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Measuring conformational stability of proteins using an optimized temperature-controlled capillary electrophoresis approach

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Abstract

The thermal denaturation process of a model protein, bovine β-lactoglobulin, was analyzed using capillary zone electrophoresis (CZE). For this purpose, a commercial CE apparatus was improved, allowing efficient control and accurate measurement of the temperature up to 95°C. Under various pH conditions, transition temperature (T_m), enthalpy change (ΔH) and entropy change (ΔS) associated with the thermal denaturation were determined. Moreover, the technique is unique in its ability to estimate the heat capacity change (ΔC_p). This work shows that CZE, performed even when electroosmotic flow occurs, is an innovative approach for determining the stability curves of proteins. Accordingly, CZE is a powerful tool to study protein unfolding/folding quickly and with minimal sample requirements. © 1999 Elsevier Science B.V. All rights reserved.

Keywords: Thermodynamic parameters; Stability studies; Proteins; Lactoglobulins

1. Introduction

The mechanism of protein folding is one of the most fascinating problems. Depending on the environmental or physiological conditions some proteins are able to adopt either a benign or a pathogenic conformation [1], and besides, improper folding of recombinant proteins frequently occurs in expression cells. Moreover, for industrial, pharmaceutical and biomedical applications, a major goal is to design the more stable enzymes having the desired activity. For this purpose, methods for measuring conformational stability of proteins are required. These considerations are sufficient to prompt to develop new methodologies for studying protein folding and stability.

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Although capillary electrophoresis (CE) is particularly well suited for the study of proteins, it has been scarcely used to study denaturation and folding, or to estimate stability. Most techniques used for investigating protein folding/unfolding processes depend on direct measurements of physical properties sensitive to conformational changes, such as UV-absorbance and fluorescence. Theoretically, due to its high resolution, high sensitivity, high speed and accuracy, CE is an attractive alternative providing access to populated molecular states within folding/unfolding equilibria. A very promising first attempt in this field was carried out on lysozyme at low pH, allowing to estimate the unfolding transition temperature, T_m and the apparent thermodynamic parameters, ΔH and ΔS, associated with this transition [2,3]. Unfortunately, to date no other experimental studies were available. A recent application of CE at low pH for monitoring the effects of additives on the thermal denaturation of ribonuclease A was de-

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scribed. Meanwhile, this study was performed using an unmodified CE instrument and it failed to provide T_m at temperatures lower than 50°C [4].

The aim of the present work was to extend application of CE to analysis of thermal denaturation of proteins at different pH. The efficiency of CE for analyzing thermally induced denaturation of a model protein, bovine β-lactoglobulin (β-Lg), existing in various oligomeric states depending on the protein concentration, the pH and the temperature [5,6] was investigated. The prerequisite for a robust study was the improvement of the CE system, for efficient control and accurate measurement of the temperature, up to 95°C. The unfolding of β-Lg was carried out at different pH. The thermodynamic parameters of unfolding were calculated according to the CE data. This new approach enabled us to calculate protein stability by drawing stability curves.

2. Materials and methods

2.1. Chemicals and samples

Bovine β-Lg variant B, used without further purification, sodium phosphate and sodium hydroxide were obtained from Sigma (Saint Louis, MO, USA). The electroosmotic flow marker was dimethylformamide (DMF) from Pierce (Rockford, IL, USA). All other chemicals were of analytical grade. Buffers and solutions were prepared using water from a Milli-Q water purification system (Millipore, Waltham, MA, USA), and filtered through 0.45 μm disposable filters (Schleicher & Schuell, Dassel, Germany), prior to use. β-Lg B stock solutions of 20 mg ml^{-1} were prepared in water and stored at −20°C. Protein samples for CE analysis (2 mg ml^{-1}) were prepared daily by diluting stock solutions in the appropriate running buffer, complemented with DMF (0.01%, v/v, final concentration). Samples were incubated for 1 h at the given temperature prior analysis. Separation buffers were a set of 100 m*M* sodium phosphate buffers ranging from pH 6.2 to 8.2.

2.2. Equipments and modification of the CE apparatus

A Beckman (Fullerton, CA, USA) CZE System (Model P/ACE 5500), equipped with a diode array detection (DAD) system, a fluid-cooled column cartridge, a sample thermostating accessory and a P/ACE Station 1.0 software, was used for CE experiments. Fused-silica capillary tubing of 47 cm (40 cm length to the detector)×50 μm of I.D. was used. For precise temperature control in the range 20–95°C, the CE apparatus was modified (Fig. 1). Samples and buffer in the auto-sampler tray were thermostated by an external circulating bath (Julabo, Seelbach, Germany), having the following specifications: working temperature range −28 to 100°C, temperature stability ±0.02°C, pump flow-rate 9 l min^{-1}. An external thermal-exchange unit immersed in the thermostated circulator was assembled in the laboratory. It was used with a Beckman fluoroorganic fluid (boiling point >97°C) and a circulating pump similar to the initial internal pump. The temperature during migrations was measured into the coolant compartment of the capillary cartridge by implanting a thermocouple microprobe (Physitemp Instruments, Clifton, NJ, USA) type IT-

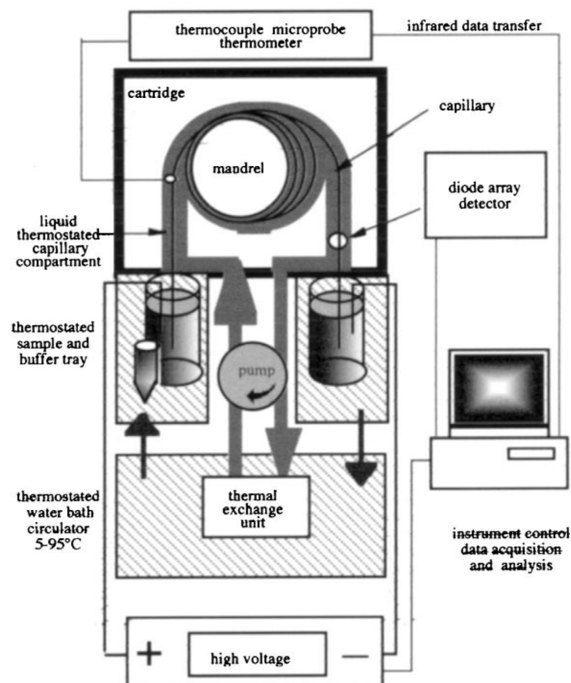

Fig. 1. Schematic diagram of the modified Beckman P/ACE 5000 capillary electrophoresis instrument. The capillary is surrounded by a fluoroorganic fluid which is thermostated by an externally controlled bath.

23 (time constant=0.005 s), connected to a digital thermometer Digi-Sense (Cole-Parmer, Chicago, IL, USA). Temperature data (up to 1000 readings/run) were transfered from the thermometer's infrared output to an RS-232 serial port of the computer.

2.3. Electrophoresis

The CE was performed at constant voltage in the normal polarity mode with the negative electrode at the detector (outlet) end of the capillary. Prior to sample injection, the capillary was rinsed with the running buffer for 2 min. The β-Lg samples were pressure-injected into the capillary. The duration of injection (15 s at 30°C) was decreased (2% per °C) to correct for the decrease in viscosity with increasing temperature [7]. Peak detection was carried out by monitoring the change in UV absorbance at 200 nm.

The apparent mobilities for β-Lg and DMF were calculated as follows:

$$\mu_{app} = (L_t \times L_d)/(t_m \times V) \quad (1)$$

where μ_{app} is the apparent electrophoretic mobility ($cm^2\ V^{-1}\ s^{-1}$), L_t is the total length of the capillary, L_d is the length of capillary to detector, V is the applied voltage, and t_m is the migration time. The true mobility, μ, is related to the apparent mobility by:

$$\mu = \mu_{app} - \mu_{eo} \quad (2)$$

where μ_{eo} is the mobility due to endoosmotic flow.

2.4. Analysis of thermal unfolding

Analysis of unfolding transitions of β-Lg was conducted assuming a reversible two-state mechanism, and that the rate of interconversion between native (N) and unfolded (U) forms was slow compared to the separation time. Under these conditions (known as the slow-time regime), N and U are assumed to be in equilibrium, the direct UV quantitation of CZE allowed the relative concentrations of the two populations to be calculated. Thus, the ratio of the peak areas is: $K_D = [U]/[N]$. Since van't Hoff plots for thermal denaturation of proteins are linear in the transition region, the enthalpy change (ΔH_m) of unfolding at the transition temperature (T_m) can be estimated. On the other hand, because the change in free energy (ΔG) is 0 at T_m, the entropy of unfolding (ΔS_m) at the transition midpoint can be calculated from:

$$\Delta S_m = \Delta H_m / T_m \quad (3)$$

The experimental ΔG values as a function of temperature (T), were fitted to a form of the Gibbs-Helmholtz equation:

$$\Delta G_{(T)} = \Delta H_m (1 - T/T_m) - \Delta C_p [(T - T_m) + T \ln (T/T_m)] \quad (4)$$

where ΔH_m is the enthalpy change at T_m and ΔC_p is the change in heat capacity between the native and the denatured states. ΔC_p for the unfolding process was calculated using the Kirchoff equation:

$$\Delta C_p = \partial(\Delta H)/\partial(T) \quad (5)$$

as the slope of the plot of ΔH_m vs T_m, measured at different pH.

3. Results

3.1. Validation of the improved system and of the experimental settings

The temperature was measured in situ using a thermocouple microprobe introduced inside the capillary jacket. The improved CE system, with externally controlled capillary temperature, was tested by carrying out electrophoretic runs between 40 and 95°C. A linear regression was obtained by plotting the temperature measured in the capillary cartridge against the temperature set on the thermostating water bath circulator (Fig. 2A). Another linear regression was obtained by plotting the current intensity in the capillary against the temperature in the capillary during migrations (Fig. 2B). The values of the regression coefficient for both plots validated the efficiency of the modifications. The improvement allowed both efficient control, with temperature fluctuations lower than 0.05°C, and accurate measurement of the temperature (±0.1°C) near by the outer capillary wall.

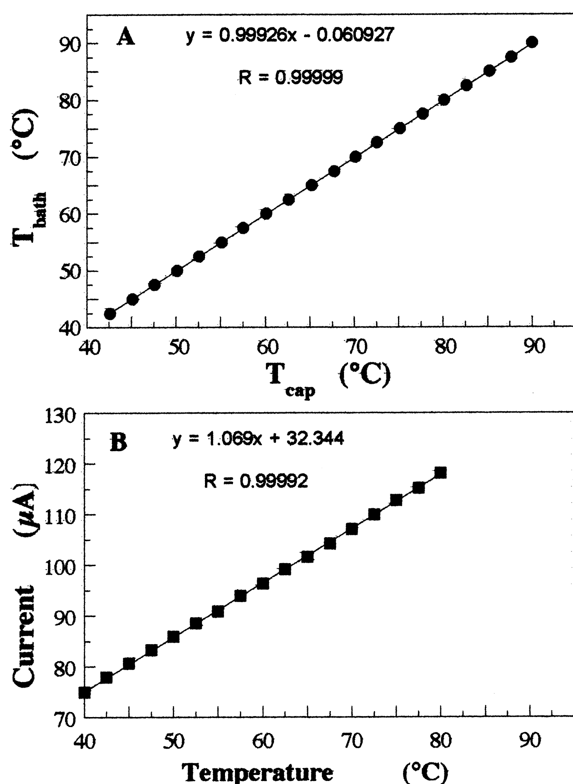

Fig. 2. Effectiveness of the improved CE system. (A) Correlation between temperatures set on the thermostated bath (T_{bath}) and measured by the sensor in the capillary cartridge (T_{cap}). (B) Temperature-dependence of the current intensity observed with 100 mM phosphate buffer at pH 7.2; separation voltage 10 kV.

3.2. Thermal unfolding of β-Lg at various pH monitored by capillary electrophoresis

To evaluate whether CE would offer sufficient resolution under various pH conditions for most temperature-induced conformations of β-Lg, analysis was performed in the pH range 6.2–8.2 at different temperatures between 40 and 95°C. The ionization heat of phosphate is small and, accordingly, the temperature coefficient is small: $\partial pK_a/\partial T = -\Delta H/2.3\ RT^2 = -0.28$, with $pK_a = 7.2$ and $\Delta H = 4.8$ kJ mol^{-1}. Thus, the pH of phosphate buffer is almost constant with rising temperature. Moreover, phosphate buffer is well-suited for use in CE separations of proteins due to its protein compatibility and good transmission characteristics. When the analysis was performed at increasing temperatures the following findings were observed (Fig. 3). At pH 7.2 and 62°C for example, two peaks were seen; the first was DMF and the second was β-Lg. At higher temperatures, due to the decrease in buffer viscosity, the mobility of both DMF and β-Lg increased with temperature. An additional peak appeared above 62°C, and its area increased as the temperature reached the upper limit of the CE system (95°C). A concomitant decrease in the peak area of β-Lg occurred. The unfolding transition of β-Lg was thought to be between 71 and 72°C. In this temperature range, the two peaks were presumed to correspond to native

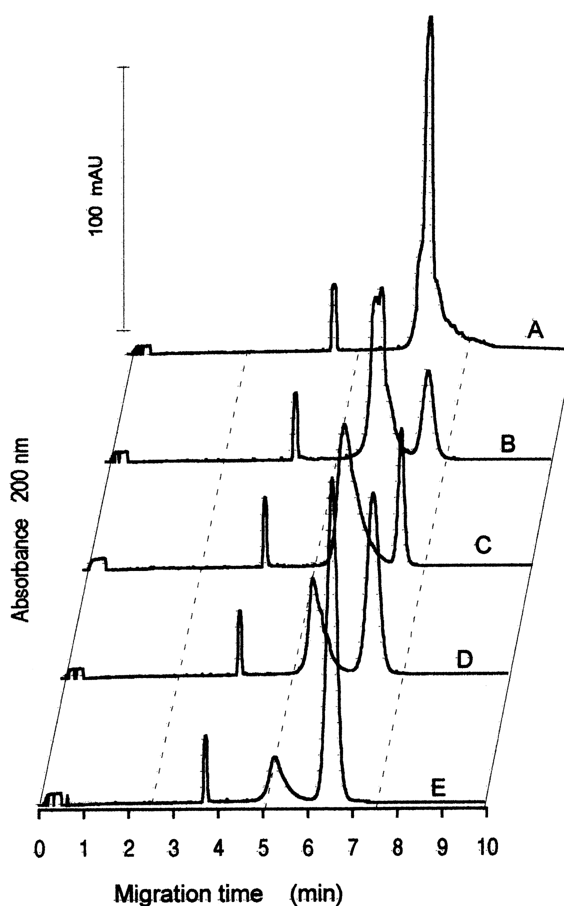

Fig. 3. Representative electropherograms for β-Lg B at (A) 62°C, (B) 67°C, (C) 71°C, (D) 72°C, and (E) 78°C. The sample and run buffer was 100 mM sodium phosphate at pH 7.2; electrophoresis was conducted at 10 kV. The first peak corresponds to the EOF marker and the others to β-Lg.

(N) and unfolded (U) β-Lg, respectively. Within the transition temperature range, the rapid mobility change of the main peak did not provide enough values to draw the transition curve. Fortunately, below and above the transition temperature, T_m, the progressive change in the main peak area and the appearance of the additional slow-migrating peak at the expense of the first one, reflect the concentration change of the N and U species as a function of temperature. Assuming N and U in equilibrium, the direct UV quantitation of CZE allowed calculation of the relative concentrations of the two protein populations. Characterization of the transition was made possible by fitting the relative peak areas to the ratio $K_D=[U]/[N]$ at different temperatures. In this way, the true thermodynamic parameters for the thermal unfolding transition were determined. For example, at pH 7.2, the calculated values were: $\Delta H=350.4\pm0.5$ kJ mol^{-1}, $T_m=71.0\pm1.0$°C and $\Delta S=1.02\pm0.5$ kJ mol^{-1} K^{-1}.

Similar determinations at different pH were carried out. Change in pH affected the heat stability, thus allowing analysis of the temperature dependence of the enthalpy change. The slope of plots of ΔH_m vs. T_m at different pH was used to estimate the ΔC_p (Fig. 4). These values were fed into Eq. (4) to plot the free energy of unfolding, ΔG, as a function of temperature. This permitted us to build the stability curves for β-Lg B at the different pH. Fig. 5 shows a representative stability curve determined at pH 6.2. These curves allowed to obtain the temperature of

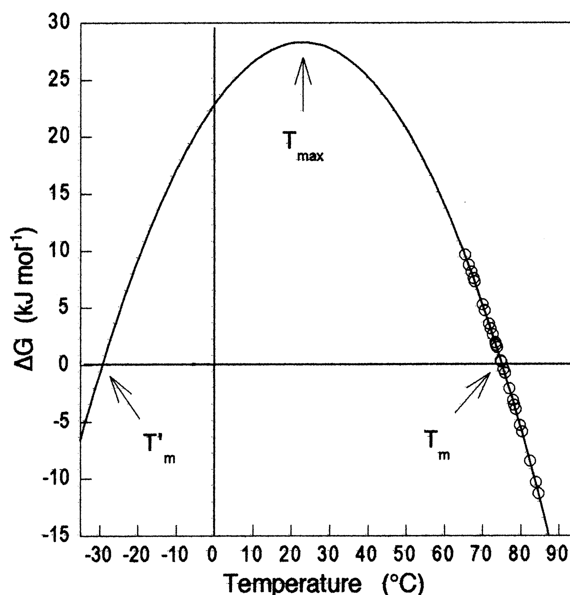

Fig. 5. Stability curve for β-Lg B established by the temperature-dependence of the free energy change for the thermal unfolding of β-Lg B in 100 mM sodium phosphate at pH 6.2. The line represents the best fit to Eq. (4) with CZE values of T_m and ΔH_m from peak area data and of ΔC_p estimated from Fig. 4.

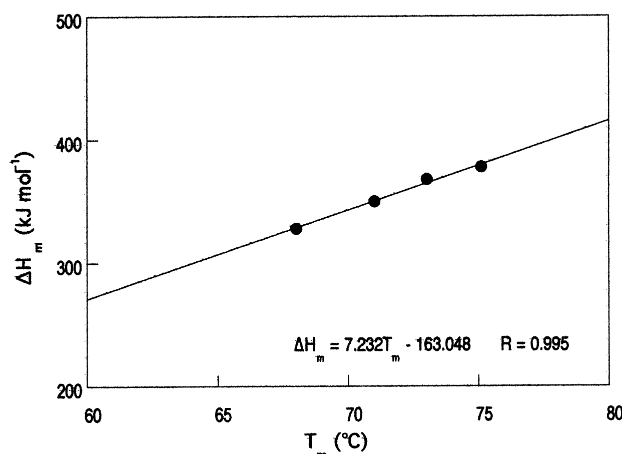

Fig. 4. Determination of the heat capacity change for the denaturation of β-Lg B by plotting the denaturation enthalpy (ΔH_m), as a function of the temperature of denaturation (T_m), determined from the data of CZE of β-Lg B solutions in phosphate buffer of pH between 6.2 and 7.7.

Table 1
Effect of pH on stability of β-Lg B in 100 m*M* sodium phosphate; heat and cold denaturation transition temperatures (T_m and T'_m), respectively, and temperature at which the stability of the protein is maximal (T_{max}) were determined using Eq. (4)

Phosphate buffer pH	T'_m (°C)	T_{max} (°C)	T_m (°C)
6.2	–29.2	23.1	75.1
6.7	–28.5	22.3	73.1
7.2	–26.1	22.7	70.9
7.7	–22.6	22.7	67.9

maximum stability (T_{max}) and the cold denaturation transition temperature (T'_m). Table 1 summarizes temperature data at the different pH used, estimated according to theses curves.

4. Discussion

4.1. Conditions for accurate monitoring by CE of protein thermal denaturation

The main thermal characteristics of a CE equipment are the ambient column temperature, heat generation rate and heat dissipation characteristics of the cooling system. Commercial CE apparatus generally fulfill the conditions required for controlling these three characteristics, under current conditions of use, i.e. in the temperature range 15–50°C. Comprehensive works have detailed the theory of heat generation and dissipation in CE [8,9]. The most important for studying the thermal denaturation of proteins is to minimize temperature fluctuations and to accurately measure the temperature. For this purpose, we used an implantable microprobe-thermocouple. This probe enabled to record (every 10 s), and to computerize temperature data.

Thermostating of commercial CE equipments designed for studies at constant temperature in the range 15–50°C, is controlled by using a circulating fluid such as halogenated alkanes, or air. Although air thermostating is as effective as liquid thermostating for current experimental conditions [10], a fluid with a kinetic viscosity/thermal conductivity ratio smaller than that of air results in more effective heat dissipation [9]. Thus, liquid thermostating was thought to provide the best temperature control over a wide temperature range. For the CE analysis of lysozyme unfolding [2,3], it was assumed that temperature fluctuations within the capillary did not exceed 0.2°C, but the location and characteristics of the temperature probe were not mentioned. For the work on ribonuclease, the use of a low pH buffer (2.3) and the properties of the protein were both supposed to provide a transition temperature interval (28–42°C) compatible with the working temperature range of the CE instrument (from ambient to 50°C) [4]. A modified apparatus allowed CZE at subzero temperatures; temperature was measured with a precision of ±0.1°C using a thermocouple plugged at the cartridge outlet [11].

Our experimental device enabled to estimate temperature close to the capillary outside wall within the thermostated cartridge, with a precision of ±0.1°C and with fluctuations during electrophoretic runs less than 0.05°C. This permitted run reproducibility and measurement accuracy. By choosing an intermediate capillary inner diameter (50 μm), separation efficiency and decrease of the temperature drop from buffer to the outside wall of the capillary were balanced. This improved the heat transfer to the cooling system. On the other hand, the design of the cartridge interface provided liquid thermostatization of the entire length of the capillary, from the inlet vial to the detection windows, except 0.5 cm between the buffer meniscus and the vial cap. Finally, the applied voltage (10 kV) together with the moderate–high ionic strength buffer used (100 m*M*) allowed short migration times (lower than 15 min) and applied power values (0.3–0.7 W m^{-1}) in agreement with the theoretical optimum efficiency (1 W m^{-1}).

4.2. CE analysis of thermal denaturation of β-Lg at various pH values

Since the conformational stability of bovine β-Lg has long been investigated [12] and because it has been shown that the unfolding process of this protein is complex, β-Lg was particularly attractive for CE analysis of heat denaturation. Moreover, little is known with regard to its dimeric association and process at neutral pH, since calorimetric studies were preferentially performed at pH$<$3, where formation of high order aggregates do not occur. Thus, limited

information is available on changes occurring at physiological pH values, which represent the common conditions for industrial processing of this protein, and for its biological role.

The present study validates the use of CZE for monitoring protein unfolding of β-Lg in the physiological ranges of pH and protein concentration (pH≈ 6.8, β-Lg concentration ≈3 mg ml^{-1} in bovine milk), and for estimating true thermodynamic parameters of denaturation even in the presence of electroosmosis. The abrupt transition observed for the dependence of mobility of β-Lg with temperature did not allow us to determine the apparent thermodynamic parameters of denaturation, as for lysozyme [2]. Notwithstanding, peak area measurements allowed T_m, ΔH, ΔS and ΔG to be estimated. Indeed, electrophoresis of proteins in slow equilibria allows direct quantitation of population of each form from the area under peaks. Moreover, since it was emphasized that true thermodynamic parameters are obtained by this way even though they are obtained by a van't Hoff analysis [3], the thermodynamic parameters of unfolding determined by our CZE approach represent true parameters. Besides, our approach allowing to perform CZE analysis at different pH provided accurate determination of the heat capacity change (ΔC_p) using the temperature-dependence of the enthalpy change (Eq. 5). Finally, all these values were used to plot the free energy of unfolding (ΔG) as a function of temperature. The stability curves predicted, for example at pH 7.2 and 7.7, both the cold denaturation temperatures of β-Lg B (T'_m= −26.1 and −22.6°C) and the maximal stability temperatures (T_{max}=22.4 and 22.7°C). These values are in agreement with the values obtained by differential scanning calorimetry (DSC) (T'_m=−25°C; T_{max}=20°C) for β-Lg AB at pH 7.5 [13].

The T_m values of β-Lg experimentally determined in the present work (Fig. 6) compare favorably with the literature values [13–15]. The pertinence of our results provided evidence that CE is now a useful tool for studying thermal denaturation processes of proteins under different conditions. The higher thermostability of β-Lg A at protein concentration lower than 25 mg ml^{-1} [16], and the higher resistance to denaturation of individual A and B variants compared with the mixture of both variants were confirmed. In addition, results obtained at pH 2.0 in citrate buffer (data not shown) confirmed the protective effect of phosphate as previously observed [17].

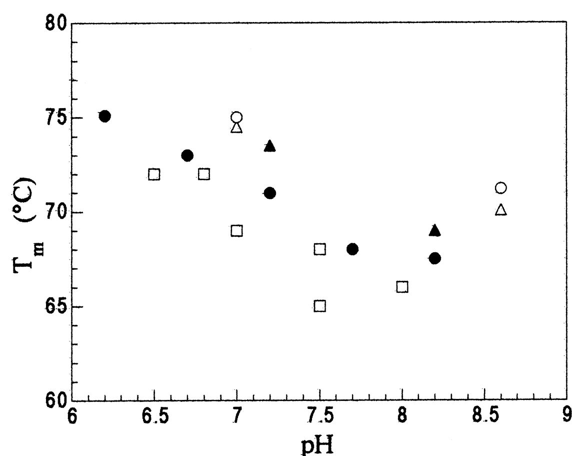

Fig. 6. Denaturation temperature at different pH of β-Lg A (triangles) and β-Lg B (circles) estimated by CE (closed symbols), compared to values in the literature [13–15] obtained by other methods (open symbols; squares correspond to mixture of variants A and B). All determinations were on samples prepared in phosphate buffer.

4.3. Advantages and limitations of CE for analysis of thermal denaturation

Our study of the unfolding of β-Lg induced by temperature and followed by CZE confirms our previous results [18] and demonstrates that this approach can be very fruitful. When comparing the unfolding studies of lysozyme [2], ribonuclease [3] and β-Lg [this work], it is obvious that the more complex the structure of the protein, the more abundant are the data obtained by CE. However, interpretation of the CE data becomes more difficult. β-Lg displays various structural changes depending on protein concentration, solvent composition, pH and temperature. Some of them are described in Fig. 7.

In addition to its complex dissociation/unfolding processes β-Lg is particularly resistant to heat and pH denaturation. Notwithstanding, our CZE approach allows to investigate almost all heat-induced structural changes in a wide range of pH (1–11) and temperature (4–95°C). These results show the potential use of the CZE analysis in this field. Electro-

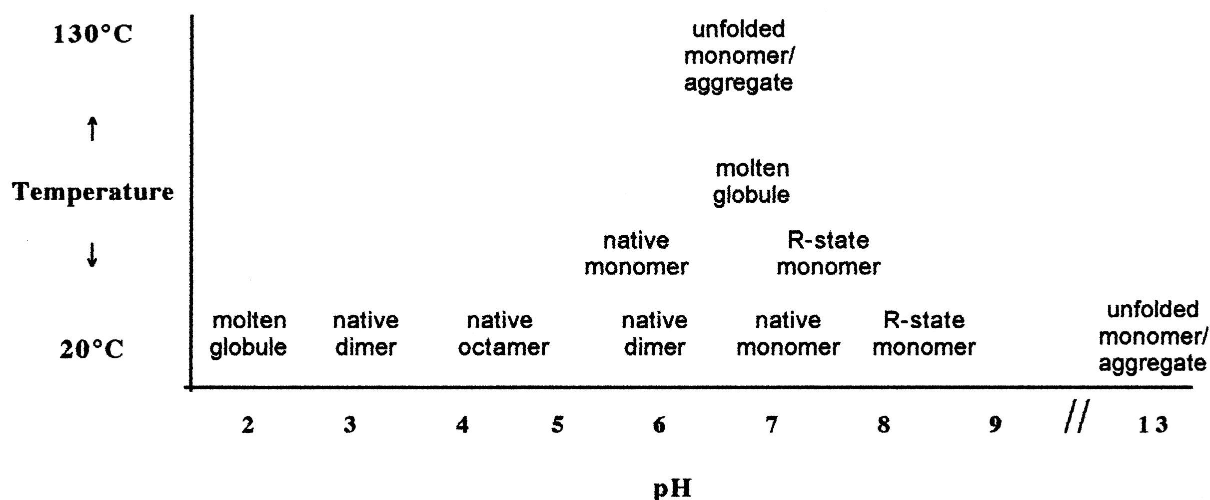

Fig. 7. Schematic representation of the pH- and temperature-dependence of the association/dissociation/unfolding balances in the quaternary structure of β-Lg.

phoretic runs in a wide range of very accurately controlled temperature were performed at low cost improvement of a commercial CE apparatus. T_m, ΔH and ΔS could have been determined. Besides, it was possible to determine the free energy change (ΔG) through its temperature-dependence, using a modified form of the Gibbs-Helmholtz relation (Eq. 4). This was done by estimating the change in heat capacity for unfolding, (ΔC_p) by the Kirchoff relation (Eq. 5) using thermodynamic parameters obtained by analysis of thermo-electropherograms. To date, as far as we are aware of, this result is the first description of determination of ΔC_p using CZE. It should be remembered that ΔC_p plays a central role in the energetics of protein stabilization [19].

The parameter introduced in CE analysis of thermally induced unfolding of protein is the temperature variation from run to run. Finally, the limitation of the applicability of CE analysis could be only inherent to the cooperativity of protein denaturation. Besides, although the characterization of protein populations that are only detected by their UV absorbance and their migration time remains a constant limitation of the CE analysis, the use of a diode array detector allowing spectral analysis of molecular states separated by CZE, could provide more information.

5. Conclusion

Usually known as an analytical separation technique, capillary zone electrophoresis can also be used to determine the thermodynamic parameters of thermal unfolding of proteins under different pH conditions. The ability to perform this type of analysis on minute amounts of samples (3–4 orders of magnitude lower than that needed for DSC analysis), should facilitate the exploration of the folding/unfolding processes of proteins of pharmaceutical or biological interest. Obvious applications are to determine the stability of mutant or recombinant proteins, or of proteins in different conditions of solvent or stabilizers. There is a need to estimate stability differences between natural and recombinant proteins and stability of engineered proteins. The results obtained with an oligomeric protein model displaying complex dissociation/unfolding processes suggest that CZE can become a technique of choice for studying protein denaturation.

Acknowledgements

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