Capillary Electrophoretic Monitoring of the Folding and Unfolding of β-Lactoglobulin B

Kevin R. Skelsey and **Michelle M. Bushey***
Department of Chemistry, Trinity University, 715 Stadium Drive, San Antonio, Texas 78212

Abstract

Capillary electrophoresis is used to monitor the change in peak mobility and shape that results from the folding and unfolding of β-lactoglobulin B. The protein is unfolded with denaturing solutions containing either urea or dithiothreitol or both denaturants. Samples are analyzed after varying exposure time to the denaturing solutions. When only urea is used as the denaturant, unfolded protein is refolded after dialysis, and the electrophoretic changes again suggest that a conformational change takes place as a function of time. The short analysis time required by capillary electrophoresis allows for observation of non-native conformations that are difficult to observe by conventional methods of electrophoresis or high-performance liquid chromatography.

Introduction

Despite the considerable growth in the field of protein folding and unfolding studies, the nature of factors involved in the folding process is still largely unknown. If the three-dimensional conformation of a protein could be accurately predicted from the primary structure of amino acids, a wide range of applications would open in the field of genetic engineering. Jaenicke (1) discussed the importance of local structures and their placement along the folding pathway and pointed out that the half-life of in vitro folding can be on the order of several hours, whereas in vivo folding usually occurs over the time range of seconds to minutes. Gething and Sambrook (2) also indicated the role of enzymes during in vivo folding. Whatever steps may be involved in the folding pathway, it is still necessary to detect the different intermediates to gain insight into the processes involved. A variety of techniques has been exploited to study protein conformation (3). Intermediates along the ribonuclease A (RNase) folding pathway have been characterized (4,5). In one of these studies (4), the rate of disulfide reduction of native RNase was reported to be approximately 10 h, and the primary product was fully reduced RNase. Little accumulation of intermediate species was seen, which indicates that reduction of the first disulfide bond is the rate limiting step.

The folding and unfolding of several proteins, including β-lactoglobulin B, have been studied by urea gradient polyacrylamide electrophoresis (6). In this study, protein was applied across a gel that incorporated a urea gradient. Protein structure changed during the electrophoresis depending on the concentration of urea found in that area of the gel. All conformational changes took place in the gel during electrophoresis. Unfolded protein was uniformly found to have a slower mobility than the more compact native forms. β-Lactoglobulin B exhibited complex conformational changes depending on pH, and the refolding was found to occur over a similar urea concentration as the unfolding. The protein was found to refold very slowly. Another report (7) confirms that urea-induced unfolding of β-lactoglobulin B is very complex and that the -SH/-SS- interchange can become appreciable. Monomers, dimers, and other configurations may be present as well.

Still another report (8) investigates the folding and unfolding of β-lactoglobulins in urea solutions at alkaline pH. This report shows that exposure to 7M urea solutions at pH 8.9 results in slow migrating bands within 1 h. These bands are of larger apparent molecular weight than native protein and are believed to result at least in part from -SH/-SS- interchange. This report also shows that prolonged dialysis of the unfolded products causes a reappearance of protein with the same electrophoretic mobility as the native protein; extensive conformational changes can occur during electrophoresis if urea is present in the gel; slow moving products are not formed when sulfhydryl reagents are present during denaturation. Optical rotatory dispersion measurements, performed at pH 7.5 on size fractionated reaction products, show differences in the spectra of these samples both with respect to reaction time and with respect to product size. The spectrum for the largest protein product lacks the Cotton effects associated with the native protein, and the major trough is shifted to a lower wavelength. The smallest protein product has a spectrum that changes as a function of urea exposure time from one resembling the native protein to one intermediate between the native protein and the largest product's spectrum. Morr (9) also showed that urea-treated

* Author to whom correspondence should be addressed.

protein can partially return to the faster electrophoretic mobility of control protein when the denaturation is performed at pH 6.98. Morr also stated that the appearance of slow migrating components supports the idea of a number of intermediate states between native and fully unfolded protein.

Electrophoretic analysis may take several hours using these traditional methods. In the case of McKenzie and Ralston (8), the electrophoresis time ranged from 6 to 11 h. In addition, use of urea gradient gels requires that polyacrylamide gradients be used to adjust for urea effects. Thus, migration of proteins through these gels can be complicated by a number of factors. As pointed out by Creighton (6), this type of approach to understanding protein folding and unfolding has several flaws. First, it is often assumed that conformational states other than fully folded or fully unfolded, detected in equilibrium populations at intermediate denaturant concentrations, are intermediate in conformational properties and are assumed to be intermediate on the folding pathway. Kinetic sequences are thus often implied when no such approach is taken in the analysis. Second, these intermediate conformations are often detected in the presence of the denaturant. The denaturant may impart stability to these intermediates that is not inherent in the conformation itself.

Capillary electrophoresis (CE) holds more promise than conventional techniques, such as slab-gel electrophoresis, for the analysis of pathways because of its high resolution and shorter analysis times. Recent studies of protein folding and unfolding have shown that CE is applicable to this type of protein analysis. Kilar and Hjerten (10) investigated the urea-induced denaturation of human serum transferrin by CE. Protein was as fully denatured as possible at specific urea concentrations before electrophoresis. Urea was included in the capillary buffer. The corrected migration time was plotted as a function of urea concentration to simulate the data obtained by conventional electrophoresis in urea gradient gels. Strege and Lagu (11) investigated the urea induced denaturation of bovine serum albumin using methods similar to those of Kilar and Hjerten. Strege and Lagu (12) also studied the refolding of reduced bovine trypsinogen. In this study, protein was fully reduced, and the reformation of disulfide bonds was studied as a function of time after quenching the process with acid, dialysis, and concentration of the partially refolded protein. Observation of the refolded protein and intermediates was accomplished by CE and a variety of other techniques. Approximately 15% recovery of activity was observed after 22 h of refolding.

We have chosen to study the folding and unfolding behavior of β-lactoglobulin B. This is a well-studied protein with two disulfide bonds that exhibits complex conformational change behavior as described previously. In addition, this protein can successfully undergo electrophoresis in bare fused-silica capillaries. Both the folding and unfolding behavior of this protein is investigated by CE. The fast analysis time of CE allows for the electrophoretic monitoring of possible intermediate conformational forms of the protein. These forms can be monitored as a function of time that the folded protein is exposed to denaturing solutions or as a function of time that the unfolded protein is free from denaturants. This is not possible by conventional electrophoretic methods because of the experimental time frame. Also, because protein is separated from denaturing agents early in the electrophoretic process, it seems unnecessary to quench reactions with the addition of acid or reagents such as iodoacetate (13). Another advantage of this analytical approach is that it is not necessary to incorporate urea in the electrophoretic buffer system because most of the conformational changes are slow with respect to the electrophoretic time frame. This simplifies detection and eliminates the need for viscosity corrections in mobility measurements. More importantly, this also eliminates the possibility that the denaturant may artificially stabilize intermediate conformations.

Experimental

Equipment

The CE system was assembled in-house from components. A 50-µm i.d fused-silica capillary was used for separations. The total capillary length was 50 cm, and the separation length was 35 cm. The capillary was purchased from Polymicro Technologies (Phoenix, AZ). The polyimide coating was removed in a hood with a drop of fuming sulfuric acid to create an on-column detection window. A linear UVis 200 detector operated at 215 nm was positioned at the capillary window. A 316SX Dell PC (Austin, TX) was used to collect and store data via a National Instruments multifunction interface board (Austin, TX). The computer program was written in-house using Microsoft Quickbasic (Redmond, WA) and Lab Windows software (National Instruments). The computer also controlled the Spellman high voltage DC power supply (Plainview, NY), which had an output range of ±30 kV. The user was protected from the high-voltage electrode by the electrode and buffer vial being enclosed in a Plexiglas box fitted with an interlock system.

A Pharmacia PhastSystem (Pleasant Hill, CA) was used to determine isoelectric points of the native and dithiothreitol (DTT) denatured protein.

Reagents and procedures

Deionized water was obtained from a Barnstead Nanopure system (Dubuque, IA). The electrophoresis buffer consisted of 0.05M tris(hydroxymethyl)aminomethane, prepared by mixing equimolar solutions of Trizma base and Trizma hydrochloride (Sigma; St. Louis, MO) until the approximate pH was reached. Final pH adjustments to 8.2 were made with dilute HCl, and 0.005% NaN_3 (Anachemia, Champlain, NY) was added. The resulting solutions were filtered through Acrodisc 0.2-µm filters from Gelman Sciences (Ann Arbor, MI).

Injections were performed electrokinetically by applying +5 kV for 5 s. Run voltages were +15 kV. The capillary was rinsed between runs with 1.0M NaOH, water, and running buffer for about 30 s each using a Nalgene hand vacuum (Rochester, NY). Capillaries were stored in water overnight. In order to observe any conformational changes occurring on a fast time scale, samples containing both urea and DTT were injected onto the capillary on the short, grounded end (15 cm)

after reversing the power supply controls to output negative voltage. This reduced run times from 7 to 4 min.

Denaturing and refolding conditions

Unfolding studies used approximately 0.2 mg β-lactoglobulin B dissolved in 1 mL of the denaturing solution. Protein was obtained from Sigma and used without further purification. Denaturants were added to the 0.05M tris running buffer, and protein was dissolved in the resulting solutions. Denaturants investigated were 8M urea, 0.1M DTT, and a mixture of urea and DTT. Both the urea and DTT were obtained from Sigma. All solutions were filtered through an Acrodisc low protein binding filter (0.2 μm) from Gelman before injection onto the capillary. Denaturing solutions were prepared fresh each day. Samples were injected onto the capillary as a function of the time that the protein was exposed to the denaturing solutions at room temperature.

To determine the isoelectric points of the native and DTT-denatured proteins, four samples were made at 30-min intervals as follows: approximately 5 mg of protein was dissolved in 1 mL of the tris buffer containing 0.1M DTT. These four samples and protein dissolved in 1 mL of the tris buffer without DTT were applied to the precast gels (PhastGel IEF 4-6.5) along with two lanes of protein standards (Pharmacia Low pI Kit). Gels were prepared, run, and developed with coomassie staining (PhastGel Blue R tablets) all according to the Pharmacia literature.

Protein refolding was monitored by allowing a 1.0-mg/mL sample of protein to completely denature as already described, after which dialysis was done using Millipore (Bedford, MA) membrane filters (0.25 μm). The filters were floated on deionized water, and a 20-μL denatured protein sample droplet was placed on the filter as instructed in the company literature. The sample was allowed to sit for 30 min at room temperature. Droplets were collected and combined in a vial, and samples were injected onto the capillary as a function of time. The 30-min dialysis time is included in the sampling times reported later in this paper. No attempts were made to reform disulfide bonds. No attempts were made to prevent air oxidation.

Results and Discussion

All electrophoretic results were compared with an electropherogram of a protein sample that contained no denaturants, as is shown in Figure 1. Any protein peak corresponding in shape and mobility to the peak seen in this electropherogram is referred to as native protein. It should be noted however, that some denatured forms of the protein may in fact have the same mobility as the native form.

After exposure to 8M urea, the mobility and peak shape of the protein began to change. Initially, the peak resembled the native protein peak, but as time progressed, the native peak decreased in magnitude, while other peaks began to appear at slightly earlier times (Figure 2). Between the exposure times of 2 min and 1 h 35 min the relative percent area of the native

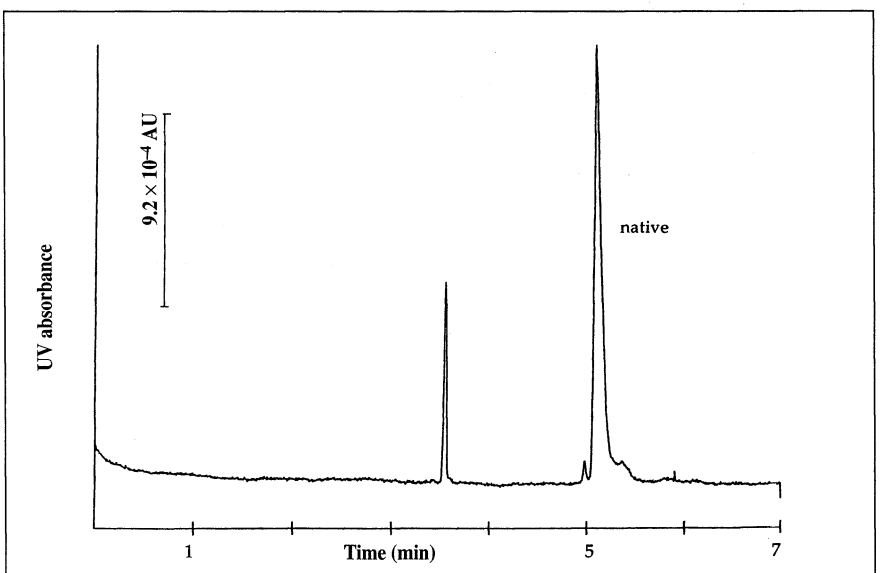

Figure 1. Electropherogram of native β-lactoglobulin B. Formamide is used to monitor the electroosmotic flow.

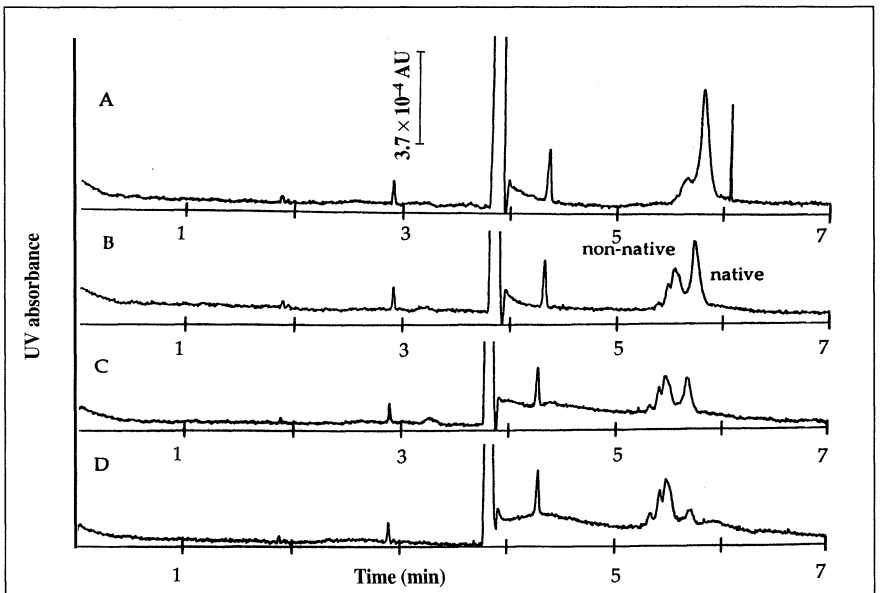

Figure 2. Electropherograms of β-lactoglobulin B obtained after exposure to 8M urea solution for various times: A, 2-min exposure time, native peak area/non-native peak area is approximately 62%; B, 18-min exposure time, native area/non-native area is 36%; C, 31-min exposure time, native area/non-native area is 28%; D, 95-min exposure time, native area/non-native area is 16%. Urea marks the electroosmotic flow time.

peak decreased from 62% to 16% of the total protein peak area. Multiple forms of the unfolded protein were detected. The disulfide bonds were not broken in this procedure; unfolding was incomplete.

The electrophoretic buffer does not contain urea. Urea greatly increases the viscosity of the solution and thus decreases the electroosmotic flow. When 8M urea was included in the electrophoretic buffer, the electroosmotic flow decreased from 5.5 $\times 10^{-4}$ cm^2/V· s to 4.3 $\times 10^{-4}$ cm^2/V · s, and the current dropped from 15 to 6 μÅ. In order to perform experiments on a faster time frame, urea was removed from the electrophoretic buffer system and was instead included only in the sample solutions.

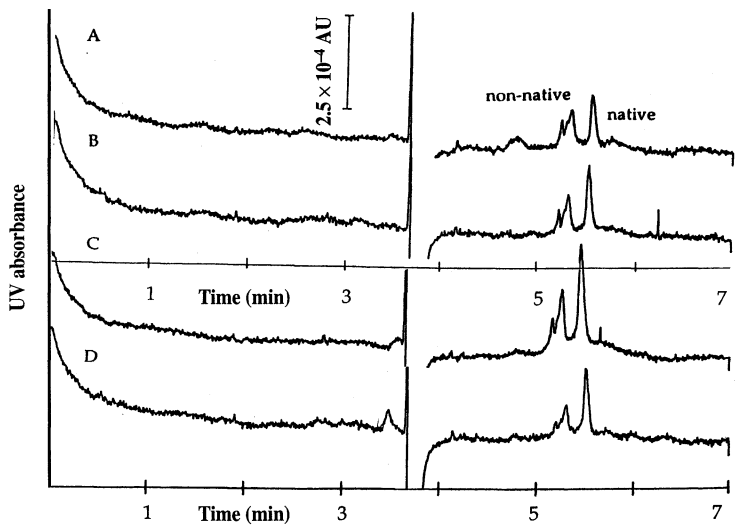

Figure 3. Electropherograms of β-lactoglobulin B obtained after removal from 8M urea solution for various times: A, 65-min exposure time, native peak area/non-native peak area is approximately 28%; B, 81-min exposure time, native area/non-native area is 46%; C, 112-min exposure time, native area/non-native area is 64%; D, 133-min exposure time, native area/non-native area is 65%. Absence of buffer salts, due to removal by dialysis, marks the electroosmotic flow time.

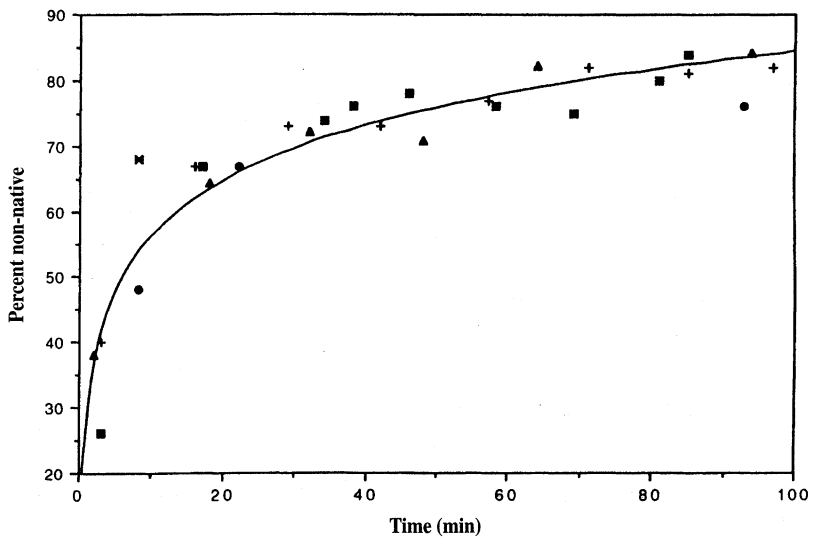

Figure 4. Plot of percent non-native peak versus time for urea-induced unfolding for five separate experiments.

It appears as though the conformational changes that were observed here are slow with respect to the electrophoretic time frame. The two small peaks present just before 3 min and just after 4 min are related to the buffer system and can be seen in some subsequent figures as well. They are not present in dialyzed samples but are present in blank samples containing no protein. The large peak appearing just before 4 min is due to the presence of urea in the sample solution and marks the electroosmotic flow time. The relative standard deviation (RSD) of the native peak mobility is 1.24% for a series of electropherograms occurring over a time period of 95 min.

Because urea interferes with hydrophobic forces and should therefore loosen the protein structure, which in turn increases the hydrodynamic radius of the protein, we should expect unfolded protein to have a slower mobility than tightly folded protein. The formation of protein aggregates would also produce a slower migrating species. Because the peaks formed after exposure to the urea solution were closer to the electroosmotic flow peak, slower migrating species are formed upon reaction with urea. The native protein peak did not disappear completely before sample degradation prevented further analysis. These results are consistent with previous work at alkaline pH (8).

Protein denatured with urea can be partially refolded to its apparent native conformation. The electropherograms obtained from refolding β-lactoglobulin B are shown in Figure 3. When the protein is removed from a urea environment, refolding begins and appears to be complete after a little less than 2 h. We found this time frame typical for this type of refolding experiment. The RSD of the native peak mobility is 1.67%. Reproducibility complications associated with electrokinetic injections are evident in the differences in peak heights from one electropherogram to another. Similar to the unfolding study, refolding was never observed to be 100% effective. This is again consistent with earlier studies (8,9). Incomplete refolding may be due to lost information, such as chemical modification of the protein, the presence of very stable unfolded protein forms, or incorrectly refolded protein. If the electrophoretic changes that were observed in the unfolding case were due simply to a perturbation of the system caused by injecting a urea-containing sample onto a non-urea capillary, this reversal of the process shown in Figure 3 should not be observed because urea has been removed from the sample before injection on the capillary. Based on diagonal electrophoretic results, earlier workers (8) also concluded that protein species detected after denaturation

with urea were real reaction products and were not formed simply as a result of interactions with buffer components.

A plot of percent non-native conformation versus time is shown in Figure 4 for the urea unfolding experiments. Data from five separate denaturing experiments are included. A logarithmic fit is included on the plot for visual clarity. Although the specific distribution on the individual non-native peaks can vary slightly from experiment to experiment in terms of peak height, the overall change in percent non-native peak area seems to follow a reproducible trend. McKenzie and Ralston (7) also investigated the kinetics of β-lactoglobulin B conformational changes in urea solutions, but their work was done at lower pH, where the protein behaves differently. However, they did find that the protein exhibits complex behavior and does not follow simple first- or second-order behavior. Our plot suggests that it may be possible to use CE to extract kinetic information from folding and unfolding experiments. A full kinetic analysis is unavailable at this time, partly because of variations associated with manual injection techniques that prevent a more extensive quantitative evaluation of these results. The corresponding information on the refolding process is not available because the dialysis procedure prevents data collection before 30 min.

When the disulfide bonds are disrupted with DTT, the unfolding process appears to be different from the urea results as observed by electrophoretic changes. When DTT is used alone as a denaturant, only two peaks are observed. These results are shown in Figure 5. A non-native protein peak appears just after the native peak. It is not fully resolved. The non-native peak becomes visible in the electropherogram after only a 1-min exposure time to the DTT solution. Again, the native peak does not completely disappear even after 90 min of exposure. The non-native protein has a longer migration time than the native protein. The RSD for the native peak mobility is 0.83%. Isoelectric focusing performed with the Pharmacia PhastSystem shows that the isoelectric point (pI) of the protein changes from 5.38 for the native species to 5.22 for the DTT-treated species as measured against the standard protein kit. A very faint second band appears in the treated sample at a pI of 5.15. This change would impart a slightly more negative charge to the denatured species at the CE-operating pH of 8.2, which accounts for the slightly longer migration time of the denatured species relative to the native peak.

When both urea and DTT are used to denature the protein, a two-peak system is again observed (Figure 6). In this case, however, the non-native protein peak has a shorter migration time than the native protein. The presence of urea either influences the degree of ionization change caused by use of DTT alone by altering the exposure of buried residues or forces the protein to acquire a less compact configuration than when DTT is used alone. Again, even after approximately 80 min, a native protein peak is still seen as a shoulder on the non-native peak. The two small peaks eluting late in the electropherogram are buffer system peaks unrelated to the protein itself. The RSD of the mobility of the non-native peak is 1.39%. Isoelectric focusing with the Phastgel system was attempted with the DTT–urea denatured samples. The results were similar to those obtained with the DTT-denatured samples except that the faint second band was absent. This is not surprising as the Pharmacia precast gels do not contain urea, and the changes that we observed seemed to occur on a time scale comparable with that of the IEF time scale. Thus, the destabilizing effects of the urea were not maintained during the electrophoretic time frame, and only the permanent modifications due to the DTT were observed in the IEF study.

Attempts were made to plot percent non-native protein area versus reaction time for both DTT and DTT–urea unfolding sys-

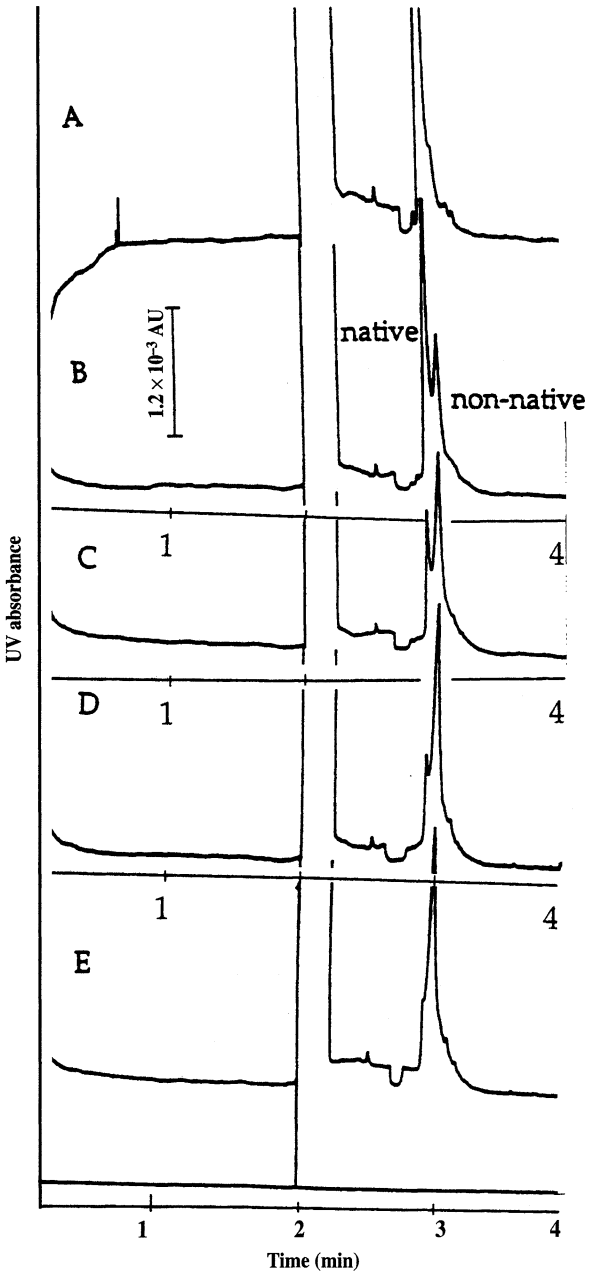

Figure 5. Electropherograms of β-lactoglobulin B obtained after exposure for various times to solutions containing dithiothreitol: A, 1-min exposure time; B, 14-min exposure time; C, 31-min exposure time; D, 70-min exposure time; E, 90-min exposure time. Sample components mark the electroosmotic flow time. Injections were performed on the short end of the capillary to decrease analysis time (50-cm total length, 15-cm separation length). The power supply was operated in the negative voltage mode.

tems. However, these results were not as reproducible throughout the reaction time frame as the urea-induced unfolding study. This is probably partly because of more extensive peak overlap and variability inherent in manual electrokinetic injections as was mentioned previously. This is a limitation of this technique that perhaps could be solved with an autosampler system using hydrodynamic or pressure injections. Visual inspection of the results involving DTT denaturation suggests that the unfolding process occurs on roughly the same time scale whether urea is used in the unfolding process or not. The lack of multiple non-native species after reaction with DTT is again consistent with earlier work with this protein (8).

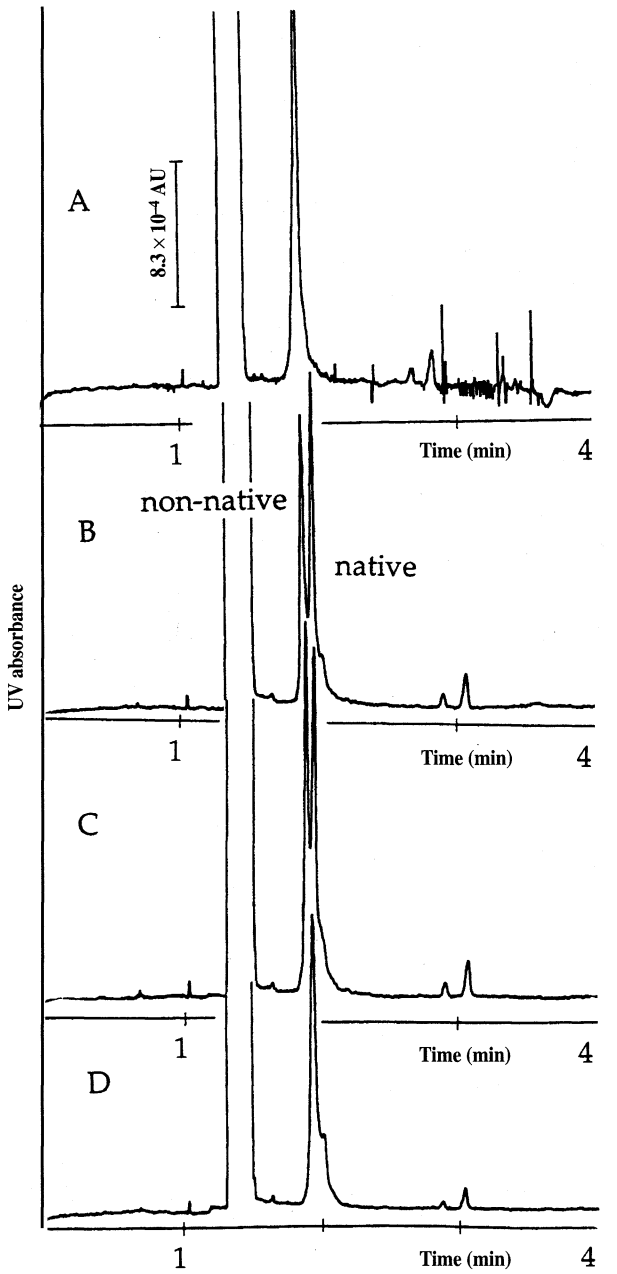

Figure 6. Electropherograms of β-lactoglobulin B obtained after exposure for various times to solutions containing dithiothreitol and urea: A, 1-min exposure time; B, 41-min exposure time; C, 62-min exposure time; D, 81-min exposure time. Sample components mark the electroosmotic flow time. Injections were performed on the short end of the capillary to decrease analysis time (50-cm total length, 15-cm separation length). The power supply was operated in the negative voltage mode.

Refolding of β-lactoglobulin B was attempted after unfolding involving exposure to DTT and to DTT combined with urea. The process was the same as that used for the urea case. No evidence of refolding was observed by this method, as was expected. No attempts were made to properly reform the disulfide bonds, and air oxidation appears to be inadequate in this example for allowing correct refolding.

Conclusions

It has been shown that it is possible to monitor the folding and unfolding of a protein by CE using unmodified fused-silica capillaries. This approach suggests that it may be possible to study the denaturing effects of other reagents by CE. This approach has several advantages over urea gradient gel methods of monitoring protein folding. First, this approach is faster, which allows for observation of possible intermediates that cannot be seen over longer time frames. Second, it is possible to observe changes in the protein as a function of time and not denaturant concentration. This analytical approach will also allow for comparison of the concentration method and the time method to determine if species observed as a function of urea concentration are comparable with species observed as a function of time of exposure. This approach may allow for a kinetic analysis of in vitro protein folding and unfolding. Finally, and most importantly, the protein is separated from the denaturant early in the electrophoretic process; thus, the protein can be observed in the absence of denaturant, which might otherwise stabilize intermediates. Experiments in this area are continuing in our laboratory.

Acknowledgments

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