Journal of Chromatography, 369 (1986) 143–149
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROM. 18 917

ISOLATION OF HUMAN HAEMOGLOBIN VARIANTS WITH ALTERED BOHR EFFECT

APPLICATION TO HAEMOGLOBIN RAINIER

J. ROCHETTE*
Laboratoire de Biochimie Genetique et INSERM U 15, CHU Cochin, 24 rue du Faubourg Saint Jacques, 75014 Paris (France)
and
V. BAUDIN, B. BOHN, C. POYART and H. WAJCMAN
INSERM U 299, 42 rue Desbassayns de Richemont, 92150 Suresnes (France)
(Received June 24th, 1986)

SUMMARY

Isoelectric focusing on polyacrylamide gel in the absence of haem ligands represents a useful, convenient and rapid procedure to isolate silent Hb variants in their native forms, provided that they exhibit an abnormal Bohr effect. The amount of material which is eluted is sufficient for both a limited functional study and a structural determination using microscale high-performance liquid chromatography. This is exemplified by the isolation and the study of Hb Rainier.

INTRODUCTION

Almost 500 human haemoglobin (Hb) variants have been described[1] but the isolation of those with electrophoretic mobility identical to that of HbA remains technically difficult.

The probability of detecting these neutral Hb variants is very low when they are not associated with haematological disorders. Abnormal Hbs are mainly discovered by systematic electrophoretic screening of blood samples during population studies, and neutral substitutions are therefore not detected. The discovery of these neutral substitutions may be of interest for genetic reasons, as is the case for HbF Sardinia which is linked to specific molecular forms of β thalassemia[2]. In populations in which other Hb disorders such as sickle cell anaemia are common, it may be also important to know whether some silent variant is interacting with the more frequent ones modifying the course of the disease.

By contrast, in patients suffering from haemolytic anaemia, erythrocytosis or cyanosis, a haemoglobin abnormality is carefully looked for, even if the standard electrophoretic study exhibits a normal pattern. Additional electrophoretic methods are necessary, easy ones such as citrate agar gel electrophoresis, or more discrimi-

0021-9673/86/$03.50

native ones, such as isoelectric focusing (IEF) on polyacrylamide gels[3] or, even, immobilized pH gradient IEF[4]. When the abnormal component is separated from the normal one by any of these techniques, this difference in electrophoretic behaviour, small as it is, can form the basis of a preparative separation of the mutant Hb[5].

Other abnormal properties, like a decrease in stability or solubility, may reveal a neutral Hb variant. These properties may be used for purifying the variant. However, this results in the isolation of a non-functional Hb.

In some cases the abnormality is demonstrated through a study of the lysate by reversed-phase high-performance liquid chromatography (RP-HPLC). Normal and abnormal subunits will be observed in the elution pattern[6]. Nevertheless, the separation of the tetrameric native form cannot be achieved by this method.

Oxygen equilibrium curves for red blood cells may also indicate the presence of a variant Hb. An alteration in the oxygen binding properties, when it modifies the exposure of the Cys β 93 SH group, has been used for isolating Hb Creteil[7] after selective binding of cystamine.

The isoelectric point differs between the two quaternary conformations of the Hb molecule in accord with the alkaline Bohr effect[8]. Therefore when an electrophoretically silent Hb variant displays an increased oxygen affinity and a decreased Bohr effect, its purification by IEF in the deoxy form is expected.

In this paper we demonstrate the use of this property for isolating a variant having high oxygen affinity, Hb Rainier, α_2 β_2 145 (HC2) Tyr → Cys[9]. Another variant possessing high oxygen affinity, but with a normal Bohr effect, Hb San Diego, α_2 β_2 109 (G11) Val → Met, the preparation of which by immobilized pH gradient IEF we have described previously[5], could not be distinguished by this procedure.

MATERIALS AND METHODS

Haematological studies

Blood was collected on EDTA. Haematological studies were performed according to standard procedures.

2,3-Diphosphoglycerate (2,3-DPG) in the red blood cells (RBCs) was measured by the phosphoglycerate method (Boehringer Kit, Mannheim, F.R.G.).

Haemoglobin studies and analytical electrophoreses

RBCs were lysed with water, extracted with toluene and centrifuged at 12000 *g* for 45 min.

Standard electrophoreses were performed at pH 8.6 in Tris–EDTA–borate buffer on cellulose acetate plates (Helena Labs., Beaumont, TX, U.S.A.). Citrate agar gel electrophoreses were done at pH 6.3 (Corning, Palo Alto, CA, U.S.A.).

Isoelectric focusing studies were carried out on a 0.2-mm polyacrylamide gel containing a mixture of Ampholines pH 3.5–10, 6–8, 7–9 (LKB, Bromma, Sweden) and of separators (6-aminocaproic acid and alanine) as described by Cossu *et al.*[10].

The alkali denaturation was tested with 0.083 *M* sodium hydroxide as described by Singer *et al.*[11]. A kinetic study was performed on the patient's haemolysate in comparison with a normal one.

Preparative IEF in the deoxy form

The Hb solutions were deoxygenated in a glass tonometer under pure

argon until the ratio of absorbances at 554.5 and 540 nm was at least equal to 1.25. To complete the deoxygenation of the high affinity variant, a two-fold molar excess of dithionite was added. IEF was performed in cylinders of polyacrylamide gel using the technique of Bunn[12] as modified by Poyart *et al.*[8]. To each tube, 40–50 μl (corresponding to about 200–250 μg Hb) were applied. At the end of the experiment the bands were cut and the Hb was eluted from the gel in 50 m*M* bis(2-hydroxyethyl)-iminotris(hydroxymethyl)methane (bis-tris), pH 7.2 buffer.

Functional studies

Oxygen equilibrium curves for red blood cell suspensions were determined with an Hemox-Analyzer (T.C.S., Southampton, PA, U.S.A.) as described[13]. For the isolated abnormal component, such curves were obtained by the discontinuous equilibrium technique[14] using a Cary 219 spectrophotometer, in 0.1 *M* sodium chloride, 50 m*M* bis-tris, pH 7.2 buffer at 25°C. The amount of metHb present in the sample was measured from the ratio of absorbances at 576 and 500 nm under an atmosphere of pure oxygen.

The Bohr effect was estimated by measuring the difference in isoelectric points between the deoxy form and the fully liganded carbonyl form.

Structural characterization

The pure Hb fractions isolated as described above were concentrated under vacuum. The subunits were separated by RP-HPLC on an Aquapore RP 300 column (C_8, porosity 300 Å). A 1-mg amount of globin was chromatographed using a gradient of acetonitrile–trifluoroacetic acid–methanol as described by Baudin and Wajcman[15]. The fractions were collected manually and dried. The chains were then hydrolyzed with trypsin.

The tryptic digest (0.2–0.5 mg) was dissolved in 10% acetic acid and applied on a RP-HPLC column. In order to obtain a good resolution of all the peptides, including the small ones, a Microbondapak column (C_{18}, porosity 100 Å; Waters Assoc.) was selected and the elution performed as described previously[16].

In parallel, a structural study was undertaken using more conventional methods including globin precipitation with acid acetone, subunit separation by CM-cellulose chromatography in 8 *M* urea, aminoethylation and peptide analysis as described[17].

The amino acid composition of the peptides was determined by HPLC according to a modification of the procedure described by Heinrikson and Meredith[18]. The retention time of phenylthiocarbamoyl(aminoethyl)cysteine (PTC-AECys) was determined by the analysis of normal Cys-containing peptides after aminoethylation. PTC-AECys is slightly more strongly retained than PTC-Lys.

RESULTS

Case report

The patient, 56 years old, was referred to the hospital as exhibiting polycythaemia. The haematological parameters were as follows: RBC, $6.46 \cdot 10^{12}\ l^{-1}$; Hb, 20.9 g/dl; haemocrit, 0.59. The leukocyte and platelet counts were normal. This polycythaemia had been found in several other members of the patient's family. The

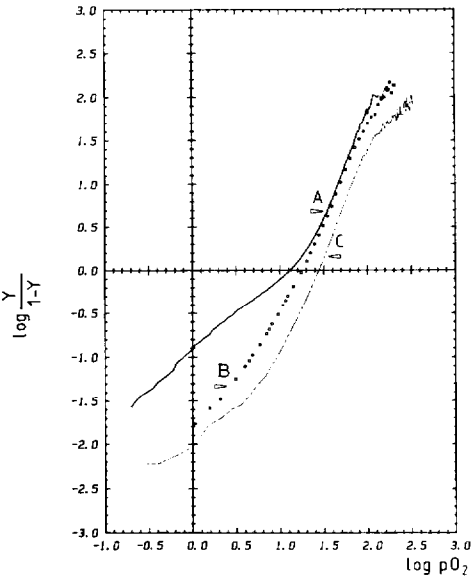

Fig. 1. Oxygen equilibrium curves obtained for red blood cell suspensions. Experimental conditions: 50 m*M* bis-tris, 140 m*M* sodium chloride 10 m*M* glucose, pH 7.4, 37°C. (A) Heterozygote HbA/Hb Rainier; (B) heterozygote HbA/Hb San Diego; (C) normal control. Y represents the fractional saturation of haemoglobin with oxygen.

erythrocytic 2,3-DPG level was normal (0.9 mmol per Hb tetramer, normally 0.85 ± 0.05). The oxygen affinity of red blood cell suspensions was increased two-fold [P50 (the partial pressure of oxygen at half saturation in mmHg) = 13 mmHg, normally = 26 ± 1 mmHg at pH 7.4 and 37°C]. These facts argued for the presence of an abnormal haemoglobin.

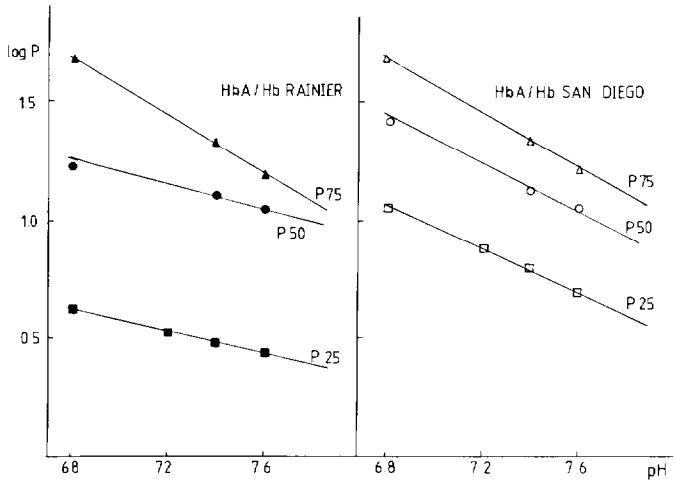

Fig. 2. Variation of log pO_2 with pH at different oxygen saturation levels. A decreased alkaline Bohr effect is observed in the bottom of the oxygen equilibrium curve of the HbA/Hb Rainier red blood cell suspension.

Haemoglobin studies

(a) Electrophoreses. No abnormal component nor HbF could be detected by alkaline pH electrophoresis of the lysate and the stability of the lysate was found to be normal. Citrate agar electrophoresis showed an abnormal Hb migrating like HbF. The resistance to alkali denaturation was increased (9.6% in 2 min, *versus* 0.8% in the control), suggesting the presence of Hb Rainier.

Standard IEF of the propositus haemolysate showed no abnormal band.

(b) Blood oxygen binding properties. Fig. 1 illustrates the oxygen binding curve for the propositus red blood cell suspension compared to that of a patient heterozygous for Hb San Diego and to that of a normal individual. For both abnormal red blood cells the oxygen affinity was increased. A biphasic Hill plot and a lower cooperativity at P50 were found in the red cells of the propositus.

The variation of log pO_2 with pH, at different oxygen saturation levels, showed a decreased alkaline Bohr effect in the bottom portion of the oxygen equilibrium curve. By contrast, a constant and normal value was observed for the red cells of the patient heterozygous for Hb San Diego (Fig. 2).

On this basis the separation of the abnormal fraction was attempted by isoelectric focusing in the deoxy form of the haemolysate.

IEF of the deoxy form (Fig. 3)

The IEF pattern of the deoxygenated lysate demonstrated the presence of a component with a slightly lower isoelectric point than deoxyHbA (7.04 *versus* 7.12 for the control) and a content of approximately 30%. The difference in isoelectric point (0.09 against 0.17 pH units for the normal case) suggests a 40% reduction in the alkaline bohr effect.

When the lysate was fully liganded with CO, only one band could be seen with a p*I* of 6.95.

Hb San Diego, which exhibits a normal Bohr effect[19], showed a similar difference in isoelectric points, between the deoxy and the liganded forms as compared to HbA.

From eleven tubes, 1 mg of abnormal component was recovered.

Oxygen binding properties of the isolated fractions

Only one experiment could be performed due to the limited amount of Hb recovered from the IEF gels.

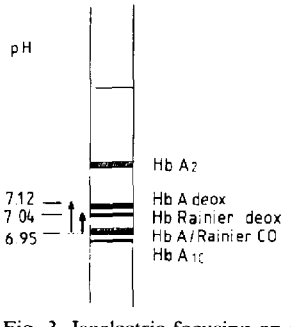

Fig. 3. Isoelectric focusing on polyacrylamide gel. The lysate was applied both in the deoxy and in the carbonyl form; the decrease in ΔpI upon deoxygenation of Hb Rainier is clear (0.09 instead of 0.17 pH units).

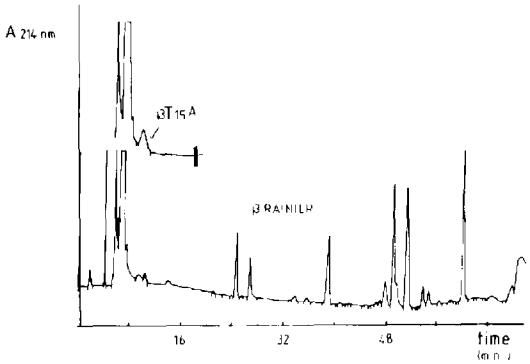

Fig. 4. RP-HPLC analysis of tryptic peptides. In the β chain of the isolated abnormal component, the peptide T15 is missing.

The visible spectrum of the oxygenated abnormal component was identical to that of HbA.

The P50 was 0.2 mmHg and the n50 (the slope of the Hill plot at half ligand salination) = 0.8 (0.1 *M* sodium chloride, 50 m*M* bis-tris buffer at 25°C and pH 7.2, 20 μg/ml catalase, 60 μM haem). Under identical experimental conditions, the oxygen binding parameters for HbA, eluted from the gels, were P50 = 3 mmHg and n50 = 2.4. In both samples, 6–8% metHb was present at the end of the experiments.

The abnormal functional properties for Hb Rainier have been previously studied in detail by Imai[20]. The values of P50, haem haem interaction and Bohr effect reported in the present study correspond closely to those given by this author.

Structural determination

The abnormal subunit could not be isolated from the normal one by CM-cellulose chromatography in 8 *M* urea, and therefore a preliminary study was performed on a mixture containing the normal and abnormal β chains. After aminoethylation and digestion with trypsin, the RP-HPLC elution pattern on an Aquapore RP 300 column showed no abnormal peptide.

In agreement with the hypothesis of Hb Rainier heterozygoty, a peak containing only His and AE-Cys and another one corresponding to normal T15 were found in the front of the chromatogram using a Microbondapak column.

A study of the IEF-purified haemoglobin components showed no difference in the RP-HPLC elution pattern of the subunits between the normal and abnormal haemoglobins. After digestion with trypsin the peptides were analyzed by HPLC (Fig. 4). The β T15 peptide was missing from the abnormal sample, identifying the variant as Hb Rainier.

DISCUSSION

In many cases the size or charge properties of a protein do not allow its isolation from a mixture. Therefore separation methods based on biological properties have to be used. We can distinguish the purification of a protein either by true biospecific affinity, by non-biospecific affinity methods or by conformational specificities.

In the first case, affinity chromatography methods are widely used; a ligand which may be a substrate, a cofactor, a product or a stereospecific receptor is bound to the matrix. Immunoreactive properties may also be used. In the second case the ligand is a synthetic or a natural molecule displaying a less specific interaction; examples have been reported of the use of hydrophobic chromatography, boronate chromatography or agar gel electrophoreis for haemoglobin separation[21].

In this paper we have demonstrated that Hb molecules can be separated on the basis of their oxygen binding properties. It is known that there is a difference in the surface charges between the oxy and the deoxy forms. In variants with altered Bohr effects this difference will be reduced. Isoelectric focusing, being one of the most sensitive methods for separating Hbs on both analytical and preparative scales, can be applied to this problem.

ACKNOWLEDGEMENTS

We thank J. Kister for his valuable technical assistance, J. Delaunay for fruitful discussion and A. Najman for providing the blood sample of the patient. This work was made possible through financial support from the INSERM and from the Fondation pour La Recherche Médicale.

REFERENCES

1 International Hemoglobin Information Center, *Hemoglobin*, 10 (1986) 259–351.
2 C. Belfjord, M. Arbane, C. Lapumeroulie, Ph. Rouyer-Fessard, M. Benabadji, D. Labie and Y. Beuzard, *Mol. Biol. Med.*, 2 (1984) 301–306.
3 J. W. Drysdale, P. G. Righetti and H. F. Bunn, *Biochim. Biophys. Acta*, 229 (1971) 42–50.
4 B. Bjellqvist, P. G. Righetti, E. Gianazza, A. Görg, R. Westermeier and W. Postel, *J. Biochem. Biophys. Methods.*, 6 (1982) 317–339.
5 J. Rochette, P. G. Righetti, A. Bianchi-Bosisio, F. Vertongen, G. Schneck, J. P. Bossel, D. Labie and H. Wajcman, *J. Chromatogr.*, 285 (1984) 143–152.
6 L. Leone, M. Monteleone, V. Gabutti and C. Amione, *J. Chromatogr.*, 321 (1985) 407–419.
7 M. C. Garel, M. Cohen-Solal, Y. Blouquit and J. Rosa, *FEBS Lett.*, 43 (1974) 93–96.
8 C. Poyaart, P. Guenon and B. Bohn, *Biochem. J.*, 195 (1981) 493–501.
9 J. Greer and M. F. Perutz, *Nature New Biol.*, 230 (1971) 261–263.
10 G. Cossu, M. Manca, P. M. Galvina, R. Bullita, A. Bianchi-Bosisio, E. Gianazza and P. G. Righetti, *Am. J. Hematol.*, 13 (1982) 149–157.
11 K. Singer, A. I. Chernoff and L. Singer, *Blood*, 6 (1951) 413–415.
12 H. F. Buhn, in N. Catsimpoolas and J. Drysdale (Editors), *Biological and biomedical Appliocation of Isoelectric Focusing*, Plenum, New York, London, 1977, pp. 29–55.
13 J. Rochette, C. Poyart, B. Varet and H. Wajcman, *FEBS Lett.*, 166 (1984) 8–12.
14 R. Benesch, G. MacDuff and R. E. Benesch, *Anal. Biochem.*, 11 (1965) 81–87.
15 V. Baudin and H. Wajcman, *J. Chromatogr.*, 299 (1984) 495–497.
16 H. Wajcman and J. P. Boissel, in H. Wajcman (Editor), *HPLC en Chimie des Proteines (INSERM editions*, Vol. 115), 1983, pp. 139–150.
17 J. Rochette, B. Varet, J. P. Boissel, K. Clough, D. Labie, H. Wajcman, B. Bohn, P. Magne and C. Poyart, *Biochim. Biophys. Acta*, 785 (1984) 14–21.
18 R. L. Heinrikson and S. C. Meredith, *Anal. Biochem.*, 136 (1985) 65–74.
19 D. Loukopoulos, C. Poyart, J. Delanoe-Garin, C. Matsis, N. Arous, J. Kister, A. Loutradi-Anagnostou, Y. Blouquit, Ph. Fessas, J. Thillet, J. Rosa and F. Galacteros, *Hemoglobin*, 10 (1986) 143–159.
20 K. Imai, *Allosteric Effects in Haemoglobin*, Cambridge Univ. Press, Cambridge, 1982.
21 W. P. Winter and J. Yodh, *Science (Washington, D.C.)*, 221 (1983) 175–177.