Journal of Chromatography, 361 (1986) 223–229
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROM. 18 585

DETECTION OF ELECTROPHORETICALLY SILENT MUTATIONS BY IMMOBILIZED pH GRADIENTS

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(Received February 20th, 1986)

SUMMARY

The detection of neutral amino acid mutants, by isoelectric focusing in immobilized pH gradients (IPGs), is exemplified by the separation of hemoglobin (Hb) Beirut (126β Val → Ala) from Hb A in a shallow pH 7.2–7.6 IPG gradient with 2% Ampholine pH 6–8. The mechanism of these separations appears to involve minute alterations in the p*K* values of ionizable groups bordering the mutation site, which are in turn reflected in tiny alterations in the net surface charge, ΔpI. The ΔpI values are of the order of 0.01 to 0.001 pH units, outside the resolving limits of conventional isoelectric focusing, and correspond to changes of the order of 0.1–0.01 unit charge (a proton or an electron).

INTRODUCTION

In a recent survey[1], 457 hemoglobin (Hb) variants were listed. Of the 134 alpha chain mutants, only 11 bear neutral → neutral substitutions; in the case of beta chains, out of 229 variants only 59 are neutral. Of the delta chains, no neutral mutants are described amont the 15 reported and, in the case of gamma chains, only four neutral mutants out of a total of 39 have been detected. By averaging these data, it appears that barely 15% of all Hb mutants listed 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 would be expected so that, in a population at equilibrium, *ca.* 70% of the variants should be neutral and 30% charged. Thus, if the total number of charged Hb mutants is 383, there should be of the order of 893 neutral ones and close to a total of 1276 overall existing Hb phenotypes, rather than the value of 457 reported.

Most of these variants have been detected by conventional electrophoretic techniques, like cellulose acetate, starch, citrate–agar or polyacrylamide gel electro-

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phoresis, which would be generally unable to detect neutral mutants. Conventional isoelectric focusing (IEF) has substantially increased our ability to resolve mutants with similar p*I* values, but is still unable to separate neutral variants with minute p*I* values, lest than 0.01 pH units. We report here that, with immobilized pH gradients (IPGs), it is possible to resolve even these silent mutations, and most neutral variants, as long as the difference in surface charge of these variants corresponds to a Δp*I* not less than 0.001 pH unit, the present resolution limit of IPGs. This investigation was prompted by the finding, in an healthy adult male of Lebanese origin, of an abnormal Hb band in a shallow IPG gradient. While, upon high-performance liquid chromatographic (HPLC) analysis, this mutation was found to be already known (Hb Beirut)[2], this, and similar separations obtained with IPGs of other neutral Hb mutants, has allowed us to propose a mechanism for the IPG separation of otherwise "electrophoretically silent" variants.

MATERIALS AND METHODS

Materials

Acrylamide, N,N'-methylenebisacrylamide (Bis), ammonium persulphate and N,N,N',N'-tetramethylethylenediamine (TEMED) were from Bio-Rad Labs. (Richmond, CA, U.S.A.). β-alanine (β-Ala), 6-aminocaproic acid (6-ACA) and bromophenol blue were from BDH (Poole, U.K.). Ampholines pH 6–8, 7–9 and 3.5–10, Immobilines p*K* 3.6 and 7.0, Gel Bond PAG and Bind Silane were from LKB Produkter (Bromma, Sweden). The red blood cell collection and Hb preparation were as described[3].

Conventional IEF

For routine analysis, the procedure was as outlined[3]. Briefly, 12.5 cm × 12.5 cm, 0.5 mm thick, polyacrylamide gels were cast onto silanized glass plates. For silanization, the glass was dipped for 30 s in 0.2% Silane A-174 in anhydrous acetone and then left to dry in the air[4]. The sample was usually applied at the cathodic side, in a *ca.* 10-μl volume (corresponding to *ca.* 50 μg total protein load) in slots precast in the gel. The matrix concentration was 5% T and 4% C, 2% Ampholine pH 6–8 and 0.2% Ampholine pH 3.5–10. The anodic and cathodic solutions were 0.5 *M* orthophosphoric acid and 0.5 *M* sodium hydroxide, respectively. The conditions for a typical experiment were as follows: 15 min at 400 V, then the samples were loaded and the experiment continued for 90 min at 15 W (limiting voltage 1500 V, reached 20 min after sample application). An LKB 2117 Multiphor cell with a constant wattage power supply (LKB 2103) was employed, with the thermostat at 4°C. For IEF in the presence of separators (an equimolar mixture of 0.2 *M* β-Ala and 6-ACA), see ref. 5.

Immobilized pH gradients

The basic, step-by-step methodology was as detailed[6]. A 0.7 mm thick gel was cast on the hydrophilic side of Gel Bond PAG, and was made to contain 4% T and 4% C, since diluted gels had been found to produce sharper bands due to increased charge density along the polymer coil[7]. The levels of the buffering ion (Immobiline p*K* 7.0) were chosen so as to obtain an average concentration of 10 m*M* in the gel,

corresponding to an average buffering power, β, of 5 mequiv. l^{-1} pH^{-1} and to an average ionic strength, μ, of 4.5 mequiv. l^{-1}. The chambers of the gradient mixer were each filled with 8 ml of a solution containing acrylamide–bis (4% T, 4% C) and Immobilines of p*K* 7.0 (buffering species) and p*K* 3.6 (titrant) at concentrations calculated to give a pH gradient from pH 7.2 to 7.6. The gel was obtained by a linear gradient of a dense, acidic solution containing 418 μl of Immobiline p*K* 7.0 and 175 μl of Immobiline p*K* 3.6 (0.2 *M* stock solutions, in a total of 8 ml of gelling mixture) and of a light, basic solution containing 471 μl of Immobiline p*K* 7.0 and 101 μl of Immobiline p*K* 3.6. The catalysts (8 μl of 40% ammonium persulphate and 6 μl of TEMED per chamber) were added directly to the gradient mixer immediately before packing the gel into the cassette. All the gels were polymerized for 1 h at 50°C[8]; after their removal from the cassette, they were washed in 1 l of distilled water for 1 h with continuous shaking. The gels were then dried and reswollen to their initial weight in a 2% Ampholine pH 6–8 solution[9,10]. For the experiments, the gels were thermostatted at 10°C and were overlaid with an anodic strip containing 10 m*M* glutamic acid and a cathodic strip containing 10 m*M* lysine. Conditions: 60 min at 20 W (starting values 1333 V and 15 mA) with an LKB Macrodrive power supply; after sample application (at the anodic side), the separation was continued for 300 min at 5000 V (maximum power 5-W and limiting amperage 5 mA). Total amount of sample applied per track: 200 μg in 20 μl.

RESULTS

When analyzing the unknown Hb mutation (which later turned out to be Hb Beirut) by conventional IEF in carrier ampholytes, even in non-linear pH gradients (in the presence of separators)[11] no separation was obtained between Hb A and Hb X (Fig. 1). As a control, a diabetic patient, having 15% glycosylated hemoglobin (Hb A1c), was used (Fig. 1, left track), in order to test the separation capability of

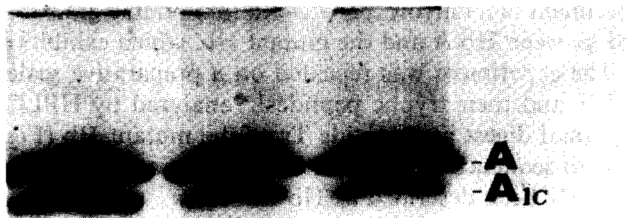

Fig. 1. IEF of Hb A and Hb X in a 5% T, 4% C polyacrylamide gel containing 2% Ampholine pH 6–8 and an equimolar (0.2 *M*) mixture of β-Ala and 6-ACA. Left: control lysate from a diabetic patent. Centre: 1:1 mixture of Hb A and Hb X. Right: lysate containing Hb X. Conditions: 15 min at 400 V, sample application at the cathode (50 μg total protein per track), followed by 90 min at 15 W (limiting voltage 1500 V). The gel was stained with 0.1% bromophenol blue in 50% methanol and 10% acetic acid.

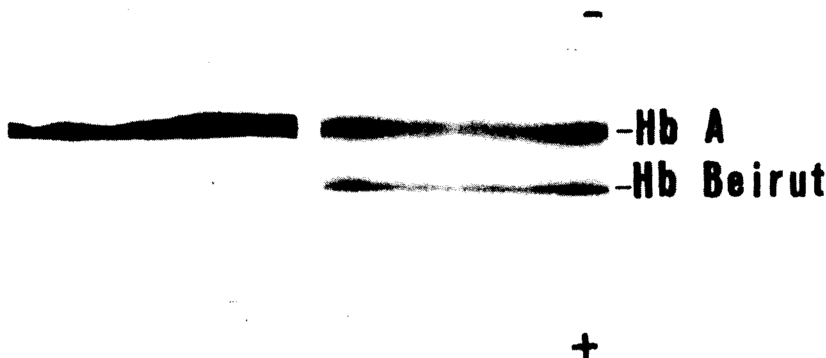

Fig. 2. Separation of Hb A and Hb X in an immobilized pH gradient. A 4% T, 4% C matrix was prepared, containing an IPG pH 7.2–7.6 interval. After washing and drying, the matrix was reswollen in 2% Ampholine pH 6–8. Left: control sample from a diabetic patient. Right: lysate of Hb X. Due to the very shallow gradient utilized, the glycosylated components (Hb A1c or Hb X1c) are lost at the anodic gel extreme. Conditions: 60 min at 20 W (starting values 1333 V and 15 mA); after anodic sample application (total protein 200 μg) the separation was continued for 300 min at 5000 V (maximum power 5 W and limiting amperage 5 mA). The gel was not stained.

the pH 6–8 gradient added with 0.2 *M* β-Ala and 6-ACA (which essentially transform a 2- into a 0.7-pH unit span). The central lane contains a 1:1 mixture of Hb A and Hb X, while the right track has been loaded with pure Hb X. Compared with the two side lanes, the central sample shows a thickening of the main band, suggesting that it might be an envelope of more than one compdnent, but no hint of a separation.

When repeating this experiment in a narrow, pH 7.2–7.6 Immobiline gradient, ample separation was obtained between Hb A and the mutant Hb, which exhibits a lower p*I* than Hb A (Fig. 2). The experiment was repeated on a preparative scale, pure β chains were prepared[12,13] and their tryptic peptides[14] analyzed by HPLC. Peptides T13 and T14 of the normal digest and peptide Tx of the mutant Hb (Fig. 3) were recovered and subjected to sequence analysis. The result was a 126β Val → Ala mutation, which had previously been described as Hb Beirut.

DISCUSSION

We have recently started an extensive investigation on the resolution of neutral Hb mutants by IEF in IPGs: it appears that all the "unseparable" neutral → neutral variants are in fact fully separated by IPGs; this was the case for Hb San Diego[15], Hb F Sardinia[11], Hb Beirut (present investigation), Hb Hamilton and the two phenotypes common in newborns, Hb F-Gγ and Hb F-Aγ[16]. Here we propose a likely mechanism for these separations which, by involving non-charged amino acids, should normally be the province of HPLC rather than of electrophoretic techniques. There has to be a "transducing element" which converts an hydrophobic (non-polar

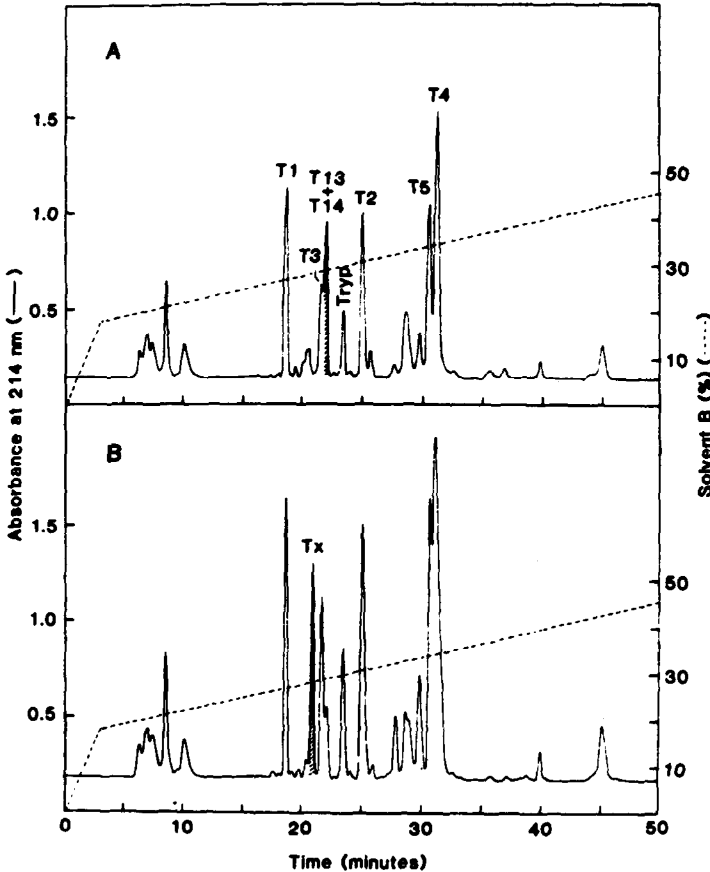

Fig. 3. Reversed-phase HPLC separation of tryptic peptides of β-A and β-X. The normal and variant tetramers (each 20 mg) were isolated from a preparative IPG experiment. Globin chains were prepared and β-A and β-X obtained by preparative IEF of globin chains[12]. The purified β-A and β-X globins (20 and 25 nmol, respectively) were lyophilized and digested with trypsin (TPCK, Miles) for 20 h. Tryptic peptides were separated by reversed-phase HPLC on a μBondapak C_{18} column operated at room temperature. The column was equilibrated in acetonitrile–water (40:60) to which 0.2% (w/v) trifluoroacetic acid (TFA) was added. Elution of peptides was achieved with a linear gradient of solvent A (acetonitrile–water, 60:40, with 0.05% TFA) as follows: 0–18% A in 3 min, then to 46% A in 47 min, at a flow-rate of 0.6 ml/min. The labelled peaks designate tryptic peptides from the β chain of Hb. These fractions were collected and identified by amino acid analysis. Sequence data on T13 + T14 and on the Tx peaks demonstrated a 126 β Val → Ala mutation, typical of Hb Beirut. (A) Tryptic peptides from 20 nmol of β-A; Tryp = trypsin. (B) Tryptic peptides from 25 nmol of β-X.

or non-charged) signal into an electric signal, which is reflected as a feeble change in the net charge at the surface of the polymer. This "transformer" can only be another amino acid, with an ionizable side group, bordering the mutation site, which senses the hydrophobicity change brought about by the mutation, and changes its p*K* accordingly. If this hypothesis is correct, it is not true that a neutral → neutral amino acid substitution is "electrophoretically silent" (in the sense that it does not alter the protein mobility in an electric field): it does indeed alter the net surface charge of the

native macromolecule, but to such a minute extent as to be generally undetected by conventional electrophoretic techniques and often even by more sophisticated ones, like conventional IEF. These changes are in general quite subtle and produce p*I* changes generally well below 0.01 pH unit, taken as the resolution limit of conventional IEF[17]; that is why quite a few of them have escaped even IEF fractionation. In fact, by IEF, we have never been able to resolve fetal Hb (Hb F) from umbilical cord blood into its two naturally occurring constituents, the Gγ and Aγ tetramers, the former containing glycine, the latter alanine in position 136 of the gamma chains[10]. Obviously the Δp*I* between the two species must be well below the resolving limit of Δp*I* = 0.01 pH units. Needless to say, if our hypothesis is correct, once the tetramer is denatured and disaggregated into its constituent chains, even this minute Δp*I* will completely disappear, as the chains will now be random coils in an 8 *M* urea solution and no p*K* changes can be expected in ionizable side groups as a result of the mutation. In fact, when analyzing heme-free, denatured globin chains by plain IEF in 8 *M* urea, we have never been able to resolve the Gγ and Aγ polypeptides. The spectacular separation we have reported[10,12,18] is due to a new phenomenon, a sort of affinity or charge-shift isoelectric focusing: when adding to the 8 *M* urea, IEF gels a neutral detergent (Nonidet P-40, NP-40), the latter seems to bind preferentially to the Aγ mutant, probably along the hydrophobic stretch of the mutation, from Met-133 to Leu-141, thus masking the residue Lys-142 and producing a charge shift (loss of one proton unit) in this phenotype (in fact Gγ exhibits a p*I* of 6.95, *cf.* p*I* = 6.85 for Aγ in a 1% NP-40 solution; Δp*I* = 0.1 pH units corresponds, in the case of Hbs, to a change in surface charge of 1 proton unit)[19].

There is another general conclusion we can draw from our results. With a resolution of Δp*I* = 0.001 pH units[20], it should be possible, in principle, to separate 1000 proteins in a 1 pH-unit span. Given the fact that we can now utilize pH 3–11 IPGs (over 8 pH units), if the proteins had p*I* values evenly distributed along the pH axis, we should be able to separate 8000 protein bands. In fact, this is not the case[21], as 60% of all possible phenotypes focus in the range pH 4–7; thus, this capability is reduced to about 4000 bands over the entire pH axis. Eukariotic cells are thought to be able to express 40 000–60 000 different polypeptide chains[22]. However, in practice, at any given time no more than 4000 spots are detected in two-dimensional maps[23], suggesting that, during normal activity, the cells express only 10% of their potential gene products (the other alternative being that many more are expressed, but in such low amounts as to be undetectable even by highly sensitive staining techniques, such as silver stain or autoradiography). If this is so, when working in ultra-shallow IPG gradients (0.1 pH-unit spans), with a resolution of Δp*I* = 0.001, a band focusing in a given p*I* position is likely to be truly homogeneous, *i.e.* uncontaminated by a different protein band co-focusing in the same position.

CONCLUSIONS

Neutral amino acid variants, once the realm of HPLC, are now amenable to detection by electrophoretic techniques, in particular by high-resolution probes such as immobilized pH gradients. The strategy of analysis, though, will be completely different: while in HPLC the protein mutant has to be denatured, so as to separate the protomers, in case of a quaternary structure, and expose the mutated site to the

solvent, in IPGs the macromolecule has to be analyzed in its native form, so as to preserve the minute alteration in surface charge, ΔpI, induced by the "transducer", *i.e.*, by the charged amino acid altering its pK in proximity of the mutation. The advantage of working with native proteins, together with the large sample-processing ability (at least 30 samples per gel slab), renders IPGs an attractive alternative to HPLC analysis.

ACKNOWLEDGEMENTS

This work was supported by grants from Regione Sardegna (G.C.), by Ministeri della Sanita' e della Pubblica Istruzione (Rome, P.G.R.) and by National Institutes of Health grant Hl-16008 (S.M.H.).

NOTE ADDED IN PROOF

The manuscript might give the false impression that we found a new family carrying the Beirut mutation. In fact, the variant was found in a member of the same family reported in ref. 2.

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