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Journal of Chromatography A, 838 (1999) 131–138

JOURNAL OF CHROMATOGRAPHY A

Protein folding observed by capillary electrophoresis in isoelectric buffers

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Abstract

Capillary zone electrophoresis measurements in acidic isoelectric buffers provide a sensitive and rapid method for comparison of the folding and stability of wild type, mutant or post-translationally modified proteins. The potential of the method is illustrated using the small globular protein cytochrome *c*.

Keywords: Protein folding; Buffer composition; Proteins; Cytochromes

1. Introduction

The ability of a newly synthesized polypeptide chain to spontaneously fold into its biologically functional conformation is an important attribute of proteins. Unfortunately, polypeptide chains can also fold into alternative nonfunctional conformations or aggregates, such as inclusion bodies, depending upon the integrity of the polypeptide chain and upon the folding conditions. This report describes a rapid sensitive assay of protein folding using capillary zone electrophoresis (CZE) which can be used to screen polypeptide chains and folding conditions for their ability to generate compactly folded functional proteins.

The linear velocity of a protein in a unit electric field is termed its electrophoretic mobility, μ. The electrophoretic mobility of a protein is directly dependent upon the net charge of the protein, Z, and inversely dependent upon the hydrodynamic size of the protein, as described by its frictional coefficient, f, and upon the viscosity of the solvent through which the protein moves, η, as indicated by the equation $\mu = Z/f\eta$. Folding a polypeptide chain into its compact biofunctional conformation will decrease the frictional coefficient of the polypeptide chain and may change its net charge if one or more of its ionic groups acquire abnormal pK values in the folded conformation. Accordingly, the electrophoretic mobility of a unfolded polypeptide chain will likely [1,2], though not necessarily [3], increase upon folding into a compact globular conformation.

Rapid measurement of protein electrophoretic mobility by CZE is complicated by two problems, electroosmotic flow (EOF) and Joule heating. EOF results from the negatively charged silanol groups on the capillary wall. The hydrated, positively charged counterions in the diffuse double layer migrate toward the anode when the electric field is applied, creating a net flow of solvent which perturbs the intrinsic mobility of a protein. This perturbation can be minimized either by using a coated capillary

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0021-9673/99/$ – see front matter
PII: S0021-9673(98)01048-6

whose charged groups have been masked or by using an uncoated capillary and acidic buffers in which the silanol groups are uncharged. The latter option is considerably less expensive and more permanent.

Joule heating results from the current developed in ionic buffers by high electric fields. Failure to dissipate this heating results in measurement of an apparent mobility whose value is larger than the intrinsic mobility, owing to the decrease in the viscosity of the aqueous solvents with increasing temperature. Joule heating can be minimized by reducing the ionic strength of the buffer solution in the capillary. Unfortunately, this often results in a significant loss in buffering capacity. One solution to this dilemma is to employ an isoelectric buffer which can maintain significant buffering capacity in solutions of low ionic strength [4].

In this report we utilize isoelectric aspartic acid, denoted as Asp, as the buffer for CZE. The pH of this isoelectric buffer ranges from 3.36 to 2.80 as the concentration of Asp is increased from 5 m*M* to 50 m*M* at 25°C [4]. Cytochrome *c* is used as a representative globular protein because its unfolded polypeptide chain is known to rapidly and quantitatively refold in acidic solvents [5]. A few control measurements were performed using alternative small globular proteins, lysozyme, ribonuclease and thioredoxin.

2. Experimental

2.1. Protein samples

Purified lyophilized horse heart ferricytochrome *c*, bovine pancreatic ribonuclease and egg white lysozyme were purchased from Sigma, St. Louis, MO, USA. Purified *E. coli* thioredoxin was purchased from MBI Fermentas, Buffalo, NY, USA. All protein solutions contained about 2.5 mg/ml protein unless noted otherwise.

2.2. Electrophoresis

CZE was performed using a BioFocus 3000 system purchased from Bio-Rad Labs., Hercules, CA, USA. Routine measurements were obtained using a 50 μm (inner diameter) uncoated capillary having a total length, L_t, of 24.0 cm and a length to the observation window, L_d, of 19.6 cm. Protein samples were routinely injected into the capillary for 1 s using 0.069 mbar pressure. Electrophoresis was routinely performed in a capillary containing 40 m*M* Asp using a constant electric field of 10 kV. The capillary was thermostatted at a selected temperature between 15 and 65°C by a circulating fluid. These conditions generated a current which ranged from 7 to 12 μA, dependent upon the thermostatted temperature, during electrophoresis.

Electropherograms were obtained by continuous monitoring of 214 nm absorbance at the observation window. Representative electropherograms for cytochrome *c* obtained using conditions described in this report are illustrated in Fig. 1. The apparent mobility of a protein, μ_{app}, having the units $cm^2\ (V\ s)^{-1}$, was calculated using the relationship $\mu_{app}=L_tL_d/tV$, where t is the migration time of the maximum ordinate in seconds and V is the applied constant

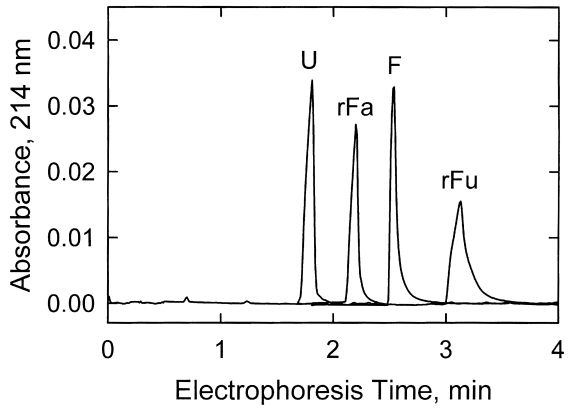

Fig. 1. Representative electropherograms of cytochrome *c*. This figure is a superposition of four individual measurements, each obtained by injection of protein into a capillary containing 40 m*M* Asp. The peaks labeled F and U were obtained in dynamic unfolding measurements initiated by injection of an aliquot of folded cytochrome *c*. The peak labeled F represents the peak for the folded protein observed in the capillary thermostatted at 15°C. The peak labeled U represents the peak for the unfolded protein generated in the capillary thermostatted at 65°C. The peaks labeled rFa and rFu were obtained in dynamic refolding measurements initiated by injection of unfolded cytochrome *c* into the capillary thermostatted at 25°C. The peak labeled rFa represents the peak for the refolded protein generated in the capillary following injection of cytochrome *c* unfolded in 10 m*M* HCl. The peak labeled rFu represents the peak for the refolded protein generated in the capillary following injection of cytochrome *c* unfolded in 20 m*M* Asp containing 6 *M* urea.

electric field in volts. Apparent mobility measurements were reproducible within $0.03 \cdot 10^{-4}$ cm^2 $(V\ s)^{-1}$. All apparent mobility measurements represent the average of at least two successive measurements.

3. Results

3.1. Joule heating

If an isoelectric buffer is effective in reducing Joule heating, then the observed mobility for a protein should be relatively independent of the applied electric field and of the diameter of the capillary employed. A test of this premise is illustrated in Fig. 2, using folded ribonuclease as a representative protein and 40 m*M* Asp as a representative isoelectric buffer, having a pH of 2.85, and a thermostatted temperature of 25°C. Panel A of Fig. 2 indicates that the apparent mobility of ribonuclease is increased by Joule heating as the electric field or the capillary diameter is increased. Panel B indicates that these variables combine to generate a common dependence of the apparent mobility on the electrophoretic power, the product of the applied electric field and the current generated. These results indicate that the apparent mobility measured using a field of 10 kV and a 50 μm capillary is less than 10% larger than the intrinsic mobility. These conditions were routinely employed in the experimentation described in this report.

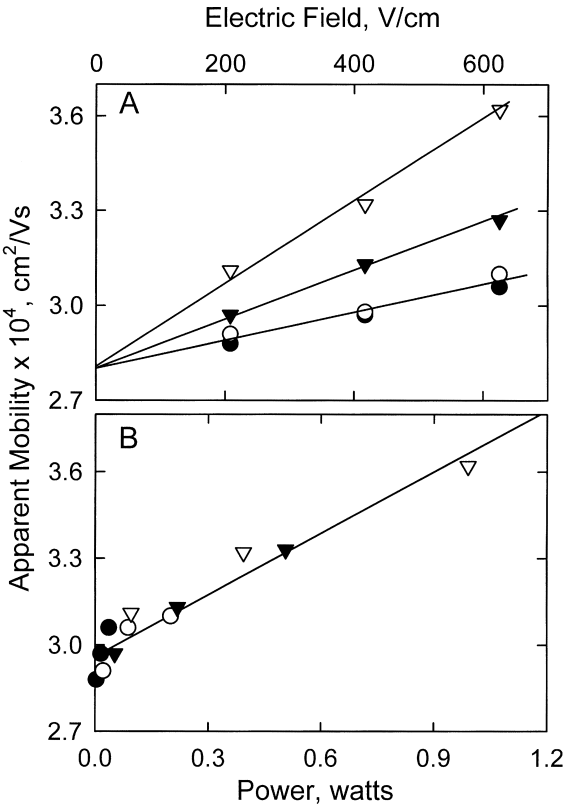

Fig. 2. The effect of Joule heating on the electrophoretic mobility of folded ribonuclease measured in 40 m*M* Asp maintained at 25°C. The filled circles, open circles, filled triangles and open triangles indicate measurements obtained using uncoated capillaries having diameters of 25 μm, 50 μm, 75 μm and 100 μm, respectively.

3.2. Electroosmotic flow

EOF was measured in a 50 μm uncoated capillary using non-ionic acrylamide as the sample, an electric field of 10 kV and a thermostatted temperature of 25°C. Acrylamide was detected in the observation window after 6.35 min of electrophoresis in 40 m*M* Tris–acetate buffer, pH 8.0. This migration time corresponds to an apparent mobility of $1.23 \cdot 10^{-4}$ cm^2 $(V\ s)^{-1}$, comparable to that exhibited by proteins. By contrast, no acrylamide was detected in the observation window after 110 min of electrophoresis in 40 m*M* Asp, pH 2.85, or in 20 m*M* Asp containing 6 *M* urea, pH 3.88. This indicates that EOF contributes an apparent mobility of less than $0.07 \cdot 10^{-4}$ cm^2 $(V\ s)^{-1}$ to the measurements described in this report.

3.3. Equilibrium unfolding

The effect of urea on the equilibrium conformation of cytochrome *c* was examined in 20 m*M* Asp in order to maintain the solubility of the isoelectric buffer in excess urea. Cytochrome *c* was equilibrated with a series of solutions containing increasing concentrations of urea in 20 m*M* Asp at 25°C. Aliquots from each of these solutions were injected into a capillary containing the same Asp/urea concentration thermostatted at 25°C and subjected to electrophoresis.

Fig. 3A illustrates the dependence of the apparent mobility of the single peak observed for cytochrome

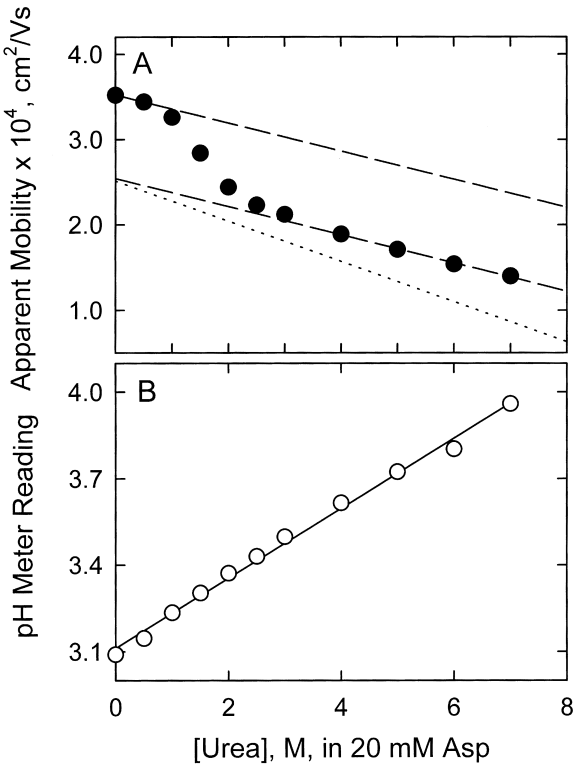

Fig. 3. Equilibrium unfolding measurements of cytochrome *c* in 20 m*M* Asp containing variable concentrations of urea maintained at 25°C. The filled circles in panel A indicate the dependence of the apparent mobility of cytochrome *c* on the concentration of urea in the 20 m*M* Asp. The dashed lines represent the observed effects of urea on the apparent mobility of the folded (upper dashed line) and unfolded (lower dashed line) protein. The dotted line predicts the effect of the relative viscosity of urea on the apparent mobility of the unfolded protein. The open circles in panel B indicate the pH meter readings for solutions of 20 m*M* Asp containing the indicated concentrations of urea.

c on the concentration of urea in the capillary. The measured apparent mobility values describe a transition between the two conformational baselines indicated by the dashed lines. The upper dashed line is the baseline for the folded protein and the lower dashed line is the baseline for the unfolded protein. The difference between these baselines indicates that unfolding reduces the apparent mobility of cytochrome *c* by $1.0 \cdot 10^{-4}$ cm^2 (V s)$^{-1}$. It is likely that this reduction principally reflects the larger frictional coefficient of the unfolded protein. Analysis of the urea dependence of the apparent mobility in terms of a two-state transition indicates that the folded and unfolded conformations of cytochrome *c* are equally populated in 1.5 *M* urea.

The contribution of the viscosity of urea solutions [6] to the apparent mobility of unfolded cytochrome *c* is predicted by the dotted line in Fig. 3A. This prediction has a greater slope than the baseline for unfolded cytochrome *c*, the lower dashed line. The difference between these two lines likely reflects the pH dependence of isoelectric Asp on urea concentration, illustrated in Fig. 3B. A similar pH dependence of isoelectric iminodiacetic acid, IDA, on urea concentration has been reported [7].

3.4. Dynamic unfolding

In the equilibrium unfolding measurements described above, unfolding occurs prior to CZE. In the dynamic unfolding measurements described in this section, all unfolding occurs during CZE. Aliquots of a solution of folded cytochrome *c* in 40 m*M* Asp at 25°C were injected into a capillary containing the same solvent thermostatted at a series of different temperatures. If the thermal unfolding of the protein occurs within this temperature range, then a change in protein mobility should be detected. As shown by the filled circles in Fig. 4A, cytochrome *c* exhibits a discontinuous decrease in its apparent mobility consistent with the thermal unfolding of the injected folded protein during its migration through the capillary. Only a single narrow peak is observed in each electropherogram, as shown in Fig. 1, indicating that unfolding is fast relative to the electrophoresis time scale, a few minutes.

In contrast to cytochrome *c*, the thermal dependence of apparent mobility of the single peak observed for lysozyme and for thioredoxin exhibits no discontinuities indicative of a conformational transition, as shown in Fig. 4B. The regular increase in the mobility of each of these proteins likely represents the effect of temperature on the viscosity of the solvent. However, the thermal dependence of the viscosity of water [8], the principal solvent component, predicts a much steeper mobility dependence, as illustrated by the dotted line for lysozyme in Fig. 4B. The disparity between the predicted and observed mobilities for lysozyme cannot be ascribed

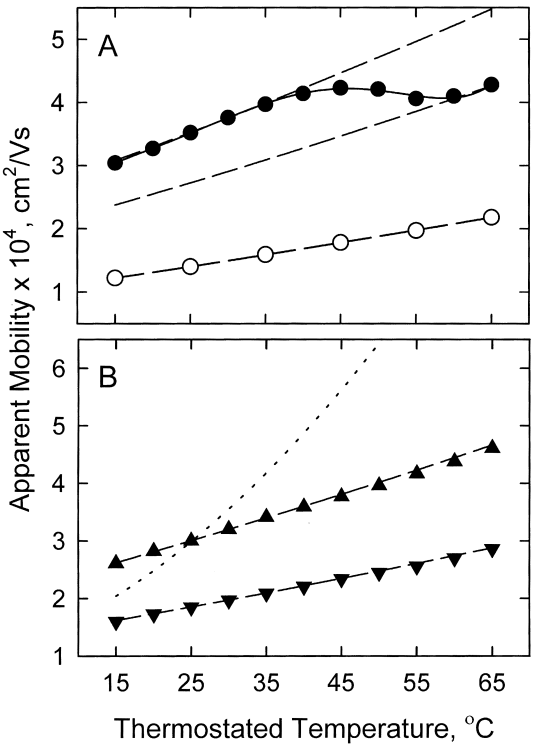

Fig. 4. Effect of thermostatted temperature on protein mobility. The filled symbols indicate the apparent mobility values measured for capillaries filled with 40 m*M* Asp. The filled circles, filled triangles and filled inverted triangles denote values measured for cytochrome *c*, lysozyme and thioredoxin, respectively. The open circles denote values measured for unfolded cytochrome *c* in a capillary filled with 20 m*M* Asp containing 7 *M* urea. The dotted line predicts the effect of the relative viscosity of water on the apparent mobility of lysozyme. The dashed lines predict this effect assuming that only 55% of the length of the capillary between the injection point and the observation window, L_d, is maintained at the thermostatted temperature.

to pH effects since the p*K* for a carboxylate group is essentially temperature independent.

We suggest that the disparity results from the design of the cartridge holding the capillary. This design is such that only a portion of the capillary is thermostatted to the selected temperature while the rest of the capillary remains at ambient temperature. As shown by the dashed lines in Fig. 4B, the thermal dependence of the apparent mobility values for lysozyme and for thioredoxin can each be mimicked if it is assumed that only 55% of the capillary dimension L_d is maintained at the thermostatted temperature, while the remaining 45% of the capillary remains at the ambient temperature, assumed to be 25°C.

As shown by the lowest dashed line in Fig. 4A, the thermal dependence of the apparent mobility of unfolded cytochrome *c* in 20 m*M* Asp containing 7 *M* urea, the open circles, is also fit assuming that only 55% of the capillary dimension L_d is maintained at the thermostatted temperature. Accordingly, this formulation was used to generate the dashed baselines for the thermal dependence of the apparent mobility of the folded and unfolded conformations of cytochrome *c* in 40 m*M* Asp. As shown in Fig. 4A, these baselines fit well with the observed mobility values for cytochrome *c*, the filled circles. These baselines indicate that dynamic unfolding of cytochrome *c* reduces its apparent mobility by $0.8 \cdot 10^{-4}$ cm^2 $(V\ s)^{-1}$, a reduction similar to that obtained from the equilibrium unfolding measurements illustrated in Fig. 3A. Two-state analysis of the thermal dependence for the mobility for cytochrome *c* using the illustrated baselines indicates that the protein has a melting temperature of about 50°C in 40 m*M* Asp.

3.5. Dynamic refolding

Dynamic refolding is akin to dynamic unfolding in that the conformational transition occurs during migration through the capillary. In this case, a sample of an unfolded protein is injected into a capillary containing a folding solvent. Two different samples of unfolded cytochrome *c* were utilized, the protein unfolded in 10 m*M* HCl [5] and the protein unfolded in 20 m*M* Asp containing various concentrations of urea, Fig. 3A. The capillary folding solvent was 40 m*M* Asp thermostatted at a series of different temperatures.

Measurement of the dynamic refolding of cytochrome *c* from 10 m*M* HCl is illustrated in Fig. 5A. Each electropherogram contained only a single narrow peak, Fig. 1, indicating that refolding of cytochrome *c* is rapid on the electrophoresis time scale and that a single refolded electrophoretic product is generated. The thermal dependence of the apparent mobility of cytochrome *c* obtained by dynamic refolding, the open triangles in Fig. 5A, is identical with the thermal dependence of the apparent mobility of cytochrome *c* obtained by dynamic unfolding, the

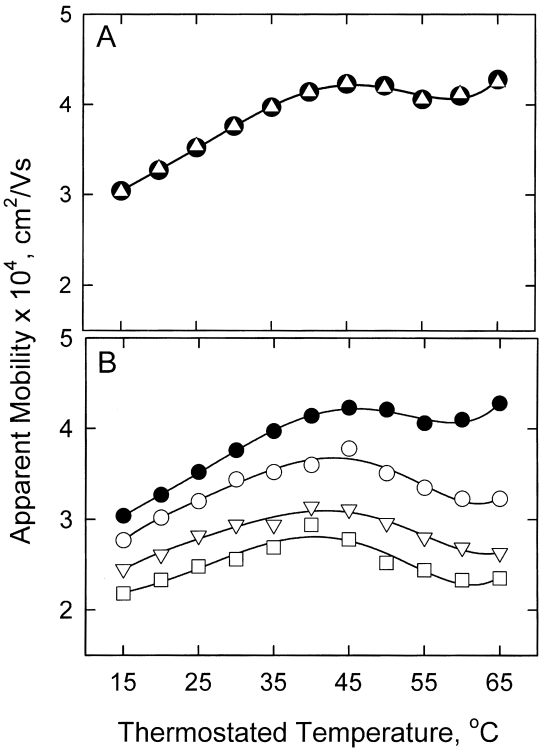

Fig. 5. Dynamic refolding of cytochrome *c* during CE. In all measurements, the capillary was filled with 40 m*M* Asp. The filled circles indicate the apparent mobilities observed following injection of folded cytochrome *c*. The open symbols indicate the apparent mobilities observed following injection of unfolded cytochrome *c*. In panel A, the cytochrome *c* was unfolded in 10 m*M* HCl. In panel B, the cytochrome *c* was unfolded in 20 m*M* Asp containing either 2 *M* urea, open circles, 4 *M* urea, open inverted triangles, or 6 *M* urea, open squares.

filled circles. This identity suggests that the folded and refolded proteins have the same conformation.

Measurements of the dynamic refolding of cytochrome *c* from 20 m*M* Asp containing different concentrations of urea are illustrated in Figs. 1 and 5B. Several aspects of these dynamic refolding measurements are anomalous relative to the remaining measurements described in this report. Firstly, the current exhibited abrupt excursions during electrophoresis. Secondly, the peak shapes in the observed electropherograms, illustrated by peak rFu in Fig. 1, are broader and more asymmetric. Thirdly, the thermal dependence of the apparent mobility of the maximum ordinate in each of these refolding electropherograms, the open symbols in Fig. 5B, is depressed relative to the thermal dependence of the apparent mobility of folded cytochrome *c*, the filled circles in Fig. 5B.

Two additional features of the measurements illustrated in Fig. 5B are noteworthy. Firstly, the contours of all the thermal profiles illustrated in Fig. 5B are similar. Each contour can be fit with a two-state transition having a melting temperature of about 50°C. This suggests that the urea unfolded cytochrome *c* has refolded during electrophoresis in 40 m*M* Asp and that the refolded protein has the same thermostability as folded cytochrome *c*. Secondly, the extent of the depression of the thermal profiles described by the open symbols is proportional to the concentration of urea present in the unfolded sample prior to dynamic refolding. This suggests that the urea in the sample plug is responsible for the depressed mobility of refolded cytochrome *c*.

A likely source of this depression is the lower conductivity of the urea plug, 0.35 mS for 6 *M* urea, compared with the buffer in the remainder of the capillary in a dynamic refolding measurement, 1 mS. Accordingly, the voltage gradient will be higher in the sample plug and lower in the remainder of the capillary. Since the urea remains in the sample plug during electrophoresis, the protein refolded from urea will exhibit a depressed mobility and the extent of this depression will be dependent upon the urea concentration in the plug.

As a test of this explanation, samples of unfolded cytochrome *c* containing 6 *M* urea were inserted into the capillary by electrokinetic rather than pressure injection. In contrast to pressure injection, electrokinetic injection should not place any urea in the unfolded protein sample plug. The thermal profile for the apparent mobility of the electrokinetically injected samples was found identical with that shown by the filled circles in Fig. 5B. Accordingly, the injected urea appears responsible for the depressed apparent mobility values for dynamically refolded cytochrome *c*.

4. Discussion

These measurements of the dynamic unfolding and

refolding of cytochrome *c* indicate that CZE can be used to advantage to investigate the comparative conformational integrity of mutant proteins generated both in vivo and in vitro. Only a few ng of protein are required using the far ultraviolet absorbance of the peptide bonds which are common to all proteins. Measurement of an individual mobility value is rapid, requiring only a few minutes in an isoelectric buffer. Preliminary results suggest that the time for a mobility measurement can be shortened further by using a larger electric fields up to and including 25 kV. Measurement of the mobilities of a series of protein samples can also be automated at a number of preselected temperatures.

An isoelectric buffer need be selected such that the thermal transition of a reference wild type protein can be completely observed. Unfortunately, electroosmosis constrains the available pH range for measurements in uncoated capillaries. However, several isoelectric buffers exist whose pH values fall within this available range. Additionally, the thermal transition of a given protein can be manipulated to advantage using modest concentrations of urea. An example is illustrated in Fig. 6. Dynamic unfolding measurements of cytochrome *c* in 40 m*M* Asp indicate that the conformational transition is just barely completed in the accessible temperature range, as shown in Fig. 4A. Addition of 0.7 *M* urea to the Asp buffer places the conformational transition of cytochrome *c* in a more central location in the accessible temperature range, as shown in Fig. 6.

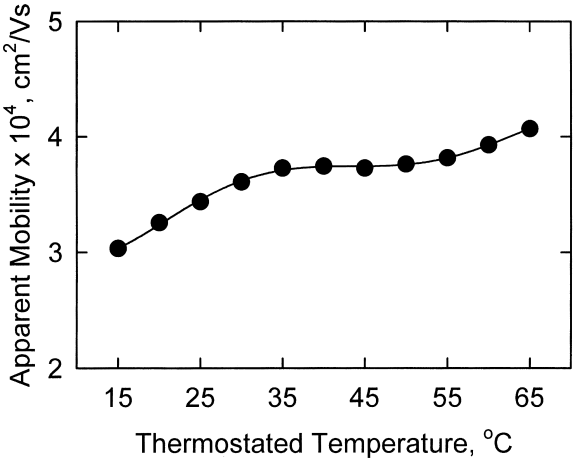

Fig. 6. Dynamic unfolding of cytochrome *c* during CE. The injected samples of cytochrome *c* was equilibrated with 40 m*M* Asp at 25°C. The capillary was filled with the same buffer containing 0.7 *M* urea.

While the conformational transition of cytochrome *c* occurs within a few milliseconds, larger proteins unfold and refold on a more leisurely time scale involving seconds or minutes. The kinetics of such conformational transitions could be directly observed by CZE in isoelectric buffers, particularly in high electric fields at low temperatures. The time scale for such measurements could be further diminished by shortening the capillary distance L_d, provided the difference between the mobility of the folded and unfolded conformations is adequate.

The capillary distance L_d may be effectively shortened by pressure injection of buffer following injection of the sample prior to electrophoresis. For example using the results illustrated for cytochrome *c* in Fig. 4A and an L_d of 1 cm, the migration times for folded and unfolded cytochrome *c* should be resolved and observed in less than 20 s, as illustrated in Table 1. Such times should facilitate observation of the mechanism and folding kinetics of many protein, particularly at low capillary temperatures. The precise value for the L_d in each measurement could be established by addition of a marker to the protein sample, such as a peptide, whose mobility does not exhibit a conformational transition and whose mobility is slower than either the folded or unfolded conformations of the protein. It may also be necessary to add an inert stabilization reagent, such as sucrose or polyethylene glycol [3], to the protein sample to minimize convective stirring during positioning of the sample plug by buffer injection prior to electrophoresis.

Table 1
Predicted migration times of cytochrome *c* for an L_d of 1 cm

Electric field (V/cm)	Migration time (s) at 25°C	
	Folded protein	Unfolded protein
208	13.7	17.2
417	6.9	8.6
625	4.6	5.7
833	3.4	4.3
1042	2.7	3.4

Acknowledgements

This investigation was supported by grants to P.G.R. from Agenzia Spaziale Italiana (No. ARS-98-179) and from MURST (Coordinate Project 40%, Biologia Strutturale).

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