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# Monitoring the refolding pathway for a large multimeric protein using capillary zone electrophoresis

Z. Hugh Fan[a], Pamela K. Jensen[a], Cheng S. Lee[a,*], Jonathan King[b]

[a]*Department of Chemistry and Ames Laboratory USDOE, Iowa State University, Ames, IA 50011, USA*

[b]*Department of Biology, Massachusetts Institute of Technology, Cambridge, MA 02139, USA*

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**Abstract**

Rapid identification of transient partially folded intermediates formed during protein refolding and aggregation has been difficult, particularly with separation methods relying on solid matrices. Capillary zone electrophoresis equipped with laser-induced fluorescence detection provides a fast sensitive means of identifying folding and aggregation intermediates using the intrinsic tryptophan fluorescence. The in vitro refolding of the trimeric P22 tailspike, a model system for the study of protein folding, misfolding and aggregation, has been monitored after dilution out of denaturant. Both monomeric and trimeric folding intermediates were resolved. The refolding kinetics and yields measured by capillary zone electrophoresis were in good agreement with those obtained via fluorescence spectrophotometry and polyacrylamide gel electrophoresis. In comparison with typical UV detection, laser-induced tryptophan fluorescence increased detection sensitivity. In addition, the fluorescence signal carries information on the packing of the tryptophan residues in the folding intermediates. For tailspike and many other proteins, the off pathway aggregation reactions proceed from a thermolabile intermediate at the junction with the productive pathway. By monitoring refolding intermediates after temperature shifts, the structured monomeric intermediate was identified as the thermolabile junctional intermediate between the productive and aggregation pathways.

*Keywords:* Proteins; Endorhamnosidase, P22 tailspike; Enzymes

## 1. Introduction

Newly synthesized polypeptide chains within cells pass through a series of partially folded intermediates in reaching their native state. Association of these intermediates into the aggregated inclusion body state often competes with productive folding into the native state in both prokaryotic and eukaryotic cells [1]. The generation of misfolded and generally aggregated chains has emerged as a practical problem both in the biotechnology industry and in biomedical research. It has been particularly, though not exclusively, associated with expression of cloned genes in heterologous hosts [2,3].

Many proteins can fold in vitro from a completely denatured state [4,5], providing evidence that a polypeptide chain may contain all the information required for folding. Various refolding studies performed to characterize the effects of denaturant, redox reagent and protein concentration have improved the efficiency and yield of the refolding process. The study of protein refolding is also crucial

*Corresponding author.


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to understand the underlying mechanisms, as well as for the elucidation of "the second half of the genetic code" in the prediction of protein structure.

Most techniques for characterizing protein folding reactions usually depend on direct measurements of some average physical properties that are sensitive to the change in the protein structure. Examples include the changes in optical density and steady state fluorescence intensity [6–10]. Capillary zone electrophoresis (CZE) is an attractive alternative to experimental observation and provides the access to a cross section of the population of molecular states within an equilibrium system during a protein refolding reaction. Additionally, separation in CZE is a function of intrinsic molecular properties and does not involve the interaction and mass transfer between a mobile and a stationary phase, such as in size-exclusion [11,12] and ion-exchange [11–14] HPLC.

CZE has been used to monitor the unfolding of human serum transferrin in urea [15] and the temperature-dependent unfolding transitions of lysozyme at low pH [16]. The unfolding of transferrin isoforms is dependent on the iron content, but not the carbohydrate content of isoforms. The mobility data obtained from CZE were used to estimate the apparent thermodynamic parameters of enthalpy and entropy changes associated with the unfolding transition of lysozyme. Furthermore, CZE was utilized for the analysis of bovine trypsinogen, which underwent oxidation from a fully reduced molecule through a distribution of intermediate species until it reached the disulfide bond conformation corresponding to the native structure [17]. In comparison to the results obtained from size-exclusion HPLC, slab gel electrophoresis and gel isoelectric focusing, CZE provides an effective technique for the analysis of protein refolding.

In addition to protein unfolding studies, CZE has been applied for the analysis of point mutations in DNA using temporal thermal gradients in the capillary filled with sieving liquid polymers [18]. The temperature gradient was used to exploit the differences in melting temperature ($T_m$) of DNA molecules differing by a single nucleotide [18]. When the temperature in the capillary equaled a DNA fragment's $T_m$, the double helix partially unwound and resulted in a marked retardation of its electrophoretic mobility. The method was demonstrated for the identification of three point mutations located in the cystic fibrosis transmembrane conductance regulator gene.

In the biotechnology industry, it is often important to be able to monitor species which associate off pathway into insoluble aggregates. The absence of a solid matrix, and the speed of separation, suggests that CZE may be a method of choice for such monitoring. In this study, CZE equipped with native protein fluorescence detection is employed for monitoring the refolding of phage P22 tailspike endorhamnosidase, a model system for the study of folding and aggregation processes. Refolding intermediates separated in CZE can be monitored by their tryptophan fluorescence at 340 nm. The current CZE–UV absorbance measurements not only suffer from the strong background absorbance of denaturant, but also exhibit higher concentration detection limits in comparison with laser-induced fluorescence detection (LIFD). Additionally, tryptophan fluorescence reflects the compactness of the molecule during the refolding reaction.

The P22 tailspike endorhamnosidase is an oligomeric protein and consists of three identical monomers containing 666 amino acids. Six tailspikes assemble onto the phage capsid to form the cell recognition and attachment apparatus of the phage. The native protein is thermostable, with a $T_m$ of 88°C, and is resistant to sodium dodecyl sulfate (SDS) and proteases [19,20]. Refolding experiments, starting from unfolded single-chain monomer in acid-urea, have shown that the in vitro refolding pathway closely resembles the folding pathway in vivo [21,22]. Upon initiation of refolding, tailspike polypeptides rapidly fold into structured monomer intermediates with a high content of secondary structure. These monomeric species associate to form the triple-chain defined folding intermediates, the protrimers, that are susceptible to SDS and proteases. Conversion of the protrimer into the native, SDS-resistant tailspike protein is the rate-limiting step in the folding pathway [21,22].

## 2. Experimental

### *2.1. Capillary zone electrophoresis–laser-induced fluorescence detection*

The CZE apparatus shown in Fig. 1 was con-

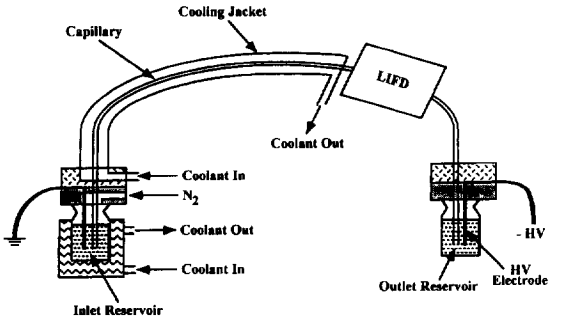


Fig. 1. Schematic diagram of capillary zone electrophoresis equipped with the cooling jacket and the laser-induced fluorescence detection. Nitrogen gas was used for purging the capillary with washing and buffer solutions.

structed in the laboratory using a CZE 1000R high-voltage (HV) power supply (Spellman High-Voltage Electronics, Plainview, NY, USA). A negative electric potential was applied at the detector end of the capillary for electrokinetic injection and for electrophoretic separation while the injection end was grounded. Fused silica capillaries of 50 μm I.D.× 150 μm O.D. were obtained from Polymicro Technologies (Phoenix, AZ) and were cut to a length of 40 cm. The separation distance was 27 cm between the injection point and the LIFD system. Except for those specified, CZE separations were carried out in 25 m*M* sodium phosphate–140 m*M* urea at pH 7.6. The CZE apparatus was enclosed in an interlock box for operator safety.

A cooling jacket (see Fig. 1) was employed to maintain a constant temperature of 10°C at the inlet reservoir and around most of the separation capillary for refolding measurements. There was a 3-cm cooling gap between the end of the cooling jacket and the LIFD system. The capillary was inserted inside a 2 mm I.D.×5 mm O.D. C-Flex tubing (Cole-Parmer, Chicago, IL, USA) where the coolant was circulated around the capillary. The coolant, a mixture of ethylene glycol and water in a volume ratio of 2 to 1, was thermostatted in a low temperature circulating bath (Fisher Scientific, Pittsburgh, PA, USA). A peristaltic pump (Cole–Parmer) was used to circulate the coolant in the cooling jacket at a flow rate of 35 ml/min.

The LIFD system originally designed by Yeung and his co-workers [23] was employed for measuring tryptophan fluorescence of refolding intermediates separated by CZE. Briefly, the 248 nm line of a KrF excimer laser (Potomac Photonics, Lanham, MD) was used for excitation. A mirror directed the laser beam through a 248 nm bandpass filter (Oriel, Stratford, CT, USA) after which a lens (Oriel) focused the beam onto the capillary window. The detection window was made by removing the polyimide coating with heated sulfuric acid. A 10× microscope objective (Edmund Scientific, Barrington, NJ, USA) collected the fluorescence emission and a second mirror directed the fluorescence through a 340 nm filter (Melles Griot, Irvine, CA, USA) and then onto a photomultiplier tube (Hamamatsu, Bridgewater, NJ, USA). Data collection was performed by an HP 35900C analog to digital interface board with the HP G1250C General Purpose Chemstation software (Hewlett–Packard, Fullerton, CA, USA).

### 2.2. Protein denaturation and refolding

The P22 tailspike endorhamnosidase was denatured in a solution containing 7 *M* urea and 25 m*M* sodium phosphate at pH 3 for 1 h at room temperature [21,22,24]. Refolding was initiated by rapidly diluting the unfolded protein with the refolding buffer of 25 m*M* sodium phosphate and 1 m*M* ethylenediaminetetraacetic acid at pH 7. The refolding buffer was pre-equilibrated at 10°C in a low temperature circulating bath (Fisher Scientific). Based on a 50-fold dilution, the final tailspike and urea concentrations in the refolding reaction were 22 μg/ml and 140 m*M*, respectively.

The progress of the refolding reaction was monitored by subjecting an aliquot of refolding sample to CZE separation at various reaction times. Additionally, the refolding samples were analyzed by slab gel electrophoresis and the results were compared with the CZE separations. For slab gel electrophoresis, a 15 μl aliquot of refolding sample was placed on ice to quench refolding. Before it was placed on the gel, the refolding sample was mixed with 7.5 μl of SDS solution containing 0.28 g/ml glycerol, 0.16 *M* tris(hydroxymethyl)aminomethane at pH 6.8, 5% SDS, 0.25 mg/ml bromophenol blue and 50 m*M* dithiothreitol.

### 2.3. Slab gel electrophoresis

A Bio-Rad Mini-Protean II dual slab cell (Her-

cules, CA, USA) was used to perform all SDS–polyacrylamide gel electrophoresis measurements. The separation and stacking gels were cast as described in the instruction manual. A protein mixture of rabbit muscle myosine, *E. Coli* β-galactosidase, rabbit muscle phosphorylase, bovine albumin, egg albumin and bovine erythrocyte carbonic anhydrase was used as the molecular mass standard. The electrophoresis voltage was increased to 150 V after the protein bands had entered the separation gel. Electrophoresis was stopped when the bromophenol blue dye front reached about 1 cm from the gel bottom. Silver staining of protein bands was performed as described elsewhere [25].

### 2.4. Tryptophan fluorescence spectrophotometry

Tryptophan fluorescence of tailspike protein during the refolding reaction was monitored by a Perkin–Elmer LS 50B spectrophotometer (Norwalk, CT, USA). Fluorescence was excited at 280 nm with a slit width of 5 nm. Fluorescence emission spectrum was scanned between 300 and 400 nm with a slit width of 5 nm. Refolding was initiated by rapidly diluting the unfolded protein with the refolding buffer in a 200 μl quartz cell with a 3 mm path length (Starna Spectrophotometer Cells, Atascadero, CA, USA). The quartz cell was thermostatted at 10°C by a low temperature circulating bath (Fisher Scientific).

### 2.5. Chemicals

The P22 tailspike endorhamnosidase with a concentration of 8.9 mg/ml was prepared and purified by standard procedures in King and Yu [26]. The protein molecular mass standard used in slab gel electrophoresis was purchased from Sigma (St. Louis, MO, USA).

L-Tryptophan received from Sigma was used as the electroosmosis marker for the CZE separations. Monobasic and dibasic sodium phosphate, urea and dithiothreitol were obtained from Aldrich (Milwaukee, WI, USA). Tris(hydroxymethyl)-aminomethane and ethylenediaminetetraacetic acid were purchased from Bio-Rad and Fisher Scientific, respectively. All solutions were prepared using water purified by a NANOpure II system (Dubuque, IA, USA) and further filtered with a 0.22 μm membrane (Millipore, Bedford, MA, USA).

## 3. Results and discussion

### 3.1. Monitoring tailspike refolding using capillary zone electrophoresis–laser-induced fluorescence detection

The near-UV detection of tailspike refolding intermediates separated in CZE suffered from the strong background absorbance of typical denaturants including guanidine hydrochloride and urea. For the far-UV detection of tailspike protein, each single-chain polypeptide of 666 amino acids contained only 7 tryptophan residues. To reduce single-chain polypeptide self-association and aggregation, a low protein concentration around 10–50 μg/ml has been required for achieving in vitro reconstitution of native tailspikes from completely unfolded polypeptides [21,22,24]. The small UV extinction coefficient of tailspike protein at 280 nm and the use of low protein concentration in productive refolding led to the selection of LIFD of refolding intermediates in CZE.

By measuring native tryptophan fluorescence emitted at 340 nm, a concentration detection limit of $1 \cdot 10^{-8}$ *M* for conalbumin in CZE was demonstrated by using the excitation at 257 nm as the result of frequency doubling from an argon ion laser [27]. An even lower detection limit of $1 \times 10^{-10}$ *M* for conalbumin was later achieved by Lee and Yeung [28] with the application of a 275 nm line of an argon ion laser. The good match of the laser line at 275 nm with tryptophan's maximum absorbance at 280 nm accounted for the improvement on the detection limit by two orders of magnitude. In this study, the 248 nm line of a KrF excimer laser was used for excitation in the measurement of native tryptophan fluorescence. A concentration detection limit of 1 μg/ml (or $1.4 \cdot 10^{-8}$ *M*) for native tailspike protein was obtained in normal CZE separation (data not shown).

As shown in Fig. 2A, the CZE separation of tryptophan (the first peak) as the electroosmosis marker and native tailspike protein (the second peak) was obtained in the electrophoresis buffer of 25 m*M*

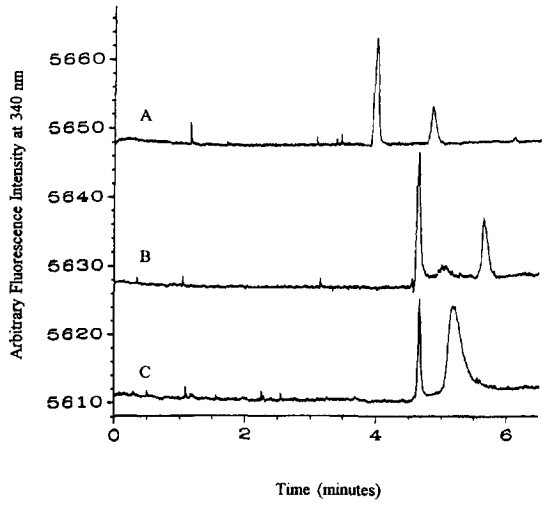


Fig. 2. Capillary zone electrophoresis–laser induced fluorescence detection of native and denatured tailspikes at 10°C. Elution order was tryptophan, followed by tailspike protein. (A) 22 μg/ml native tailspike in the electrophoresis buffer of 25 m*M* sodium phosphate and 100 m*M* urea at pH 7.6; (B) 22 μg/ml native tailspike in the electrophoresis buffer of 25 m*M* sodium phosphate and 6 *M* urea at pH 7.6; (C) 85 μg/ml denatured tailspike in the electrophoresis buffer of 25 m*M* sodium phosphate and 6 *M* urea at pH 7.6. Capillary, 40 cm (27 cm to detector)×50 μm I.D.×150 μm O.D.; applied voltage, −10 kV and 2 s for tailspike injection, −10 kV for electrophoresis; fluorescence detection at 340 nm.

sodium phosphate–100 m*M* urea at pH 7.6. At pH 7.6, the tailspike protein with a p*I* of 5.2 exhibited a net negative charge. The high thermostability of phage P22 tailspike protein was also reflected in its resistance to unfolding by chemical denaturants. At neutral pH, the tailspike protein trimer withstood urea up to the limit of solubility of the denaturant at room temperature. By using a combination of urea and acid pH (6–7 *M* urea at pH 3), tailspike trimers were dissociated and completely unfolded.

To maintain the denatured state of tailspike polypeptide chain and compare its electrophoresis behavior with that of native tailspike in CZE, the electrophoretic separations of native and denatured tailspike proteins were carried out in the electrophoresis buffer containing 25 m*M* sodium phosphate and 6 *M* urea at pH 7.6, as shown in Fig. 2B and Fig. 2C, respectively. The increase in the migration times of tryptophan and native tailspike in Fig. 2A and Fig. 2B presumably reflects the increase in the solution viscosity due to the addition of 6 *M* urea. In the presence of 6 *M* urea, the absolute value of negative electrophoresis mobility of tailspike endorhamnosidase was $0.69 \cdot 10^{-4}$ $cm^2/V$ s for native tailspike (see Fig. 2B) and decreased to $0.39 \cdot 10^{-4}$ $cm^2/V$ s for denatured single-chain polypeptide (see Fig. 2C). In comparison with the contracted chains of native tailspike, the random coil configuration of denatured tailspike resulted in a greater frictional drag and a smaller electrophoretic mobility under the influence of an electric field. Additionally, the peak broadening of denatured tailspike might reflect the slowness of the interchange reactions among various denatured forms and the greater protein-wall interactions due to the hydrophobic nature of unfolded protein.

The formation of native tailspikes during reconstitution from denatured polypeptides occurred very slowly at 10°C [21]. Thus, a constant temperature of 10°C was selected for tailspike refolding and for refolding monitoring by CZE equipped with LIFD. As shown in Fig. 1, nearly 90% of the separation capillary between the injection point and the LIFD was thermostatted at 10°C by using a cooling jacket.

The refolding reaction was performed at 10°C by rapidly diluting the unfolded tailspike protein with the refolding buffer of 25 m*M* sodium phosphate and 1 m*M* ethylenediaminetetraacetic acid at pH 7. Based on a 50-fold dilution, the final tailspike and urea concentrations in the refolding reaction were 22 μg/ml and 140 m*M*, respectively. The refolding conditions, including refolding temperature and final denaturant and tailspike concentrations, were selected for the enhancement of productive refolding over aggregation. As shown in Fig. 3, a series of tailspike refolding samples taken at various reaction times were analyzed by CZE–LIFD. Two partially resolved protein peaks were observed in the electropherograms and their tryptophan fluorescence intensities all increased during the first hour of tailspike refolding (see Fig. 3A–C). The fluorescence intensity of the early eluting peak at 9.6 min reached its maximum during the first hour of refolding reaction and continuously decreased to its disappearance at 48 hours (see Fig. 3C–G). The early eluting peak was assumed to be the formation of structured monomers which formed rapidly at the beginning of reconstitution [21,22,24].

In contrast, the fluorescence intensity of the late eluting peak at 10.0 min continued to increase and

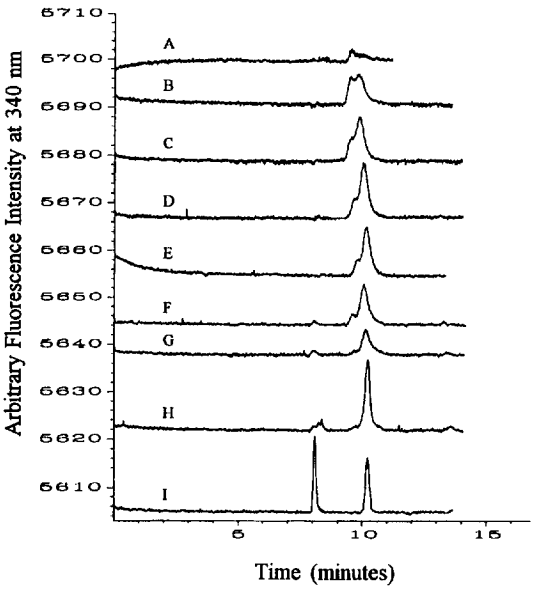


Fig. 3. Monitoring refolding of tailspike protein by capillary zone electrophoresis–laser induced fluorescence detection at 10°C. Refolding samples were taken at (A) 1 min, (B) 15 min, (C) 1 h, (D) 3 h, (E) 5 h, (F) 22 h and (G) 48 h after the initiation of tailspike refolding. The electropherograms marked as H and I contained the refolding sample at 48 h spiked with native protein and the mixture of tryptophan and 22 μg/ml native tailspike, respectively. Electrophoresis buffer, 25 m*M* sodium phosphate and 140 m*M* urea at pH 7.6; capillary, 40 cm (27 cm to detector)×50 μm I.D.×150 μm O.D.; applied voltage, −6 kV and 10 s for refolding sample injection, −6 kV for electrophoresis; total tailspike protein concentration, 22 μg/ml; fluorescence detection at 340 nm.

reached its maximum around the refolding time of 3 h (see Fig. 3D). The late eluting peak was formed by association from the monomeric polypeptides, and represented a precursor (the protrimer) of the native tailspike protein trimer [19,22]. Its late migration time, in comparison with that of the monomeric intermediate, was attributed to the relative compactness of protrimer structure. The observed refolding kinetics in the formation of monomeric and trimeric intermediates during the first three hours of refolding reaction were in good agreement with those reported in the literature [21,22,24].

The tryptophan fluorescence intensity of the late eluting peak decreased slowly after the first three hours of refolding reaction (see Fig. 3D–G). The conversion of the protrimer into the native, SDS resistant tailspike protein is the rate-limiting step in the folding pathway [21,22] and occurs very slowly at temperatures below 20°C [29]. In this study, the protrimer and the formation of native tailspike coeluted during the CZE separations. The coelution of native tailspike with trimeric precursor was illustrated by spiking the refolding sample with native tailspike (see Fig. 3H). The decrease in fluorescence intensity of the late eluting peak was contributed by the maturation of tailspike trimers and the difference in tryptophan fluorescence between the protrimer and native tailspike. By assuming the presence of only native tailspike in the refolding sample at 48 hours (see Fig. 3G), the comparison of peak intensities between the refolding sample and the authentic tailspike (see Fig. 3I) indicated a refolding yield of ~50% of total denatured protein.

### *3.2. Monitoring tailspike refolding using tryptophan fluorescence spectrophotometry*

Fluorescence emission spectra of tailspike refolding samples excited at 280 nm are summarized in Fig. 4. The denatured tailspike protein had an emission maximum of 355 nm. Tailspike protein refolded by rapid dilution had a higher fluorescence intensity and an emission maximum that was shifted to 342 nm, indicating that some of the tryptophans became inaccessible to the solvent during refolding. The fluorescence intensity continued to increase for the first three hours of tailspike refolding and remained relatively steady for the next three to six hours. The increase in tryptophan fluorescence during the early stage of tailspike refolding corresponded to the formation of structured monomeric intermediates and the association of monomeric intermediates into the protrimer.

The maturation of native tailspike from the protrimer was extremely slow at 10°C and accounted for the decrease in the fluorescence intensity during the late stage of tailspike refolding. The decrease in the fluorescence intensity was most likely due to a rearrangement of the hydrophobic environment surrounding some of the tryptophans from the protrimer to the native, SDS-resistant tailspike. Similar changes in tryptophan fluorescence were also observed during in vitro folding of the phage P22 coat protein [30]. The spectroscopic results illustrated in

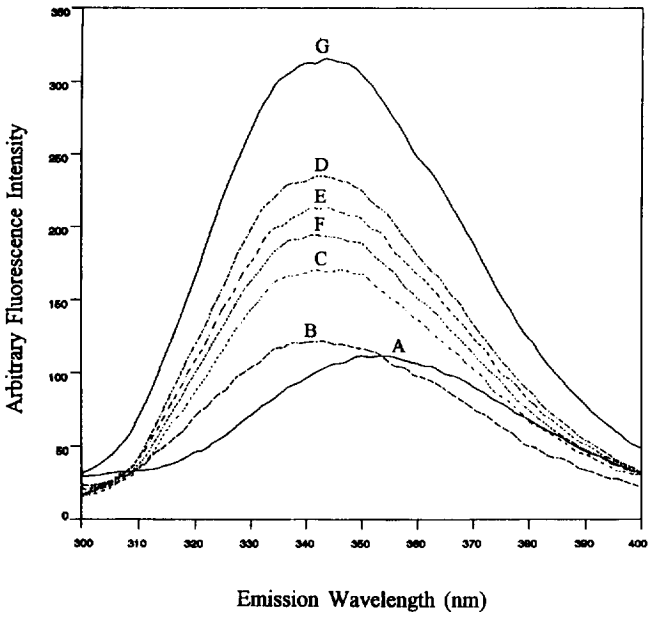


Fig. 4. Monitoring refolding of tailspike protein by tryptophan fluorescence spectrophotometry at 10°C. All protein concentrations were the same at 22 μg/ml. Fluorescence emission spectra are shown for (A) denatured tailspike, (B) 1 min, (C) 15 min, (D) 3 h, (E) 24 h, (F) 48 h after the initiation of tailspike refolding and (G) native tailspike.

Fig. 4 were in good agreement with the CZE electropherograms summarized in Fig. 3 for tailspike refolding monitoring.

### *3.3. Monitoring tailspike refolding using slab gel electrophoresis*

The mature native tailspikes remained native in the presence of SDS and migrated much more slowly than the denatured extended SDS polypeptide chain complex. Those refolding species that have not yet matured into native trimers, including single-chain and protrimer intermediates, were denatured by the detergent and easily distinguished from the native tailspikes. As shown in Fig. 5, the presence of native tailspike appeared as early as 2 h after the initiation of tailspike refolding. Still, this observation confirmed our previous assumption that the two species partially resolved by CZE during the first three hours of tailspike refolding were mainly contributed by the structured monomers and the protrimers (see Fig. 3A–D). The increase in the amount of native tailspike was clearly accompanied with the decrease in the band intensity of denatured monomers contributed by both the single-chain and protrimer intermediates. By comparing the band intensity of refolding sample at 48 h (lane 8) with that of native tailspike control (lane 2), a refolding yield of ~63% was estimated, indicating a reasonable agreement with the result of CZE–LIFD measurement.

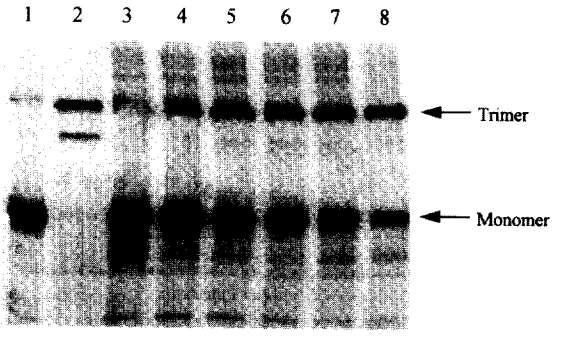


Fig. 5. Monitoring refolding of tailspike protein by slab gel electrophoresis. Lanes: 1=denatured tailspike control; 2=native tailspike control at 22 μg/ml; 3–8=refolding samples taken from 1 h, 2 h, 6 h, 10 h, 24 h and 48 h after the initiation of tailspike refolding.

### 3.4. Temperature dependence of productive vs. aggregation pathways

As shown in Fig. 3E–G, a minor peak was observed in the electropherogram with the migration time very close to that of tryptophan as the electroosmotic flow marker. This minor component might be the early intermediate in the aggregation pathway. To test this, protein reconstitution could be performed at protein concentrations above 50 μg/ml, or at elevated temperatures, which increases off pathway aggregation. To further illustrate the effect of temperature on tailspike refolding, the refolding was initially carried out at 10°C, followed by a temperature shift at different stages of tailspike refolding. As shown in Fig. 6A, an increase of refolding temperature from 10 to 30°C during the formation and accumulation of structured monomers significantly affected the distribution and competition among misfolding (aggregation) and productive folding. In contrast, the aggregation intermediate remained in a relatively small amount when the refolding temperature was increased during or after the accumulation of protrimer tailspike (Fig. 6B). These preliminary results seemed to indicate that the monomeric species were the critical intermediates at the junction between the productive and aggregation pathways. By comparing with the results shown in Fig. 3, the increase in the migration times of refolding and aggregation intermediates was caused by the slower electroosmotic flow in this particular capillary.

Refolding yield of tailspike endorhamnosidase was strongly dependent on the reaction temperature. High refolding temperatures promoted protein self-association and aggregation. By shifting from high temperature to permissive temperature, the formation of protrimer from structured monomer in the folding pathway appeared, and then the native protein. The observation was consistent with the refolding being blocked at the stage of the single-chain polypeptide. Thus, all experimental results based on temperature shifts supported the role of monomeric species as the critical intermediates at the junction between productive folding and aggregation. Furthermore, high refolding yield of tailspike protein, around 60–80% of the total protein, has been reported [24,31] and demonstrated in this study by initiating protein refolding at 10°C.

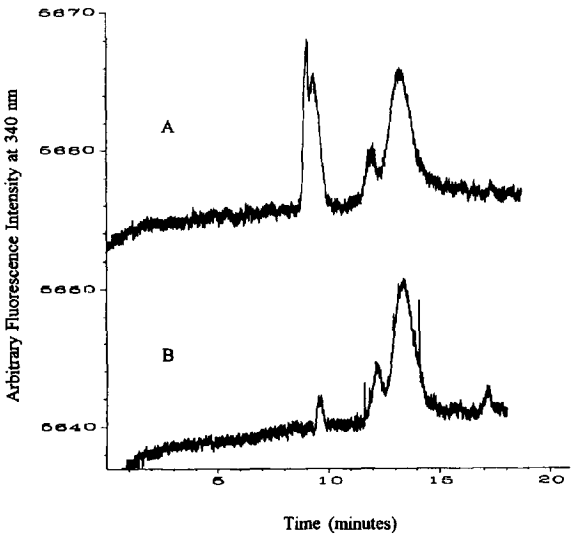


Fig. 6. Effect of temperature shift on tailspike refolding. (A) Refolding occurred at 10°C for 45 min, and then shifted to 30°C for 30 min. (B) Refolding occurred at 10°C for 2 h and then shifted to 30°C for 30 min. The separation conditions for performing capillary zone electrophoresis–laser induced fluorescence detection of samples (A) and (B) were the same as in Fig. 3.

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