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Conformational transformations of the fusion protein BR96 sFv-PE40 as monitored by micellar electrokinetic capillary chromatography and circular dichroism

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Abstract

Three isoforms of BR96 sFv-PE40, a single-chain fusion protein, were separated by micellar capillary electrokinetic chromatography (MECC) using cholic acid as a micelle-forming surfactant. Two well-known modifiers of protein structure guanidine hydrochloride and trifluoroethanol altered the separation profile in a concentration-dependent manner. In parallel, conformational changes of BR96 sFv-PE40 were monitored by near- and far-UV circular dichroism measurements. In general, a good correlation was found between structural transformations and alterations in the electrophoretic profile. It was suggested that differences in the electrophoretic mobilities of closely-related species, such as isoforms and/or conformers, may result from differences in exposure of polar and hydrophobic elements on the exterior of the protein globule via formation of distinct complexes between the micelles and protein molecules. Application of MECC to protein analysis offers a unique tool for separation and quantitation of closely related species such as isoforms and conformers.

Keywords: Circular dichroism; Proteins; Fusion proteins

1. Introduction

In recent years capillary electrophoresis (CE) has emerged as a powerful and versatile analytical technique due to its small sample requirements, high sensitivity, speed, reproducibility, high theoretical plate count and automated instrumentation. Following the explosive growth of biotechnology, CE has found new applications in the rapid separation and analysis of a wide range of biologically important molecules, in particular, proteins and peptides. Procedures are available that allow separation of native and denatured forms of proteins by free-solution CE [1–3], MECC [4,5] and other techniques. The second technique was also successful in the separation of different glycoforms of a number of glycoproteins [6].

BR96 sFv-PE40 is a single-chain immunotoxin fusion protein targeted to a carbohydrate receptor composed in part by the Le^y (Lewis-Y) antigen. The polypeptide chain includes the randomly linked variable regions of BR96 antibody (V_H and V_L connected via a 15 amino acid flexible linker), followed by a seven amino acid spacer and domains II, Ib and III of *Pseudomonas* exotoxin (PE40) [7]. The high potency, excellent antitumour activity, and mechanism of action distinct from that of drug conjugates make BR96 sFv-PE40 a very promising

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agent in treatment of human carcinomas. Monitoring structural integrity constitutes a significant part in structural identification and control of the recombinant proteins.

Our earlier study [5] showed that application of the MECC technique to a monoclonal human antibody BR96 resulted in a four-peak pattern at room temperature and pH 9.4. The separated species were shown to be not a result of carbohydrate moiety heterogeneity or partial oxidation/deamidation, but rather different forms of the same primary structure. Heat treatment with and without sodium dodecyl sulfate induced interconversions between the species, and this result correlated well with the observed changes in the antibody secondary structure. One of the possible explanations of the separation of a homogeneous protein population into multiple species is that the detected isoforms are different conformers of the same primary structure. It was also shown that the equilibrium between the isoforms was preserved during migration of the antibodies through the micelle-containing running buffer within the capillary at room temperature both for intact and heat-stressed immunoglobulin molecules.

In this study, we compare the effects of two different secondary and tertiary structure modifying agents on the BR96 sFv-PE40 electrophoretic migration pattern and circular dichroism spectra. Guanidine (GdmCl) and trifluoroethanol (TFE) were chosen because of their pronounced, but different and even in some respects opposite, effects on protein conformations.

2. Experimental

2.1. Reagents and materials

Sodium hydroxide 10 *M* solution, potassium phosphate and sodium borate crystals were obtained from Fisher Chemical (Fair Lawn, NJ, USA). Dodecyl sulfate, sodium salt and 2,2,2-trifluoroethanol, NMR grade were from Aldrich (Milwaukee, WI, USA). Guanidine hydrochloride, sequanal grade, was purchased from Pierce (Rockford, IL, USA). Pre-treated capillary cartridges were purchased from Beckman Instruments (Palo Alto, CA, USA). The protein used in this investigation was BR96 sFv-PE40 (Bristol-Myers Squibb, Syracuse, NY, USA). HPLC-grade water was from Baxter Healthcare (McGaw Park, IL, USA) was used in the preparation of the sample and buffer solutions.

2.2. CD instrumentation and method

Structural changes in the BR96 sFv-PE40 were monitored using near- and far-UV circular dichroism as previously described [5]. In brief, spectra were taken with a Jasco spectropolarimeter, Model J-710 (Jasco International, Easton, MD, USA). A cylindrical cuvette of 0.1 mm light path length was used for the far UV and a cylindrical cuvette of 1 cm light path length was used for the near-UV range measurements. The BR96 sFv-PE40 was dissolved in 50 m*M* phosphate buffer (pH 7.4) at concentrations 0.7–1.0 mg/ml. The protein was incubated for at least 10 min at each GdmCl or TFE concentration before CD measurement. The spectra, taken at a time constant of 16 s and a scanning rate of 10 nm/min were signal-averaged four times for both samples and buffer blanks. After the buffer spectrum had been subtracted, the results were converted into the molar ellipticity using a molecular mass of the BR96 sFv-PE40 of 67 106 Da and a total number of amino acid residues of 622. UV absorbance spectra of the protein solutions, containing the desired GdmCl and TFE concentrations, were obtained by converting channel 2 voltage into absorbance units using Jasco data analysis software. BR96 sFv-PE40 concentrations in each solution were calculated using the absorbance at 280 nm and an absorptivity of 1.30.

2.3. CE instrumentation and method

All CE separations were performed on a Beckman P/ACE 2100 capillary electrophoresis system (Palo Alto, CA, USA) with a personal computer IBM PS/2 utilizing P/ACE software and a Microsoft Windows interface. Pre-treated capillary cartridges were purchased from Beckman Instruments (Palo Alto, CA, USA). Separations were carried out with fused-silica capillary cartridges 57 cm in length (50 cm to detector) with an internal diameter of 75 μm (Beckman Instruments, rinsed by the manufacturer with 0.1 *M* NaOH). All electrophoretic separations were performed using a 12 m*M* borate buffer (pH 9.6)

containing 16 m*M* cholic acid. Samples were transferred to P/ACE microvials contained in a sample holder and applied to the capillary as described below. Upon completion of each sample analysis, the capillary was cleaned with a high-pressure rinse of 0.1 *M* sodium hydroxide solution for 0.5 min, followed by a high-pressure rinse of the separation buffer solution for 0.5 min. Samples were injected by a positive nitrogen pressure of 6.2×10^5 Pa (90 psi) for 2 s. The components of the BR96 sFv-PE40 samples were then separated by a voltage of 30 kV (600 V/cm) at 25°C.

3. Results and discussion

In our earlier study, application of the MECC technique to the analysis of the BR96 antibody led to an interesting observation that a homogenous population of the immunoglobulin manifested itself on the electropherograms as a multiple peak pattern [5]. The peaks can be interconverted and transformed into a single species upon thermal denaturation. The exact identity of the separated species remains unknown, although multiple isoform presence is shown to result not from microheterogeneity of the carbohydrate moiety or partial deamidation of Asn/Gln residues, but rather from conformational diversity.

Guanidinium ion is a well-known denaturant, which disrupts a protein's structural stability when added to aqueous solutions; at high concentrations it generates true random coils from the vast majority of globular proteins [8]. Despite a broad use and numerous studies, the exact mechanism of GdmCl-induced unfolding is not well understood due to its multiple effects on protein molecules and surrounding solvent. Nevertheless, it is clear that the denaturant interacts with both polar and nonpolar surfaces of proteins, decreasing the magnitude of the hydrophobic interactions and weakening intramolecular hydrogen bonds. Based on the hypothesis that the MECC-separated isoforms of BR96 sFv-PE40 are various conformers, we expected that GdmCl treatment would result in the formation of new protein states which are electrophoretically distinct from the native conformers or would shift an equilibrium between the existing species.

Unlike our previous work, the attempts to separate BR96 sFv-PE40 isoforms by using sodium dodecyl sulfate micelle-containing buffer were unsuccessful; the protein under this condition migrated as a single species. Utilization of cholic acid as a micelle-forming surfactant resulted in a typical MECC electropherogram of BR96 sFv-PE40 represented by a three-peak pattern (Fig. 1). An effect of GdmCl on migration time of 1 MECC-separated species can be observed starting at concentrations as low as 0.1 *M*. It is important to emphasize that in all MECC experiments the running buffer composition remained the same as described in Section 2 and all perturbations of the protein structure were carried out only in the sample buffer. Within the GdmCl concentration range of 0.1–0.5 *M* the slowest migrating isoform (isoform 3) gradually transforms into the fastest species (isoform 1). When the GdmCl concentration increased to 1 *M*, the three-peak pattern is replaced by a single broad asymmetrical peak, with the electrophoretic mobility about 2% lower than the isoform 1 but higher than that for the second species (isoform 2) of the intact BR96 sFv-PE40. The second isoform apparently merged with the preceding peak contributing to the broadening of its base. Concentrations of 3 *M* and higher transform the single peak into a later migrating irregular-shaped bump.

Conformational changes in BR96 sFv-PE40 were monitored by measurement of the protein circular dichroism (CD) spectra in the near- and far-ultraviolet (UV) region. The far-UV CD spectrum demonstrates a pronounced alpha-helical component with a characteristic minimum at 220 nm, attributed to the PE40 protein part of the molecule. The remaining part of the BR96 sFv-PE40 is composed of variable regions of heavy and light chains of the BR96 antibody and contributes to the beta-structured component of the secondary structure. The guanidinium ion effect on the BR96 sFv-PE40 secondary structure becomes noticeable at concentrations of 0.25–0.5 *M* (Fig. 2A). In aqueous GdmCl solutions with increasing concentrations, the ellipticity minimum at 220 nm shifts to a shorter wavelength characteristic of random coil structure, thus indicating complete loss of the original ordered structure at GdmCl concentrations of 3 *M* and higher. Interestingly, CD spectra in the near-UV region reveal the two-phasic

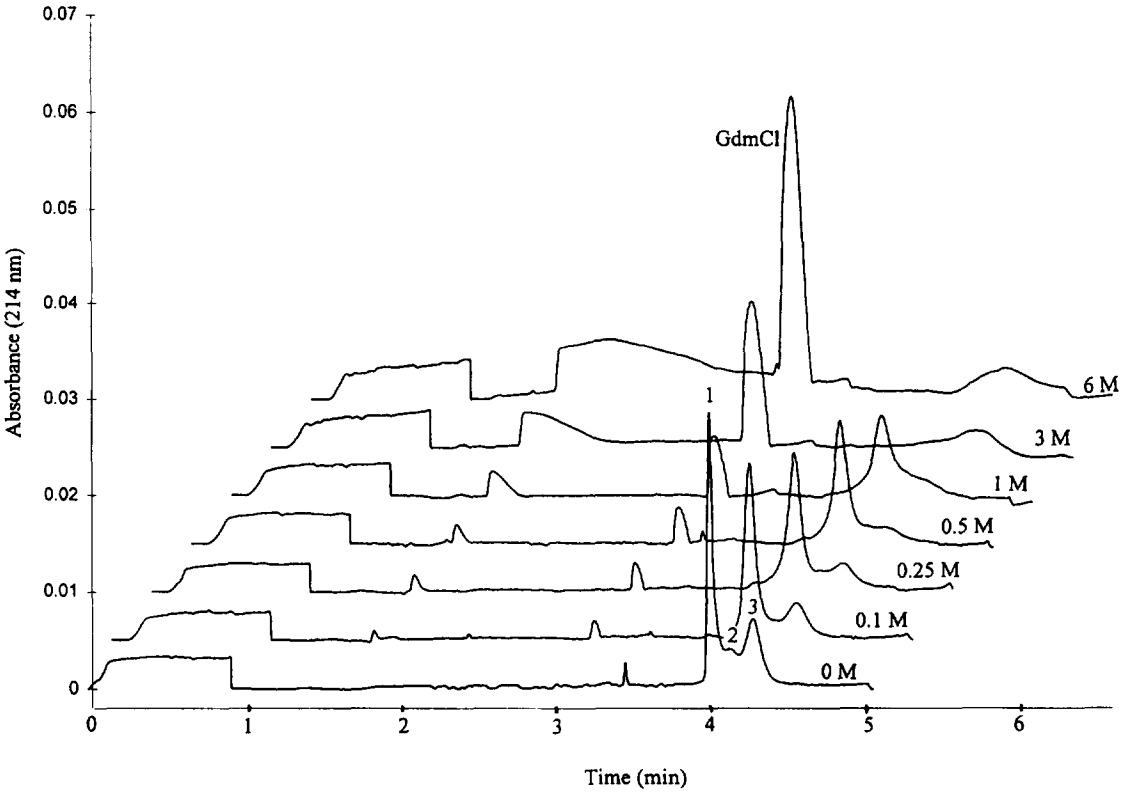

Fig. 1. Electropherograms of BR96 sFv-PE40 in the presence of different concentrations of guanidinium chloride.

effect of GdmCl on the tertiary structure of BR96 sFv-PE40: a slight increase in the aromatic residue band intensities within the concentration range 0.1–0.5 *M* with subsequent weakening of the CD signals at GdmCl concentrations of 1–6 *M* (Fig. 2B). In the 255–310 nm spectral zone, the CD band centered at 291 nm (related to tryptophan chromophore) was less sensitive to the presence of low concentrations of guanidinium ion than that of the CD bands at 260–275 nm (related to tyrosine and phenylalanine residues). Apparently, the tyrosine and phenylalanine chromophore surroundings were more readily accessible to the perturbants than the tryptophan groups. The complex band pattern observed in the near-UV CD spectrum almost completely vanished in the corresponding spectra of the GdmCl-denatured BR96 sFv-PE40 at a guanidine hydrochloride concentration of 3 *M* and above. The resulting CD spectrum does not, however, coincide with the baseline, indicating some residual asymmetric environment around the various aromatic amino acids. Four disulfide bonds remaining intact during GdmCl-denaturation probably prevented the protein molecules from complete unfolding.

Comparison of the guanidinium ion effects on electrophoretic migration and structural characteristics of BR96 sFv-PE40 reveals a strong parallelism, since both techniques demonstrate biphasic, concentration-dependent effects. The presented electropherograms (Fig. 1) show that peak 3 transforms into isoform 1 within the same GdmCl concentration range with the increase in the CD band intensities of the aromatic amino acid residues (Fig. 2B). Subsequent increases in guanidinium ion concentration reverse both these trends. Disordered at higher GdmCl concentrations (1–6 *M*), BR96 sFv-PE40 molecules (Fig. 2) appear as an irregularly shaped bump on the electropherograms (Fig. 1).

2,2,2-Trifluoroethanol (TFE) is widely used as an alpha-helix promoting cosolvent. The dielectric con-

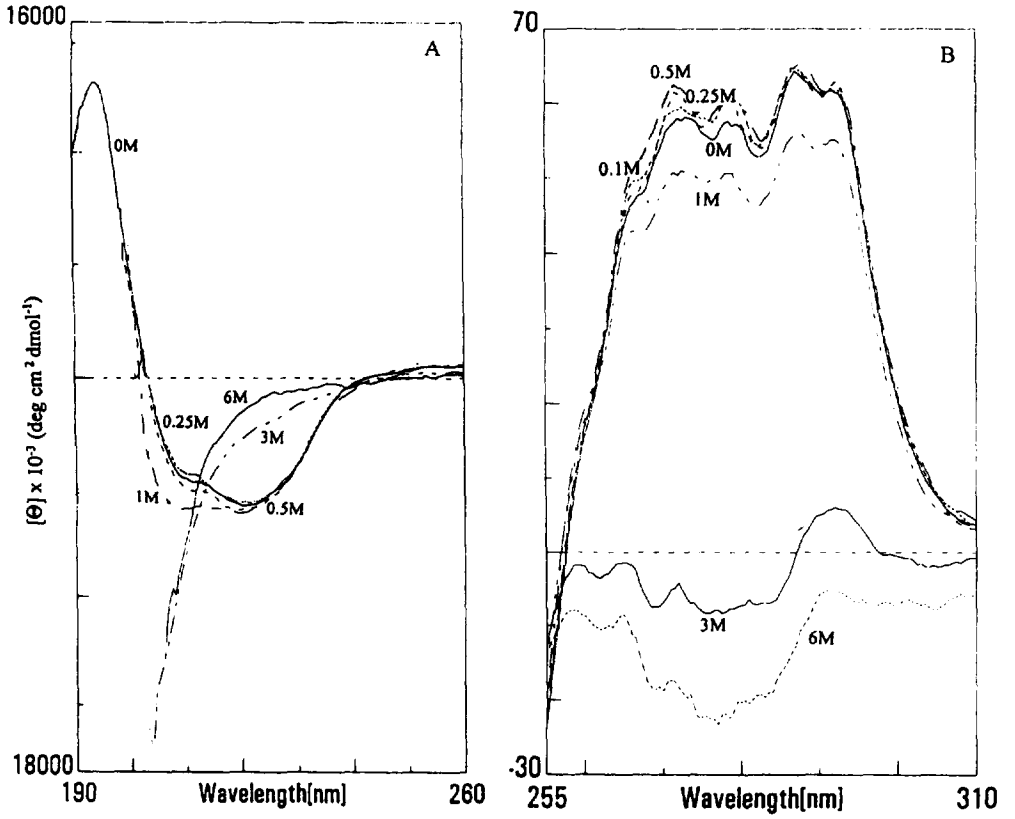

Fig. 2. The effect of different concentrations of guanidinium chloride on molar ellipticity spectra of BR96 sFv-PE40 in the (A) far-UV, and (B) near-UV regions.

stant of TFE is about one-third of that of water, which more closely approximates the hydrophobic interior of a typical protein. The lower dielectric constant strengthens interactions between charged groups. In diluted aqueous solutions, TFE increases the proteins' helicity by selectively destabilizing solvent-exposed amide groups with the consequence that more favourable compact conformations (such as helixes that maximize intramolecular amide–amide hydrogen bonding and minimize amide solvent exposure) are selectively favoured [9]. Nevertheless, the predominant effect of TFE is caused by its significantly weaker basicity. Hydrogen bonding of amide protons to the solvent is decreased, which strengthens intramolecular hydrogen bonds, and, therefore, stabilizes secondary structure. Furthermore, TFE is a less polar and more hydrophobic solvent than water. It interrupts hydrophobic interactions and can act as a denaturant of tertiary and quaternary structure. Therefore, unlike the guanidinium ion, TFE has a destabilizing effect on hydrophobic elements and increases the helical component of the secondary structure, which in concert would be expected to result in conformations different not only from the native forms, but also from the GdmCl-denatured forms.

Fig. 3 demonstrates differences in the protein distribution between MECC-separated species upon the addition of different concentrations of TFE to the sample buffer solution. TFE concentrations below 2% have no effect on the distribution pattern. Significant changes can be observed at TFE concentration of 4% and higher. The fastest-migrating species 1 gradually transforms into the slowest-migrating isoform 3 upon incremental increase in TFE content, while the concentration of the intermediate species 2 remains practically unchanged (Fig. 3). At a TFE concentration of 7.5%, isoform 3 exceeds the

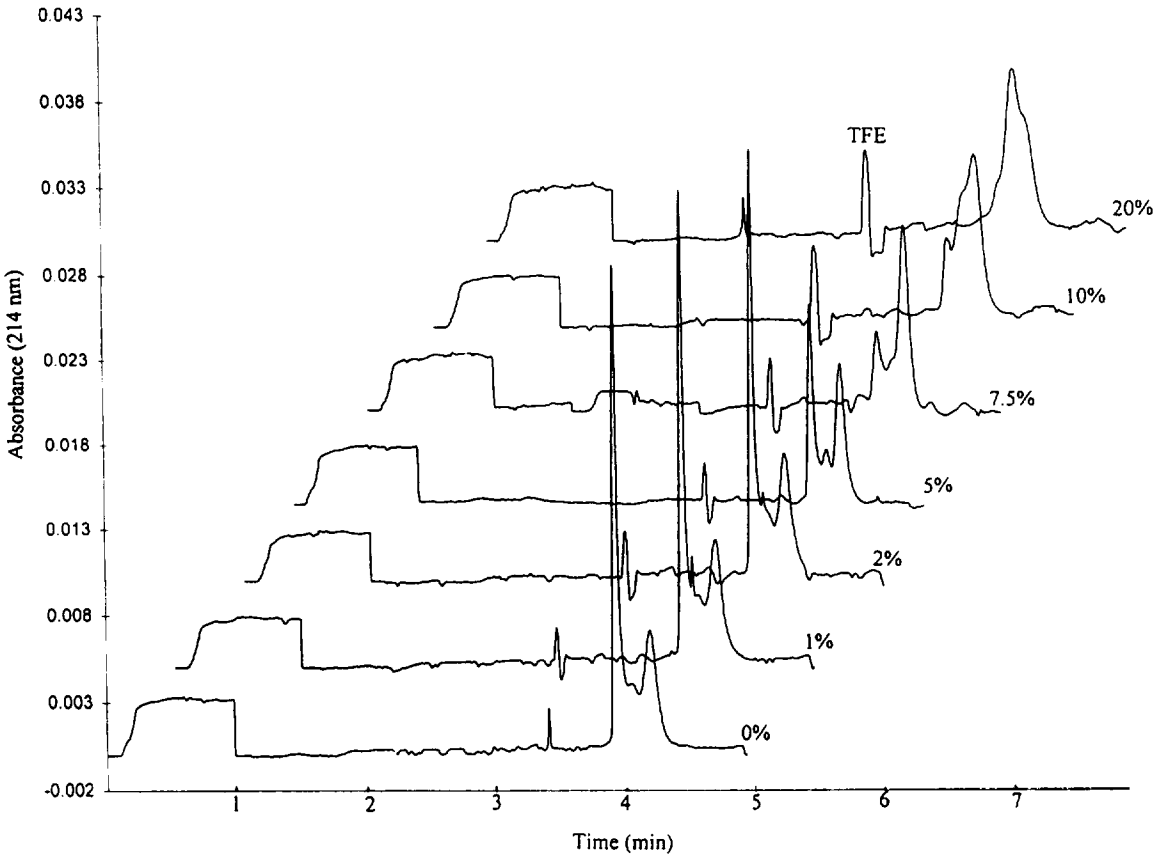

Fig. 3. Electropherograms of the BR96 sFv-PE40 in the presence of different concentrations of TFE.

abundance of the two other faster-migrating species. Additional increases in TFE concentrations up to 10% further diminished the relative abundance of species 1 and promoted growth of isoform 2 which appeared as a shoulder to species 3. This trend continues at 20% TFE, where isoform 1 completely disappeared, and species 2 dominated the separation pattern (Fig. 3). These TFE-induced transformations of the BR96 sFv-PE40 isoform separation pattern are markedly distinct from the ones induced by the guanidinium ion.

Fig. 4A shows the far-UV CD spectra of the BR96 sFv-PE40 as a function of increasing concentrations of TFE. The minima near 208 and 220 nm are characteristic of helical secondary structure. Interestingly, the BR96 antibody-derived portion of the molecule was shown to possess a pronounced beta-structure [5,10]. No changes in the BR96 sFv-PE40 secondary structure were observed in the presence of low concentrations (1–2% v/v) of TFE. A slight increase in the negative band intensities became noticeable at a concentration of 5% TFE. At concentrations above 5% TFE, the bands negativity grows within the wavelength range 203–240 nm, apparently due to increasing helicity. The molar ellipticity of BR96 sFv-PE40 at wavelength 220 nm in 20% TFE-containing buffer was about 1.5 fold more negative than that of the native conformation of the protein. Furthermore, incremental addition of TFE resulted in an increase of the intensity of the 194 nm positive band with a slight shift of this band to shorter wavelengths. The isodichroic point near 203 nm suggests that TFE-induced secondary structure transitions are mainly cooperative. CD measure-

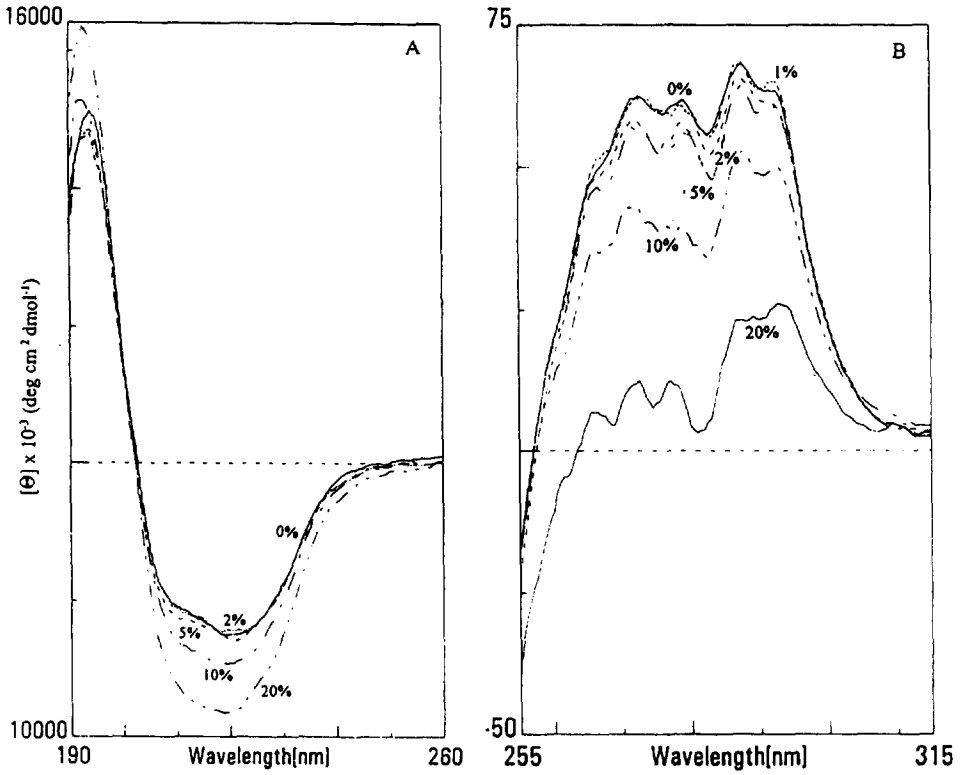

Fig. 4. The effect of different concentrations of TFE on the molar ellipticity spectra of BR96 sFv-PE40 in the (A) far-UV, and (B) near-UV regions.

ments at higher TFE concentrations were not possible to carry out due to precipitation of the protein under these conditions.

The CD spectra of BR96 sFv-PE40 in the near-UV range demonstrated a gradual decrease of the tertiary structure signals as the TFE concentration increased (Fig. 4B). Up to TFE concentrations of 2% no effect on the CD spectra of the aromatic amino acid surroundings could be detected. A noticeable decrease in the positive band intensities was observed at a TFE concentration of 5%. The positive CD bands at 260–275 nm were almost completely lost at 20% TFE concentration, whereas the signal around 291 nm (related to the tryptophan absorbance) decreased in its magnitude by a factor of ca. 2.5. Apparently, the hydrophobic environments in the vicinity of tyrosine and phenylalanine chromophores were more readily susceptible to the TFE perturbations than the tryptophan surroundings. The obtained CD data demonstrate that the TFE-induced helicity of the secondary structure coincides with the disruption of the hydrophobic regions in the protein molecules. These effects find strong parallelism with the changes in electrophoretic mobilities of the MECC-separated species.

The observed alterations in the MECC separation pattern and structural characteristics of BR96 sFv-PE40 occur in the same concentration intervals of TFE or GdmCl. It is reasonable to suggest that the observed changes in the electrophoretic migration rates and CD spectra resulted from the same structural modifications of the protein. Throughout the entire concentration intervals the effects of both utilized modifiers on the protein secondary and tertiary structure are profoundly different, whereas their effects on the electrophoretic separation profile are more complicated. At low concentrations, TFE increases the content of isoform 3 while GdmCl

increases the content of the faster-migrating species. Both these facts correlate well with the different transformations incurred by the agents in the BR96 sFv-PE40 structure. However, at high concentration, TFE reverses the trend, and at a TFE concentration of 20% the MECC separation profile appears to be similar to that in the presence of 1 *M* GdmCl (Figs. 1 and 3), although there may be a slight difference in the migration time midpoints and shapes of the major peaks. Under these conditions the structural characteristics of BR96 sFv-PE40 are significantly different (Figs. 2A, 2B, 4A and 4B); therefore, the similarity in the migration profiles of these two structurally different states can be attributed to the limitations in the separation power of the applied MECC method.

We believe that interactions between detergent-formed micelles and protein molecules represent a decisive factor in MECC separation. Differences in exposure of polar and hydrophobic structural elements to the solvent may ensue in the formation of disparate protein micelle complexes and subsequently result in different electrophoretic migration rates. In principle, any two conformers can be separated providing an appropriate detergent is found. However, technically, finding the optimal conditions for separation of the closely related species represents a challenging task. We were unable to attain sufficient separation of the BR96 sFv-PE40 isoforms by applying sodium dodecyl sulfate as a micelle-forming surfactant, although this approach was successful in the separation of five isoforms of the monoclonal antibody BR96 [5] and three isoforms of CTLA4Ig, an immunoglobulin fusion protein [11]. Obviously, any given detergent has its own limitations in its ability to discriminate between closely related species; therefore, it is unrealistic to expect development of a universal approach for electrophoretic separation of all isoforms or conformers. Instead, it seems more likely that MECC separates protein fractions which do not necessarily represent individual conformers, but rather are composed of mixtures of conformers which form similar complexes with micelles and are indistinguishable under a particular set of separation conditions. For example, in the presence of 20% TFE, the BR96 sFv-PE40 is characterized by increased helicity and significant perturbation of its hydrophobic elements (Fig. 4). In the presence of 1 *M* GdmCl, the BR96 sFv-PE40 aromatic amino acids are perturbed to a much lesser extent, but this is accompanied by a substantial unfolding of the polypeptide backbone (Fig. 2). Both effects may lead to the formation of a similar polar/hydrophobic balance on the exterior of the protein globule to the balance in the 20% TFE-containing buffer, resulting in similar protein–micelle bindings. In general, interaction of the detergent-formed micelles with proteins is not considered to be structure specific, although some differences in surface charge distribution and hydrophobicity result in the formation of several different types of micelle-protein complexes, and, therefore, can be electrophoretically separated. Apparently, the number of the types of such complexes is limited for a given protein and a selected surfactant and this number determines the number of electrophoretically separable species.

4. Conclusion

Application of MECC for protein analysis offers a unique and powerful tool capable of separating structurally similar isoforms and/or conformers. Despite obvious limitations in separation and difficulties in species identification, the technique enables the separation and quantitation of closely-related species which would be indiscernible using other analytical methods.

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