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The application of capillary electrophoresis for monitoring effects of excipients on protein conformation[1]

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Abstract

Studies were conducted to assess the utility of free solution capillary electrophoresis (CE) for monitoring the effects of selected excipients on the thermal denaturation of a model protein (Ribonuclease A, RNase A) at low pH. Thermal denaturation/unfolding experiments were conducted via temperature-controlled CE using a run buffer of 20 mM citric acid in the pH range of 2.3–3.1, with a marker peptide incorporated to correct for temperature-induced changes in endoosmotic flow. The effects of selected excipients on the thermal unfolding of RNase A were then evaluated by adding either sorbitol, sucrose, polyethylene glycol 400 (PEG 400) or 2-methyl-2,4-pentanediol (MPD) to the electrophoretic run buffer (pH 2.3). Confirmatory denaturation experiments were conducted under the same solution conditions using circular dichroism (CD) spectropolarimetry. Using temperature-controlled CE, an increase in solution pH from 2.3 to 2.7 and 3.1 resulted in an increase in transition temperatures of RNase A by approximately 8 and 13°C, respectively. Similar shifts in transition temperatures were observed when thermal denaturation transitions were monitored by far-UV CD. Sorbitol (0.55–1.1 M) and sucrose (0.55 M) each shifted the denaturation transition temperatures of RNase A to higher values, whereas PEG 400 and MPD had minimal effect on the unfolding transition midpoint at the concentrations evaluated (0.55 M for each). The observed changes in the transition temperatures for RNase A as a function of pH and selected excipients were similar when measured by either CE or far-UV CD. These results support the utility of CE for monitoring the effects of neutral excipients on the thermal denaturation of a model protein under selected conditions. The widespread utility of the technique may be limited by the narrow temperature range of most commercial CE instruments and the need to use extreme pH conditions to monitor the complete denaturation transition.

Keywords: Ribonuclease A; Capillary electrophoresis; Excipients; Thermal unfolding

1. Introduction

Excipients are routinely incorporated into protein pharmaceuticals to enhance the stability of solution formulations or lyophilized preparations. Albumin, amino acids, surfactants, polyhydric alcohols, carbohydrates and fatty acids are

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examples of excipients which have been used to enhance the physical stability of proteins [1]. An understanding of protein stability, and the effects of added excipients on conformational transitions, is essential for the successful formulation of protein drugs. In the case of solution preparations, it is particularly important to investigate temperature-dependent changes in conformation as elevated temperatures are often used when screening the stability of potential formulations. Therefore, it is necessary to have independent analytical methods available which are capable of monitoring changes in protein conformation in the presence of excipients.

Spectroscopic methods, such as circular dichroism spectropolarimetry, UV and fluorescence spectroscopy, are the most common techniques used to monitor protein unfolding transitions. The advantages of these techniques include sensitivity, small sample requirements, and a non-destructive method of analysis. Since spectroscopic techniques rely upon changes in the molecular environment of chromophoric groups upon denaturation, these methods do not allow direct measurement of the energetics of protein unfolding transitions. In contrast, calorimetry allows direct determination of the thermodynamic parameters associated with protein folding/unfolding transitions and is preferred over spectroscopy for this purpose.

Free solution capillary electrophoresis (CE) has recently been proposed as an additional independent method for characterising protein unfolding transitions [2]. In these studies, the thermal unfolding of lysozyme at low pH was measured by CE using a heptapeptide to correct for viscosity-related changes in the electroosmotic flow. Thermodynamic parameters associated with unfolding of lysozyme under these conditions were comparable when the transition was monitored by either CE or microcalorimetry.

The objective of the current study was to extend the application of CE as a novel analytical method for assessing excipient-induced changes in the thermal denaturation profile of a model protein, ribonuclease A (RNase A). In order to examine the effect of structural changes on electrophoretic mobility with increasing temperature, a marker peptide was used to correct for temperature-dependent viscosity changes in the run buffer [2]. A low pH buffer was required to ensure that the unfolding transition of RNase A occurred within the temperature range of the available CE instrument (approximately 18–50°C). The unfolding transition was initially assessed by CE over the pH range of 2.3–3.1, after which the results were compared to those obtained by far-UV CD. Selected neutral excipients, including sorbitol, sucrose, PEG 400, and MPD, were then individually added to the electrophoretic run buffer to determine their effect on the thermal unfolding profile.

2. Materials and methods

2.1. Materials

Ribonuclease A (type III-A) was obtained from Sigma Chemicals (St. Louis, MO) and used without further purification. The CE endoosmotic flow marker was the heptapeptide Arg-Lys-Arg-Ser-Arg-Lys-Glu (Perkin Elmer, Norwalk, CT). All buffers and solutions were prepared using water from a Milli-Q water purification system (Millipore, Bedford, MA) and filtered through 0.2 μm cellulose acetate microcentrifuge filters (Lida Corp., Kenosha, WI) prior to use. Buffer reagents and excipients were of analytical grade.

2.2. Solution preparation

RNase A solutions were prepared fresh on the day of each experiment. The concentration of RNase A stock solutions was determined by the absorbance at 278 nm using an extinction coefficient of 9800 M^{-1} cm^{-1} [3]. A citrate buffer, consisting of 20 mM citric acid adjusted to pH 2.3, 2.7, or 3.1 with HCl or NaOH (after the addition of excipients), was used for all CE and CD experiments.

2.3. Capillary electrophoresis

CE studies were conducted using a Beckman P/ACE System 2100 HPCE equipped with a UV detector and liquid temperature control. The CE

was operated in the 'normal' polarity mode with the negative electrode at the detector (outlet) end of the capillary. A 57 cm (50 cm to detector) fused silica capillary with an internal diameter of 75 μm was used throughout these studies. Peak detection was carried out by monitoring the UV absorbance at 200 nm. The temperature of the liquid coolant was measured by placing a calibrated thermometer into the coolant reservoir, and a calibration curve was constructed to relate the measured temperature to that displayed on the instrument readout. The resulting temperature correction was applied to all CE data. For the purpose of these studies, it was assumed that the difference in temperature between the inside of the capillary and the surrounding liquid coolant was minimal [4].

Samples for CE analysis consisted of a mixture of RNase A (~ 200 μg ml^{-1}) and marker peptide (~ 70 μg ml^{-1}) and were prepared in electrophoretic run buffer. Samples were maintained at ambient temperature in the sample carousel prior to injection. Electrophoresis was performed at 16 different temperatures, increasing by 2°C between each injection over the temperature range of approximately 20–50°C. Prior to each sample injection, the capillary was rinsed for 0.25 min with 0.1 M HCl followed by a 2 min rinse with run buffer. A waiting period was incorporated to allow the capillary to reach the designated temperature. A 10 min voltage separation (15 kV) was then conducted without sample introduction to further ensure temperature equilibration prior to sample injection. The RNase A sample was then introduced into the capillary with a 5 s pressure injection, and electrophoresis was conducted with an applied voltage of 15 kV.

The apparent mobilities for RNase A and the marker peptide were calculated using the following equation:

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

where μ_{app} is the apparent electrophoretic mobility (cm^2/V.s), 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_r 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. Since the endoosmotic flow is constant for a given temperature and solution condition, the difference in the true mobilities between the marker and protein is equal to the difference in their apparent mobilities. Therefore, the difference in apparent mobilities ($\Delta\mu$) was calculated to account for temperature (viscosity) associated changes in the endoosmotic flow as follows:

$$\Delta\mu = \mu_{app}(\text{peptide}) - \mu_{app}(\text{RNaseA}) \quad (3)$$

Individual values for $\Delta\mu$ were plotted against temperature to determine the unfolding transition profile for RNase A under the different solution conditions.

2.4. Circular dichroism spectropolarimetry

CD studies were conducted using a JASCO J-710 Spectropolarimeter (Japan Spectroscopic Company, Tokyo) calibrated with ammonium *d*-camphorsulfonate. Sample temperature was controlled using a Neslab RTE 111 (Newington, NH) recirculating water bath with jacketed, stoppered cylindrical quartz CD cells. Cell temperatures were calibrated using a thermistor probe placed directly in the sample cell. The far-UV CD spectra were recorded from 250 nm to 200 nm using a 0.1 cm quartz cell. Spectra represented the average of 4 accumulations obtained with 0.1 nm resolution and a 20 nm/min scan speed. A response time of 1 s was used with a sensitivity of 50 mdeg and a band width of 1 nm.

The solution conditions used to obtain the CD thermal unfolding data were identical to those used for the CE experiments. Spectra were acquired over the temperature range of approximately 20–70°C in 3–4°C increments with a 10 min equilibration time at each temperature. A buffer baseline spectrum (at ambient temperature) was subtracted from each RNase A spectrum and noise reduction calculations were performed using the J710 processing software. Molar ellipticity ($[\theta]$, deg cm^2 $dmole^{-1}$) values were calculated using a mean residue weight of 110.48. Ellipticity values at 222 nm were plotted against temperature to determine the unfolding transition of RNase A.

2.5. Analysis of thermal unfolding profiles

Analysis of the unfolding transition of RNase A was conducted assuming a two-state transition mechanism. The fraction of protein unfolded (f_U) at each temperature was calculated as:

$$f_U = (y_F - y)/(y_F - y_U) \quad (4)$$

where y is the observed signal (either $\Delta\mu$ or molar ellipticity) at a given temperature and y_F and y_U are signals characteristic of the folded (F) and unfolded (U) protein, respectively. Data for y_F and y_U were obtained by linear regression of data in the pre- and post-transition regions of the profiles, respectively, as previously described by Shirley [5]. Fraction unfolded versus temperature profiles were fit using a non-linear curve fitting program (SigmaPlot, Jandel Scientific, San Rafael, CA) to a general sigmoidal expression of the form:

$$f(x) = \frac{a}{1 + \exp[-b(x-c)]} + d \quad (5)$$

where a and d are the asymptotic maximum and minimum values, respectively, b is the slope coefficient, and c is the transition midpoint (T_m).

3. Results and discussion

The electrophoretic mobility (μ) of a protein is directly related to the Stokes radius (r) and net charge (q) according to the following relationship:

$$\mu = q/6\,\pi\, r\, \eta \quad (6)$$

where η is the solvent viscosity. Therefore, thermally induced structural changes in protein molecules give rise to changes in electrophoretic mobility based upon the relative changes in the size and charge characteristics which occur upon unfolding.

The thermal unfolding profile of RNase A at low pH has been extensively characterized and shown to proceed via a reversible, 2-state transition [6,7]. While some reports have indicated the presence of residual secondary structure in thermally unfolded RNase A [8], a recent study using carefully controlled conditions indicated the absence of regions of native-like secondary structure in the heat denatured state [9]. Calorimetric data have also shown the population of intermediates to be less than 5% at any temperature [3].

3.1. Thermal unfolding of RNase A monitored by capillary electrophoresis

To assess the thermal unfolding of RNase A by CE, a low pH buffer was required for the complete unfolding transition to be assessed within the temperature range of the available CE instrument (ambient through 50°C). Fig. 1 displays representative electropherograms for the marker peptide (first eluting peak) and RNase A (second peak) obtained using a run buffer at pH 2.3. With increasing temperature, the electrophoretic mobility of each species increased reflecting a decrease in solvent viscosity. The increase in peak height and decrease in peak width with increasing capillary temperature were likely to be due to the combined effect of reduced band broadening with earlier elution of the peaks and increased injection volumes with increasing temperature resulting from reduced sample viscosity [10].

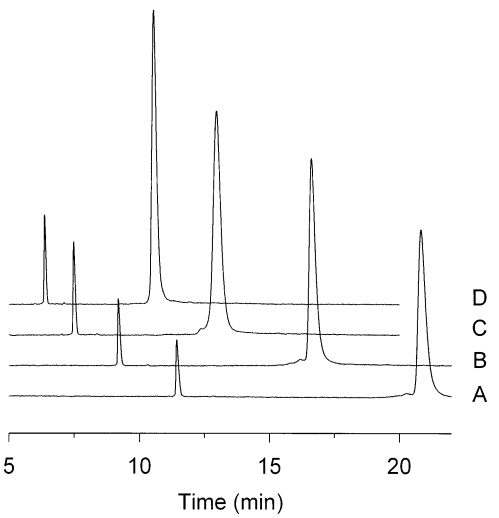

Fig. 1. Representative electropherograms for RNase A at (A) 21.2°C, (B) 31.7°C, (C) 42.1°C, and (D) 52.5°C. The sample and run buffer was 20 mM citric acid, pH 2.3 and electrophoresis was conducted at 15 kV. The first peak corresponds to the marker peptide and the second peak to RNase A.

The apparent mobility of the marker peptide increased linearly with increasing temperature under all conditions consistent with previous results using the same marker peptide [2]. Although the apparent mobility of RNase A also increased with temperature, the slope of the line in the pre-transition region was less than that observed for the marker peptide. Based on Eq. (6), this trend is consistent with the larger size of the protein relative to the peptide. In the post-transition region, the mobility of RNase A was greater than at lower temperature with the individual data values at temperatures greater than approximately 38°C lying above the extrapolated line from the pre-transition region (not shown). This observed trend was opposite to that predicted based upon an expected increase in the Stokes radius with unfolding resulting in a relative decrease in mobility. Since the electrophoretic mobility of a protein reflects the contribution of both charge and size characteristics (Eq. (6)), a net increase in the mobility of RNase A in the post-transition region most likely indicates that unfolding resulted in an increase in overall positive charge due to protonation of residues which were largely 'buried' in the folded state.

The $\Delta\mu$ data for RNase A, calculated according to Eq. (3), at pH 2.3 resulted in a sigmoidally-shaped protein unfolding transition when plotted as a function of temperature (Fig. 2). The profile was characterized by linear pre- and post-transition regions reflecting the mobility characteristics of the folded and unfolded protein species, respectively. In the transition region (approximately 28–42°C), the non-linearity was attributed to changes in the size and charge characteristics which occurred upon unfolding.

Fig. 3 represents the fraction unfolded (f_U) of RNase A plotted as a function of temperature for RNase A at pH 2.3, 2.7, and 3.1. An increase in pH from 2.3 to 2.7 and 3.1 led to an increase in the transition temperature (T_m) from 35.7 to 43.7°C and approximately 48.7°C, respectively. The transition observed at pH 3.1 was shifted to a sufficiently high temperature that the linear post-transition region could not be accurately defined. Under these conditions, y_U was estimated using the slope for the pre-transition region since pre- and post-transition slopes were similar for pH 2.3 and 2.7.

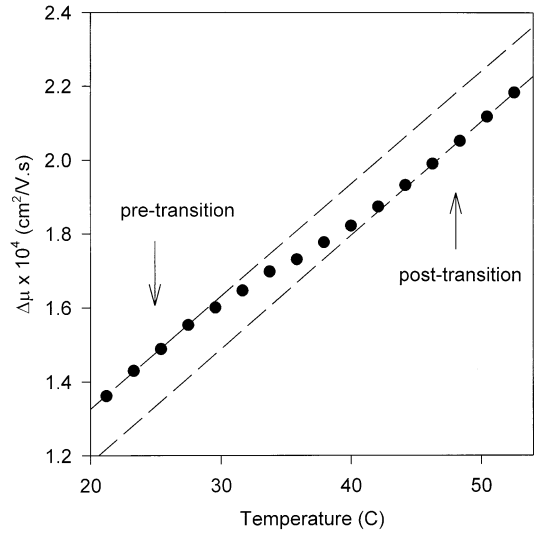

Fig. 2. Effect of temperature on the difference in electrophoretic mobility ($\Delta\mu$) between the marker peptide and RNase A. Data were obtained by CE using 20 mM citric acid (pH 2.3) as the sample and run buffer.

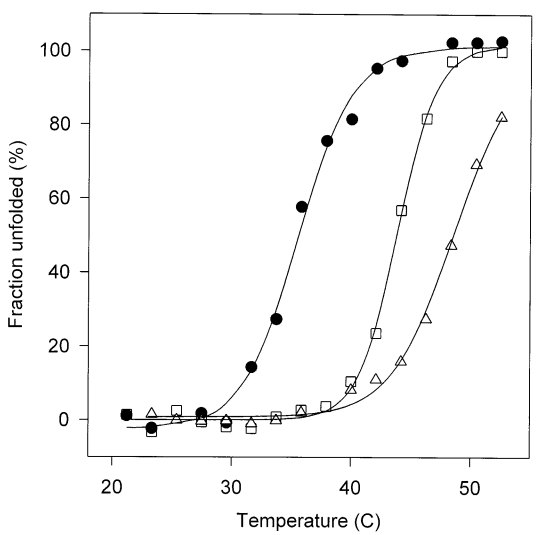

Fig. 3. Thermal unfolding profiles for RNase A determined by CE at pH 2.3 (●), pH 2.7 (□), and pH 3.1 (△). Symbols represent data points and the solid lines represent the lines of best fit to a sigmoidal equation.

Table 1
Estimated transition temperatures (°C), and changes in transition temperatures (ΔT_m), for the thermal unfolding of RNase A under various solution conditions determined from capillary electrophoresis (CE) and Far-UV circular dichroism (CD) experiments[a]

Solution conditions	Transition temperature (T_m,°C)		Change in transition temperature (ΔT_m,°C)[b]	
	CE	Far-UV CD	CE	Far-UV CD
pH 2.3	35.5 ± 0.3	38.7 ± 0.2	–	–
0.55 M sorbitol	40.5 ± 0.4	43.7 ± 0.2	5.0	5.0
1.1 M sorbitol	43.2 ± 0.1	46.0 ± 0.2	7.7	7.3
0.55 M sucrose	44.9 ± 0.1	47.0 ± 0.2	9.4	8.3
0.55 M PEG 400	36.1 ± 0.1	38.6 ± 0.2	0.6	−0.1
0.55 M MPD	36.0 ± 0.2	40.1 ± 0.1	0.5	1.4
pH 2.7	43.9 ± 0.1	46.3 ± 0.2	8.4	7.6
pH 3.1	48.6 ± 0.5[c]	49.9 ± 0.1	13.1	11.2

[a]Transition temperatures and corresponding standard errors were estimated by non-linear least squares curve fitting.
[b]ΔT_m is the difference between the measured T_m value and the reference value at pH 2.3.
[c]Value estimated as described in the text.

When the unfolding experiment at pH 2.3 was conducted in triplicate to estimate the reproducibility of the method, the mean ($\pm$S.D.) T_m value for three separate experiments was 35.5 ± 0.6°C. The variability was most likely due to temperature differences within the capillary between the different runs. The transition temperature at pH 2.3 was also measured using an applied voltage of 10 kV to increase the migration time and therefore increase the time of exposure of RNase A to elevated temperature. Under these conditions, the measured T_m was 34.8°C which compared within error to the results obtained at 15 kV. All further experiments were conducted using an applied voltage of 15 kV. Table 1 presents the T_m values for RNase A at pH 2.3, 2.7, and 3.1 determined by CE. The data compare favorably to literature values determined using other independent techniques [11–13].

3.2. Thermal unfolding of RNase A monitored by circular dichroism spectropolarimetry

Circular dichroism spectropolarimetry was used as a confirmatory method for assessing the thermal unfolding profile of RNase A under solution conditions identical to those employed in the CE experiments. The thermal transition of RNase A under these conditions was completely reversible when the sample was cooled to room temperature and the spectrum re-scanned. The transition temperatures measured by far-UV CD at pH 2.3, 2.7, and 3.1 are also presented in Table 1.

The transition temperatures determined by CD were consistently 2–3°C higher than those measured by CE. Near-UV CD analysis of the thermal transition gave results similar to those obtained in the far-UV region suggesting that the difference between the CD and CE results was not due to population of intermediate states. The basis for the observed difference in transition temperatures measured by CE and CD has not been fully defined, although it may result from differences in temperature calibration between the different instruments. Although the temperature of the coolant reservoir of the CE was measured using a calibrated thermometer, the temperature difference between the capillary cartridge and reservoir (two points separated by approximately 30–40 cm tubing) was not known and could not be readily measured. A temperature drop between the capillary and coolant reservoir would indicate that the actual temperature within the capillary would have been higher than the expected temperature, thereby accounting for the observed differences in transition temperatures measured by the two methods.

Transition temperature data obtained by CE and CD, as a function of solution pH or added excipient, were analysed as a change in individual

transition temperatures (ΔT_m) using the transition temperature at pH 2.3 as a reference value. The similarity in calculated values for the ΔT_m data obtained from the CE and CD methods demonstrated the utility of CE for assessing relative changes in thermal stability.

3.3. Excipient effects on the thermal unfolding of RNase A

Sugars and polyhydric alcohols are well known for their effects on protein structural stability [14]. The stabilising effect of many sugars and polyols has been attributed to preferential exclusion of the additive from the immediate solvent domain of the protein which thermodynamically favors the folded conformation ([14], and references therein). Stabilisation of the folded conformation of a protein is manifested as an increase in the transition temperature associated with unfolding. Selected polyols, such as MPD and PEG, have been shown to specifically interact (e.g., bind) with the denatured state of several proteins even though they are preferentially excluded from the native state [15]. Under these circumstances, MPD and PEG are classified as destabilising excipients which lower the unfolding transition temperature.

To investigate the potential utility of CE for studying excipient effects on protein stability, quantities of sorbitol, sucrose, MPD and PEG 400 were directly incorporated in the electrophoretic run buffer. In the presence of either 0.55 M sucrose, 0.55 M PEG 400, or 1.1 M sorbitol, the apparent electrophoretic mobilities of the marker peptide and RNase A were significantly reduced due to the large increase in the buffer viscosity in the presence of these agents. The stabilising effect of sorbitol on the thermal unfolding transition of RNase A is demonstrated by the data shown in Fig. 4. The T_m increased from 35.5°C in buffer to 39.9 and 40.5°C in 0.55 and 1.1 M sorbitol, respectively. The increase in T_m determined by CE, relative to that determined at pH 2.3 in the absence of sorbitol, was similar to the relative increase determined by CD analysis (see Table 1). These results are consistent with previous reports in the literature which indicated a 5°C increase in the T_m of RNase A in the presence of 0.55 M sorbitol and a 7–8°C increase in the presence of 1.4 M sorbitol when unfolding was conducted at pH 2.3 [12].

Fig. 5 compares the effect of equimolar concentrations (0.55 M) of sorbitol, sucrose, PEG 400, and MPD on the thermal unfolding of RNAse, and the resulting transition temperatures for each of the buffer systems are shown in Table 1. Sucrose had the greatest stabilising effect resulting in an increase in the T_m value by 9.4°C relative to buffer. At a concentration of 0.55 M, neither PEG 400 nor MPD notably affected the thermal unfolding transition temperature of RNase A.

MPD has previously been shown to lower the T_m of RNase A, although under different solution conditions than employed in this study. At pH 5.8, 2.5 M MPD resulted in a reduction in T_m from 59°C in buffer to 49°C [15]. At concentrations ranging from 0.1 to 0.3 M, PEG 1000 was found to have minimal effect on the T_m of RNase A at pH 2.9 [16]. Both PEG and MPD are known to be strongly repelled from charged residues [16,17] resulting in their preferential exclusion from the folded structure of most proteins. At the same time, PEG and MPD each have a high

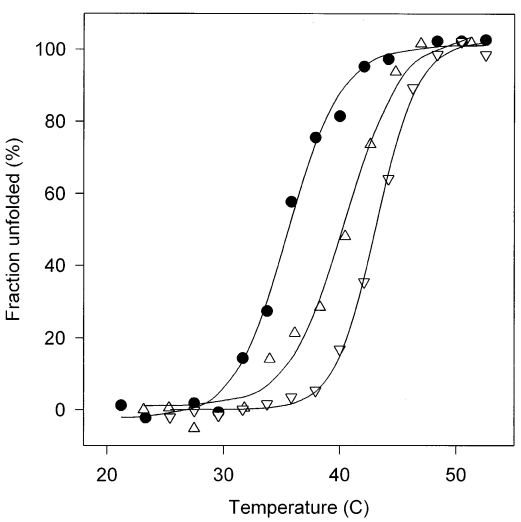

Fig. 4. Thermal unfolding profiles for RNase A determined by CE in 20 mM citric acid, pH 2.3 containing 0 M (●), 0.55 M (△), and 1.1 M (▽) sorbitol. Symbols represent data points and the solid lines represent the lines of best fit to a sigmoidal equation.

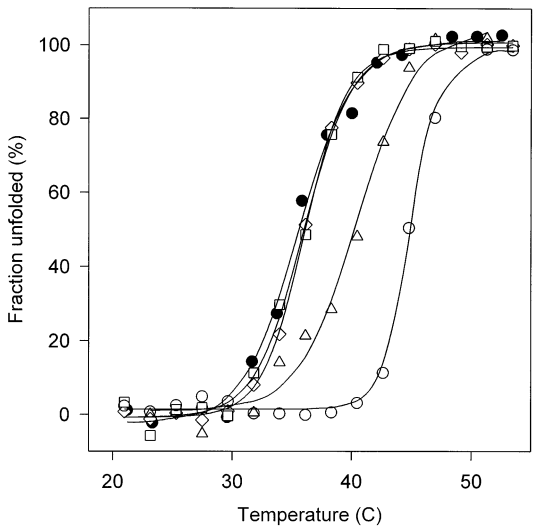

Fig. 5. Thermal unfolding profiles for RNase A determined by CE in 20 mM citric acid, pH 2.3 (●), and buffer containing 0.55 M sorbitol (△), 0.55 M PEG 400 (□), 0.55 M MPD (◇), and 0.55 M sucrose (○). Symbols represent data points and the solid lines represent the lines of best fit to a sigmoidal equation.

affinity for nonpolar residues [16] and in many cases, are structure destabilising due to binding with nonpolar regions exposed in the denatured state. Specific binding interactions of this nature are highly sensitive to the surface charge characteristics of the protein which vary with changes in solution pH. The lack of a significant destabilising effect of PEG 400 and MPD on the unfolding transition of RNase A at pH 2.3 is likely to reflect the absence of a significant interaction with the unfolded protein due to charge repulsion at low pH.

In studying excipient effects on protein conformational stability, it is preferable to determine thermodynamic parameters associated with the unfolding transition as changes in stability may not be adequately concluded on the basis of transition midpoints alone. In the case of RNase A transitions monitored by CE, the steepness of the transition region (which reflects the entropy and enthalpy of unfolding) was found to be highly sensitive to the assignment of the pre- and post-transition regions due to the small differences in $\Delta\mu$ values for the folded and unfolded species. In several of the buffer systems studied, only 3–4 points were available for assignment of the post-transition region since the transition occurred in the upper temperature range of the instrument. For that reason, no attempt was made to derive thermodynamic parameters from the unfolding profiles determined by CE. Since the transition midpoint of the unfolding profiles did not show this same sensitivity, T_m values were used to assess the relative effects of different excipients.

3.4. Limitations of capillary electrophoresis for assessing thermal unfolding profiles

The utility of CE in studies of this nature is restricted to the use of neutral excipients or very low concentrations of charged excipients in order to avoid excessively high background currents. Another limitation in the use of CE to examine thermal unfolding of proteins is the temperature range over which the unfolding transition can be monitored. Most commercially available CE instruments have a relatively narrow temperature operation range and have been designed to maintain a constant temperature rather than to accurately vary the temperature. CE instruments which are thermostatted by forced air convection are capable of operating over a much wider temperature range providing greater flexibility in the choice of solution conditions, but have the disadvantage of high temperature differences between the capillary and the environment due to less efficient heat dissipation [4]. In the present studies, low pH buffers were incorporated to ensure that the unfolding transition of RNase A occurred within the temperature range of ambient to 50°C. The use of pH extremes to study excipient effects is common and can yield valuable information regarding the rank order effectiveness at a more neutral pH, provided the excipient does not interact with the protein surface and therefore, is not sensitive to changes in protein surface charge. The use of low pH run buffers has the advantage over higher pH conditions of minimising the endoosmotic flow due to the decrease in charge on the surface of the capillary. The decrease in capillary surface charge may also serve to reduce potential adsorption of some proteins onto the capillary wall.

In conclusion, the utility of CE for monitoring excipient-induced changes in protein structural stability has been demonstrated using RNase A as a model protein, with the effects confirmed using CD spectropolarimetry. Although the addition of CE to the range of available techniques provides another independent approach for the evaluation of excipient effects on protein physical stability, there are limitations associated with the range of temperatures and conditions over which the studies can be conducted. The ability to derive thermodynamic parameters from CE-based unfolding profiles is likely to be protein specific and will depend upon the accuracy with which the pre- and post-transiton regions can be determined, the temperature range of the transition, and the difference between the mobilities for the folded and unfolded species.

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