A new hemoglobin variant altering the $\alpha_1\beta_2$ contact: Hb Chemilly $\alpha_2\beta_2$ 99(G1)Asp→Val

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Received 9 November 1983

Hemoglobin Chemilly ($\alpha_2\beta_2$ 99(G1)Asp→Val), a high oxygen affinity variant, was uncovered in the red blood cells of a polycythemic patient who reported to the hospital concerning periodic headaches. We describe the molecular abnormality and functional studies of this new abnormal Hb. β 99(G1)Asp, an invariant residue of hemoglobin, is considered a key amino acid for conformational changes between the R⇆T quaternary structures responsible for the allosteric behavior of hemoglobin. Hb Chemilly exhibits a high O_2 affinity, very low cooperativity and reduced Bohr effect. Its functional abnormalities are compared to the 5 other Hb variants at site β 99(G1) described up to now of the 7 single base substitutions predictable from the genetic code.

Hb variant *Erythrocytes* *Oxygen affinity* *Reversed phase HPLC*

1. INTRODUCTION

Accurate functional studies of Hb variants as well as X-ray diffraction analyses of the R and T quaternary conformational states in Hb [1,2] have stressed the important role of the $\alpha_1\beta_2$ interface in the cooperative mechanism of O_2 binding to tetrameric hemoglobin. Among various different amino acids the β_1 99(G1)Asp residue has been assigned a key role in stabilizing the T structure through hydrogen bonding to the neighboring α_2 (C_7)(42)Tyr. Substitution of β 99(G1)Asp by other residues has been described in 5 Hb variants [3–7] which have in common a high oxygen affinity, low cooperativity and more or less reduced alkaline Bohr effect. We describe below a sixth Hb variant, β 99(G1)Asp→Val whose abnormal functional properties confirm the prerequisite role of this residue in functional properties of hemoglobin.

2. MATERIALS AND METHODS

2.1. *Hematological and electrophoretic studies*

Blood was collected on heparinized tubes and the hematological studies were performed by routine procedures. The red cell mass was measured using ^{99}Tc as the label [8]. Electrophoresis at alkaline and acidic pH and isoelectric focusing in polyacrylamide gels were performed as in [9].

2.2. *Functional studies*

The abnormal hemoglobin was separated from HbA by DEAE-Sephadex chromatography. Oxygen binding curves in intact abnormal red cells (RBC), on the stripped hemolysate and in the purified abnormal component were carried out with a Hemox-Analyzer (TCS, Southampton, PA) according to a technique described in detail in [10].

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This apparatus has been now interfaced with a microcomputer (HP85) which allows one to store on-line up to 500 points of the curve. The recordings were made from $p_{O2} = 500$ mmHg after equilibration with pure oxygen down to $p_{O2} = 0$ mmHg, during at least 30–40 min. Conditions of pH and temperature were as indicated in the figure legends.

2.3. *Structural studies*

Globin was prepared by the acid-acetone method and the globin chains were separated by CM-cellulose chromatography in 8 M urea [11]. After aminoethylation the isolated chains were submitted to tryptic digestion [12]. Peptides were purified by reverse phase high-performance liquid chromatography (RP-HPLC) in a Chromatem 800 apparatus (Touzart et Matignon, Ivry) using an Aquapore RP 300 column (25 × 0.4 cm) (Brown Lee Labs). One mg of tryptic digest was applied on the column and elution was obtained by developing a non-linear gradient of acetonitrile (HPLC Carlo Erba) from 5 to 50% (as shown in fig.2) in 0.01 M ammonium acetate buffer (pH 6.0) [13] with a flow rate of 1.5 ml/min. The eluent of the column was monitored at 220 nm and fractions were collected every 0.7 min.

After 22–72 h hydrochloric acid hydrolysis the amino-acid composition of the peptides were determined on a Chromaspek aminoacid analyser (Rank Hilger J 180).

3. RESULTS

3.1. *Case report*

The abnormal hemoglobin was found in a 35-year-old Caucasian women who reported to the hospital showing clinical symptoms of polycythemia. Hematological data were as follows: RBC count, 6.3×10^{12}/l; Hb, 18.7 g/dl; PCV, 55%; MCV 88 fl. The red cell mass was increased to 46 ml/kg (normal 31 ml/kg) and 2,3-diphosphoglycerate (2,3-DPG) concentration amounted to 1.47 mmol/mmol Hb tetramer (normal value = 0.9 mmol/mmol Hb_4).

3.2. *Electrophoresis studies*

At alkaline pH a component migrating as HbF was observed. The alkaline denaturation test and the electrophoresis on citrate gel were both normal. Isoelectric focusing showed that this hemoglobin was slightly more alkaline than HbF (fig.1). The abnormal component amounted to about 40% of the total Hb.

3.3. *Structural studies*

Abnormal β chains were clearly isolated from the normal ones by CM-cellulose chromatography suggesting one charge difference at pH 6.9 between both components. Fig.2 shows the elution profile of the amino-ethylated tryptic digest. As compared to the normal β chain, the variant digest showed a supplementary peak between βTp12b and βTp10. In parallel we observed a decrease of the peak eluted before βTp13 and containing, in this gradient system, βTp3 and βTp11. The amino-acid composition of the supplementary peak showed the disappearance of an aspartyl residue replaced by a valine as demonstrated by kinetics of hydrochloric acid hydrolysis. The substitution β 99(G1)Asp→Val has not yet been reported and we propose the name of hemoglobin Chemilly for this variant.

3.4. *Functional studies in intact RBC*

Fig.3 depicts the biphasic shape of the oxygen binding curve (Hill plot) of the propositus' red cells. The overall affinity determined at 50% saturation is markedly increased under the physiological conditions of arterial blood in vivo. This curve clearly indicates the presence of two

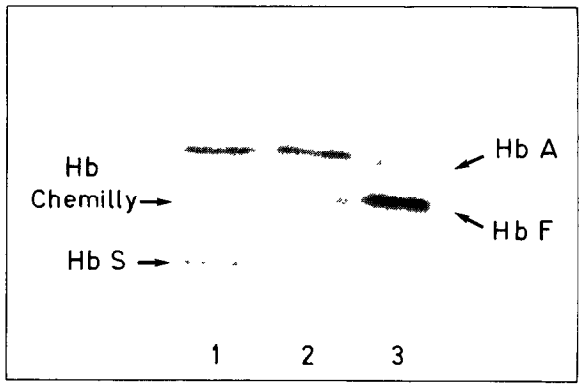

Fig. 1. Isoelectric focusing (pH gradient 6–8) of 3 erythrolysates: (1) heterozygous A/S patient, (2) propositus blood, (3) cord blood. Hb Chemilly migrates at a slightly more alkaline position than HbF_{II} and amounts to about 40% of the total Hb.

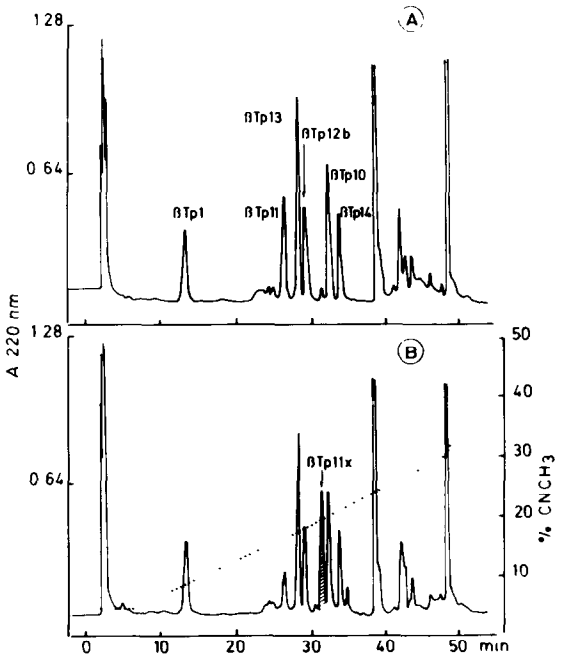

Fig. 2. Elution profile of the tryptic digest of βA (A) and β Chemilly (B) chains. The hatched area in B indicates the abnormal βTp11 peak.

components: one predominating at the upper part of the oxygen binding isotherm with normal oxygen affinity and normal cooperativity; the abnormal component expresses itself at the lower part of the curve with high affinity and low cooperativity. Fig.3 also shows for comparison a normal AA RBC O_2 binding curve ($p_{50} = 26$ mmHg $n_{50} = 2.55$). From these two curves we calculated by difference the Hill plot for the abnormal component. Its p_{50} was estimated to be 2.73 mmHg and $n_{50} = 1.17$ at pH 7.40 and 37°C in conditions of tetramer concentration prevailing inside the RBC (2.1 mM of the abnormal Hb). Similar calculations were made from the Hill plots recorded from oxygen dissociation curves in RBC suspensions at pH 7.05 and 7.60. In both cases cooperativity was close to unity (0.96 and 1.07, respectively). Values of log p_{50} indicated a small influence of pH on the O_2 affinity of Hb Chemilly: $\Delta \log p_{50}/\Delta$pH being equal to -0.25 or half the alkaline Bohr effect measured in normal adult RBC under the same conditions of temperature and ionic strength. We conclude from these results that Hb Chemilly tetramer has a high O_2 affinity, reduced Bohr effect and much decreased cooperativity indicative of its stabilization in the R quaternary structure.

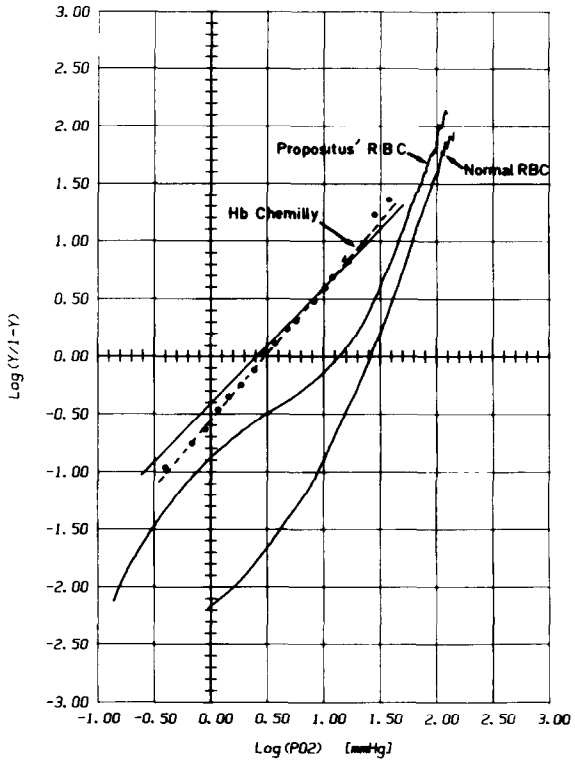

Fig. 3. Experimental Hill plots of the propositus red cells compared to that of normal adult erythrocytes. The curve (Hb Chemilly) results from the subtraction of the normal AA curve from that of the abnormal red cells (see text). Points are calculated coordinates. The continuous line has a slope of unity. Conditions were: 0.14 M NaCl, in 0.05 M Bistris buffer, pH 7.40 at 37°C.

These conclusions were confirmed in analyzing O_2 binding of the purified Hb Chemilly as illustrated in fig.4. Curve C is the Hill plot for purified stripped HbAo measured at 25°C in the presence of 0.1 M chloride in Bistris buffer ($p_{50} = 4.6$ mmHg, $n_{50} = 2.80$). Curve A is the Hill plot for Hb Chemilly determined under the same conditions ($p_{50} = 1$ mmHg, $n_{50} = 1.13$). Curve B is the Hill plot for Hb Chemilly in 0.1 M chloride and 2 mM inositol hexaphosphate ($p_{50} = 1.2$ mmHg, $n_{50} = 1.40$). Clearly this potent allosteric effector of tetrameric Hb A does not modify the abnormal function of Hb Chemilly. Similar conclusions were drawn from O_2 binding curves of the abnormal red cells which had been depleted of their 2,3-DPG content after incubation at 37°C for 18 h.

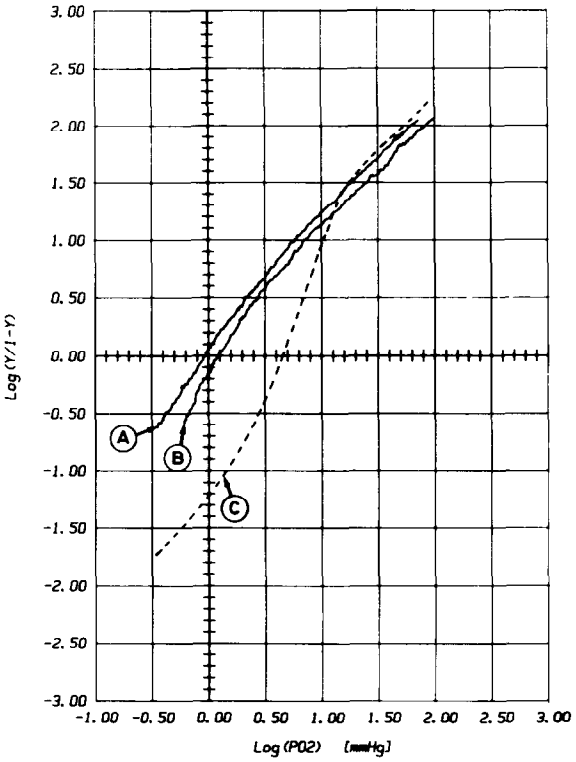

Fig. 4. Experimental Hill plots of oxygen binding to: (A) purified Hb Chemilly, (C) purified HbA and (B) purified Hb Chemilly plus 2 mM IHP. Otherwise conditions were: 0.1 M NaCl, 0.05 M Bistris buffer, pH 7.20 at 25°C.

4. DISCUSSION

Hb Chemilly is a new Hb variant bearing a substitution β 99(Gl)Asp residue and leading to high oxygen affinity, almost zero cooperativity and reduced alkaline Bohr effect. These abnormalities were not due to instability of the tetrameric form leading to increased dimer formation. This was demonstrated from comparison of the curves calculated in the RBC suspension experiments (fig.3) and those of the purified component (fig.4) for which comparable affinity and cooperativity are observed if one takes into account the effect of the difference in temperature at which measurements were made (37 and 25°C, respectively).

These abnormalities in the functional properties of Hb Chemilly are a common feature but with varying degrees of severity in all mutant Hbs [3–7] with substitution at the β 99(Gl) site. Several differences in the functional abnormalities of Hb Chemilly and other mutants Hb at the β 99(Gl) site were observed. For example Hb Radcliffe [6] (β 99Ala) showed only a minor decrease of the alkaline Bohr effect which was supported by X-ray analyses indicating that deoxy Hb Radcliffe crystals were isomorphous to those of deoxy HbA. Unlike Hb Kempsey [14,15] and Hb Hotel Dieu [7], which have a similar reduced Bohr effect to that of Hb Chemilly, the latter was intensitive to the effect of organic phosphates. Some of these discrepancies may be due, at least in their quantitative values, to the difficulty of measuring accurately p_{O2} values in solution of the order of 1–1.5 mmHg. More likely they could be related to the differences at the $\alpha_1\beta_2$ contact provoked by the various substituted residues (His, Asn, Tyr, Ala, Gly and Val). It was obvious upon examination of the 3D model of deoxy HbA in Cambridge that in Hb Chemilly, the γ-methyl of β_2 99(Gl)Val is less than 2 Å from the OH group of α_1 (C_7) 42 Tyr. This excludes any contact between the α_1 and β_2 interface and should lead to a destabilization of the T structure in such a way that it cannot even properly be restored upon addition of IHP. It is also likely that due to their hydrophobicity, the two methyl groups of β 99(Gl)Val contribute to these chemical abnormalities. Lastly Hb Chemilly is the sixth Hb variant described at the β 99(Gl) site. According to the genetic code only one remains to be found (Asp(GAU)→Glu(GAA/G)).

ACKNOWLEDGEMENTS

We thank Professor M.F. Perutz for helpful discussions, B. Bohn, C. Gautheron and J. Grellier for their help in preparing this manuscript. We are grateful to Dr Desvignes for his clinical help. This work was supported by funds from INSERM.

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