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IMMOBILIZED pH GRADIENTS AND REVERSED-PHASE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY: A STRATEGY FOR CHARACTERIZATION OF HAEMOGLOBIN VARIANTS WITH ELECTROPHORETIC MOBILITY IDENTICAL TO THAT OF Hb A

THE CASE OF Hb SAN DIEGO

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SUMMARY

The preparative aspects of immobilized pH gradients applied to abnormal haemoglobins (Hb) is described. As shown with the example of Hb San Diego, this method is successful even with as small a difference in pHi as 0.01 pH unit. For characterization of such neutral variants, reversed-phase high-performance liquid chromatography is demonstrated to be a very efficient tool.

INTRODUCTION

The characterization of haemoglobin (Hb) variants with electrophoretic mobilities identical with that of Hb A by conventional methods requires the analysis of all the peptides in a mixture of normal and abnormal chains. In some fortunate cases the amino acid replacement may involve a residue giving a specific staining on the fingerprint.

In this paper we describe the use of two recent separation techniques that could

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be of general suitability for this purpose: isoelectric focusing (IEF) in immobilized pH gradients (IPG) for the separation of the Hb components, and reversed-phase high-performance liquid chromatography (RP-HPLC) for the isolation of the abnormal peptides. We shall describe the use of these two techniques in the case of Hb San Diego.

In a 16-year-old boy of Tunisian origin, presenting with a polycythaemia (Hb 18.3 g/dl, RBC count $6.2 \cdot 10^{12}$/l, haematocrit 54.2%), the electrophoresis results for Hb, under standard conditions, were normal but a clear increase in the oxygen binding properties of the blood was found (P_{50} = 17 mmHg against 26 ± 1 mmHg in the control). On IEF and abnormal band migrating between Hb A and Hb A_{1c} was observed; these bands were, nevertheless, too close to each other to allow the recovery of pure fractions. The preparative fractionation of this electrophoretically "silent" variant was easily performed with IPG methodology.

EXPERIMENTAL

Blood was drawn in heparinized tubes and the lysate prepared according to routine procedures. Oxygen binding properties of the blood were studied using the Hemoscan (Aminco, Silver Spring, MD, U.S.A.).

IEF studies

Apparatus. IEF experiments were carried out using an LKB 2217 Ultrophor electrofocusing unit together with a constant power d.c. supply, Model 2103, and an LKB Multitemp thermostatic circulator (LKB, Bromma, Sweden). To generate immobilized pH gradients in 1–2 mm thick gels, a 2117-901 2-D and gradient kit was used, containing cassettes and gradient mixer. For the electrophoretic recovery of proteins from the Immobiline matrix, an LKB Multiphor chamber was used as described by Ek *et al.*[1].

Chemicals. Immobilines of p*K* 3.6, 6.2, 7.0 and 8.5 were obtained from LKB. Acrylamide, N,N'-methylenebisacrylamide (Bis), N,N,N',N'-tetramethylethylenediamine (TEMED) and ammonium persulphate were purchased from Bio-Rad Labs. (Richmond, CA, U.S.A.). Gel Bond PAG film was obtained from Marine Colloids (Rockland, ME, U.S.A.). Agarose A 37 and hydroxyapatite (HA) Ultrogel were supplied by IBF (Villeneuve La Garenne, France).

Casting of 1 pH unit wide gradients with the "tandem" principle. The dimensions of the gels were 125 × 110 mm with a thickness of 1 mm (for analytical purposes) or 2 mm (for small-scale preparative purposes), with a total acrylamide–Bis concentration (%*T**) of 5% for analytical and 3.3% for preparative gels. During the pouring of the solutions into the casette and the polymerization step, the pH gradient was stabilized by a co-linear density gradient of glycerol (0–20%).

The moulding cassette was assembled using as a cover a Plexiglass sheet coated with a thin layer of vaseline, to prevent gel sticking. On the opposite side, the gel was made to adhere to the hydrophilic surface of Gel Bond PAG. Acrylamide and Bis were used as mixed stock solutions with concentrations of 28.8 and 1.2% (w/v), respectively. For 1 mm thick gels the chambers of the gradient mixer were each filled

* %*T*: total concentration of monomers (acrylamide and Bis) in 100 ml solution.

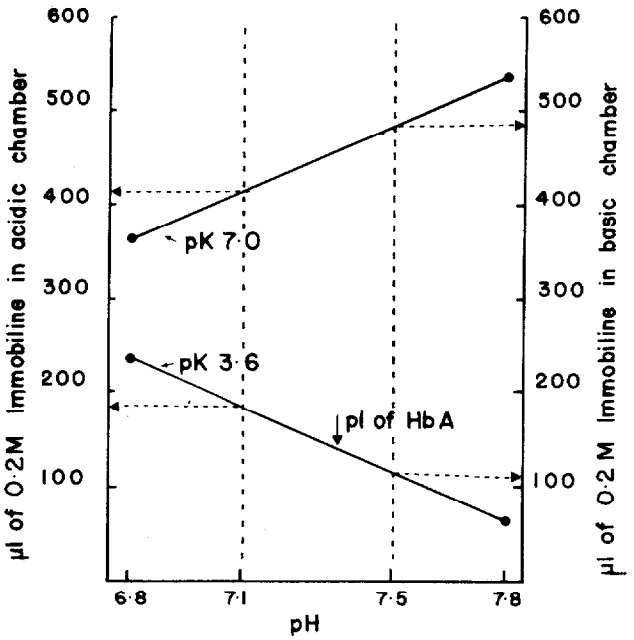


Fig. 1. Graphical representation of the preparation of narrow (up to 1 pH unit) IPG gradients on the "tandem" principle. The limiting molarities of p*K* 7.0 (buffering species) and p*K* 3.6 (titrant) Immobilines needed to generate a pH 6.8–7.8 interval are calculated with the aid of nomogram II in LKB Application Note No. 321. These points are joined by straight lines and the new molarities needed to generate any narrower pH gradient within the stated intervals are then obtained by simple linear interpolation (broken vertical and horizontal lines). In this example, a narrow pH 7.1–7.5 gradient is derived graphically.

with 8 ml of a solution containing acrylamide–Bis (to a final concentration of 5% *T*, 4% C*) and Immobilines of p*K* 7.0 (buffering species) and pH 3.6 (titrant) at concentrations calculated to give a pH gradient from 6.8 to 7.8 (this gradient has as its mid-point the p*I* of Hb A, which in Immobiline gradients, at 10°C, has been found to be *ca.* 7.3; therefore, it is ideal for separating mutants and species with p*I*s values in the neighbourhood of pH 7.3). The amount of Immobilines needed to generate this pH interval was calculated with the aid of nomogram II in LKB Application Note No. 321. For the 125 × 110 × 1 mm gel dimensions, in the acidic chamber 364 µl of 0.2 *M* Immobiline of p*K* 7.0 and 236 µl of 0.2 *M* Immobiline of p*K* 3.6 were used; for the basic chamber the corresponding amounts were 536 and 64 µl, respectively. Once the extremes of this pH interval have been calculated, any narrower pH range within the range 6.8–7.8 can be derived by a simple linear interpolation of intermediate Immobiline molarities. Fig. 1 gives a graphical representation of the method employed: for resolving Hb San Diego from Hb A it was found necessary to operate over a narrow pH interval of 0.4 pH unit (pH 7.1–7.5). The limit molarities of the two Immobilines in the 1 pH unit interval are joined by a straight line (because the pouring of the gradient from the two-chamber mixer is carried out linearly) and then the new pH interval is defined according to experimental needs (in

* %C: concentration of crosslinker per 100 g of *T*.

this instance, pH 7.1–7.5). Two lines are drawn from the two new limits of the pH interval, parallel to the ordinates (broken vertical lines). When they intersect the two sloping lines defining the two Immobiline molarities, four new lines (broken lines with arrow heads) are drawn parallel to the abscissa and the four new molarities of the two Immobilines defining the new pH interval are read directly on the ordinates. This process can be repeated for any desired pH interval, down to ranges as narrow as 0.1 pH unit. Within these limits (up to 1 pH unit) we prefer to work on a "tandem" principle, *i.e.*, with only one buffering and one non-buffering Immobiline.

The catalysts (8 μl of 40% ammonium persulphate and 6 μl of TEMED per chamber) were added directly to the gradient mixer immediately before filling the gel into the cassette. The gel solutions were not degassed as the gradient mixer was used with both chambers open. All the gels were polymerized for 1 h at 50°C[2] and then stored at 4°C for 30 min. After removing the gels from the cassette, the 1 mm thick gels were washed in 1 l of distilled water with continuous shaking, and the 2 mm thick matrices were washed twice under the same conditions. All the gels were then brought back to their initial weight by using a heater with a fan placed 1 m from the vertically standing gel.

Casting of 2 pH units wide gradients with multiple buffering species. For Hb analysis and for screening of unknown samples, it might often be necessary to use a wide pH 6–8 gradient, as is customarily done in conventional IEF with carrier ampholytes[3]. The problem, which had not found an immediate solution when the IPG technique was first described[4], has now been solved with the aid of computer programs developed by Dossi *et al.*[5] and Gianazza *et al.*[6]. The data, for generating

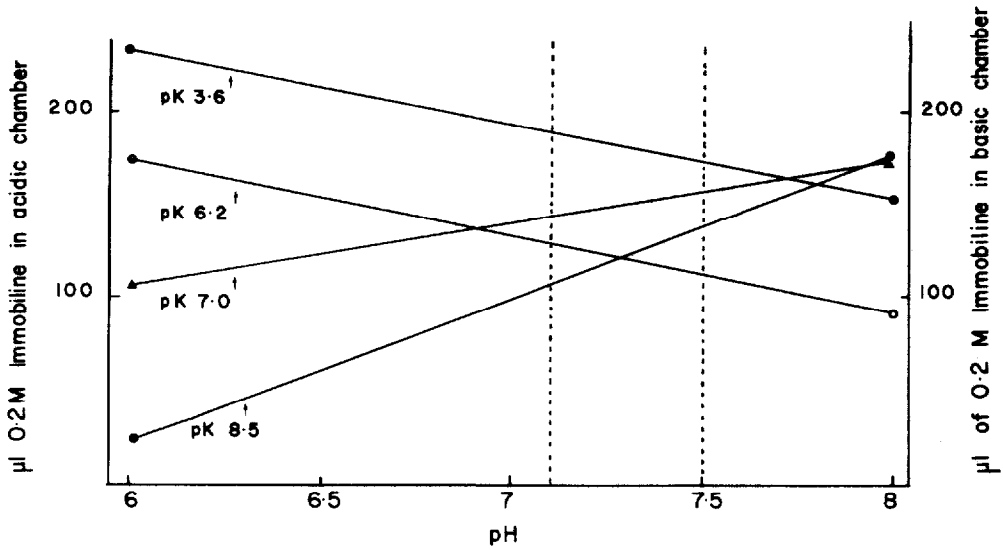


Fig. 2. Graphical representation of the preparation of wide (2 pH units) IPG with multiple buffering species. The limiting molarities of the three buffers (p*K* 6.2, 7.0 and 8.5) and of the titrant (p*K* 3.6) needed to generate a pH 6–8 interval have been tabulated by Righetti *et al.*[7]. By the same principle as described in Fig. 1, once these points have been connected by straight lines, any narrower pH gradient within these limits can be derived by simple linear interpolation (the two vertical lines would represent the same narrow pH 7.1–7.5 interval shown in Fig. 1). For clarity, the eight horizontal lines starting from the eight intercepts and going to the two ordinates, for calculating the new Immobiline molarities, have been omitted.

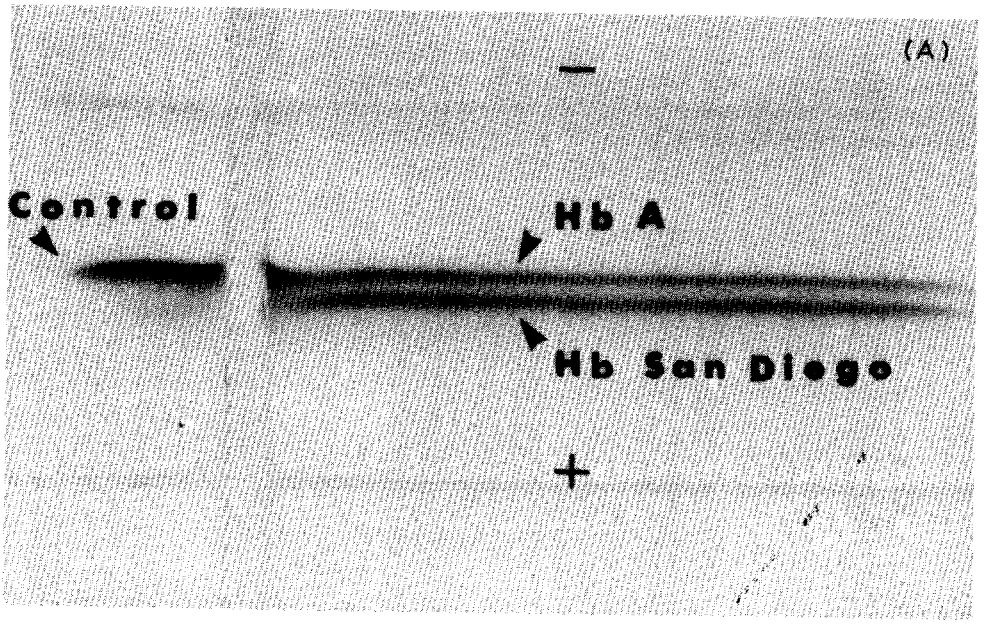
2 and 3 pH units wide gradients, have been tabulated by Righetti *et al.*[7] and in LKB Application Note No. 322. Fig. 2 shows graphically how to generate an Immobiline pH 6–8 gradient with the aid of three buffering species (p*K* 6.2, 7.0 and 8.5) and one titrant (pH 3.6). The rationale in choosing the relative molarity ratios of the three buffers is to try to keep the buffering power within the stated pH interval as constant as possible: this will automatically ensure minimum or no deviation from linearity of the generated pH gradient[6]. For 8 ml of solution in the acidic chamber (pH 6) the following volumes of buffers were used: 235 μl of p*K* 3.6, 177 μl of p*K* 6.2, 108 μl of p*K* 7.0 and 24 μl of p*K* 8.5 Immobilines; for the corresponding 8 ml of solution at the other extreme (pH 8), the volumes were 156 μl of p*K* 3.6, 95 μl of p*K* 6.2, 177 μl of p*K* 7.0 and 180 μl of p*K* 8.5 Immobilines. Again, by a linear interpolation of these limiting molarities, any narrower pH gradient within these two extremes can be derived graphically. In Fig. 2, the computation of the same narrower pH 7.1–7.5 gradient as described above is shown (broken vertical lines). Experiments performed in these two different types of narrow pH gradients have given identical results (data not shown), although it is much simpler, below a 1 pH unit interval, to work with the "tandem" approach. For all other experimental conditions the reader is referred to the previous section.

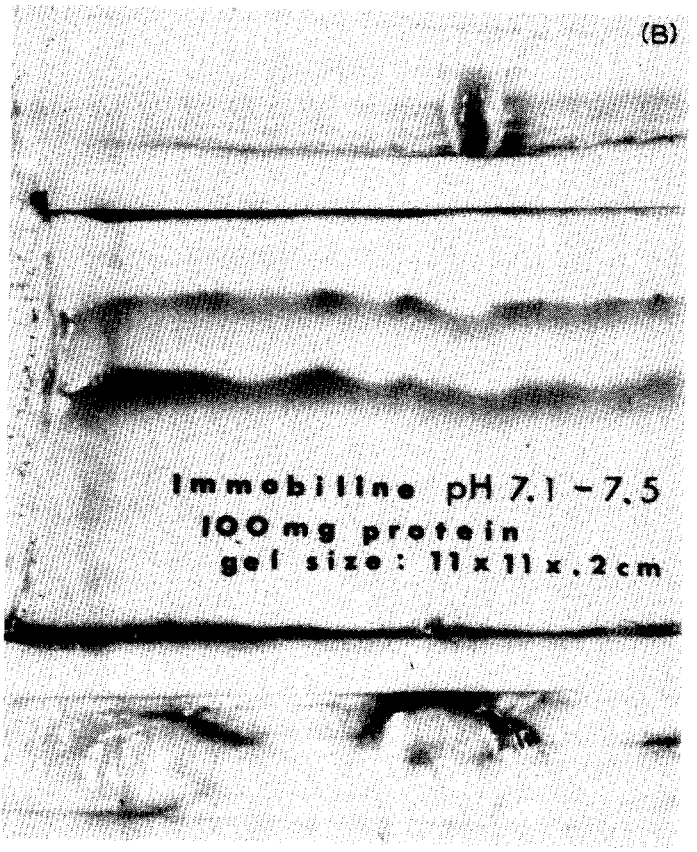
Protein recovery from the Immobiline matrix. For preparative Hb runs, a more dilute matrix (3.3% *T*) was used, as this allows a considerable increment of protein load per unit gel volume (60 mg of protein per ml of gel volume in a 3% *T* matrix compared with 40 mg in a corresponding 5% *T* matrix)[8]. At the end of the IPG run, the Immobiline gel strips containing the Hb bands of interest are cut out and embedded in 1% agarose A 37, in 100 m*M* Tris–Gly buffer (pH 9.1), gelled in an LKB 9000 0157 glass tray[1]. A 2 cm wide central layer of agarose is removed and the trench filled with a slurry of HA-Ultrogel. Protein recovery into the hydroxyapatite beds is performed by a 1-h electrophoretic run at 10 W (initial voltage *ca.* 440). Elution of Hb from the resin is performed by six washings (5 ml each) with 0.2 *M* phosphate buffer (pH 6.8)[1]. These purified Hb bands are then used for tryptic digestion and subsequent HPLC and amino acid analysis. The rationale for optimizing preparative IPG runs has been discussed by Righetti and Gelfi[8,9].

Structural determination

The structural abnormality was characterized as follows. The globin was prepared by the acid–acetone method and the polypeptidic chains were separated by CM-cellulose chromatography in 8 *M* urea according to Clegg *et al.*[10]. After aminoethylation[11], the chains were digested with trypsin as described by Schroeder *et al.*[12].

The peptides were separated by RP-HPLC on a Chromatem 800 machine (Touzart et Matignon, Vitry, France) equipped with two Altex pumps. About 1 mg of tryptic digest, dissolved in 10% acetic acid, was applied on an Aquapore RP-300 (Brownlee Labs., Santa Clara, CA, U.S.A.) analytical column protected with an RP-300 guard column. The elution was obtained using a gradient of acetonitrile (RS HPLC, Carlo Erba) in 0.05 *M* ammonium acetate (pH 6.0)[13] as shown in Fig. 4. The isocratic period (16% acetonitrile) was necessary to isolate the βT 12a peptide from the large peak corresponding to βT 2. The effluent of the column was monitored at 220 nm using a Shimazu UV detector. Fractions were collected every 0.7 min, dried


(A)
−
Control
Hb A
Hb San Diego
+


(B)
Immobiline pH 7.1 - 7.5
100 mg protein
gel size : 11 x 11 x .2 cm

and submitted to hydrolysis with 6 *N* hydrochloric acid containing 0.05% of β-mercaptoethanol for 22 h. The amino acid compositions of the peptides were determined on a Rank-Hilger J 180 analyser.

RESULTS

Hb separation

Fig. 3A shows the analytical separation in an IPG of 0.8 pH unit (pH 6.9–7.7) of the Hbs from the blood of a patient heterozygous for Hb San Diego (as latter assessed by RP-HPLC and amino acid analysis). The abnormal Hb was identified as the lower p*I* species by running, in the left-hand pocket, a normal human lysate as a control. As the distance between the two Hb bands (1.5 mm), although allowing for an unambiguous characterization of the two species, would not have permitted high protein loads for preparative purposes, the experiment was repeated in a considerably shallower pH gradient (pH 7.1–7.5). The gel for the preparative run was also made 2 mm thick and contained a reduced amount of matrix (3.3% *T*). With these modifications, it was possible to apply 100 mg of total protein, while still maintaining ample resolution between Hb A and Hb San Diego (Fig. 3B). The two major zones were cut along the band borderline with scissors (with the precaution of cutting the supporting plastic foil also for easy manipulation of the soft gel) and eluted electrophoretically into HA beads, as described under Experimental. The purified fractions were then digested with trypsin and subjected to HPLC analysis.

Characterization of the structural modification

When starting the study with a mixture of normal and abnormal Hbs, the two β-chains could not be distinguished from one another by cation-exchange chromatography. On the purified abnormal component the chain separation resulted, of course, in a normal elution pattern.

Without aminoethylation no difference could be observed when analyzing the tryptic digest by RP-HPLC. After aminoethylation in both the mixture of normal and abnormal β-chains and in the pure abnormal chain, an extra peak was found, eluting between βT 2 and βT 9 (Fig. 4). In a first set of experiments using a 1-h programme with a linear gradient between 5 and 30% of acetonitrile, this zone of the chromatogram was poorly resolved; in order to increase the resolution it was necessary to introduce an 8-min isocratic step at 16% acetonitrile. Under these experimental conditions, in the mixture of normal and abnormal subunits the peak

Fig. 3. (A) Separation of Hb A–Hb San Diego by IPG. The analytical gel was 125 × 110 mm, 1 mm thick, and contained 5% *T* and Immobilines of p*K* 7.0 and 3.6 in such ratios as to generate a 0.8 pH unit span (see Fig. 1) from pH 6.9 to 7.7. About 8 mg of total protein were loaded on the right-hand trench. In the left-hand pocket, 1.5 mg of Hb from a normal adult lysate were applied. The p*I* between Hb A and Hb San Diego was calculated to be 0.01 pH unit. (B) Separation of Hb A–Hb San Diego by small-scale preparative IPG. The gel was 125 × 110 mm, 2 mm thick, and contained 3.3% *T* and Immobilines of p*K* 7.0 and 3.6 calculated to give a pH 7.1–7.5 interval (see Fig. 1). 100 mg of total Hb (in a volume of *ca.* 1 ml) were loaded in a continuous trench on the anodic side. The run was overnight at 10°C, 2000 V and 800 μA. In order to bypass the conductivity dip in the trench, once the sample had moved toward the centre of the gel, the anodic filter-paper strip was moved on the cathodic edge of the pocket. Anolyte, 10 m*M* glutamic acid; catholyte; 10 m*M* sodium hydroxide solution.

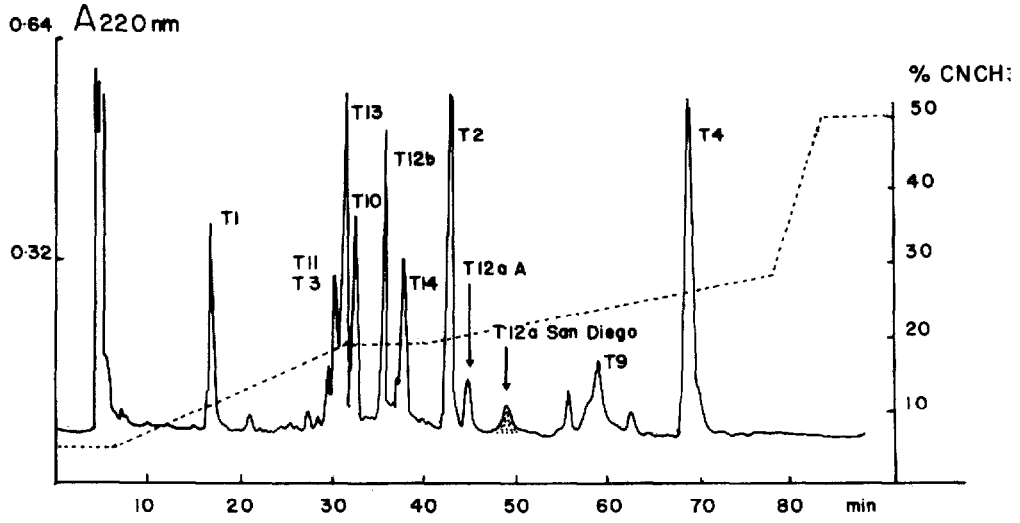


Fig. 4. Elution pattern of the tryptic digest of a mixture of aminoethylated normal and San Diego β-chains. Column, Aquapore RP-300; gradient, acetonitrile ($CNCH_3$) in ammonium acetate, 0.05 *M* (pH 6.0); flow-rate, 1.5 ml/min. A supplementary peak is observed between the normal T 12a (the size of which is halved in comparison with a normal sample) and T9. The amino acid analysis of this extra peak demonstrated that it was a T 12a in which a methionine was substituted for a valine. On the abnormal chain only the substituted T12a was present.

corresponding to the normal βT 12a was clearly separated from that of T2 and was found to be reduced in comparison with a normal tryptic digest.

The amino acid composition of the supplementary peak indicates that it was a βT 12a peptide in which a valine residue was replaced with a methionine. Normally, the βT 12a peptide contains two valine residues, one at position 105 and the other at 111. As it is known that these two residues are coded by distinct sequences at the DNA level[14], GTG and GTC, respectively, the only one that can lead to a methionine residue by a single base change is the former. This substitution has already been described as Hb San Diego[15], which displays a high oxygen affinity[16]. Moreover, the substitution of the second valine residue, found in Hb Peterborough[17], leads to decreased oxygen binding properties.

DISCUSSION

The novelty of our approach is not so much that of having separated for the first time Hb A from Hb San Diego (when first described[14], this mutant could not be separated from Hb A by any electrophoretic or chromatographic means), but of having combined the most resolving electrophoretic technique available (immobilized pH gradients) with one of the most powerful chromatographic techniques (RP-HPLC). The proper use of these two methods should allow the resolution, preparative fractionation and chemical analysis of any genetic mutant, no matter how minute the p*I* difference from the wild type.

As this combined approach is novel, we should like further to evaluate critically their respective experimental parameters.

Immobilized pH gradients

Conventional IEF with carrier ampholytes was virtually unable to resolve Hb A from Hb San Diego. Only when resorting to non-linear pH gradients, with the use of separators (in this particular instance, an equimolar mixture of 6-aminocaproic acid and β-alanine, as described by Cossu *et al.*[18], we were able to split the two species but, owing to the still low resolution and limited load capacity of conventional IEF, any attempts to scale up the separation were disastrous. It was only on resorting to IPG methodology that we could couple a high load capacity with the resolution of analytical gels. IPGs have in fact been demonstrated to have a load ability at least ten times higher than conventional IEF gels and to be compatible with concentrations, in the focused zone, of the order of 40 mg of protein per ml of gel[1,9]. Moreover, with the discovery of the high protein-carrying ability of highly diluted gels[8], this apparent upper limit has been increased to as much as 90 mg of protein per ml of gel, an extremely high protein concentration to be reached in a single zone by any electrophoretic or chromatographic technique. What is even more impressive is that these very large protein loads are fully compatible with the resolution found in analytical runs, thus permitting their scaling up in an easy and reproducible manner. For the analytical and preparative aspects of IPGs, the reader is referred to ref. 4 and refs. 1, 8 and 9, respectively.

RP-HPLC

In these electrophoretically "silent" Hb variants the abnormal peptide is not expected to be distinguished from the normal variant in a system based on the charge of the molecule. In the original description of Hb San Diego, both βT 12a peptides were found in the same spot of the fingerprint; the abnormal peptide could even not be detected by specific staining with methionine, as it also contains an AE-cysteine residue. The amino acid analysis of all the peptides was therefore required.

Although small, the change in polarity between a valine and a methionine was sufficient to increase the retention time of the abnormal peptide. An identical result has been recently found in another case of Hb San Diego[19].

In preliminary experiments the separation of the peptides was performed using the same conditions but with a 100 Å porosity column (μBondapak C18; Waters Assoc., Milford, MA, U.S.A.) instead of the macroporous Aquapore column. A better resolution of the short peptides (T7, T8, T6 and T15) was observed, the retention time of all the peptides was increased and, as a consequence, the peaks were much broader. Although the short peptides are eluted in the front of the column, the overall resolution was found to be much better when using the 300 Å porosity phase.

RP-HPLC may be of general interest for this category of haemoglobins. In our laboratory this method was successfully applied to the separation of the abnormal peptide T 14 of Hb Saint Jacques in which residue Ala β 140 is replaced by a Thr[20] and to the isolation of the abnormal β T3 in Hb Knossos[21], both variants being electrophoretically "silent". In these instances a 60-min gradient was developed; as illustrated with the example of Hb San Diego, the gradient can easily be modified in order to improve the separation of various peptides.

The peptides recovered by RP-HPLC are reasonably pure: starting with 1 mg of tryptic digest, approximately 20 nmol of peptides are obtained, which is a sufficient amount for sequencing when required.

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