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RESOLUTION OF G_γ AND A_γ FOETAL HAEMOGLOBIN TETRAMERS IN IMMOBILIZED pH GRADIENTS

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SUMMARY

Intact tetramers of foetal haemoglobin (G_γ, A_γ and the mutant $A_\gamma T$) can be separated by isoelectric focusing in immobilized pH gradients over a very shallow pH interval (pH 7.35–7.55). The G_γ tetramer exhibits a lower p*I* (7.450) than the A_γ tetramer (p*I* 7.453); the ΔpI between the two species is barely 0.003 of a pH unit, close to the theoretical resolution limit of the technique, $\Delta pI = 0.001$. Haem-free, denatured γ chains exhibit a reversal in p*I* order, the A_γ chain being more acidic than the G_γ chains: this is attributed to preferential binding of detergent micelles to the more hydrophobic A_γ polypeptide. The advantage of the present technique is the simultaneous analysis of several samples (30–40 per gel slab) and the recovery of intact, haemoglobin tetramers for subsequent studies.

INTRODUCTION

At birth, umbilical cord blood contains an average of 70% foetal haemoglobin (Hb F), 20% of adult haemoglobin (Hb A) and 10% of acetylated foetal haemoglobin (Hb Fac)[1]. Amino acid analysis of normal cord blood has shown that both alanine and glycine are present at position 136 in the γ chain (A_γ and G_γ). G_γ chains represent 70–80% of the total γ chains in the blood of the foetus and of new-born babies[2], but this fraction falls to about 40% by 5 months of age[3]. The two tetramers A_γ and G_γ are the products of two non-allelic genes. More recently, Ricco *et al.*[4] found that the blood of up to 40% of normal new-born and premature babies contained yet another type of Hb F in which the isoleucine residue at position 75 of A_γ had been substituted by threonine ($A_\gamma T$) (Hb F Sardinia). The amount present varies between only traces and 40% of the total haemoglobin fraction.

These native tetramers cannot be resolved by high-resolution electrophoretic techniques, such as isoelectric focusing (IEF)[5] or by high-performance liquid chromatography (HPLC) on cation exchangers[6]. However, the haem-free, disaggregated

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globin chains can readily be separated, on an hydrophobicity scale, by reversed-phase HPLC[7] or, on a charge scale, by IEF in urea and detergent[8]; the latter technique exploits the differential binding of the detergent micelle to the more hydrophobic chain, A_γ.

Neutral-to-neutral amino acid variants are not supposed to be amenable to charge fractionation, as the mutation will in general alter the hydrophobicity of the protein rather than its surface charge. Yet, with the advent of IEF with immobilized pH gradients[9] we were able to separate all the "electrophoretically silent" mutants we had available: this was the case with Hb San Diego[10], Hb Beirut[11] and, recently, even with Hb Sardinia[12]. Moreover, different haemoglobin species, co-focusing in the same p*I* zone, could be resolved, *e.g.*, Hb A2 *vs.* Hb E[13].

In this report, we describe the separation of G_γ *vs.* A_γ foetal tetramers, with the Immobiline technique, using extremely shallow pH gradients. The novelty of our fractionation method is the direct analysis of intact, native haemoglobin tetramers, instead of denatured, haem-free globin chains. The recovered species are thus available for additional physiological studies.

MATERIALS AND METHODS

Ampholine and Pharmalyte carrier ampholytes were from LKB (Bromma) and Pharmacia (Uppsala), respectively. Immobilines with p*K* 7.0 (buffering ion) and 3.6 (titrant), a Multiphor II cell with a Multitemp thermostat and a Macrodrive 5 power supply were from LKB Produkter (Bromma). Lysates were prepared by mixing packed red cells from cord blood, washed three times with 0.9 g/dl sodium chloride solution, with an equal volume of distilled water and 0.4 volume of carbon tetrachloride. Membranes and other debris were removed by centrifugation. The haemolysate was adjusted to 2 gHb/dl and stored under carbon monoxide.

IEF of native haemoglobins

Conventional IEF in carrier ampholytes[14] was performed in 0.7-mm 5% T polyacrylamide gels containing 2% Ampholine pH 6–8 and an equimolar mixture (each 0.2 *M*) of β-alanine(β-Ala) and 6-aminocaproic acid (6-ACA) to flatten the pH gradient from 2 to *ca.* 0.7 pH units[1].

Immobilized pH gradients

IEF in immobilized pH gradients was performed in 0.5-mm, 3% T polyacrylamide gels containing the standard amount of buffering ion (12 m*M* Immobiline p*K* 7.0) and the required amount of titrant (Immobiline p*K* 3.6) to generate a gradient of pH 7.35–7.55 along the separation axis[15]. In the experiment shown in Fig. 2B (performed over a separation distance of 20 cm) the gel was impregnated with 6% Pharmalyte pH 5–8[16].

IEF of globin chains

IEF of haem-free, globin chains (acid–acetone powder) was performed in 0.5-mm 5% T polyacrylamide gels containing 2% Ampholine pH 6–8, 8 *M* urea and 2% Nonidet P-40, cast onto silanized glass plates[8].

In IEF in carrier ampholytes, *ca.* 50 μg total sample were applied per track,

while in immobilized pH gradients up to ten times as much sample could be applied without overloading effects.

RESULTS

Fig. 1 shows the best separation obtainable for the three major components of umbilical cord blood (Hb F, A and Fac) in conventional IEF with separators (an equimolar mixture of β-Ala and 6-ACA), which reduce the gradient from 2 to *ca.* 0.7 pH units. The three components are well separated, but the Hb F zone still comprises at least two major bands, the G_γ and A_γ tetramers. In two cases (marked with an asterisk), cord blood from new-born babies homozygous for the Hb F Sardinia variant was analyzed: this tetramer is seen to be separated at slightly more alkaline pH than the main Hb F band, but complete resolution is not achieved. When the same samples were analyzed in an immobilized pH gradient spanning barely 0.2 pH units (pH 7.35–7.55), ample resolution was obtained between $A_\gamma T$ and G_γ from the homozygous new-born baby, and reasonable resolution could be achieved between the A_γ and G_γ zones from a normal cord blood lysate (Fig. 2A). Owing to its relative abundance, the lower p*I* component (p*I* 7.45 at 10°C) was tentatively assigned as G_γ. When the same experiment was repeated over the same pH gradient but with double the gel length (20 cm *vs.* the customary 10 cm) a sharp resolution was obtained between the two phenotypes (Fig. 2B). Note that the difference in isoelectric points between the two species is barely 0.003 of a pH unit (7.450 for G_γ *vs.* 7.453 for A_γ) which means that our system has a resolution of $\Delta pI = 0.001$ pH unit, as predicted[9].

The identity of the two resolved bands in Fig. 2B was confirmed as shown in Fig. 3. The two zones were eluted from the immobilized pH gradient gel, haem-free chains were prepared (acid–acetone powder) and analyzed by conventional IEF in the presence of 8 *M* urea and 2% neutral detergent (Nonidet P-40). As shown in Fig. 3, the upper band contained only the A_γ chains (left side), while the lower p*I* component essentially comprised G_γ chains (central lane); the right lane represents the analysis of infractionated Hb F.

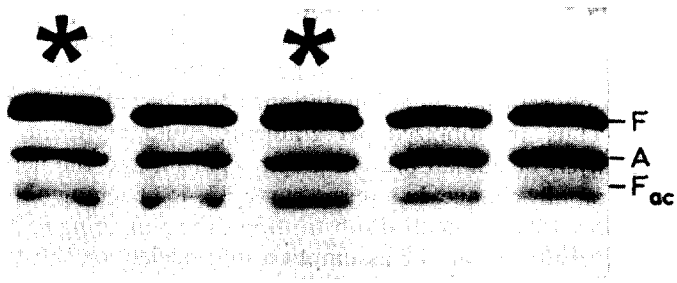

Fig. 1. Analysis of haemoglobin components from umbilical cord blood at birth on 5% polyacrylamide gel, 0.7 mm, containing 2% Ampholine pH 6–8, 0.2 *M* β-Ala and 0.2 *M* 6-ACA. Focusing for 3 h at 1200 V (at equilibrium), 4°C. Sample: 50 μg Hb applied cathodically per lane (the cathode is uppermost). Unstained gel. The two samples marked with an asterisk from new-born babies homozygous for $A_\gamma T$, while the other three are from normal new-born babies.

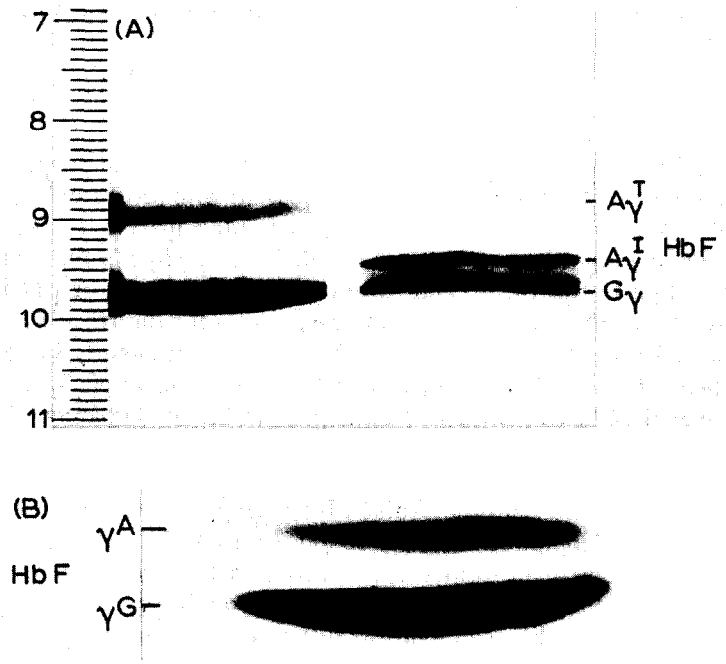

Fig. 2. Resolution of the Hb F bands in immobilized pH gradients on 3% polyacrylamide gel, 0.5 mm, containing gradient of pH 7.35–7.55. (A) The gel (10 cm) was subject to focusing for 10 h at 5000 V, 10°C. Left sample: from baby homozygous for $A_\gamma T$. Right sample: from normal new-born baby. (B) Same gradient as in (A), except that the gel was 20 cm long (0.01 pH unit/cm) and 6% Pharmalyte pH 5–8 was added. Focusing time: 6 h at 5000 V, 10°C. Unstained gels. The cathode is uppermost.

DISCUSSION

To our knowledge, this is the first report of the fractionation of native, intact G_γ and A_γ tetramers. The separation was the most difficult to achieve of any neutral haemoglobin mutant, as the ΔpI between the two phenotypes was barely 0.003 of a pH unit, *i.e.*, very close to the resolving limit of the immobilized pH gradient technique, presently $\Delta pI = 0.001$. This opens up the intriguing possibility that most, if not all, of the so-called "electrophoretically silent" mutations could be amenable to electrophoretic fractionation. Thus, immobilized pH gradients could represent a powerful probe for genetic analysis of human protein polymorphism, and the field of haemoglobin analysis could rapidly change. In a recent survey[17], 457 haemoglobin mutants were listed, of which only 15% are neutral substitutions, the remaining 85% involving charged amino acids. However, on the assumption that nucleotide substitutions in human genes occur at random, a preponderance of neutral mutants is expected, so that, in a population at equilibrium, *ca.* 70% of the variants should be neutral and 30% charged. If this is the case, there could be several hundred mutants still undetected in human populations.

The advantage of the present technique over HPLC analysis is that the sample

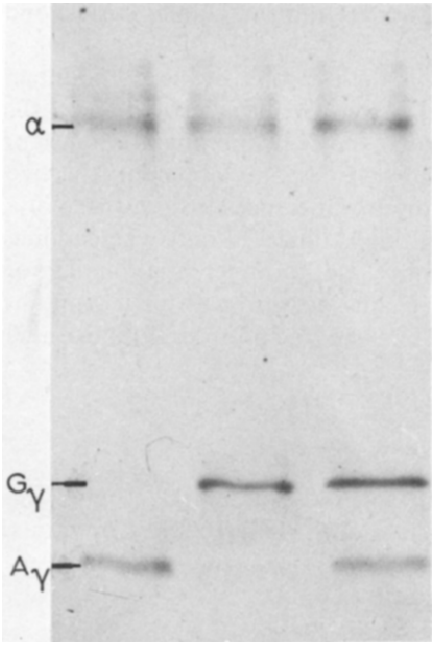

Fig. 3. Focusing of globin chains in urea–detergent gels. 5% T polyacrylamide gel, 0.7 mm, containing 2% Ampholine pH 6–8, 8 *M* urea and 2% Nonidet, P-40. The two zones of Fig. 2B (and a control HbF) were eluted and haem-free globin chains prepared (acid–acetone powder); *ca.* 50 μg sample were applied (anodically) per lane and focused for 4 h at 12°C, 1500 V at equilibrium. Coomassie Blue stain. Lanes: left, upper band (higher p*I*) of Fig. 2B; centre, lower band (lower p*I*) of Fig. 2B. The cathode is uppermost.

can be analysed intact, without any prior treatment, with a considerable saving in time and handling procedures. An extra bonus of any gel slab technique is the inherent large sample capacity: as many as 30–40 lysates can be analysed simultaneously side by side in a single slab, drastically reducing the costs of each analysis. Last, but not least, is the possibility of recovering the sample in a native form, which renders it amenable to additional physiological studies; work is in progress on the oxygen-binding curves of the two isolated tetramers. This simply cannot be achieved with previous techniques. In principle, conventional IEF could attain the same degree of resolving power as immobilized pH gradients, provided true "pH gradient engineering" could be arrived at, *i.e.*, flattening of any pH gradient or the creation of any new pH interval for specific separation purposes. Chen-Marotel *et al.*[18] were able, by subfractionating carrier ampholytes, to separate Hb F Sardinia from Hb F, both in the native state; the latter band, however, was still a mixture of A_γ and G_γ tetramers. However, owing to the progressive improverishment of the mixture of carrier ampholytes in the shallow pH gradient, distorted bands and poor separations are in general achieved (*cf.*, Fig. 1 in ref. 18 with Fig. 2A in the present report). In addition, it is curious that those authors could separate these two species in a gradient of 1.3 pH units when we barely achieved a separation in a gradient of 0.7–0.8 pH units (see our Fig. 1). Perhaps their separation was accidental, *e.g.*, due to a drift in pH gradient owing to the long focusing times (5 h) and high voltages applied (not

reported, but 16 W on a 0.5-mm gel, in a shallow pH gradient, should correspond to *ca.* 2000 V).

An intriguing aspect of the present work is the fact that, while in the intact foetal haemoglobins the A_γ has an higher p*I* than the G_γ tetramer (Fig. 2B), the denatured, haem-free chains focus in reverse order, the G_γ exhibiting an higher p*I* than the A_γ chain (Fig. 3). This discrepancy, however, is only apparent: the two chains, existing as random coils, do not show any p*I* differences and co-focus in a single band in the presence of 8 *M* urea. The splitting is observed only when adding 2% Nonidet, which we attribute to a "charge-shift", *i.e.*, to preferential binding of the detergent micelle to the more hydrophobic A_γ chains, possibly with a concomitant masking of the charge on the Lys-146 residue[8]. Thus, the true p*I* order is represented by the fractionation in Fig. 2B.

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