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Utility of isotachophoresis–capillary zone electrophoresis, mass spectrometry and high-performance size-exclusion chromatography for monitoring of interleukin-6 dimer formation

J. Gysler[a,*], M. Mazereeuw[a], B. Helk[b], M. Heitzmann[b], U. Jaehde[c], W. Schunack[c], U.R. Tjaden[a], J. van der Greef[a]

[a]*Leiden/Amsterdam Center for Drug Research, Division of Analytical Chemistry, P.O. Box 9502, 2300 RA Leiden, The Netherlands*
[b]*Novartis Pharma AG, Biotechnological Development and Production, 4002 Basel, Switzerland*
[c]*Institute for Pharmacy, Free University of Berlin, Kg. Luise Strasse 2+4, 14195 Berlin, Germany*

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Abstract

The utility of isotachophoresis–capillary zone electrophoresis (ITP–CZE) and high-performance size-exclusion chromatography (HPSEC) was investigated for determination of dimeric and monomeric recombinant human interleukin-6 (rhIL-6). Using ITP–CZE heterogeneity of dimeric rhIL-6 could be revealed resolving two peaks in the electropherograms, while with HPSEC dimeric rhIL-6 eluted as one homogeneous fraction. Both protein forms were monitored during incubation of monomeric rhIL-6 at different pH and temperature. The selectivity of counterflow ITP–CZE in conjunction with the low concentration determination limits enabled reanalysis of HPSEC fractions for identification of the dimer in the electropherograms. Both ITP–CZE and HPSEC were shown to be suitable to monitor the dimerization of rhIL-6, similar monomer-to-dimer peak area ratios were obtained throughout the incubation. Dimer formation kinetics increased with decreasing pH and with increasing temperature, it was entirely suppressed at neutral pH and room temperature. In contrast to HPSEC, ITP–CZE enabled separation of further still unidentified artifacts apparently formed during incubation of rhIL-6. CZE analysis in conjunction with electrospray ionization mass spectrometry revealed the non-covalent binding character of the dimeric rhIL-6 complex and facilitated interpretation of the electropherograms.

Keywords: Isotachophoresis–capillary zone electrophoresis; Interleukins; Proteins

1. Introduction

Since the rapid growth of large scale production of pharmaceutically relevant proteins after the introduction of modern recombinant technology in the 1980s, convenient survey of protein purity and stability has attracted attention in the field of analytical chemistry. Chemical as well as physical instabilities, e.g., deviations in the three-dimensional structure of proteins leading to oligomer formation or denaturation are frequently observed during protein handling.

Recombinant human interleukin-6 (rhIL-6) from *E. coli* is a 185 amino acid non-glycosylated cyto-

*Corresponding author. Tel.: +31-71-527-4320; fax: +31-71-527-4277.
E-mail address: gysler@chem.leidenuniv.nl (J. Gysler)

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kine with an isoelectric point (p*I*) of 6.3. In recent studies rhIL-6 could be associated with the classical four-helix-bundle family of cytokines, structurally closely related to other cytokines like G-CSF, GH or LIF [1,2]. It acts as a pleiotropic growth factor regulating differentiation of immature blood cells [3]. Native IL-6 also plays an important role as a mediator of inflammation [4]. In their recently published study Matthews et al. described the use of high-performance size-exclusion chromatography (HPSEC) and analytical ultracentrifugation to monitor the urea-induced dissociation of a non-covalently bound dimeric association form of a rhIL-6 fusion protein [5].

Analytical techniques routinely applied in purity analysis of proteins mainly comprise selective methods based on chromatographic and electrophoretic principles. Highly specific and highly sensitive techniques like enzyme-linked immunosorbent assay (ELISA) or bioassays measure sample activity but cannot discriminate between several active analytes. Furthermore, mass spectrometry (MS) is commonly employed to identify analytes by their molecular mass [6].

During the last decade, capillary electrophoresis (CE) has matured from a novel technique to a powerful alternative to conventional slab gel electrophoresis of proteins, and is already routinely used in many laboratories [7]. Most commonly, protein analysis is performed in the capillary zone electrophoretic mode using coated capillaries to minimize loss of efficiency as a consequence of adsorption to the capillary walls [8,9].

Sensitivity problems of CE have reasonably been overcome by coupling of capillary zone electrophoresis (CZE) with on-line isotachophoretic sample preconcentration. In isotachophoresis (ITP) large diluted samples are focused to narrow and bands with high concentration using discontinuous electrolyte systems [10,11]. Several strategies proved successful for trace analysis of proteins by ITP–CZE [12–14]. Interfacing CE systems to various types of mass spectrometers (CE–MS) provided structural information on substances following their separation, and often enables clear identification of selected analytes within complex mixtures [15,16].

HPSEC is governed by a separation principle complementary to that of CZE. Sieving takes place with smaller molecules being more easily retained in the pores of a three-dimensional gel than larger molecules, which are therefore eluted faster by a mobile phase [6,17].

Besides qualitative structural information, however, reliable quantitative data are essential for the surveillance of the quality of a pharmaceutical product. Often, data collected from several analytical systems operating with orthogonal principles are placed into perspective to examine the quality of a product, or to trace a dynamic process taking place during the production.

In this report we investigated the utility of ITP–CZE and HPSEC to separate dimeric and monomeric rhIL-6. The potential of both techniques for quantitative monitoring of a this physical protein instability was evaluated. Dimer formation was recorded over a wide range of incubation pH and temperature.

2. Methods and materials

2.1. Chemicals

Anhydrous ammonium acetate, ammonium persulphate, potassium hydrogen phosphate, 2,4,6,8-tetramethyltetravinylcyclotetrasiloxane, *N*,*N*,*N*′,*N*′-tetramethylenediamine (TEMED), vinyltriacetoxysilane and cytochrome *c* (from horse heart, p*I* 10.2, M_r 12 400) were obtained from Sigma (St. Louis, MO, USA). Acrylamide, ammonia, anhydrous diethylether and glacial acetic acid were purchased from Merck (Darmstadt, Germany). Non-glycosylated rhIL-6 (from *E. coli*, p*I* 6.3, M_r 20 977) was supplied by Novartis, Basle, Switzerland. Water for the preparation of all solutions was taken from a Milli-Q UF Plus water purification system (Millipore, Bedford, MA, USA).

2.2. ITP–CZE

All ITP–CZE experiments were carried out on a programmable injection system for CE (PrinCE, Lauerlabs, Emmen, The Netherlands) equipped with a high-voltage power supply (Spellman, Plainview, NY, USA). Fused-silica capillaries, 75 μm I.D.×360 μm O.D. (SGE, Ringwood, Victoria, Australia), were cut to a total length of 70 cm (distance to the

detector 58 cm), and coated following the procedure established by Schmalzing et al. [18]. The capillary and the sample solution were held at ambient temperature. The UV detector (Spectra Physics, San Jose, CA, USA) was set to 200 nm. Peak integration was achieved by a Chromatopac C-R3A integrator (Shimadzu, Kyoto, Japan).

Single capillary ITP–CZE was performed applying a modified method introduced previously [13,19]. In brief, the separation capillary was entirely filled with leading electrolyte (20 m*M* ammonium acetate buffer, pH 4.2). Next, sample dissolved in terminating electrolyte (10 m*M* acetic acid, pH 4.2) was injected for 1.5 min at 75 mbar, corresponding to an injection volume of approximately 1 μl. Afterwards, isotachophoretic focusing was accomplished at 7 kV for 2 min with the inlet and outlet of the capillary dipping in terminating and leading electrolyte, respectively. For the reanalysis of HPSEC fractions the ITP focusing time was extended to 30 min while a simultaneous counterflow of 3 mbar was applied to the outlet vial to remove the excess of highly mobile salts originating from the eluent. Subsequently the counterflow was increased to 40 mbar to transport the analyte zones back to the capillary inlet [14,20].

In either case the final CZE separation step took place at 30 kV with both ends of the capillary placed in leading electrolyte. Between the runs the capillary was rinsed with leading electrolyte (LE) at 1 bar for 2 min.

2.3. HPSEC

HPSEC analyses were performed on a FPLC system (Pharmacia, Uppsala, Sweden). It was equipped with a Progel G2000SWXL column, 30× 0.78 cm (Supelco, Bellefonte, PA, USA). The elution buffer was 150 m*M* potassium phosphate, pH 6.8, the flow-rate was adjusted to 0.5 ml/min. A 25-μl volume of each sample was pressure-injected, for detection the UV absorbance was monitored at 214 nm. For quantification a D-200 integrator (Merck-Hitachi, Darmstadt, Germany) was used.

2.4. Mass spectrometry

In the CZE–electrospray ionization (ESI) MS experiments a volatile 20 m*M* ammonium acetate buffer, pH 4.2, was used for separation. Coated fused-silica capillaries of 75 μm I.D. were cut to a total length of 75 cm. A programmable modular capillary electrophoresis system (PrinCE, Lauerlabs) was connected to a SSQ 710 mass spectrometer (Finnigan MAT, Bremen, Germany) via an ESI interface (Analytica, Branford, CT, USA). The sampling capillary of the ESI interface was held at ground potential and heated to 180°C. Mass analysis was performed on a single quadrupole with an upper mass limit of 2000 u.

A stainless steel ESI needle (300 μm I.D.) was positioned at approximately 1 cm distance, in-line with the sampling capillary and set to +3 kV. The sheath liquid consisted of methanol–water (80:20, v/v) containing 1% (v/v) acetic acid and was pumped with a flow-rate of 2 μl/min using a syringe pump (Harvard Apparatus, South Natick, MS, USA). The outlet of the fused-silica capillary was placed in the ESI needle to establish electrical contact at the cathodic side. Analytes were sprayed at atmospheric pressure and measured as multiply protonated ions in the m/z range between 800 and 1600.

2.5. Incubation

To avoid thermal denaturation occurring above 50°C leading to insoluble material the incubation temperature was limited to 45°C. For the HPSEC study monomeric rhIL-6 was incubated at pH 4.0, 5.0, 6.0 and 7.0. Incubation buffers were prepared from a stock solution of 20 m*M* sodium triphosphate–sodium acetate and adjusted to the respective pH with concentrated phosphoric acid. Samples to be analyzed by ITP–CZE were incubated in 20 m*M* ammonium acetate buffer adjusted to pH 4.0, 5.0, 6.0 and 7.0, respectively, with acetic acid or ammonia (5%, v/v).

In order to evaluate the impact of different incubation temperatures on dimer formation by ITP–CZE, 100 μl of monomeric rhIL-6 solution (4.5 mg/ml) was mixed with 200 μl of acetic acid (0.5%, v/v) and incubated over 48 h at 20, 30 and 45°C, respectively. Hereby, addition of extra salts to the sample was avoided, which would cause uncomfortably long extension of the required ITP focusing time. All incubation experiments were performed with three different samples.

2.6. Sampling

Serial samples of 50 μl were drawn before the start and after 2, 4, 7 and 21 h of incubation and immediately analyzed by HPSEC without further preparation (see Section 2.3). For ITP–CZE analysis (see Section 2.2) 10 μl samples were drawn, mixed with 5 μl of internal standard and finally dissolved in 485 μl of terminating electrolyte to give a nominal rhIL-6 concentration of 30 μg/ml ($1.4 \cdot 10^{-6}$ *M*).

2.7. Precision and calibration

For ITP–CZE weighted linear regression ($w=1/y^2$) of rhIL-6 monomer concentration (3.3–30.0 μg/ml) versus peak area was performed in order to create calibration graphs. Calibration was renewed daily, only graphs with $r>0.990$ were accepted to calculate rhIL-6 concentrations.

Cytochrome *c* was selected as internal standard for several reasons. First, it was well separated from all rhIL-6 peaks, secondly its peak shape helped to indicate the quality of the coating of the capillary, because cytochrome *c* easily adsorbs to bare fused-silica due to its basic isoelectric point.

For HPSEC calibration was carried out with proteins from a calibration kit (Pharmacia) containing bovine serum albumin (M_r 67 000), ovalbumin (M_r 43 000), chymotrypsinogen A (M_r 25 000), and ribonuclease A (M_r 13 700). Log M_r was plotted against retention time and the molecular masses of the rhIL-6 peaks were calculated from this calibration curve.

3. Results and discussion

3.1. HPSEC

Representative chromatograms of monomeric (1) and dimeric (2) rhIL-6 are presented in Fig. 1. As expected, the larger dimeric form elutes faster (elution time 15.1 min) than the smaller rhIL-6 monomers. Before acidic stressing the monomeric form was dominantly present (Fig. 1A), while after 21 h incubation of monomeric rhIL-6 at pH 4.0 and 45°C a protein of double mass could be detected in the chromatograms (Fig. 1B). None of the chromatograms indicated the presence of trimers or even higher order agglomerates (expected elution time 14.1 and 11.4 min, respectively). After reneutralisation the monomer was again found in large excess (not shown). The reversibility of the acid induced dimerization indicated a non-covalent binding character within the dimeric associates. In order to further characterize the mechanism dimerization of rhIL-6 was monitored during incubation at four different pH values at 45°C. The data are summarized in Table 1. We chose the monomer-to-dimer (peak area) ratio as a measure for dimerization, because dimeric rhIL-6 was not available as a standard. Dimer formation is efficiently suppressed when the pH approaches neutral. At pH 7.0 no significant increase of dimer content could be observed after 21 h compared to the initial situation even at elevated temperature during incubation. HPSEC measurements of rhIL-6 incubated at room temperature generally revealed much slower dimerization kinetics (not shown).

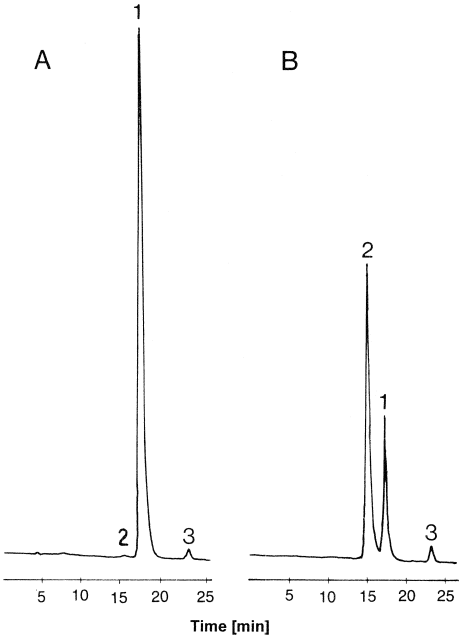

Fig. 1. Representative HPSEC chromatograms of rhIL-6 (A) before, (B) after 21 h of incubation at pH 4.0 at 45°C. A 25-μl volume was injected, the sample concentration was 1.5 mg/ml. Peak identification: 1=dimeric, 2=monomeric rhIL-6, 3=acetate.

HPSEC was suitable to separate monomeric and

Table 1
Dependency of the dimer formation of rhIL-6 on incubation pH at 45°C determined by quantitative HPSEC

pH	Concentration (mg/ml)	Monomer/dimer ratio at 45°C (%)				
		0 h	2 h	4 h	7 h	21 h
4.0	1.5	99/1	41/59	33/67	32/68	32/68
5.0	1.5	99/1	66/34	59/41	57/43	55/45
6.0	1.5	99/1	n.d.	98/2	n.d.	97/3[b]
7.0	1.5	99/1	n.d.	n.d.	99/1	99/1[b]
7.0[a]	1.0	32/68	70/30	82/18	n.d.	98/2

[a] In these reequilibration experiments, pure monomer samples previously incubated at pH 4.0 were readjusted to pH 7.0 and reincubated at 45°C.
[b] Final equilibration not reached due to slow reaction rates.
n.d.=Not determined.

dimeric rhIL-6 and monitor the reversible dimerization process. Absolute protein amounts in the lower nanomolar range were required for a single analysis leading to high costs in routine quality control analysis. Alternative analytical methods should therefore be considered to reduce the sample consumption.

3.2. ITP–CZE

The ITP–CZE electropherogram of the untreated rhIL-6 solution is shown in Fig. 2A. Before the injection the rhIL-6 stock solution (4.5 mg/ml) was diluted to a concentration of 30 μg/ml adding terminating electrolyte (10 m*M* acetic acid). Besides the internal standard (I.S.) rhIL-6 gave one main peak (3) besides two small side peaks with higher electrophoretic mobility (1, 2). During the incubation of monomeric rhIL-6 in diluted acetic acid (pH 4.0, 45°C) the size of peaks (1) and (2) increased while the original peak decreased (Fig. 2B). This reaction was reversible upon reneutralisation (Fig. 2C). Comparing these results with the HPSEC data (see Section 3.1) this observation lead to the assumption that the double peak stands for two physically different forms of dimeric rhIL-6 which could not be separated using HPSEC. Using ITP–CZE much lower protein quantities only in the lower picomolar range were required for a single analysis when compared to HPSEC. Despite the reduction of sample concentration and injection volume several small peaks (marked with asterisks) were observed in the electropherograms after reneutralisation. They might indicate cleavage products of the protein back-bone during the incubation stress. The identification of these by-products is currently under investigation.

3.3. Analysis of HPSEC fractions by counterflow ITP–CZE

In order to ensure the interpretation given above ITP–CZE electropherograms both protein-containing fractions collected from the HPSEC separations (see Section 3.1) were further analyzed by counterflow ITP–CZE. The use of a counterflow was unavoidable because the time required for ITP focusing was highly extended due to the presence of large amounts of highly mobile salts from the HPSEC eluent. Transient ITP–CZE failed to resolve any peaks in the electropherograms, even after five-fold dilution of the samples with terminating electrolyte (not shown). The use of a 3 mbar back-pressure permitted extension of the effective ITP focusing time and ensured removal of salts with a mobility above that of the leading electrolyte. Sufficient focusing and sample cleanup was indicated by the decrease of the current [19]. After 30 min the back-pressure was increased to 40 mbar to transport the analytes back to capillary inlet. Fig. 3A shows the counterflow ITP–CZE reanalysis of HPSEC 2 fraction containing dimeric rhIL-6. The double peak dominating the electropherogram is comparable to that in Fig. 2B and was therefore associated with dimeric rhIL-6. The peak size of the third peak increased upon addition of HPSEC fraction (1) to the sample which was further evidence that the dimeric form of rhIL-6 was represented by the double peak.

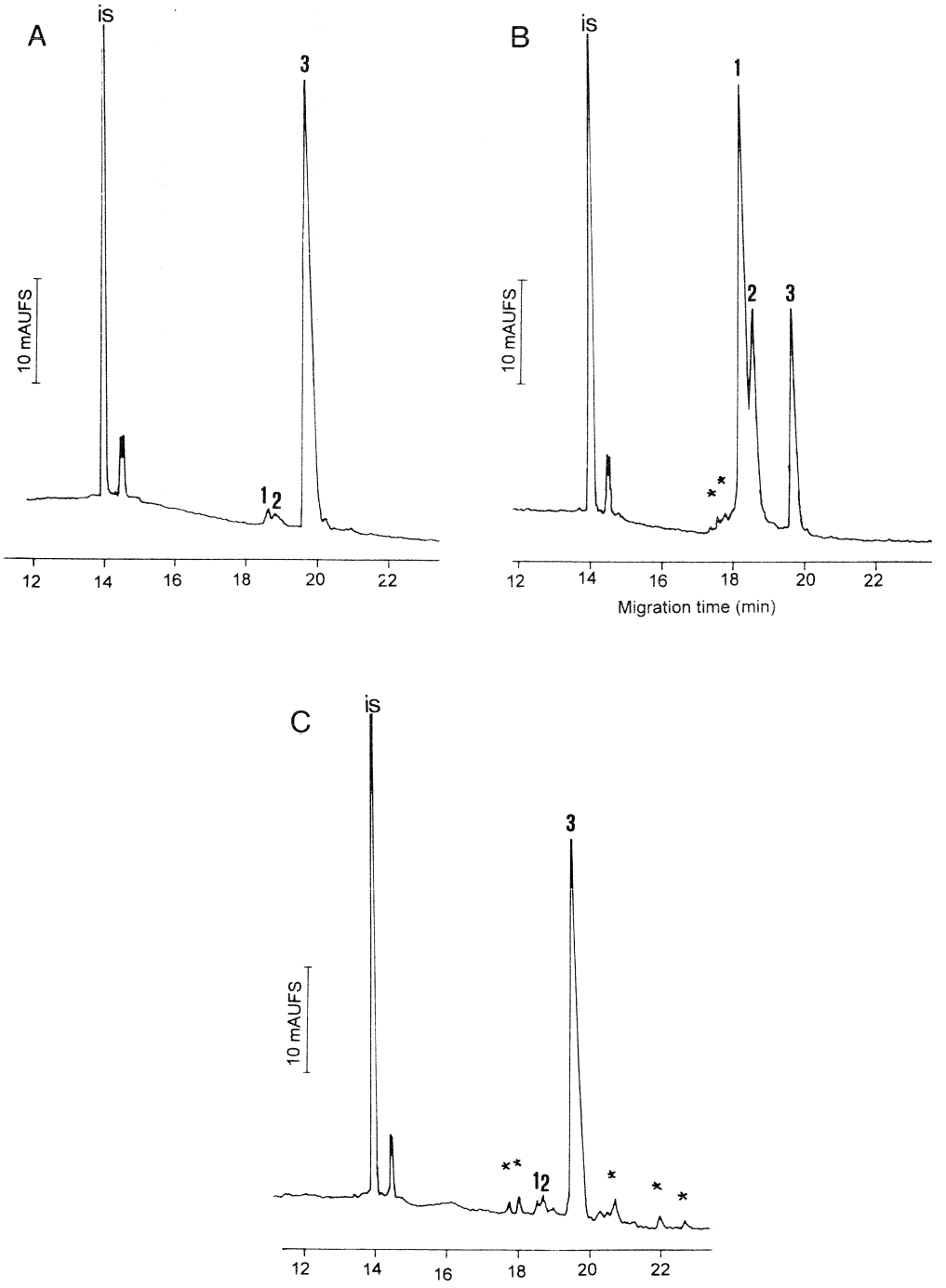

Fig. 2. Representative ITP–CZE electropherograms of dimeric (1, 2) and monomeric (3) rhIL-6 (A) before start of the incubation, (B) after 21 h of incubation in diluted acetic acid at 45°C, and (C) after reneutralisation of (B). Cytochrome *c* (I.S.) was utilized as internal standard. The concentration of rhIL-6 was 30 μg/ml, 1 μl of sample was injected. Peaks marked with an asterisk are unidentified artifacts appearing during the course of incubation with diluted acetic acid.

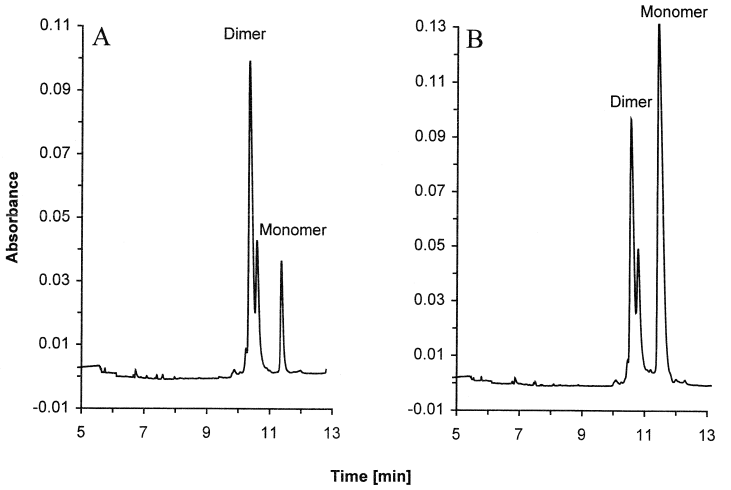

Fig. 3. Reanalysis of HPSEC fractions by counterflow ITP–CZE after five-fold dilution with terminating electrolyte. (A) Fraction (2) containing dimeric rhIL-6 collected after incubation at pH 4 and 45°C for 25 h, (B) shows an electropherogram of a mixture of fractions (1) and (2) of sample incubated with acetic acid. The injection volume was 1 μl. ITP focusing was extended to 30 min to remove salt from the sample and to complete the focusing process. The abscissa shows the time from the start of the CZE step.

3.4. CZE with ESI-MS detection

ESI-MS was interfaced to the CZE system to receive structural information about the peaks. Fig. 4A shows the CZE–ESI-MS electropherogram of rhIL-6 after pH 4 incubation at 45°C. The mass spectra recorded at the migration times are depicted in Fig. 4B–D. Equal mass spectra revealed that all of the observed peaks contained only monomeric rhIL-6. This finding can be explained with dissociation of the non-covalently bound dimeric rhIL-6 complexes during the mixing with the methanol-containing sheath liquid. This suggestion was supported by the observation that the double peak had completely disappeared from the electropherograms when 30% of methanol was added to samples containing dimeric rhIL-6 (not shown). The simultaneous increase of the monomer peak indicated that hydrophobic forces play an important role in stabilizing the rhIL-6 dimers.

The combination of these findings strongly supports the concept that rhIL-6 forms two different dimeric complexes. They require an electrophoretically-based separation mechanism for their separation.

Conformational changes initiated by acidification might expose hydrophobic domains of the protein molecule to its surface. Following this assumption physically varying sub-classes might form within the dimer fraction, when monomers are attached to each other at different moieties. At the same time our results lead to the assumption that reversibility and suppression of dimerization at elevated pH are caused by coulombic repulsion of negatively charged carboxylic groups of the monomers. Our interpretation was supported by a recently published study from Matthews et al. [5]. They used conventional sedimentation velocity analysis to indicate that dimer formation might be based on a domain-swapping mechanism.

3.5. Quantitative aspects – dimerization kinetics at different pH and temperature

In Fig. 5 the rhIL-6 monomer concentration, the monomer-to-dimer peak area ratio and the total electropherogram peak area were plotted against incubation time, exemplary for a pH 4.0 incubation at 45°C. As dimeric rhIL-6 was not available as standard material to establish independent calibration graphs for this component the monomer-to-dimer peak area ratio was calculated as a measure to

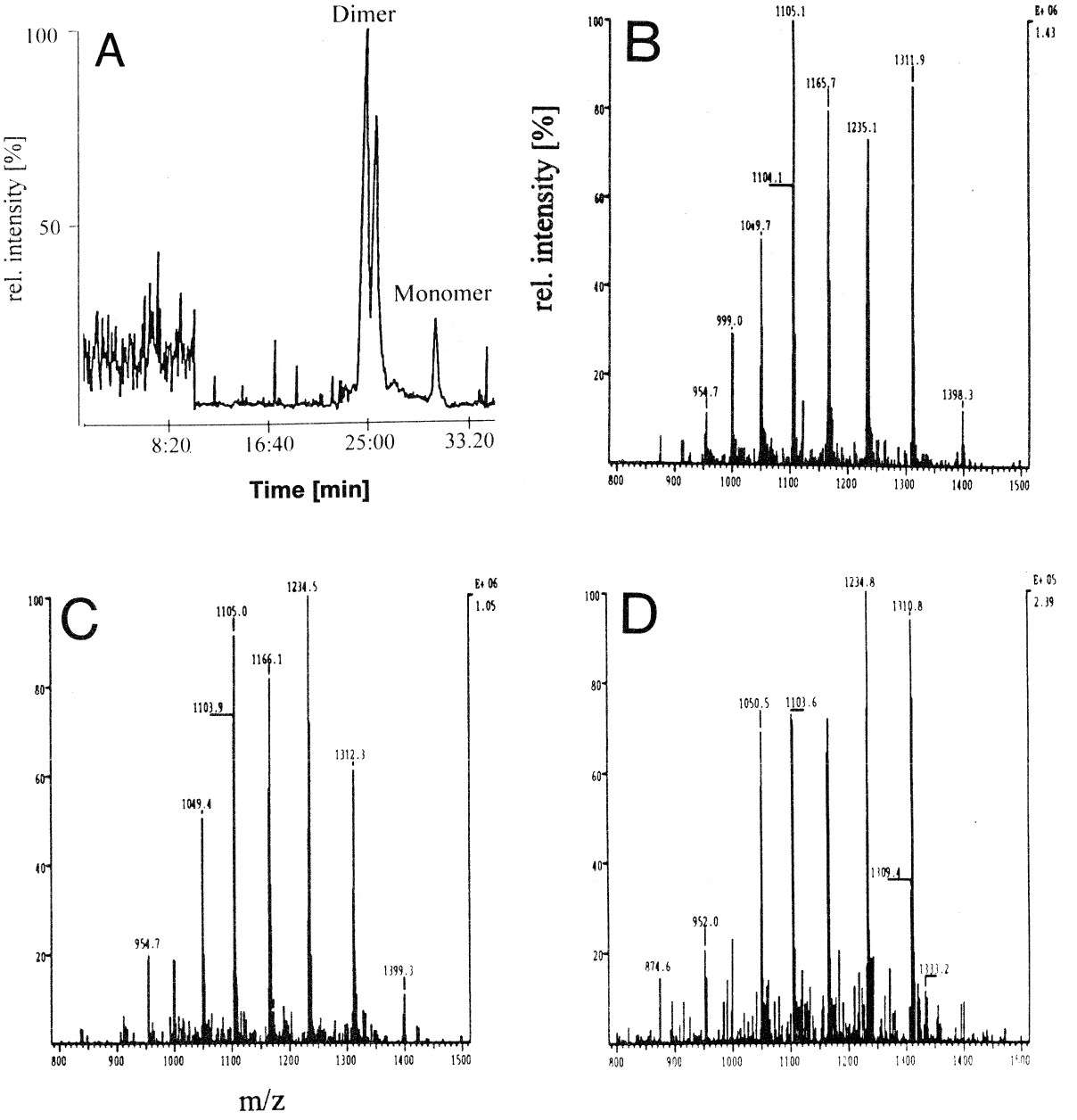

Fig. 4. (A) CZE–ESI-MS electropherogram of a 20-nl injection of rhIL-6 treated with acetic acid at 40°C over 25 h. The running electrolyte was 10 m*M* ammonium acetate buffer, pH 4.2, to guarantee sufficient volatility. The sheath flow consisted of methanol–water (80:20, v/v) with 1% (v/v) acetic acid (B–D). The similar pattern of the mass spectra at different migration times indicates that dimeric rhIL-6 dissolves into monomers under the spraying conditions.

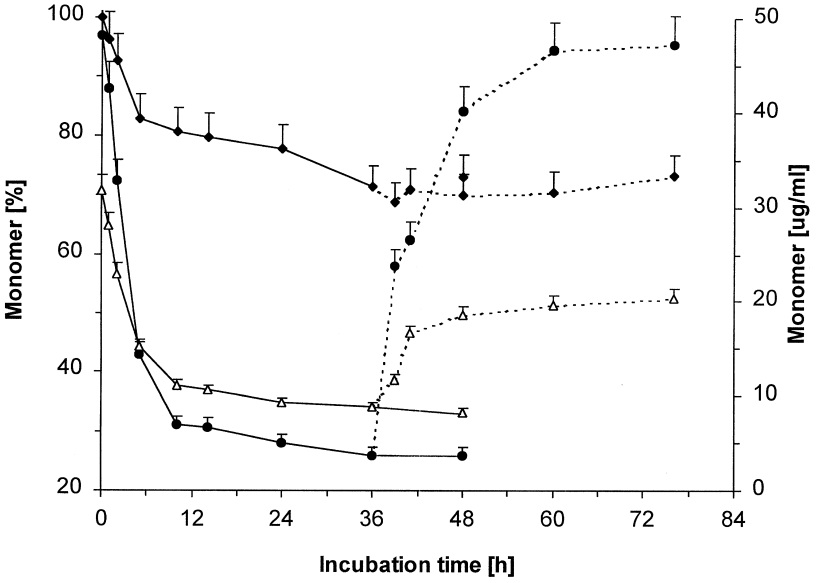

Fig. 5. ITP–CZE: Dynamic change of the peak area percentage of monomeric rhIL-6 (●) during incubation of monomeric rhIL-6 with diluted acetic acid at 45°C. ♦=The total area of all peaks observed in the electropherograms including the internal standard. The concentration values (μg/ml) of monomeric rhIL-6 (△) are read on the right ordinate. Solid lines stand for measurements during acidic incubation, dashed lines depict the course after reneutralisation. All curves show average values of three incubation experiments±SD (n=3).

express dynamic changes during the incubation. Moreover, we monitored the total peak area of each electropherogram including all other peaks. The decrease of total peak area during the first 36 h of incubation indicated a loss of total protein (Fig. 5). Nonspecific and irreversible adsorption to the walls of the incubation vials was made responsible, yet no visible aggregation occurred applying the selected mild conditions. After about 36 h the total peak area remained stable which was taken as an indicator for saturation of nonspecific binding sites. The internal standard (cytochrome *c*), added directly before the injection, showed no relevant decrease in signal with assay time indicating good stability of the separation system. After 48 h of incubation at room temperature the total protein loss was less than 10% (not shown).

The size of the unidentified characteristic side peaks formed during incubation at pH 4.0 (see Fig. 2C) was also increasing with incubation temperature and time. It should be mentioned that higher incubation pH induced formation of peaks not found after incubation at pH 4.0 and 5.0, exhibiting longer retention times than the parent compound rhIL-6 (not shown). Chemical instabilities, preferably occurring at elevated pH but not in acidic media could provide an explanation for these observations [21].

The decrease of the peak area percentage of monomeric rhIL-6 during 48 h of incubation at pH 4.0 at 20, 30 and 45°C is depicted in Fig. 6. The loss of monomers, equivalent to the formation of dimers, was significantly higher at 45°C (graph A) than at 30°C (graph B), while at 20°C less than 10% of monomers had formed dimeric complexes after 48 h (graph C).

If ionic and hydrophobic interaction forces are assumed to effect the non-covalent binding of the rhIL-6 dimers dimer formation should depend manly on incubation pH. To reduce the positive charges on one hand and to deprotonate carboxylic functions on the other incubation was also carried out at pH approaching neutral. Table 2 summarizes the monomer/dimer peak area ratio obtained with ITP–CZE analysis during incubation at four different pH values (4.0, 5.0, 6.0, 7.0). Obviously, the dimerization was effectively suppressed at pH values approaching neutral, even at elevated temperature. These findings stand in good agreement to our predictions. The deviations to the values determined by HPSEC

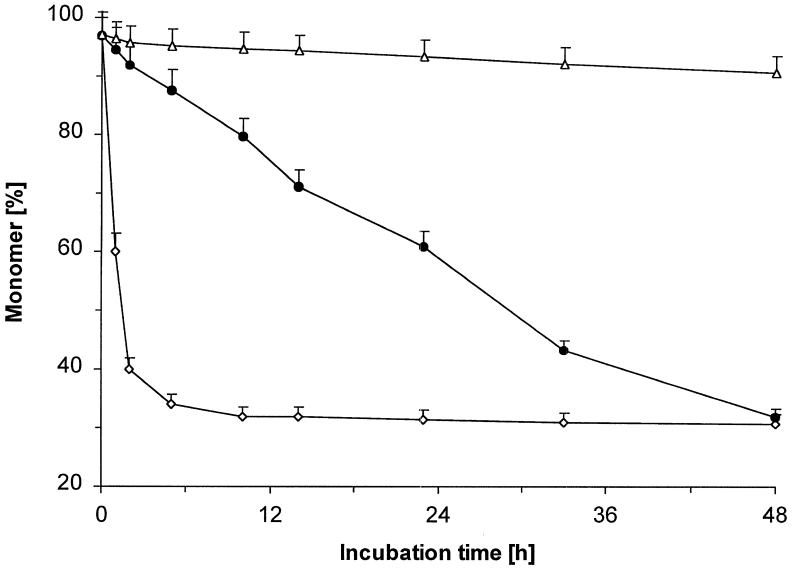

Fig. 6. ITP–CZE: Percentage of monomers measured during incubation of monomeric rhIL-6 solution with acetic acid (pH 4.0) at three different temperatures, (△) 20°C, (○) 30°C and (◊) 45°C. All curves show average values of three incubation experiments±SD (n=3).

(Table 1) might be caused by slight differences of the incubation pH. Furthermore, the ionic strength during analysis has to be taken into consideration when comparing data.

4. Conclusions

RhIL-6 dimer formation during acidic incubation was successfully monitored by ITP–CZE and HPSEC. While two different dimeric association forms could be separated by ITP–CZE, the dimeric fraction eluted as homogeneous fraction from the HPSEC column. The heterogeneity in shape among these forms was not sufficient to achieve separation based on the size-exclusion mechanism. The complementary CZE separation mechanism, based on charge-to-mass ratio rather than on size exclusion, along with its tremendous separation power enabled discrimination of two different dimeric rhIL-6 association forms.

The 20-fold reduced sample volume, along with much lower required sample concentration compared to HPSEC makes ITP–CZE very attractive for fast

Table 2
Dependency of the rhIL-6 dimer formation on incubation pH at 45°C determined by quantitative ITP–CZE

pH	Concentration (mg/ml)	Monomer/dimer ratio at 45°C (%)				
		0 h	2 h	4 h	7 h	21 h
4.0	1.5	98/2	40/60	36/56	32/68	31/69
5.0	1.5	98/2	68/32	65/35	64/36	62/38
6.0	1.5	99/1	98/2	n.d.	98/2	96/4[b]
7.0	1.5	99/1	99/1	n.d.	99/1	98/2
7.0[a]	1.0	32/68	63/37	74/26	86/14	97/3

[a] In these reequilibration experiments, pure monomer samples previously incubated at pH 4.0 were readjusted to pH 7.0 and reincubated at 45°C.

[b] Final equilibration not reached due to slow reaction rates.

n.d.=Not determined.

and efficient analysis of expensive proteins or for samples available in minute quantities only.

The applicability of the ITP–CZE system for the characterization of other dimer- or oligomer-forming proteins is currently under investigation.

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