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Structural and conformational variants of human β_2-microglobulin characterized by capillary electrophoresis and complementary separation methods

Niels H.H. Heegaard[a,*], Luca Rovatti[b], Mogens H. Nissen[c], Mahmoud Hamdan[b]

[a]*Department of Autoimmunology, Statens Serum Institut, Copenhagen, Denmark*
[b]*Computational, Analytical and Structural Sciences, GlaxoSmithKline, Verona, Italy*
[c]*Institute of Medical Anatomy, Copenhagen, Denmark*

Abstract

The small (M_r=11 729) serum protein β_2-microglobulin is prone to precipitate as amyloid in a protein conformational disorder (PCD) that occurs in a significant number of patients on chronic hemodialysis. Analyses by capillary electrophoresis (CE) were undertaken to study β_2-microglobulin conformations under native separation conditions and showed an apparent heterogeneity of purified preparations when the sample matrix included organic solvents such as acetonitrile, trifluoroethanol and ethanol. We here present LC–MS, CE–MS, and CE studies of changes of separation profiles as a function of capillary temperature, organic solvent concentration, and analysis time. The results suggest that the apparent β_2-microglobulin heterogeneity observed by CE is caused by two distinct protein conformations that are present in β_2-microglobulin under partly denaturing conditions and that Met^{99}-oxidized and normal (i.e. nonoxidized) β_2-microglobulin behave similarly with respect to the potential to attain this alternative conformation. CE is an attractive method to study early and intermediate soluble folding variants that may be involved in PCDs and CE thus may have an important role as a tool for understanding other PCDs characterized by amyloid deposition.
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1. Introduction

Despite its numerous specific virtues [1–8] capillary electrophoresis (CE) in its usual configurations is not a preparative technique and does not readily allow specific probing of separated analytes, e.g. with antibodies, to identify the components of an analyte mixture. This is aggravated by the superior and unique resolving capabilities of CE that often reveal inhomogeneities in preparations deemed homogeneous by other analytical separation techniques such as gel electrophoresis and liquid chromatography. Also, a low detector wavelength is usually necessary because of the mediocre concentration detection limits offered by standard CE–UV-absorbance detectors and many small molecules (e.g. sample solvent salts) absorb at these wavelengths and are not necessarily separated from larger sample molecules because separation in CE is mainly dependent on charge [9–12]. The unique separation characteristics of CE [1–3] also mean that other

*Corresponding author. Bldg. 81, Rm. 536 Statens Serum Institut Artillerivej 5 DK-2300 Copenhagen S, Denmark. Tel.: +45-3-268-3378; fax: +45-3-268-3876.
E-mail address: nhe@ssi.dk (N.H.H. Heegaard).

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inhomogeneities may be revealed only by CE because there are not sufficient differences with respect to mass and/or hydrophobicity to allow discrimination by traditional techniques.

Thus, it is often seen that a single peak isolated by RP-HPLC, e.g. from a tryptic digest of a protein, contains multiple peptides when subsequently analyzed by CE [13,14]. Conversely, it is conceivable that an analyte appearing homogeneous by CE is heterogeneous by another separation technique. This point is illustrated and investigated in the present study of native β_2-microglobulin (β_2m) and organic solvent-treated β_2m that complements previously published work on conformations of β_2m and β_2m-variants [15–17]. β_2m is of clinical interest because it is involved in complications such as carpal tunnel syndrome and joint disease arising in patients on long-term hemodialysis [18–20]. It is one example from the group of diverse proteins and polypeptides that are involved in protein conformational disorders (PCD), a group of diseases characterized by proteins attaining pathological, nonsoluble conformations leading to destructive precipitates such as amyloid in joints, tissues, and organs [21–25].

Multidimensional analytical approaches involving CE such as CE–mass spectrometry (MS) [26–31], chip-CE–MS [32–34], CE–NMR spectroscopy [35–39], and CE combined with affinity techniques [40,41], fraction collection [42] or pre-CE or on-line sample enrichment approaches [43–48] increase the analytical power of CE significantly. RP-HPLC and mass spectrometry (MS) in conjunction with CE is here used to resolve the issue of why a purified β_2m preparation appeared homogeneous by CE while being heterogeneous by RP-HPLC and how treatment with organic solvent induces heterogeneity by CE [15]. We conclude that conformational variants (conformers) of β_2m are distinguished by CE but not by MS or RP-HPLC while, conversely, RP-HPLC separates—and MS identifies—nonoxidized and oxidized species that are not readily separated by CE.

2. Materials and methods

2.1. Reagents

HPLC-grade water and acetonitrile were from Merck (Darmstadt, Germany). A synthetic marker peptide: Ac-PSKD-OH, was synthesized by Schafer-N (Copenhagen, Denmark). Buffer salts and all other chemicals were of analytical grade from Sigma (St. Louis, MO, USA). Precast polyacrylamide gels from Novex (San Diego, CA, USA) were used for sodium dodecylsulfate–polyacrylamide gel electrophoresis (SDS–PAGE).

2.2. β_2-Microglobulin

β_2m was purified from a pool of urine from uremic patients as previously described [9]. Total protein concentrations were estimated using a bicinchoninic acid protein assay from Pierce (Rockford, IL, USA) with bovine serum albumin as a standard. The preparations only showed one band each at approximately $M_r = 12\,000$ upon analysis by SDS–PAGE followed by Coomassie blue staining. The purified proteins in phosphate-buffered saline, pH 7.4, were kept at -20 °C until use.

2.3. LC–MS

A Q-TOF 2 mass spectrometer from Micromass (Manchester, UK) on-line with an HPLC system equipped with a Vydac C_{18} (Hesperia, CA, USA) protein and peptide column was used. A 20-μl volume of a 0.5 mg/ml β_2-microglobulin sample was injected onto the C_{18} column and eluted into the mass spectrometer at 50 μl/min using a gradient from 27.5 to 54.5% (v/v) aqueous acetonitrile in 0.1% (v/v) trifluoroacetic acid over 60 min. Spectra were acquired in the positive ion mode after electrospray ionzation using a source temperature of 100 °C while monitoring the total ion current (TIC).

2.4. CE

CE was performed on a Beckman P/ACE system 2050 equipped with sample cooling. Unless otherwise noted UV detection at 200 nm and an uncoated fused-silica capillary of 57 cm (50 cm to the detector window) × 50 μm I.D. were used and the electrophoresis buffer was 0.1 *M* phosphate, pH 7.3–7.5. Separations were carried out at different voltages as specified. Data were collected and processed by the Beckman SYSTEM GOLD software. The capillary cool-

ing fluid was thermostatted at 20 °C unless specified otherwise. The capillary was rinsed after electrophoresis for 1 min with each of the following: 0.1 *M* NaOH, water, and electrophoresis buffer. β_2m samples and marker peptide (Ac-PSKD-OH) were analyzed after pressure injection at the dilutions and injection times given in the figure captions. In some experiments evaporation of samples and sample solvents were inhibited by placing a layer (typically 20 μl) of mineral oil on top of the samples as previously described [17].

2.5. CE–MS

CE–MS measurements were conducted on a Beckman P/ACE system 5000 connected via a triaxial probe to a Quattro II (Micromass) triple quadrupole mass spectrometer operated in positive electrospray ionization mode with a source temperature of 60 °C. Electrophoresis buffer was 0.10 *M* ammonium acetate, pH 7.3, and the sheath fluid (30 μl/min) was 0.02 *M* ammonium acetate, pH 7.3. Samples were pressure injected at 0.5 p.s.i. (1 p.s.i. = 6894.76 Pa) for 60 s into an uncoated fused-silica capillary of 95 cm (20 cm to the UV detector). The CE separation took place at 30 kV at 20 °C and detection at 200 nm was employed. Sample concentrations were 1.45 and 0.73 mg/ml for β_2-microglobulin and β_2-microglobulin in 50% acetonitrile, respectively.

3. Results

3.1. LC–MS analysis of purified, native β_2m

β_2m purified from urine to apparent homogeneity when analyzed by CE under native conditions (Fig. 1A, insert) displays heterogeneity by reversed-phase HPLC using a shallow gradient on a C_{18} column [17]. As seen in Fig. 1A two partially resolved peaks dominate the TIC chromatogram obtained during an LC–MS analysis of native β_2m. In different β_2m preparations the less abundant early peak constituted from 20 to 45% of the total peak area (29% in Fig. 1A). The mass determined for this peak was 16 mass units higher than that of the second peak which had a mass value (11 729.5 u) in good agreement with the calculated mass (11 729.1 u) of β_2m [49] (Fig. 1B and C). The results indicate that a substantial part of the purified β_2m molecules are oxidized, most likely on their exposed C-terminal methionine residue (Met^{99}), leading to the less hydrophobic, earlier eluting fraction in the RP-HPLC analyses.

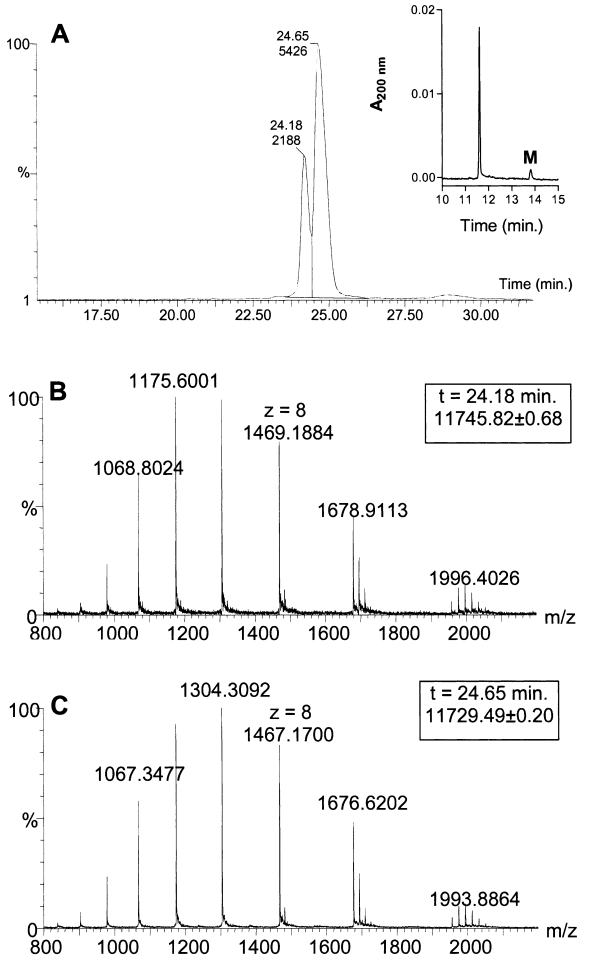

Fig. 1. LC–MS of purified β_2m. (A) Total ion current chromatogram; (B) and (C) are the mass spectra associated with the peaks seen in A at 24.18 and 24.65 min, respectively. For the analysis 20 μl of 0.5 mg/ml β_2m in water was injected onto a C_{18} column and eluted into the mass spectrometer at 50 μl/min as described in Materials and methods. Charge states (z) and some peak *m*/*z* values have been indicated and the deconvoluted masses of the two peaks are also given. The insert in (A) shows part of the CE analysis of purified β_2m. A sample of 0.4 mg/ml β_2m together with 0.05 mg/ml of a marker peptide was injected for 2 s (at 0.5 p.s.i.) into the capillary and analyzed at 15 kV under the conditions listed in Materials and methods. There is no indication of heterogeneity of the β_2m-peak. *M* indicates the peptide marker.

3.2. CE analysis of β_2m and Met^{99}-oxidized β_2m purified by RP-HPLC or total β_2m pretreated with denaturants

As illustrated by the insert in Fig. 1A the separation of Met^{99}-oxidized β_2m from unmodified β_2m by RP-HPLC (Fig. 1) was not accomplished by CE. However, when β_2m was first subjected to an RP-HPLC analysis and the two fractions then were analyzed individually by CE a marked heterogeneity in the form of two components [fast (f) and slow (s)] in both the Met^{99}-oxidized β_2m fraction (peak 1, Fig. 2) and the β_2m fraction (peak 2, Fig. 2) was observed [17]. The major f peak component migrates as native β_2m and is connected to the s component by an elevated baseline. Exactly the same apparent

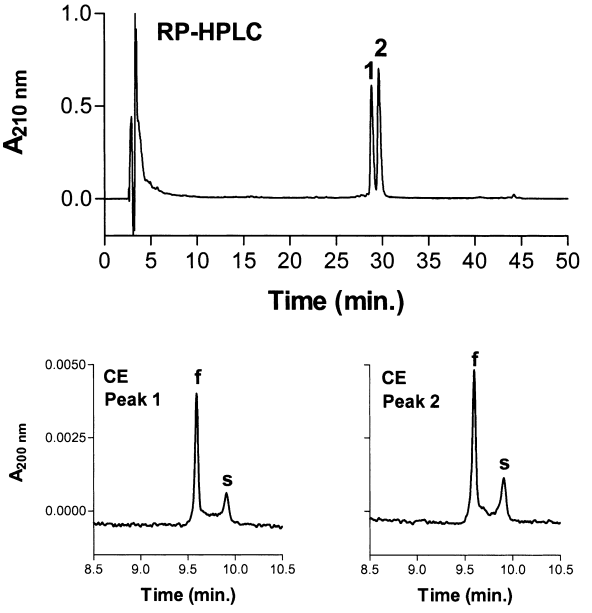

Fig. 2. RP-HPLC-fractionated β_2m analyzed by CE. A sample of native β_2m (250 μl of 0.5 mg/ml) was separated on a C_{18} column using a flow-rate of 1 ml/min and a gradient of 28–49% (v/v) acetonitrile over 60 min. The eluate was monitored at 210 nm (upper panel marked RP-HPLC) and the two main peaks labelled 1 and 2 were collected separately and dialyzed at 4 °C against dilute PBS (diluted 1:10 in water). After centrifugal concentration on an M_r 5000 cut-off filter to 10× less volume the samples were analyzed by CE (lower panels) under the conditions detailed in Fig. 1 except that the sample was injected for 6 s and 17 kV was used as the separation voltage. The material from both HPLC-fractions separates into a fast (f) and a slow (s) component in the CE analysis.

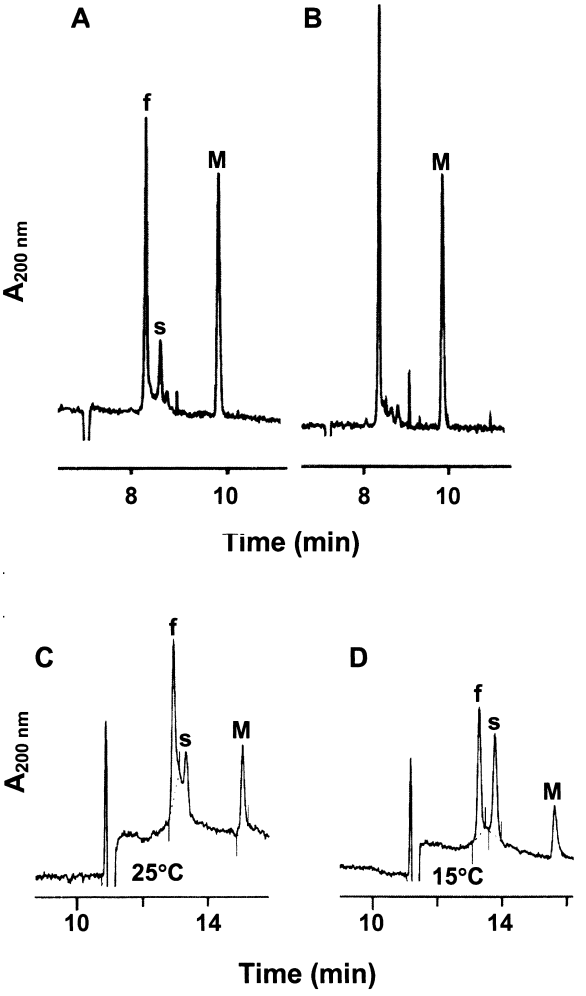

Fig. 3. β_2m analyzed by CE: effect of sample solvent evaporation and resolubilization and capillary temperature on peak profiles. CE analyses at 20 kV in 47 cm capillary with 40 cm to the detector. Capillary thermostatted at 22 °C. Sample was injected for 4 s at 0.5 p.s.i. and consisted of 0.11 mg/ml β_2m and 0.08 mg/ml marker peptide (M) dissolved in electrophoresis buffer. (A) sample analyzed 30 s after sample (20 μl) was dried completely down by vacuum evaporation in a Speed-Vac and resolubilized in 20 μl of water; (B) same sample injected 48.5 min after resolubilization. (C) and (D) show the CE analyses at two different capillary thermostatting temperatures (25 and 15 °C) of a sample consisting of 0.5 mg/ml β_2m in dilute PBS (diluted 1:20 in water)–acetonitrile (50:50, v/v). The sample (40 μl) was overlayed with 20 μl of mineral oil, injected for 4 s at 0.5 p.s.i. and subjected to 15 kV separating voltage at the capillary temperatures indicated.

β_2m heterogeneity could be induced by adding organic solvents such as acetonitrile (cf. below), trifluoroethanol, and ethanol directly to native β_2m samples in phosphate buffers at neutral pH [16,17]. The area of the s peak was found to be directly proportional to the percentage (0–50%) of organic solvent present in the samples while the total peak area was constant except that over time the total amount of soluble material is reduced [16,17]. A transient s peak was also observed in the absence of any organic solvent when a β_2m sample (in phosphate buffer) was simply dried down and then analyzed just after resolublization in aqueous buffer. This s peak disappeared over time as illustrated by repeated analyses of the same sample (Fig. 3A and B). With the decrease of the s peak the native f form increased exactly as was seen in repeatedly analyzed samples where the added acetonitrile was allowed to evaporate [17]. The effect of capillary temperature on this apparent reversibility was investigated (Fig. 3C and D). It is seen that a β_2m sample (kept at a constant temperature of 15 °C in 50% acetonitrile in the sample tray) reverts faster to the f form when being separated in a capillary kept at 25 °C than when being separated at 15 °C. The first very narrow peak represents the organic solvent (acetonitrile) that migrates with the electroosmotic flow and thus is separated from the other sample components immediately after application of the separation voltage.

3.3. CE–MS analyses of native β_2m and acetonitrile-treated β_2m

In an attempt to confirm or reject the presence of β_2m in both the f and s components observed in the CE analyses both native and acetonitrile-treated

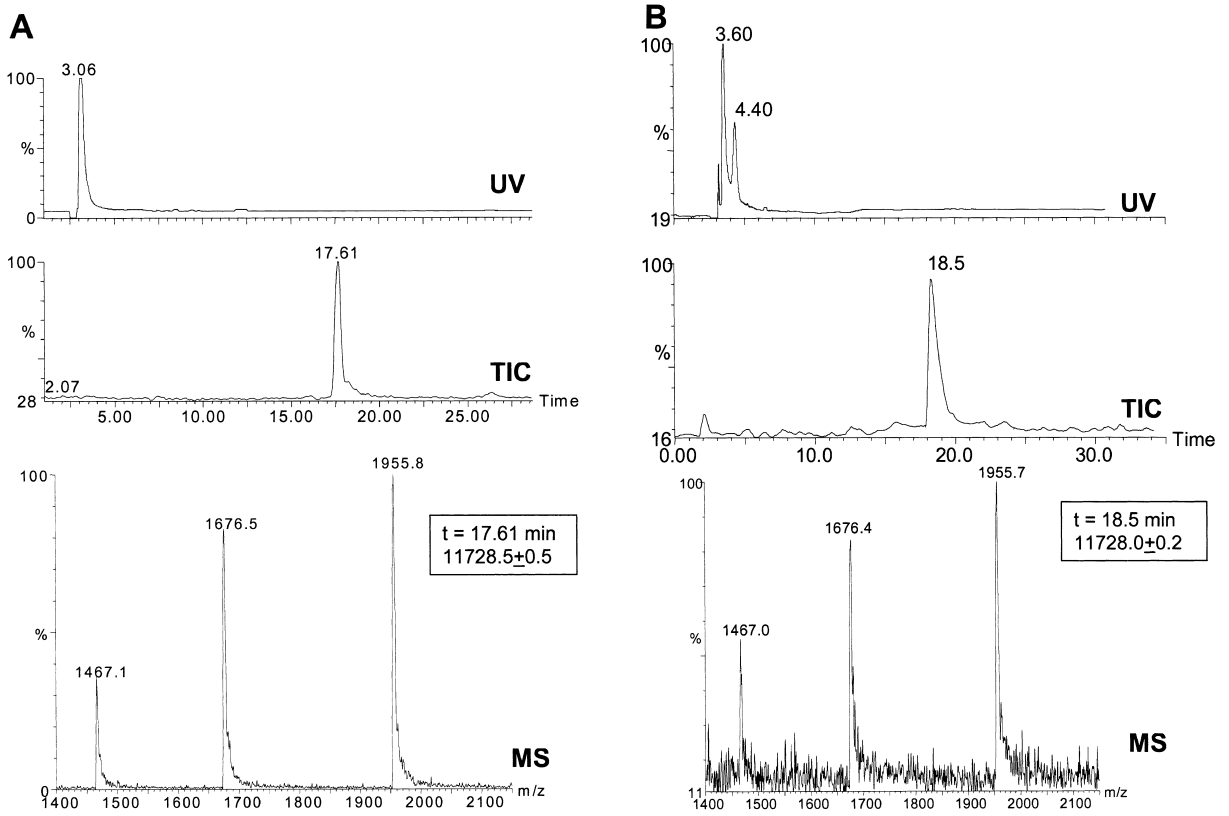

Fig. 4. CE–MS of purified β_2m. (A) native β_2m; (B) β_2m in 50% acetonitrile. For each experiment (A and B) the figure illustrates the CE–UV trace (detector at 20 cm), the TIC electropherogram (mass spectrometer at 95 cm), and the mass spectrum including the deconvoluted mass value associated with the TIC peaks at 17.61 and 18.5 min in experiments (A) and (B), respectively.

samples were analyzed by CE–MS (Fig. 4). To ensure minimal ion suppression and volatility a 100 m*M* ammonium acetate electrophoresis buffer was used instead of 0.1 *M* phosphate in these experiments. Still, the UV trace clearly reveals two distinct f and s peaks in the CE analysis of a β_2m sample containing 50% acetonitrile (Fig. 4B, UV). The TIC trace of the sample, however, does not resolve the two peaks originating from the CE column and entering the mass spectrometer 75 cm after the UV detector. The TIC shows a broad and slightly asymmetric peak at 18.5 min. The mass spectra associated with this peak only showed components corresponding to the same ($[m+6-8\mathrm{H}]^{6-8+}$) charge states observed in the native β_2m sample (Fig. 4A). No evidence of stable multimers or the presence of contaminating molecules could be found.

3.4. CE peak analyses at different β_2m concentrations in trifluoroethanol (TFE)

To assess the possible involvement of β_2m aggregation in the appearance of an s-peak in the CE separation profiles a series of CE analyses at a fixed TFE concentration added to increasing total β_2m concentrations were performed. The ratio of the s-peak area to the total peak area was recorded for each β_2m concentration. The results (Fig. 5) show that the peak ratio is independent on the analyte concentration. This supports the idea that conformational factors and not β_2m multimer formation cause the differential mobility of the s peak.

3.5. CE peak reversibility as a function of analysis time in different sample solvents

The reversibility between the s and f peaks were investigated in both TFE- and acetonitrile-treated samples (Fig. 6). Data on the reversibility as a function of peak appearance time for samples in 50% acetonitrile were previously reported [16] but here the study is extended to organic solvent concentrations of 13 and 33% and the effects of both TFE and acetonitrile are studied. A reversal to the f peak is observed with decreasing field strengths (i.e. with longer analysis times) for both 15 and 33% acetonitrile-treated samples and for the 15% TFE-treated sample. However, the sample containing 33% TFE behaves distinctly different (Fig. 6) since the s peak area increases relatively to the total peak area with increasing analysis time. Thus, the s form appears not to be reversible under conditions of high (i.e. 33%) concentrations of TFE in the sample.

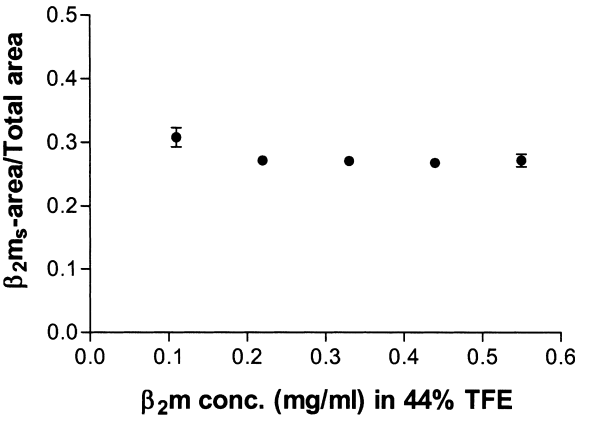

Fig. 5. β_2m peak distribution in native CE as a function of β_2m concentration and capillary temperature. Shown is the ratio of the area of the s-peak (cf. Fig. 2) to the whole peak area as a function of total β_2m sample concentration. Values are the means±the standard deviations of triplicate experiments. Samples [0.11–0.55 mg/ml β_2m in 44% (v/v) TFE] were injected for 4 s at 0.5 p.s.i. and separated at 17 kV as described in Materials and methods.

4. Discussion

Analyses of preamyloid β_2m conformations [16,17] require that the preparations of molecules are well-characterized with respect to purity and structure. We therefore have used different separation techniques with complementary characteristics. The present combination of CE, LC and MS reveal that although a substantial fraction of β_2m molecules is modified by oxidation, probably of the C-terminal amino acid residue, this does not influence the propensity of β_2m to attain a distinct alternative conformation that is readily observed by CE. In the absence of direct proof of the identity of the s peak the analyses and combination of analyses presented here support the absence of contaminating molecules, other structural β_2m variants, and lack of aggregation and thus support the notion that a variant

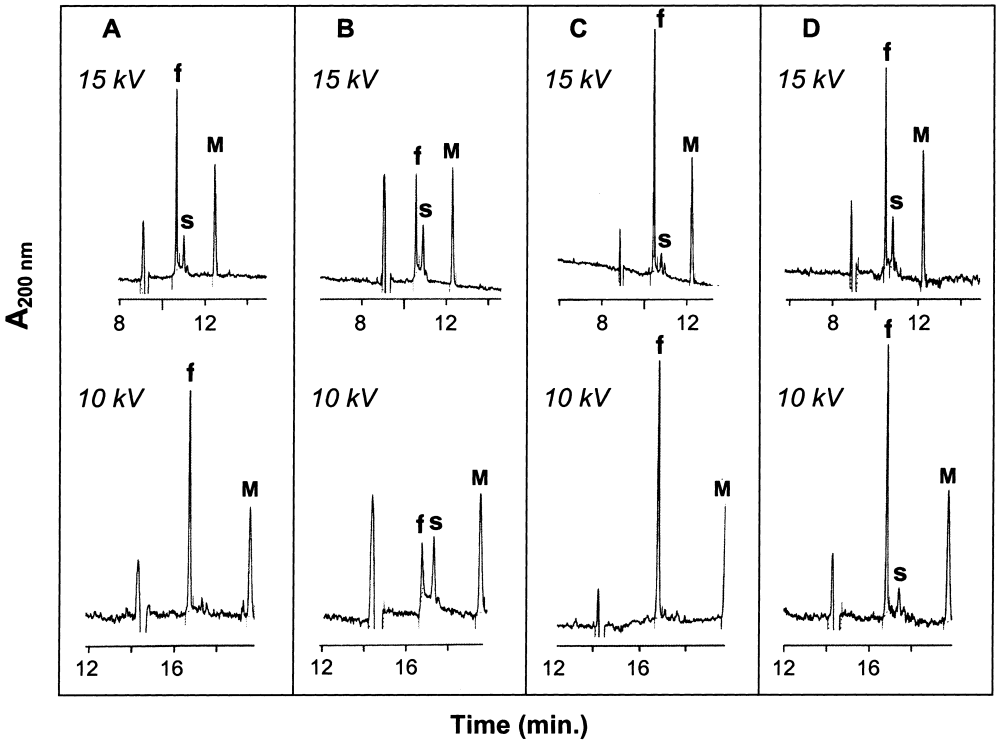

Fig. 6. Reversibility of β_2m peaks in CE as a function of analysis time and solvent added to samples. Analyses of β_2m samples (0.25 mg/ml with 0.06 mg/ml marker peptide (M)) containing: (A) 13% (v/v) TFE and (B) 33% (v/v) TFE or (C) 13% (v/v) acetonitrile and (D) 33% (v/v) acetonitrile. Samples (15 μl) were covered by 20 μl mineral oil and were cooled in the sample tray at 15 °C. Injection volumes were 5 s at 0.5 p.s.i. and separations took place at 15 kV (263 V/cm, upper row) or 10 kV (175 V/cm, lower row) to evaluate the distribution of the s and f forms as a function of analysis time.

conformation readily reverting back to the native fold under physiological conditions is responsible for the observations. Reversion into a single f-component in slow analyses (low field strengths) where the analyte spend longer time in the native electrophoresis buffer before reaching the detector [16] and where the denaturant (e.g. acetonitrile or trifluoroethanol) is removed from the analytes by the CE analysis itself makes it possible to study renaturation directly on line including characterizing conditions that will accelerate refolding. An intriguing finding was that TFE- and acetonitrile-treated samples despite having similar s peaks and despite similar concentration-dependent changes in β_2m circular dichroism spectra [16] did not behave similarly in the refolding experiments. Thus, at any acetonitrile concentration tested increased analysis times—equivalent to the longer residence time in native buffer after sample injection—resulted in increased proportions of the native f peak. In contrast, we found the opposite to be the case with TFE at 33% in the sample. Under these conditions the s peak failed to revert with longer analysis times and the native peak decreased. This may point to the existence of a threshold effect above which irreversible conformational changes begin to occur. Since the solvent plug length in the capillary was shown to be directly proportional to the degree of change in the peak profile [16] it may be that the broader TFE plugs (as compared with the acetonitrile plugs that give rise to very narrow peaks) is responsible for the effect. This will be part of the focus of future studies aimed at characterizing further steps along the misfolding pathway leading to insoluble proteins.

5. Conclusions

The combination of separation and detection methods used in the present study has made it possible to establish the presence of a distinct folding intermediate of the potentially amyloidogenic protein β_2m. Repeated quantitative measurements of reversible folding reactions in small sample volumes is not easily accomplished by other techniques than CE. The unequivocal determination of structural alterations, however, heavily relies on MS measurements. These tools will be indispensable for the further study of molecular pathology involved in protein conformational disease and for the discovery of means to inhibit initiation and progression of aberrant folding.

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