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Congophilicity (Congo red affinity) of different β_2-microglobulin conformations characterized by dye affinity capillary electrophoresis

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Abstract

The amyloidogenic protein β_2-microglobulin was characterized by affinity capillary electrophoresis (CE). CE could separate conformational variants of β_2-microglobulin and with the amyloid-specific dye Congo red as a buffer additive it was possible to measure different Congo red-affinities of native and abnormally folded β_2-microglobulin. We find that native β_2-microglobulin has an intermediate affinity for Congo red at pH 7.3 and that binding involves electrostatic interactions. The conformational variant of β_2-microglobulin that appears in acetonitrile solutions binds Congo red more strongly. Affinity CE using Congo red as a buffer additive is a new, simple, fast, and quantitative micromethod for the characterization of soluble conformational intermediates of amyloidogenic proteins.

Keywords: Affinity capillary electrophoresis; Dyes; Congo red; Microglobulins; Globulins; Proteins; Amyloids

1. Introduction

Several devastating diseases are associated with the accumulation of fibrillar polypeptide aggregates (amyloid) in tissues and organs [1]. It is not known why otherwise soluble proteins and polypeptides precipitate in these disorders but conformational changes precede amyloid fibril formation [2–5]. Fibrils in different types of amyloid are ultrastructurally similar [6,7]. They appear to be composed of antiparallel β-strands or related structures oriented perpendicular to the fibril axis [8–11]. The dye Congo red (CR) is bound by such fibrils in an specifically aligned fashion so that characteristic shifts in the dye absorption spectrum and an enhanced birefringence and dichroism in polarized light is observed upon binding [2,12]. Congo red binding to amyloid proteins appears to be directly related to the content of β-sheets [13,14]. The amyloidogenicity of a given protein conformation may thus be reflected by its CR-affinity (congophilicity). As an example, the degree of toxicity toward neuronal cells of different preparations of the β-amyloid peptide involved in Alzheimer's disease has been shown to be quantitatively correlated to the degree of Congo red binding in a simple dye assay [13].

Monomeric, soluble intermediates on the folding pathway to amyloid fibrils have been demonstrated for some amyloidogenic proteins including the prion protein [14,15], β-amyloid peptide [16], and transthyretin [4,17] by the use of circular dichroism and analytical gel filtration and ultracentrifugation techniques. Folding intermediates and soluble aggregates have also been characterized in other proteins [18]

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using nondenaturing gel electrophoresis [19] and quasi-elastic light scattering [20]. However, these methods generally consume much sample and few of them are suited for the study of folding intermediates under physiological pH and ionic strength conditions and do not allow simultaneous assessment of ligand binding interactions.

Free-solution capillary electrophoresis (CE) appears to be an attractive alternative technique to study protein folding as reviewed in Ref. [21] because no secondary equilibria are involved in the separation and because separations are highly efficient and in a number of cases have been shown to be sensitive to changes in protein conformation [21–24]. With the application of the affinity CE approach [25] interactions of CE-separated conformational variants with Congo red would be expected to be readily detectable because binding of this anionic dye would give the analyte–dye complex a significantly different electrophoretic mobility as compared to the free ligand [25]. We report here that CE may be used to separate conformational variants of the potentially amyloidogenic protein β_2-microglobulin (β_2m) and to quantitatively measure the increased affinity for Congo red of specific conformations. β_2m forms the amyloid encountered in patients on long-term hemodialysis therapy and the precipitated protein appears normal and intact [26]. Using this protein as a model system the results presented here suggest that Congo red affinity CE may be useful for the evaluation of the congophilicity of conformational variants of other amyloidogenic proteins as well.

2. Materials and methods

2.1. Chemicals and materials

All chemicals unless otherwise stated were of analytical grade from Sigma (St. Louis, MO, USA). High-performance liquid chromatography (HPLC)-grade water and acetonitrile were from Merck (Darmstadt, Germany) while trifluoroacetic acid (TFA) was from Fluka (Buchs, Switzerland). A marker peptide: Ac-PSKD-OH, was synthesized by Schafer-N, Copenhagen, Denmark. Precast polyacrylamide gels from Novex (San Diego, CA, USA) were used for sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE). β_2m was purified from a pool of urine from uremic patients as previously described [27]. The preparations (5–10 mg/ml) showed one band at approximately M_r 14 000 upon analysis by SDS–PAGE followed by Coomassie blue staining. The purified proteins in phosphate-buffered saline (PBS), pH 7.4 (6.5 m*M* Na_2HPO_4, 1.5 m*M* KH_2PO_4, pH 7.4, 137 NaCl, 2.7 m*M* KCl_2) were kept at −20°C until use. Total protein concentrations were estimated using a bicinchoninic acid protein assay from Pierce (Rockford, IL, USA) with bovine serum albumin as a standard. Congo red (Sigma C-6767) had a dye content of 50% as estimated by measurements of the 488 nm absorbance of Congo red solutions at pH 7.3 and using an extinction coefficient of 595 (10 mg/ml, 1 cm, 488 nm) [28].

2.2. Capillary electrophoresis

CE was performed on a Beckman P/ACE 2050 equipped with sample cooling. Conditions were as follows unless noted otherwise: electrophoresis buffer was 0.1 *M* phosphate, pH 7.3, detection was at 200 nm, and an uncoated fused-silica capillary of 57 cm (effective length 50 cm)×50 μm I.D. was used. The capillary housing had an aperture of 800×100 μm. Separations were carried out at 15 kV with the anode at the sample injection end of the capillary. Data were collected and processed by the Beckman System Gold software. The capillary cooling fluid was thermostatted at 20°C and samples were cooled at 10 or 15°C using a circulating water bath. The capillary was rinsed after electrophoresis for 1 min with each of the following: 0.1 *M* NaOH, water, and electrophoresis buffer. This prerinse was followed by a 0.1 min rinse with electrophoresis buffer from a separate vial to minimize carry-over into the sample of ligand (Congo red) when that was added to the electrophoresis buffer. Electrophoresis subsequently took place using a set of independent vials that were not used for any washing/prerinse steps. The β_2m samples containing an internal marker peptide (M= Ac-PSKD-OH) were analyzed in the dilutions given in the figure texts. The internal marker is a measure of sample volume and ensures that the migration shifts are specific measures of β_2m–Congo red interactions. Samples (typically 10–20-μl volumes) containing acetonitrile were protected against evapo-

ration by a 10-μl oil layer (mineral oil, Sigma M-3516) on top of the sample (cf. Results).

2.3. Data handling

Mobility shifts were expressed as the difference $[\Delta(1/t)]$ between the $1/t$ values (t=analyte peak appearance time) obtained at various concentrations (c) of additive (Congo red) and the $1/t$ value of the reference experiment with no additive in the electrophoresis buffer. All inverse peak appearance time ($1/t$) values were normalized by subtracting the $1/t$ value of the marker peak from the $1/t$ value of the β_2m peak before calculating $\Delta(1/t)$. Plots of $\Delta(1/t)$ as a function of c provided for estimates of dissociation constants, K_d after fitting a one-site binding hyperbola directly to the experimental data (GraphPadPRISM, v. 2.01, GraphPad Software, San Diego, CA, USA) or after linearizing the data in a plot of $\Delta(1/t)$ as a function of $\Delta(1/t)/c$ that yields $-K_d$ as the slope of the best fit straight line [29,30].

2.4. Mass spectrometry of fractions from reversed-phase HPLC

Reversed-phase HPLC separations were accomplished using an analytical C_{18} reversed-phase column (5 μm particle size, 250×4.6 mm) (Vydac, Hesperia, CA, USA) equilibrated in 0.1% (v/v) aqueous TFA. Separation of β_2m subfractions took place at 1 ml/min using a 60 min linear gradient of 28–49% acetonitrile in 0.1% TFA. Peaks were detected at 210 and 280 nm, collected manually, and dried down in a vacuum evaporator. Masses were measured using electrospray ionization time-of-flight mass spectrometry (MS) on a Mariner Biospectrometry Workstation (PerSeptive Biosystems, Framingham, MA, USA) after solubilizing the samples in 30% aqueous acetonitrile containing 0.1% formic acid.

3. Results

3.1. Congo red affinity CE of native β_2m

Purified human β_2m was evaluated for its ability to interact with the anionic dye Congo red (Fig. 1A)

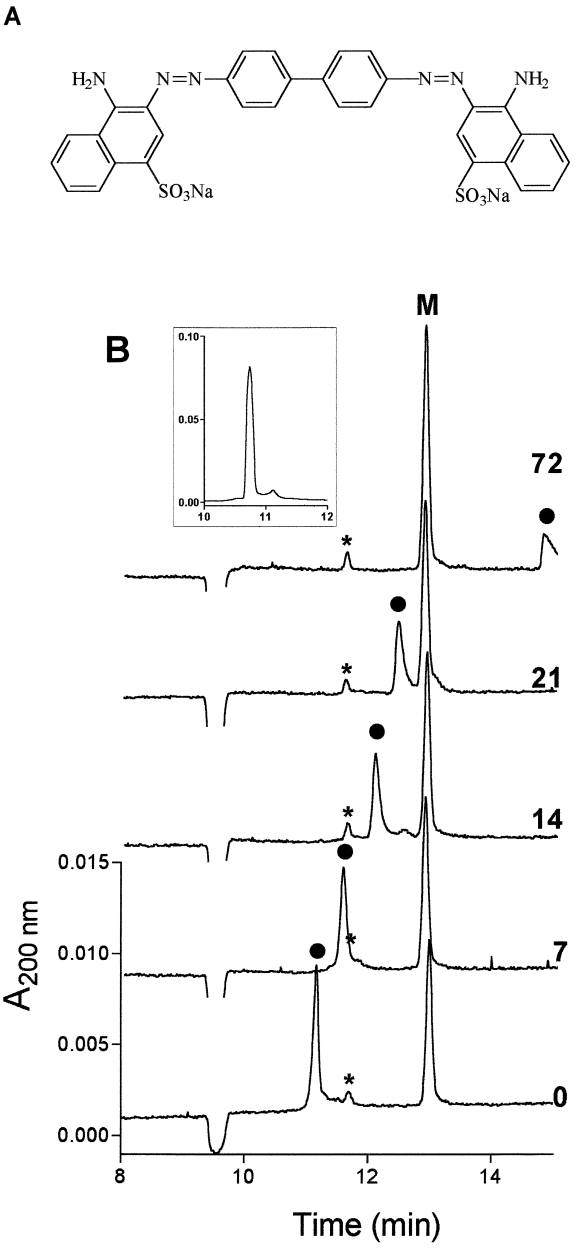

Fig. 1. Dye affinity CE of native native β_2m. (A) Structure of Congo red. (B) CE analyses of β_2m (0.37 mg/ml, diluted from a stock of 7.4 mg/ml in PBS with water) injected for 4 s and a marker peptide [(M), Ac-PSKD-OH, 0.2 mg/ml in water; (*) is a marker impurity] co-injected for 1.5 s. Separation at 5 kV in a 37 cm long capillary (30 cm to the detector). Performed in the presence of different amounts of Congo red added to the electrophoresis buffer as given (in μM) on the figure. The filled circles indicate the position of the β_2m peak. Insert is the CE analysis of purified β_2m alone with no marker added.

by affinity CE in 0.1 *M* phosphate, pH 7.3. The dye was added to the electrophoresis buffer and peak appearance times of the components of the injected mixture of β_2m and a synthetic peptide marker (M) were determined at 7–72 μ*M* Congo red concentrations (Fig. 1B). β_2m appeared as a single, symmetrical peak with an occasional small additional anionic peak appearing (Fig. 1B, insert). Clear anionic shifts of β_2m proportional to the concentration of Congo red present during electrophoresis were observed while the marker peptide (M) and its impurity (*) were unaffected by the presence of the dye at any concentration tested. In addition to the migration shifts the β_2m peak also broadened and decreased in height with increasing Congo red concentrations.

The results of Fig. 1B indicated that specific interactions between Congo red and native β_2m took place. The dye affinity CE method was subsequently used to evaluate the dependence of this binding on pH and ionic strength.. The results are plotted in Fig. 2. By non-linear regression analysis all peak appearance shift data fitted well (R^2>0.98 for all) to a one-site binding hyperbola with 27–240 μ*M* dissociation constants as specified on the figure. The dissociation constant values were found to increase with increases in ionic strength and pH (Fig. 2).

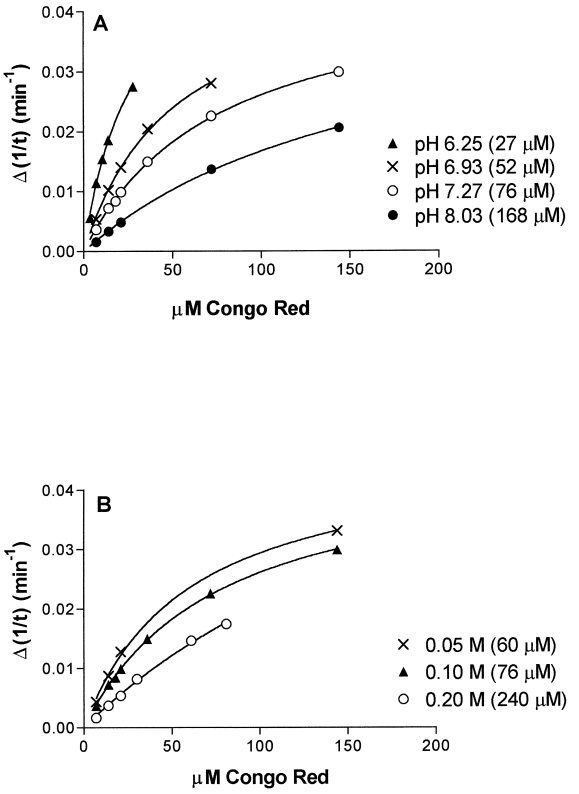

Fig. 2. Dye affinity CE of native β_2m. Characterization of the dependence of interactions between β_2m and Congo red on buffer pH (A) and ionic strength (B). CE performed as described in Fig. 1B in (A) 0.1 *M* phosphate buffers of the stated pH or (B) in phosphate buffers at pH 7.3 at the stated concentrations. Corrected migration shifts as a function of concentration of Congo red added to the electrophoresis buffer are shown in the graphs. The best fit one-site binding hyperbolas have been calculated by the GraphPad software and are depicted together with the binding constants (dissociation constants, K_d) derived from the best fit equations (R^2>0.98 in all instances).

3.2. Reversed-phase HPLC fractionation of β_2m

The purified β_2m was further analyzed by C_{18} reversed-phase HPLC using a shallow gradient (Fig. 3). Two main peaks (A and B) appeared. By MS the mass of the later peak (B) was 11 728 while the first peak (A) had a mass of 11 744. The mass of the (B) peak agreed with the theoretical mass (11 729.1) of the protein calculated on the basis of its amino acid sequence [31]. The higher mass form (A) (approximately 25% of the total amount) that eluted earlier than the main component differed by +16 mass units. This difference could be due to oxidation of the C-terminal methionine residue [Met(99)] in β_2m which would give a theoretical mass of 11 745.1. No evidence of dimers or higher-order multimers was seen in the mass spectra. However, upon CE analysis of (A) and (B) resolubilized in 50% acetonitrile both fractions were clearly electrophoretically heterogeneous with a distinct second peak in addition to the main β_2m-peak (inserts in Fig. 3). Thus, the structural heterogeneity (oxidized and non-oxidized β_2m) that was resolved by the reversed-phase HPLC analysis was not resolved by the CE analysis. However, the CE analysis revealed another heterogeneity in both fractions that was not detected by reversed-phase HPLC and which may represent a conformational variant of β_2m appearing in acetonitrile.

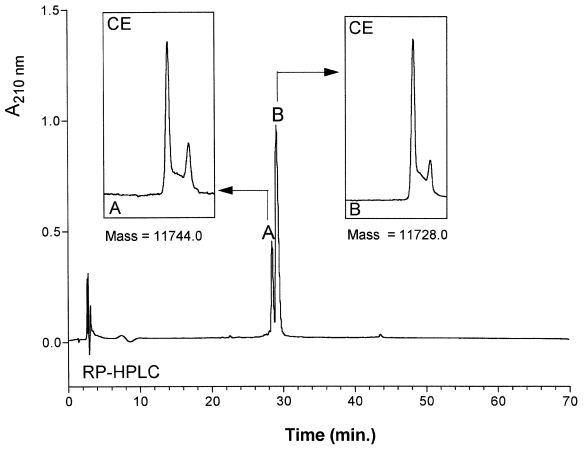

Fig. 3. CE and reversed-phase HPLC separation of purified β_2m using a shallow HPLC gradient from 28 to 49% acetonitrile in 0.1% TFA over 60 min at 1 ml/min. The two components that were separated by the reversed-phase HPLC analysis were collected separately and dried down. The samples were then resolubilized in 50% aqueous acetonitrile and subjected to CE analysis in plain electrophoresis buffer as given for Fig. 1B except that separation at 15 kV and a 57 cm capillary with the detector at 50 cm were used. A 40-μg amount of purified β_2m was injected for the reversed-phase HPLC analysis. The dried material from peaks (A) and (B) that were collected separately was resolubilized in 20 μl 50% aqueous acetonitrile and injected for 8 s for the CE separation shown in the inserts. Aliquots of these fractions were also analyzed by electrospray ionization time-of-flight mass spectrometry as described in Materials and methods. The deconvoluted peak masses of peaks (A) and (B) are given under the inserts.

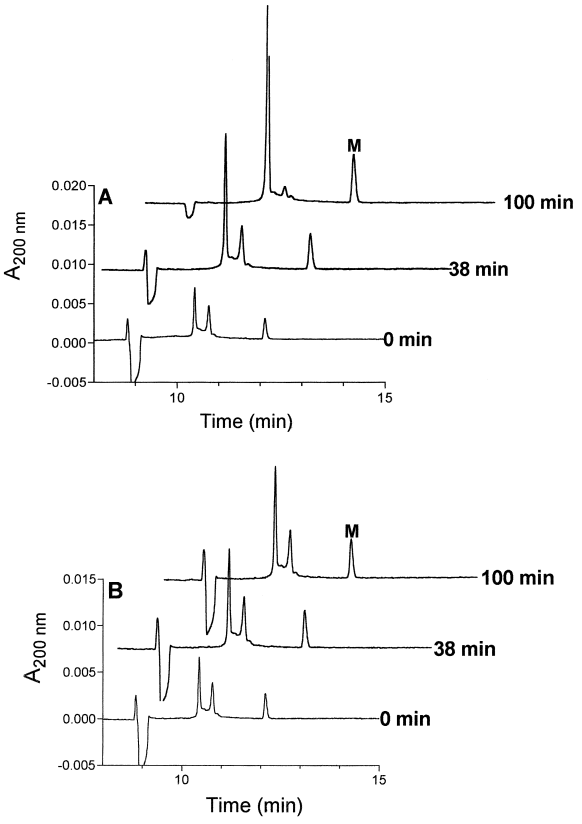

Fig. 4. CE analysis of acetonitrile-treated β_2m samples. Protection against evaporation accomplished by adding a layer of mineral oil on top of the sample in (B) as compared with (A). Repetitive CE analyses of a β_2m sample (0.09 mg/ml, diluted in water to 0.18 mg/ml from a 10 mg/ml preparation in PBS and then diluted 1:1 in acetonitrile) containing 0.05 mg/ml of the marker peptide (M). CE performed with sample cooling at 15°C and capillary thermostatting at 21°C as described in Materials and methods and in Fig. 3. Samples were 20-μl volumes and were injected for 8 s in three repetitive runs either unprotected (A) or protected by a 10-μl layer of mineral oil (B). Sample incubation times are given on the individual electropherograms.

3.3. Congo red affinity CE of acetonitrile-treated β_2m

The behavior of the conformational variant of β_2m was further characterized by dye affinity CE. To perform multiple analyses of μl-volume β_2m-samples in 50% acetonitrile it proved necessary to cover the sample with a layer of oil as previously reported in work with nanoliter samples [32] (Fig. 4). As seen in Fig. 4B this measure considerably reduced the increase in sample concentration due to acetonitrile evaporation and made it possible to ensure a constant acetonitrile concentration in multiple analyses from the same sample mixture. It was observed (Fig. 4A) that the distribution between the two β_2m species is highly dependent on the amount of acetonitrile present in the sample. Without acetonitrile (the sample at 100 min) almost no detectable β_2m in the variant conformation is present while there is an increased peak corresponding to native β_2m. The conformational variant of β_2m therefore appears to be reversible.

With samples protected against evaporation it was possible to assess the affinity of the conformational variant of β_2m for Congo red in dye affinity CE

experiments carried out as those shown for native β_2m in Fig. 1B (Fig. 5). It was found that the conformational variant had an increased affinity for Congo red as illustrated by the experiments in Fig. 5 which are performed in the presence of 0.9 to 7.2 μM Congo red. This range is 10-times lower than the concentration range used in Fig. 1 but still gives roughly the same magnitude of migration shifts. At the highest concentrations of Congo red used in this experiment it can be observed that also the native β_2m peak as expected begins to move anodically, i.e., as a more negatively charged species. In addition to the migration shifts there are the same decreases in peak area with increased Congo red concentration as were observed for native β_2m. Using the normalized inverse migration times (cf. Materials and methods) the migration shift plots also suggested a change in binding behavior of native versus the conformational variant of β_2m (Fig. 6). The data from the experiments with native β_2m give straight lines that fit very well to the experimental points while no correlation to a one-site binding hyperbola could be obtained using the migration shift data of the conformational variant of β_2m. This is illustrated by the lack of linearity of the plot in Fig. 5D as compared to Fig. 5B where the linearization of the migration shifts obtained with native β_2m is shown. No apparent K_d based on the experimental data for the conformational variant of β_2m could thus be estimated when using a simple one-to-one binding model with fast interaction kinetics.

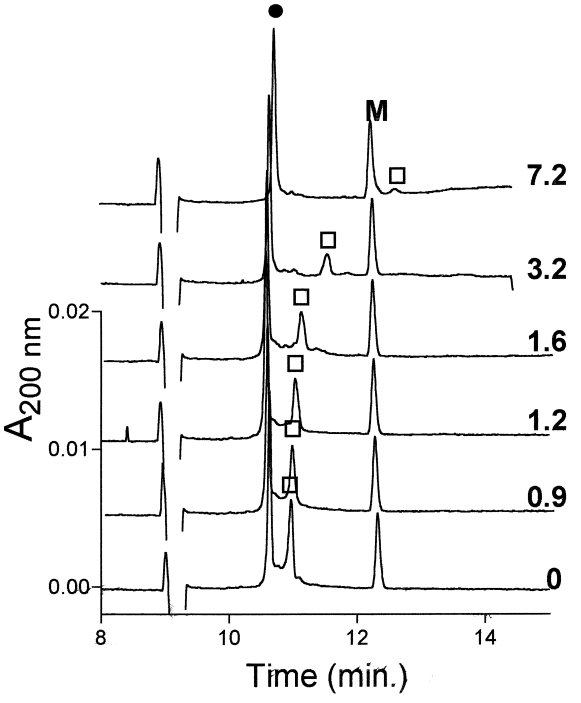

Fig. 5. Probing affinity for Congo red of a conformational variant of β_2m by dye affinity CE. CE analyses of a mixture of β_2m and marker peptide (M) under oil as detailed in Fig. 4 performed in the presence of the indicated concentrations (μM) of Congo red added to the electrophoresis buffer. Filled circle marks the peak corresponding to native β_2m while an open square marks the new β_2m peak that appears in an acetonitrile-treated sample.

4. Discussion

The amyloid fibril structure, e.g., the cross β-sheet [33] that appears to be characteristic of many, if not all [9,34], amyloid materials has an affinity for the planar diamino, disulfonated diazo dye Congo red [35]. Amyloid binds and aligns this dye in an optically active configuration [36]. β_2m is a potentially amyloidogenic protein that precipitates and attaches to collageneous structures in patients on long-term hemodialysis [37,38]. Unlike many other amyloid proteins it has not been possible to demonstrate that the precipitated β_2m contains alterations in primary structure or that the protein to a substantial degree is processed into fragments [26,39,40].

The dye affinity CE analyses of native β_2m and a distinct β_2m conformational variant that occurs in the presence of acetonitrile indicate that both native and conformational variants of β_2m interact with Congo red. Further, the binding of the β_2m conformational variant to Congo red is of a higher affinity than the binding of native β_2m and the dye affinity CE patterns show changes in migration times and peak shapes that indicate that an equilibrium is not established during the electrophoretic run. Thus, off-rates were slow – especially for the conformational variant of β_2m (even though no complex peak was observed) – compared with the time for electrophoresis. The migration shift data therefore could not be linearized according to the usual type of equations used in affinity CE [41–43].

The electrophoretic separation of the β_2m conformers presented here does not require buffer additives

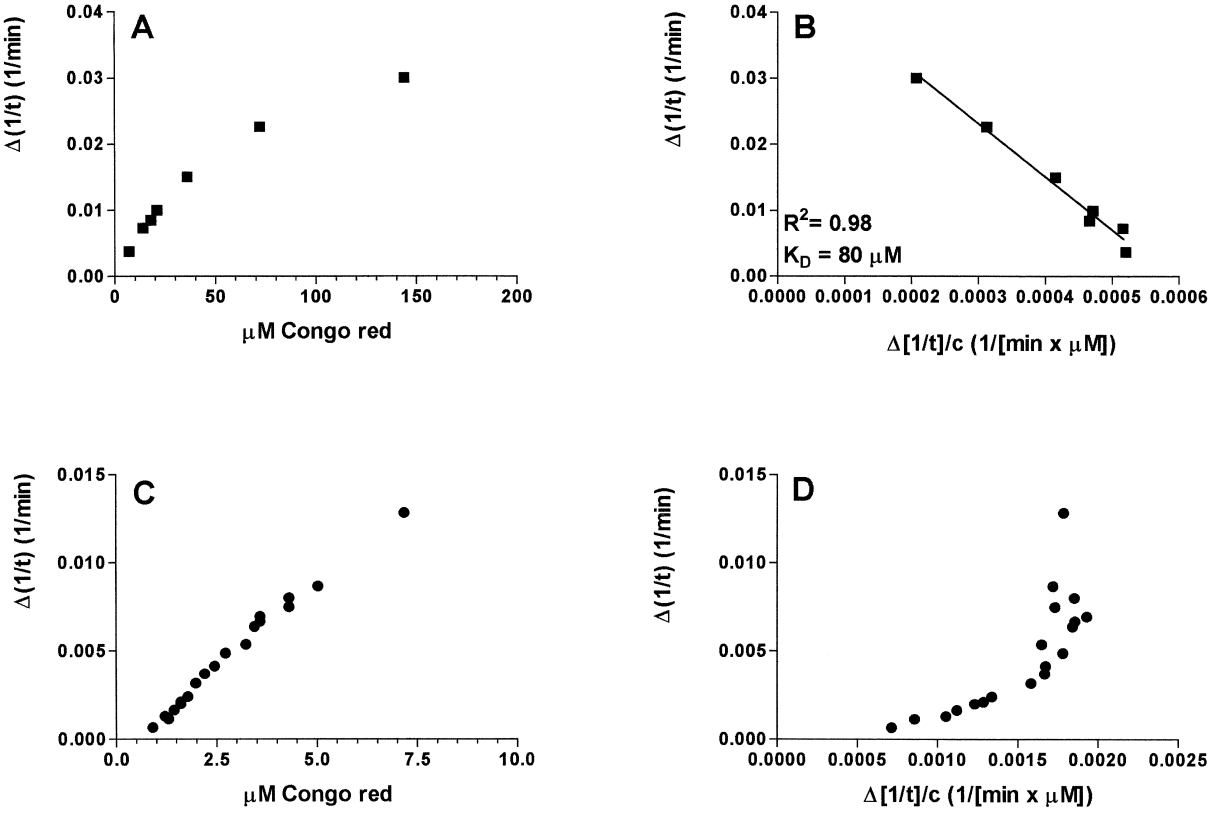

Fig. 6. Summary of migration shift data from dye affinity CE experiments such as those shown in Figs. 1 and 5 performed in 0.1 *M* phosphate, pH 7.3. Data from the analysis of the native (A and B) and variant (C and D) β_2m peaks. A and C are the direct migration shift data while B and D represent linear transformations of the same data. Only for the data in A it is possible to linearize and thus extract a binding constant estimate based on the assumption of a one-to-one non-cooperative binding.

but takes place in plain 0.1 *M* phosphate buffer at physiological pH. The amount of the conformational variant of β_2m was proportional to the acetonitrile concentration in the sample when acetonitrile evaporated from 50% over time in unprotected samples, i.e., the conformational change was reversible and the technique will allow monitoring the time course of refolding.

The use of CE to study interconversion kinetics between conformers of proteins has been discussed before [21] and applied to molecules such as salmon calcitonin, bovine β-lactoglobulin, and *cis–trans* isomers of small peptides [24,44,45]. While the CE analysis in the present study appears to be able to separate conformational variants of β_2m the heterogeneity corresponding to a C-terminally Met(99)-oxidized β_2m subfraction was not resolved in the CE system while it was readily demonstrated by MS in combination with reversed-phase HPLC. Final proof of two distinct conformations of β_2m in the presence of acetonitrile will depend on the application of spectroscopic methods such as circular dichroism and/or nuclear magnetic resonance measurements but it is difficult to explain our observations in any other way. However, the relationship between the acetonitrile-induced conformational β_2m variant demonstrated here and the conformation existing in β_2m-amyloids also remains to be further elucidated.

5. Conclusions

We have shown that two distinct β_2m conformations can be discerned by CE and have demonstrated

by dye affinity CE that the two conformational isoforms have a differential affinity for the amyloid-specific dye Congo red. In comparison with other structure-specific separation methods the present approach has a much smaller sample consumption since the same sample can be used repetitively. Also, the combination of separation and affinity measurements in one operation is unique for the methods used here.

The approach should be useful for characterizing the kinetics of denaturation and refolding of β_2m which is of interest for the understanding of amyloidogenicity of this and other proteins. In addition, as demonstrated by the present work the method can be used to measure the affinity of Congo red and Congo red analogs for various analytes including amyloid fibril precursors, i.e., to assess analyte congophilicity. Thus, it may also be a helpful tool for discovering and quantitatively measuring molecular candidates for inhibition of abnormal protein and polypeptide folding.

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