Modification of capillary electrophoresis selectivity in hydro-organic solutions
Dissociation constants and Stokes radius measurements of peptides in water–2,2,2-trifluoroethanol mixtures

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Abstract

Peptide electrophoretic mobilities were measured at different acidic pH in water–2,2,2-trifluoroethanol mixtures. Fit of experimental mobilities according to binding equations allowed the calculation of peptide C-terminus dissociation constants and the Stokes radius of the differently protonated forms. Analysis of the data showed that the use of hydro-organic solvents in capillary electrophoresis offers the following principal advantages: (a) a modification of dissociation constants and Stokes radii that are strongly dependent upon peptide sequence and that can be conveniently utilised for selectivity manipulation; (b) an increase of separation performance arising from the stabilisation of particular peptide conformations; (c) a greater solubility of large apolar peptides with respect to aqueous solutions. © 1997 Published by Elsevier Science B.V.

Keywords: Selectivity; Dissociation constants; Stokes radius; Buffer composition; Peptides; Trifluoroethanol

1. Introduction

The use of mixed hydro-organic solution as conductivity media in capillary electrophoresis (CE) is continuously increasing. In fact, the addition of an organic solvent to CE separation buffers can offer various advantages over aqueous media [1–4]. Since the electric double layer of the capillary wall is modified, electroosmosis is greatly reduced [5]. Furthermore, the general increase in viscosity reduces and stabilises the running current and also facilitates heat exchanges, thus improving the num-

ber of theoretical plates. Organic solvents strongly modify both charge and Stokes radius of peptides, adding a chance in manipulation of separation selectivity. Sahota and Khaledi [6] established that formamide exhibits the best characteristics with respect to other solvents, including methanol and acetonitrile. Recently, the use of non-aqueous media in capillary electrophoresis was widely reviewed [7]. We have demonstrated that one of the most promising options is the use of 2,2,2-trifluoroethanol (TFE) [8]. This solvent increases the solubility of apolar peptides. Moreover, TFE induces stable secondary conformations in peptides which are otherwise unstructured in aqueous solutions, contributing to transitions from random to defined and limited structures [9], with a

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consequent decrease in peak dispersion and improvement of CE separation. Furthermore, it is commonly assumed that TFE forces peptide towards conformations similar to those assumed in membrane environment [10]. An aqueous environment promotes conformational rearrangements by mediating hydrogen bond exchange and by inducing dynamics at the solvent exposed surface [11,12]. Consequently, even minimum-energy conformational states are marginally stable. On the contrary, apolar environments do not mediate hydrogen bond exchange or promote dynamics and conformational states are stabilised. A suitable composition is TFE–water (30:70, v/v) [13], in order to exploit these TFE structuring properties. For this reason we have chosen this concentration as a reference solution to gain information about the peptide conformational transition connected to the proton loss in hydro-organic media. In fact, the dependence of electrophoretic mobility on pH can be used to calculate the proton dissociation constants and the Stokes radii of the different protonated peptide forms [14]. The comparison between peptide behaviours in aqueous and TFE–water solutions can be utilised to modify CE selectivity in peptide separations.

2. Material and methods

2.1. Reagents and instruments

All common chemicals used were from Farmitalia-Carlo Erba (Milan, Italy) or from Merck (Darmstadt, Germany). Standard peptides were purchased from Bachem (Bubendorf, Switzerland) and Serva (Heidelberg, Germany). Capillary electrophoretic separations were performed on a Beckman (Fullerton, CA, USA) PACE system 2100, equipped with a Beckman Gold 711 system for automated apparatus control and data acquisition. Fused-silica capillaries were provided by Beckman. They were coated with a layer of linear polyacrylamide directly on the electrophoretic apparatus [15] and coating was stabilised as previously described [8]. By this coating, at acidic pH values no appreciable electroosmosis was observed, as acetanilide transit was not observed after more than 6 h of CE run performed under the conditions described in the next section.

2.2. Methods

The electrophoretic runs were performed with a coated silica capillary of 56.5 cm (50.0 cm to detection window)×75 μm I.D., at a constant applied voltage of 25 kV. The running temperature was 25°C. The buffer solutions in water as well as in TFE–water (30:70, v/v) mixtures (corresponding to a 0.0531 molar fraction) were prepared at the desired pH value from stock solutions in order to obtain a final sodium phosphate concentration of 80 mM. The measured decrease of pH values in TFE–water (30:70, v/v) mixtures accounted for 0.24±0.01. The sample solutions were usually prepared from a stock solution ranging between 2–3 mg/ml, diluted with the running buffer in order to obtain a final concentration of about 0.2–1.0 mg/ml in sodium phosphate concentration of 8.0 mM. The injection was performed by pressure for 2 s, corresponding to about 5–8 nl. The detection absorbance wavelength was set at 200 nm. Each experimental result is the mean of at least duplicate measurements. Experimental electrophoretic mobility values were analysed according to the equations described in the theory section. The pK_{app} values and Stokes radius were calculated by parametric best fits using minimisation procedures based on Marquard's algorithm. The error on measurement was assumed as two times the standard deviation.

2.3. Theory

In the absence of electroosmotic flow the experimental electrophoretic mobility is simply controlled by the basic equation:

$$\mu_{ep} = \frac{qZ}{6\pi\eta r_S} \qquad (1)$$

where q is the electron charge ($1.60\cdot10^{-19}$ coulomb), η the solution viscosity, r_S the Stokes radius (i.e. the radius of the sphere equivalent to hydrated peptide) and Z corresponds to the number of elementary charges (dimensionless). Thus, electrophoretic mobility is simply related to the ratio Z/r_S. For weak electrolytes, such as peptides, the charge and the Stokes radius are affected by running buffer properties, particularly by proton activity. In a

solution at high proton activity, i.e. low pH values, the peptide is quite fully protonated and has a positive charge approximately equal to the total number of the basic groups.

If a generic peptide ($X^{(\text{charge})}$) having j basic groups and i acidic groups is considered, its dissociation can be described as follows:

$$X^j \underset{K_1}{\to} X^{j-1} \underset{K_2}{\to} \cdots \underset{K_{j+i}}{\to} X^{-i} \tag{2}$$
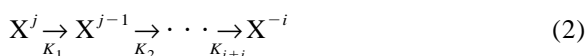
Since proton exchange is much faster than the amount of time required for the analysis, electrophoretic mobility observed at a given pH may be regarded as a means of the mobility of any k different charged form. Each form contributes with its molar fraction x_k:

$$\mu_{\text{obs}} = \mu_j x_j + \mu_{j-1} x_{j-1} + \cdots + \mu_{-i} x_{-i}$$
$$= \sum_{k=j}^{k=-i} \mu_k x_k \tag{3}$$

and from Eqs. (1) and (3):

$$\mu_{\text{obs}} = \frac{q}{6\pi\eta} \sum_{k=j}^{k=-i} \frac{k}{r_{s_k}} \cdot x_k \tag{4}$$

The molar fraction of any form can be expressed as a function of proton activity and of dissociation constants, as follows:

$$x_{j-n} = \frac{[X^{j-n}]}{[X^j] + [X^{j-1}] + [X^{j-2}] + \cdots + [X^{-i}]}$$
$$= \frac{\beta_n / [H^+]^n}{P} \ (n = 0, 1, 2, \ldots, j+i) \tag{5}$$

where P is the binding polynomial

$$P = \beta_0 + \beta_1 / [H^+] + \beta_2 / [H^+]^2 + \cdots$$
$$+ \beta_{j+i} / [H^+]^{j+i} \tag{6}$$

and

$$\beta_0 = 1; \ \beta_1 = K_1; \ \beta_2 = K_1 K_2; \ldots;$$

$$B_{j+i} = K_1 K_2 K_3 \ldots K_{j+i} \tag{7}$$

Eqs. (4)–(7) account for the difference in the Stokes radius of the various charged forms.

If the peptide is small and the pH modifications are restricted to a limited range in order to provide

the dissociation of single groups, the theoretical treatment described in Eqs. (4)–(7) can provide a measurement of dissociation constants and of Stokes radius at different protonation stages [14]. For example, at acidic pH range and for peptides having more than one basic group and C-terminus as the only acidic group (which were defined Class II peptides [14]) Eqs. (4)–(7) may be simplified as follows:

$$\mu_{\text{obs}} = \frac{q}{6\pi\eta} \left[\frac{j}{r_{(j)}} \cdot \frac{1}{1 + K/[H^+]} \right.$$
$$\left. + \frac{j-1}{r_{(j-1)}} \cdot \frac{K/[H^+]}{1 + K/[H^+]} \right] \tag{8}$$

where K is the dissociation constant of the C-terminus carboxylic group.

Eq. (8), upon simple algebraic modification, changes into:

$$\mu_{\text{obs}} = \frac{q}{6\pi\eta} \left[\frac{j}{r_{(j)}} \cdot \frac{[H^+]}{[H^+] + K} + \frac{j-1}{r_{(j-1)}} \cdot \frac{K}{[H^+] + K} \right] \tag{9}$$

and, in order to transform it into a function of pH:

$$\mu_{\text{obs}} = \frac{q}{6\pi\eta} \cdot \left[\frac{j}{r_{(j)}} \cdot \frac{10^{-\text{pH}}}{10^{-\text{pH}} + 10^{-\text{p}K}} \right.$$
$$\left. + \frac{j-1}{r_{(j-1)}} \cdot \frac{10^{-\text{p}K}}{10^{-\text{pH}} + 10^{-\text{p}K}} \right] \tag{10}$$

Eq. (10) for pH < pK reduces to:

$$\mu_{\text{obs}} = \frac{q}{6\pi\eta} \cdot \frac{j}{r_{(j)}} \tag{11}$$

and for pH ≫ pK to:

$$\mu_{\text{obs}} = \frac{q}{6\pi\eta} \cdot \frac{j-1}{r_{(j-1)}} \tag{12}$$

The following three possibilities may occur, each with different consequences on mobility, as shown in Fig. 1. When $r_{(j)}$ is equal to $r_{(j-1)}$ Eq. (10) can be transformed into:

$$\mu_{\text{obs}} = \frac{q}{6\pi\eta r_s} \cdot \left[\frac{j \cdot 10^{-\text{pH}}}{10^{-\text{pH}} + 10^{-\text{p}K}} \right.$$
$$\left. + (j-1) \cdot \frac{10^{-\text{p}K}}{10^{-\text{pH}} + 10^{-\text{p}K}} \right] \tag{13}$$
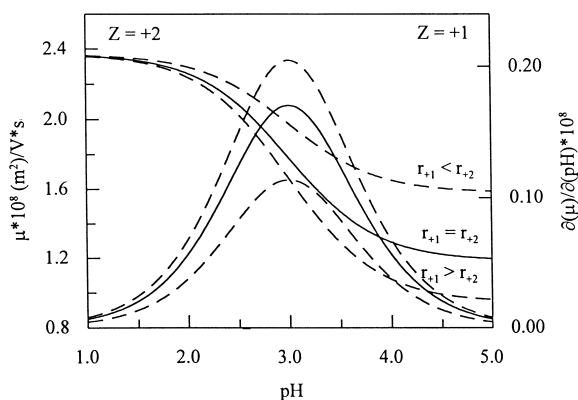
Fig. 1. pH dependence of mobility values of a generic peptide with a Stokes radius of 8.0 Å and C-terminus pK value of 3.00, according to Eq. (10) and corresponding derivatives obtained according to Eqs. (14) and (16). Continuous lines correspond to a peptide Z +2 charged at full saturation that does not change its Stokes radius by proton loss. Dashed lines correspond to peptides showing either a 2.0 Å decrease or a 2.0 Å increase of the Stokes radius.

Eq. (13) obviously does not account for Stokes radius changes. In this case, i.e. at a constant radius, curve shape is invariable. This is evidenced by the derivative of this equation as a function of pH:

$$\left(\frac{\partial \mu}{\partial \mathrm{pH}}\right)_r = -\frac{q}{6\pi\eta}\cdot\frac{1}{r_{\mathrm{s}}}\cdot\ln_e(10)\cdot\frac{10^{(\mathrm{pH}+\mathrm{p}K)}}{(10^{\mathrm{pH}}+10^{\mathrm{p}K})^2}$$

(14)

This function assumes the maximum absolute value at pH=pK (when saturation reaches 50%):

$$\left|\left(\frac{\partial \mu}{\partial \mathrm{pH}}\right)_r\right|_{(\mathrm{pH}=p\mathrm{K})} = \frac{q}{6\pi\eta}\cdot\frac{\ln_e(10)}{4r_{\mathrm{s}}}$$

$$= 9.48\cdot 10^{-18}\cdot\frac{0.576}{r_{\mathrm{s}}}$$

(15)

On the contrary, when $r_{(j)}$ is different from $r_{(j-1)}$ the curve is stretched or compressed (Fig. 1), since the mobility of X^{j-1} form is either lower or higher than expected, respectively. In these cases, partial derivative of Eq. (10) as a function of pH is:

$$\left(\frac{\partial \mu}{\partial \mathrm{pH}}\right)_r = -\frac{q}{6\pi\eta}\cdot\left(\frac{j}{r_j}-\frac{j-1}{r_{(j-1)}}\right)\cdot\ln_e(10)$$

$$\cdot\frac{10^{(\mathrm{pH}+\mathrm{p}K)}}{(10^{\mathrm{pH}}+10^{\mathrm{p}K})^2}$$

(16)

The curves corresponding to Eqs. (14) and (16) are represented in Fig. 1.

The absolute value of the above equation at pH equal to pK is dependent upon Stokes radius modifications:

$$\left|\left(\frac{\partial \mu}{\partial \mathrm{pH}}\right)_r\right|_{(\mathrm{pH}=p\mathrm{K})}$$

$$= \frac{q}{6\pi\eta}\cdot\frac{\ln_e(10)}{4}\cdot\left(\frac{j}{r_j}-\frac{j-1}{r_{j-1}}\right)$$

$$= 9.48\cdot 10^{-18}\cdot 0.576\cdot\left(\frac{j}{r_j}-\frac{j-1}{r_{j-1}}\right)$$

(17)

Paradoxically, if proton loss provides a very strong Stokes radius decrease, mobility modification as a function of pH cannot be observed at all.

The basic Eq. (1) establishes an inverse proportion between mobility and Stokes radius. The hyperbolic shape of this relationship suggests that beyond a certain value mobility measurements are not able to detect Stokes radius transitions deriving from the proton loss. Namely, the peptide under examination could be too voluminous in order to appreciate the differences existing between the Stokes radius of the different protonated forms by mobility measurements. This Stokes radius limit can be established assuming that the variation of mobility due to Stokes radius variation (namely the partial derivative) with respect to the radius itself must be higher than the detection limit of a peptide CE separation. The partial derivative of mobility with respect to the radius is:

$$\left|\frac{\partial \mu}{\partial r_{\mathrm{s}}}\right| = \frac{qZ}{6\pi\eta r_{\mathrm{s}}^2}$$

(18)

This function, calculated in water ($\eta=8.95\cdot 10^{-4}$ N s m^{-2} at 25°C) and TFE–water (30:70, v/v) ($\eta=13.8\cdot 10^{-4}$ N s m^{-2} at 25°C [16]) is reported in Fig. 2, where the continuous lines correspond to peptides having a net +2 charge. On the other hand, the detection limit can be established assuming a 1% error on mobility determinations and the possibility to detect a 0.2 Å Stokes radius modification. The detection limit values are also reported in Fig. 2 (dashed lines) as a function of the peptide Stokes radius. When separations performed in water are considered, the detection limit curve crosses the derivative at a value of about 21 Å. At Stokes radius
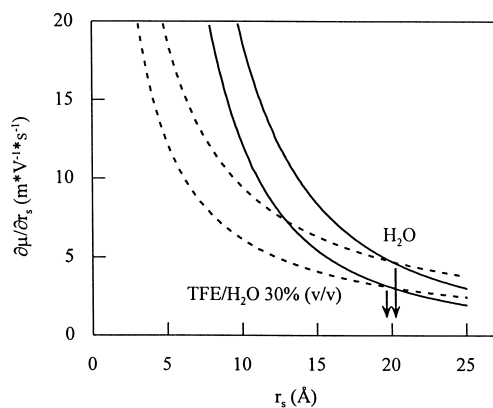
Fig. 2. Variation of the electrophoretic mobility due to variations of peptide Stokes radius values ($\partial\mu/\partial r_s$) as a function of radius itself. When this function assumes values lower than the detection limit of CE separation (arrows), the conformational transitions linked to the proton loss is not determinable. The detection limit was established considering a 1% error on mobility and a 0.02 Å Stokes radius variation due to the proton loss (dashed lines).

3. Results and discussion

In order to compare the physico-chemical properties of small peptides in hydro-organic solvents with those obtained in aqueous solutions and to avoid differences arising from distinct viscosity values, the utilisation of the product of mobility by viscosity is useful. This product can be defined as 'intrinsic mobility' (i.e. the analyte electrophoretic mobility in whatever electrophoretic solution) and its dimensions are obviously either $N\,V^{-1}$ or $C\,m^{-1}$. The value of water viscosity is $8.95\cdot10^{-4}\,N\,s\,m^{-2}$ at 25°C. The measurements in TFE–water solution were always performed at 30% (v/v) (corresponding to a 0.0531 molar fraction) [9,10], since this value is commonly accepted as the best compromise in order to profit of TFE structure-forming capability [13]. The viscosity value of TFE–water (30:70, v/v) mixture is $13.8\cdot10^{-4}\,N\,s\,m^{-2}$ at 25°C [16]. Furthermore, in order to respect the limitations imposed by Eqs. (10) and (19), we have performed the mobility measurements only in acidic pH range using a coated capillary. Under these conditions the use of an opportune marker (acetanilide) ensured that the electroosmotic flow can be considered negligible. Moreover, at pH values less than 2.2, the current instability did not allow reliable results to be obtained. The use of TFE–water mixtures allowed the extension of the pH range reaching an apparent pH value of about 1.9 pH unity.

The C-terminus apparent pK values obtained in water and TFE–water mixtures are reported in Table 1. Even though the pH measurements in TFE–water mixture have not the same meaning of those performed in water alone, from the differences of pK_{app} values (Table 1) it can be generalised that Class I peptides decrease the acidity of the terminal carboxylic group, probably for a decrease of solution dielectric constant, whereas Class II peptides increase their acidity probably for increased field effect of the positively-charged side chains. Namely, the decrease of dielectric constant provides an increase of the interaction between the proton and carboxylate anion, but a contemporaneous increase of the repulsive effect between proton and the positive charges of side-chain basic peptide groups. In agreement with this interpretation, HFRW peptide (α-MSH fr. 6–9), which bears two positive side chain charges,

values greater than this limit, mobility measurements cannot detect a Stokes radius modification less than 0.2 Å. This limit value obviously increases for Stokes radius modification greater than 0.2 Å and decreases for experimental error greater than 1%. Furthermore, as shown for the TFE–water curves, this limit slightly decreases when the viscosity increases. All the peptides selected in this study displayed a value of Stokes radius which is comprised into the dynamic range both of water and TFE–water mixtures.

For peptides which have only dissociable C- and N-terminus (which were defined Class I peptides [14]) the second term of Eq. (10) is zero and the equation reduces to:

$$\mu_{obs} = \frac{q}{6\pi\eta} \cdot \frac{1}{r_{(+1)}} \cdot \frac{10^{-pH}}{10^{-pH} + 10^{-pK}} \qquad (19)$$

The above equation at a pH<pK becomes:

$$\mu_{obs} = \frac{q}{6\pi\eta} \cdot \frac{1}{r_{(+1)}} \qquad (20)$$

Eqs. (10) and (19) can be utilised by best fitting multiparametric procedures in order to provide a measurement of dissociation constants and peptide Stokes radius.

Table 1
Values of apparent C-terminus dissociation constants of selected peptides in water and TFE

Peptide	Sequence	Class	pK_{app} (C-term)[a]	
			Water	TFE
Met-enkephalin	YGGFM	I	3.52	3.82
Leu-enkephalin	YGGFL	I	3.69	4.15
	YGGF	I	3.30	3.62
	GGNA	I	3.65	3.72
	GGQA	I	3.67	3.73
	GGRA	II	2.75	2.41
Bradikinin (fr. 1–5)	RPPGF	II	2.64	2.28
α-MSH (fr. 6–9)	HFRW	II	2.77	2.25

[a] The error on pK_{app} determination is always less than 0.02 pH unity.

shows a pK_{app} modification approximately double of those observed for GGRA and RPPGF (bradikinin fr. 1–5) peptides. Due to the high polarity of Class I peptides, sensible TFE-induced modification of Stokes radii were not observed (Table 2), suggesting that at TFE–water (30:70, v/v) mixture, water molecules bind to peptide preferentially with respect to TFE molecules. On the contrary, the measure-

Table 2
Values of Stokes radii of the protonated form of selected Class I peptides determined by best fitting procedures according to Eq. (19)

Peptide	Sequence	r_{+1} (Å)[a]	
		Water	TFE
Met-enkephalin	YGGFM	8.2	8.2
Leu-enkephalin	YGGFL	8.2	8.4
	YGGF	7.4	7.4
	GGNA	6.0	5.8
	GGQA	6.1	6.1

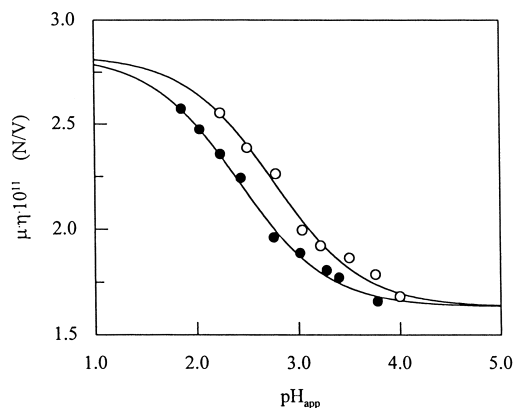[a] The error on Stokes radius determination is always less than 0.2 Å.

Fig. 3. Value of intrinsic mobility ($\mu\eta$) as a function of apparent pH for GGRA peptide in water (open symbols) and in TFE–water (30:70, v/v) (closed symbols). The continuous lines correspond to the curves obtained by best fitting procedures applying Eq. (10) to experimental data. Throughout this minimization multiparametric procedure the determination of Stokes radii at different protonation stages and of the C-terminus dissociation constant was possible (Tables 1 and 3).

ments performed on Class II peptides clearly showed that Stokes radius modifications were strongly connected to peptide sequence (Table 3). The polar GGRA peptide, similarly to that observed for Class I peptides, did not modify the Stokes radii of the +2 and +1 forms (Fig. 3). RPPGF peptide in water showed a Stokes radius decrease, deriving from proton loss, of 1.2 Å. This decrease can be attributed to the formation of an intramolecular ion pair [14]. In the TFE–water mixture, the Stokes radius of +2 and +1 forms is stabilized and did not change upon proton loss (Fig. 4). This puzzling observation could be justified only by performing a conformational analysis of this peptide in TFE–water mixtures. The behaviour of HFRW peptide appears to be different from the other ones. HFRW in water showed a 0.4 Å Stokes radius decrease upon proton loss, whereas in

Table 3
Values of Stokes radii of different charged forms of selected Class II peptides determined by best fitting procedures according to Eq. (10)

Peptide	Sequence	Water			TFE		
		r_{+3}[a]	r_{+2}	r_{+1}	r_{+3}	r_{+2}	r_{+1}
	GGRA		6.2	5.2		6.0	5.2
Bradikinin (fr. 1 5)	RPPGF		8.2	7.0		7.3	7.3
α-MSH (fr. 6–9)	HFRW	9.4	9.0		9.0	10.5	

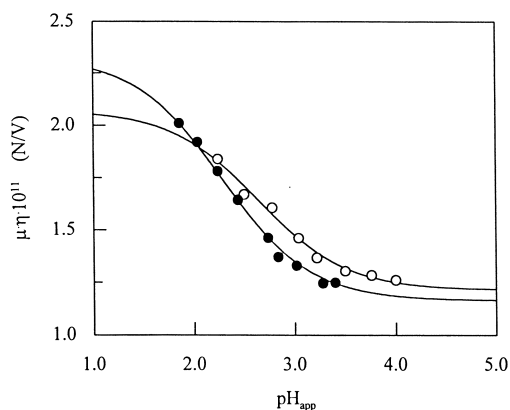[a] The error on Stokes radius determination is always less than 0.2 Å.

Fig. 4. Value of intrinsic mobility ($\mu\eta$) as a function of apparent pH for RPPGF peptide (Bradikinin, fr. 1–5) in water (open symbols) and in TFE–water (30:70, v/v) (closed symbols). The continuous lines correspond to the curves obtained by best fitting procedures applying Eq. (10) to experimental data. Throughout this minimization multiparametric procedure the determination of Stokes radii at different protonation stages and of the C-terminus dissociation constant was possible (Tables 1 and 3).

TFE–water mixture showed a 1.5 Å Stokes radius increase (Fig. 5). This fact should indicate that, after the proton loss, several TFE molecules could bind to the apolar peptide molecule.

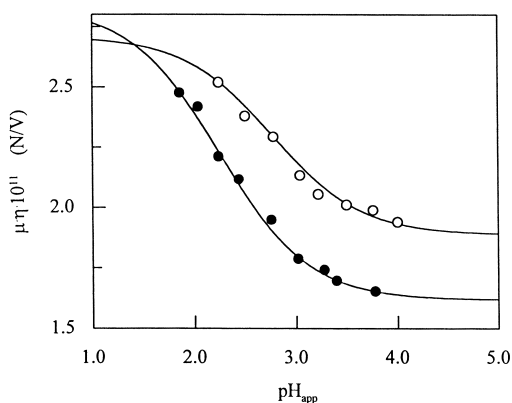These TFE-induced modifications of peptide prop-

Fig. 5. Value of intrinsic mobility ($\mu\eta$) as a function of apparent pH for HFRW peptide (α-MSH, fr. 6–9) in water (open symbols) and in TFE–water (30:70, v/v) (closed symbols). The continuos lines correspond to the curves obtained by best fitting procedures applying Eq. (10) to experimental data. Throughout this minimization multiparametric procedure the determination of Stokes radii at different protonation stages and of the C-terminus dissociation constant was possible (Tables 1 and 3).

erties can be properly utilized for selectivity manipulation. As pointed out in a previous study [14], peptide CE can be performed in two different pH regions. The first region corresponds to very acidic pH values (approximate pH range $2.0 \div 2.5$), where mobility differences are mainly linked to the differences of Stokes radius values, since similar peptides are quite completely proton saturated and have similar charges. The second region corresponds to pH values approximately near to pK_{app} values (approximate pH range $3.2 \div 3.8$), where mobility differences are principally linked to differences in peptide dissociation constants which reflect on different peptide saturation and charges. The middle region (approximate pH range $2.5 \div 3.2$) should be avoided since a change in mobility control can provide low resolution.

The three Class I enkephalin related peptides illustrate this mobility behaviour in water well [14], since the differences in dissociation constants provide the best CE separations at pH near to pK values. The C-terminus pK_{app} modification in TFE–water solutions can offer a good chance for a further improvement of resolution. The pK_{app} of Leu-enkephalin in hydro-organic mixture shows an increase more pronounced than those observed for Met-enkephalin and YGGF peptide. The pK_{app} in the TFE–water mixture are enhanced, with respect to water mixtures. Thus, the use of TFE in CE separations performed at pH_{app} near to pK_{app} values provides a sensible increase of resolution (Figs. 6 and 7). At these pH values the smaller YGGF peptide, according to the higher acidity of its C-terminus (Table 1), shows the lowest mobility. On the contrary, at pH values corresponding to high saturation, YGGF peptide shows the highest mobility, according to its dimension.

At variance with enkephalin-related peptides, the equivalence of the pK_{app} of GGNA and GGQA peptides did not allow separation of them at pH_{app} values near to the pK_{app}. However, at acidic pH values, the two peptides are well separated at the baseline both in water and in TFE–water mixture (Figs. 8 and 9), where the separation is principally connected to Stokes radius difference. In fact, as also mentioned in the theory section, a minimal structural difference ($-CH_2$) in small peptides is sufficient for a baseline separation. In TFE–water mixture the
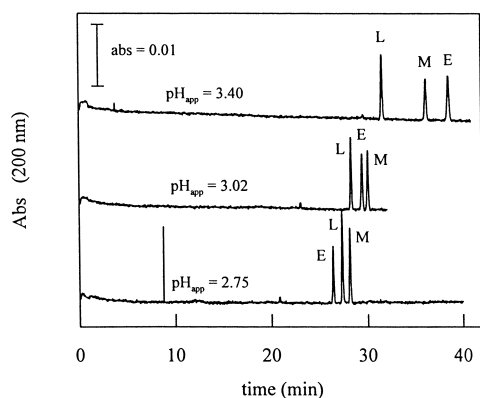
Fig. 6. Electropherograms of Met-enkephalin (M; YGGFM), Leu-enkephalin (L; YGGFL) and YGGF peptide (E) at three different apparent pH values in TFE–water (30:70, v/v). The mobility of YGGF is the highest at an apparent pH value of 2.75, due to its smaller dimension with respect to M and L, whereas it is the lowest at an apparent pH value of 3.40, due to its more acidic C-terminus dissociation constant. The best resolution is obtained at pH near to pK_{app}.

resolution observed (Fig. 9) is sensibly better than that observed in water (see also Ref. [8]).

The plot of intrinsic mobility versus apparent pH values of enkephalin-related peptides in TFE–water mixture shows an unusual increase at strong acidic pH values (Fig. 7). Unfortunately, due to current instability it is impossible to verify whether or not the same event is observable in water solution. The simplest interpretation is the presence of an additional very acidic proton binding site common to the three peptides.

The results obtained in TFE–water mixtures with respect to water solution (Figs. 3–5) clearly show that the modification of C-terminus dissociation constants and Stokes radii of Class II peptides are connected to their sequence. For these reasons the TFE effect on a particular peptide separation is not easily predictable a priori. However, for the same reasons, the use of TFE–water mixtures can provide a convenient opportunity for selectivity manipulation.

Overall, and as also partially mentioned in previous studies [8], the advantages of the use of TFE–water mixtures for CE peptide separations can be summarised as follows:
1. A current lowering that permits better temperature control and extended ranges of ions activities (i.e. proton activity) with respect to aqueous solutions.
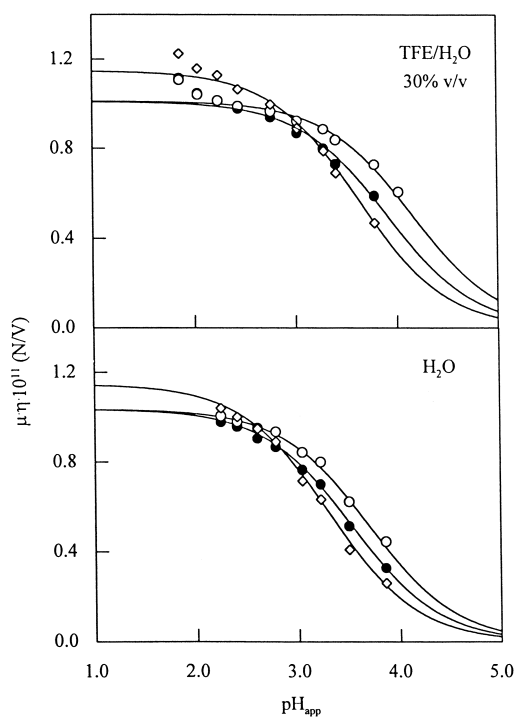

Fig. 7. Value of intrinsic mobility ($\mu\eta$) as a function of apparent pH for Met-enkephalin (YGGFM; closed circles), Leu-enkephalin (YGGFL; open circles) and YGGF peptide (open squares) in water (bottom panel) and in TFE–water (30:70, v/v) (top panel). The continuous lines correspond to the curves obtained by best fitting procedures applying Eq. (19) to experimental data. Throughout this minimization multiparametric procedure the determination of Stokes radius and of the C-terminus dissociation constant was possible (Tables 1 and 3).
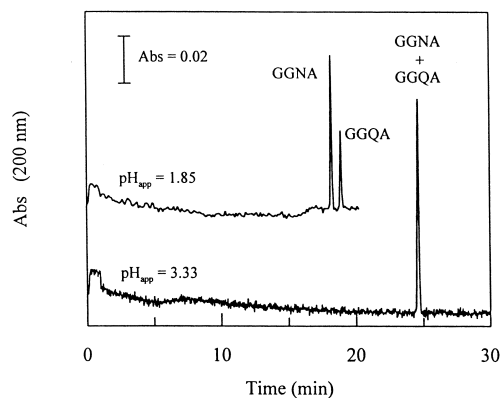

Fig. 8. Electropherograms of GGNA and GGQA peptide at two different apparent pH values in TFE–water (30:70, v/v). The best resolution is obtained at very acidic apparent pH values, due to the Stokes radius differences, whereas at pH_{app} values near to pK_{app} the two peptides migrate together due to the very close C-terminus dissociation constants.
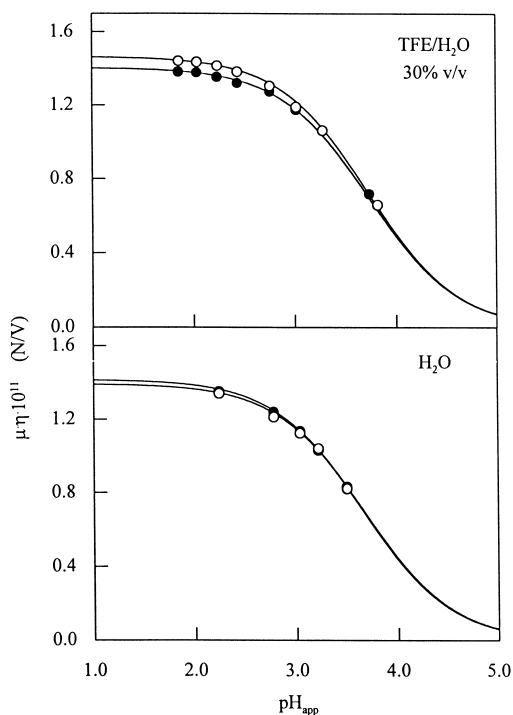
Fig. 9. Value of intrinsic mobility ($\mu\eta$) as a function of apparent pH for GGNA (open symbols), and GGQA peptides (closed symbols) in water (bottom panel) and in TFE–water (30:70, v/v) (top panel). The continuous lines correspond to the curves obtained by best fitting procedures applying Eq. (19) to experimental data. Throughout this minimization multiparametric procedure, the determination of Stokes radius and of the C-terminus dissociation constant was possible (Tables 1 and 3).

2. A modification of dissociation constants and Stokes radii strongly dependent upon peptide sequence which can be utilised with profit for selectivity manipulation.

3. An increase of separation performance deriving from the stabilisation of particular peptide conformations.

4. A greater solubility of large apolar peptides with respect to aqueous solutions [8].

5. A coating stabilisation probably resulting from decreased hydrolysis processes and wall passivation [8].

These advantages can be often utilised in order to manipulate selectivity and performance of particular peptide CE separations, especially when separation performed in water alone are not able to provide satisfactory performance. However, it must be pointed out that this improvement could not be obtainable for every peptide separation. In fact, the TFE-induced peptide modification in several situations can impair the separation with respect to electrophoresis in water.

References

[1] Y. Walbroehl, J.W. Jorgenson, J. Chromatogr. 315 (1984) 135.
[2] Y. Walbroehl, J.W. Jorgenson, Anal. Chem. 58 (1986) 479.
[3] I. Bjørnsdottir, S.H. Hansen, J. Chromatogr. A 711 (1995) 313.
[4] M. Chiari, E. Kenndler, J. Chromatogr. A 716 (1995) 303.
[5] G.M. Janini, K.H. Chan, J.A. Barnes, G.M. Muschik, H.J. Issaq, Chromatographia 35 (1993) 497.
[6] R.S. Sahota, M.G. Khaledi, Anal. Chem. 66 (1994) 1141.
[7] I.E. Valkó, H. Siren, M.L. Riekkola, LC–GC Internat. 10 (1997) 190.
[8] M. Castagnola, L. Cassiano, I. Messana, M. Paci, D.V. Rossetti, B. Giardina, J. Chromatogr. A 735 (1996) 271.
[9] J.W. Nelson, N.B. Kallenbach, Biochemistry 28 (1989) 5256.
[10] M.D. Bruch, L.M. Gierasch, J. Biol. Chem. 265 (1990) 3851.
[11] A. Zaks, A.M. Klibanov, J. Biol. Chem. 263 (1988) 8017.
[12] B.K. Shoichet, W.A. Baase, R. Kuroki, B.W. Matthews, Proc. Natl. Acad. Sci. USA 92 (1995) 452.
[13] A.R. Van Buuren, H.J.C. Berendsen, Biopolymers 33 (1993) 1159.
[14] M. Castagnola, D.V. Rossetti, L. Cassiano, F. Misiti, L. Pennacchietti, B. Giardina, I. Messana, Electrophoresis 17 (1996) 1925.
[15] M. Castagnola, L. Cassiano, R. Rabino, D.V. Rossetti, F. Andreasi Bassi, J. Chromatogr. 572 (1991) 51.
[16] R. Palepu, J. Clarke, Termochim. Acta 156 (1989) 51.