A METHOD FOR ISOLATION OF ABNORMAL HAEMOGLOBINS WITH HIGH OXYGEN AFFINITY DUE TO A FROZEN QUATERNARY R-STRUCTURE: APPLICATION TO Hb CRETEIL $\alpha_2{}^{A}\beta_2$ (F5) 89 ASN

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1. Introduction

Familial erythrocytosis is most likely due to the presence of an abnormal haemoglobin which possesses an increased oxygen affinity. At the present time 12 different haemoglobin variants have been reported [1]. At least 4 of them: haemoglobin Olympia [2], haemoglobin Brigham [1], haemoglobin Syracuse [3] and haemoglobin Heathrow [4] cannot be separated from haemoglobin A by electrophoresis or chromatography since these haemoglobins are due to a neutral amino acid substitution. Since such abnormal haemoglobins are relatively frequent, we have tried to develop a new general method for their separation. The theoretical basis of this work is the higher reactivity of the sulfhydryl group β93 in the oxy (R) form of haemoglobin vis à vis the deoxy (T) form. Since haemoglobins responsible for congenital erythrocytosis are in a frozen quaternary R-structure they should be in absence of oxygen much more reactive to sulfhydryl reagents than haemoglobin A. They might thereby be isolated from unreacted haemoglobin A.

2. Material and methods

For this study a frozen quaternary R-structure abnormal haemoglobin was used. This haemoglobin was detected in a family with hereditary erythrocytosis in which the whole blood and the haemolysate, 2,3-diphosphoglycerate free, showed increased oxygen affinity. Several results indicated the presence in the propositus red cells of approximately the same amount of haemoglobin A and of an abnormal haemoglobin with a very high affinity for oxygen and low cooperativity. The abnormal haemoglobin cannot be separated from haemoglobin A by any electrophoretic technique. It is not an unstable haemoglobin; α and β chains have a normal electrophoretic mobility; structural studies have demonstrated that this haemoglobin was an undescribed variant. This abnormal haemoglobin will be called haemoglobin Créteil ($\alpha_2 A\beta_2$ (F5) 85 Ser → Asn, its structural study and its characteristics will be given in a subsequent paper [5] Preparations of haemolysate, control electrophoresis on cellulose acetate strips, preparative starch block electrophoresis and determination of oxygen equilibrium curves were performed following routine techniques used in our laboratory. Methods used for preparation of globin, isolation of tryptic peptides by ion exchange column chromatography and amino acid analysis were performed as described by Cohen-Solal et al. [6].

Fingerprints of tryptic hydrolysates were performed on silica gel thin layer (Schleicher and Schull G-1500). Alkylation of haemoglobin by iodoacetamide was performed according to Benesch [7] using iodoacetamide (Calbiochem) freshly recrystallized from petroleum ether. Reaction of haemoglobin with cystamine was performed according to the technique described by Wegmann [8] using cystamine dihydrochloride Merck. Specific radioactivities were measured using a Nuclear Chicago Mark I scintillation counter, after mineralization of the samples in a Packard 305 oxydizer. Incubations in

absence of oxygen were carried out in a two compartment tonometer [9] evacuated at 37°C using a Hi-Vac pump.

3. Results and discussion

3.1. *Incubation of haemoglobin A and haemoglobin Créteil with cystamine*

The first attempt to isolate haemoglobin Créteil from haemoglobin A was performed by electrophoresis after incubation of the haemolysate with cystamine. Cystamine was choosen as reagent because, as demonstrated by Taylor et al. [10] it reacts more rapidly with the β-SH 93 reactive group of globin than with deoxy-haemoglobin. Cystamine reacts with the β SH 93 group according to the following equation:

$$2\,Hb + H_2N{-}CH_2{-}CH_2{-}S{-}S{-}CH_2{-}CH_2{-}NH_2 \rightarrow 2\,Hb{-}S{-}S{-}CH_2{-}CH_2{-}NH_2$$

Consequently each cystamine bounded haemoglobin molecule becomes more positively charged. Electrophoretic patterns of the samples incubated in various conditions are presented in fig. 1. After 1 hr incubation with a 5–fold molar excess of cystamine, oxyhaemoglobin A is completely converted into a slower moving component. Incubations during shorter period of time or with cystamine/haemoglobin ratios smaller than five result in an incomplete binding of cystamine. Assays with deoxy-haemoglobin were conducted in order to avoid any conversion to the oxy form during incubation with cystamine and during stripping of unreacted cystamine at the end of the incubation time. Unfortunately even in these conditions a large amount of a slowly moving band is obtained. These results indicate that the affinity of oxyhaemoglobin A and of deoxy-haemoglobin A for cystamine is not sufficiently different to allow a separation between the two forms. The same phenomenon was observed after incubation of Créteil haemolysate; in very strict anaerobic conditions it presents only a slowly moving band, indicating that haemoglobin Créteil and haemoglobin A have both reacted with cystamine. It was impossible to obtain by a one step method a specific binding of cystamine on the R frozen abnormal haemoglobin.

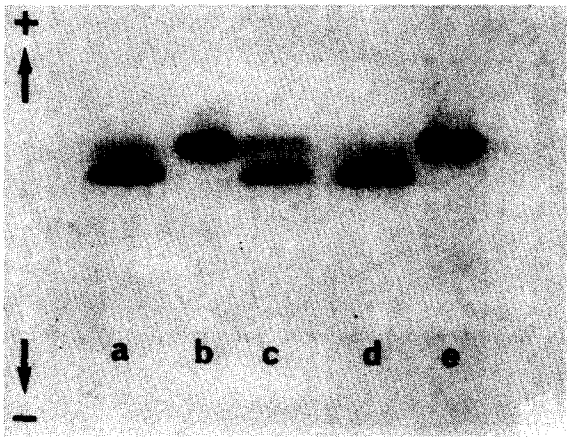

Fig. 1. Influence of cystamine on the electrophoretic mobility of haemoglobin. 4.5×10^{-4} M of haemoglobin was incubated during 1 hr. in presence of 2.25×10^{-3} M of cystamine in 0.05 M. Tris–HCl buffer pH 8.6. Electrophoresis on cellulose acetate strips was performed at pH 8.6 in a Tris–EDTA borate buffer. (a), Hb-A incubated with cystamine in presence of oxygen, (b), control Hb-A, (c), whole Créteil haemolysate incubated with cystamine in absence of oxygen, (d), Hb-A incubated with cystamine in absence of oxygen, (e), control Créteil haemolysate.

3.2. *Incubations with cystamine of haemoglobin previously reacted with iodoacetamide*

As shown by Benesch and al. [2] the sulfhydryl reagent, iodoacetamide, exhibits a very great difference in its affinity constant for the oxy and the deoxy forms of haemoglobin. Nevertheless, as this reagent is uncharged, alkylated haemoglobin cannot be separated by electrophoresis from non-alkylated haemoglobin A with either oxy and deoxy-haemoglobin.

To overcome these difficulties we decided to perform two operational steps. In the first we incubated, in anaerobic conditions, the sample with iodoacetamide. In these conditions haemoglobin molecules present in a T state do not react and thus keep their β SH groups free, while haemoglobin molecules present in a R state have their β_{93} SH groups blocked by carboxymethylation. Unreacted iodoacetamide molecules are then eliminated by addition in anaerobic conditions of an excess of cysteine. The mixture is allowed to react 5 min. and then anaerobically transferred on the top of a Sephadex G-25 column equilibrated with an oxygen free buffer. After elution haemoglobin is

incubated with cystamine in presence of oxygen. Only oxyhaemoglobin A in a normal quaternary R-structure would react with cystamine since it has kept its β SH 93 groups reactive during the first step. A mixture of haemoglobin A and abnormal haemoglobin in a frozen R state could in these conditions be separated by electrophoresis. Haemolysate Créteil containing haemoglobin A + haemoglobin Créteil was treated according to the above procedure. Electrophoresis patterns of haemolysate Créteil (fig. 2) show two bands, each having approximately the same amount of material. The slower band migrates at the same rate as a control haemoglobin A incubated in the same conditions. The other band migrates as a carboxymethylated haemoglobin A.

These results indicate that most probably the slow moving component is haemoglobin A while the fast one is haemoglobin Créteil. Additionnal proof was given using radioactive haemoglobin A. Haemolysate Créteil was mixed with a ^{14}C leucine-labelled haemoglobin A, incubated with iodoacetamide and cystamine as described, and submitted to a preparative starch block electrophoresis. It was found that more than 93% of the total radioactivity was in the slower band, indicating that in these conditions most of the haemoglobin A present in the mixture was converted to a cysteamine-Hb disulfide component. Thus the fast moving component, since it cannot be haemoglobin A, is the abnormal haemoglobin.

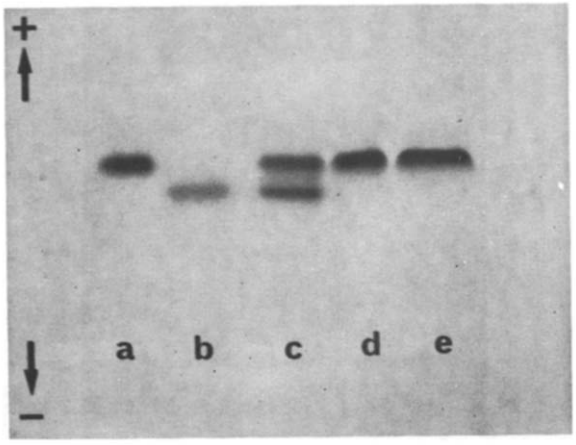

Fig. 2. Influence of cystamine on the electrophoretic mobility of haemoglobin previously treated with iodoacetamide. A mixture of 4.5×10^{-4} M of haemoglobin and of 4.5×10^{-3} M iodoacetamide in a 0.1 M Tris–HCl buffer pH 7.3 was incubated during 1 hr in a tonometer at room temperature. Excess of unreacted iodoacetamide was blocked by addition of 1.2×10^{-2} cysteine HCl. Stripped haemoglobin was obtained by chromatography on a Sephadex G-25 column and incubated with cystamine as described in legend fig. 1. (a), Hb-A incubated in non-evacuated tonometer, (b), Hb- A incubated in evacuated tonometer, (c), Créteil haemolysate incubated in evacuated tonometer, (d), control Créteil haemolysate, (e), control carboxymethylated Hb–A.

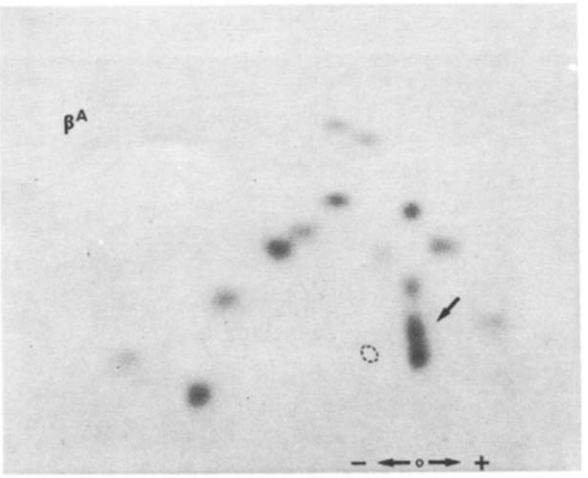

Fig. 3. Fingerprint of tryptic digest of aminoethylated β chain from an iodoacetamide treated Hb-A. Fingerprints were made on silicagel thin layer plates 20 cm × 20 cm. A 20 V/cm electrophoresis in pyridine, acetic acid, water (100:4:100, v/v/v) was followed by ascending chromatography in *n*-butanol, acetic acid, water and pyridine (40:8:21:32, v/v/v/v). Peptides were stained by ninhydrine. All the peptides were found in the normal position except for the S-carboxymethylated βT10 peptide (indicated by the arrow) which has migrated with the neutral peptides. Position of normal aminoethylated βT10 peptide is indicated by the dotted line.

3.3. *Possibility of structural studies of an abnormal haemoglobin isolated by this technique*

Chains of iodoacetamide reacted haemoglobin A can be isolated on CMC in 8 M urea. They elute at their normal position. Amino acid analysis of hydrochloride hydrolysates of α and β chains were performed. Only one S-carboxymethyl-cysteine was found in the β chain. α 104 and β 112 cysteinyl groups were then converted to aminoethylcysteine by aminoethylation of the isolated α and β chains

in order to avoid the formation of an insoluble core during tryptic digestion. Subsequent fingerprinting and ion exchange chromatography indicated that all the peptides from α and β chains were present in the normal position, except βT10. The group β 93 Cys which is localized in the peptide βT10, was converted to S-carboxymethyl cystein [11] and was therefore found in a more neutral position (fig. 3). These data demonstrate the possibility of attempting structural studies on abnormal haemoglobins isolated as described in this paper.

4. Conclusions

Most of the abnormal haemoglobins with high oxygen affinity have a neutral amino acid substitution; they cannot be isolated from haemoglobin A by any usual electrophoretic or chromatographic techniques. Recently Bunn, using isoelectric focusing electrophoresis in anaerobic conditions, succeeded in analytical isolation of haemoglobin Bethesda [12] and of haemoglobin Brigham [1]. It is however very difficult to adapt this method to preparative purpose and special equipment is necessary. The method described in this paper permits the easy isolation of large amounts of abnormal haemoglobins with frozen quaternary R-structure even if they have the same isoelectric point as haemoglobin A. Our results confirm the Perutz model [13] according to which, even after deoxygenation, some abnormal haemoglobins remain in a frozen quaternary R-structure. This method could probably be extended to the study of abnormal haemoglobins frozen in a quaternary T-structure having a low affinity for oxygen. Physicochemical and functional studies thereby become possible on pure abnormal haemoglobins without the interference of haemoglobin A. However, this method gives rise to carboxymethylated samples. Further studies will be necessary to develop a derived method which leaves free β SH 93 groups on the haemoglobin molecules.

Acknowledgements

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