structure which may correspond to the presence of different structural intermediates.

When the retention behaviour of the cytochrome *c* molecules chromatographed on the C_4 sorbent is compared with that observed for the C_{18} (Fig. 3) several important differences are evident. These differences are most noticeable in the low-temperature range 5–10°C and at the higher-temperature range 65–75°C. With the C_{18} sorbent between 5 and 10°C, decreases in the observed retention parameters indicated that the protein was less structured at 5°C, relative to 10°C, whilst on the C_4 column S and log k_0 were observed to increase slightly. Thus, in this low-temperature range the different ligands are affecting the three-dimensional structure of the holo-proteins to different degrees. Between 65 and 75°C, the interactive properties of the proteins with the C_{18} sorbent were characterised by relative increases in the retention parameters, whilst with the C_4 sorbent the structural changes were manifested as a relative decrease in the S and log k_0 values. In addition, comparison of the thermal denaturation curves obtained from the two different *n*-alkyl silicas reveal that the magnitude of the S and log k_0 values obtained with the C_4 sorbent were significantly greater than those obtained with the C_{18} sorbent at each operating temperature employed. These results suggest that the structure of the ligand can significantly affect the way in which the protein is stabilised/destabilised in a hydrophobic environment.

3.4. The retention behaviour of apocytochrome c on the C_4 sorbent

The dependence of the S and log k_0 values on temperature obtained from the chromatographic analysis of the two apocytochrome *c* molecules are illustrated in Figs. 10 and 11 together with the data for the corresponding holo-proteins. Inspection of the data in Fig. 10 for equine apocytochrome *c* again reveals more complex retention behaviour than found with the C_{18} sorbent. For both apoproteins in the lower-temperature range 5–10°C, the phenomenon of peak splitting was again observed with the C_4 ligands. For equine apocytochrome *c*, the chromato-

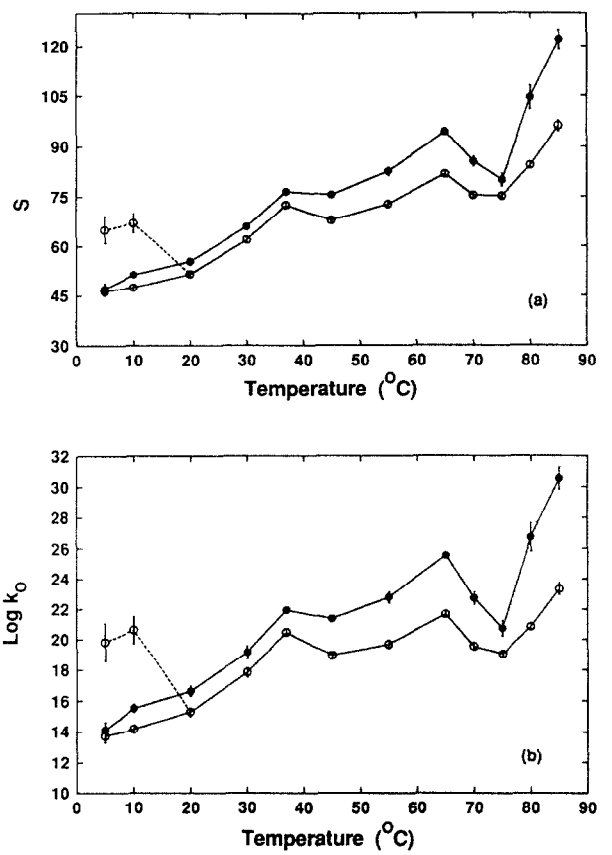

Fig. 10. Dependence of (a) S and (b) log k_0 on temperature for equine holo- (●) and apo- (○) cytochrome *c* separated on the C_4 sorbent. The data points connected by broken lines represent the S and log k_0 values for the second conformer.

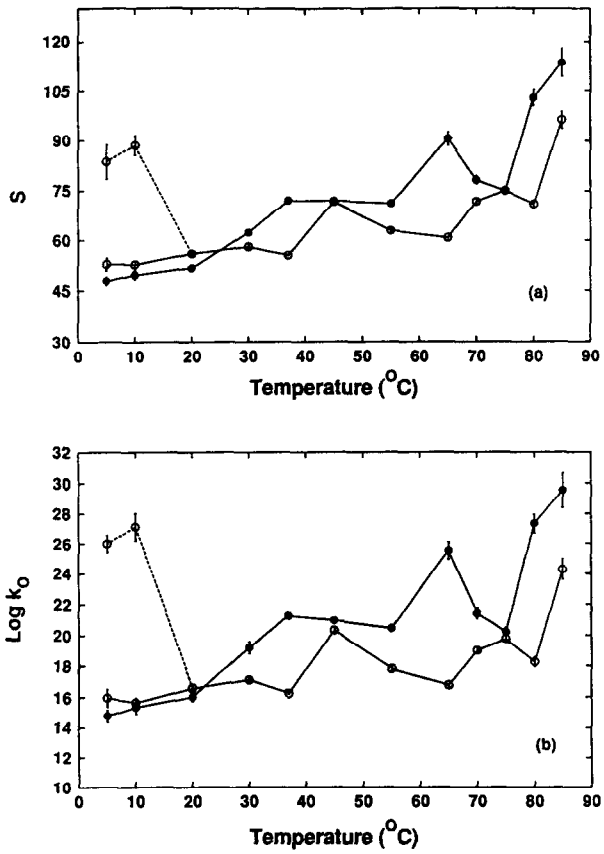

Fig. 11. Dependence of (a) S and (b) log k_0 on temperature for tuna holo- (●) and apo- (○) cytochrome c separated on the C_4 sorbent. The data points connected by broken lines represent the S and log k_0 values for the second conformer.

graphic elution profile was very similar to that shown in Fig. 5 for the C_{18} sorbent. At 5°C the presence of the second peak was detected at each of the five gradient times employed, whilst at 10°C the presence of the second peak was determined at only the 30-, 45- and 60-min gradient times. In addition, the area of the second peak diminished as that of the first peak increased. The apparent first-order rate constants for the structural interconversion for both apoproteins are listed in Table 3. In contrast to the results observed for the C_{18} sorbent, there was very little difference in the apparent rate of unfolding for each apoprotein at either temperature. In addition, at both 5 and 10°C, the second peak was only observed at shorter residency times on the C_4 compared to the C_{18} sorbent, which suggests that the n-octadecyl ligand stabilises this more denatured conformer. If the magnitude of the S and log k_0 values of the second peak are compared with the values obtained for apoprotein at 85°C, they are significantly lower, suggesting that the conformers present at the lower temperatures may not be as unfolded as the structures which exist at high temperatures.

Comparison of the data for the equine holoprotein with the equine apoprotein on the C_4 sorbent reveals a very similar dependence of the S and log k_0 values on temperature. The difference in the magnitude between the retention curves obtained for the holo-protein when compared to the apocytochrome c molecule indicates that the haem moiety is at least partially exposed at all the operating temperatures and contributes increasingly to the interactive segment on the surface of the protein which binds to the ligands as the protein unfolds. The haem is further implicated in a structurally stabilising role, as the phenomenon of peak splitting is not observed for the holo-protein. The high correlation in the retention behaviour evident in these curves suggests the secondary and tertiary structure of both the holo- and the apoprotein are very similar, and as a consequence, in the reversed-phase chromatographic environment they undergo very similar denaturation pathways. In addition, as both equine proteins progressively unfolded as indicated by the retention parameters, it is likely that they are approaching a similar denatured structure at 85°C.

Comparison of the S and log k_0 values obtained for the equine apocytochrome protein molecule chromatographed on C_4 sorbent (Fig. 10), with those obtained from chromatography on the C_{18} sorbent (Fig. 7) reveals that the magnitudes of these parameters were significantly larger at the higher temperatures with the C_4 sorbent than with the C_{18} sorbent. Between 65 and 85°C the S and log k_0 parameters also exhibited large fluctuations when chromatographed on the C_4 sorbent, whilst this was not evident on the n-octadecyl ligands. Furthermore,

the S and log k_0 values for both the first and second peaks remained essentially constant between 5 and 10°C on the C_4 sorbent but decreased over this temperature range on the C_{18} sorbent.

Fig. 11 illustrates the dependence of both S and log k_0 on temperature for tuna apocytochrome c. These retention data for tuna apocytochrome c protein molecule upon interaction with the C_4 sorbent reveals that S and log k_0 values varied over the entire range of operating temperatures to a much greater degree than was observed for equine apocytochrome c. The differences in the shapes of these denaturation curves further implicate a structural role for the prosthetic haem and suggests that the haem moiety has a significant influence on the specific properties of the interactive binding site. Further inspection of Fig. 11 reveals a local maximum in S and log k_0 values at *ca.* 65°C for the holoprotein, which may correspond to the unfolding of significant secondary structural elements, such as α-helices. Due to the lack of the stabilising influence of the haem group in the apoproteins, it is possible that these same structures melt at lower temperatures and are manifested as a local maximum in the S and log k_0 thermal denaturation curves for the apoprotein near 45°C. Between 5 and 10°C, both the equine and tuna apoproteins exhibit the formation of two resolvable peaks. However, the second peak of the equine species was still evident under conditions of longer residency, *i.e.* longer gradient times, and is characterised by larger S and log k_0 values than the corresponding second peak of the tuna species. These results suggest that the equine conformer is more stable in the C_4 chromatographic environment than the tuna conformer, has a higher affinity for the stationary phase ligands and interacts through a larger contact area.

4. Conclusions

In the present study, the retention characteristics of a family of related cytochrome c proteins, namely equine, tuna, canine and bovine along with chemically modified cytochrome c, *i.e.* equine apocytochrome c and tuna apocytochrome c have been documented. In particular, the relationship between the experimental S and log k_0 values and the conformational state of these proteins has been investigated. It has been demonstrated that these retention parameters are particularly sensitive to structural changes that occur at or near the interactive binding region on the surface of the protein, as well as in response to manipulation of several chromatographic variables (*i.e.* column residency time, operating temperature, n-alkyl ligand structure). Comparisons of the interactive characteristics of the holo-protein *versus* the apoprotein has revealed that the prosthetic haem molecule plays an important role in the structural integrity of the protein in the n-alkyl silica chromatographic environment. In particular, the absence of the haem group at low temperatures resulted in the formation of resolvable conformers with otherwise homogeneous preparation of apocytochrome c. The results of the present study in terms of the effect of various amino acid substitutions and the putative role of the haem moiety in the interactive behaviour of the cytochrome c molecules have laid the foundation for more detailed analysis of the nature of the chromatographic contact region. For example, it can be postulated from these results that the stability of the C-terminal helix significantly determines the interactive binding region on the surface of the molecule. As the C-terminal helix forms part of the hydrophobic crevice, and as the retention behaviour of the holo-protein *versus* the apoprotein demonstrates that the haem moiety may be involved in the contact region, then it can be proposed that the protein is orientated with the haem crevice aligned with the sorbent-phase ligands. Confirmation of this proposal through the application of *in situ* proteolytic techniques will be described in an associated paper [39]. Overall the analysis of the changes in the experimental S and log k_0 retention parameters in response to changes in amino acid sequence and other experimental operating parameters thus provides a powerful tool to elucidate the struc-

tural stability of globular proteins as they interact with hydrophobic surfaces.

5. Acknowledgement

The financial support of the Australian Research Council is gratefully acknowledged.

6. References

[1] M.A. Stadalius, H.S. Gold and L.R. Snyder, *J. Chromatogr.*, 296 (1984) 31.
[2] X. Geng and F.E. Regnier, *J. Chromatogr.*, 296 (1984) 15.
[3] M.I. Aguilar, A.N. Hodder and M.T.W. Hearn, *J. Chromatogr.*, 327 (1985) 115.
[4] M.T.W. Hearn and M.I. Aguilar, *J. Chromatogr.*, 359 (1986) 31.
[5] M.T.W. Hearn and M.I. Aguilar, *J. Chromatogr.*, 392 (1987) 33.
[6] M.T.W. Hearn and M.I. Aguilar, *J. Chromatogr.*, 397 (1987) 47.
[7] M. Kunitani, D. Johnson and L.R. Snyder, *J. Chromatogr.*, 371 (1986) 313.
[8] A.W. Purcell, M.I. Aguilar and M.T.W. Hearn, *J. Chromatogr.*, 593 (1992) 103.
[9] M.I. Aguilar, S. Mougos, J. Boublik, J. Rivier and M.T.W. Hearn, *J. Chromatogr.*, 646 (1993) 53.
[10] P. Oroszlan, S. Wicar, G. Teshima, S.L. Wu, W.S. Hancock and B.L. Karger, *Anal. Chem.*, 64 (1992) 1623.
[11] M. Hanson, K.K. Unger, C.T. Mant and R.S. Hodges, *J. Chromatogr.*, 599 (1992) 65.
[12] K. Benedek, S. Dong and B.L. Karger, *J. Chromatogr.*, 317 (1984) 227.
[13] S.A. Cohen, K. Benedek, Y. Tapuhi, J.C. Ford and B.L. Karger, *Anal. Biochem.*, 144 (1985) 275.
[14] S.A. Cohen, K. Schellenberg, K. Benedek, B.L. Karger, B. Grego and M.T.W. Hearn, *Anal. Biochem.*, 140 (1984) 223.
[15] X.M. Lu, K. Benedek and B.L. Karger, *J. Chromatogr.*, 359 (1986) 19.
[16] S. Lin and B.L. Karger, *J. Chromatogr.*, 536 (1990) 89.
[17] E. Watson and W.C. Kenny, *J. Chromatogr.*, 606 (1992) 165.
[18] R. Rosenfeld and K. Benedek, *J. Chromatogr.*, 632 (1993) 29.
[19] K. Benedek, *J. Chromatogr.*, 646 (1993) 91.
[20] K.G. Paul, *Acta Chem. Scand.*, 4 (1950) 239.
[21] I. Yarovsky, M. Zacchariou, M.I. Aguilar and M.T.W. Hearn, in preparation.
[22] Cs. Horváth, W. Melander and I. Molnár, *J. Chromatogr.*, 125 (1976) 129.
[23] M. Ohgushi and A. Wada, *FEBS Lett.*, 164 (1983) 21.
[24] S. Potekhin and W. Pfeil, *Biophys. Chem.*, 34 (1989) 55.
[25] Y. Kuroda, S. Kidokoro and A. Wada, *J. Mol. Biol.*, 223 (1991) 1139.
[26] G. Williams, G.R, Moore, R. Porteous, M.N. Robinson and R.J.P. Williams, *J. Mol. Biol.*, 183 (1985) 409.
[27] R.E. Dickerson, T. Takano, D. Eisenberg, O.B. Kallai, l. Samson, A. Cooper and E. Margoliash, *J. Biol. Chem.*, 246 (1971) 1511.
[28] G.W. Bushnell, G.V. Louie and G.D. Brayer, *J. Mol. Biol.*, 214 (1990) 585.
[29] M.T.W. Hearn, A.N. Hodder and M.I. Aguilar, *J. Chromatogr.*, 327 (1985) 47.
[30] S.A. Cohen, K. Benedek, S. Dong, Y. Tapuhi and B.L. Karger, *Anal. Chem.*, 56 (1984) 217.
[31] A. Walter, D. Margolis, R. Mohan and R. Blumenthal, *Membr. Biochem.*, 6 (1987) 217.
[32] W. Jordi, Z. Li-Xin, M. Pilon, R. Demel and B. de Kruijff, *J. Biol. Chem.*, 264 (1989) 2292.
[33] H.H. de Jongh and B. de Kruijff, *Biochim. Biophys. Acta*, 1029 (1990) 105.
[34] A. Muga, H.H. Mantsch and W.K. Surewicz, *Biochemistry*, 30 (1991) 2629.
[35] H.H. de Jongh, J.A. Killian and B. de Kruijff, *Biochemistry*, 31 (1992) 1636.
[36] I. Yarovsky, M.I. Aguilar and M.T.W. Hearn, *J. Chromatogr. A*, 660 (1994) 75.
[37] E. Bayer, A. Paulus, B. Peters, G. Laupp, J. Reniers and K. Albert, *J. Chromatogr.*, 364 (1986) 25.
[38] J. Nawrocki and B. Buszewski, *J. Chromatogr.*, 449 (1988) 1.
[39] M.I. Aguilar, D. Clayton and M.T.W. Hearn, in preparation.