CHROMSYMP. 898

THERMAL BEHAVIOR OF PROTEINS IN HIGH-PERFORMANCE HYDRO-PHOBIC-INTERACTION CHROMATOGRAPHY

ON-LINE SPECTROSCOPIC AND CHROMATOGRAPHIC CHARACTERIZATION

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SUMMARY

The thermal behavior of a series of standard proteins in hydrophobic interaction chromatography on a previously developed weakly hydrophobic ether-bonded phase column has been studied. Depending on the temperature and protein, conformational changes can occur in the chromatographic system. Methods for recognizing these conformational effects are presented, including retention and peak width variations with temperature, and Z values (the slope of the plot of log k' *vs.* log %B solvent. The Z value is shown to be a general index characterizing protein retention as a function of salt concentration. In addition, on-line UV spectroscopic analysis, (absorbance ratios and second derivative spectroscopy) with a photodiode array detector, is shown to corroborate chromatographic trends. Lysozyme maintains a stable conformation over the temperature range 10–40°C, whereas β-lactoglobulin A has a conformational transition between 25°C and 40°C. Calcium-depleted α-lactalbumin, a rather labile species, maintains a stable conformation up to *ca.* 20°C, and then undergoes structural changes. Finally, cytochrome c appears to be relatively stable up to *ca.* 65°C. Since conformational changes for this protein occur at *ca.* 35°C on more hydrophobic phases, the extent of hydrophobicity of the stationary phase is important for maintenance of the native state. Based on this work, hydrophobic-interaction chromatography at sub-ambient temperatures appears promising.

INTRODUCTION

High-performance liquid chromatography (HPLC) of biopolymers is currently an area of rapid development. Whereas separations of biopolymers within hours or days were typical in the past, today separations are readily achievable in only a few minutes. At present, the most widely used general procedure for resolution of biopolymers is ion-exchange chromatography (IEC). However, recently, hydrophobic-

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interaction chromatography (HIC) has developed into a high-performance general separation method[1-6], as a result of earlier work[7-10].

HIC is based on a mild adsorption process, yielding protein fractions in a biologically active state. This contrasts with reversed-phase HPLC where most proteins elute in a denatured state[11-13]. It needs to be recognized, however, that protein structural changes (*i.e.* changes in secondary, tertiary and/or quarternary structure) can occur on any surface, depending on (1) the protein, (2) the mobile phase composition and pH, and (3) the column temperature[14]. Thus, we can anticipate that labile species may be altered during either HIC[15] or IEC[16], depending on the conditions imposed.

Some of the structural changes of proteins upon adsorption will be subtle and reversible, yielding biologically active material. Such changes might be considered as native-state "breathing"[17], in which the protein retains thermodynamic properties close to those in the solution state. Some changes will result in an intermediate structure in which the protein possesses properties intermediate between the native and denatured state. Other changes will be extensive, leading to significant alteration in the thermodynamic and biological properties of the molecule. Often, the latter effects will be cooperative and occur dramatically over a short temperature range[18]. Such alterations may or may not be reversible in a reasonable time span.

In this paper we shall examine simple, monomeric proteins and focus on conformational alterations in their tertiary structure. It is important to study conformational changes of proteins during HPLC for several reasons. First, an examination of such effects for well characterized proteins, particularly as a function of temperature or denaturing agent, can yield insights into the adsorption process of the protein. Secondly, systematic studies of standard proteins can be one of the means of categorizing stationary phases. If relatively stable, standard proteins can be unfolded, we may anticipate difficulties with more labile species. Clearly, other measures of characterization are also required, *e.g.*, selectivity and capacity. However, the ability of a surface or stationary phase to alter protein conformation must be an important consideration, whenever protein isolation is undertaken. In addition, the catalytic effect of a surface may be especially significant when unknown or poorly characterized mixtures are being resolved. Thirdly, conformational changes may lead to multiple peaks or distorted peak shape[15,16,19-21]. An understanding of protein conformational behavior can result in optimized chromatographic conditions for the separation of biologically active species.

The methodology used for recognition of conformational effects in HPLC is also very important. Spectroscopy is a well developed method for characterization of protein conformational changes[22-24]. It is possible to collect peak fractions and examine the solutions spectroscopically in an off-line mode. However, it may be anticipated that some structural changes will be rapidly reversible and, hence, important information may be lost by off-line spectroscopy. For this reason and for convenience, we have decided to explore on-line spectroscopy. In this work, we have employed a photodiode array detector, using UV absorbance ratioing and second-derivative spectroscopy.

Chromatographic methodology can also be useful for assessing the thermodynamic consequences of conformational changes. In this paper, we examine the use of retention and peak width changes as a function of chromatographic conditions in

order to elucidate structural effects. In addition, we show that Z values, which represent the slope of the plot of log capacity factor (k') $vs.$ log %B solvent can be a sensitive index of unfolding.

Using the above methodologies, we explore conformational changes of standard proteins in HIC as a function of column temperature, employing a previously developed weakly hydrophobic ether column[5]. The results of this investigation, by themselves, and in comparison with results obtained with other HIC phases[15], provide insights into the role of the stationary phase in effecting conformational changes.

EXPERIMENTAL

Equipment

For on-line spectroscopic analysis, a 25-μl computer-controlled injection loop, designed in this laboratory was connected to a series 8800 gradient controller with a three head chromatographic pump (DuPont Instruments Product Division, Wilmington, DE, U.S.A.). A Hewlett-Packard (HP) (Palo Alto, CA, U.S.A.) Model 1040A photodiode array detector with a HP85B personal computer, DPU multichannel integrator, HP 9121 P/S disc drives, HP 7470 A graphics plotter and HP think-jet printer were used in this work. The chromatographic data were processed with the HP Data Evaluation II software package.

For isocratic measurement of Z values, a second liquid chromatograph was used, consisting of a Model 5000 solvent delivery pump and solvent programmer (Varian Assoc., Palo Alto, CA, U.S.A.), a Rheodyne 7125 injection valve (Rheodyne, Cotati, CA, U.S.A.), a Model 440 fixed-wavelength UV detector (280 nm) (Water Assoc., Milford, MA, U.S.A.) and a BD40 recorder (Kipp & Zonen, Amsterdam, The Netherlands). The chromatographic data were processed with a Nelson Analytical (Cupertino, CA, U.S.A.) Model 2600 chromatography software package in conjunction with an IBM (Boca Raton, FL, U.S.A.) personal XT computer. Both chromatographic systems (Du Pont and Varian) also included a Model NBE water bath (Haake-Buechler, Saddle Brook, NJ, U.S.A.), which maintained the temperature within $\pm 0.1°C$.

The chromatographic column was similar to that previously used, containing an ether-bonded, silica-based stationary phase[5,24]. The column dimensions were 100 mm × 4.6 mm I.D., and a precolumn (66 mm × 4.6 mm I.D.) containing the same phase was inserted between the pump and injector. A gradient delay volume of 3.0 ml was observed for the Varian system and a gradient delay volume of 6.45 ml for the Du Pont system. This volume (or time) was subtracted from the retention data.

Chemicals

HPLC-grade water was purchased from Baker (Philipsburg, NJ, U.S.A.). Ammonium acetate, Grade III ammonium sulfate, as well as various high-quality protein standards, were obtained from Sigma (St. Louis, MO, U.S.A.) and used as received. The proteins used are α-lactalbumin (milk) (α-LACT) (calcium depleted to contain less than 0.3 mol of Ca^{2+} per mol of α-LACT), cytochrome c (horse heart) (CYT C), lysozyme (chicken egg white) (LYSO), and β-lactoglobulin A (milk) (β-LA). N-Acetyl-L-tryptophanamide (N-AcTrpNH$_2$), N-acetyl-L-tyrosinamide (N-Ac-TyrNH$_2$), and N-acetyl-L-phenylalanine ethyl ester (N-AcPheOEt) were also purchased from Sigma.

Chromatographic procedures

Mobile phases were prepared by adding the correct weight of salt and buffer to a volumetric flask, containing HPLC-grade water, previously degassed under vacuum. The pH was adjusted with either glacial acetic acid or ammonium hydroxide, and a small amount of HPLC-grade water (degassed) was added to the mark. Solutions containing high concentrations of salt were not allowed to remain in the column or pump for long periods of time. The standard mobile phases were: solvent A, 2 *M* ammonium sulfate, 0.5 *M* ammonium acetate (pH 6); solvent B, 0.5 *M* ammonium acetate (pH 6). A 20-min linear gradient from 100% A (0% B) to 100% B (0% A) was used in most experiments with a flow-rate of 1.0 ml/min. Exceptions to these standard conditions are noted in the paper. Protein solutions (5–10 mg/ml) were freshly prepared. When not in use, the samples were stored at $-20°C$. Z values were determined by isocratic elution of the proteins, where Z is the slope of the plot of log k' *vs.* log %B, k' is the capacity factor of the protein and %B is the volume percentage of mobile phase B mixed with mobile phase A. The minimum amount of sample needed for a good signal was always used. The experimental error for Z was $\pm 10\%$.

RESULTS AND DISCUSSION

On-line spectroscopy

The UV spectrum of a protein in solution is broad and featureless, lacking qualitative structural information. However, subtle changes in the UV spectrum may occur as proteins undergo conformational changes. Aromatic amino acid residues [tryptophan(Trp), tyrosine(Tyr) and phenylalanine(Phe)] may become more or less exposed to the solvent (typically water). Generally, the aromatic amino acid residues will be found in the interior of a globular, water-soluble protein, since they are relatively hydrophobic. Conformational changes may bring one or more of these residues to the surface of the protein. These changes can lead to small variations in the UV spectrum of the protein[22].

In this work, the spectroscopic analysis consisted of two procedures: (1) absorbance ratios of different wavelengths were used to assess aromatic amino acid residue exposure to the mobile phase solvent, and (2) the second-derivative spectrum was used to examine the exposure of Tyr, in particular, to the solvent.

We first studied the UV spectra of standard aromatic amino acid derivatives with the photodiode array detector. Fig. 1 displays the spectra of three model aromatic amino acid compounds: N-AcTrpNH$_2$, N-AcTyrNH$_2$, N-AcPheOEt. The spectra follow closely those published for the derivatized amino acids on standard spectrometers. Note that the extinction coefficient of Phe at the absorption maximum is much smaller than that of Trp. As a consequence, our studies at this point have focused mainly on Trp and Tyr. Note also that, depending on the number of Trp and Tyr amino acid residues available, Trp may interfere with Tyr analysis.

Fig. 1 shows that the absorption at 292 nm is mainly due to Trp, whereas at 274 nm both Trp and Tyr may contribute, and at 254 nm all three aromatic amino acids absorb. Since the photodiode array detector can provide the whole spectrum immediately, it is useful to select several absorption ratios in exploring protein conformational changes as a function of column temperature. We have selected the 292
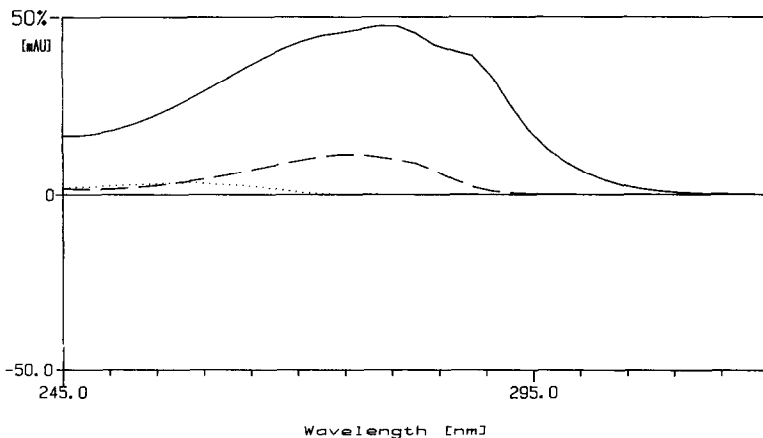
Fig. 1. Normal UV spectra measured with the photodiode array detector of an equimolar solution of N-AcTrpNH$_2$ (—), N-AcTyrNH$_2$ (– – –) and N-AcPheOEt ($\cdot \cdot \cdot$), dissolved in 0.5 M ammonium acetate (pH 6).

nm/254 nm ratio to indicate changes in Trp exposure. Increases in this ratio suggest that Trp amino acid residues have moved from the interior to the surface of the protein. Similarly, an increase in the 274 nm/292 nm ratio with a concomitant decrease in 292 nm/254 nm ratio suggests greater Tyr exposure to the mobile phase. Finally, an increase of 280 nm/254 nm (or 287 nm/254 nm) along with the above ratio could suggest either Trp or Tyr exposure to reinforce the indication of protein conformational change.

It should be noted that the HP photodiode array detector has a 2-nm difference between diodes[26]. The selected wavelengths are therefore based upon the diode situated closest to the particular value. For example, a nominal wavelength of 292 nm is actually 292.5 nm, and the others are also correspondingly increased by 0.5 nm. Using the absorbance ratios of the precise wavelengths can lead to good reproducibility. Separate chromatographic studies revealed that the interassay error in absorption ratio from run to run is ±0.03. Values greater than this can be assumed to represent a different conformation.

Since Trp can interfere significantly with Tyr measurements, a closer examination of Tyr was obtained by second-derivative spectroscopy. This procedure has recently been developed for the examination of aromatic amino acid residues in proteins[27–29]. Since the HP software provides an option for second-derivative spectroscopy, we have incorporated the latter into our on-line spectroscopic analysis of the microenvironments of the aromatic amino acid residues.

The second derivative spectrum of LYSO in the spectral region between 245 and 320 nm is shown in Fig. 2. As is observed for other proteins[30], the spectral region between 280 and 300 nm possesses two major peaks. The peak at the lower wavelength can be ascribed to Tyr and Trp, whereas the peak above 290 nm is due to Trp alone. It has been shown[30] that the a/b ratio (or γ) in Fig. 2 is an index of Tyr exposure to the aqueous solvent (the larger the value, the greater the exposure). Separate experiments revealed that the error in γ in duplicate experiments is ±0.15.
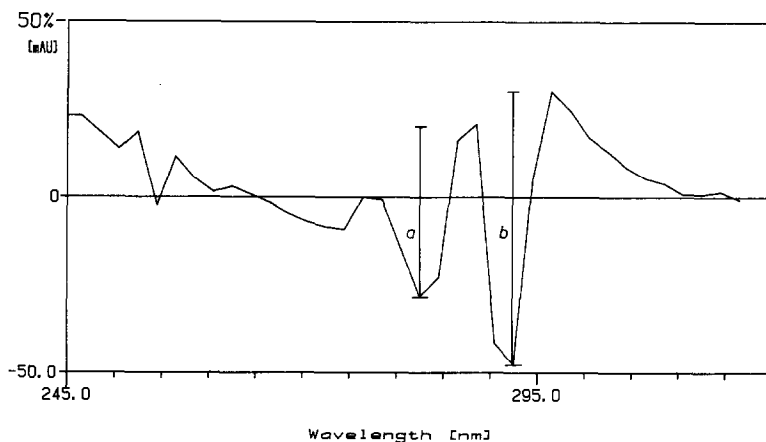
Fig. 2. On-line second-derivative spectrum of LYSO eluted from the ether HIC column at 10°C. Gradient conditions: see *Chromatographic procedures*. The peak-to-peak distance between the maximum at 288.5 nm and the minimum at 282.5 nm (a), and between the maximum at 296.5 nm and the minimum at 292.5 nm (b) defines γ (= a/b).

Thus, values greater or smaller than this would indicate a different microenvironment for the aromatic amino acid residues.

Chromatographic behavior

It is well known that a protein in the folded state is less retained than in an unfolded state[15,16,19]. This is because the number of points or areas of contact of the protein with the adsorbent surface is larger in an unfolded state. Thermodynamically, the free energy change on adsorption will be more negative for an unfolded state. Consequently, an increase in column temperature can lead to an increase in retention, if a conformational change results in a contact area change. Thus, a nonlinear change of retention with temperature can be suggestive of a conformational change in the protein. In addition, at transition temperatures, the peak may be broadened[15]. Therefore, an examination of retention and peak shape as a function of column temperature can yield information concerning unfolding processes during chromatography.

Another important measurement that can reflect contact area is the change of protein retention with solvent composition. Timasheff and co-workers have developed a thermodynamic description of various protein systems based on preferential interactions[31] and have recently applied this model to high salt solutions relevant to HIC[32,33]. The preferential interaction parameter can be incorporated explicitly into the equilibrium constant K for adsorption on the bonded phase, using the Wyman theory of linked functions[34]:

$$\frac{d (\ln K)}{d (\ln a_3)} = (v_3^s - v_3^m) - \frac{m_3}{m_{H_2O}} (v_{H_2O}^s - v_{H_2O}^m)$$

$$= \Delta v_3 - \frac{m_3}{55.5} \Delta v_{H_2O}$$

(1)

where v_3 and v_{H_2O} are, respectively, the number of salt and water molecules bound to the protein and bonded phase surface, Δv_3 and Δv_{H_2O} are the differences in those number of molecules when the protein is adsorbed relative to when it is in solution, a_3 is the activity of the salt, m_3 is the molality of the salt, and superscripts s and m refer to the stationary and mobile phases, respectively.

The adsorption of protein on the stationary phase will lead to negative values for Δv_3 and Δv_{H_2O}, since protein contact with the surface will decrease the number of salt and water binding sites. Thus, the slope of a plot of log k' ($= \varphi K$, where φ is the phase ratio) vs. log a_3 will represent the changes in salt and water that occur when the protein is adsorbed on the surface. Salts which reduce the solubility of the protein, e.g. ammonium sulfate, have been shown to be largely excluded from the protein in solution, leading to preferential hydration[32]. As a consequence, Δv_{H_2O} will be more negative than Δv_3, and from eqn. 1, adsorption will increase as the concentration of salt is increased.

For a 2:1 salt, such as ammonium sulfate, the activity of the salt[33] a_3 is equal to

$$a_3 = 4m_3^3 \gamma_\pm^3 \tag{2}$$

where $\gamma_\pm$ is the mean molal ionic activity coefficient of the salt. Since in a gradient a protein is eluted over a small salt concentration range, it is reasonable to assume that the activity coefficient is constant. Hence, a plot of log k' vs. log salt concentration can be directly related to eqn. 1.

In our studies, it was found that the slopes of the linear plots of either log k' vs. log m_3 or log k' vs. m_3 did not directly correlate with protein retention trends as a function of column temperature. However, conversion to log %B yielded a slope that was a sensitive function of retention change with temperature. A plot of log k' vs. log %B in HIC focuses on water concentration variation in the mobile phase rather than salt concentration variation. Eqn. 1 can be simply recast as

$$\frac{d(\ln K)}{d(\ln a_{H_2O})} = \Delta v_{H_2O} - \frac{m_{H_2O}}{m_3} \Delta v_3 \tag{3}$$

Assuming, as above, that the activity coefficient of water does not change over the small concentration range of protein elution in the gradient, we can rewrite eqn. 3 as

$$\frac{d(\log k')}{d(\log \%B)} = Z = \Delta v_{H_2O} - \frac{m_{H_2O}}{m_3} \Delta v_3 \tag{4}$$

For the salts which reduce protein solubility, typically used in HIC, preferential hydration occurs, and the salt term on the right hand side of eqn. 4 can be assumed to be negligible relative to the water term[32]. In such a case, the slope of the log–log plot, Z, is equivalent to Δv_{H_2O}, the number of moles of water displaced per mole of protein adsorbed on the bonded phase surface. The Z value will thus be related to the contact area of the adsorbed protein on the surface; hence, an unfolded protein should yield a higher Z value than a more folded state. The results to be presented

in this paper reveal Z to be a sensitive measure of conformational change. In a separate paper, we will explore more fully the Z value and the reasons that the above method of plotting provides a sensitive probe of protein structural change. In addition, we shall relate the Z value to the solvophobic model of HIC retention[36,37].

Conformational changes as a function of temperature in HIC

LYSO and β-LA. The influence of temperature on chromatographic behavior for standard proteins was investigated by the methods described above. HIC, in contrast to reversed-phase liquid chromatography, is a mild process in which proteins are generally eluted from the chromatographic column in a biologically active state. However, conformational changes may be induced, depending on the stability of the protein and the hydrophobicity of the column.

Fig. 3 displays the effect of temperature on the retention of LYSO, β-LA and α-LACT. A parallel experiment was performed to measure Z values by isocratic elution. In addition, the on-line spectral characteristics from 245 nm to 320 nm of these proteins were examined using the photodiode array detector. In this section we will discuss the results for LYSO and β-LA.

As seen in Fig. 3, LYSO displays a linear increase in retention with increasing temperature, as generally found in HIC[6,8,15]. There are no slope changes in the retention–temperature curve. Table I gives details on absorbance ratios, second-derivative ratios (γ) and chromatographic Z values for LYSO in the temperature range from 10 to 40°C. In agreement with Fig. 3 there are no changes in the spectral features nor in the Z values for LYSO over this temperature range. The results suggest that LYSO is quite stable under the HIC conditions and undergoes no conformational changes. LYSO is known to be a very stable protein for which the thermal denaturation transition temperature in water at pH 7 is around 70°C[38]. It can be predicted
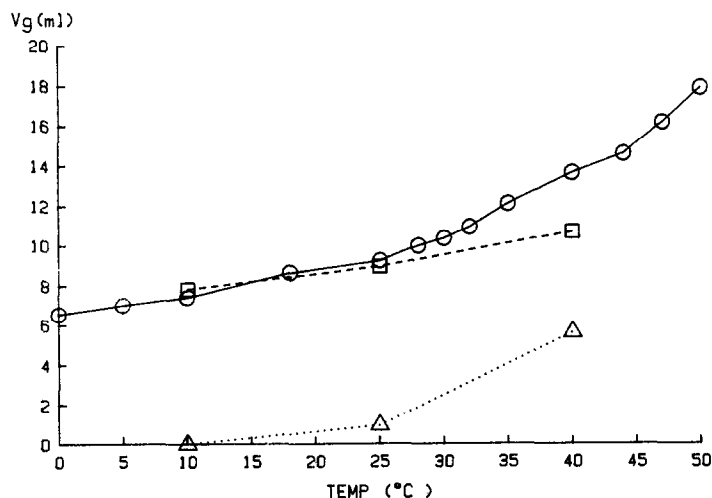

Fig. 3. Effect of temperature on retention on the ether column of α-LACT (O—O), LYSO ($\square$---$\square$) and β-LA ($\triangle$· · ·$\triangle$). HIC was carried out with a gradient described in *Chromatographic procedures*. The retention volume, V_g, is corrected for the delay volume.

TABLE I

CHROMATOGRAPHIC AND SPECTROSCOPIC CHARACTERISTICS OF LYSOZYME IN HIC AS A FUNCTION OF COLUMN TEMPERATURE

Conditions: Ether–HIC column; solvent A, 2 M ammonium sulfate and 0.5 M ammonium acetate (pH 6); solvent B, 0.5 M ammonium acetate (pH 6).

Temperature (°C)	292/254*	280/254*	274/292*	γ**	Z***
10	1.42	2.10	1.41	0.61	4.50
25	1.41	2.10	1.42	0.63	4.89
40	1.42	2.10	1.42	0.63	4.56

    * Absorbance ratio at the wavelengths (nm) indicated.
   ** Second-derivative characteristics, defined in Fig. 2.
 *** Slope of plot of log k' vs. log %B.

that the thermal transition on LYSO will be at a higher temperature under the sulfate salt elution conditions[39].

LYSO has six Trp and only three Tyr amino acid residues. The ability of absorbance ratios to measure Tyr exposure upon conformational change is therefore limited because of the interference of the greater number of Trp. While the second-derivative spectrum has been used to observe Tyr exposure for LYSO[30], the error of the photodiode array detector is too large to discern changes for LYSO. However, Z values can be rather sensitive to changes in conformation as a consequence of contact area variation. The fact that Z does not change with temperature is a good indication that LYSO remains in a stable conformation throughout the temperature range of the study.

β-LA is known to be more easily thermally denatured than LYSO. In water at pH 7, the transition temperature for β-LA is approximately 50°C[40], which can be compared to that for LYSO of 70°C. Examination of Fig. 3 reveals that there is a linear change in retention from 10°C to 25°C, followed by a sharp increase in retention to 40°C. This trend is suggestive of some structural change in β-LA from 25°C to 40°C.

The measured absorbance ratios and γ values, along with the Z values, for the three temperatures are displayed in Table II. It can be observed that the spectral

TABLE II

CHROMATOGRAPHIC AND SPECTROSCOPIC CHARACTERISTICS OF β-LACTOGLOBULIN A AS A FUNCTION OF COLUMN TEMPERATURE

Conditions and footnotes as in Table I.

Temperature (°C)	292/254*	280/254*	274/292*	γ**	Z***
10	1.45	2.50	1.64	1.24	2.11
25	1.42	2.48	1.66	1.11	2.16
40	1.30	2.30	1.71	1.63	4.36

features and Z values are the same at 10°C and 25°C but changes occur at 40°C. The Z value increases by a factor of 2, suggesting an expansion of the contact area of the adsorbed protein at the higher temperature. Changes are also observed in the absorption ratios. The 292 nm/254 nm ratio decreases from 25 to 40°C, suggesting that one or more Tyr residues are being exposed on the surface of the protein. This suggestion is further supported by the increase in the 274 nm/292 nm ratio. In addition, γ increases substantially from 25 to 40°C, a further indication of Tyr exposure. It should be noted that β-LA possesses two Trp and four Tyr amino acid residues. The Tyr/Trp ratio of 2:1 appears to offer sufficient sensitivity for conformational changes to be observed in which the microenvironment of Tyr residues are altered.

α-*LACT*. Fig. 3 displays the retention variation of calcium-depleted α-LACT as a function of temperature. This protein represents a good example of a labile species, since in aqueous solution at pH 7, the thermal denaturation transition temperature is approximately 40°C[41-43]. It is readily apparent from Fig. 3 that the retention pattern of α-LACT is different from that of LYSO and β-LA. From 0 to *ca.* 20°C there is a gradual increase in V_g with column temperature, followed by a change in slope from 25 to 40°C and, finally, a sharp increase in V_g from 40°C to 50°C. It is interesting to note that below 25°C, LYSO and α-LACT are eluted very close to
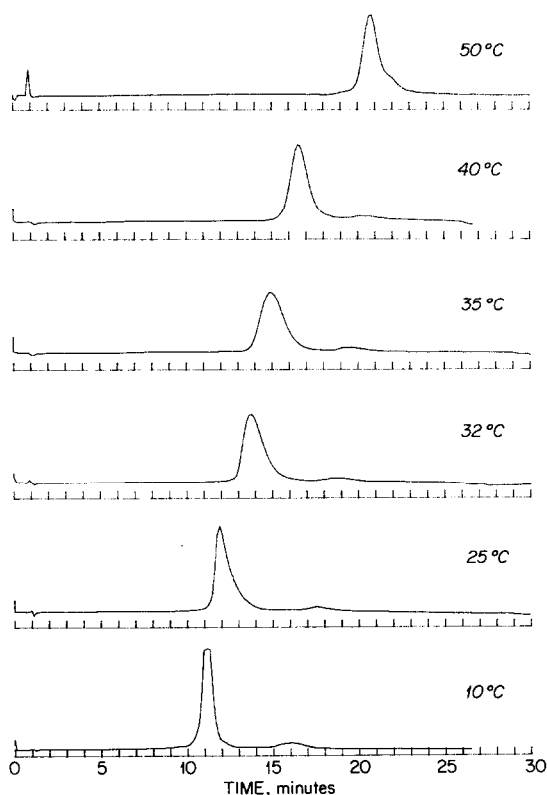

Fig. 4. Effect of temperature on the chromatographic peak width of α-LACT on the ether HIC column. For conditions: see *Chromatographic procedures*.

TABLE III

CHROMATOGRAPHIC AND SPECTROSCOPIC CHARACTERISTICS OF α-LACTALBUMIN IN HIC AS A FUNCTION OF COLUMN TEMPERATURE

Conditions and footnotes as in Table I.

Temperature (°C)	292/254*	274/292*	287/254*	γ**	Z***
0.5	1.41	1.43	1.78	0.82	2.1
5	1.42	1.42	1.79	0.83	2.2
10	1.43	1.43	1.80	0.85	2.3
18	1.40	1.44	1.77	0.89	2.6
25	1.41	1.43	1.77	0.87	5.3
32	1.45	1.41	1.87	0.70	5.6
50	1.40	1.46	1.84	0.85	8.5

one another. This result may be a consequence of the fact that the two proteins are homologous to each other[44]. However, differences clearly arise above 25°C.

Fig. 4 provides a further identification of chromatographic changes with column temperature from 10 to 50°C. It can be seen that the peak is relatively sharp at 10°C and at 50°C. In the intermediate region of 25–40°C an enhanced broadening of the band is observed. Such broadening of intermediate regions has been found in HIC for other proteins[15], as well as in other modes of chromatography[45]. Most likely the broadening is due to either multiple conformers of the protein yielding similar but not identical retention and/or kinetic processes of conformational interchange[20,45]. The behavior in the temperature range 25–40°C is currently under study.

Table III presents the absorbance ratios, γ values and Z values for α-LACT from 0.5 to 50°C. The amino acid residue ratio for Trp/Tyr is 1:1 for α-LACT and, thus, the sensitivity of the spectroscopic characteristics for conformational changes should be intermediate between those of LYSO and β-LA. From 0.5 to 18°C, no change is seen in the on-line spectroscopic characteristics of α-LACT, and only a slight change in the Z value. At 25°C, the Z value doubles from 2.6 to 5.3; but this change is not observed in the spectroscopic values. This behavior suggests that the Z value may be a more sensitive probe of a conformational change at the surface than the on-line absorbance ratios or γ values. Alternatively, the reversible rate of refolding in solution at 25°C may be so rapid that the on-line approach cannot observe the effect or that non-aromatic amino acid residues become exposed to the mobile phase.

At 32°C the peak is much broader than at 25°C (cf. Fig. 4) and at this temperature the spectroscopic criteria are also altered. The 292 nm/254 nm and 287 nm/254 nm absorbance ratios increase beyond the experimental error, suggesting exposure of Trp. This indication is strengthened by the decrease in the γ value from 25°C to 32°C. Interestingly, solution studies have demonstrated exposure of Trp during unfolding[41–43]. Note that the Z value does not change from 25 to 32°C.

At 50°C, there is another dramatic increase in Z from 5.3 to 8.5. The decrease in the 292 nm/254 nm ratio coupled with the increase in the 274 nm/292 nm ratio and the increase in γ, now suggests a further exposure of Tyr. In the previously cited
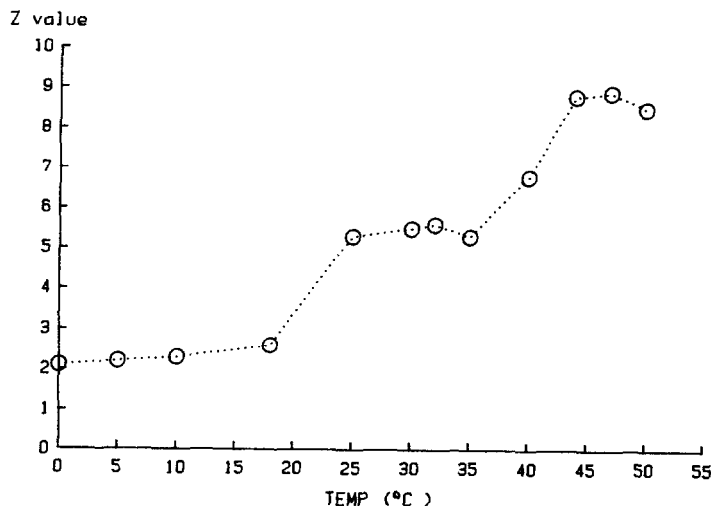
Z value

Fig. 5. Plot of Z values of α-LACT vs. temperature.

work for solution unfolding of α-LACT[41-43], no examination of Tyr exposure was undertaken, because of the interference of Trp.

Fig. 5 presents in more detail the change in Z value as a function of column temperature. As already seen in Table III, Z increases generally with temperature. However, we now observe three distinct temperature regions of constant Z. The low- and high-temperature regions can be associated with two different conformational states. The constancy of Z in the intermediate region 25–35°C might suggest a single major conformational species, intermediate between the low- and high-temperature
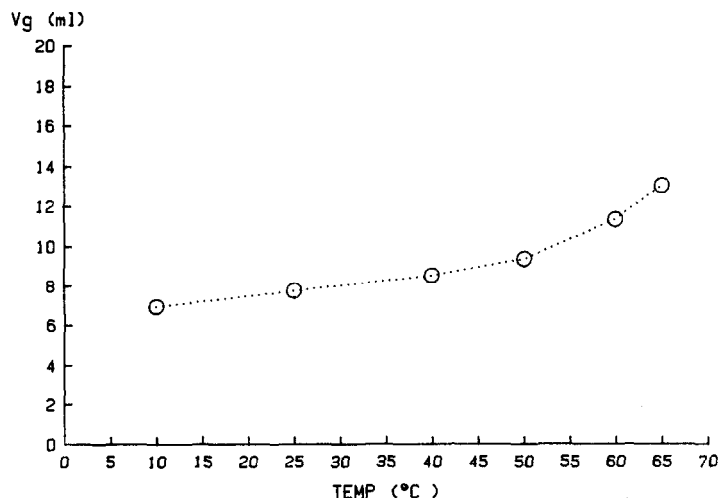
Vg (ml)

Fig. 6. Effect of temperature on retention of CYT C on the ether HIC column. For conditions, see Fig. 3, except solvent A: 3 M ammonium sulfate and 0.5 M ammonium acetate (pH 6). V_g is corrected for the delay volume.

TABLE IV

CHROMATOGRAPHIC AND SPECTROSCOPIC CHARACTERISTICS OF CYT C IN HIC AS A FUNCTION OF COLUMN TEMPERATURE

Conditions and footnotes as in Table I, except solvent A: 3 M ammonium sulfate and 0.5 M ammonium acetate (pH 6).

Temperature (°C)	292/254[*]	287/254[*]	274/292[*]	γ[**]	Z[***]
10	0.74	0.94	1.47	0.58	4.0
25	0.73	0.93	1.47	0.48	4.0
40	0.73	0.93	1.47	0.50	4.0
50	0.73	0.93	1.47	0.57	4.2
60	0.73	0.93	1.47	0.48	—
65	0.73	0.93	1.47	0.47	4.9

forms. However, other work[46] suggests that the retention time and peak shape of the broad peak is kinetically controlled, indicating the possibility of a mixture of conformational states.

α-LACT is a labile protein in HIC. Fig. 3 shows that the protein can be eluted with a stable conformation below 20°C. As will be discussed in the conclusion, subambient column temperature operation would appear to be highly promising for HIC, particularly in the elution of thermally labile species.

CYT C. In aqueous solution at pH 7, the thermal transition temperature of CYT C is 82°C[47]. Fig. 6 shows that V_g for this protein increases in a linear fashion, with a shallow slope up to 50°C, followed by a gradual increase in slope to 65°C. It should be noted that due to the hydrophilicity of CYT C, it was necessary to change solvent A to 3 M ammonium sulfate. Fig. 6 suggests the beginning of a conformational change for CYT C at *ca.* 60–65°C.

Table IV presents the absorbance ratios and γ values at 10–65°C. There does not appear to be any discernable changes in the on-line spectral features of CYT C. Since this protein possesses one Trp and four Tyr amino acid residues, the sensitivity for Tyr microenvironment changes is high, especially for the γ value[30]. The Z values, on the other hand, suggest the start of a conformational change in the 65°C range, in agreement with the V_g values. We again observe that chromatographic retention appears to be more sensitive to conformational changes than UV spectral features.

The results in Fig. 6 and Table IV suggest that the transition temperature for conformational change may be approximately 75°C. This temperature may be compared with 35°C, observed for CYT C on a more hydrophobic phase, TSK-Phenyl-PW[15]. This comparison suggests that weakly hydrophobic phases ought to be used to minimize conformational changes for labile species. This point has been emphasized by Shaltiel, who used alkylated agarose as stationary phases[48]. In addition, it will be noted that elution on weakly hydrophobic phases occurs at a higher salt concentration than on more hydrophobic phases, further stabilizing the protein[39].

CONCLUSIONS

This work is a continuation of our studies on the conformational effects of

biopolymers in HPLC. We have examined specific methods for the characterization of conformational changes and applied these methodologies to the thermal behavior of several standard proteins in HIC. We have shown that UV spectral features can, at times, be useful to corroborate suspected conformational changes, based on chromatographic results. At present, chromatographic behavior in terms of V_g, peak widths, and Z values seems more sensitive to protein structural changes. However, further improvements, in particular of the second derivative spectrum, should increase the discriminating power of the spectral approach. Moreover, other on-line spectroscopic methods, e.g., fluorescence, circular dichroism, could, in principle, provide additional sensitive characterizations of conformational changes. Moreover, the on-line approach may permit the determination of the relationship of the conformation of the protein eluted from the column relative to that in solution, even if reversible refolding takes place in a short time span.

The Z value appears to be quite sensitive to conformational changes. This value is based on the chemical potential difference of the protein between the adsorbed and solution states.

Our results suggest that the more hydrophobic the stationary phase, the greater will be the chance for protein conformational changes at a given temperature. This will clearly be a function of the protein and the mobile phase conditions as well. It should be emphasized that the structural changes observed in this paper do not necessarily represent complete unfolding or denaturation. Indeed, it has been suggested that localized conformational changes can occur[15]. Presumably, many of these changes are reversible.

An important conclusion of the present study is the promising use of sub-ambient temperatures in HIC for the stabilization of labile proteins. The results with α-LACT clearly point to the value of operating at temperatures of 5°C or lower. It should be noted that for HIC, in contrast to IEC, shorter retention times are observed at lower temperature. Another advantage of low temperature operation is the increase in the hydrolytic stability of the bonded phase. A lowering of the column temperature by 20°C will increase the phase stability or column lifetime by 4- to 5-fold[49].

Sub-ambient operation may at first seem less convenient, but most biochemistry laboratories now have cold rooms. Our measurements reveal that the viscosity of the mobile phase will increase by a factor of 2 when the temperature is decreased from 40°C to 10°C. However, since short columns are typically employed, the increased pressure drop is tolerable. In addition, since gradient elution is employed, the effect of viscosity on band broadening should not be great. The solubility of the sulfate salt will decrease by 10–15% when the temperature is lowered from 40°C to 10°C[50]. For ammonium sulfate, this decreased solubility presents no problem. Thus, HIC at low temperatures would appear quite promising.

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