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Separation of peptides in isoelectric cysteic acid buffer and hydro–organic solvents (hexafluoro-2-propanol–urea)

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Abstract

A novel amphoteric, isoelectric, acidic buffer is here reported for separation of oligo- and polypeptides by capillary zone electrophoresis: cysteic acid (Cys-A). Cys-A, at 200 m*M* concentration, exhibited an isoelectric point (p*I*) of 1.80; given a $\Delta pK=0.6$, the p*K* of the carboxyl was assessed as 2.1 and the p*K* of the sulphate group as 1.50. At 100 m*M* concentration, this buffer provided an extraordinary buffering power: $140 \cdot 10^{-3}$ equiv./l per pH unit. In presence of 30% (v/v) hexafluoro-2-propanol (HFP), this buffer did not change its apparent p*I* value, but drastically reduced its conductivity. In Cys-A–HFP buffer, small peptides exhibited a mobility closely following the Offord equation, i.e., proportional to the ratio $M_r^{2/3}/Z$). With addition of 4–5 *M* urea, there was an inversion in the mobility of some peptides, suggesting strong p*K* changes as an effect of urea addition. It was found that the minimum mass increment, for proper peptide separation, was ΔM_r=ca. 1%. In case of simultaneous M_r and p*K* changes, the minimum ΔM_r is reduced to only 0.6%, provided that a concomitant minimum $\Delta pK=0.08$ took place. When separating large peptides (human globin chains) in 100 m*M* Cys-A, 30% HFP and 7 *M* urea, the β-chain was found to co-elute with the α-chain, suggesting a subtle interplay between the helix forming (HFP) and helix breaking (urea) agents. When HFP was omitted, the original globin separation could be restored.
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1. Introduction

Capillary zone electrophoresis (CZE) is rapidly becoming a major laboratory method for the separation, analysis and characterization of peptides. Several recent studies have focused on deriving a correlation between certain physico–chemical attributes of peptides and their observed electrophoretic mobilities. Such correlations would facilitate the prediction of their expected mobilities and thus the design of optimum experimental conditions.

Due to the fact that peptides and proteins are prone to binding to ionized silanols, different strategies have been designed in order to minimize such interactions, which would have a detrimental effect in separation. A number of authors [1–12] adopted acidic buffers, typically composed of phosphate, formate or citrate ions, titrated in the pH 1.9 to 3.0 interval, since at such acidic pH values the silica wall should be extensively protonated, effectively abolishing coulombic interactions. In contrast, others optimized alkaline buffer recipes, ranging from pH

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8.0 up to pH 11.5, in the hope that such basic conditions would ensure a net negative charge on the peptide surface, thus repelling it from the highly ionized silanols on the silica wall [13–18]. A third group, finally, developed a number of buffer systems comprising modulators of peptide mobility, so as to further maximize their separation [19–27]. These include: acetonitrile and zwitterions (the latter for quenching potential binding to the wall) [19]; temperature changes [20]; ion-pairing agents, such as tetrabutyl ammonium bromide [21], triethyl ammonium phosphate [22], heptane sulfonic acid [23]; heavy water [24,25] and helix-inducing agents, such as 2,2,2-trifluoroethanol (TFE) [26,27].

Among the different strategies illustrated above, the use of acidic separation buffers for peptide mapping by CZE presents some distinct advantages. First of all, at pH values well below the pK of the free silanols on the fused-silica wall (assessed as pK=6.3, with a wall neutralization at pH=2.3) [28], their dissociation will be significantly suppressed and, in principle, the negative charge on the silica surface abolished. The electroosmotic flow (EOF) should thus be negligible and the peak capacity greatly increased. Additionally, the low pH value ensures that the vast majority of peptides will bear a net positive charge, thus allowing their migration at the cathodic end of the capillary, past the detector window. As a third bonus, the negligible negative charge on the capillary wall will also mean that the coulombic interaction between the fused-silica surface and the peptides will be markedly diminished, thereby removing the most important reason for peak broadening and skewing [29,30]. However, satisfactory separations are not always obtained using acidic buffer conditions: at low-enough pH values effectively minimizing peptide–wall interaction (i.e., at or below pH 2), the buffer conductivity (combined with that of bulk water) is so high that only low voltage gradients can be adopted (typically not higher than 100 V/cm), thus greatly lengthening the analysis times (in many reports, up to 60–70 min are required for fully developing complex peptide maps). During such long runs, peptide zones considerably broaden and peak resolution worsens. In view of these shortcomings, our group has taken a completely different route: the use of amphoteric, isoelectric buffers. Nembri and Righetti [31] recently suggested isoelectric aspartic acid as the sole background electrolyte (BGE), operating at pH=isoelectric point (pI)=2.77 (at 25°C). These authors could produce peptide maps of casein in only 10–12 min (as opposed to >70 min in standard phosphate buffer, pH 2.0) at voltage gradients as high as 800 V/cm, with much increased resolution. Adsorption of some larger peptides to the wall was completely eliminated by adding to the background, isoelectric Asp buffer, 0.5% hydroxyethyl cellulose (HEC, number-average molecular mass, M_n=27 000) and 5% trifluoroethanol (TFE).

The method has been successfully used for a number of applications: for generating peptide maps of α- and β-globin chains from tryptic digests of human adult hemoglobin [32], for analysis of gliadins in the screening of wheat cultivars [33] and of zeins in maize [34] and for human globin chain separation and quantitation [35]. The fundamental properties of such buffers have been enunciated [36] and a number of reviews have already covered the field [37–39]. Notwithstanding the increasing importance of such isoelectric buffers, some limitations are apparent. As the charge of the peptides plays a pivotal role in their separation by CZE, other pH values might be necessary in order to avoid co-migration of peptides having cross-over points at a given pH value in their titration (pH/mobility) curves. A case in point is offered by the example of α- and β-globin tryptic digests [32]: the β-T2/T9 coeluted at the pH=pI=2.77 of 50 mM Asp and could only be split at an operative pH of 3.0, as elicited by diluting the running buffer to 30 mM. It was thus apparent that more than one amphoteric buffer was needed, in order to explore larger pH windows along the titration curves of proteins and peptides. In search for additional BGEs, Bossi and Righetti [40] reported the use of iminodiacetic acid (IDA) as a unique isoelectric buffer, possessing a remarkably low apparent pI (2.23 at 100 mM concentration) and an extreme solubility both in neat water and in a number of hydro–organic solvents.

In the present report, we have extended this search and discovered a new amphoteric ion of extreme acidity: cysteic acid (Cys-A), with an apparent pI of 1.85 (at 100 mM concentration) and an extraordinary buffering power, given by its remarkably low ΔpK value (approaching the theoretical limit of 0.6 for a

molecule having two identical, independent ionizable groups).

2. Experimental

2.1. Reagents

Cys-A, IDA and all di- and tripeptides (GG, GGG, GGL, GGV, PM, AG) and TFE were purchased from Sigma (St. Louis, MO, USA). Hexafluoro-2-propanol (HFP) was obtained from Fluka (Buchs, Switzerland). Fused-silica capillaries (30 cm×50 μm I.D.× 375 μm O.D.) were from Polymicro Technologies (Phoenix, AZ, USA) and were used as such, without inner coating. Globin chains from fetal cord blood were prepared according to Ref. [35].

2.2. Capillary electrophoresis

CZE was carried out with the Waters Quanta 4000E unit equipped with automated Millenium software. Uncoated capillaries of 32 cm×50 μm I.D.were used. A few types of BGEs were tested: (a) 100 m*M* Cys-A containing 30% (v/v) HFP, either as such, or in presence of increasing amounts of urea (from 4 to 7 *M*); (b) 100 m*M* Cys-A without HFP but in presence of 7 *M* urea; (c) 50 m*M* isoelectric IDA (pH=p*I*=2.30 at 25°C) added to 7 *M* urea. In all cases, the sample and standard were loaded by hydrostatic pressure for 15 s. Separations were performed at different voltage gradients, from 250 V/cm up to 500 V/cm. Ultraviolet absorbance was monitored at 214 nm.

2.3. pH determinations

In order to assess potential pH changes with dilution of isoelectric Cys-A, the following potentiometric titration was performed. Doubly distilled, degassed water was thermostatted at 25°C and added to Cys-A, first up to a concentration of 5 m*M*, then up to 10 m*M*, subsequently at 10 m*M* increments up to 100 m*M*. At each Cys-A addition, the pH was carefully assessed with a pHM64 Research pH Meter, equipped with a GK2401C combination electrode from Radiometer (Copenhagen, Denmark). The apparent p*I* changes as a function of increasing amounts of organic solvents (such as HFP and urea) were measured in the same manner.

2.4. Buffering capacity and conductivity measurements

In order to assess the β power of free Cys-A, the following measurements were carried out. A 50 m*M* solution of Cys-A (20 ml), thermostatted at 25°C, was allowed to equilibrate in the presence of the pH electrode, until constant pH was reached. This solution was then rendered 5 m*M* in NAOH (from a carefully titrated, 1 *M* NaOH standard) and the pH variation measured. Two subsequent additions of NaOH were made and the pH increments recorded (as we are working in a linear portion of the titration curve, the three different pH increments were in fact almost identical, so they could be averaged out). By definition, $\beta = dB/d(pH)$, i.e., it is given by the amount (in equivalents) of a strong base added to the solution of the protolyte, divided by the resulting increase in pH occurring in said solution. The resulting value of β power was additionally assessed with the differential pH meter. Conductivity of neat Cys-A solutions, or in presence of various additives (TFE, urea) was measured at 25°C with an Orion conductivity meter fitted with a 1-cm cell.

3. Results

3.1. Physico–chemical characterization of Cys-A solutions

As we could not find any report on the utilization of Cys-A as a buffering ion in electrophoresis, we first set out to measure its physico–chemical parameters. Fig. 1 shows the dependence of the pH and conductivity from the molarity of Cys-A solutions. It is well known, from theoretical considerations of Rilbe [41] and from our experimental work [32,40], that indeed the p*I* of an amphotere is a limit value, which can vary between two extremes: at high-enough concentration, it will approach the true p*I* value, at infinite dilution it will reach the p*I* of distilled water (p*I*=7.0). It is in fact seen that, at rather low concentrations (5 m*M*) the apparent p*I* of Cys-A is 2.64, whereas, at high-enough levels (100

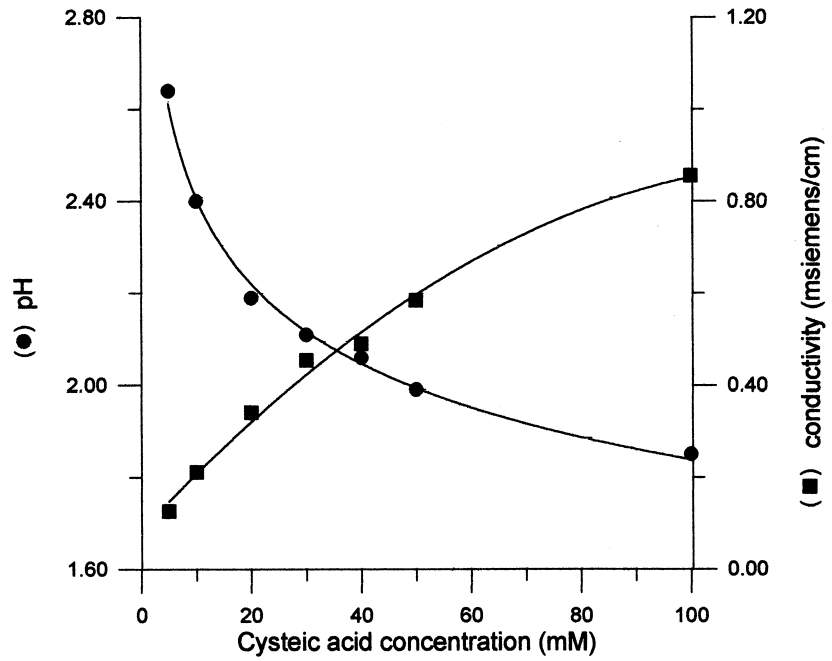

Fig. 1. Variations of pH (solid circles) and conductivity (solid squares) as a function of the concentration of Cys-A in solution, from 5 up to 100 m*M*.

m*M*), it tends to plateau at a value of 1.85, which we assume to be quite close to the real p*I* value of Cys-A (at 200 m*M* it stabilized to pH 1.80 and no further decrements were experienced upon further increments of concentration). Concomitantly, as the pH decreases at increasing concentrations of Cys-A, also the overall conductivity of the solution increases (up to $0.856 \cdot 10^{-3}$ S/cm). For practical purposes, we have then adopted a constant concentration of Cys-A of 100 m*M* (apparent p*I*: 1.85). It can be additionally appreciated that Cys-A is a super-buffer: considering that the p*K* of the carboxyl is given as 1.90 and that the p*K* of the sulfate group is 1.30 (the p*I* is equidistant between these two p*K* values; note, however, that these two p*K* values, as reported in the literature, are not compatible with our experimentally measured apparent p*I* of 1.80!) the ΔpK is thus 0.6 [48]. According to Rilbe, this ΔpK is the lowest limiting value which can be reached by two neighboring p*K*s in an amphotere. At this value, the species becomes a super-buffer, with a relative buffering power $\beta=2$ at the p*I* (note that the maximum β for a monoprotic weak ion cannot exceed 1). In our case, considering an experimentally determined p*I* value of 1.80 (at 200 m*M* concentration) and assuming the $\Delta pK=0.6$ to be a valid figure, we have tentatively set the p*K* of the carboxyl at 2.1 and the p*K* of the sulfate at 1.5. Theoretical calculations, performed with the program of Giaffreda et al. [49] and experimental data, have given us an average β value (for a 100 m*M* solution of Cys-A) of $140 \cdot 10^{-3}$ equiv./l per pH unit, a most remarkable β power, indeed. As a comparison, Lys and His, at the same 100 m*M* concentration, give a β power of 66 and $13 \cdot 10^{-3}$ equiv./l per pH unit, respectively.

Since selectivity in peptide separations can be markedly manipulated by working in hydro–organic solvents (especially in HFP), it was of interest to measure the same parameters (pH and conductivity, which greatly condition the voltage gradients applicable to the capillary separation cell) in presence of these additives. Fig. 2 gives the variations of pH and conductivity as a function of addition of HFP, from 10 to 50% (v/v). It is seen that, whereas the conductivity markedly decreases (from 0.85 to $0.10 \cdot 10^{-3}$ S/cm) the apparent p*I* of Cys-A is almost unaffected (it varies only from 1.85 in the absence of

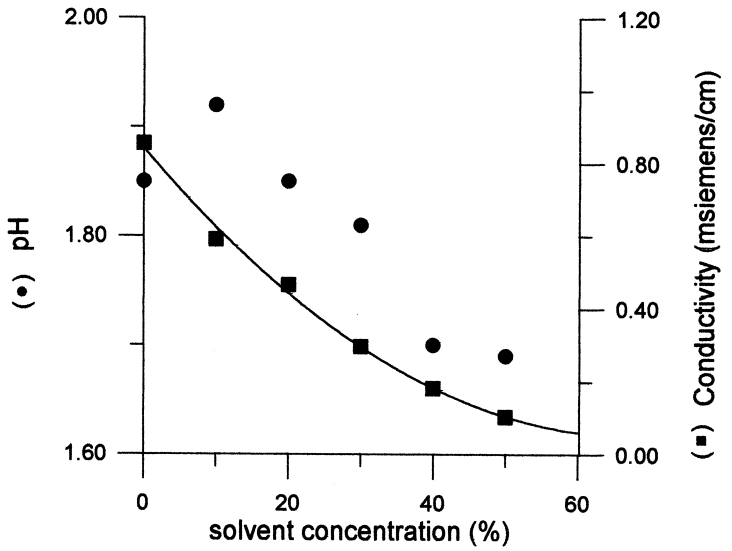

Fig. 2. Variations of pH (solid circles) and conductivity (solid squares) as a function of the concentration of hexafluro-2-propanol in solution, from 10 to 50% (v/v), at constant 100 m*M* Cys-A.

additive down to 1.70 in 50% HFP; curiously, after an initial increase to 1.92, it resumes the standard value of 1.85 at 30% HFP, which will be selected as the working concentration in all peptide separations). On the contrary, both parameters are strongly affected in presence of increasing amounts of urea, at constant values of 30% HFP (Fig. 3). In 6 *M* urea the conductivity of the mixed urea–HFP solution matches that of a 50% (v/v) HFP solution. However, contrary to the action of HFP alone, the apparent p*I*

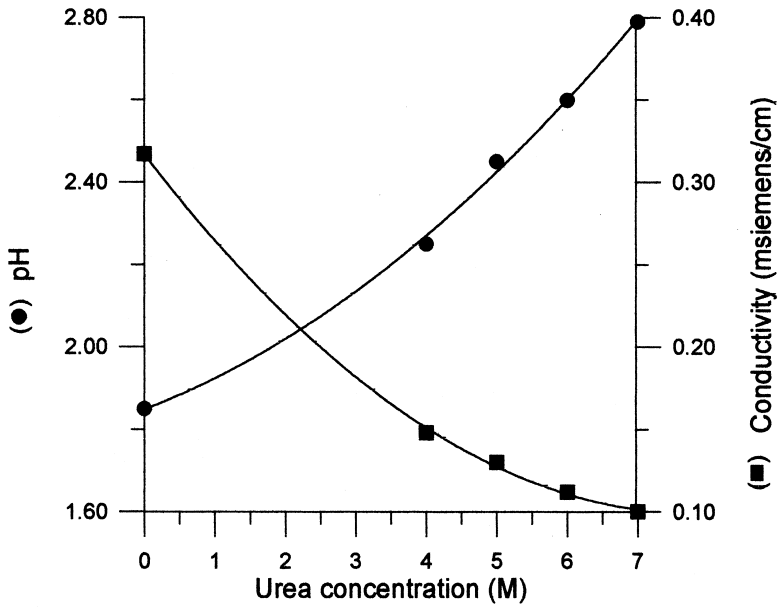

Fig. 3. Variations of pH (solid circles) and conductivity (solid squares) as a function of the concentration of urea (from 1 to 7 *M*) at a fixed concentration of 30% (v/v) hexafluro-2-propanol in solution and at constant 100 m*M* Cys-A.

increases from 1.85 to as high as 2.60 (and to pH 2.80 in 8 *M* urea).

3.2. Peptide separations

It is of interest to explore the behavior of this novel buffer in peptide separations. We have chosen a set of small di- and tripeptides, in order to see if their migration behavior would respect the Offord [42] relationship, which recently has been reevaluated as the most accurate one in predicting the mobility of peptides as a function of the ratio of molecular mass (M_r, as $M_r^{2/3}$) divided by the charge (Z) [27] (a host of relationships have been proposed, for reviews see Refs. [43,44]). Fig. 4a shows the separation of a set of six small peptides (GG,

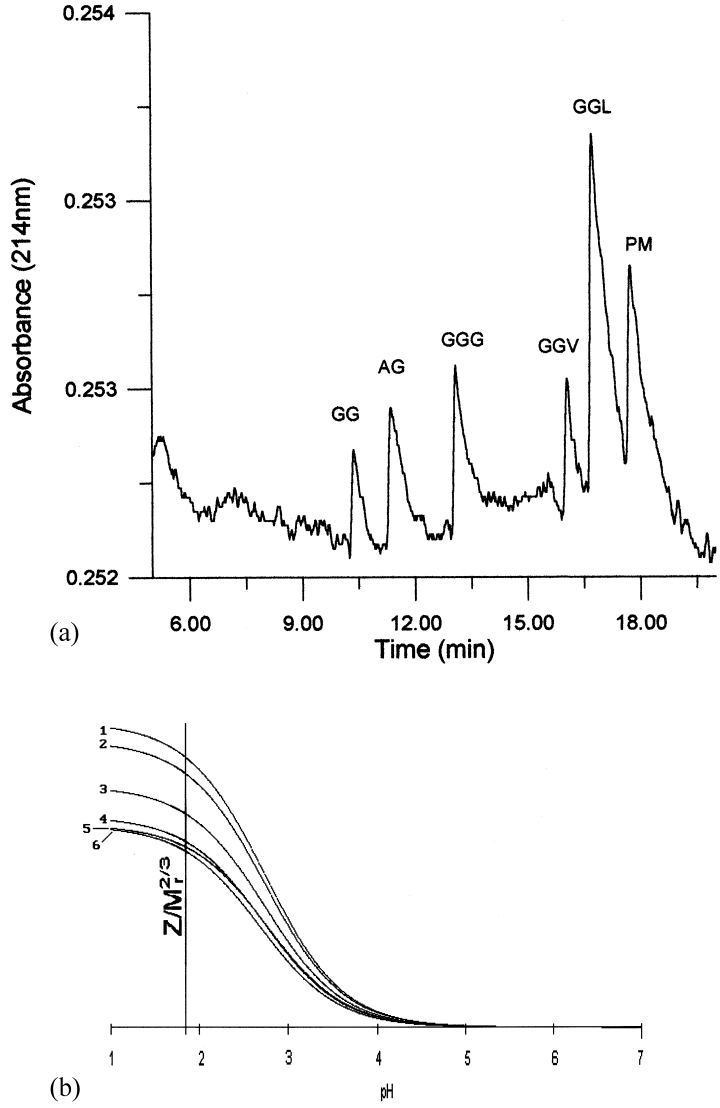

Fig. 4. Separation of six peptides by CZE in 100 m*M* Cys-A (pH=1.85) and 30% (v/v) HFP (a). Electrophoretic conditions: 250 V/cm, 20 μA, at 25°C. (b) Theoretical titration curves of the same six peptides calculated according to the Offord equation (1=GG; 2=AG; 3=GGG; 4=GGV; 5=GGL; &= PM). Note the excellent separation between GGL and PM, with a ΔM_r=1 u and ΔpK=0.08 and that the scale in the ordinate is expressed as $Z/M_r^{2/3}$, instead of $M_r^{2/3}/Z$.

M_r=133; AG, M_r=146; GGG, M_r=189; GGV, M_r=231; GGL, M_r=245; PM, M_r=246), analyzed at pH 1.85 in 100 m*M* Cys-A in presence of 30% (v/v) HFP. As shown in Fig. 4b, which plots the titration curves of these six analytes in the pH range 1–7, calculated on the basis of the reciprocal of the Offord relationship ($Z/M_r^{2/3}$) it would appear that indeed this plot predicts with accuracy the elution order of such peptides, even in the case of GGL and PM, which exhibit a minute difference in mass values (ΔM_r of only 1 u). However, the picture changes substantially upon addition of urea: in the presence of 4 *M* urea (operative buffer pH of 2.25) there is an inversion in elution order, whereby PM now preceeds GGL (Fig. 5a). An even more profound effect is seen in presence of 5 M urea (operative pH 2.45): as shown in Fig. 5b, now PM is eluted in front of GGV and GGL. We interpret that as a marked, differential p*K* change brought about by the presence of urea (see Discussion). In fact, if the pH is raised not by addition of urea, but simply by diluting the buffering ion, the elution order follows again the titration curve profiles shown in Fig. 4b, producing the same pattern of Fig. 4a at pH 2.25 (as obtained in 18 m*M* Cys-A). However, if the pH is raised to 2.40 (10 m*M* Cys-A) there is an inversion in the elution order between GGV and GGL (not shown): this, in turn, is precisely as predicted by the titration curves of Fig. 4b.

3.3. Separation of polypeptides

The separation of entire polypeptide chains is also of great interest, also considering the fact that such large polymers could interact with the few potentially ionized silanols of the silica wall. An interesting case is represented by the analysis of α-, β- and γ-globin chains, as purified from umbilical cord blood. These chains, in fact, exhibit an incredibly high net positive charge at a pH of 2–2.5 (25 for α, 24 for β and 21 for γ chains) [35] thus their interaction with the capillary wall could be a most likely event. Fig. 6a shows the separation of these chains in 100 m*M* Cys-A added only with 7 *M* urea (apparent pH of 2.70). Excellent separation is obtained among the three chains, with an elution order just as expected from the theoretical titration curves [35]. However, when the same separation is attempted in 100 m*M* IDA buffer, also added with 7 *M* urea (operative pH of 3.1) the separation of Fig. 6b is obtained, which indicates strong adsorption of such polypeptides to the wall. Another curious phenomenon can be observed in Fig. 6c: if the same buffer of Fig. 6a is adopted, but added with 30% (v/v) HFP, the separation of the chains is deeply altered, and the β-peak is co-eluted with the α-zone. The significance of these phenomena will now be elaborate upon.

4. Discussion

A number of interesting aspects emerge from the data presented, as discussed below.

4.1. On the use of Cys-A and the effects of hydro–organic solvents

Cys-A, per se, would be a valuable addition to the number of isoelectric buffers we have described so far for the analysis of peptides (IDA, p*I*=2.30, Asp, p*I*=2.80), since its very low p*I* (1.85) offers another pH window with which modulate the analyte mobilities. With these three buffers (and with the potential of covering smoothly a ca. 0.4 pH interval situated to the right of the p*I* of each amphotere) one can cover along the titration curve of peptides a pH span of >1 pH unit. This should allow to find a pH window able to separate analytes having coincident titration curves at some given pH values. However, not much is to be gained by using Cys-A alone over conventional, non-isoelectric buffers: given its very low p*I* value, the conductivity of bulk solvent will be so high as to negate any beneficial effect deriving from low-conductivity buffers. Much better results are obtained when using HFP, since this additive is, for all practical purposes, a conductivity quencher (in 50% HFP the conductivity of plain Cys-A drops by a factor of 8). However, best separations are obtained only at 30% HFP, higher levels giving blurred peptide patterns. This seems to be in line with what reported by us in the case of IDA [40], where TFE could be added at levels not higher than 35% and with the data of Castagnola et al. [26] who also reported optimal levels of TFE at 35–37%. Quite different results are obtained when urea is added, forming a mixed organic solvent. Levels of 4–5 *M*

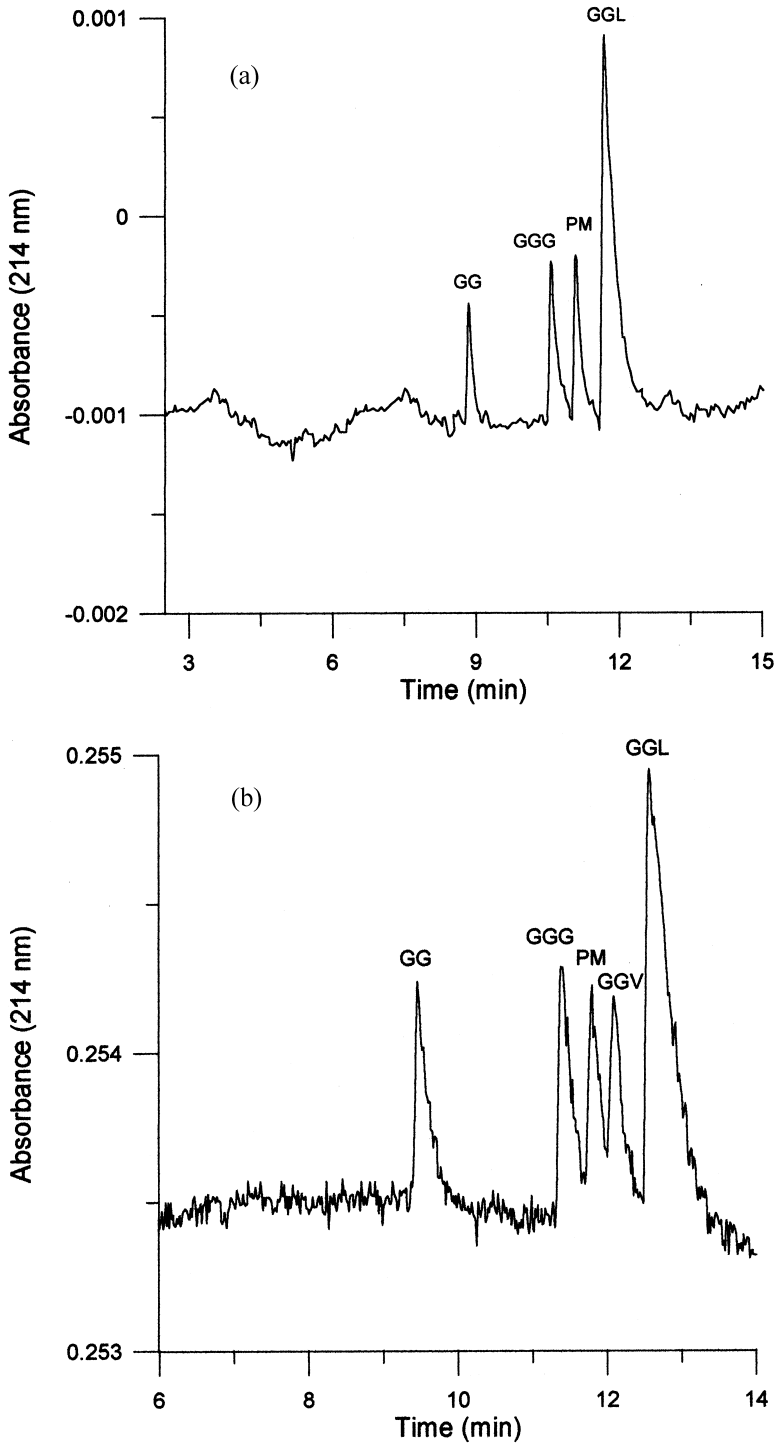

Fig. 5. Separation of different di- and tripeptides by CZE. (a) 100 m*M* Cys-A buffer, in presence of 30% (v/v) HFP and 4 *M* urea (apparent pH of 2.25). (b) 100 m*M* Cys-A in 30% (v/v) HFP and 5 *M* urea (apparent pH of 2.45). Note, in both cases, the inversion of mobility between PM and GGL (a) and among PM, GGV and GGL (b). Conditions: 480 V/cm and 18 μA (a) and 16 μA (b).

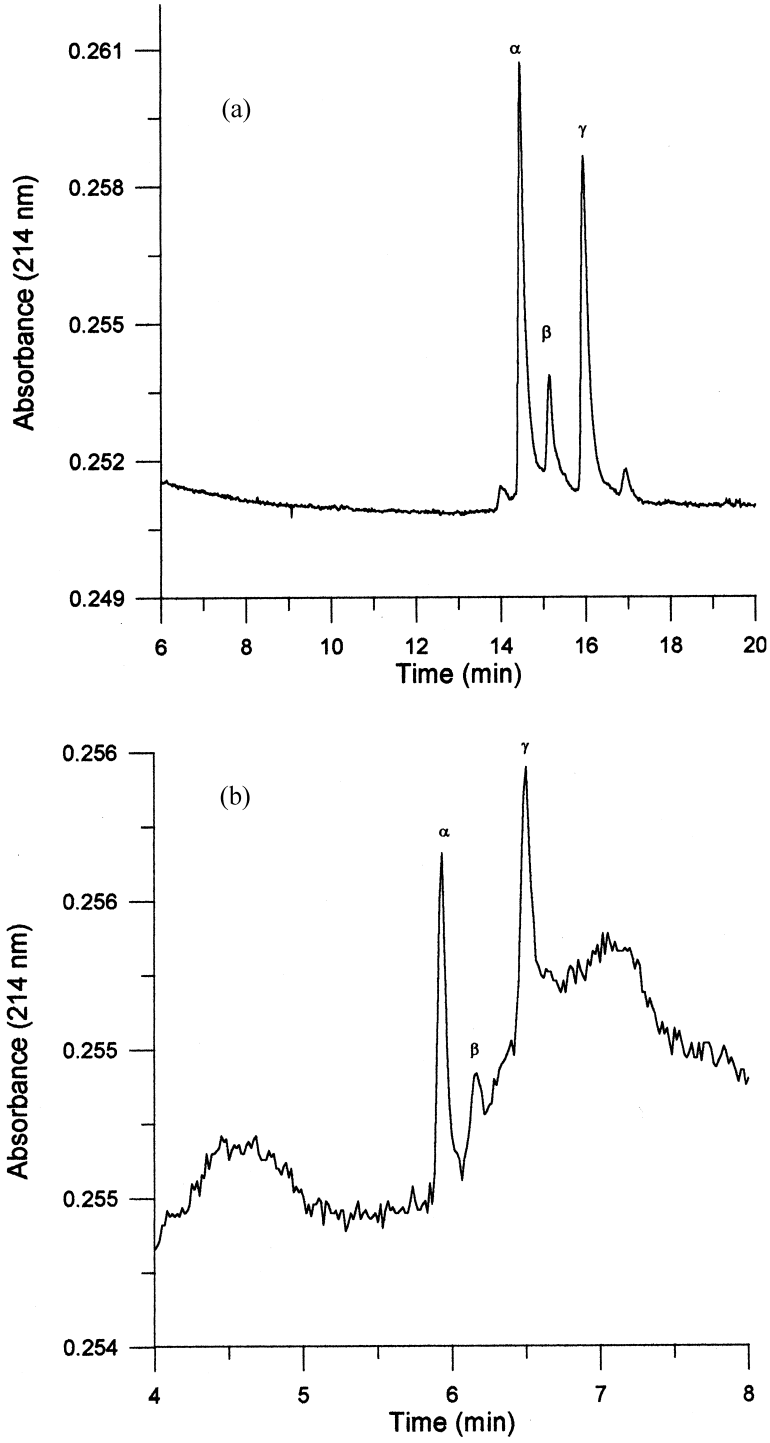

Fig. 6. CZE separation of α-, β- and γ-globin chains from umbilical cord blood. (a) 100 m*M* Cys-A buffer in 7 *M* urea (apparent pH of 2.70). (b) 50 m*M* IDA buffer in 6 *M* urea (apparent pH of 3.10). (c) 100 m*M* Cys-A buffer in 7 *M* urea and 30% (v/v) HFP (apparent pH of 2.70). Note, in this last case, the disappearance of the β-globin peak. Note additionally, in b, the strong adsorption of the globin chains to the capillary wall. Conditions: (a) 300 V/cm, 25 μA; (b) 460 V/cm, 21 μA; (c) 460 V/cm, 17 μA. All runs at 25°C.

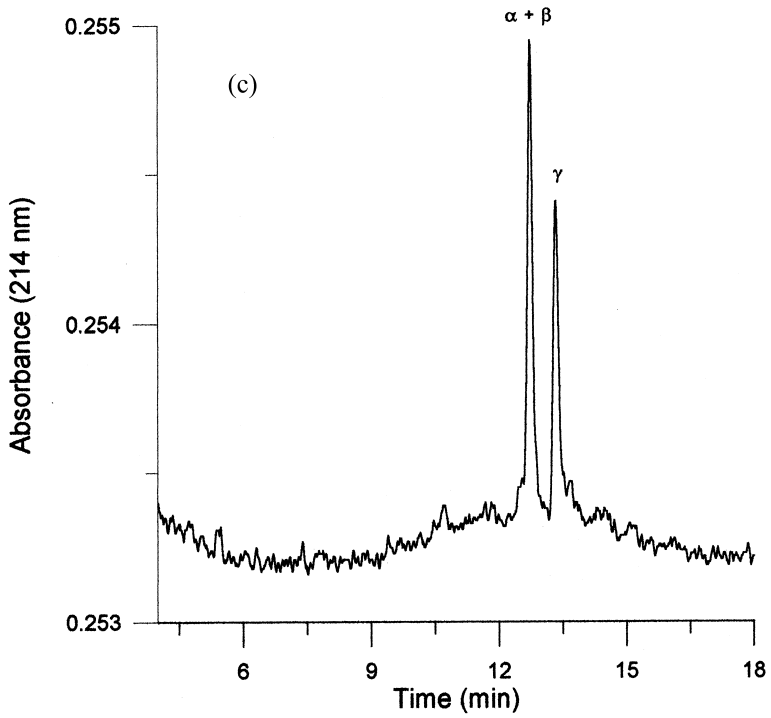

Fig. 6. (*continued*)

urea seem to alter to different extents the p*K* values of the carboxy termini, since at these levels the elution order of some peptides is altered, so that now PM migrates in front of GGL (at pH 2.25) and of GGV and GGL (at pH 2.45). In principle, also HFP should alter these p*K* values, since large p*K* alterations have been reported by Castagnola's group in the case of TFE. However, if such Δp*K* values occur in HFP, they must leave unaltered the relative p*K* differences among the different carboxy termini, since the elution order respected, in presence of HFP but in the absence of urea, is the same as that given in Fig. 4b. In addition, if such large p*K* alterations occur in presence of HFP (and of TFE as well) their remains the odd finding that in both co-solvents neither IDA nor Cys-A change their p*I* values. Since in both cases the p*I* is the arithmetical mean of the two acidic p*K* values (two carboxyls for IDA, one carboxyl and a sulphate for Cys-A) it is quite strange that the p*I* should not change at all in presence of a large alteration of these p*K* values. If this were the case, the only explanation would be that, if one carboxyl group increases its p*K*, the other acidic species should decreases its p*K* by the same extent, a unique situation indeed. On the contrary, it is quite clear that urea dramatically alters, and to different extents, probably depending on the hydrophilicity/hydrophobicity of the peptide, the p*K* values of the various carboxy termini (it will of course also alter the p*K* values of the amino groups, but at the operative pH of these separations this alteration will not affect the net surface charge of the peptide). It was in fact reported by our group, that addition of 8 *M* urea changes the p*K* of weak carboxyls (in the case of the Immobiline chemicals) by up to 1 pH unit, whereas it changes the p*K* of the corresponding alkaline Immobilines by no more that 0.5 pH units [45]. It is thus seen that these two additives serve two different purposes: HFP will be used mostly as a conductivity quencher, whereas urea will be useful both, as a conductivity quencher and as a p*K* modulator, thus influencing the selectivity of the analysis. Both of them, of course, will act also as excellent solubilizers of more hydrophobic peptides. It should be additionally emphasized that such acidic pH values (typically between pH 2.0 and 2.7) are a

must when attempting to separate oligopeptides having, as only charged residues, the amino and carboxy termini (i.e., composed solely of mono-amino, mono-carboxyl amino acids): it should be appreciated, in fact, that such oligopeptides are essentially isoelectric over the pH 4–8 range so that their separation can only be attempted either by mobility modulations around the p*K* of the carboxyl group (but below this value, where the titration curves fan out; above it, they all tend to converge towards the zero net charge value, cf. Fig. 4b) or at alkaline pH values, around the p*K* of the terminal amino group (of course, above this p*K* value!).

4.2. On the separation of polypeptides

Additional, interesting phenomena can be appreciated in the separation of polypeptides. First of all, one unique finding can be deduced from Fig. 6a: notwithstanding the high positive charges of the three globin chains, no binding to the silica wall seems to occur, since the peaks are quite symmetric and the base line returns to the origin after passage of the train of peaks. On the contrary, when the same separation is attempted in presence of IDA, strong adsorption of the polypeptides is seen to occur, as witnessed by the strong ramping in the base line and lack of return to the origin after passage of the analytes. That these chains would bind to the wall had to be expected; in fact, when these separations had originally been reported, the IDA–urea buffer solution was added also with 0.5% hydroxyethyl cellulose chains (HEC, average M_r=27 000), a polymer known to form a good dynamic coating of the silica wall. In both, Fig. 6a and b, no HEC was added, so it is surprising that no such a binding occurs in the case of Cys-A, whereas it does in IDA buffer. Considering the modest increment in apparent pH (2.7 in Cys-A vs. 3.1 in IDA) it is hard to accept that this small pH variation would be sufficient to provoke such a large interaction of the globins with the wall. We thus hypothesize that, perhaps, Cys-A per se has an additional shielding effect, thus preventing the binding of globin chains to the wall. Another unique phenomenon can be appreciated when confronting Fig. 6a and c: the mere addition of 30% HFP to the Cys-A–urea solution has the effect of completing altering the mobility of the three globin chains, to such an extent that the β-chain disappears and co-migrates with the α-zone. We have no ready explanation for it, except for resorting to the known fact that HFP is a strong helix-promoter [50]. Such conformational changes must induce different structural variations in the three chains, as a result of a competition between the helix-promoting and helix-disrupting properties of HFP and urea, respectively. The result of this interplay could be the fusion of the two zones into a single peak. Thus, our results suggest two operative strategies: for oligopeptides, HFP and urea can be used as a mixed organic solvent, since they help both in quenching the conductivity and modulating the peptide mobility, but with no structuring effects due to the small size of the peptides. For polypeptides, one would have to perform trial runs in order to assess the effects (positive or negative) of the simultaneous use of these two additives. In our case, in fact, good results could only be obtained by omitting HFP from the BGE.

4.3. On the minimum mass/charge variation required for separation of peptides

Another interesting question, which does not seem to have been addressed up to the present, is what is the minimal charge and/or mass variation requirement in order to separate two similar peptides. We have made an extensive search in the literature, and could elaborate on the following data: Jones et al. [46], recently reported the separation of β-lactoglobulin from the product of its Maillard reaction. The native protein has a M_r of 18 500, whereas the product of addition of a single lactose (as Schiff-base to a Lys residue, then rearranged via an Amadori mechanism to form a lactulosyl-lysine) shows a mass increment of 324 u. These two products can be separated, although not to baseline, by CZE at pH 3.0 in 8 *M* urea. One can thus neglect the p*K* variation of Lys at such an acidic pH, and attribute the separation solely to the mass increment. According to these data, we have calculated that the minimum mass variation for eliciting peak splitting between two peptides should be 1.5%. By looking at our data (Fig. 4a), we see ample separation between GGL and PM, two peptides differing by only 1 u over ca. 150 u, which would mean a mass difference

of only 0.67%, clearly much too small for inducing such a good separation. However, this minute ΔM_r is accompanied also by a small ΔpK between the terminal Met and Leu ($\Delta pK = 0.08$). Thus, it would appear that the two minimal values for achieving separation between two similar peptides is a ca. 1% ΔM_r (or less), in concomitance with a ΔpK of ca. 0.08. Of course, the situation is different if one analyses a series of homo-peptides, like in the work of Hamrnikova' et al. [47], who studied the behavior of oligo-Ala and oligo-Gly peptides. In this last case, it is to be expected that, since the mobility differences are due purely to constant increments in mass, the peptides will be eluted strictly in the order of this ΔM_r and at constant time increments (a situation which is largely verified up to at least the decamer).

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