STRUCTURAL AND FUNCTIONAL STUDY OF Hb NANCY
β 145 (HC 2) Tyr → Asp
A HIGH OXYGEN AFFINITY HEMOGLOBIN

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1. Introduction

In 1970, Perutz demonstrated that in deoxyhemoglobin the penultimate tyrosines (HC 2) of both α and β chains are stabilized in pockets between the F and H helices. Upon oxygenation, these tyrosines are ejected and the C-terminal residues are then able to rotate freely [1].

Two variants with substitution of this residue have been described: Hb Rainier β 145 (HC 2) Tyr → Cys [2] and Hb Bethesda β 145 (HC 2) Tyr → His [2], both of them presenting very similar hematological features, an isolated erythrocytosis. This could be explained by an increased oxygen affinity, decreased Bohr effect and impaired heme–heme interaction.

We describe here the structural characterization of a new hemoglobin variant, Hb Nancy, in which tyrosine β 145 (HC 2) is replaced by aspartate. Preliminary studies show that the pure abnormal component, like Hb Bethesda, has an oxygen affinity almost identical to the affinity of isolated chains, the heme–heme interaction is strongly impaired and the Bohr effect is reduced. The same results have been found by studying the oxygen affinity in whole cells.

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2. Materials and methods

2.1. *Structural studies*

Blood was collected and the lysate prepared as usually. The electrophoretic study was performed on cellulose-acetate plates (Helena Laboratories) in Tris EDTA buffer pH 8.8. Electrofocusing in polyacrylamide gels was done as described by Drysdale et al. [3]. The abnormal component was purified on DEAE Sephadex, Tris–HCl 0.05 M buffer, using a pH gradient from 7.8 to 6.8. After deheminization, the chains were separated according to Clegg et al. [4].

The tryptic digest was finger-printed on silica-gel thin layer. Various specific stainings were used. A larger quantity of the abnormal peptide was isolated on cation-exchange resin (Beckman PA 35) according to Jones [5]. It was further purified on Aminex AG 50 W - X 2, and its amino acid composition determined on a Beckman 120 C Aminoacid analyzer.

The digestion by carboxypeptidase A (CPA) was performed first under normal conditions [6], and, because of the very slow release of the involved residue, it was tried under more vigorous conditions, using an enzyme substrate ratio of 1:1 as described by Perutz et al. [7].

2.2. *Functional studies*

The oxygen affinity was measured by the discon-

tinuous spectrophotometric technique of Benesch et al. [8]. The red blood cells (RBC) were suspended in isotonic phosphate buffers pH 7.15 and 6.46 [9]. The lysate and pure components were stripped on a Dintzis' column [10] and studied in bis–Tris buffer 0.05 M*.

The intra-erythrocytic 2,3 DPG** was estimated enzymatically by measuring NADH formation at 340 nm (Sigma, Technical Bulletin 35-UV).

3. Results and discussion

3.1. *Structural characterization*

The abnormal hemoglobin was found incidentally in a French woman, accompanying an asymptomatic erythrocytosis (RBC = 6.76 X 10^6 per mm^3, Hb = 13.4 g per 100 ml, PCV = 48 per cent, reticulocytes = 1.3 per cent).

At alkaline pH the abnormal component was moving more anodically than Hb A, between Hb A and Hb J. The percentage of alkali-resistant Hb was below 1, the Hb A_2 was normal, the heat stability normal.

The isoelectric point of the abnormal component was found by electrofocusing to be 6.86 against 6.95 for Hb A and 6.74 for Hb J Mexico.

It was eluted on DEAE Sephadex at pH 7.48 (Hb A = pH 7.70, Hb J Mexico = pH 7.35). It amounted to 50 per cent. An abnormal β chain was also eluted faster than normal in chain separation.

On the finger-print of this β chain, no normal dipeptide βT 15 (Tyr-His) was found. The βT 14 seemed especially faint. One extra-spot more polar and more anodic was observed specifically stained for histidine, but nor for tyrosine (fig.1).

Similar abnormalities were observed on the cation-exchange pattern: absence of normal βT 15, a small amount of βT 14 and one extra peak. After further purification this peak was identified as a peptide βT 14-15 containing one Asx instead of Tyr at position 145.

The CPA digestion in normal conditions released 0.5 residue of histidine, 0.1 residue of aspartate. In the enzyme/substrate ratio 1:1, one residue of

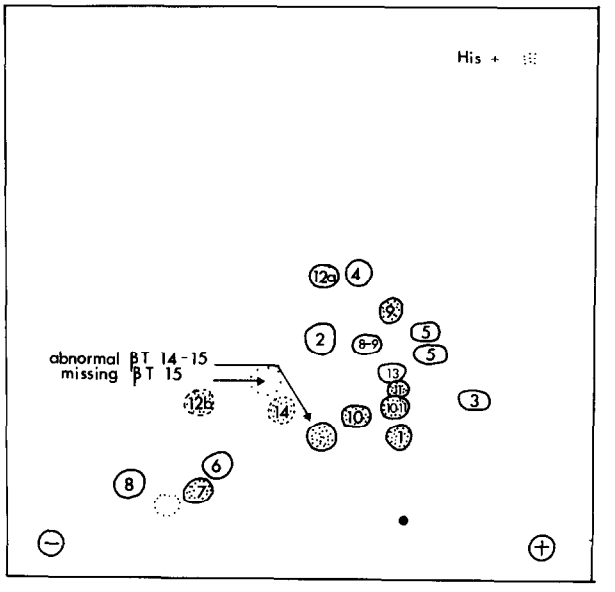

Fig.1. Finger-print of the β amino-ethylated chain on silica gel thin layer. The normal peptide βT 15 is missing. A new spot corresponding to the abnormal βT 14-15 is stained for Histidine but nor for tyrosine.

histidine and 0.4 residue of aspartate were released per $\alpha\beta$ dimer. Other contaminant residues were found identical in a digest of Hb A in the same conditions. This might be due either to autolysis of the enzyme or to a partial proteolytic cleavage of the hemoglobin.

All the other peptides were found normal. The final sequence of the abnormal β chain is then:

$$\ldots \underset{142}{\text{Ala}}\text{–His–Lys–Asp–}\underset{146}{\text{His}}$$

It is likely that the immediate vicinity of several positively charged residues may explain the smaller than expected difference in electrophoretic behavior due to a modified ionization as well as the absence of tryptic cleavage between peptides T 14 and T 15.

3.2. *Functional studies*

In intact RBC the oxygen affinity was found very high: P_{50} = 13 mm Hg at pH 7.15, versus 29.5 ± 0.5 in the control. The intra-erythrocytic 2,3 DPG was normal (13.4 μmol/g Hb). The oxygen association curve was diphasic. When plotted according to Hill's equation, two parts could be distinguished. The

* bis-tris = bis(2-hydroxyethyl)imino-tris (hydroxylmethyl)methane

** 2,3 DPG = 2,3 diphosphoglycerate

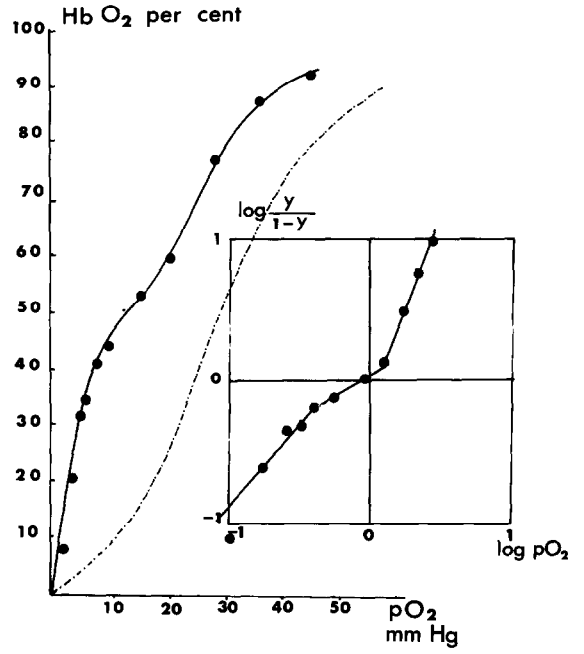

Fig.2. Oxygen affinity of intact RBC suspended in isotonic phosphate buffer pH 7.15 at 37°C. The P_{50} is increased when compared to control RBC (– · – ·) and the curve is clearly biphasic. From the Hill's plot, the slope of the lower half of the curve is equal to 1.2 corresponding to a high oxygen affinity hemoglobin without heme–heme interaction. The upper part shows a shift to the right of the oxygen affinity and a normal slope of 2.7.

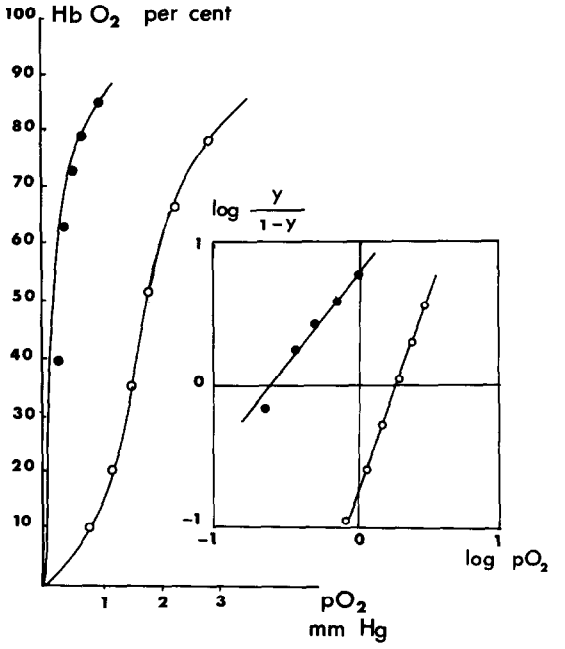

Fig.3. Oxygen affinity of the stripped pure hemoglobin isolated by DEAE-Sephadex. Bis–Tris 0.05 M buffer, pH 7.1, 25°C. Hb Nancy (–●–); Hb A (–○–). The oxygen affinity of the abnormal component is very high (P_{50} = 0.21 mm Hg), and its interaction coefficient is equal to 1.1.

slope is 1.2 in the lower part, 2.7 in the upper part, with a shift to the right of the oxygen affinity (fig.2).

This curve suggested the presence of two molecular species in approximately equal amounts and did not favor the existence of the asymetric hybrid $\alpha_2\beta^A\beta^{Nancy}$ [11].

When the cells were suspended in phosphate buffer pH 6.46, there was apparently no change in the lower part of the curve. Only the upper part was shifted to the right.

On the stripped lysate, the same phenomenon was still clearer, showing together the difference in oxygen affinity and the difference in cooperativity of both hemoglobins. This was found also on isolated components (fig.3).

The two hemoglobins were studied at several pHs varying from 7.20 to 5.90 in bis–Tris buffer, NaCl 0.1 M. The pH dependence of log P_{50} was clearly different in Hb A and Hb Nancy (table 1) confirming the data observed in the cells.

These results are very similar to what has been described concerning Hb Bethesda [11,12], the erythrocytosis being compensatory for the poor oxygen delivery. As in Hb Bethesda and in des-His hemoglobin, some restoration of the co-operativity and Bohr effect could be expected by addition of organic phosphate.

Table 1
pH dependence of log P_{50} of pure hemoglobin

	log P_{50}	
	Hb A	Hb Nancy
pH 7.20	0.82	– 0.50
pH 6.60	1.07	– 0.36
pH 6.40	1.16	– 0.28
pH 5.90	1.19	– 0.36

Bis–Tris 0.05 M, NaCl 0.1 M buffer, 25°C

Acknowledgements

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