ISOLATION AND PARTIAL CHARACTERIZATION OF HEMOGLOBIN A_{1b}

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1. Introduction

The existence of non-genetically determined minor fractions of hemoglobin has been puzzling for many years. Their understanding progressively improved with the introduction of new analytical techniques. Among these fractions, HbA_{1c} has been, so far, extensively studied and demonstrated to have N-terminal glycosylated β-chains [1].

This report concerns studies on the isolation and the partial characterization of another minor fraction called hemoglobin A_{1b}. This component has been prepared in its pure native-form starting with aged red blood cells, the final step being a preparative isoelectrofocusing. HbA_{1b} is formed during the life-span of the red blood cell and is not identical to any of the fractions described earlier. It differs from HbA_{1c} and evidence is brought forward in favour of a modification in the zone of $\alpha_1\beta_2$ contact and for its existence as an asymmetrical hybrid.

2. Materials and methods

Blood was obtained from normal adult donors. Routine procedures were used for the preparation of hemolysates and globin. Age (density)-dependent separations of RBCs were performed in phthalate solutions according to Danon et al. [2].

The pure HbA_{1b} was prepared in a three-step procedure:

(i) Enrichment in aged cells by two successive centrifugations [3]

(ii) Chromatographic separation of the lysate on DEAE–Sephadex with stepwise elution (Tris–HCl, 0.05 M, pH 7.8 and 6.9)

(iii) Isoelectrofocusing according to Drysdale et al. [4] using 15 mm inside diameter tubes followed by either electrophoretic or diffusion method of Hb-elution. 'G-6-P-hemoglobin' was prepared as described by Haney et al. [5]. Polyacrylamide gel electrophoretic analysis of globin-chains, at alkaline pH, according to Moss et al. [6] and at acid pH, following the technique of Stegink et al. [7], were performed. Their chromatographic separation was as described by Clegg et al. [8].

The techniques of Hartley [9] for N-terminal analysis by dansylation, of Blombäck et al. [10] for peptide finger-printing, of Grassetti et al. [11] for spectrophotometric titration of reactive SH-groups, of Cartier et al. [12] for enzymatic microquantitation of glucose and glucose-6-phosphate, of Chen et al. [13] for colorimetric quantitation of phosphate preceded by ashing of protein with hot perchloric acid [14], were utilised.

Presence of sialic acid was tested for by incubation with neuraminidase (*Vibrio cholerae*) [15] followed by an analytical isoelectrofocusing.

Functional studies were carried out on isolated pure hemoglobin fractions freed of organic phosphates by ion-exchange chromatography [16]. Oxygen affinity was measured by the discontinuous method of Benesch et al. [17] using bis-Tris 0.05 M buffer at 25°C. The pH-dependence of $\log P_{50}$ at 25°C was measured in bis-Tris 0.05 M/NaCl 0.1 M buffer below pH 7.5 and in Tris–HCl 0.05 M/NaCl 0.1 M buffer above this pH-value.

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3. Results and discussion

In contrast to chromatographic separation, the use of isoelectrofocusing gives a clear and reproducible resolution of the three minor hemoglobin components A_{1a}, A_{1b} and A_{1c}, the first two being present in trace amounts whereas the third represents approximately 5% of the total hemoglobin (fig.1) [4,18]. By density-dependent separation of freshly drawn red blood cells, elevated levels of HbA_{1b} (> 3%) were demonstrated to be present in aged cells with a density higher than 1.114. Conversely, in reticulocytes with a density lower than 1.086, no HbA_{1b} could be detected. Whole unfractionated red blood cells contain 0.5–1.0% HbA_{1b}. These results are in favour of post-translational nature of the formation of HbA_{1b} during the life-span of the red blood cell.

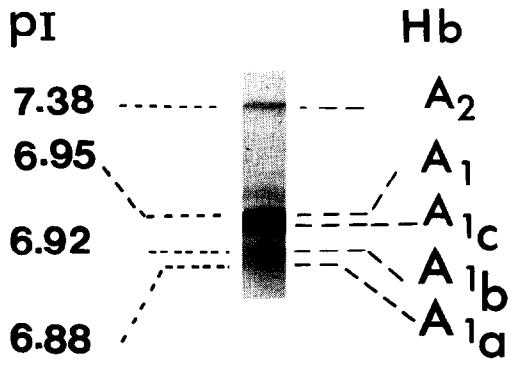

Fig.1. Polyacrylamide gel electrofocusing pattern of normal adult hemolysate in a pH-gradient 8.0–6.0 (acrylamide 4%, ampholine 2%).

This property has been used in the first step of the preparation of this fraction. A stepwise elution of DEAE–Sephadex chromatography was chosen as a second step for a good yield and a quick preparation without concomitant oxidation. The resultant fraction contains about 10% of HbA_{1b}. With this material the use of preparative isoelectrofocusing allows isolation of HbA_{1b} in pure native form in a sizable quantity. After elution from the gel, the purity was always checked by an analytical gel.

This fraction was compared with 'G-6-P hemoglobin' prepared in vitro by incubation. This compound is a possible metabolic precursor of HbA_{1c} [4] and behaves in a similar way to HbA_{1b} in chromatography. The incubated lysate was shown to contain 30% of a fraction more anodic than the major HbA fraction which focuses at a pH_i of 6.90 as compared to 6.92 for the native HbA_{1b}. The non-identity of the two components was further demonstrated by estimation of glucose-6-phosphate and phosphate. These estimations were done in parallel on HbA, HbA_{1c}, HbA_{1b} and 'G-6-P Hemoglobin', the range of sensitivity allowing the detection of quantities corresponding to a 1:1 molar ratio. The results are summarised in table 1. Concerning HbA_{1c} and 'G-6-P Hemoglobin', they confirmed the published data [1,2] but HbA_{1b} does not contain any significant amount of any of these residues.

Structural studies were performed on globin. Chromatographic chain-separation shows two β-peaks in equal amounts, one eluting with the normal one, the other in front of it. By electrophoresis at alkaline pH, the β-chain was again demonstrated to be duplicated. Conversely only one β-chain was observed at

Table 1

	HbA	HbA_{1b}	HbA_{1c}	G6P Hb
Glucose	< 5 nM/100 nM dimer	< 5 nM/100 nM dimer	35 nM/100 nM dimer	–
G-6-P[a]	< 5 nM/100 nM dimer	< 5 nM/100 nM dimer	< 5 nM/100 nM dimer	67 nM/100 nM dimer
Phosphate	< 10 nM/100 nM dimer	< 10 nM/100 nM dimer	< 10 nM/100 nM dimer	77 nM/100 nM dimer

[a] G-6-P is measured as glucose and correlated with the amount of phosphate

Globin or hemoglobin is freed of sugar by 1 N HCl at 100°C for 10 min followed by cold perchloric acid precipitation of proteins. The neutralised supernatant is used for quantitation of sugars.

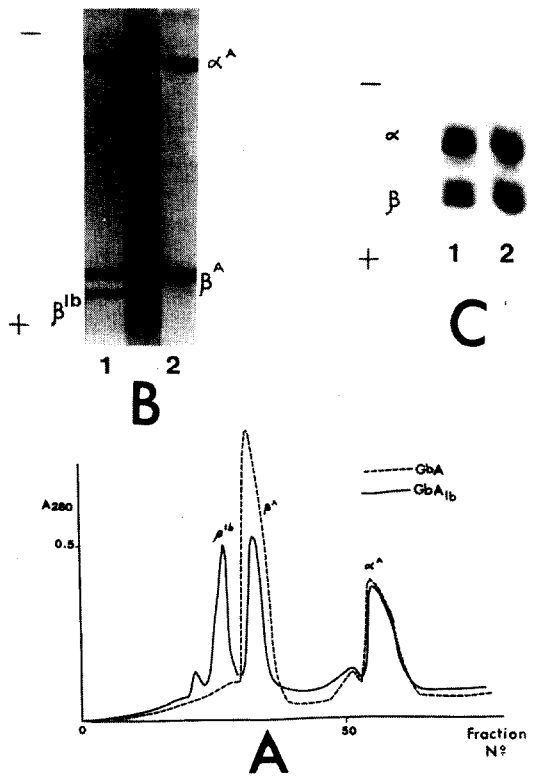

Fig.2. Separation of polypeptide chains in 8.0 M urea. (A) Carboxymethyl cellulose chromatographic pattern. Elution is obtained by Na^+ ion-gradient of phosphate buffer (ionic strength 0.007–0.03 M of gradient buffers, pH 6.9). Polyacrylamide gel electrophoresis of HbA_{1b} (1) and HbA (2) (B) at pH 9.8, (C) at pH 2.8.

acid pH (fig.2). By thin-layer finger-printing of the tryptic digest, the abnormal chain was shown to contain most of the peptides of β-chain in the right place including the characteristic β-T_3 and β-T_{10} peptides differentiating it from a δ-chain. Moreover this was confirmed by amino acid analysis of these peptides. By dansylation of normal and abnormal β-chains, the N-terminal valine was demonstrated to have a free amino group.

Taking into account the observed charge difference, some other possibilities were tested and ruled out. The reactive -SH β93 were demonstrated to be present. No phosphate group was found. The unmodified focusing pattern after incubation with neuraminidase eliminated the possibility of sialylation.

Functional studies were done comparing native HbA_{1b} and HbA prepared and stripped of phosphate in an identical way from the same gel. At neutral and slightly alkaline pH, the oxygen affinity of HbA_{1b} was found to be normal but with a reduced heme–heme interaction (n = 1.7 instead of 2.5 for HbA).

A striking fact was observed when the pH was progressively decreased. At pH 6.5 the experimental curve becomes obviously biphasic. The Hill plot shows a clear change in slope at about 40% oxygen saturation. This observation is consistent with the presence of two molecular species, the lower part corresponding to a non-cooperative high affinity component whereas the upper part tends to be normal (fig.3).

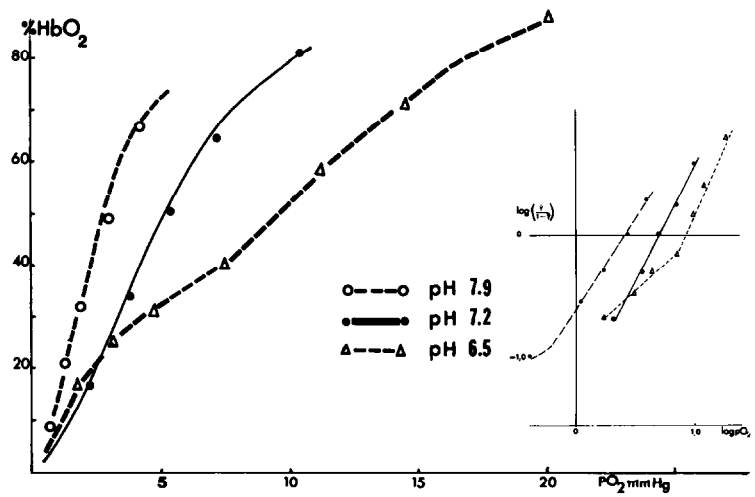

Fig.3. Oxygen dissociation curves of HbA and HbA_{1b} at different pH values. For method see the text. The insert shows a representation of these curves in Hill plots.

A possible explanation is that at acid pH, dissociation of a slightly unstable molecule into dimers might lead to a different type of reassociation resulting in functionally different non-hybrid forms as shown in the scheme below:

$$2\,\alpha_2\beta^A\beta^x \rightleftarrows 2\,\alpha\beta^A + 2\,\alpha\beta^x \rightleftarrows \alpha_2\beta_2^A + \alpha_2\beta_2^x \text{ or } (\alpha\beta^x)$$

These results together with the structural data are compatible with the hypothesis that hemoglobin A_{1b} is an asymmetrical hybrid with only one of its β-chains chemically modified. This modification might directly or indirectly perturb the quaternary contacts, this perturbation becoming more evident below a certain pH.

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