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Capillary electrophoresis as a tool for the analysis of protein folding

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ABSTRACT

The utility of capillary electrophoresis (CE) was demonstrated for the analysis of a model protein, bovine trypsinogen, as it underwent oxidation from a fully reduced molecule through a distribution of intermediate species until the disulfide bond conformation corresponding to that of the globular native structure was reached. Through the use of CE, completely refolded (native) trypsinogen was resolved from both the reduced protein and intermediate refolded conformations. In addition, the presence of polyethylene glycol in the separation buffer was determined to provide size-based protein separations of significantly higher resolution than those obtained in phosphate buffer alone. Quantitation of native trypsinogen in refolded material revealed an excellent correlation between CE determinations and analyses by established techniques such as biological activity assay and high-performance size-exclusion and cation-exchange chromatography. These results, together with a comparative consistency of CE separations with those obtained via non-denaturing slab gel electrophoresis and isoelectric focusing, suggest that CE can be an effective technique useful for the analysis of protein refolding.

INTRODUCTION

The formation of a protein inside a living cell is known to take place in two steps: the biosynthesis of the protein chain, followed by its folding into a native three-dimensional structure. While extensive work has been devoted toward the study of the first process, much less is understood concerning the latter. It is known that many proteins can fold *in vitro* from a completely unordered state [1], providing evidence that a polypeptide chain, in an aqueous environment, contains all of the information required for folding. Therefore, the study of protein folding *in vitro* is crucial to understanding the mechanism of protein self-organization.

Two fundamental experimental approaches, the determination of biological function (activity) and the determination of the state of association, have been traditionally employed to characterize the folding and association of proteins. Because of the structure–function relationship of proteins, activity represents the most sensitive criterion. However, studies monitored using UV [2–4], circular dichroism [5], fluorescence [6,7], NMR [8–10] and gel electrophoresis [11] have demonstrated that the protein denaturation–renaturation process is not always a two-state transition, *i.e.*, intermediate species can be detected. Various chromatographic techniques, such as high-performance size-exclusion [12–14], ion-exchange [13,15,16], and hydrophobic interaction chromatography [17–19], have demonstrated potential for non-denaturing protein separations, in addition to the analysis of folding intermediates. However, the number of analytical methods readily applicable to the separation, detection and quantitation of changes in covalent structure, such as those undergone by a protein during folding, remain quite limited.

Advances in the development of analytical methods for the analysis of changes undergone by a protein structure during refolding will

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impact work upon the following two issues. A primary objective of the study of protein folding is the elucidation of the "second half of the genetic code" that is assumed to govern the spontaneous transition from the one dimensional structural information (encoded in a given primary structure) to the three-dimensional biologically active state [20]. If this folding code were known, not only structural predictions would be at hand, but hypothetical functions could also be deduced from given amino acid or DNA sequences. Secondly, the advent of the recombinant DNA revolution and the new biotechnology that derives from it has brought protein folding to the forefront of the awareness of the scientific community. Recent biotechnological advances have resulted in the development of genetically engineered cells which can be viewed as factories for the production of specific polypeptides. The polypeptide product is often in the form of an inclusion body, and the solubilization, purification, and refolding of the product are processes which can provide enormous challenges for separation scientists. Analytically, it is crucial to be able to detect the different conformational states of a protein in both process development and quality assurance.

In this paper, the use of a relatively new bioseparation technique, capillary electrophoresis (CE), is reported for the analysis of proteins differing in *covalent* structure as they undergo refolding. Separations of refolded proteins by CE, a quantitative electrophoretic technique, are demonstrated to compare favorably with analyses by other analytical methods (activity assay, high-performance liquid chromatography, native gel electrophoresis and isoelectric focusing) which have previously demonstrated application for determinations of this nature. Bovine trypsinogen was chosen as the protein of interest for this investigation. The refolding biochemistry of this serine protease zymogen has been investigated extensively [21–23].

EXPERIMENTAL

Materials

Bovine trypsinogen [phenylmethylsulfonyl fluoride (PMSF)-treated], bovine trypsin, dithiothreitol (DTT), cysteine, cystine, calcium chloride, phenylmethylsulphonyl fluoride (PMSF), Nα-*p*-tosyl-l-arginine methyl ester hydrochloride (TAME), trifluoroacetic acid (TFA) and urea were purchased from Sigma (St. Louis, MO, USA). Sodium acetate, glacial acetic acid and ethylenediaminetetraacetic acid (EDTA) were from EM Science (Gibbstown, NJ, USA), polyethylene glycol (PEG; M_r 40 000) was from Serva (New York, NY, USA), and glycerin was from Fisher (Fair Lawn, NJ, USA). Formic acid was obtained from Fluka (Ronkonkoma, NJ, USA). Analytical-grade acetonitrile was purchased from Burdick & Jackson (Muskegon, MI, USA).

Reduction of trypsinogen

A 50-mg sample of trypsinogen was dissolved in 10 ml 0.1 *M* Tris–HCl pH 8.5, 8 *M* urea, 1 m*M* EDTA, 0.1 *M* DTT and 0.2 m*M* PMSF. The reduction of disulfides was carried out at ambient temperature for 3 h under a nitrogen atmosphere. The presence of PMSF, a trypsin inhibitor, was found necessary for the minimization of enzymatic degradation via trypsin contaminant activity during the reduction. Following reduction, the pH was then lowered to pH 3.0 with concentrated formic acid, and the sample was then dialyzed overnight in a Spectra/Por 2000 M_r cut-off membrane (Spectrum, Houston, TX, USA) in 400 volumes of 100 m*M* acetic acid to remove salts.

Refolding of trypsinogen

Quantities (50 mg) of reduced trypsinogen were refolded through the use of techniques described by Jaenicke and Rudolph [20] and Light and Higaki [21]. Aliquots of the folding solution were removed at various time points, and the pH of these samples was immediately dropped to pH 3 by addition of concentrated formic acid. Samples analyzed by CE, native gel electrophoresis and isoelectric focusing were first dialyzed in 100 m*M* acetic acid to remove salts, and then concentrated in a SpeedVac concentrator (Key Scientific, Mt. Prospect, IL, USA) to a final volume corresponding to a concentration of 2.0 mg/ml total protein.

Activity assay of trypsinogen

Samples of refolded trypsinogen were solvent exchanged into 50 m*M* Tris–HCl pH 8.1, 10 m*M* calcium chloride and then activated to trypsin via incubation with a low concentration of trypsin (5 units of trypsin/mg trypsinogen) at ambient temperature for 6 h. Activity toward TAME was determined spectrophotometrically according to the procedure of Hummel [24], through the use of a Model DU-7400 UV–Vis diode array spectrophotometer (Beckman, Fullerton, CA, USA).

Capillary electrophoresis

CE was performed with an automated P/ACE 2100 instrument (Beckman Instruments, Palo Alto, CA, USA) controlled by an IBM PS/2 Model 80 386 computer fitted with PACE software (Beckman) running in a Windows (Microsoft, Redmond, WA, USA) environment. The capillary cassette was fitted with a 50 μm I.D. fused-silica capillary, 37 cm total length (30 cm inlet-to-detector window). All separations were obtained through the employment of a cathodic outlet reservoir, and injection of the sample was by low pressure (0.5 p.s.i.; 1 p.s.i. = 6894.76 Pa). On-column detection was performed by UV absorption at 200 nm, and the temperature was controlled at 25 ± 0.1°C. The capillaries employed in this study were fused-silica capillaries obtained from Polymicro Technologies (Phoenix, AZ, USA).

Cation-exchange HPLC

Chromatography was performed with a gradient HPLC system (Models 303 and 305 pumps, Model 811B dynamic mixer, Model 116 variable-wavelength UV detector, and a Model 231 sample injector equipped with a 100-μl sample loop (Gilson, Madison, WI, USA). Analog data were collected directly from the absorbance detector (280 nm) on an in-laboratory centralized chromatography computer system based on the Hewlett-Packard Model 1000 minicomputer. A ProGel-TSK SP-5PW column (Supelco, Bellefonte, PA, USA) (7.5×0.75 cm), a vinyl polymer-based strong cation exchanger, was used for this investigation, together with an in-line 0.25-μm filter frit preceding the analytical column. Mobile phase A was 25 m*M* sodium acetate pH 4.0, 2 *M* urea, 20% acetonitrile and mobile phase B was 25 m*M* sodium acetate pH 4.0, 2 *M* urea, 20% acetonitrile, 1 *M* NaCl. A linear 45-min gradient from 0 to 35% B was utilized with a 1.0 ml/min flow-rate, and the column was operated at ambient temperature.

Size-exclusion HPLC

Size-exclusion chromatography was performed on a Superose 12 column (30×1.0 cm) (Pharmacia) using the chromatography system described above and a flow-rate of 0.5 ml/min. An in-line 0.25-μm filter frit preceded the column. Isocratic elution, using a mobile phase containing 100 m*M* acetic acid, 100 m*M* NaCl and 2 *M* urea, was monitored at 280 nm. Fractions of refolded trypsinogen were collected through the use of a Model 203 fraction collector (Gilson).

Native gel electrophoresis

The protein samples (2 mg/ml total protein, 1 μl volume loaded) were electrophoresed using PhastGel Homogeneous 20 gels (Pharmacia/LKB Biotechnology, Piscataway, NJ, USA) and then stained with Coosmasie Brilliant Blue using a PhastSystem equipped with a reversed-polarity electrode assembly. The samples were separated, fixed, stained, and destained according to the procedures described in the product literature.

Isoelectric focusing

The protein samples (2 mg/ml total protein, 1 μl loaded) were focused using a PhastSystem and PhastGel IEF 3–9 gels (Pharmacia). The samples were fixed, silver stained, and destained as per the product literature. A p*I* marker mixture containing, among other proteins, native trypsinogen, was also purchased from Pharmacia.

RESULTS AND DISCUSSION

In this report, the native state of trypsinogen will be referred to as that conformation possessing the correct covalent structure, *i.e.* the disulfide bond arrangement present in protein which can be converted to active trypsin. Therefore, the term "refolding" will be employed to designate the reformation and interchange of disulfide

bonds occurring during the re-oxidation of the reduced protein, a process considered distinct in this study from the instantaneous reversible tertiary conformational changes experienced by the protein in response to changes in pH [25].

Refolded samples of bovine trypsinogen were generated following reduction of the commercially available native protein via the employment of a folding protocol derived from conditions suggested by Light and Higaki [21] and Jaenicke and Rudolph [20], as described in the Experimental section. Aliquots of material quenched with acid at various time points following the initiation of refolding were analyzed for activity via conversion of the protein to trypsin. By following the protocol described in this report, approximately 15% recovery of activity was observed after 22 h refolding. The low yield may in part be attributed to the formation of insoluble protein aggregates which were visible during refolding, and also to low levels of enzymatic degradation (occurring during reduction) caused by tryptic contamination.

Bovine trypsinogen is a basic protein, possessing a p*I* of *ca.* 9.3 in its native state [26]. Proteins of this nature have demonstrated a tendency to electrostatically adsorb to the negatively charged silanol groups present on the walls of silica capillaries, thereby seriously impairing the separation efficiency of these molecules during analysis by CE [27]. This phenomenon, together with poor recovery of the reduced and intermediate refolded species (which are known to exhibit pronounced hydrophobic character relative to native proteins), prevented the achievement of successful separations of the trypsinogen samples in both polyacrylamide- and octadecylsilane-derivatized capillaries in the presence of zwitterionic surfactant and non-ionic surfactant, respectively, at neutral pH. It has been demonstrated that basic proteins are separable by CE under acidic conditions (pH < 3), where the silica capillary walls are highly protonated and electrostatic adsorption is minimized [28]. Using this approach, 5 s injections of native and reduced trypsinogen were observed to separate successfully in a 25 m*M* sodium phosphate pH 2.0 buffer used in conjunction with a 37 cm bare silica capillary, 10 kV, and 200 nm UV detection (see Fig. 1). The minor peaks present in these separations were presumed to represent impurities in the commercially available material. Under these conditions, reduced trypsinogen was observed to migrate significantly slower than did the native protein. These results provide strong evidence for the dependence of the electrophoretic mobility of proteins, as observed via free-solution CE, upon the solvent-accessible charge. It appears that in the case of bovine trypsinogen, reduction of the molecule results in diminished basic character, perhaps through the exposure of acidic residues which are not accessible to the buffer when the protein is in its native, completely folded conformation.

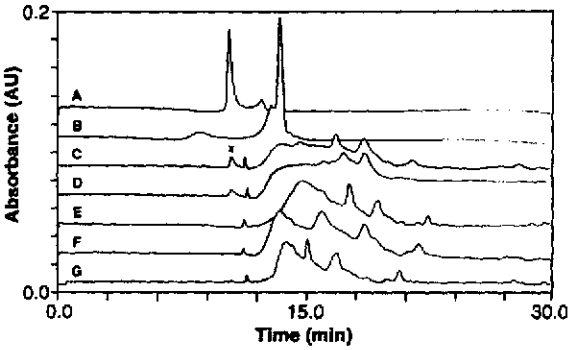

Fig. 1. Capillary electrophoretic separations of 5-s injections of 2 mg/ml samples of (A) native trypsinogen, (B) reduced trypsinogen, and (C) 22-h, (D) 7-h, (E) 1-h, (F) 30-min and (g) 10-min refolded trypsinogen obtained using 25 m*M* sodium phosphate pH 2.0 in a 37 cm, 50 μm bare silica capillary, 10 kV potential, and 200 nm detection. Refolded trypsinogen is marked by an "×".

Analyses of the refolded samples revealed a distribution of components, the majority displaying migration times equal to or greater than the reduced protein. These peaks are presumed to represent trypsinogen folding intermediates, the existence of which has been previously substantiated [21,22]. The distributions of electrophoretic mobilities displayed by the refolded species probably reflect differences in the solvent-accessible charges of these molecules. The viscous drag of the protein molecules as they migrate through the phosphate buffer appears to play at best a minor role in these separations, since trypsinogen folding intermediates have been demonstrated via size-exclusion chromatography (SEC)

to possess hydrodynamic radii significantly less than that of the completely unfolded protein [21,22]. It is important to note that a component which co-migrated with the native species (verified by spiking the refolded samples with native trypsinogen) was observed in the 1-, 7- and 22-h folded samples. This component probably represents completely refolded trypsinogen (corresponding to *ca.* 14% yield at 22 h), the presence of which was verified through the use of the activity assay.

Addition of 5% glycerine to 25 m*M* sodium phosphate pH 2.0 buffer facilitated an increase in the resolution of 10-s injections of the trypsinogen samples. These effects are visible in Fig. 2 where electropherograms (obtained using 10 kV) of the native, reduced, and 22-h refolded samples are displayed. The impact of the increased buffer viscosity seemed to be most evident in its effect upon the migration of the reduced protein, which possesses an extended unfolded conformation. In free solution the electrophoretic mobility of a macromolecule is expected to be inversely related to its frictional coefficient [29]. The frictional coefficient, in turn, depends upon not only the size and shape of a macromolecule, but also upon its interactions with the solvent (glycerine may associate hydrophilically with proteins). In the presence of 5% glycerine, reduced trypsinogen was observed to display a migration time approximately twice that of the native species.

Upon substitution of PEG, a hydrophilic nonionic polymer, for the glycerine in the separation buffer, resolution between the native and reduced trypsinogen increased to an even greater degree, and all of the partially refolded species displayed intermediate migration times (separations obtained using 30 kV), as presented in Fig. 3. The presence of PEG not only promoted a high viscosity, but may have also formed a network of intermeshed polymers which has been suggested to be capable of facilitating size-based macromolecule electrophoretic separations via sieving [30]. During the folding transition of a protein, the reduced form is converted to a distribution of rapidly interconverting conformations, some of which eventually may reach the compact and relatively fixed native state. At this time, the majority of these intermediate conformations are much more extended than the native conformation, but less extended than the reduced form. The order of migration of the native, reduced, and partially refolded trypsinogen appeared to correlate directly with the hydrodynamic radii of these species, as had been determined by studies employing SEC [21,22]. The viscosities of the phosphate buffer alone, the phosphate buffer with 5% glycerol, and the phosphate buffer with 5% PEG were determined

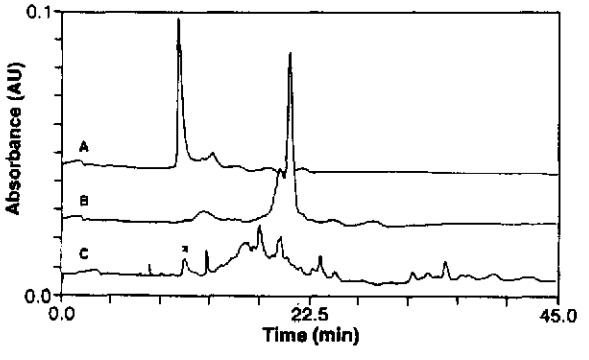

Fig. 2. Capillary electrophoretic separations of 10-s injections of 2 mg/ml samples of (A) native trypsinogen, (B) reduced trypsinogen and (C) 22-h refolded trypsinogen obtained using 25 m*M* sodium phosphate pH 2.0, 5% glycerine in a 37 cm, 50 μm bare silica capillary, 10 kV potential, and 200 nm detection. Refolded trypsinogen is marked by an "×".

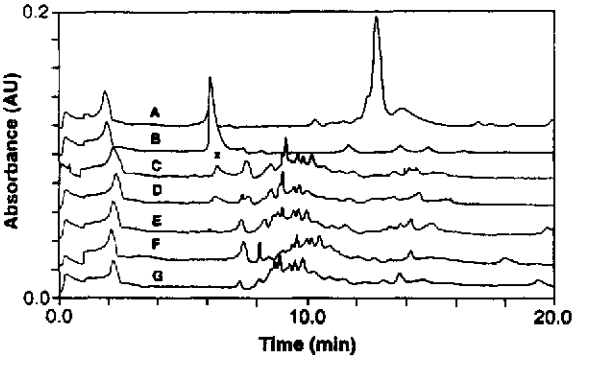

Fig. 3. Capillary electrophoretic separations of 15-s injections of 2 mg/ml samples of (A) reduced trypsinogen, (B) native trypsinogen, and (C) 22-h, (D) 7-h, (E) 1-h, (F) 30-min and (G) 10-min refolded trypsinogen obtained using 25 m*M* sodium phosphate pH 2.0, 5% PEG in a 37 cm, 50 μm bare silica capillary, 20 kV potential, and 200 nm detection. Refolded typsinogen is marked by an "×".

through the use of Poiseuille's law [31] and the CE instrument (the refractive index change occurring at the UV detector, zeroed *vs.* air, was used to measure the time required to pump buffer from the capillary inlet to the detector window) to equal 0.89, 1.01 and 4.60 cP, respectively (the viscosity of water at 25°C is 0.8904 cP [31]). From this data, it was not possible to determine whether viscosity effects or sieving, or a combination of the two, were responsible for the apparent size-based separation occurring in the buffer containing PEG.

Since Corbett and Roche [14] first demonstrated the displacement of sperm whale myoglobin toward shorter elution times following urea denaturation, SEC has been utilized to monitor the denaturation and refolding of a variety of proteins. Conformational changes in proteins are resolvable via SEC through changes in retention time, where distribution coefficients correlate with Stokes radii of protein conformers [14,32,33]. To compare high-performance SEC elution profiles of refolded trypsinogen with the CE electropherograms discussed earlier, acid-quenched refolded trypsinogen samples (50 μg/ml protein, 100-μl injections) were separated using a Superose 12 column and a mobile phase containing 100 m*M* acetic acid, 100 m*M* sodium chloride and 2 *M* urea at a flow-rate of 0.5 ml/min (absorbance monitored at 280 nm UV). The presence of urea was found to improve recovery of both the reduced protein and the partially refolded intermediates. Cysteine and cystine, two components present in the solutions of refolded material, appeared in the SEC chromatograms as large peaks appearing at *ca.* 35 min, following the elution of the trypsinogen conformers. The elution profiles displayed in Fig. 4 demonstrate the ability of the Superose packing to baseline resolve the native and reduced trypsinogen, and to provide partial resolution of the components present in the samples of refolded protein. Approximate quantitation of the material in the refolded samples demonstrated a 13% yield of completely refolded trypsinogen present after 22 h, in good agreement with both the activity assay and CE analysis.

A direct comparison of the SEC *vs.* CE analyses of refolded trypsinogen was achieved

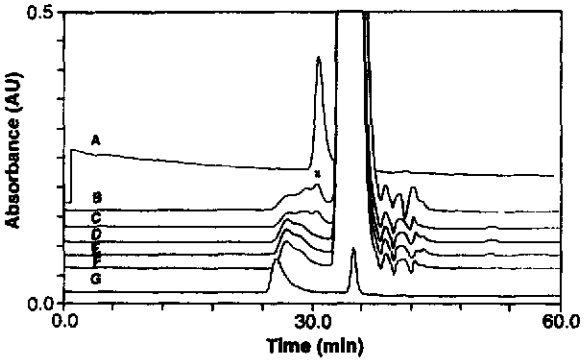

Fig. 4. SEC profiles of 50 μg/ml samples (100-μl injections) of (A) native trypsinogen, (B) 22-h, (C) 7-h, (D) 1-h, (E) 30-min, (F) 10-min refolded trypsinogen, and (G) reduced trypsinogen obtained via elution through a Superose 12 packing (30.0 × 1.0 cm) in the presence of 100 m*M* acetic acid pH 3.3, 100 m*M* sodium chloride, 2 *M* urea at a flow-rate of 0.5 ml/min monitored at 280 nm. Refolded trypsinogen is marked by an "×".

via isolation of the material corresponding to a 22-h refolded sample [concentrated 50-fold using a 150 ml OmegaCell 150 stir cell concentrator (Filtron, Northborough, MA, USA)] in four fractions (*ca.* 10 mg total protein collected), as indicated in Fig. 5a. The two later eluting peaks in the SEC profile were not fraction-collected, since they were determined to represent low-M_r components present in the fold solution (cysteine, cystine, urea, etc.). The fractions were dialyzed against 100 volumes of 1% acetic acid and then concentrated down to 1.0 ml final volume prior to analysis by CE. The CE buffer used for this study was 25 m*M* sodium phosphate pH 2.0, 5% PEG. The electropherograms of the SEC fractions displayed in Fig. 5b demonstrate, under these conditions, the dependence of protein electrophoretic migration time upon hydrodynamic volume. Refolded trypsinogen species appear to be present in fractions A–C (native trypsinogen is probably represented by the early-eluting peak in the electropherogram of fraction C), while the peaks generated by fraction D may represent peptides (from enzymatic degradation), or also cysteine or cystine.

High-performance ion-exchange chromatography has also demonstrated the ability to effect separations of various protein conformers under non-denaturing conditions [34–36]. The samples

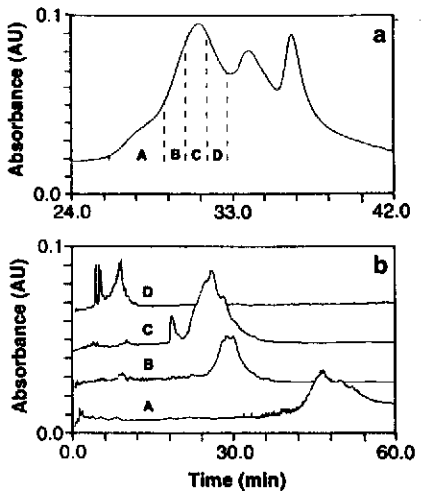

Fig. 5. (a) Fractionation of 0.5 mg of 22-h refolded trypsinogen, chromatographed using the conditions described in Fig. 4. (b) Capillary electrophoretic separations of 15-s injections of the dialyzed and concentrated SEC fractions (2.5 mg/ml final approximate total protein concentration) obtained using 25 m*M* sodium phosphate pH 2.0, 5% PEG in a 37 cm, 50 μm bare silica capillary, 10 kV potential, and 200 nm detection.

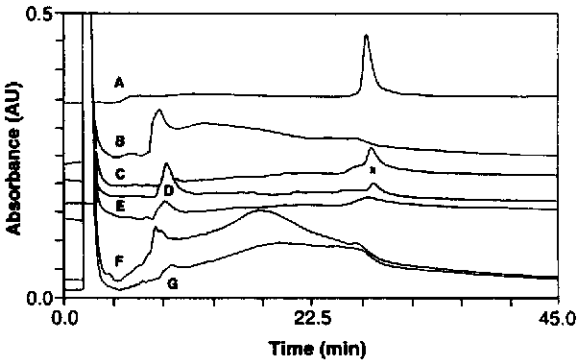

Fig. 6. Cation-exchange HPLC profiles of 50 μg/ml samples (100-μl injections) of (A) native trypsinogen, (B) reduced trypsinogen, (C) 22-h, (D) 7-h, (E) 1-h, (F) 30-min and (G) 10-min refolded trypsinogen obtained via elution through a Progel-TSK SP-5PW packing (7.5 × 0.75 cm) in the presence of 25 m*M* sodium acetate pH 4.0, 2 *M* urea, 20% acetonitrile and a 45 min 0.00–0.35 *M* sodium chloride gradient at a flow-rate of 1.0 ml/min monitored at 280 nm. Refolded trypsinogen is marked by an "×".

of refolded trypsinogen made for this study were separated using a Progel-TSK SP-5PW cation-exchange packing, a 25 m*M* sodium acetate pH 4.0, 2 *M* urea, 20% acetonitrile mobile phase with a 45-min linear gradient from 0 to 0.35 *M* NaCl, and a 1.0 ml/min flow-rate at ambient temperature. The presence of both the urea and acetonitrile was found to be necessary for the recovery of the reduced protein and the partially refolded intermediates. The separation profiles obtained under these conditions are presented in Fig. 6. The chromatograms reveal that the reduced protein elutes much earlier than the native conformer, suggesting that, in agreement with the CE results discussed earlier, reduced trypsinogen possesses significantly less basic character than does the native protein. The refolded samples appear in these separations as a distribution of components eluting between the reduced and native species. Quantitation of the peak co-eluting with the native protein (at *ca.* 29 min) indicated a 17% yield of completely refolded material in the 22-h sample.

Perhaps the technique most comparable to the use of viscous/sieving buffers employed in conjunction with CE for the separation of proteins is non-denaturing slab gel electrophoresis. Non-denaturing (native) gel electrophoresis has been successfully employed for the analysis of the conformations of proteins. Studies using non-denaturing gel electrophoresis have demonstrated that the reduction of the disulfide bonds in a protein lead to the formation of an extended unfolded conformation with reduced electrophoretic mobility [37,38]. Non-denaturing slab gel separations of the refolded trypsinogen samples generated for this study are displayed in Fig. 7. The outer lanes on both gels were loaded with a 2 mg/ml native trypsinogen standard, and the lanes adjacent with 2 mg/ml reduced protein. It was evident that the native conformer migrated much further through the gel matrix than did the reduced. A distribution of folding intermediates appeared as a stained haze migrating within the region between the native and reduced protein, and a band co-migrating with the native standard, probably representing completely refolded conformers, was visible in the lanes representing the 7- and 22-h folded samples. These observations correlated well with the CE and chromatography results discussed previously.

The majority of charged groups in native globular proteins, both acidic and basic, are

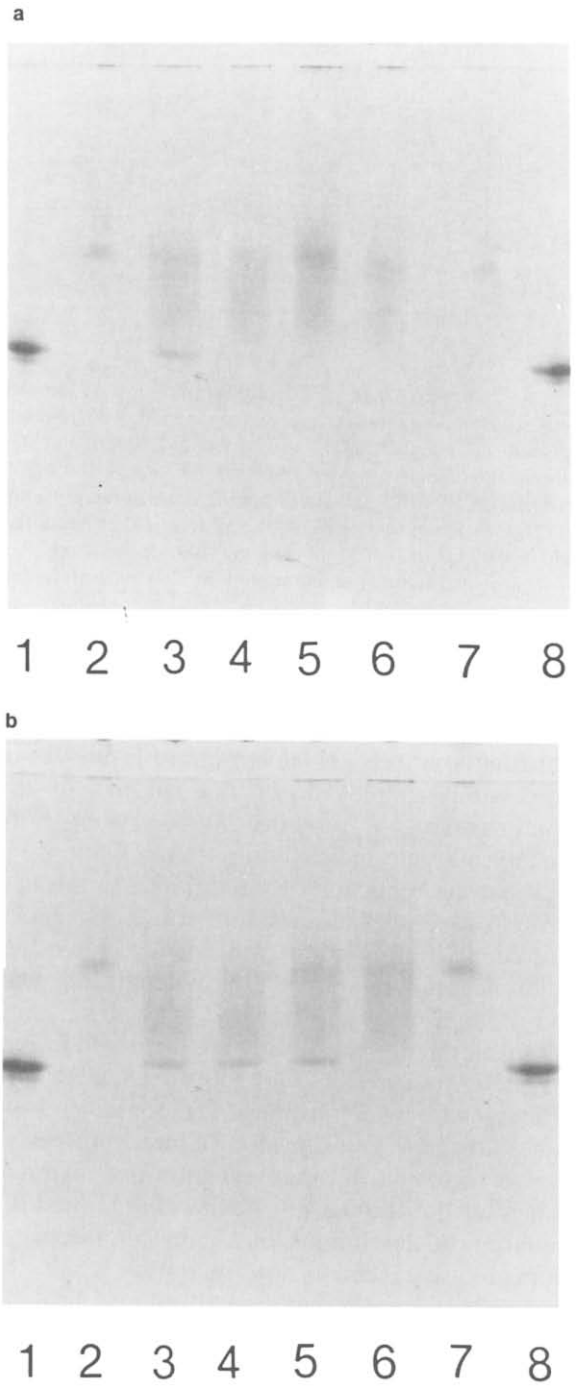

Fig. 7. Non-denaturing gel electrophoresis of samples (2 mg/ml, 1 μl loaded) of (lanes a1, a8, b1, b8) native trypsinogen; (a2, a7, b2, b7) reduced trypsinogen; (a6) 10-min refolded; (a5) 30-min refolded; (a4, b6) 1-h refolded; (a3, b5) 7-h refolded; (b3, b4) 22-h refolded trypsinogen obtained in Homogenous 20 PhastGels.

located on the surface of the molecules and are freely accessible to the solvent. However, some may be buried within the interior and only become accessible when the molecule is unfolded or denatured. In such cases the isoelectric point will then be altered as the protein is unfolded and these groups become exposed. Thus isoelec-

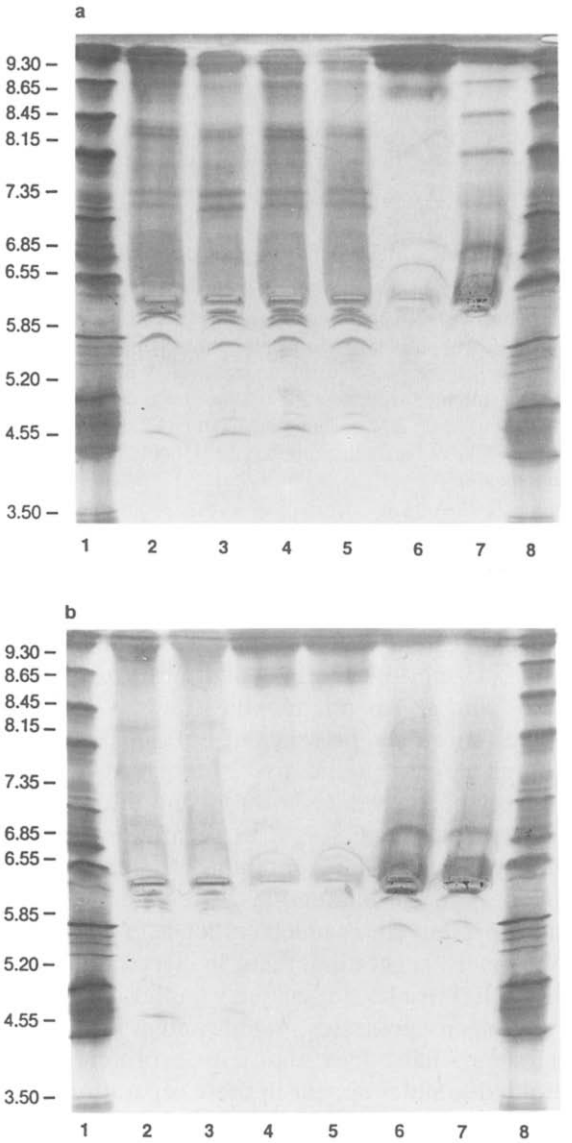

Fig. 8. Isoelectric focusing of samples (2 mg/ml, 1 μl loaded) of (lanes a1, a8, b1, b8) p*I* markers; (a7, b6, b7) reduced trypsinogen; (a6, b4, b5) native trypsinogen; (a5) 10-min refolded; (a4) 30-min refolded; (a3) 1-h refolded; (a2) 7-h refolded; (b2, b3) 22-h refolded trypsinogen obtained in PhastGel IEF 3–9 gels.

tric focusing (IEF), which is a highly sensitive technique in which changes in p*I* as small as 0.001 can be distinguished, can be used to study conformational changes [21,39–41]. The refolded trypsinogen samples of this study were analyzed by focusing on a p*I* 3–9 IEF PhastGel, and the results are presented in Fig. 8. Reduced trypsinogen (p*I* 6.5) was well resolved from native trypsinogen (p*I* 9.3). A dark well-focused band representative of the native species was evident at the top of the gels, its position aligning well with the trypsinogen present in the commercially available p*I* marker mixture. The minor bands observed in the native and reduced trypsinogen standards may represent impurities also observed in the CE electropherograms (see Figs. 1–3) (the reduced trypsinogen in Fig. 8a may also have been contaminated with the p*I* markers run in the adjacent lane). A number of components in the p*I* range of 6.5–9.3, and also several with p*I* values <6.0, were detected as the reduced trypsinogen underwent folding. A minor component focusing at p*I* ≈ 9 appeared in the reduced and early time point refolded samples, and perhaps corresponded to a contaminant possessing a p*I* ≥ 9; however, the bands focusing in this region darkened significantly as folding time increased, providing qualitative evidence for refolding of the protein to its native form.

CONCLUSIONS

By monitoring the re-oxidation of reduced bovine trypsinogen, CE proved to be an effective method capable of separating and quantitating native and reduced protein, and also intermediate conformations present during the refolding. The presence of components such as glycerine and PEG in the CE buffer was observed to facilitate size-based separations via viscosity and/or sieving effects. The progress curves for the regeneration of native trypsinogen calculated from four methods of analysis, enzyme activity, CE, SEC and cation-exchange HPLC, were in excellent agreement with one another, as displayed in Fig. 9. CE analyses also correlated well with qualitative separations obtained via non-denaturing gel electrophoresis and IEF. The results of this study suggest that

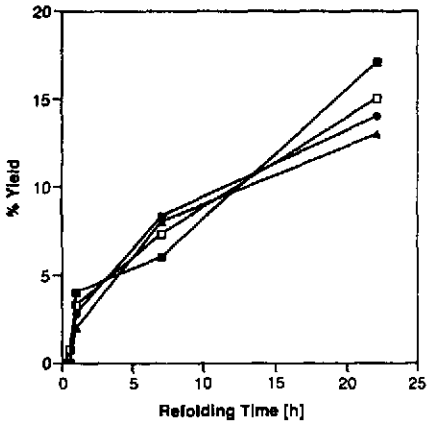

Fig. 9. Rate of appearance of refolded trypsinogen from analyses via (□) enzymatic activity following activation with trypsin, (▲) SEC, (■) cation-exchange HPLC, (●) CE in 25 m*M* sodium phosphate pH 2.0, 5% PEG.

CE can serve as a simple, rapid, and powerful analytical method capable of detecting and resolving changes in protein covalent structure. In this capacity, it may provide information crucial to the analysis of the protein folding process.

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