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Effect of metal ions on the unfolding kinetics of α-lactalbumin on weakly hydrophobic surfaces

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ABSTRACT

The effect of metal ion [Ca^{2+} and $Zn(NH_3)_4^{2+}$] on the unfolding kinetics of bovine-α-lactalbumin (α-LACT) on a weakly hydrophobic chromatographic surface (ethyl polyether phase bonded on porous silica, C_2 ether) has been studied using surface intrinsic fluorescence spectroscopy and liquid chromatography. Chromatographic results on the C_2 ether phase revealed two peaks for α-LACT, the first being the folded and the second an unfolded conformation, as determined by fluorescence spectroscopy. The retention time for the second peak was found to depend on the specific metal additive in the mobile phase. Fluorescence studies showed a slow change in emission maximum from *ca.* 330 nm to 350 nm and a 5-fold increase in emission intensity for the adsorbed protein in the unfolded state. By following the fluorescence emission intensity at a given wavelength during the unfolding process, biphasic kinetics were observed with the kinetic constants depending on the specific metal-ion additive. In addition, solution refolding rates of the desorbed, unfolded species were measured and found to be consistent with literature refolding rate constants.

INTRODUCTION

The adsorption of proteins to liquid–solid interfaces is a subject of broad interest, including the design of biocompatible materials, bioanalytical sensors and the chromatographic separation of biomolecules [1,2]. From the separation point of view, an understanding of the protein adsorption process can aid in the optimization of conditions for selectivity, resolution and the minimization of the loss of biological activity of the protein [3]. One of the most important aspects of this adsorption process is the degree of alteration of the three-dimensional protein structure associated with solute contact with the chromatographic surface. The particular protein structure which interacts with the solid support can significantly influence retention. In addition, asymmetrically broadened or multiple peaks have been observed, depending on the rate and extent of structural alteration during adsorption and desorption [4–8].

To date, most studies of protein structural alteration upon adsorption have focused on the protein in solution after desorption [8,9]. Obviously, more detailed information can be obtained by exploring the protein while in contact with the surface of the adsorbent. Methods to study proteins on surfaces include antibody binding [10], ellipsometry [11], Raman [12], circular dichroism [13], Fourier-transform infrared

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(FT–IR) [14], total internal reflectance fluorescence (TIRF) [15,16] and intrinsic fluorescence spectroscopy [17].

Recently, this laboratory has developed a direct intrinsic fluorescence spectroscopic method combined with gradient elution chromatography for the study of adsorbed proteins on chromatographic surfaces [3,18–20]. Our studies have demonstrated that conformational changes of proteins on typical chromatographic surfaces used in reversed-phase liquid chromatography (RPLC) and hydrophobic interaction chromatography (HIC) can be followed by an examination of the fluorescence changes as a function of contact time of the protein with the surface. This approach has been shown to provide significant insight into the real-time, on-column structural change of proteins in contact with the surface. In a previous paper, detailed methodologies have been defined to elucidate kinetic processes of change of α-LACT on two HIC adsorbents [19].

Subsequent to the discovery that α-LACT was a calcium metalloprotein [21], extensive studies have been made on the binding properties and induced conformational changes caused by a variety of metal ions [22]. In contrast to calcium binding, which stabilizes the native conformation, Zn^{2+}, on binding to apo α-LACT, appears to stabilize the so-called *A* conformation, *i.e.*, a conformation closely resembling that of the acid-denatured α-LACT, which possesses a flexible molten globule structure [23] and exposed hydrophobic patches [24]. These metal binding characteristics and induced conformational changes of α-LACT have been utilized in low-pressure HIC to affect separation. Lindahl and Vogel [25] reported that Ca^{2+} eluted adsorbed α-LACT from Phenyl-Sepharose by inducing a less hydrophobic conformation, whereas elution could not be achieved by addition of Zn^{2+}. In high-performance liquid chromatography (HPLC), addition of Ca^{2+} has also been used to manipulate the retention and maintain biological activity of other calcium-binding proteins [26]. Salt addition to the mobile phase would appear to be a general approach for the manipulation of selectivity in the chromatographic separation of proteins [27,28].

Previous chromatographic work in this laboratory has demonstrated [8,19] that calcium-depleted α-LACT eluted as two well separated peaks from an ethyl polyether column (C_2 ether). The kinetics of conversion of the first peak to the second peak were observed by varying the time and temperature while the protein was in contact with the surface [7]. It was found that the second peak of α-LACT, identified as the unfolded species by on-line second-derivative UV spectroscopy, could undergo refolding in solution [8]. Conformational alteration of α-LACT has also been shown to occur on other commercially available HIC columns, *i.e.*, poly(alkyl) aspartamide columns [29]. Our chromatographic results further indicated that addition of a small concentration of calcium to the mobile phase caused a different HIC elution profile of α-LACT [8]. This paper examines further the effect of metal ions on the structural changes of adsorbed α-LACT using on-column intrinsic fluorescence.

EXPERIMENTAL

Equipment

The instrumental components for the on-column fluorescence measurements have been previously described in detail [19]. Briefly, a Suprasil quartz spectroscopic flow cell (35 μl, 11 × 2 mm I.D.) was utilized as both a cell for the surface fluorescence

studies using an SPF-500 spectrofluorometer (SLM-Aminco, Urbana, IL, U.S.A.) and as a microcolumn attached to a Series 410 BIO LC liquid chromatographic pump (Perkin-Elmer, Norwalk, CT, U.S.A.). The outlet of the flow-cell column was connected to a 1046A fluorescence LC detector (Hewlett-Packard, Palo Alto, CA, U.S.A.) *via* an empty Suprasil quartz spectroscopic flow cell. The sample compartment, injector and tubing (PTFE, 1.5 mm O.D. × 0.3 mm I.D.) attached to the column were thermostated at 5.8 ± 0.2°C by an Exacal and FTC Model 350A flow-through cooler system (Neslab, Newington, NH, U.S.A.), controlled by a thermocouple (Omega, Stanford, CT, U.S.A.). The chromatographic and spectroscopic data were processed with a data acquisition system (Nelson Analytical, Cupertino, CA, U.S.A.) and Spectrum Processor software (SLM Instruments, Urbana, IL, U.S.A.).

The packing in the flow cell (cell I) was made from Vydac silica gel (particle size 6 μm, pore diameter 300 Å, specific surface area 90 m^2/g) (Separations Group, Hesperia, CA, U.S.A.) bonded with an ethyl polyether phase (C_2 ether) and prepared as described elsewhere [30]. The surface coverage was 4.0 $\mu mol/m^2$ as determined by elemental analysis (assuming a binding stoichiometry to the silica of 2 ethoxy groups per silane molecule). The packing of the silica gel into the flow cell was carefully performed by hand to obtain a homogeneous distribution of the support in the column. Column conditioning was accomplished by injecting 10 μl aliquots of a 5.0-mg/ml protein solution 20 times, followed each time by elution under HIC-gradient conditions. The reproducibility of the gradient retention time for independent injections was 0.3% coefficient of variation (C.V.) ($n=5$) from run to run, and was 0.5% C.V. ($n=3$) from column to column.

Chemicals

Calcium-depleted bovine-milk α-LACT was purchased from Sigma (St. Louis, MO, U.S.A.). The purity of the protein was found to be sufficient for the chromatographic and fluorescence experiments [19]. High-purity-grade ammonium sulfate (treated to reduce the content of heavy metals) was the product of Sigma. HPLC-grade water and reagent-grade ammonium acetate were purchased from J. T. Baker (Philipsburg, NJ, U.S.A.).

The protein samples (5.0 mg/ml) were freshly prepared in 1 *M* ammonium sulfate–0.5 *M* ammonium acetate, pH 6.3. For the metal additive experiments, 1 m*M* $CaCl_2$ or $ZnCl_2$ was added to the same sample buffer.

Procedures

Mobile phase solutions of 3 *M* ammonium sulfate–0.5 *M* ammonium acetate (mobile phase A) and 0.5 *M* ammonium acetate (mobile phase B) were prepared by dissolving the correct weight of salt in degassed HPLC water and adjusting the pH to 6.3. Metal-ion-containing mobile phases were prepared by pipeting the correct volume of 0.5 *M* $CaCl_2$ or $ZnCl_2$ into the non-metal-containing mobile-phase solutions. These solutions were filtered by passing through an 0.45-μm membrane filter and then degassed under vacuum before use.

A 10-μl injected volume of a 5.0-mg/ml sample (50 μg) of α-LACT was determined as a convenient amount, and 0.3 ml/min a convenient flow-rate for the surface fluorescence and chromatographic measurements; the surface kinetics were independent of injected protein concentration (0.5–5.0 mg/ml) and mobile-phase

flow-rate (0.1–0.5 ml/min) [19]. Upon injection, the incubation solvent (mobile phase A) transported the sample in a 10-μl loop into the flow-cell column (cell I). The time of solute travel from the injector (including the sample loop) to the column was 12 s, at a flow-rate of 0.3 ml/min. Thus, 12 s after injection was defined as the initial contact time of the protein to the surface, *i.e.*, zero time. A 10-min linear gradient from 100% mobile phase A to 100% mobile phase B was used to elute the protein from the flow-cell column after a given period of incubation. For the solution-refolding experiments, the second empty flow cell (cell II) was switched into the light path, and the fluorescence was monitored in this cell. The time from cell I to cell II was determined to be 6 s and to the chromatographic fluorescence detector to be 34 s, at a flow-rate of 0.3 ml/min. The reproducibility of maximum emission intensity at fixed sampling time for independent injections of the same protein solution on the column was 0.5% C.V. ($n = 5$). The procedure for the fluorescence data acquisition and manipulation have been detailed in the previous paper [19].

RESULTS AND DISCUSSION

It is important to note that at high concentrations of NH_4^+ and nearly neutral pH, significant amounts of NH_3 can be present in the mobile phase. Based on an extrapolated formation constant of $2 \cdot 10^{10}$ for $Zn(NH_3)_4^{2+}$ at 5.8°C [31], 98, 58 and 0.2% of added $ZnCl_2$ (1 m*M*) will be in the complex form of $Zn(NH_3)_4^{2+}$, if the solution conditions are 3.0, 1.0 and 0 *M* ammonium sulfate plus 0.5 *M* ammonium acetate, pH 6.3, respectively. Thus, in the incubation mobile phase (mobile phase A, 3 *M* ammonium sulfate and 0.5 *M* ammonium acetate, pH 6.3), the added 1 m*M* $ZnCl_2$ will exist predominately as $Zn(NH_3)_4^{2+}$. In mobile phase B (0.5 *M* ammonium acetate, pH 6.3), almost all the added $ZnCl_2$ will be in the free ionic form Zn^{2+}. Note that in the HIC gradient from 3 *M* to 0 *M* $(NH_4)_2SO_4$, the surface tension of the solution not only decreases, but also $Zn(NH_3)_4^{2+}$ is converted to Zn^{2+}.

Hydrophobic interaction chromatography

Fig. 1 presents the HIC chromatograms for α-LACT on the C_2 ether phase in the packed flow cell (cell I) under gradient-elution conditions from 3 *M* to 0 *M* ammonium sulfate. The chromatograms are a function of incubation time of the adsorbed protein with an isocratic hold of mobile phase A. In agreement with previous results [8,9], two peaks are observed, the second peak increasing with the incubation time at the expense of the first. By following the decrease in area of the first peak as a function of incubation time, the apparent first-order rate constant was determined to be $6.9 \cdot 10^{-2}$ min^{-1} ($r^2 = 0.998$). It should be noted that this value was larger than that reported in the earlier paper [19], *i.e.*, $7.4 \cdot 10^{-3}$ min^{-1}; however, two different C_2 ether bonded phases were used in these two studies. The synthesized adsorbent in the present work had a larger surface area (90 *vs.* 72 m^2/g) and a higher absolute carbon loading than that of the previous study. The overall higher hydrophobicity of the surface was thus presumably a significant factor in the faster unfolding kinetics of the protein on the surface. In addition, the slightly higher column temperature used in this work (5.8 *vs.* 4.0°C) also contributed to the faster kinetics. Nevertheless, the ten-fold difference in the rate of unfolding demonstrates that this parameter can be a sensitive function of surface characteristics and thus could be useful in the characterization of chromato-

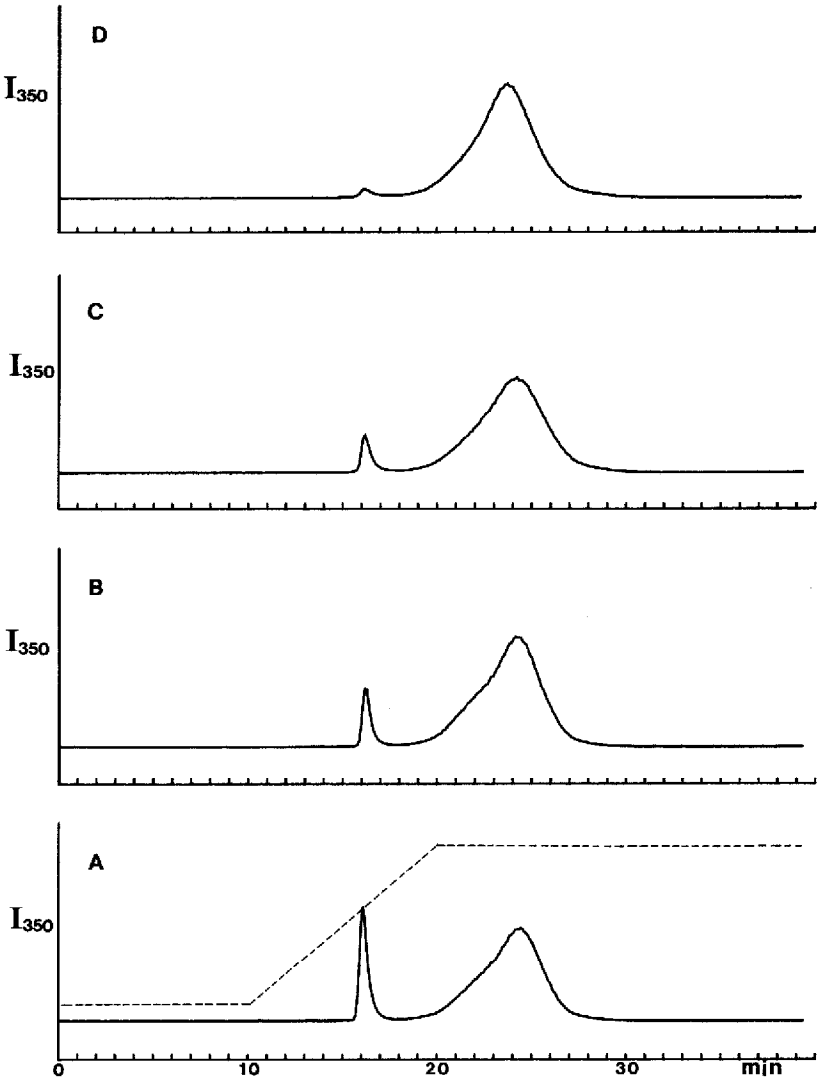


Fig. 1. Gradient-elution chromatograms of α-LACT as a function of incubation time (10 min gradient delay time not included) with mobile phase A. (A) 0 min; (B) 10 min; (C) 20 min; (D) 50 min. Conditions: 10-min gradient from 100% mobile phase A (3 *M* ammonium sulfate–0.5 *M* ammonium acetate, pH 6.3) to 100% mobile phase B (0.5 *M* ammonium acetate, pH 6.3). Sample: calcium-depleted bovine α-LACT, 5.0 mg/ml in 33% A, 67% B, 10-μl injection. Fluorescence detection wavelength: 350 nm, flow-rate 0.3 ml/min, temperature 5.8°C.

graphic adsorbents. The broad and asymmetric second peak in Fig. 1 will be considered shortly.

The influence of added metal ion on the elution profile is shown in Fig. 2. In all three cases, *i.e.*, no metal-ion additive, 1 m*M* Ca^{2+} and 1 m*M* $Zn(NH_3)_4^{2+}$ in the

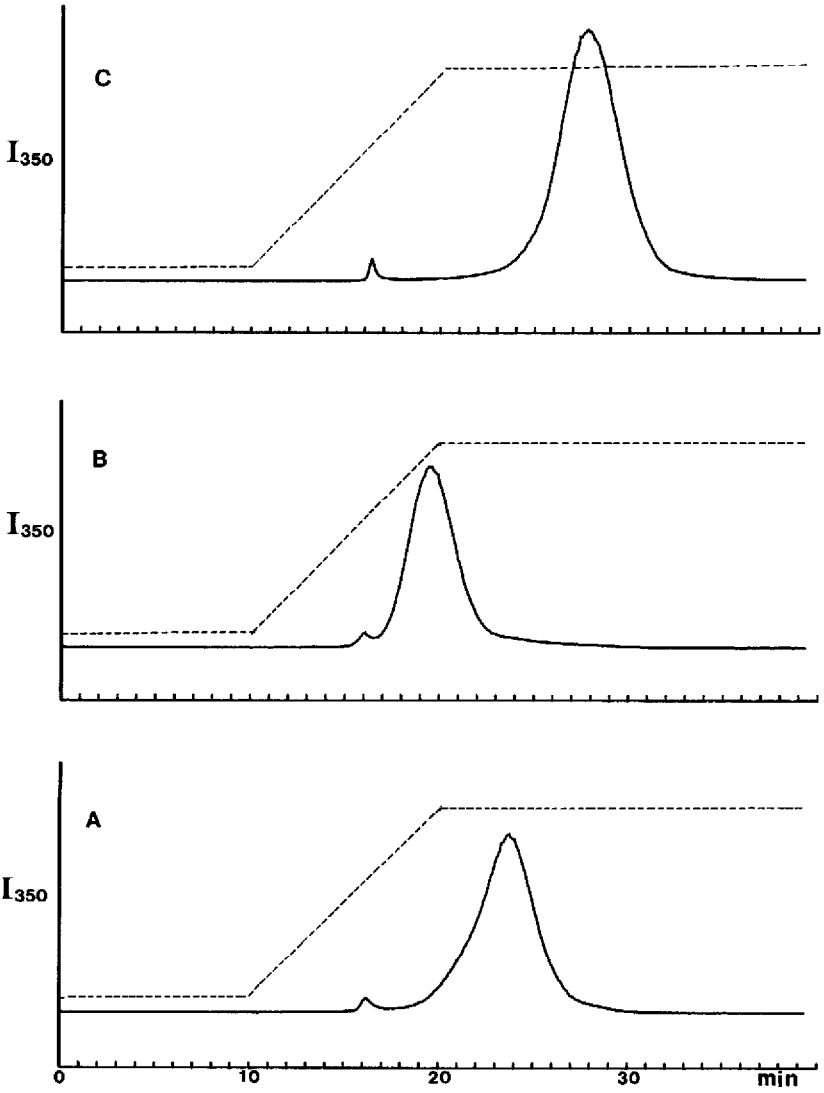


Fig. 2. Gradient-elution chromatograms of α-LACT as a function of metal-ion additives in the mobile phase. (A) no metal-ion additive, incubation 50 min; (B) 1 m*M* $CaCl_2$ added in both mobile phases A and B as well as the sample solution, incubation 80 min; (C) 1 m*M* $ZnCl_2$ added in both mobile phases and the sample solution, incubation 80 min. Other conditions are the same as in Fig. 1.

mobile phase, two peaks were observed, with the first peak in all cases eluting at the same position of the gradient (1.26 *M* ammonium sulfate) while the second peak eluted at different gradient positions. With the Ca^{2+}-containing mobile phase, the second peak was eluted at the end of the gradient whereas without metal ion and with the zinc-added mobile phase, the second peak was eluted 4 min and 8 min after the end of the gradient, respectively. As will be shown later, the differences in the retention time of

the second peak for the various mobile phases are a consequence of the differences in the refolding rates of the desorbed species under the second peak.

Fig. 2 further shows that a slower conversion of the first peak into the second was observed in the presence of Ca^{2+} (80 min incubation time), relative to that of the metal-ion-free condition (50 min incubation time). Small amounts of the first peak were still seen after 80 min incubation with the calcium-containing mobile phase, while a shorter incubation time (50 min) had to be used in order to maintain roughly the same amount of the first peak in the absence of Ca^{2+}. Interestingly, with $Zn(NH_3)_4^{2+}$ in the mobile phase, conversion of the first peak to the second was also slower than the non-metal-ion-additive case.

Since the chromatographic method can only probe the gross unfolding kinetics, we next explored the surface unfolding of the adsorbed protein for a more detailed kinetic picture. This examination was accomplished by *in situ* intrinsic fluorescence of the protein on the chromatographic surface.

Kinetic analysis of the surface-unfolding of α-LACT

Fig. 3 shows the emission spectra of α-LACT on the C_2 ether phase as a function of incubation time with and without metal-ion additive. In order to minimize photodecomposition, a discrete method of data collection was employed [19]. The fluorescence emission spectra were collected by scanning for 10 s intervals from 325 to 355 nm at 30, 60 and 300 s after injection. Two major features of the three mobile phases in this figure are: (1) the general increase in emission intensity with incubation time and (2) the 16-nm red shift in the emission maximum (from 333 nm in the first minute of surface contact to 349 nm after 60 min) in the absence and presence of Ca^{2+} or $Zn(NH_3)_4^{2+}$. Similar to the fluorescence behavior of α-LACT in solution reported by others [32], the increase in emission intensity and the red shift in the emission maximum strongly suggest unfolding of the adsorbed protein.

The surface kinetics of structure change of the adsorbed protein can be derived from the plot of fluorescence intensity *vs.* incubation time at a given wavelength. Fig. 4 presents the on-column fluorescence emission intensity at 350 nm as a function of incubation time of the adsorbed α-LACT with and without 1 m*M* Ca^{2+} or 1 m*M* $Zn(NH_3)_4^{2+}$ in the mobile phase. As can be seen, the fluorescence intensity of α-LACT in the presence of Ca^{2+} increased at a slower rate compared to the metal-ion-free mobile phase, in agreement with the chromatographic results of Fig. 2. Interestingly, in the presence of 1 m*M* $Zn(NH_3)_4^{2+}$ in the incubation mobile phase, the rate of increase in fluorescence intensity was reduced even further.

Curve fitting of the plots in Fig. 4 revealed that in all three cases the unfolding process could be fitted to a biphasic kinetics model (Fig. 3). The macroscopic rate constants were derived from curve fitting according to the equation [19]

$$I(t) = I_\infty - I_1^* e^{-\lambda_1 t} - I_2^* e^{-\lambda_2 t} \quad (1)$$

and the results are shown in Table I. As can be seen, the macroscopic rate constants for the slow kinetic phase (λ_2) are of the same magnitude as the rate constants derived from chromatography, while the fast kinetic phase (λ_1) can be monitored only by on-column fluorescence. Addition of Ca^{2+} in the incubation mobile phase reduced the macroscopic rate constants of unfolding of α-LACT by 30 and 40% for the fast and

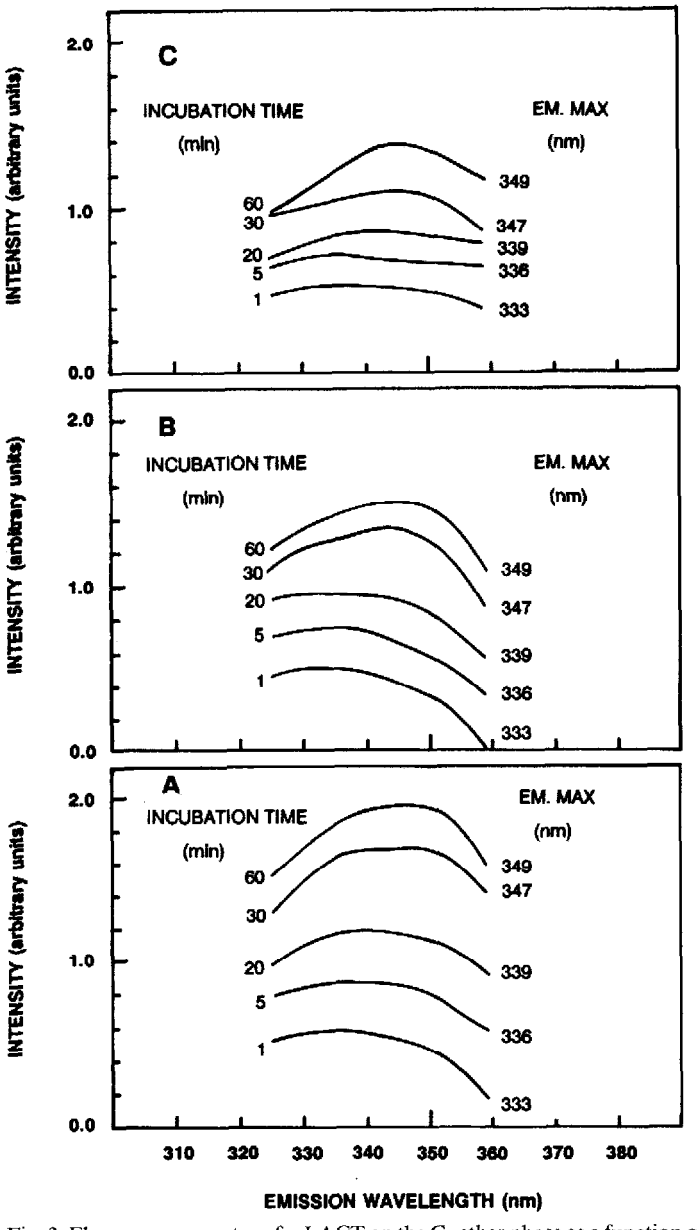


Fig. 3. Fluorescence spectra of α-LACT on the C_2 ether phase as a function of incubation time (see Fig. 1 for other conditions). (A) No metal-ion additive; (B) 1 m*M* $CaCl_2$; (C) 1 m*M* $ZnCl_2$ added in the incubation solvent. Excitation wavelength: 295 nm; emission scan: 2 nm/s; resolution: 0.5 nm; incubation solvent: mobile phase A (3 *M* ammonium sulfate–0.5 *M* ammonium acetate, pH 6.3); temperature 5.8°C.

slow kinetic phases, respectively. In the presence of $Zn(NH_3)_4^{2+}$, the rate constants for the two kinetic phases were even smaller, both by a factor 3, relative to the non-metal-ion case.

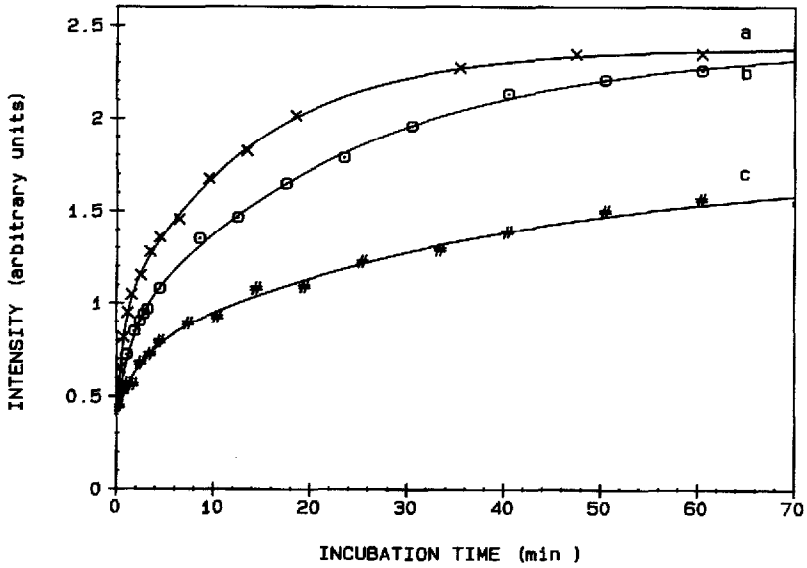


Fig. 4. Fluorescence emission intensity (at 350 nm) of α-LACT adsorbed on the C_2 ether phase as a function of incubation time. Conditions are the same as in Fig. 3. Lines are fitted to a biphasic equation (eqn. 1 in the text). Curves: (a) no metal ion additive, (b) 1 m*M* $CaCl_2$, (c) 1 m*M* $ZnCl_2$ added in the incubation solvent.

Kinetic analysis of the microscopic unfolding process was performed using a kinetic model of

$$F \underset{k_2}{\overset{k_1}{\rightleftarrows}} X \xrightarrow{k_3} U \tag{2}$$

where *F* denotes the initial folded state, *U* the final unfolded state and *X* a kinetic intermediate [19]. The validity of this model is seen in that neither parabolic

TABLE I

BIPHASIC KINETIC PARAMETERS FOR THE UNFOLDING OF α-LACT ON THE C_2 ETHER PHASE

Experimental conditions are the same as in Fig. 3. I_1^* and I_2^* are the dimensionless amplitudes for the two exponential phases. $I(0)$ is the intercept corresponding to the $I(t)$ value at $t=0$. See Fig. 4 and eqn. 1.

Additive		$I(0)$	I_1^*	I_2^*	I_∞	λ_1 (min^{-1})	λ_2 (min^{-1})
None		0.40	0.68	1.71	2.83	1.25	0.073
	$\pm\sigma$	0.04	0.03	0.01	0.20	0.03	0.003
1 m*M* Ca^{2+}		0.38	0.44	1.50	2.32	0.81	0.043
	$\pm\sigma$	0.03	0.02	0.03	0.10	0.02	0.001
1 m*M* $Zn(NH_3)_4^{2+}$		0.42	0.21	0.97	1.60	0.44	0.028
	$\pm\sigma$	0.04	0.08	0.03	0.20	0.06	0.001

TABLE II

MICROSCOPIC RATE CONSTANTS FOR THE UNFOLDING OF α-LACT ON THE C_2 ETHER PHASE; $F \underset{k_2}{\overset{k_1}{\rightleftarrows}} X \overset{k_3}{\rightarrow} U$

Experimental conditions are the same as in Fig. 3.

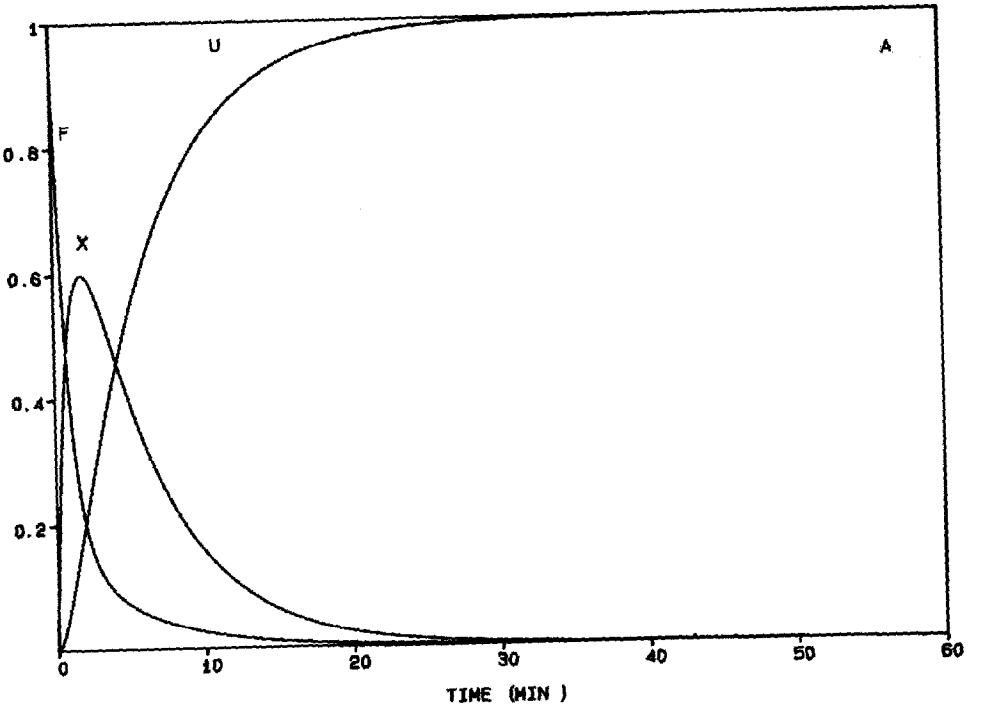
Additive	k_1 (min^{-1})	k_2 (min^{-1})	$K_e = k_1/k_2$	k_3 (min^{-1})
None	0.97 ± 0.04	0.13 ± 0.04	7.6 ± 0.3	0.22 ± 0.02
1 m*M* Ca^{2+}	0.42 ± 0.02	0.27 ± 0.04	1.5 ± 0.1	0.16 ± 0.02
1 m*M* $Zn(NH_3)_4^{2+}$	0.12 ± 0.03	0.23 ± 0.04	0.52 ± 0.1	0.12 ± 0.01

($F \rightarrow X \rightarrow U$) nor sigmoidal ($F \rightarrow X \rightleftarrows U$) curves of fluorescence intensity I *vs.* time t were observed. The microscopic rate constants, k_1, k_2 and k_3 were derived from the macroscopic parameters in Table I and are tabulated in Table II.

The microscopic parameters reveal that the rate constant of the early unfolding stage, $F \rightarrow X$, decreased when Ca^{2+} was added to the system, whereas the surface refolding process, $X \rightarrow F$, was accelerated by the presence of Ca^{2+}. Microscopic reversibility permitted the calculation of the constant, K_e, for the process $F \rightleftarrows X$. A value of $K_e = 7.6$ was obtained for the case of the non-metal-ion mobile phase (Table II). A K_e value of 1.5 in the presence of Ca^{2+} showed that the formation of the kinetic intermediate was thermodynamically less favorable with added Ca^{2+}, in agreement with literature reports that in solution calcium binding stabilized the folded state F [21]. In the presence of $Zn(NH_3)_4^{2+}$, a K_e value of 0.5 was obtained, indicating that the formation of the intermediate was unfavorable under these conditions.

The relative amounts of each species, F, X and U, as a function of time can be obtained from the microscopic rate constants using the integrated rate law for each component. The results of this calculation for the three mobile phases are shown in Fig. 5. As can be seen, the decay of the folded species F in the presence of Ca^{2+} was slower relative to the non-metal-ion case, while in the presence of $Zn(NH_3)_4^{2+}$, the decay of the folded state F was even slower, with a smaller accumulation of the intermediate X.

It is well known that Zn^{2+} binding to α-LACT favors a more flexible conformation in solution [24], whereas our results have shown that $Zn(NH_3)_4^{2+}$ appeared to stabilize the folded state on the surface. Since a significant difference in the ionic radius, charge density and coordination exists between the two forms of zinc in solution, it is not surprising that $Zn(NH_3)_4^{2+}$ may interact with α-LACT in a different way than Zn^{2+}. The effect of $Zn(NH_3)_4^{2+}$ appears to be consistent with the known cation stabilization of α-LACT in solution [22]; in the case of Zn^{2+}, specific binding is known to occur to cause destabilization [24]. (The inaccessibility of the zinc binding site on the adsorbed protein to components of the solution may also play a role.) It is further interesting to note that $Zn(NH_3)_4^{2+}$ slowed the surface-induced unfolding of α-LACT to an even greater extent than Ca^{2+}.



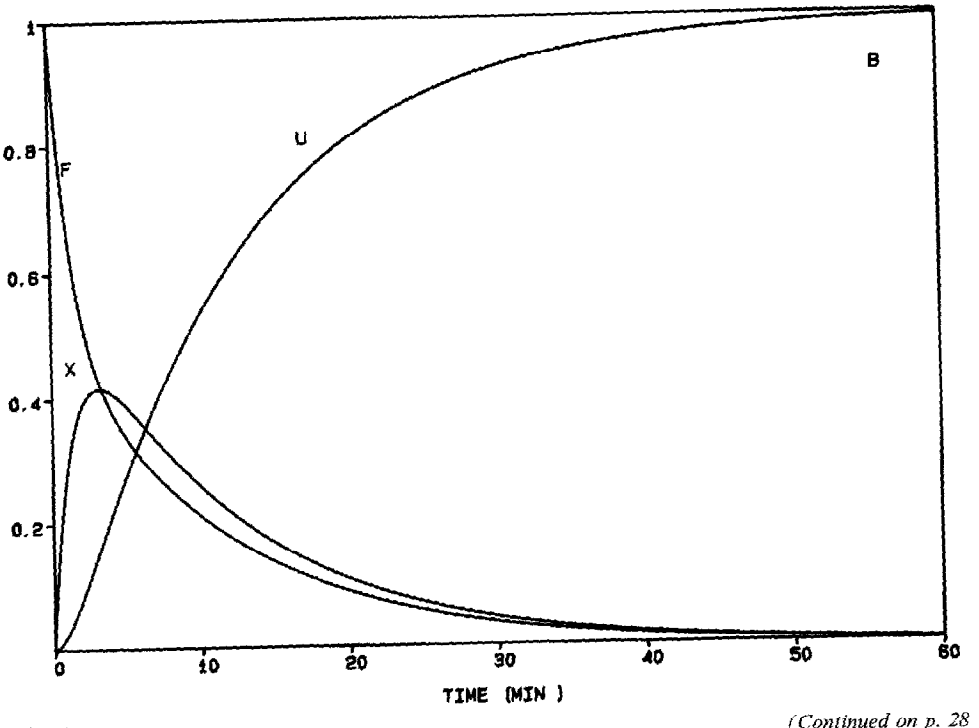


Fig. 5

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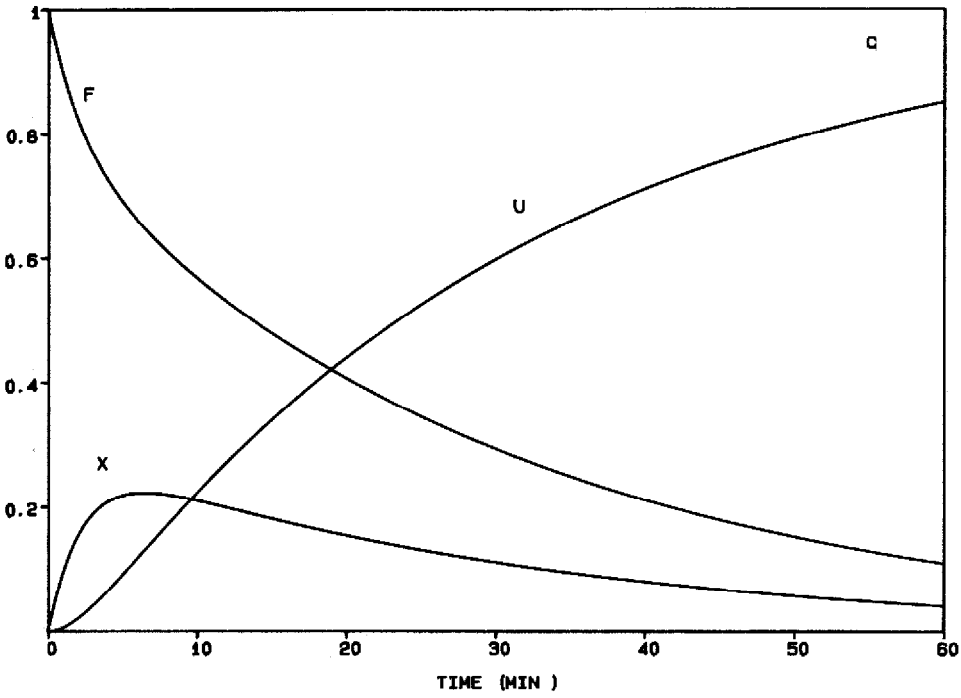

Fig. 5. Relative amounts of F (folded), X (kinetic intermediate) and U (unfolded) states as a function of time for α-LACT, adsorbed on C_2 ether phase at 5.8°C in the absence (A) and presence of 1 mM Ca^{2+} (B) and 1 mM $Zn(NH_3)_4^{2+}$ (C). Conditions as in Fig. 3. The model is based on biphasic kinetics.

Solution refolding upon desorption

In the previous work [19], the solution-refolding kinetics of α-LACT of the second peak was measured by following the change in intrinsic fluorescence of the desorbed species in a second empty flow cell (Cell II) connected in series. We again measured the solution-refolding rate constants in the absence and presence of Ca^{2+} or Zn^{2+}. The mobile-phase flow was stopped when the second eluted peak reached the empty flow cell, and the fluorescence emission spectra were collected.

The plots of natural logarithm of the fluorescence intensity at 350 nm (background corrected) *vs.* time were linear ($r^2 = 0.995$) with first-order rate constants of refolding of 0.83 min^{-1} ($t_{\frac{1}{2}} = 50$ s) and 0.48 min^{-1} ($t_{\frac{1}{2}} = 86$ s), for the metal-ion-free mobile-phase condition and in the presence of 1 mM Zn^{2+}, respectively. The refolding process in the presence of Ca^{2+} was too fast to be followed by the present method. This is reasonable since the half life for refolding at 4.5°C in the presence of Ca^{2+} as measured by circular dichroism [33], was $t_{\frac{1}{2}} = 0.25$ s, whereas the time needed to transport the eluted peak to flow cell II was 6 s. The fast refolding with Ca^{2+} is expected since this metal ion stabilizes the folded conformation in solution. The refolding rate constant for α-LACT in the metal-free case is in agreement with the value reported by others [18,33], as can be seen from Table III. On the other hand,

TABLE III

REFOLDING RATE CONSTANTS AND HALF LIVES FOR α-LACT

Additive	This work[a]		Lit. [33][b]	
	k_r (min^{-1})	$t_{\frac{1}{2}}$ (s)	k_r (min^{-1})	$t_{\frac{1}{2}}$ (s)
None	0.83 ± 0.04	50 ± 1	0.78	55
1 m*M* Ca^{2+}	⩾20	⩽6	162	0.25
1 m*M* Zn^{2+}	0.48 ± 0.02	86 ± 3	–	–

[a] 5.8°C, pH 6.3, 0.5 *M* ammonium acetate; fluorescence emission.
[b] 4.5°C, pH 7.0, 0.05 *M* sodium cacodylate; CD decay.

Zn^{2+} is destabilizing [22], and thus, the surface-unfolded species after desorption can only refold more slowly than in the case of no metal-ion additive.

The retention of α-LACT (see Fig. 2) in the absence and presence of the metal ion additives can be interpreted in terms of the different refolding rates. With the Ca^{2+}-containing mobile phase, once the unfolded protein desorbed, it rapidly refolded in solution. Since the folded form was unretained under the elution condition of the second peak, the migration of the second peak was accelerated. In the absence of metal ion in the mobile phase, the half life for the refolding of the desorbed species was comparable to the unretained time (t_0 = 39 s) of the system, yielding a peak in the isocratic region. In the presence of Zn^{2+} in mobile phase B, the refolding of the desorbed species was even slower and the peak eluted later in the isocratic region.

CONCLUSIONS

This work has extended previous studies on the on-column conformational changes of α-LACT in HIC. The crucial role of spectroscopic measurements in elucidating detailed chromatographic behavior of proteins has been further shown. The interesting effects of metal-ion additives on the surface-unfolding and solution-refolding rate constants have been characterized. Because of different solution-refolding kinetics, the retention of the unfolded species varied with the specific additive. Moreover, one metal ion, zinc, was found to be stabilizing in 3 *M* $(NH_4)_2SO_4$ and destabilizing in buffer alone (0.5 *M* NH_4OAC, pH 6.3).

The results also demonstrate the danger in generalizing retention patterns of proteins from the retention models of small molecules. In the case of proteins, complications can arise from the fact that biopolymers can readily adopt different conformations with different strengths of interaction to the adsorbent surface. At the same time this flexibility of proteins can provide great potential for manipulating separation. For example, as already noted the addition of Ca^{2+} to α-LACT adsorbed to an HIC support caused elution, after a great deal of background matrix material was first removed without Ca^{2+} in the mobile phase [25]. In addition, isocratic separation of two variants of growth hormone was possible by determining a specific elution condition under which the conformational stability of the two species differed from one another [20]. These examples point to the need to understand protein-HPLC retention as fully as possible.

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