Journal of Chromatography, 638 (1993) 269–276
Elsevier Science Publishers B.V., Amsterdam

CHROM. 24 923

Unfolding of human serum transferrin in urea studied by high-performance capillary electrophoresis

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ABSTRACT

High-performance capillary electrophoresis (HPCE) was used to monitor the progress of the unfolding of human serum transferrin in urea. Denaturation curves of the transferrin forms were constructed plotting the migration times corrected for the viscosity *vs.* the concentration of urea in the buffer. The practical advantage of capillary zone electrophoresis is the short analysis time, 5–15 min, as compared with slab-gel experiments, which require overnight runs for similar purposes. The resolution increased with the urea concentration, and hence high concentrations are beneficial for quantitative and qualitative analysis of mixtures of transferrin forms. Unfolding intermediates of the isoforms, which interconvert to the unfolded state slowly compared with the time scale of the electrophoretic separation, and also the completely unfolded isoforms were resolved and detected simultaneously when iron-free transferrin was subjected to denaturation by urea at concentrations between 3 and 6 *M*. However, no unfolding intermediates were observed with transferrin isoforms containing two iron atoms (*i.e.* diferric transferrin molecules), which accordingly are strongly resistant to urea denaturation. The unfolding of the transferrin isoforms depends on the iron content of the complexes, but not the carbohydrate content. HPCE in the presence of urea in this mode has the potential to become an analytical tool for diagnosis of diseases in which the transferrin patterns change.

INTRODUCTION

Monitoring the unfolding of proteins by denaturants by measuring one or more physical parameters as a function of the denaturant concentration gives information about the conformational stability of the molecules. Gel electrophoresis in urea gradients has been shown to be a convenient method for visualization of the conformational transition states of proteins [1,2]. The electrophoretic pattern changes as the unfolding proceeds [3]. However, if the process of unfolding is fast compared with the duration of the electrophoresis run, the possible unfolding intermediates cannot be seen in the electropherogram.

Human serum transferrin, a glycoprotein, has two lobes (N- and C-terminal ones), each having a binding site for iron(III) (see review by Brock [4]). A high degree of homology (42%) is observed between the amino acid compositions of these lobes. The carbohydrate chains are located on the C-terminal lobe. The conformational stabilities of the two lobes are different, as shown by Evans and co-workers [5,6], but measurements of changes in the optical properties of transferrin in urea do not indicate a multistep unfolding [7,8].

The transferrin isoforms (asialo, mono-sialo, 2-sialo, 3-sialo, 4-sialo, 5-sialo, 6-sialo molecules, which contain different carbohydrate chains) and the molecular forms of these isoforms (*i.e.* diferric transferrin, monoferric transferrin with iron bound in the N-terminal or in the C-terminal lobe, and iron-free transferrin, designated Fe_NTfFe_C, Fe_NTf, $TfFe_C$ and Tf, respectively) have different p*I* values and can be separated by

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0021-9673/93/$06.00

isoelectric focusing [9]. The major component in normal human serum is 4-sialo-transferrin. The amounts of the other components are significantly lower. The transferrin isoforms do not exhibit differences in iron-binding properties [10]. In an earlier report we showed that the iron-free transferrin isoforms (with only one charge unit difference) can be separated by zone electrophoresis in capillaries using a low ionic strength buffer [11].

In this study the unfolding of iron-free and iron-containing isoforms of transferrin in the presence of urea was monitored by high-performance electrophoresis in capillaries. Actually, the original purpose of this study was to develop a rapid method for the analysis of mixtures containing different molecular forms of transferrin.

EXPERIMENTAL

Materials

Human serum transferrin was purchased from Behring Werke (Marburg, Germany) and was used without further purification. Iron nitrilotriacetate and iron citrate solutions were prepared as described previously [11,12]. The desired degree of iron saturation was achieved by mixing these iron chelate solutions with the calculated volumes of a 150 mg/ml iron-free transferrin solution in a 20 m*M* HEPES buffer (pH 7.5) that contained 20 m*M* sodium hydrogencarbonate. The solutions were left at room temperature for 2 h and then dialysed overnight against 20 m*M* sodium hydrogencarbonate (pH 7.5) in the cold.

The urea was deionized on a mixed-bed ion-exchanger resin column [AG 501-X8(D) Bio-Rad, Richmond, CA, USA] and was stored in a refrigerator for up to 2 weeks in the presence of the resin. The urea-containing buffer was prepared from stock solution of 1.8 *M* Tris–1.8 *M* borate–30 m*M* EDTA (pH 8.4) diluted with an 8.08 *M* urea solution and deionized water to the desired concentration (0–8 *M*). Transferrin samples were prepared in 6 m*M* Tris–6 m*M* borate–0.1 m*M* EDTA (pH 8.4) buffer containing the same amount of urea as the electrophoresis buffer.

Capillary zone electrophoresis in the presence of urea

The electrophoresis experiments performed in glass capillaries (Modulohm, Herlev, Denmark) [13] with 0.1 mm I.D., were conducted in a 18 m*M* Tris–18 m*M* borate–0.03 m*M* EDTA (pH 8.4) buffer containing 0–8 *M* urea. The capillaries were coated as described in ref. 12 to eliminate adsorption and electroendosmosis. The lengths of the capillaries varied between 190 and 200 mm. The samples were applied into the tube by capillary force as previously described [11]. A modified Spectroflow 783 HPLC monitor (Kratos Division, Ramsey, NJ, USA) was used for on-tube detection at 280 nm. The monitoring was performed at a distance of 171–178 mm from one end of the capillary. The electrophoresis was carried out at 8000 V, which gave a current in the tube between 4.5 and 11 μA (the higher the urea concentration, the lower was the current). The relative viscosity values of the urea-containing buffers were taken from the literature [14]. All experiments were repeated 3–10 times to assess the reproducibility.

RESULTS

Iron-free and iron-saturated transferrin samples were investigated by zone electrophoresis in capillaries in the absence and in the presence of urea. In the absence of urea the characteristic pattern of iron-free transferrin was observed, showing the 4-sialo transferrin as the major component and the 2-sialo-, 3-sialo-, 5-sialo- and 6-sialo forms as minor ones (Fig. 1a). The separation order of these isoforms is reflected in the differences in their net surface charge densities caused by the differences in the sialic acid contents as discussed earlier [11].

The same number of transferrin isoforms were resolved in the ranges 0–3 *M* and 6–8 *M* urea (Fig. 1b, c, g and h). However, each isoform gave two peaks in the interval 3–6 *M* urea (Fig. 1d–f). The patterns in Fig. 1d–f show that all isoforms of the iron-free transferrin appeared in two different conformations characterized by different migration times. (It is easy to assign the major component, 4-sialo-transferrin, and the surrounding minor ones.) A change in the

5-sialo- and 4-sialo-transferrin peak sizes with time was observed. These changes occur slowly in 4 *M* urea, the ratios changing by only about 10% during 3 h (not shown). Observations made after larger periods in urea solutions were not meaningful since the expressed sulphydryl groups easily become oxidized [15], leading to the formation of "transferrin gels", as has been observed for albumin and ovalbumin [16,17]. However, the ratio of the peak areas in 5 *M* urea changed quickly (see Fig. 1e and f). After 2 h only one conformation of each isoform was detected (not shown).

Fig. 2 shows the electropherograms obtained by analysis of diferric human serum transferrin. The isoforms of the transferrin were clearly resolved. No splitting of the peaks was observed between 0 and 8 *M* urea. The resolution of the transferrin isoforms increased with the urea concentration, as was the case for the experiments presented in Fig. 1 for iron-free transferrin.

In order to follow the unfolding of transferrin, the migration times of the different components were plotted *vs.* the urea concentration. Since the migration rates decrease in the viscous urea solutions, the migration times were corrected by dividing them by the relative viscosity of the buffer. The corrected migration times are thus plotted for the 4-sialo iron-free and diferric transferrin *vs.* the urea concentration in Fig. 3. The curves show that unfolding of the iron-free transferrin is not a simple process. An intermediate conformation of iron-free transferrin exists between 3 and 6 *M* urea. As pointed out above, protein molecules in these intermediate states convert slowly to the unfolded conformation. No unfolding intermediates were observed in the case of diferric transferrin (Fig. 3).

Fig. 4a and b show denaturation curves for all the iron-free and diferric isoforms, respectively.

Since the original purpose of these capillary electrophoresis experiments was to develop a rapid method for the analysis of mixtures containing iron-free and iron-saturated transferrin molecules, artificial transferrin mixtures were analysed in 8 *M* urea. In one sample (Fig. 5a) the mixture was prepared with the intention of having the four molecular forms in a ratio of 1:1:1:1. Fig. 5 shows that the unfolded molecular forms of the different isoforms were well resolved in 8 *M* urea. Upon calculation of the peak

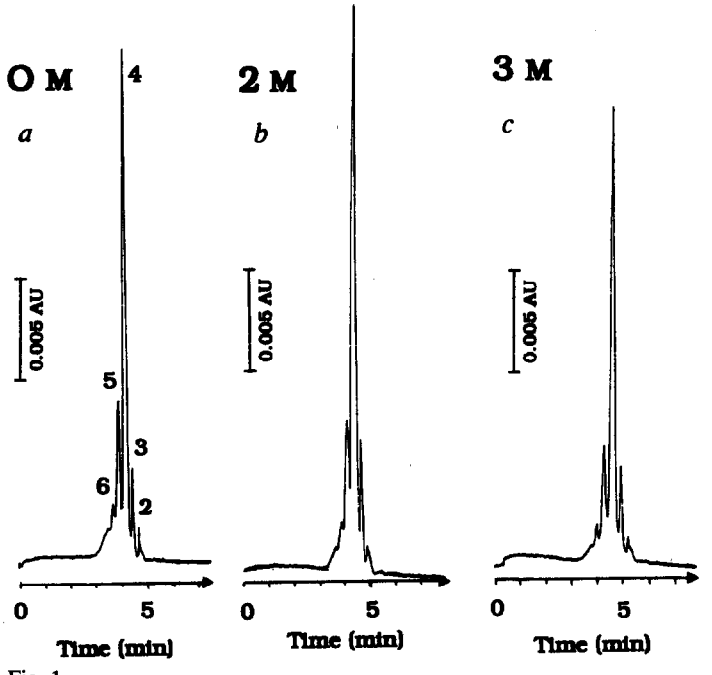

Fig. 1.

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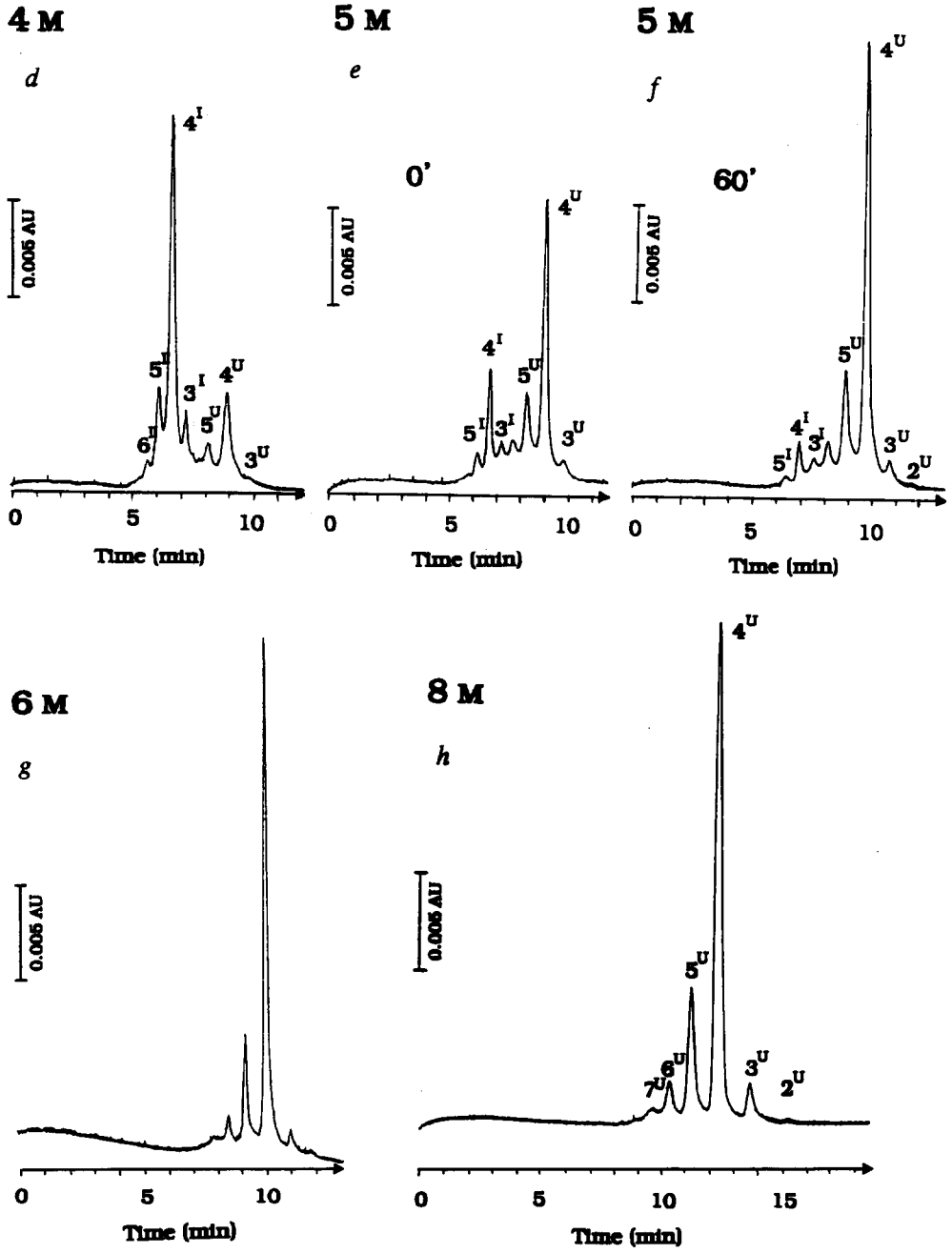

Fig. 1. High-performance electrophoresis of human serum iron-free transferrin in free solution in the absence (a) and presence (b–h) of urea. Experimental conditions: electrophoresis buffer, 18 m*M* Tris–18 m*M* boric acid–0.3 m*M* EDTA, pH 8.4; tube dimensions, 0.1 (I.D.) × 0.3 (O.D.) × 200 mm; voltage, 8000 V; on-tube detection at 280 nm. The current decreased with the increase of the urea concentration from 10 μA to 4.5 μA. Transferrin isoforms (2-sialo, 3-sialo, 4-sialo, 5-sialo and 6-sialo marked by 2, 3, 4, 5, 6, respectively) are better resolved at higher urea concentrations. The electrophoresis shows two conformations of the isoforms (I, intermediate; U, unfolded) in 4 *M* (d) and 5 *M* (e and f) urea. The ratio of the amounts of the conformational states changed significantly after 1 h in 5 *M* urea.

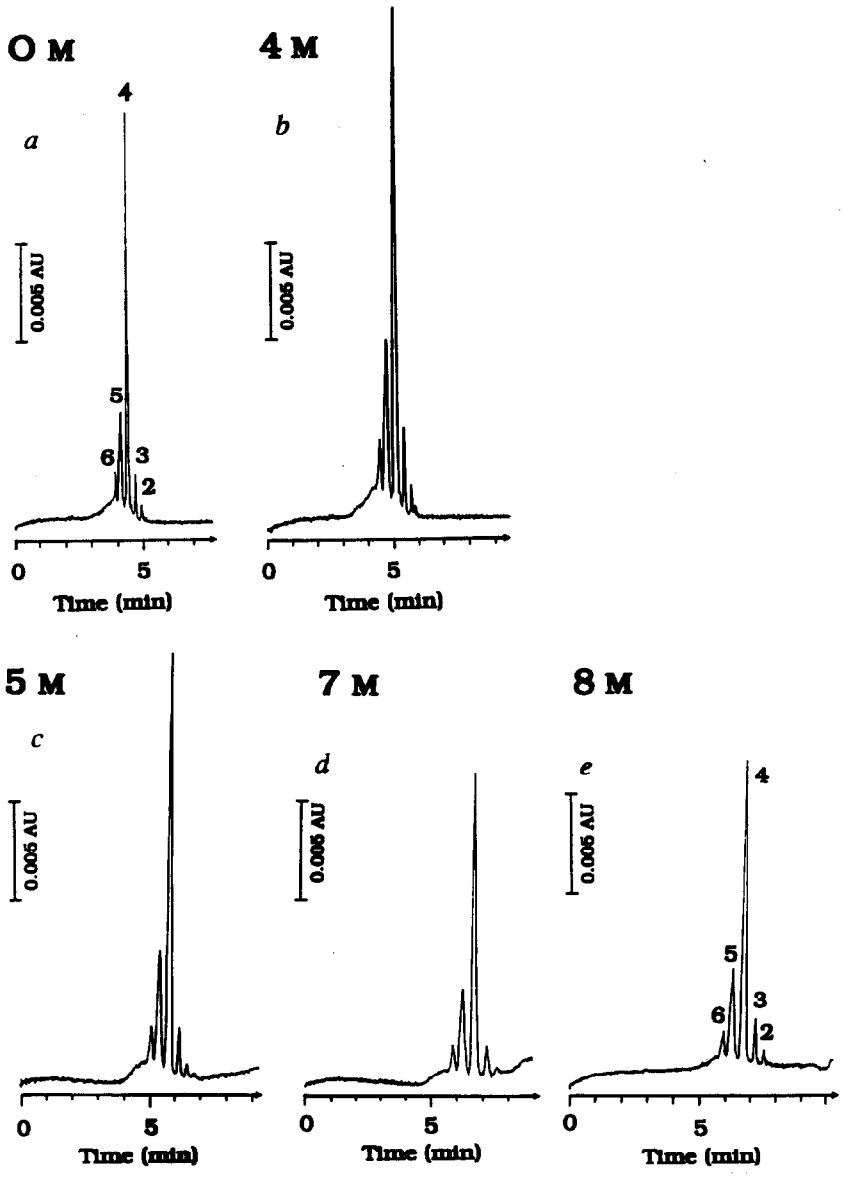

Fig. 2. High-performance electrophoresis of diferric human serum transferrin in free solution in the absence (a) and presence (b–e) of urea. The experimental conditions are the same as in the legend to Fig. 1. Transferrin isoforms (marked by 2, 3, 4, 5 and 6) are better resolved at higher urea concentration. The electrophoretic patterns are the same for all the experiments, although the resolution increases with urea concentration. The migration times of the isoforms increase with an increase in the urea concentration (a–e).

areas of the 4-sialo-transferrin components, we found that the mixture actually contained 30%, 30%, 20% and 20% of Fe_NTfFe_C, Fe_NTf, $TfFe_C$ and Tf, respectively, with an error of 5%. In another sample (Fig. 5b), the ratios of the different iron-containing isoforms were different, but all of the components were observable (compare with Fig. 5a).

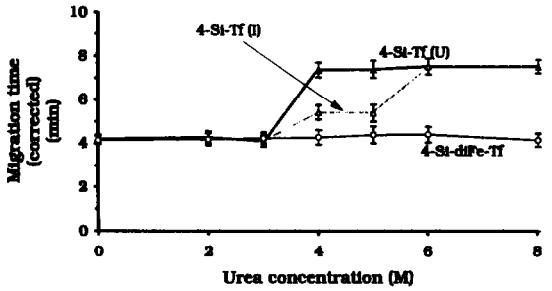

Fig. 3. Denaturation curves of the 4-sialo forms of iron-free (4-Si-Tf) and diferric (4-Si-diFe-Tf) human serum transferrin between 0 and 8 *M* urea (triangles and circles, respectively). The migration times of the 4-sialo isoforms in Figs. 1 and 2 were corrected for the increasing viscosity caused by the presence of urea and plotted *vs.* the concentration of urea in the buffer. An intermediate transition state (I) of the iron-free transferrin exists between 3 and 6 *M* urea together with the unfolded state (U). No unfolding intermediate transition state is observed for the diferric transferrin.

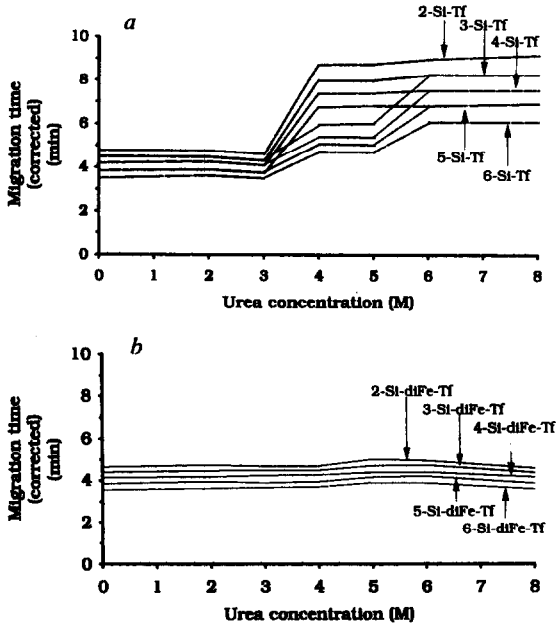

Fig. 4. Denaturation curves of the human serum (a) iron-free and (b) diferric transferrin isoforms between 0 and 8 *M* urea. Corrected migration times for the 2-sialo, 3-sialo-, 4-sialo-, 5-sialo- and 6-sialo-transferrin forms (marked as 2-Si-Tf, 3-Si-Tf, 4-Si-Tf, 5-Si-Tf, 6-Si-Tf or 2-Si-diFe-Tf, 3-Si-diFe-Tf, 4-Si-diFe-Tf, 5-Si-diFe-Tf, 6-Si-diFe-Tf, respectively) *vs.* urea concentration are plotted as in Fig. 3 (*cf.* Fig. 3). Intermediate transition states of the iron-free transferrin (a) isoforms exist between 3 and 6 *M* urea together with the unfolded states. The iron-saturated (diferric) isoforms of transferrin (b) are resistant to urea denaturation.

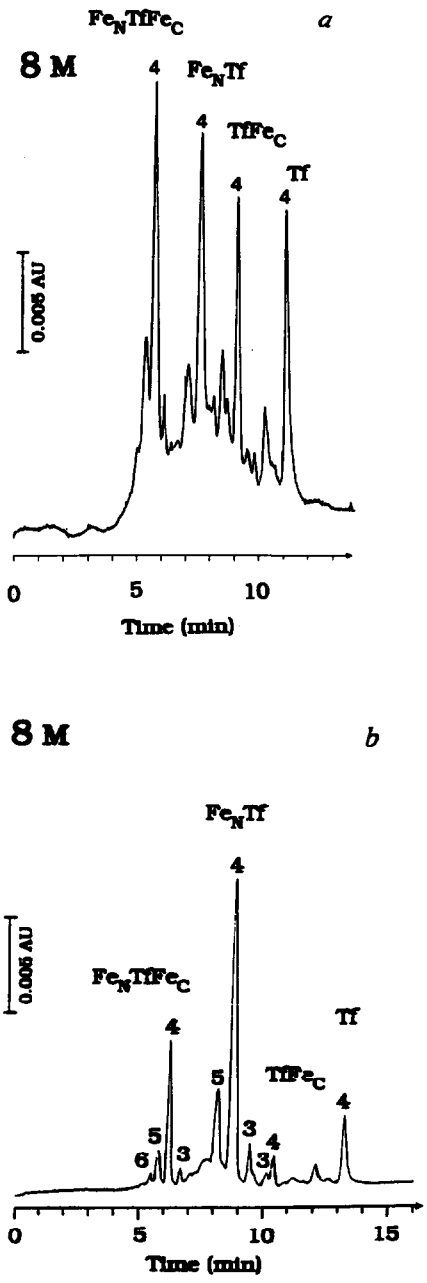

Fig. 5. High-performance capillary electrophoresis of mixtures containing iron-free (Tf), monoferric (Fe_NTf and $TfFe_C$, having iron bound in the N- and C-terminal lobe, respectively), and diferric (Fe_NTfFe_C) transferrin components (a) in approximately equal concentrations and (b) with a large amount of monoferric transferrin having iron bound at the N-terminal binding site and with lower amounts of the other forms. The peak sizes of the 4-sialo-transferrin components can be used for determination of the relative amounts of the different iron-containing transferrin forms.

DISCUSSION

Studies of the unfolding of proteins, for instance using electrophoresis in the presence of urea [3], gives information about the conformational stabilities of the molecules [18,19]. Electrophoresis has an advantage over other methods in that the relative amounts of the components may be determined easily from the electropherograms. However, conventional urea gel electrophoresis is a time-consuming procedure involving gel casting, running, staining and destaining. The use of urea gradient gels makes the procedure even more laborious. Therefore, we have utilized the advantages of capillary zone electrophoresis in free solution to analyse the unfolding of transferrin as well as for the quantitative and qualitative analysis of artificial mixtures of different species of transferrin. With the high-performance capillary electrophoresis technique the speed of the analysis in the presence of urea was increased by a factor of about 100 and the high resolution permitted detection of the unfolding intermediates of the iron-free transferrin isoforms.

The original gel electrophoresis method of Makey and Seal [20] for the analysis of the iron saturation of transferrin was adapted for capillary zone electrophoresis experiments in free solution with a few modifications. For instance, the buffer concentration was decreased to the value given in the Experimental section in order to speed up the analysis.

The unfolding of iron-free transferrin is a two-step procedure [5,6]. The transitions between the folded, unfolding (intermediate) and unfolded states occur between 3 and 6 *M* urea. Fig. 3 shows that the intermediate and the unfolded states of the iron-free transferrin coexist during a certain period of time. It has been proposed [5,6] that in the intermediate conformation the C-terminal lobe of transferrin is unfolded, while the N-terminal lobe remains in the native state. Since gel electrophoresis requires more than 15 h, the intermediate state could not be observed when the run was conducted in 4–6 *M* urea. Only the slowly interconverting conformation could be detected when the urea concentration was 3–4 *M* [5,6]. Measurement of the optical parameters of the transferrin [7,8] did not reveal an intermediate state, probably because the time scale was short compared with the speed of the interconversion.

Iron-saturated transferrin molecules have a more compact conformation than the iron-free forms [21,22] and are therefore more resistant to urea denaturation, as shown earlier [5,6] and also demonstrated in this study (see Figs. 3 and 4b). Accordingly, neither the polypeptide nor the carbohydrate chains are affected by the presence of urea.

Gel electrophoretic techniques are unable to resolve the transferrin isoforms [20,23]. Therefore, we have used capillary electrophoresis in free solution to compare the unfolding properties of the isoforms. Fig. 4a shows that the unfolding follows the same pattern for all of the isoforms in the iron-free transferrin. The differences between the migration velocities of the isoforms, which exist already in buffer without urea, are not caused by differences in the conformation of the carbohydrate moiety, as pointed out above for the iron-saturated isoforms. Hence, and as expected, only the conformation of the polypeptide chain is changed by urea, causing an alteration in net charge density, *i.e.* a change in migration time.

It has been shown previously that one can separate the two monoferric transferrin species in the presence of urea by polyacrylamide gel electrophoresis [20], which means that these species represent different conformational states. This conclusion is in agreement with small-angle X-ray scattering data [21]. However, it is not known how the differences in the conformations of the monoferric species influence the net surface charge density of the proteins and thereby their electrophoretic mobilities. As expected, also capillary electrophoresis in the presence of urea also permitted separation of the two types of the monoferric components. However, the isoforms could be resolved as well, as shown in Fig. 5 (where both monoferric transferrin components of each transferring isoform are separated).

The analytical separation of the isoforms and their species with different iron content shown in Fig. 5a and b is of diagnostic interest in the case

of, for example, alcoholism and analysis of blood from workers exposed to organic solvents [24,25] because these electropherograms can be utilized, for instance, for the quantitation of the 4-sialo- and 2-sialo-transferrin isoforms. Furthermore, it may now be possible to determine the iron content of these isoforms as well.

ACKNOWLEDGEMENTS

This work was supported by the Swedish Natural Science Research Council and by Grants OTKA No. 1981 and OTKA No. T5218 (Hungary).

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