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PROTEIN CONFORMATIONAL EFFECTS IN HYDROPHOBIC INTERACTION CHROMATOGRAPHY

RETENTION CHARACTERIZATION AND THE ROLE OF MOBILE PHASE ADDITIVES AND STATIONARY PHASE HYDROPHOBICITY

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SUMMARY

We have studied the conformational behavior of α-lactalbumin (α-LACT) in hydrophobic interaction chromatography (HIC). Retention characterization in terms of Z (slope of plot of ln k' *vs.* ln φ_B, where k' is the capacity factor and φ_B is the volume fraction of mobile phase B) has been explored, and the relationship of Z to other slopes, such as S (slope of the plot of ln k' *vs.* φ_B) has been derived. The reasons for the sensitivity of Z to conformational change are discussed. The enhanced broadening of α-LACT in a temperature transition region of conformational change has been studied by spectral analysis using on-line photodiode array detection. The influence of Ca^{2+} and Mg^{2+} addition to the mobile phase is further explored. Since α-LACT is a calcium binding protein, addition of this metal leads to stabilization, *i.e.* higher column temperatures are required for conformational change. On the other hand, addition of Mg^{2+} appears to destabilize the protein. We have explored the use of a more hydrophobic support, C_2-(ethyl) ether phase, for the elution of α-LACT. In this case, two widely separated peaks are observed. By spectral analysis the first peak is shown to be native and the later eluted, broad second peak to be an unfolded mixture of species. As previously observed in reversed-phase liquid chromatography, the second peak grows at the expense of the first, as the column temperature is raised. The second peak also grows as the contact time of the protein with the surface increases. This behavior can be ascribed to the conformational change of α-LACT in the column, the late eluted species under the second peak binding significantly more strongly to the phase than the native peak. Reinjection of the late eluted fraction reveals that reformation of the native species takes place in solution within 30 min. As before, addition of Ca^{2+} reduces the extent of unfolding under any specific condition. These results add further to our understanding and ability to control conformational changes in high-performance liquid chromatography.

INTRODUCTION

Over the past few years high-performance hydrophobic interaction chromato-

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graphy (HPHIC) has become an important method for the separation of proteins[1–6]. Current efforts in HIC result from earlier developments using organic gels[7–10] and from protein separation studies by reversed phase chromatography[11–15].

An important aspect of protein high-performance liquid chromatography is the role of solute conformation on chromatographic behavior. Changes in the secondary, tertiary or quaternary structure can have a major impact on retention and peak width and shape. An examination of the role of chromatographic parameters in conformational change can provide information on conditions most conducive to maintenance of the biologically active, native state and provide insight into mechanistic details of the adsorption/desorption process[16]. In addition, such studies are of general interest for protein interactions with various surfaces, where it is well known that protein conformational changes at interfaces can occur[17].

Our laboratory has conducted studies on conformational behavior of proteins in reversed-phase liquid chromatography (RPLC)[18–21]. Others have also been active in RPLC[12,13,22–25], as well as in ion exchange chromatography[26,27]. The importance of conformational changes affecting retention and peak shape in HIC was recognized early by Shaltiel[7] and in HPHIC by our laboratory[16] and others[28–30]. In spite of the fact that HIC is conducted with weakly hydrophobic surfaces and in general with high concentrations of stabilizing salts (*e.g.*, ammonium sulfate), conformational effects can be exhibited, depending on the mobile phase, the column temperature, the hydrophobicity of the stationary phase, and, especially, the lability of the protein.

In a previous paper[16], we developed procedures for studying and characterizing conformational changes in HPHIC. These procedures included an examination of the thermal behavior of proteins (both stable and relatively labile species) on a weakly hydrophobic bonded phase (C_1-ether phase). From a chromatographic point of view, retention, peak width variation and Z values (slope of the plot of ln k' *versus* ln φ_B, where k' is the capacity factor and φ_B is the volume fraction of mobile phase B in the solvent mixture) as a function of temperature were examined. These chromatographic parameters were complemented by on-line UV spectroscopic analysis using a photodiode array detector. It was shown that the hydrophobicity of the stationary phase is an important parameter in maintaining native state conditions upon elution from the chromatographic column. In addition, column operation at subambient temperatures was suggested as a useful procedure for HPHIC.

This paper is an extension of our previous work. Its main focus is on the relatively labile protein, calcium depleted α-lactalbumin (α-LACT), the chromatographic behavior of which is a sensitive function of column conditions. We will first explore the meaning of Z and its relationship to other chromatographic parameters, such as S, the slope of the plot of ln k' *vs.* φ_B. We will next demonstrate that the broad bands that may occur in a temperature transition region of conformational change can be spectroscopically characterized. Such examples illustrate the potential of on-line photodiode array detection, particularly with the use of absorbance ratios and second derivative spectroscopy. We will next show that subambient temperature operation on non-ionic, weakly hydrophobic bonded phases yields high-performance resolution, even with 3-h gradient elution.

We will then examine the effect of metal ions such as Mg^{2+} and Ca^{2+}, added to the mobile phase, on retention. It will be shown that in HIC these metal ions can either stabilize or destabilize protein native state structure, depending on metal–pro-

tein bonding characteristics. The importance of the addition of Mg^{2+} on retention has also recently been reported[31]. Finally, we will explore the role of the hydrophobicity of the stationary phase by using a C_2-(ethyl) ether phase. Here, we will observe the two peak phenomenon for α-LACT that was previously observed in RPLC[18]. The results of this paper provide further understanding of conformational changes in chromatography in general and HPHIC in particular and of the role various parameters play in these changes.

EXPERIMENTAL

Equipment

A 25-μl computer-controlled injection loop, designed in this laboratory, was connected to a Series 800 Du Pont gradient controller with a three-head chromatographic pump (Du Pont Instruments Product Division, Wilmington, DE, U.S.A.). A Hewlett-Packard (Palo Alto, Ca, U.S.A.) Model 1040A photodiode array detector with a HP 9000 work station, HP 7470A graphics plotter, HP 9153 disc drives, and HP think jet printer was used in this work.

A second gradient liquid chromatograph was also employed, consisting of a Model 5000 solvent delivery pump and solvent programmer (Varian, Palo Alto, CA, U.S.A.); a 7125 injection valve (Rheodyne, Cotati, CA, U.S.A.); Model 2600 chromatography software package (Nelson Analytical, Cupertino, CA, U.S.A.) in conjunction with an IBM (Boca Raton, FL, U.S.A.) XT personal computer. Both chromatographic systems (Du Pont and Varian) also included a Model NBE water bath (Haake-Buechler Instrument, Saddle Brook, NJ, U.S.A.), which controlled the temperature to ±0.1°C.

The chromatographic columns were similar to those previously published, *i.e.* the ether-bonded, silica-based stationary phase II (C_1-ether phase) and IV (C_2-ether phase)[2]. 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 (V_D) of 3.0 ml was measured for the Varian system and 6.5 ml for the DuPont system. This volume (or time) was subtracted from all chromatographic retention (V_g).

Chemicals

HPLC-grade water was purchased from J. T. Baker (Phillipsburg, PA, U.S.A.). Ammonium acetate, Grade III ammonium sulfate, magnesium chloride hexahydrate and calcium chloride dihydrate, as well as various high quality protein standards, were obtained from Sigma (St. Louis, MO, U.S.A.) and used as received. The proteins and abbreviations used are: α-lactalbumin (α-LACT) (milk), calcium depleted to less than 0.3 mole Ca^{2+} per mole protein, cytochrome *c* (CYT C) (horse heart), lysozyme (LYSO) (chicken egg white), ribonuclease A (RNase A) (bovine), and α-chymotrypsinogen A (CHMG) (bovine pancreas).

Chromatographic procedures

Mobile phases were prepared by adding the correct weight of salt and buffer or buffers, containing metal ion additives (Ca^{2+}, Mg^{2+}) to a volumetric flask containing previously vacuum degassed HPLC water. The pH was adjusted to the ap-

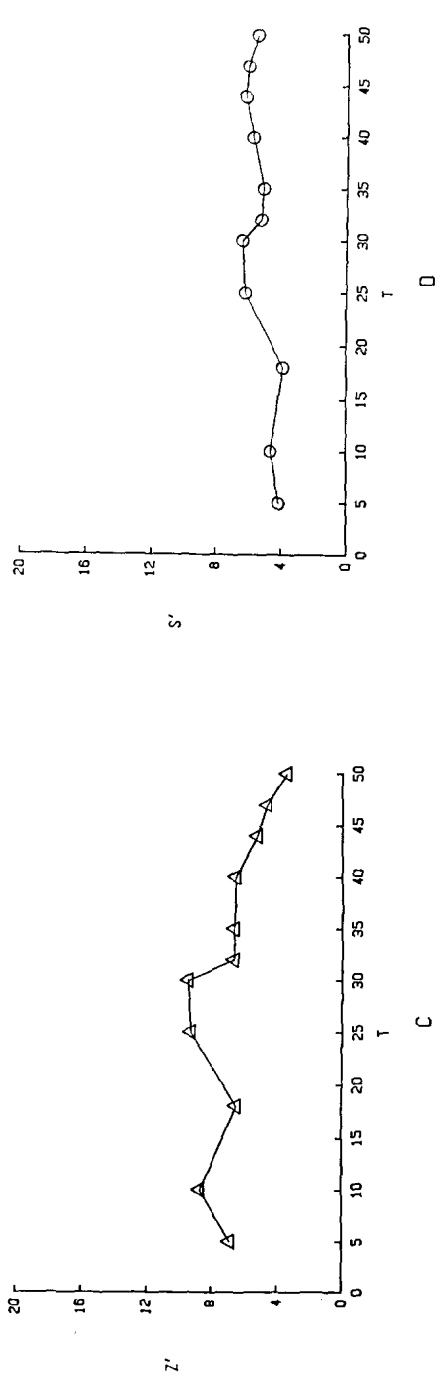

Fig. 1. Dependence of the four different slopes of retention *vs.* solvent composition as a function of temperature for α-LACT. (A) Z' *vs.* T, (B) S' *vs.* T, (C) S *vs.* T, and (D) Z *vs.* T. See Table I for definitions. Conditions: mobile phase A, 2.0 *M* ammonium sulphate, 0.5 *M* ammonium acetate (pH 6); mobile phase B, 0.5 *M ammonium acetate.*

propriate value with either glacial acetic acid or ammonium hydroxide, and a small amount of degassed HPLC water was added to the mark. Solutions containing high salt concentrations were not allowed to remain in the column or pump for long periods of time. Standard mobile phase conditions consisted of 2 *M* ammonium sulfate, 0.5 *M* ammonium acetate (pH 6) as mobile phase A and 0.5 *M* ammonium acetate (pH 6) as mobile phase B. A standard 20-min linear gradient from 0 to 100% B was used in most experiments with a flow-rate of 1.0 ml/min. Exceptions to these conditions are noted in the paper. Protein solutions (5–10 mg/ml) were freshly made up. When not in use, the samples were stored at −10°C. Z values were determined by isocratic elution of the proteins. Z is the slope of the plot of ln k' *vs.* ln φ_B, k' is the capacity factor of the protein and φ_B the volume fraction of mobile phase B in the solvent mixture. The minimum amount of sample to yield a reasonable signal was always used. The experimental error for Z was ±10%.

Spectroscopic procedures

The normal UV spectrum and its second derivative were recorded on the HP 1040A photodiode array detector. The wavelength absorbance and second derivative γ (ref. 16) ratios were processed via the HP 9000 work station. The standard deviation of the absorbance ratio was 0.03 and of the second derivative γ ratio was 0.15. Care was taken in wavelength calibration of the photodiode array, since subtle spectral changes were studied. We chose α-LACT and a typical HIC elution salt condition (1 *M* ammonium sulfate, 0.5 *M* ammonium acetate, pH 6) as a standard solution for wavelength calibration. In order to assess any intensity or wavelength shifts, we also used the flow cell rather than the testing cell provided by Hewlett-Packard. We found that the position of the flow cell must remain constant, since the wavelength absorbance and second derivative γ ratios are affected by its position. If one uses the testing cell during calibration and then replaces it with the flow cell, even a small change in position can alter the results.

RESULTS AND DISCUSSION

Retention and peak profile characterization of α-LACT

α-LACT is a calcium binding protein[32]; removal of the metal makes the protein much less stable, *e.g.* the transition temperature for the thermal denaturation of the apo form is 31°C, whereas it is 58°C for the Ca^{2+} form[33]. Thus, the calcium depleted form of α-LACT can be considered as a labile species for study in HIC. Previously, we have explored the retention behavior of this protein on the C_1-ether phase as a function of column temperature[16]. A complex retention pattern was observed (see Fig. 3 of ref. 16 or Fig. 5 of this paper), and the behavior was attributed to conformational changes of the protein occurring as a function of temperature. We shall now explore this behavior in more detail.

We have previously suggested that the slope of the linear plot of ln k' *vs.* ln φ_B, *i.e.* the Z value, was a sensitive function of protein structural changes, related to retention changes of proteins with column temperature[16]. There are at least three other slopes that could, in principle, be employed for retention characterization: ln k' *vs.* ln m_3 (Z'); ln k' *vs.* m_3 (S'); ln k' *vs.* φ_B (S), where m_3 is the salt molality and the symbols in the parentheses represent the various slopes. Fig. 1 shows plots of the

four slopes *vs.* column temperature from 5 to 50°C for α-LACT on the C_1-ether phase. It can be seen that Z', S', and $|S|$ do not correlate very well with the increasing retention of the protein as the temperature is raised. On the other hand, $|Z|$ does generally increase with temperature. In order to understand the lower sensitivity of Z', S' and $|S|$ relative to $|Z|$, it is necessary to explore the meanings and relationships of the various slopes.

In the Appendix, using a thermodynamic treatment of the equilibrium distribution of the protein with the stationary phase and assuming no specific model of retention, we show that Z' can be directly related to $\Delta\nu_3$ and $\Delta\nu_1$, eqn. A10, the respective changes in the number of moles of salt and water per mole of protein that are associated with the protein and bonded phase surface upon solute adsorption. Thus, for HIC

$$Z' = \frac{\mathrm{d}(\ln k')}{\mathrm{d}(\ln m_3)} = 3\left(\Delta\nu_3 - \frac{m_3}{m_1}\Delta\nu_1\right) = 3\Delta\nu \tag{1}$$

where m_3 and m_1, are the molalities (moles of solute per 1000 g of water) of salt and water, respectively, and $\Delta\nu$ is the combined term. This equation is derived directly from the theory of preferential interactions[34], based on linked functions[35]. The coefficient 3 arises from the relationship between activity and molality of ammonium sulfate, assuming that the activity coefficient is constant over the concentration range of salt used to measure Z'.

From eqn. 1, it is evident that the value of Z' will be a combination of the changes in preferential hydration and preferential salt interaction of the protein upon adsorption. In order to focus on protein changes with temperature, it is necessary that the solvation of the bonded phase surface does not significantly change with temperature. One approach to demonstrating that surface solvation changes with temperature are small is to examine the retention of a very stable protein unlikely to unfold over the temperature range of study. If the retention volume change is small for this species, then it can be assumed that surface changes are small. Previously, lysozyme, a very stable species, was shown to yield only a small increase in retention with temperature[16]. Thus, any larger retention changes with temperature observed for other proteins are most likely due to the protein themselves and not to the surface.

Eqn. 1 shows that Z' is a direct measure of the changes in salt and water bound to the protein, upon adsorption. As discussed in the Appendix, it is possible to derive a relationship between Z' and the other three slopes, S', S, and Z. These relationships are summarized in Table I. S', which is equivalent to λ employed by Melander *et al.*[36] can thus be written as

$$S' = \frac{3\Delta\nu_3}{m_3} - \frac{3\Delta\nu_1}{m_1} = \frac{3\Delta\nu}{m_3} \tag{2}$$

The S parameter that can be used for gradient optimization[37,38] is simply corrected by a, a parameter closely related to the initial salt concentration (see Appendix). Finally, the Z value contains the factor φ_B in its relationship to Z'.

TABLE I

RELATIONSHIP OF VARIOUS SLOPES OF RETENTION ($\ln k'$) *VERSUS* MOBILE PHASE COMPOSITION PLOTS IN HIC

m_3 = molality of salt, assumed to be ammonium sulfate; φ_B = volume percentage of mobile phase B; $a = \frac{-dm_3}{d\varphi_B}$, see eqn. A17.

$$Z' = \frac{d(\ln k')}{d(\ln m_3)} = 3\left[\Delta v_3 - \frac{m_3}{m_1}\Delta v_1\right] = 3\Delta v$$

$$S' = \frac{d(\ln k')}{dm_3} = \frac{Z'}{m_3} = \left(\frac{6.1 + \varphi_B}{14.4\ (1 - \varphi_B)}\right)Z'$$

$$S = \frac{d(\ln k')}{d\varphi_B} = \frac{-aZ'}{m_3} = \left(\frac{-7.1}{(6.1 + \varphi_B)\ (1 - \varphi_B)}\right)Z'$$

$$Z = \frac{d(\ln k')}{d(\ln \varphi_B)} = \frac{-a\varphi_B Z'}{m_3} = \left(\frac{-7.1\varphi_B}{(6.1 + \varphi_B)\ (1 - \varphi_B)}\right)Z'$$

At any temperature, eqn. 1 can be integrated to yield

$$\ln k' = A \ln m_3 + Bm_3 \tag{3}$$

where A is equal to $3\Delta v_3$ and B is equal to $-3\Delta v_1/m_1$. Using non-linear regression analysis, we have solved eqn. 3 for A and B over the whole temperature range of study for α-LACT to obtain Δv_1 and Δv_3. The values obtained for Δv_1 and Δv_3 reveal that the water term is larger than the salt term; however, both terms need to be considered in eqn. 1 due to the m_3/m_1 factor. Thus, Δv should be viewed as a combined term and not as due to water alone[16].

Returning to the various slopes, at any given temperature, the retention k' of α-LACT can be written as

$$\ln k' = S\varphi_B + I_S \tag{4}$$

where I_S is the intercept of $\ln k'$ *vs.* φ_B plot at $\varphi_B = 0$ (*i.e.* mobile phase A). In Fig. 2A we show a plot of I_S or $\ln k'_A$ *vs.* T, and it is readily apparent that the plot is similar to that of Z *vs.* T (Fig. 1D). Since $Z = \varphi_B S$ (see Table I) eqn. 4 can be written as

$$Z = \ln k' - I_S = \ln k' - \ln k'_A \tag{5}$$

where k'_A is the hypothetical capacity factor of α-LACT in mobile phase A (in this case 2 *M* ammonium sulfate) at the column temperature T and for the state (*e.g.* conformation) that exists under the isocratic conditions in which Z is measured. The value of $\ln k'$ can be adjusted to be small relative to $\ln k'_A$ in which case Z approaches S

$$Z \cong -I_S \cong -\ln k'_A \tag{6}$$

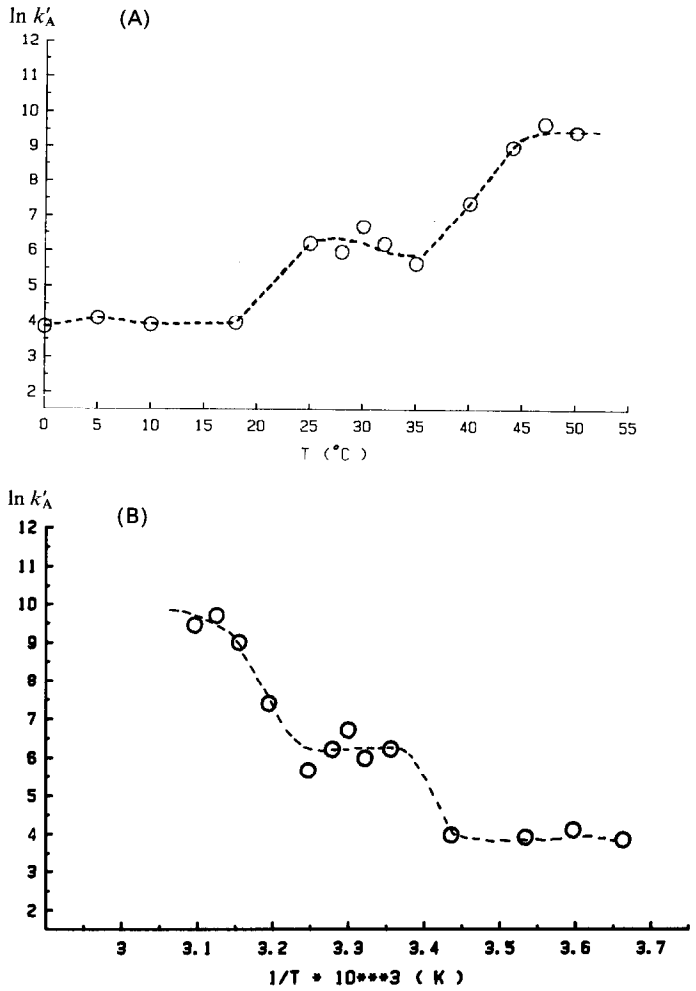

Fig. 2. Dependence of ln k'_A (the intercept of ln k' *vs.* φ_B plot) as a function of temperature for α--LACT: (A) ln k'_A *vs.* T (°C), (B) ln k'_A *vs.* $(1/T) \cdot 10^3$ (K). See Fig. 1 for conditions.

Thus, with ln k' small relative to ln k'_A, Z will reveal the same temperature trend as I_S.

We can convert ln k'_A into the free energy of adsorption ΔG^0

$$\Delta G^0 = - RT \ln K_A = - RT \ln k'_A - RT \ln \theta + lRT \ln[L] \quad (7)$$

where K_A is the adsorption constant in mobile phase A, θ is the phase ratio, [L] is the stationary phase ligand concentration and l is a stoichiometric coefficient (see eqn. A2). Substitution of the enthalpy ΔH^0 and entropy ΔS^0 of adsorption yields

$$\Delta H^0 - T\Delta S^0 = - RT \ln k'_A - RT \ln \theta + lRT \ln[L] \quad (8)$$

and rearranging

$$\ln k'_A = -\frac{\Delta H^0}{RT} + \frac{\Delta S^0}{R} - \ln \theta + I \ln[L] \quad (9)$$

Fig. 2B is a plot of $\ln k'_A$ *vs.* $1/T$ and three plateau regions are again observed. From eqn. 9 these plateau regions represent temperature ranges over which $\Delta H^0/R$ is small. The magnitude of the plateau equals $\Delta S^0/R - \ln \theta + I \ln[L]$, and the results therefore suggest entropy controlled adsorption, which is expected in HIC[2,37]. Moreover, the entropy change is greatest in the high temperature range. This result is again reasonable, since it is known that retention is greatest at the highest temperature (see Fig. 5). If conformational unfolding increases with column temperature, then the entropy change upon adsorption in HIC would be greater the more unfolded the species, as expected.

Z can be thus viewed as a measure of the free energy change of adsorption under the condition of constant salt concentration as a function of temperature. It needs to be recognized that various factors could cause binding strengths to be affected: ionization, association, conformation, etc. Thus, a change in Z does not by itself mean a conformational change. However, through a variation in temperature, knowledge of thermal unfolding transitions in solution, and on-line spectral analysis, a body of evidence can be accumulated which is consistent with conformational change. Moreover, in this study low concentrations of α-LACT were used. Increasing the concentration by a factor of 10 (to > 1 mg) did not affect retention or spectral characteristics. This result is suggestive that association/aggregation is not the direct cause of the phenomena observed. In addition, if association were to occur, some conformational change will probably first take place to expose hydrophobic groups[39–40].

The fact that the other slopes are less sensitive to free energy changes (see Fig. 1) can be understood from the relationships of Table I. An increase in free energy of adsorption leads to an increase in φ_B for elution to occur (see Fig. 5). Since $Z = \varphi_B S$, the increase in Z for the conformational change is compensated by the increase in φ_B. Since S and S' differ by only the value a, which is approximately constant (see Table I), the dampening of S' is also understood. Finally, the concentration relationship of Z to Z', *i.e.* φ_A/φ_B, also is a dampening function.

Returning to Fig. 1D, Z appears to be fairly constant over the temperature range of 25–35°C; however, we have previously noted enhanced broadening of the band in this transition region[16]. Other workers have found similar broadening in the transition region of conformational changes and have attributed the effect to a mixture of conformers binding with differing strengths to the surface[28,31].

Table II presents data of a spectral analysis across the peak profile at 32°C, the temperature of maximum broadness. Absorbance and second derivative γ ratios are presented for a position before the peak maximum 1, at the peak maximum 2, and after the maximum 3. The spectral analysis has been conducted at three flow-rates (F) (0.5, 1.0 and 2.0 ml/min), while the gradient volume ($F \cdot t_G$, where t_G is the gradient time) and thus the average k' value in the gradient $\bar{k}'$ was maintained constant[37]. At the highest flow-rate, the spectral characteristics are similar across the peak and are identical with those obtained at low temperature (< 25°C) (see ref. 16).

TABLE II

SPECTRAL CHARACTERISTICS OF THE CHROMATOGRAPHIC PROFILE OF α-LACT AT DIFFERENT FLOW-RATES

Conditions: C_1-ether phase, 32°C, variable flow-rate, variable t_G, linear gradient. Mobile phase A: 2 *M* ammonium sulfate, 0.5 *M* ammonium acetate, pH 6. Mobile phase B: 0.5 *M* ammonium acetate, pH 6.

Flow-rate (ml/min)	t_G *(min)*	*Position**	*Absorbance ratio*			*Second derivative ratio, γ*
			274/292	*292/254*	*287/254*	
2	10	1	1.42	1.43	1.87	0.75
		2	1.43	1.44	1.86	0.82
		3	1.42	1.42	1.85	0.80
1	20	1	1.41	1.45	1.87	0.69
		2	1.45	1.39	1.81	0.95
		3	1.46	1.38	1.79	1.00
0.5	40	1	1.47	1.38	1.80	1.02
		2	1.46	1.39	1.81	0.93
		3	1.45	1.39	1.81	0.94

* Spectral characteristics were measured at before (1), at (2) and after (3) the peak maximum.

For the 1 ml/min flow-rate, the spectral characteristics at position 1 remained the same, but positions 2 and 3 now produce a higher γ ratio, suggestive of a greater tyrosine exposure to the solvent. The increase in the 274 nm/292 nm and the decrease in the 292 nm/254 nm absorbance ratios are consistent with this interpretation. If the species with the γ ratio close to 1.00 is considered to be a more unfolded form, than that with γ of *ca.* 0.80 (γ = 0.80 for the protein in solution at 40°C and γ = 1.00 for the protein in solution at 50°C), the fact that the more unfolded form is eluted as the last peak of the band is reasonable in the HIC mode. Finally, at 0.5 ml/min, the γ ratio appears to be constant across the peak at a value of *ca.* 1.00, and the absorbance ratios are also the expected values. Thus, the more unfolded form seems to prevail.

Table II demonstrates that the on-line spectral analysis is able to reveal at least two species in the band. This illustrates one of the great values of on-line analysis. The results are further suggestive of kinetic conversion on the surface, from the folded to a less folded form, since the incubation time for α-LACT remaining on the ether phase is four times longer for the 0.5 ml/min flow-rate than for the 2.0 ml/min flow-rate. We have previously shown that contact time of the protein with the bonded phase surface in RPLC can influence the extent of conformational change[20]. The broad peak may represent the combined retention of the two states with the differences in binding strengths being so small that there is band overlap. At the same time, some broadening could arise from slow interconversion in the mobile phase[19,21], since the band consists of a mixture of conformers, the population of which appears to be kinetically determined. This point simply emphasizes that caution must be exercised in relating absolute Z (or Z') values to relative contact areas.

We tested for reversibility of protein structure from a partially unfolded state, eluted at high temperature, to the low temperature form. We collected a sample, eluted at 40°C, and measured the spectral characteristics of the band in solution at 5°C. Small changes in spectral features occurred as the protein relaxed to the folded

state. After 2 h, spectral characteristics identical with those at 5°C were obtained. We then injected this sample into the HIC column at 5°C and obtained a retention identical with that of a freshly prepared sample. The change thus appears reversible. Indeed, even for more fully unfolded forms, temperature reversibility was found (see C_2-ether phase results).

Finally, we used the photodiode array to measure changes in spectral features of α-LACT in solution as a function of temperature. In all cases, the spectra were taken with the elution mobile phase composition. Between 40 and 50°C we found changes in spectral characteristics, *e.g.* $\gamma = 0.80$ at 40°C and 0.98 at 50°C, suggestive of conformational changes. Since such changes occurred at lower temperatures with the column (*ca.* 25°C), this suggests that the weakly hydrophobic bonded phase has

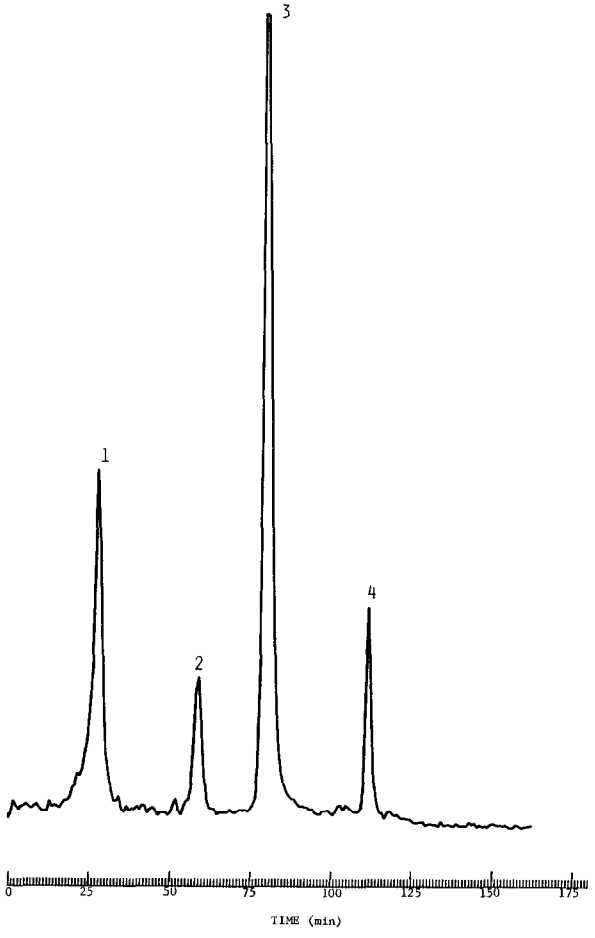

Fig. 3. Separation of four standard proteins on the C_1-ether phase at 5°C. Peaks: 1 = CYT C, 2 = RNase A, 3 = LYSO, and 4 = CHMG. Conditions: mobile phase A, 3.0 *M* ammonium sulfate 0.5 *M* ammonium acetate (pH 6); mobile phase B, 0.5 *M* ammonium acetate (pH 6); gradient time, 180 min (linear); flow-rate, 1 ml/min.

a catalytic effect on unfolding. However, as we have previously noted, the temperature difference for conformational effects to be observed from the column relative to solution is much less than on more hydrophobic phases[16]. The extent of the catalytic effect is clearly dependent on the hydrophobicity of the phase. This point will be emphasized with the study of the C_2-ether phase.

Subambient column temperature operation

The results of conformational behavior of α-LACT on the C_1-ether phase led to the conclusion that subambient temperature operation should be beneficial in HPHIC. First, conformational unfolding of a protein should be less at low temperatures relative to room temperature. Secondly, retention should be shorter at lower temperatures, in contrast to other chromatographic modes of interaction. Thirdly, the lifetime of columns should be greater. One question that could be raised is

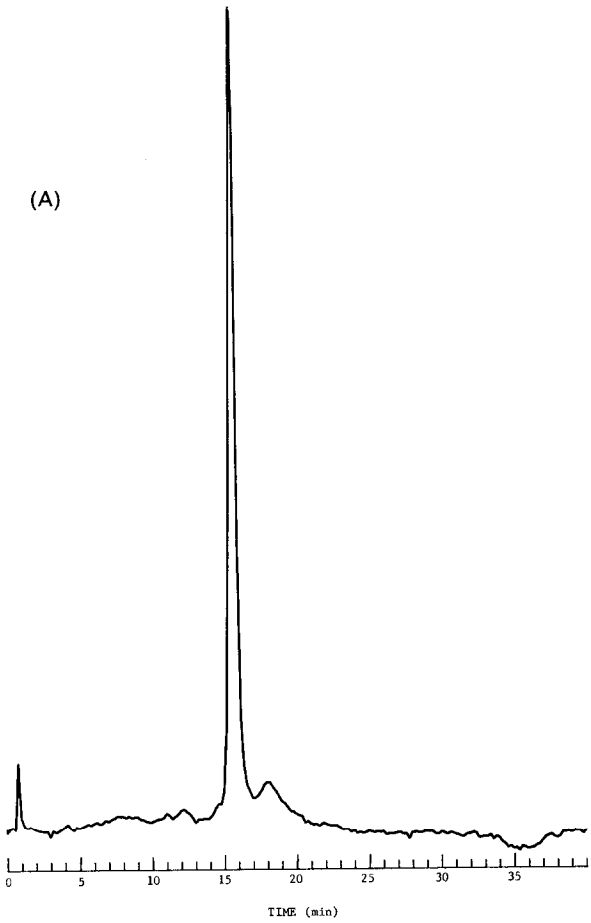

Fig. 4.

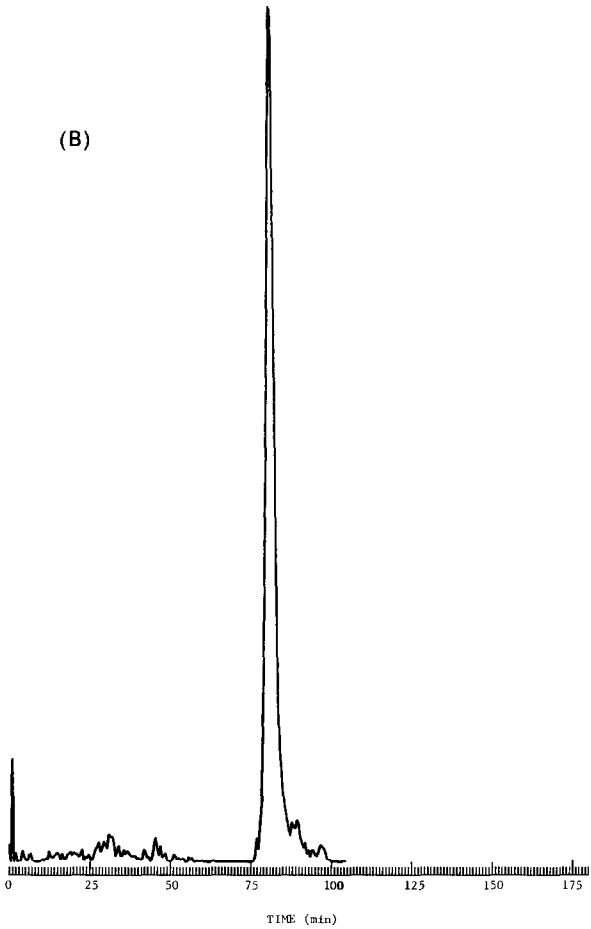

Fig. 4. Elution profiles of α-LACT on C_1-ether phase. (A) All conditions as in Fig. 3, except gradient time 20 min (linear), and mobile phase A, 2.0 *M* ammonium sulfate 0.5 *M* ammonium acetate (pH 6). (B) All conditions as in (A) except gradient time 180 min (linear).

whether chromatographic performance is affected as a consequence of subambient operation.

In order to demonstrate the high-performance capabilities of low temperature operation, we have examined the separation of standard proteins at 5°C on the C_1-ether bonded phase. Fig. 3 shows the separation of standard proteins (CYT C, RNase A, LYSO and CHMG) using a 3-h gradient. Relatively sharp peaks are obtained with peak widths comparable to that of a previously published 3-h gradient of the same mixture at 25°C[38]. Fig. 4 illustrates the elution at 5°C of the conformationally sensitive protein, calcium depleted α-LACT. Here again, reasonable band widths are observed, even for the 3-h gradient. From Figs. 3 and 4, it can be concluded that high performance is achieved at low column temperatures, even when a labile protein is in contact with the bonded phase for several hours (as in a 3-h gradient). It is

interesting to note that when ion exchange columns are used, broadened bands are observed at low temperature[42].

Addition of Mg^{2+} and Ca^{2+} to the mobile phase

The alkaline earth metals, Mg^{2+} and Ca^{2+}, have in the past been added to the mobile phase in HPLC to manipulate retention and maintain biological activity. For example, in RPLC, calcium-binding proteins have been observed to maintain biological activity even under harsh chromatographic conditions when calcium is present[43]. In HIC, we have previously shown that the magnesium salts do not in general follow the relationship of mobile phase surface tension with respect to retention, due to the salting-in characteristics of the Mg^{2+} ion[38]. Moreover, other workers have shown that magnesium can alter the slope of the log *k' versus* molality of salt plot for several proteins[31]. The addition of calcium has been used to manipulate retention for α-LACT in low pressure HIC[44]. In this case, the apo form of the protein binds to the hydrophobic surface. Addition of Ca^{2+} induces a conformational change to a less hydrophobic species and elution occurs. Interestingly, with other calcium binding proteins, addition of the alkaline earth metal most often leads to a more hydrophobic species[44]. We decided to examine further the influence of magnesium and calcium on α-LACT in HPHIC, using the C_1-ether column.

Fig. 5 presents the change in retention of α-LACT as a function of temperature for three conditions: (i) the normal HIC mode, (ii) the addition of 0.6 *M* magnesium chloride to mobile phase B, and (iii) the addition of 1 m*M* calcium chloride to mobile phases A and B. Table III presents *Z* values determined at various temperatures for the three mobile phase conditions.

It can be observed that from 0 to 15°C the influence of the metal ion salt on retention and *Z* value is minimal, and it would thus appear that a similar conformational species (i.e. the native species) is being eluted up to this temperature. At

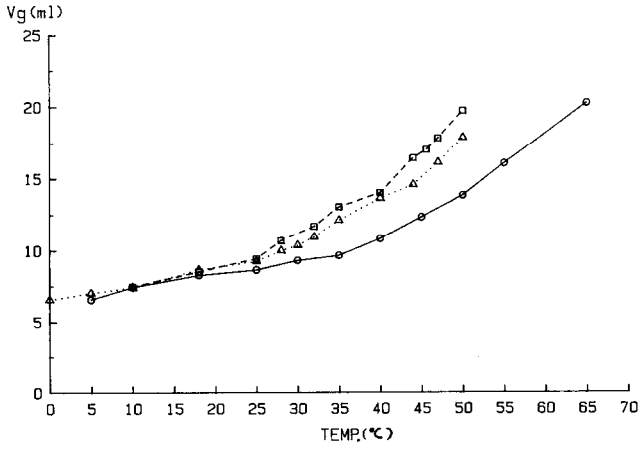

Fig. 5. Effect of temperature on retention of α-LACT on the C_1-ether phase as a function of metal ion additive. Standard HIC gradient with no metal added (△...△), see Fig. 4A, with the addition of 0.6 *M* magnesium chloride to mobile phase B (□---□), and with the addition of 1 mM calcium chloride to mobile phases A and B (○---○).

TABLE III

Z VALUES OF α-LACT AT DIFFERENT TEMPERATURES AS A FUNCTION OF MOBILE PHASE ADDITIVE

Conditions: C_1-ether phase; flow-rate 1 ml/min; variable temperature; mobile phase A, 2 *M* ammonium sulfate, 0.5 *M* ammonium acetate, pH 6; mobile phase B, 0.5 *M* ammonium acetate, pH 6. Mobile phase A and mobile phase B were mixed in different proportions for isocratic elution.

T (°C)	*HIC*	*Mobile phase additive*	
		*1 mM $CaCl_2$**	*0.6 M $MgCl_2$***
10	2.1	2.1	1.8
25	5.3	3.2	5.3
40	6.7	3.4	7.8
50	8.5	6.2	11.3
60	–	10.0	–

* Added to mobile phases A and B.
** Added to mobile phase B.

25°C, as shown in Fig. 5, the retention of α-LACT is identical with no metal added and with the addition of Mg^{2+}. However, retention with Ca^{2+} is less, suggesting a more stable conformer in which hydrophobic amino acid residues are less exposed[44]. This is further seen in Table III where the *Z* value is increased to 5.3 for the former cases and only 3.2 for the latter case. Indeed, in the case of added Ca^{2+}, the *Z* value is maintained constant until at least 40°C, whereas for the other systems, the *Z* value is increased substantially at 40°C. With added Mg^{2+}, we observed enhanced broadening of the chromatographic peak as previously seen for α-LACT under the standard HIC conditions (*i.e.* without metal). Spectral analysis again revealed at least two species represented by the peak.

It can be further observed that the *Z* value increases dramatically above 40°C for the case of Mg^{2+} added to mobile phase B, *i.e.* from 7.8 at 40°C to 11.3 at 50°C. On the other hand, for HIC without metal ion additive, lower *Z* values of 6.7 and 8.5, respectively, are observed. Examination of Fig. 5 also reveals that retention is longer with the magnesium additive than with standard HIC. Note that calcium maintains a lower *Z* value even at 50°C.

These data can be explained on the basis of the strong affinity of calcium for an active site in α-LACT ($K_f \cong 3 \cdot 10^9$)[45]. Presumably, calcium is acting as a stabilizing metal ion due to its specific binding. On the other hand, magnesium most likely cannot stabilize α-LACT due to its weak binding constant, $K_f \cong 10^3$ (ref. 33), and the high salt concentration of the mobile phase. Both calcium and magnesium are known to be generally destabilizing when not binding to specific sites[46]. Thus, magnesium is acting as a destabilizing metal ion in Fig. 5, whereas calcium is acting as a stabilizing ion. Since the effects of conformational change are reversible in the case of α-LACT, it is apparent that both calcium and magnesium can be used to manipulate retention as a function of temperature.

A further indication of the stabilizing influence of Ca^{2+} on α-LACT and the destabilizing effect of this metal ion for non-binding proteins can be seen in Table IV. In this table, gradient retention volume, $V_g - V_D$, is presented as a function of

TABLE IV

INFLUENCE OF Ca^{2+} ADDED TO THE MOBILE PHASE ON RETENTION OF α-LACT AND LYSO AS A FUNCTION OF pH

Conditions: C_1-ether phase; 25°C; flow-rate, 1 ml/min; 20-min linear gradient. Mobile phase A: 2 *M* ammonium sulfate, 0.5 *M* ammonium acetate, variable pH; mobile phase B: 0.5 *M* ammonium acetate, variable pH.

Protein	$V_g - V_D$ *(ml)*					
	pH 7		*pH 6*		*pH 4.7*	
	HIC	*1 mM* $CaCl_2$*	*HIC*	*1 mM* $CaCl_2$*	*HIC*	*1 mM* $CaCl_2$*
α-LACT	9.7	9.4	9.2	9.0	13.6	10.2
LYSO	9.4	9.4	9.0	9.0	6.6	8.2

* Added to mobile phases A and B.

mobile phase pH, with and without the addition of 1 m*M* calcium chloride. α-LACT is compared to LYSO, two proteins which possess significant sequence homology[47]. At pH values of 7 and 6 and a column temperature of 25°C, retention for the two proteins is closely similar and there does not appear to be a significant effect of added Ca^{2+}. (The slight effect at pH 6 for α-LACT on addition of Ca^{2+} has already been noted in Fig. 5.)

At pH 4.7 significant changes in retention are observed. For α-LACT, $V_g - V_D$ is increased to 13.6 ml which may be due to conformational change and possible association[40]; addition of Ca^{2+} reduces this retention volume to 10.2 ml, the reduction being suggestive of a more folded form, due to the binding of Ca^{2+}. This metal binding may be expected to be weaker than at pH 6 and 7 due to the reduction in negative charge on the protein (p*I* = 5.1), and this may in part explain the fact that $V_g - V_D$, with addition of Ca^{2+} at pH 4.7, is still larger than at pH 6 and 7.

For LYSO, retention is substantially less at pH 4.7 under the HIC condition. This result agrees with that found by other workers for the same protein as a function of pH[31] and has been attributed to protonation of His residues on the surface of the protein. Addition of Ca^{2+} significantly increases the retention of LYSO. Presumably, Ca^{2+} facilitates the unfolding of LYSO at this pH where the protein would in general be expected to be less stable than at physiological pH[48]. In addition, Ca^{2+} may neutralize some negative charges on the protein surface, thus affecting retention. Table IV thus indicates that the influence of Ca^{2+} will depend on the specific protein and the conditions under which chromatographic elution is carried out.

Influence of stationary phase hydrophobicity

We have previously noted that conformational changes of proteins as a function of column temperature appear to be a sensitive function of the stationary phase hydrophobicity[16]. It is well known from adsorption studies[17] and classical HIC studies on agarose gels[7] that proteins are more likely to unfold the more hydrophobic the support.

We decided to explore this effect of the stationary phase by examining the

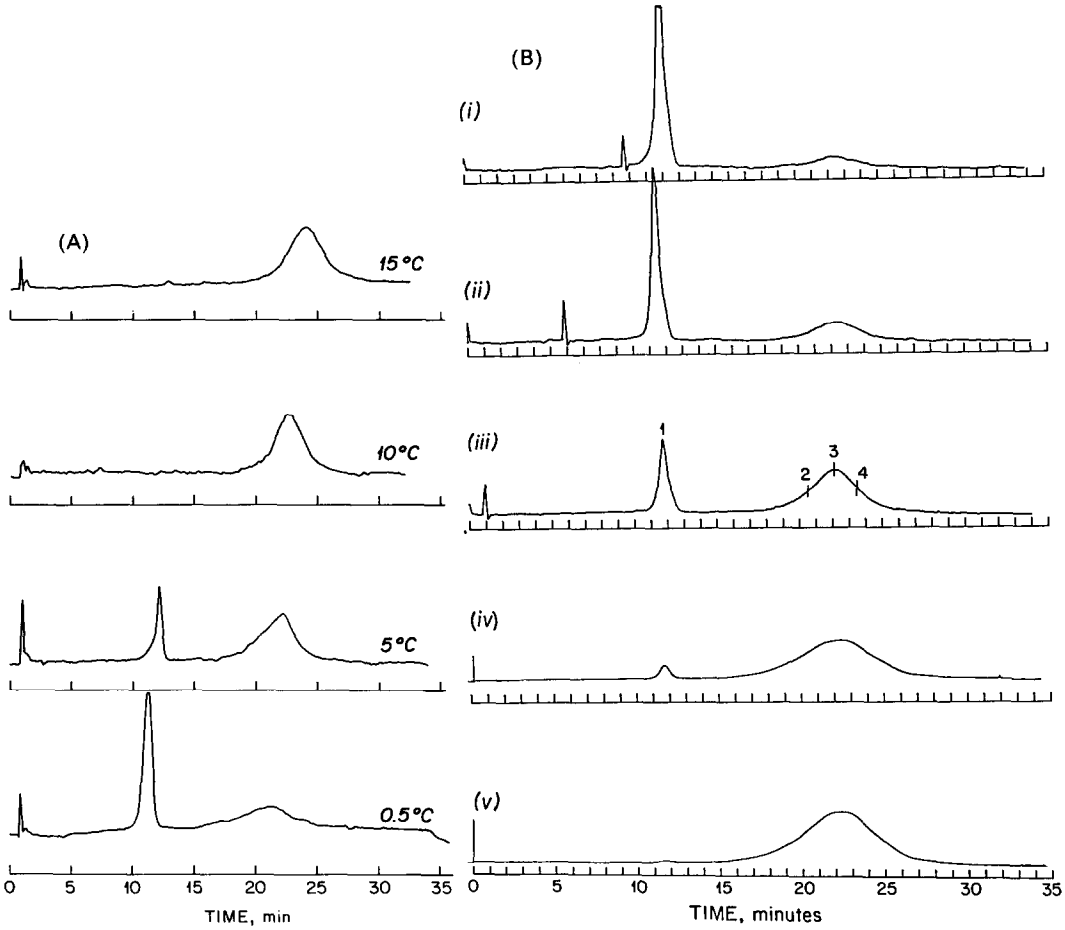

Fig. 6. (A)Effect of temperature on chromatographic behavior of α-LACT on the C_2-ether phase. See Fig. 4A for conditions. (B) Effect of contact time of α-LACT on the C_2-ether column at 5°C. The mobile phase condition was the same as in Fig. 4A. (i) Injection 9 min after start of gradient, (ii) injection 5 min after start of gradient , (iii) injection at start of gradient (standard conditions), (iv) 15 min incubation prior to start of gradient; and (v) 30 min incubation prior to start of gradient.

behavior of the test molecule, α-LACT, on the C_2-ether phase, where an ethyl group is substituted for a methyl group at the end of the polyether chain. Fig. 6A presents chromatograms of α-LACT at four temperatures from 0.5 to 15°C using a standard 20-min linear gradient from 2 to 0 *M* ammonium sulfate. Two distinct and well separated peaks are observed, with the later eluting peak growing at expense of the early eluting band as temperature is increased. Above 10°C only the second peak is observed. The behavior in Fig. 6A is similar to that previously found in RPLC for proteins such as papain and lysozyme[20]. In RPLC, we ascribed the behavior to protein unfolding in the column. (Refolding was presumed to be slow on the time scale of elution.) The second broad peak of α-LACT was collected and after 30 min rein-

jected into the column. The chromatogram showed the first and second peaks. This result means that the fraction represented by the second peak can reversibly refold in solution within a reasonable time span. In this sense, the HIC behavior of α-LACT on the C_2-ether phase is related to the behavior of lysozyme in RPLC, chromatographed at low temperatures[20]. As expected, reinjection of the material in the first peak yields both bands.

Fig. 6B shows further similarity of the results of α-LACT on the C_2-ether column with previous results obtained in RPLC. Incubation of the sample on the column at 5°C (*i.e.* an isocratic hold under mobile phase A conditions) caused a decrease of the first peak and an increase of the second peak, the extent of the change being a function of the contact time with the surface. This is further seen when the sample is injected after the start of the gradient. The shorter the contact time (*i.e.* the later the injection) the larger the first peak. These results further point to an unfolding of the protein in the chromatographic system, the first peak being folded and the later eluted peak being unfolded to some extent. As in previous work[20], we measured the first order rate constant of unfolding of α-LACT on the C_2-ether phase by varying the incubation (*i.e.*, isocratic hold) time prior to the start of the gradient. A plot of log area of the first peak *vs.* incubation time yielded a linear slope of $23.3 \cdot 10^{-4}$ s^{-1} or $t_{1/2} \cong 5$ min at 5°C, a value comparable to that found in RPLC for papain[20].

The behavior of Fig. 6 was dependent on the lability of the protein studied. Thus, lysozyme, a more stable species than α-LACT, did not show any peak distortion or double peaks over the temperature range of 0.5–25°C on the C_2-ether phase. Moreover, the peak was eluted close to the first band of α-LACT under the standard gradient condition (see Fig. 6B, iii). This result points to lysozyme remaining in the folded state throughout the temperature range of study. The effect of stationary phase hydrophobicity on conformational changes will thus clearly be dependent on the protein studied.

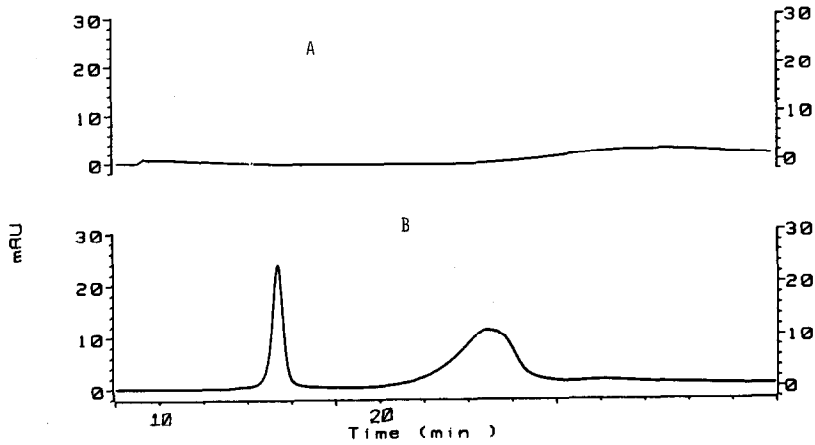

Fig. 7. Refolding of α-LACT on the C_2-ether column after lowering column temperature. (A) Injected α-LACT at 25°C. The mobile phase condition was the same as Fig. 4A. No elution observed. (B) The column was cooled from 25 to 0.5°C in Fig. 7A, followed by gradient elution as in Fig. 4A. The time interval of cooling from 25 to 0.5°C was *ca.* 40 min.

Returning to Fig. 6A, at temperatures above 20°C, no elution of α-LACT from the C_2-ether column was observed. While it is not clear as to the actual cause of this result, one possible explanation is that the higher temperature led to a further unfolding of the protein and possibly aggregation. Interestingly, α-LACT also could not be eluted from a TSK-phenyl-PW HIC column at 25°C[49], a more hydrophobic phase than the C_1-ether.

Fig. 7 shows that when the column was cooled from 25 to 0.5°C (40 min) and eluted with the HIC gradient, the two peaks were again observed. These results suggests that whatever causes α-LACT to bind strongly at 25°C can be reversibly altered when the temperature is lowered. Interestingly, a similar time behavior to Fig. 6 is observed with the first peak decreasing with incubation time of the protein on the surface, after lowering the temperature from 25 to 0.5°C.

In order to examine further the two species in Fig. 6, we used on-line spectral analysis of the peaks. We determined absorbance ratios and second derivative γ ratios at the four positions shown in Fig. 6B (iii), the standard gradient condition at 5°C. The results are shown in Table V. Position 1 is at the peak maximum of the first peak. The absorbance and second derivative ratios at this position are identical to those obtained on the C_1-ether phase at 5°C, see Table III of ref. 16. This result is consistent with the model of the first peak being a folded form of α-LACT.

Spectral characteristics were determined at three positions for the broad late eluted peak. As seen in Table V, positions 2 and 3, *i.e.*, an early eluting point and the peak maximum, were identical, but significantly different from the spectral characteristics of the folded form. The lower γ ratio for positions 2 and 3, relative to 1, is suggestive of tryptophan exposure. Position 4 reveals a dramatic spectral change from the earlier positions of the broad peak. This result suggests that at least two species are present in the broad peak, as previously observed for the broad band at 32°C for α-LACT on the C_1-ether phase.

We next added 1 m*M* calcium chloride to mobile phases A and B, and Fig. 8 shows the results on the C_2-ether phase at 10°C, with and without the metal ion additive. It is readily apparent that the folded form of α-LACT (earlier eluted peak) possesses a greater peak area when Ca^{2+} is present. (In both cases, the spectral

TABLE V

SPECTRAL CHARACTERISTICS OF THE TWO PEAKS OF α-LACT ON THE C_2-ETHER PHASE

Conditions: C_2-ether phase; temperature, 5°C; flow-rate, 1 ml/min; 20-min linear gradient. Mobile phase A: 2 *M* ammonium sulfate, 0.5 *M* ammonium acetate, pH 6; mobile phase B: 0.5 *M* ammonium acetate, pH 6.

*Position**	*Absorbance ratio*			*Second derivative ratio, γ*
	274/292	*292/254*	*287/254*	
1	1.42	1.43	1.81	0.82
2	1.34	1.45	1.70	0.65
3	1.34	1.45	1.69	0.64
4	1.77	1.18	1.45	1.38

* See Fig. 5B (iii) for definition of retention positions.

features of this early eluting band were identical to those of α-LACT, eluted from the C_1-ether column at 10°C.) This result can be explained on the basis of the stabilizing effect of the metal ion with α-LACT.

The late eluting peak is broad in both cases; but retention is significantly lower when Ca^{2+} is present. This suggests that the extent of unfolding is less when the calcium ion is added to the mobile phase. This suggestion is consistent with the spectral characteristics of the bands (Table VI). First, as can be seen for both cases, with and without Ca^{2+} present, the γ ratio in the tail end of each band is significantly different from that of either the front side or the peak maximum. The broad peak is again seen to be the result of a mixture of species. However, the γ ratio on the tail end is 1.58 for α-LACT without Ca^{2+} and 1.00 with Ca^{2+}. Given a γ value of 0.82 for the folded form at 10°C, this result suggests that in the presence of Ca^{2+}, conformational unfolding occurs to a lesser extent. We did not study the influence of kinetics in this case.

CONCLUSION

This paper is a continuation of our examination of protein conformational changes in HPHIC. The meaning of the Z values and their relationship to other retention characterization parameters (Z', S' and S) have been presented. The sensitivity of Z to conformational change is seen from this analysis .

We next explored the broadened peaks that appear in the temperature transition region of 25–35°C. On-line photodiode array spectral analysis revealed the peak to consist of at least two species. Moreover, the larger the contact time the more the protein appears to be driven into a partially unfolded conformer. The influence of adding metal ions such as Ca^{2+} and Mg^{2+} to the mobile phase has also been studied. Ca^{2+} appears to stabilize a folded conformation of α-LACT, in agreement with literature findings. On the other hand, Mg^{2+} appears to destabilize α-LACT under the HIC condition. Finally, the influence of the stationary phase hydropho-

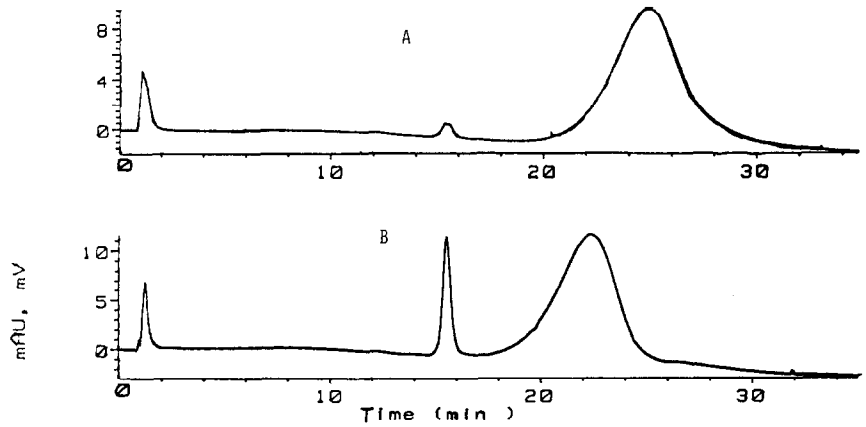

Fig. 8. Effect of Ca^{2+} on the chromatographic behavior of α-LACT on the C_2-ether column at 10°C. (A) The mobile phase condition was the same as in Fig. 4A. (B) The mobile phase condition was the same as in Fig. 4A, except that 1 mM calcium chloride was added to mobile phases A and B.

TABLE VI

COMPARISON OF SPECTRAL CHARACTERISTICS OF SECOND PEAK OF α-LACT ON C_2-ETHER COLUMN AT 10°C WITH AND WITHOUT Ca^{2+} ADDED TO MOBILE PHASE

Conditions, see Table V, with and without Ca^{2+} added.

*Position**	*Absorbance ratio*			*Second derivative ratio, γ*
	274/292	*292/254*	*287/254*	
No calcium chloride added				
1	1.31	1.47	1.68	0.71
2	1.33	1.47	1.66	0.77
3	1.60	1.17	1.44	1.58
1 mM calcium chloride added to mobile phases A and B				
1	1.33	1.46	1.68	0.58
2	1.35	1.44	1.68	0.56
3	1.51	1.17	1.44	1.00

* Spectral characteristics were measured before (1), at (2) and after (3) the peak maximum.

bicity on conformational effects is dramatically seen in the elution of α-LACT on the C_2-ether phase. Results similar to those previously found for RPLC of papain[20] are observed. The addition of Ca^{2+} appears to stabilize the native protein to some extent, even with the strong hydrophobic surface.

This work provides an illustration of the usefulness of the methodology developed in our previous paper[16] for studying conformational changes. The roles of temperature, mobile phase additive and stationary phase hydrophobicity are clearly seen. Calcium depleted α-LACT may be representative of labile species to be separated by HIC. As we have seen, subambient temperature operation on weakly hydrophobic phases permits elution of a native species, even with a very shallow gradient.

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APPENDIX

Relationship of various retention versus mobile phase composition slopes to preferential interaction parameters

The preferential interaction parameter in molal units ($\partial m_3/\partial m_2$), as described by James *et al.*[50] is given by

$$\left[\frac{\partial m_3}{\partial m_2}\right]_{T,\mu_1,\mu_3} = \frac{MW_2}{MW_3}\left[\frac{\partial g_3}{\partial g_2}\right]_{T,\mu_1,\mu_3} = \nu_3 - \frac{m_3}{m_1}\nu_1 \qquad \text{(A1)}$$

where v_i is the total amount of component i "bound" to component 2, expressed in moles of component i per mole of component 2, g_i is the amount of component i (g) present in 1 g water, MW is the molecular weight, and m_i is the concentration of i in molal units. Here, subscript 1 refers to the main solvent (water), subscript 2, where appropriate refers to the protein and subscript 3 to the salt component of the system.

The preferential interaction parameters can be applied to the elution of a conformationally constant protein by considering the distribution equilibrium that occurs during the adsorption process as

$$Q + lL \leftrightharpoons C \tag{A2}$$

where C, Q, and L are the complex of the protein-bonded phase, the protein in the mobile phase, and the stationary phase itself, respectively, and l is a stoichiometric coefficient. Assuming the temperature (T) and the pressure (P) are constant in a small section of the column length (∂L), the dependence of the adsorption equilibrium constant, K on any external agent, *e.g.*, salt, can be expressed by the Wyman theory of linked functions as[35,50]

$$\left(\frac{\partial \ln K}{\partial \ln a_3}\right)_{T,P} = \left(\frac{\partial m_3}{\partial m_C}\right)_{T,\mu_1,\mu_3} - \left(\frac{\partial m_3}{\partial m_Q}\right)_{T,\mu_1,\mu_3} - l\left(\frac{\partial m_3}{\partial m_L}\right)_{T,\mu_1,\mu_3} \tag{A3}$$

where $\left(\frac{\partial m_3}{\partial m_j}\right)$ is the salt preferential interaction with each species j and a_3 is the molal activity of the salt. Substituting eqn. A1 into eqn. A3 yields

$$\left(\frac{\partial \ln K}{\partial \ln a_3}\right)_{T,P} = \nu_{3,C} - \nu_{3,Q} - l\,\nu_{3,L} - \frac{m_3}{m_1}(\nu_{1,C} - \nu_{1,Q} - l\nu_{1,L}) \tag{A4}$$

$$\left(\frac{\partial \ln K}{\partial \ln a_3}\right)_{T,P} = \Delta\nu_3 - \frac{m_3}{m_1}\Delta\nu_1 \tag{A5}$$

where $\Delta\nu_3$ and $\Delta\nu_1$ are the differences in the number of moles of salt and water, respectively, per mole of protein when the protein is adsorbed into the mobile phase relative to when it is bound to the stationary phase. Similar relationship have been described for other types of equilibria[35].

The activity of the salt can be expressed as

$$a_3 = \varepsilon\gamma^x m_3^y \tag{A6}$$

where γ is the activity coefficient and ε, x and y are constants. We can note that

$$\frac{d(\ln a_3)}{d(\ln m_3)} = y + m_3\frac{d(\ln \gamma_3)}{d(\ln m_3)} \cong y \tag{A7}$$

For the case of ammonium sulfate, y is equal to 3 (ref. 34). Substituting eqn. A7 in eqn. A5 yields

$$\left(\frac{\partial \ln K}{\partial \ln m_3}\right)_{T,P} = 3\Delta\nu_3 - \frac{m_3}{m_1} 3\Delta\nu_1 \tag{A8}$$

Since $K = \theta k'/[\mathrm{L}]^i$ where θ is the phase ratio, k' is the capacity factor and [L] is the concentration of the ligand in the stationary phase

$$\mathrm{d}(\ln K) = \mathrm{d}(\ln k') \tag{A9}$$

and eqn. A8 becomes:

$$\left(\frac{\partial \ln k'}{\partial \ln m_3}\right)_{T,P} = 3\Delta\nu_3 - \frac{m_3}{m_1} 3\Delta\nu_1 = Z' \tag{A10}$$

Eqn. A10 is the slope, Z', of the plot of ln k' *vs.* ln m_3. As can be seen, Z' depends on m_3, and the slope will be approximately constant only for small changes in m_3.

The second method of plotting the chromatographic data is ln k' *vs.* m_3, as was described by Melander *et al.*[36]. The slope of this plot is S' and will be equivalent to λ. By substituting $\partial \ln m_3 = \partial m_3/m_3$, eqn. A10 becomes

$$S' = \left(\frac{\partial \ln m_3}{\partial m_3}\right)_{T,P} = \frac{Z'}{m_3} \tag{A11}$$

The relationship between Z' or S' and S (the slope of a plot of ln k' *vs.* volume percentage of mobile phase B, φ_B (ref. 38), can be obtained from m_3 as a function of φ_B. By definition m_3 is the number of moles of the solute (n_3) present in 1000 g water. The number of moles of the solute present in a volume, V, and the weight of water (w_1) in the same volume will be

$$\begin{aligned} n_3 &= M_3 \cdot V \\ w_1 &= M_1 \cdot V \cdot 18 \end{aligned} \tag{A12}$$

where M_i are the respective final molar concentrations. Because the gradient system produces a given mixture of mobile phases A and B by volume, the change in volume in HIC upon mixing is negligible. Therefore, the final molar concentration, M_i, can be expressed as

$$\begin{aligned} M_3 &= M_3^{\mathrm{A}}(1 - \varphi_B) + M_3^{\mathrm{B}} \varphi_B \\ M_1 &= M_1^{\mathrm{A}}(1 - \varphi_B) + M_1^{\mathrm{B}} \varphi_B \end{aligned} \tag{A13}$$

where M_i^{A} and M_i^{B} are the initial molar concentrations of component i in mobile phases A and B, respectively, and φ_B is the fraction of mobile phase B necessary for the elution of the given protein. From eqns. A12 and A13 the molality of the salt becomes

$$m_3 = \frac{[M_3^A(1 - \varphi_B) + M_3^B \varphi_B]1000}{[M_1^A(1 - \varphi_B) + M_1^B \varphi_B]18} \tag{A14}$$

If the total concentration of salt in mobile phase B is zero($M_3^B = 0$), then

$$m_3 = \frac{m_1 \cdot M_3^A(1 - \varphi_B)}{(M_1^B - M_1^A)\left(\dfrac{M_1^A}{M_1^B - M_1^A} + \varphi_B\right)} \tag{A15}$$

We can write that

$$\frac{dm_3}{d\varphi_B} = - a \tag{A16}$$

where

$$a = \frac{m_1 M_3^A M_1^B}{(M_1^B - M_1^A)^2}\left[\frac{1}{\dfrac{M_1^A}{M_1^B - M_1^A} + \varphi_B}\right]^2 \tag{A17}$$

It is to be noted that a is approximately the molality of the salt in mobile phase A. Substituting eqn. A16 into eqn. A11 we have

$$S = - aS' \tag{A18}$$

The fourth method for plotting the data is by means of the Z value[16] ($Z = \partial \ln k'/\partial \ln \varphi_B$). The relationship between Z and S ($Z = \varphi_B S$) is found by replacing $d \ln \varphi_B = \dfrac{d\varphi_B}{\varphi_B}$ in the definition of S. (Previously[16], the concentration of water was allowed to vary with φ_B. In this paper, in order to intercompare the various slopes, m_{H_2O} has been maintained at 55.5 molal over the whole gradient range.) A summary of the relationships between the different slopes and Z' appears in Table I. The values utilized for the expressions are: $m_1 = 55.5$, $M_1^A = 46.3$, $M_1^B = 53.9$ and $M_3^A = 2$, $M_3^B = 0$.

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