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Detection of neutral and charged mutations in α- and β-human globin chains by capillary zone electrophoresis in isoelectric, acidic buffers

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Abstract

A simple and reliable method, for screening for point mutations in α- and β-human globin chains, is reported here, utilizing capillary zone electrophoresis in isoelectric, acidic buffers. A solution of 50 m*M* iminodiacetic acid (p*I* 2.23) containing 7 *M* urea and 0.5% hydroxyethylcellulose (apparent pH 3.2) is used as background electrolyte for fast separation of heme-free, denatured globin (α and β) chains. Due to the low conductivity of such buffers, high voltage gradients (600 V/cm) can be applied, thus reducing the separation time to only a few minutes. In presence of neutral to neutral amino acid substitutions, it is additionally shown that the inclusion of 3% surfactant (Tween 20) in the sample and background electrolyte induces the separation of the wild-type and mutant chains, probably by a mechanism of hydrophobic interaction of the more hydrophobic mutant with the detergent micelle, via a mechanism similar to ''micellar electrokinetic chromatography''. At this low operative pH, however, charged mutants, involving substitutions of acidic amino acids (Glu and Asp) are not detected, since these residues are extensively protonated. Curiously, however, they are still separated in presence of detergent, due to the large variation in hydrophobicity involved in such mutations. Of the 19 mutants analyzed, all but one were resolved: Hb St Nazaire (β103 Phe→Ile). This is due to the fact that the Δ*G* (in kcal/mol) in the substitution Phe→Ile is zero, thus no separation can possibly take place between two chains exhibiting the same hydrophobicity parameter.

Keywords: Globins; Haemoglobin; Proteins

1. Introduction

Hemoglobins (Hbs) are polypeptide tetramers consisting of two pairs of unlike globin chains (α, β, δ, γ), each bound to a heme group. Hb in normal adult human blood consists of >96% HbA ($\alpha_2\beta_2$), ca. 2-3% Hb A_2 ($\alpha_2\delta_2$) and <1% Hb F (fetal Hb, $\alpha_2\gamma_2$). Normal newborn blood contains Hb F as the major constituent (60–80%), the rest being Hb A. Analysis of the composition of human blood is of major clinical interest in several congenital defects associated with abnormal Hb content. These hemoglobinopathies are grouped into defective variants of

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Hbs (such as Hb S and >600 other variants) and thalassemias, which are characterized by abnormal proportions of normal globin chains [1].

In the 1970s, conventional isoelectric focusing (IEF) in gel slabs became perhaps the most popular technique for screening and detecting a number of abnormal Hbs and for performing physico–chemical studies on the behaviour in solution of Hb tetramers (for reviews, see Refs. [2–6]). Although the resolving power of IEF has been estimated at 0.01 pH units, difficult separations, such as the resolution of Hb A and Hb F_{ac} (acetylated fetal Hb) in cord blood [7] or that of Hb A from Hb A_{1c} (glycated Hb) [8] for screening of diabetic conditions, could still be achieved in non-linear pH gradients. With the advent of immobilized pH gradients (IPG) in the 1980s, Hb separations received a new impetus, since the resolution was incremented by one-order of magnitude, down to 0.001 pH unit [9]. Due to this exquisite resolving power, even neutral mutants, such as Hb San Diego (β109 Val→Met) [10] or Aγ, Gγ HbF (fetal Hbs with a Ala to Gly substitution in γ136) [11] could be resolved as intact, native tetramers without resorting to denaturation and chain separation.

Although IEF is still a widely adopted technique, it suffers from the typical drawbacks of gel-slab methodologies: it is labour intensive and requires too many steps for obtaining final results: gel preparation, electrophoretic step, staining, destaining and densitometry or photographic recording. The advent of capillary zone electrophoresis (CZE) in the 1990s has greatly simplified electrophoretic procedures, by permitting automatic protocols for sample loading and data acquisition and storage [12]. The extra bonus of CZE is also the rapid sample analysis, due to the possibility of delivering high voltage gradients in narrow-lumen separation channels. Several articles have already appeared dealing with IEF of Hbs, either in coated or uncoated capillaries [13–18]; the method also found applications for the study of the interaction between Hb and haptoglobin [19]. Difficult separation cases, as discussed above, were again solved by resorting to non-linear pH gradients in the pH 6–8 range, obtained by adding a mixture of amphoteres able to flatten the pH slope around pH 7 (typically β-alanine and 6-amino caproic acid) [17,18].

Although all the studies reported above deal with IEF of native, intact Hb tetramers, a valid alternative in the study of hemogobinopathies is separation and quantitation of de-heminized human globin chains, as first reported in 1966 by Clegg et al. [20], via a chromatographic step on carboxymethyl cellulose. The method had widespread applications for the study of thalassemias and in the analysis of ontogenesis of globin chains for at least a decade until, in 1979, some of us proposed, as a fast and reliable alternative, IEF of heme-free, denatured globin chains dissolved in 8 *M* urea and a reducing agent (2-mercaptoethanol) [21]. It was also observed that the addition of a neutral surfactant (Nonidet P-40) to the solubilization mixture brought about a unique phenomenon: it greatly improved the separation between β- and γ-(fetal) chains and additionally induced the splitting of the γ zone into two peaks, identified as the products of two genes, coding for Ala (Aγ) or for Gly (Gγ) in position 136 of the fetal chains [22,23]. This surfactant effect, on the resolution of the two genetic variants of fetal chains, Aγ and Gγ, could also be reproduced by zone electrophoresis on cellulose acetate strips [24].

A few reports have already appeared on the CZE separation of denatured, heme-free globin chains. Whereas most of them adopt CZE in strongly acidic buffers [25–28], one communication deals with separations in very alkaline (pH 11.8) buffers [29]. CZE, if carried out in an acidic milieu, has some distinct advantages over capillary IEF: it can be performed in uncoated capillaries, since at such pH values (typically 2.0 to 2.5) essentially all silanols of fused silica are protonated and thus unable to adsorb proteins and polypeptides. However, one drawback still remains: due to the very high conductivity of these buffers, only low voltage gradients can be applied, resulting in long separation times. In order to improve the situation, we have reported separations in acidic, isoelectric buffers (notably aspartic and imino diacetic acids): in such amphoteric buffer solutions, much higher voltage gradients (600 V vs. 100 V) can be delivered, eliciting separations of the order of a few minutes [30]. In a first communication, we could demonstrate excellent separations of human α-, β- and γ-globin chains, taken from umbilical cord blood at delivery [31]. In the present report, we demonstrate that this technique can be

successfully used for detecting point mutations in α- and β-globin chains. The interest on the present method is not so much the detection of charged mutants, but the analysis of electrophoretically silent mutations, made possible by the use of neutral surfactants which induce a sort of ''micellar electrokinetic chromatographic'' type of separation.

2. Experimental

2.1. Reagents

Iminodiacetic acid (IDA) and urea were obtained from Fluka (Buchs, Switzerland). Fused-silica capillaries (50 μm I.D.×375 μm O.D.) were from Polymicro Technologies (Phoenix, AZ, USA) and were used as such, without inner coating. Hydroxyethyl cellulose (HEC, number-average molecular mass, M_n, of 27 000) was from Aldrich (Milwaukee, WI, USA). Tween 20 was from Sigma (St. Louis, MO, USA). The 18 human adult Hb mutants, as listed in Table 1, were collected and purified at the Hopital Henri Mondor, Creteil, France.

2.2. CZE

CZE was carried out with a Waters Quanta 4000E instrument (Milford, MA, USA). 33 cm (25 cm to detector)×50 μm I.D. uncoated capillaries were used, bathed in the following electrolyte solution: 50 m*M* IDA (pH=p*I*=2.23), with 0.5% HEC and 7 *M* urea (apparent pH 3.2). The conductivity of this buffer (at 25°C) was 176 μS/cm. The globin samples (typical concentration 1–2 mg/ml), dissolved in the same buffer (but supplemented with 2% β-mercaptoethanol and containing only 5, instead of 50, m*M* IDA) were injected for 12 s. Separations were performed at 600 V/cm (ca. 25 μA current) and detection was at 214 nm. The anolyte and catholyte were compose of the same background electrolyte filling the capillary, except that in these reservoirs the urea concentration was lowered to 6 *M*, in order to prevent potential crystallization due to some solvent evaporation upon prolonged runs. Urea crystals, when forming, would quickly occlude the capillary extremities. For separation of neutral mutants, both the sample and the background electrolyte were supplemented with 3% Tween 20.

Table 1
List of the 18 hemoglobin mutants investigated

Name of hemoglobin	Type of mutation
Hb Olympia	β20 Val→Met
Hb St. Nazaire	β103 Phe→Ile
Hb Debrousse	β96 Leu→Pro
Hb Ty Gard	β124 Pro→Gln
Hb Knossos	β27 Ala→Ser
Hb Little Rock	β143 His→Gln
Hb Sitia	β128 Ala→Val
Hb Aubenas	β26 Glu→Gly
Hb Brie Conte Robert	β36 Pro→Ala
Hb Harrow	β118 Phe→Cys
Hb Khartoum	β124 Pro→Arg
Hb Ohio	β142 Ala→Asp
Hb Westmead	α122 His→Gln
Hb M Iwate	α87 His→Tyr
Hb Mosella	α111 Ala→Thr
Hb Les Andelys	α83 Leu→Pro
Hb Chad	α23 Glu→Lys
Hb Roanne	α94 Asp→Glu

2.3. Globin chain preparation

After washing the red blood cells in physiological saline and lysing in hypotonic solution, the ghosts were pelletted by centrifugation. The concentrated Hb solution (20 μl) was added dropwise to 1.5 ml of acetone containing 2% HCl at room temperature in an Eppendorf tube. After centrifugation, the supernatant was removed and the pellet redissolved in 20 μl of water. In order to obtain the white globin powder, the precipitation procedure was repeated. The final pellet was resuspended in 20 μl of water and dried in a Speed Vac. The structural abnormality of all these variants was controlled by determination of the amino acid replacement. Prior to analysis, it was dissolved in background electrolyte (here too supplemented with 2% β-mercaptoethanol and containing only 5, instead of 50, m*M* IDA) at a concentration of 1–2 mg/ml.

3. Results

Fig. 1 shows the separation of an individual heterozygous for a neutral mutation in the β-chains: β20 Val→Met (Hb Olympia). The upper panel gives

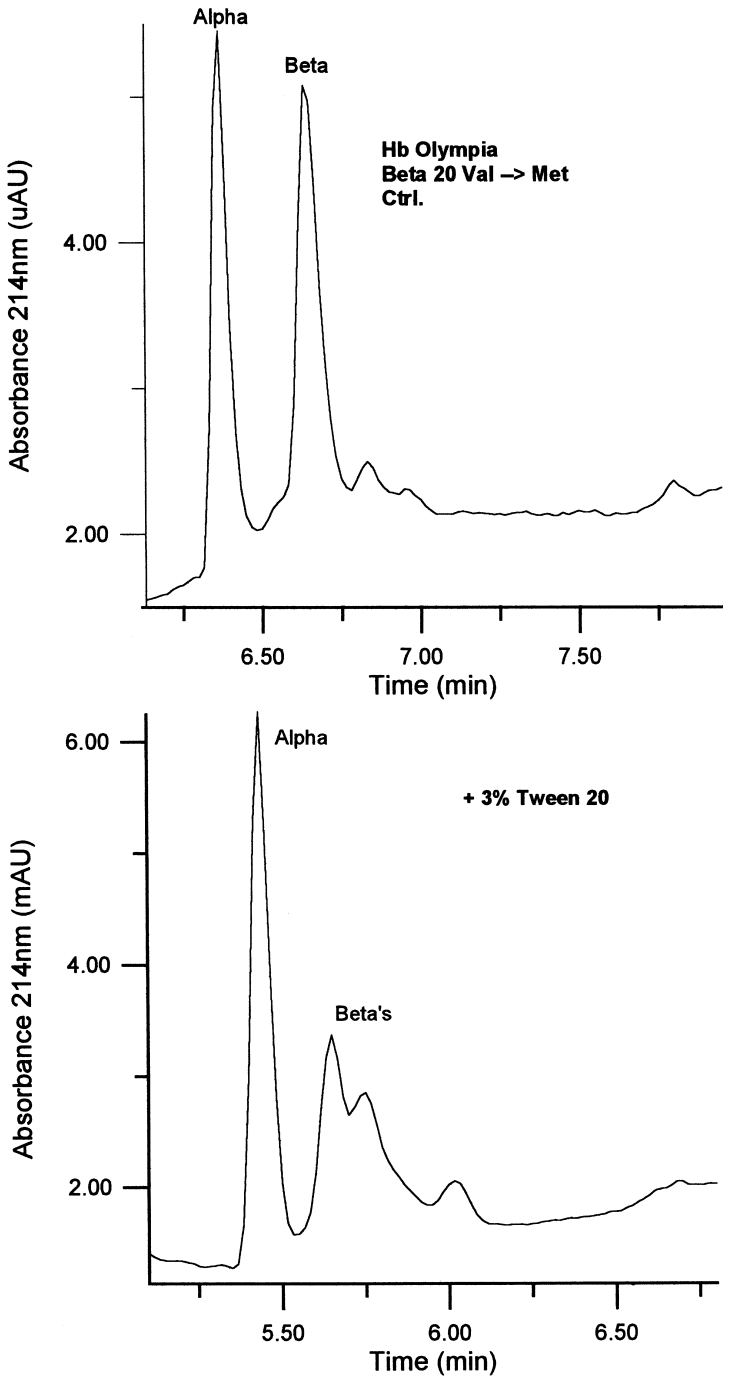

Fig. 1. CZE separation of Hb Olympia (β20 Val→Met). Here, and in all subsequent figures, the CZE conditions are as follows: 50 m*M* IDA buffer, added with 7 *M* urea and 0.5% hydroxyethyl cellulose (apparent pH 3.2) in the absence (control, Ctrl., upper panel) or in presence (lower panel) of 3% surfactant (Tween 20). Capillary: uncoated, 33 cm (length to detector 25 cm)×50 μm I.D.×375 μm O.D. Run: in a Waters Quanta 4000E, at 600 V/cm (ca. 25 μA current) and 15°C. Sample injection at the anodic side for 12 s; detection at 214 nm.

the pattern obtained in isoelectric IDA and 7 *M* urea (apparent pH 3.2) in the absence of surfactant; the lower panel shows the electropherogram of the same sample in presence of 3% Tween 20. The identification of each band was performed by running individual, purified α- and β-chains. Moreover, from the shape of the respective titration curves (see Fig. 5 in Ref. [31]) this elution order was expected since, at pH 3.0, α-chains bear an extra positive charge (24) as compared with β-chains (23). It is seen that, in presence of the detergent, the zone of β-chains is split into two bands, which should correspond to the normal and mutated species (although the precise location of each band cannot be predicted with certainty, since one would have to elute each zone and analyse it by fingerprinting; nevertheless, the retarded chain should be the one interacting more strongly with the surfactant, since this would induce a higher apparent mass, lower the charge/mass ratio and thus slow down its migration). That this splitting could be an artefact is excluded by the fact that, when analyzing a normal adult sample, no such splitting occurs; moreover, the zone of α-chains remains intact after surfactant addition.

Figs. 2–4 show the same phenomenon occurring for a series of neutral mutants in the β chains: Hb Ty Gard (β124 Pro→Gln; Fig. 2); Hb Knossos (β27 Ala→Ser; Fig. 3); Hb Debrousse (β96 Leu→Pro; Fig. 4). In all cases, the ratio of the normal and mutated chains is seen to approximate a 1:1 ratio, as it should, given the heterozygous state of all these individuals. All neutral to neutral amino acid substitutions in β-chains, as listed in Table 1, could be resolved by this method, except one: Hb St Nazaire (β103 Phe→Ile). The significance of this failure will be discussed below. An interesting phenomenon can be observed in Fig. 5: in this particular case, a charged mutant was analyzed (Hb Aubenas, β26 Glu→Gly), thus one would have expected resolution of these two chains in the control run (upper panel), in the absence of surfactant. This was not the case, however, and the splitting only occurred in presence of 3% Tween 20 (lower panel). In a way, this should have been expected (see also discussion): given the operative pH of our isoelectric buffer, the Glu residue should be extensively protonated, so that the charged amino acid substitution went undetected, but it could still be revealed in presence of surfactant due to the huge difference in hydrophobicity of these two residues.

Fig. 6 shows some interesting data obtained with two charged mutants in the α-chains: Hb Iwate (α87 His→Tyr; upper panel) and Hb Chad (α23 Glu→Lys; lower panel). Here the peak assignment was quite easy: in the upper electropherogram, the normal chain must be the first eluted peak, since it bears an extra positive charge as compared to the mutant (labelled Alpha-M). Conversely, in the lower panel, the mutant chain must be the first eluted peak, since it bears an extra positive charge. Note, however, that in this last case, the distance between normal and mutated peaks does not correspond to a double charge difference (loss of a negative charge and acquisition of a positive one) due to the same phenomenon described in Fig. 5: at the operative pH, the Glu residue is mostly protonated, thus its loss does not correspond to an appreciable charge difference. The two α peaks were in the expected ratio for an heterozygous α-chain variant since this chain is encoded by two genes in each chromosome 16 (α-1 and α-2) which is responsible for the biosynthesis of 15–20% and 30–35%, respectively.

4. Discussion

Some interesting findings of the present research are worth discussing, as they offer some insight on the advantages and limitations of the present technique.

4.1. On the mechanism of separation of neutral mutants in surfactant solutions

The principle of this separation is per se not new, since it was disclosed for the first time by our group in 1979 [21], when screening cord blood lysates for thalassemic conditions by IEF in gel slabs. We reported that the presence of Nonidet P-40 (here abandoned in favour of a UV-transparent detergent such as Tween 20) in globin chains solutions brought about two marked effects: it increased the resolution between the β- and γ-chains (which had very close p*I* values) and, surprisingly, split the zone of γ globins into two peaks, later on demonstrated to correspond to the Aγ and Gγ phenotypes normally

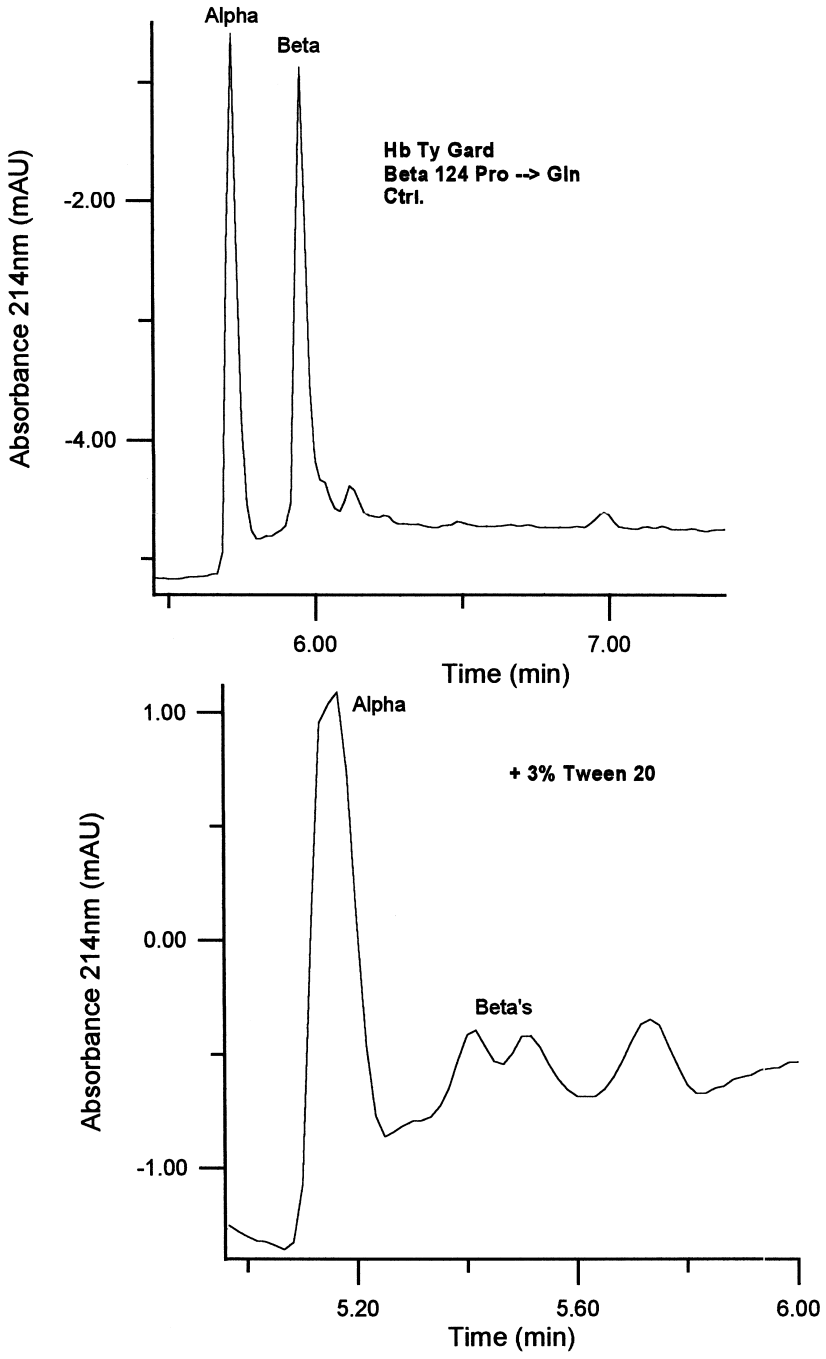

Fig. 2. CZE separation of Hb Ty Gard (β124 Pro→Gln). Upper panel: control run; lower tracing: added with 3% Tween 20. All other conditions as in Fig. 1.

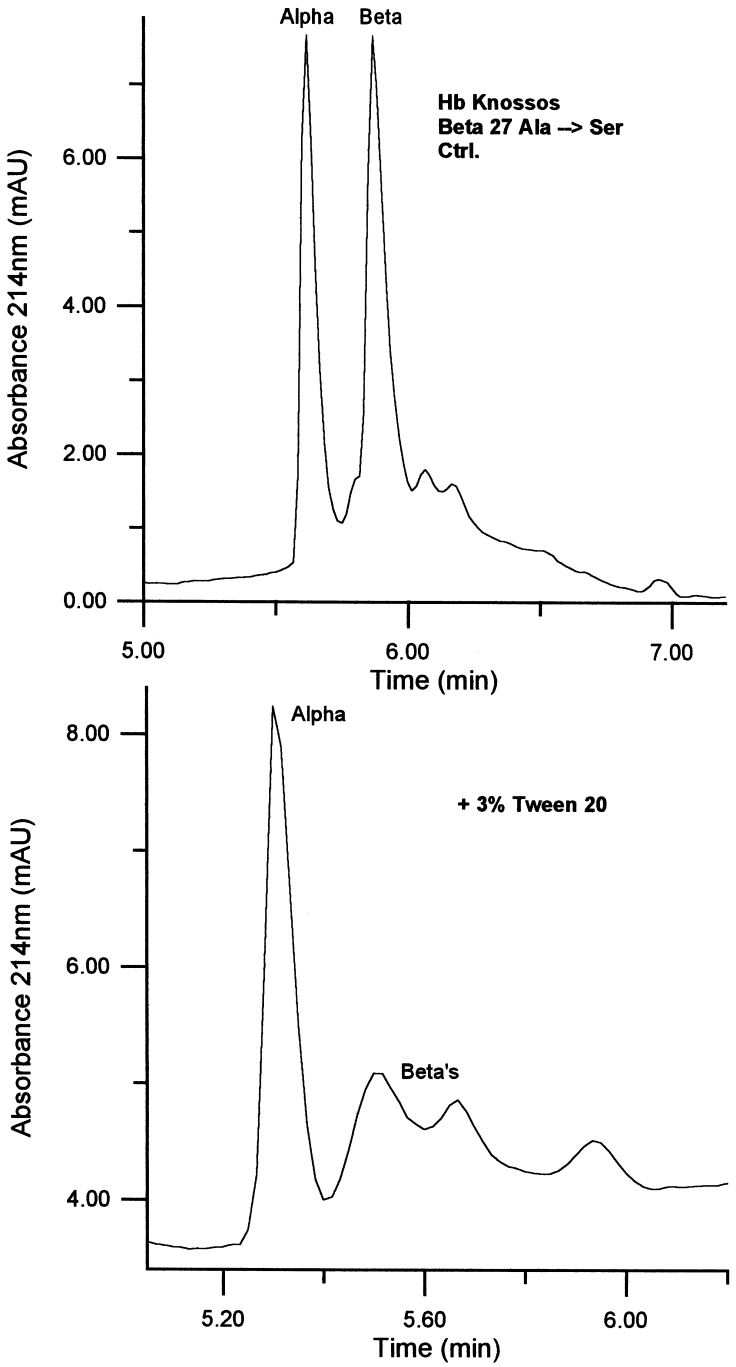

Fig. 3. CZE separation of Hb Knossos (β27 Ala→Ser). Upper panel: control run; lower tracing: added with 3% Tween 20. All other conditions as in Fig. 1.

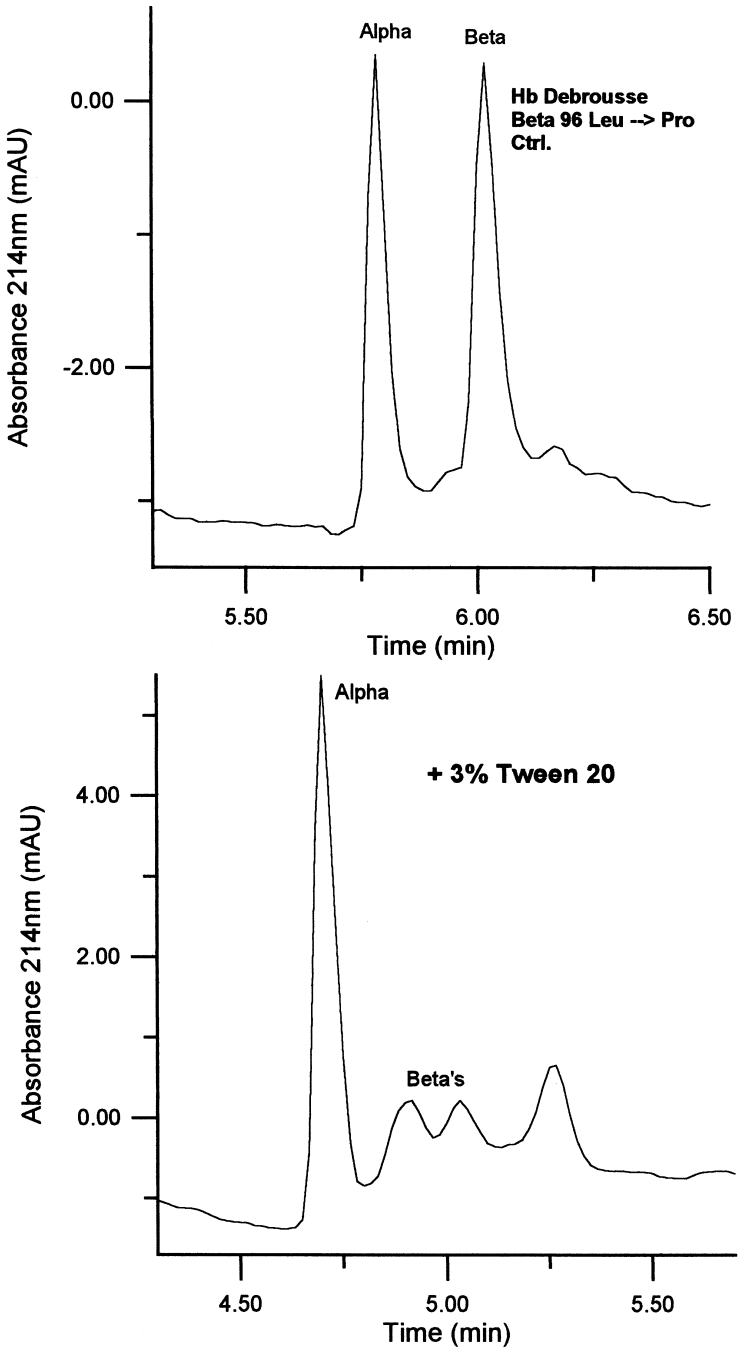

Fig. 4. CZE separation of Hb Debrousse (β96 Leu→Pro). Upper panel: control run; lower tracing: added with 3% Tween 20. All other conditions as in Fig. 1.

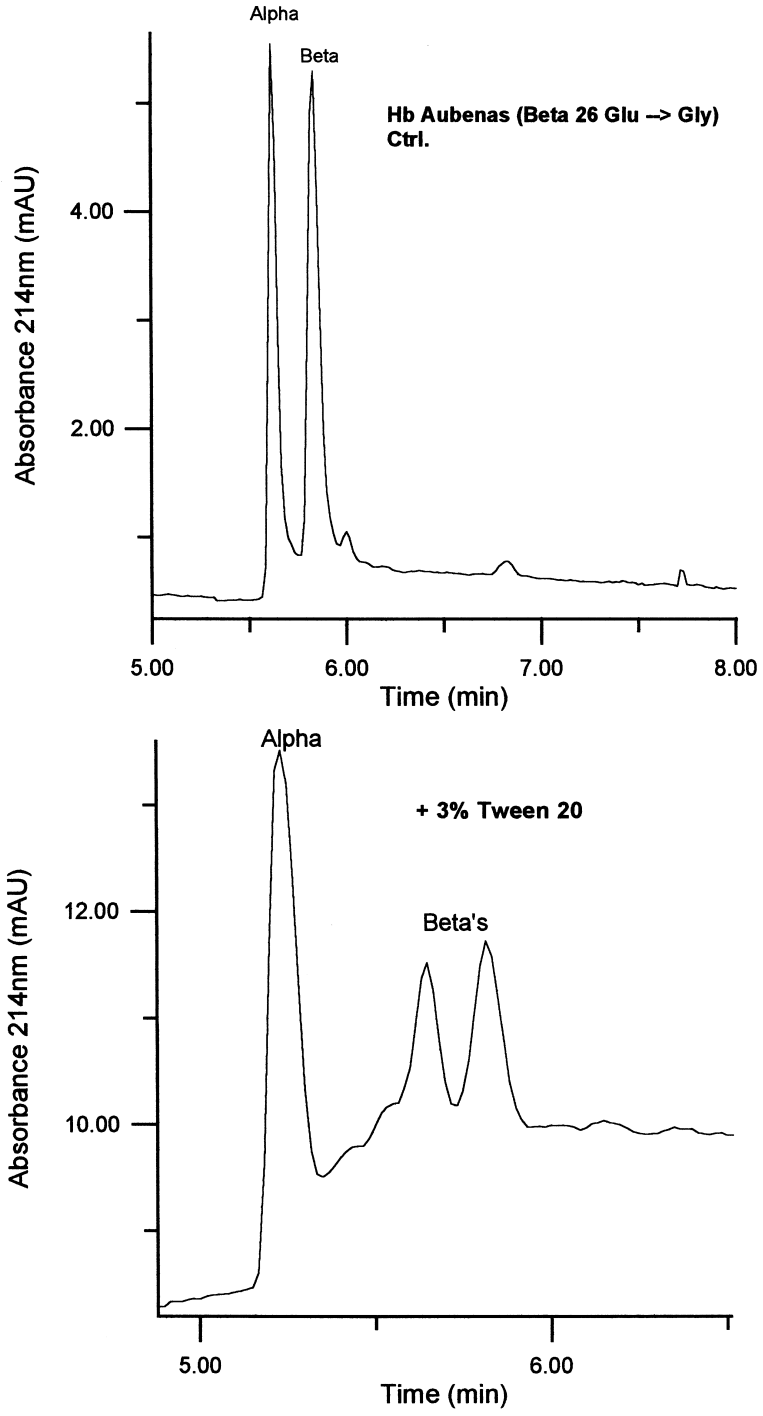

Fig. 5. CZE separation of Hb Aubenas (β26 Glu→Gly). Upper panel: control run; lower tracing: added with 3% Tween 20. All other conditions as in Fig. 1. Note that, although this mutation involves a charged amino acid, it is not detected in the control run, but only when adding the surfactant.

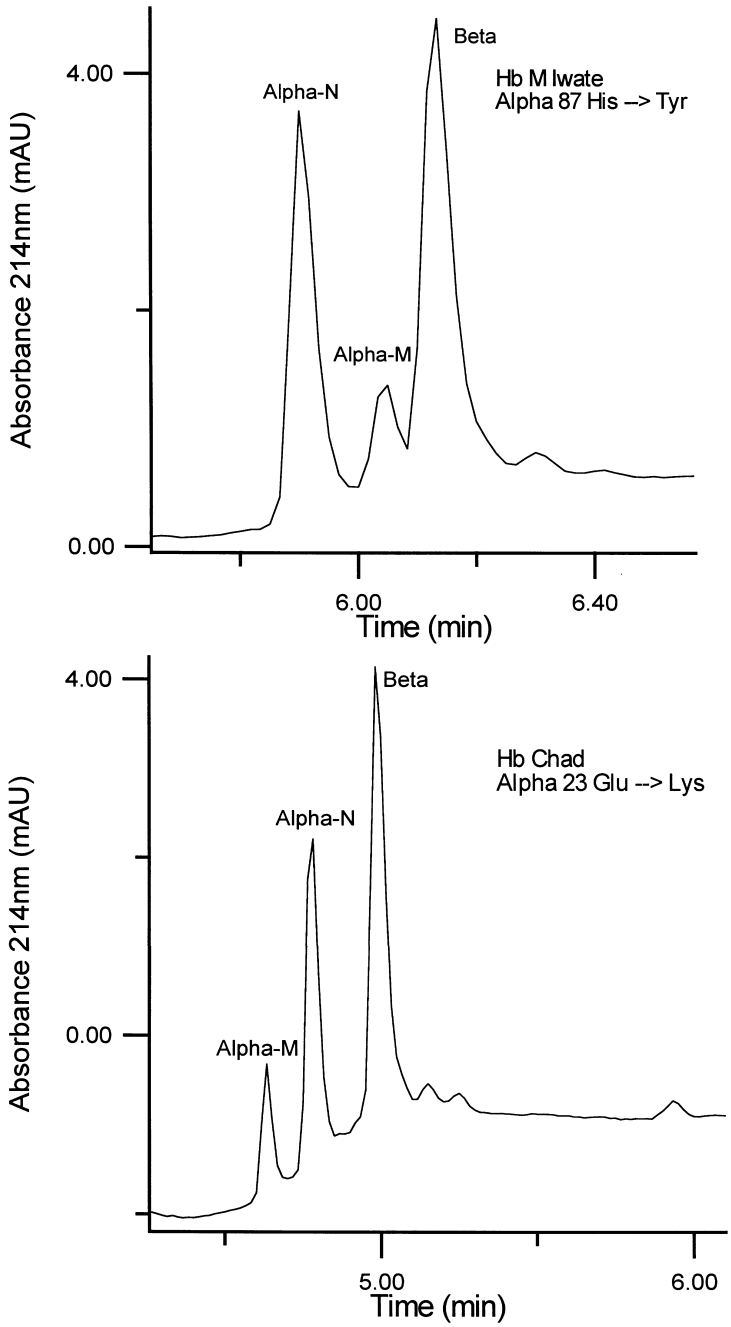

Fig. 6. CZE separation of Hb Iwate (α87 His→Tyr; upper panel) and of Hb Chad (α23 Glu→Lys; lower panel). Note that the mutated α-chains represent only 30% of the total pool of α-globins. Alpha-N: normal α-chain; alpha-M: mutated α-globin. In both cases, due to the fact that the mutants are charged, no Tween 20 was added to the background electrolyte.

present in cord blood [22–24]. Since IEF is a steady-state technique, the only likely explanation was that there had been a differential adsorption of the more hydrophobic chain (Aγ) into the Nonidet micelle. We suggested that, in Aγ globins, the detergent micelle could bind preferentially to the hydrophobic stretch ^{133}Met to ^{141}Leu. Upon binding, the detergent could sorb the ^{132}Lys in its Stern layer or bury it within the micelle, thus inducing a total loss of one proton from otherwise charge-identical phenotypes. To our knowledge, no one had reported the same phenomenon in case of α- and β-chains, thus it is quite comforting to see that the same mechanism seems to be operative for the other globins as well. However, since in our case the separation mode is a free zone electrophoretic approach, and not a steady-state procedure such as IEF, the separation principle could be different from that hypothesized for IEF. Whereas IEF can only separate on the basis of net surface charge (p*I*) differences, CZE separates analytes on the basis of the charge/mass ratio. Thus the splitting of wild-type and neutral mutants into two zones might or might not involve the masking of a charge, as suggested for IEF, but certainly it will involve a change of apparent mass of the two analytes, due to differential residence time of the two species into the surfactant micelle (the more hydrophobic mutant having, in principle, a longer interaction time). Thus, since the detergent micelles are stationary in the electric field, the species with a higher residence time will be retarded and be eluted after the less retained globin (it cannot be excluded, in fact, that also the wild-type globin will interact to some extent with the detergent). In a way, the phenomenon we are describing is analogous to ''micellar electrokinetic chromatography'' (MEKC), invented by Terabe et al. [32], but with some subtle differences. Terabe's method makes use of charged micelles and is typically adopted for separating uncharged compounds. In this methodology, charged micelles (e.g., sodium dodecyl sulphate) migrate in the electric field at an electrophoretic velocity that is proportional to their charge-to-size ratio. Uncharged solutes with different micelle–water partition coefficients can then be separated. Thus, this is viewed as a chromatographic technique with migrating charged micelles acting as pseudostationary phases. MEKC can be therefore considered as an hybrid of reversed-phase liquid chromatography and capillary electrophoresis, as the separation process incorporates hydrophobic and polar interactions, a partitioning mechanism and electromigration. In our case, it is the charged polypeptide that migrates in virtue of its intrinsic charge and it is the stationary micelle which interacts, to some extent, with the moving particle. The interaction most probably follows a hydrophobicity scale, and this explains why it was impossible to separate the wild-type and mutant chains of Hb St Nazaire (β103 Phe→Ile). According to the hydrophobicity scale of Table 2 [33,34], the Δ*G* (in kcal/mol) in the substitution Phe→Ile is zero, thus no separation can possibly take place between two chains exhibiting the same hydrophobicity parameter.

Are there other examples in the literature of such resolution-enhancing phenomena? We have located some interesting cases worth discussing. In one approach, Rathore and Horvath [35] have reported a quite unique phenomenon: addition of 20 m*M* carboxymethylated-β-cyclodextrin (CMBCD) dramatically enhanced the resolution of four standard, rather alkaline proteins: α-chymotrypsinogen

Table 2
Hydrophobicity scale of amino acids, as measured by partitioning

Amino acid[a]	Relative hydrophobicity (kcal/mol)[b]
Alanine	0.5
Arginine	−11.2
Asparagine	−0.2
Aspartic acid	−7.4
Cysteine	−2.8
Glycine	0
Glutamine	−0.3
Glutamic acid	−9.9
Histidine	0.5
Isoleucine	2.5
Leucine	1.8
Lysine	−4.2
Methionine	1.3
Phenylalanine	2.5
Proline	−3.3
Serine	−0.3
Threonine	0.4
Tryptophan	3.4
Tyrosin	2.3
Valine	1.5

[a] Data from Refs. [33,34].
[b] Negative values indicate a preference for water, positive values for organic solvents.

A, cytochrome *c*, lysozyme and ribonuclease. Furthermore, in presence of the same additive, the cis-trans isomers of angiotensin could be separated at room temperature, whereas such separation occurred only at −20°C in the absence of CMBCD. They hypothesize that CMBCD interacts with accessible hydrophobic moieties of proteins forming an adduct; the charges and hydrodynamic radii of such adducts and consequently their electrophoretic migration velocities would thus be different from those of uncomplexed proteins. In another example, Castagnola and co-workers [36,37] reported that addition of 2,2,2-trifluoroethanol (TFE) to water solutions (up to 37.5%, v/v), modified the Stokes radii of some peptides, in a manner dependent on their sequence, to such an extent that a marked increase in resolution could be obtained. Although this seemed to apply mostly to small peptides (4–10 amino acids long), a marked improvement was obtained also when running a tryptic digest of myoglobin, suggesting that even larger peptides could benefit from TFE addition.

4.2. Advantages and limitations of CZE in acidic, isoelectric buffers

The method here reported has some advantages, but also some limitations the readers should be aware off. Perhaps one of the main improvements is the high charge imparted to peptides and proteins at such a low operative pH. By exploring their respective titration curves (see Fig. 5 in Ref. [31]), one can appreciate that α-chains bear a total of 24 positive charges, β-globins 23 and γ 21 at pH 3.0. Given the fact that these three chains have very similar mass values, this results in a very favourable charge to mass ratio (on the average, one positive charge every 700 Da), which calls for very fast separations (of course, also favoured by the high voltage gradients applied, typically 600 V/cm). However, the disadvantage is that, at such low pH values, one looses charge modulation, since essentially all negatively charged amino acids (Glu and Asp residues) are extensively protonated. This results in the paradox of Fig. 5: here a charged mutation was missed, since the Glu residues did not contribute to charge at pH 3 (however, note that this mutation was still spotted by addition of surfactant). Thus, if one desires to resolve directly charged mutants (especially those involving acidic residues) a better pH value for the background electrolyte would be around pH 5, where the three titration curves maximally diverge and where a substantial ionization of Asp and Glu residues occurs. This increment of resolution will come, of course, at the expenses of longer running times, due to much lower net positive surface charge on the polypeptides, and with the added risk of adsorption of the globin chains to the ionized silica wall (we use uncoated capillaries at our low operative pH!). Another paradox can be found in the separation of Hb Khartoum (β124 Pro→Arg; not shown): although the mutant chain could be detected, due to the gain of an extra positive charge, it moved dangerously close to the α peak, giving only apex resolution. Here too, if a better resolution was sought, a pH 5 buffer should have been chosen. As an additional remark, we would like to emphasize that the separations here reported represent a delicate balance of binding of the globin polypeptides to the surfactant micelles. First of all, the presence of 7 *M* urea (needed for globin solubilization) tends to drastically alter the size of the micelle and to disrupt its interaction with the polypeptides. Secondly, also temperature changes dramatically alter this complexation. In order to improve chain separation, we use the highest possible level of detergent (3%) and adopt the lowest possible temperature during the electrophoretic run. It was found that at 25°C no detergent binding would occur; separations should thus be carried out below 20°C, possibly at 15°C or lower, temperatures not always compatible with present-day CZE instrumentation. As a final comment, diagnostic value in this kind of separations can only be brought about by a differential analysis, i.e., by admixing the wild-type and presumptive mutant in the same sample. Thus the splitting in two zones of the α- or β-globin would indicate the occurrence of a mutation. Expecting absolute reproducibility of transit times in protein and peptide analysis is a chimera: even conditions of suppressed electroendoosmotic flow, as in the present case, do not prevent some shifts in peak position from run to run. There might always be some minute analyte (or impurity) adsorption to the wall, which will change to some extent the charge state of the silica surface. In addition, changing from a control background

electrolyte to one containing surfactants brings about adsorption of micelles to the silica wall, again altering the original state of the surface. Migration times are bound to change again and the switching from one type of buffer to another one often requires long a tedious equilibration procedures aimed at restoring the original conditions of the silica surface.

4.3. Note added

At the request of one referee, we would like to add some comments to the fact that, upon addition of Tween 20, a third, relevant peak appears in most electropherograms, eluting after the doublet of β-chains (see, e.g., Figs. 1–4, lower panels). We believe, although we have no direct proof, that it could represent a (possibly) hetero-dimer of β-chains, linked via an –S–S– bridge. The latter could be generated via slow oxidation of the sample, upon prolonged storage in the 70% ethanol solution (see Section 2.3), favoured by the very high sample concentration. In fact this third peak disappeared when the sample was prepared and analysed immediately, without any prior storage, as in Fig. 5 (lower panel). If this third peak were indeed a hetero-dimer, its position as a late-eluting peak would be justified by the higher retention in the detergent micelle, due to its larger mass. Note, however, that in the absence of surfactant this additional species should not, per se, be separated from normal or mutant β-chains, since it would have essentially the same charge-to-mass ratio. This phenomenon is presently under investigation.

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