Hemoglobin Ohio (β142 Ala→Asp): A New Abnormal Hemoglobin With High Oxygen Affinity and Erythrocytosis

By Winston F. Moo-Penn, Rose G. Schneider, Tzu-bi Shih, Richard T. Jones, Sugantha Govindarajan, P. G. Govindarajan, and Leslie C. Patchen

Hemoglobin Ohio [β142 (H2O) Ala→Asp] was found in three members of a white family, all of whom showed erythrocytosis. The variant hemoglobin has a high oxygen affinity, a reduced Bohr effect, and diminished cooperativity. The functional abnormalities of Hb Ohio are explained by the proximity of the substituent β142 residue, both to β143 His, which is involved in the DPG binding site of hemoglobin, and to the critical C terminal region of the β chain, which participates in the stabilization of the deoxy (T) conformation.

ABOUT 60 variant hemoglobins with various types of structural alterations related to changes in oxygen affinity have been found to date. Those with high oxygen affinity may be associated with familial erythrocytosis.1 High affinity hemoglobins (which can be either stable or unstable) do not readily release oxygen to the tissues, and the resulting anoxia may stimulate production of erythropoietin and produce compensatory erythrocytosis.

This report describes the structural and functional characterization of Hb Ohio, which exhibits high oxygen affinity and is associated with erythrocytosis.

CASE REPORT

The proband is a 40-yr-old white male of Scottish-English ancestry with a history of alcoholism, recurrent upper respiratory infections, sinusitis, intermittent frontal headaches, and dizziness. There is a history of erythrocytosis in his father, brother, and paternal aunt. Physical examination was unremarkable except for a liver span of 12 cm. Current laboratory findings revealed the following values: Hb 18.6 g/dl, packed cell volume (PCV 54.4), reticulocyte count 2.3%, and erythropoietin 75 mIU/ml (normal range 15-59 mIU/ml). The whole blood volume was normal, but the plasma volume was slightly decreased. Blood gas studies gave values of P02 71 mm Hg, Pco2 37.6 mm Hg, and the oxygen saturation was 94%.

Pulmonary function tests, basic blood chemistries, chest roentgenogram, and ECG were normal. Other hematologic data on this family were analyzed by methods previously described.4 Electrophoretic analyses of the hemolysates were performed on cellulose acetate (Tris-EDTA-borate buffer, pH 8.5), on citrate agar (pH 6.0), and in urea-mercaptoethanol buffers (pH 8.9 and 6.0). Erythrocytes were examined for inclusion bodies before and after treatment with the reduct dye, brilliant cresyl blue (BCB). Hemoglobin stability was determined by treating the blood sample with heat and isopropanol.1 Hemoglobin A2 was quantitated on microcolumns,2 and Hbf was determined by the method of alkali denaturation.8

Structural Studies

For structural analysis, the abnormal β chain, isolated by the method of Clegg et al.,9 was chemically modified with ethyleneimine10 and was digested with trypsin.11 The tryptic digest was fingerprinted with a chromatographic solvent of isoeamyl alcohol:pyridine:water (7:7:6), and electrophoresed at pH 6.4 for 150 min at 35 V/cm.12 Peptides were also separated by column chromatography on Aminex A-5 resin.13 Isolated peptides were hydrolyzed in 6 M HCl at 110°C for 24 hr in vacuo and were analyzed on a Beckman 121 amino acid analyzer.14 A Beckman 890C sequencer was used to determine the sequence of peptides, according to the program (no. 102974) recommended by the manufacturer. The phenylthiohydantoin derivatives (PTH) were identified and quantitated by gas15 and high performance liquid chromatography.16

Oxygen Binding Studies

For oxygen binding studies, heparinized blood samples from the proband and a normal control were shipped on ice by air freight to Portland, and oxygen equilibria of the blood cell suspensions were measured within 48 hr after collection by the method of Hayashi et al.17 Simultaneously, the concentration of 2,3-diphosphoglycerate (DPG) in the blood cells was quantitated by an enzymatic assay.18 Most of the blood was then hemolyzed, and the hemolysate was stripped of organic phosphates and other ions by passing it through an ion-exchange column at 4°C.19 Hemoglobin Ohio was isolated from the other hemoglobin components on a DEAE-Sephadex column, with a pH gradient of 0.05 M Tris-HCl, pH 7.9-7.2, at 5°C.20 Oxygen equilibrium curves were measured by the automatic recording method of Imai et al.21 employing a Gilford 250 spectrophotometer and a Clark O2 electrode. Oxygen equilibrium data were recorded and analyzed with a PDP 11-V03 Digital Computer according to Adair’s stepwise oxygenation scheme,22 and the results were either printed out on a Digital Decwriter II or drawn with a Hewlett-Packard 7200 A Graphic Plotter. The isotonic phosphate buffer used for the equilibrium measurements of red cell suspensions was composed of 0.15 M Na2HPO4 and 0.15 M KH2PO4 at pH 7.4. Measurements of isolated hemoglobin fractions were done in 0.05 M Tris or bis-Tris buffers containing 0.1 M NaCl at pH 6.5, 7.4, and 9.0.

MATERIALS AND METHODS

Hematologic Studies

Hematologic data were obtained by standard methods, including Coulter Counter determinations. The erythrocytes and hemolysates were analyzed by methods previously described.4 Electrophoretic analyses of the hemolysates were performed on cellulose acetate (Tris-EDTA-borate buffer, pH 8.5), on citrate agar (pH 6.0), and in urea-mercaptoethanol buffers (pH 8.9 and 6.0). Erythrocytes were examined for inclusion bodies before and after treatment with the reduct dye, brilliant cresyl blue (BCB). Hemoglobin stability was determined by treating the blood sample with heat and isopropanol.1 Hemoglobin A2 was quantitated on microcolumns,2 and Hbf was determined by the method of alkali denaturation.8

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The electrophoretic patterns are illustrated in Fig. 1. On cellulose acetate in Tris-EDTA-borate (TEB) buffer (pH 8.5), the hemolysate resolves into approximately equal amounts of HbA and a fraction moving between HbA and HbJ Baltimore, and slightly anodically to HbK Woolwich. In citrate agar electrophoresis (pH 6.0), the fraction is again slightly anodic to HbK Woolwich. The respective β-globin chains, (β Ohio and β K Woolwich) move similarly on globin electrophoresis at pH 8.9 and with only slight differences at pH 6.0. Analysis of the hemolysate by column chromatography\(^{2,6}\) gave the following values: 49.8% HbA, 47.7% Hb Ohio, and 2.5% HbA\(_2\). Hemoglobin biosynthesis studies\(^{2,4}\) revealed a balanced α/non-α synthetic ratio of 1.19 and a β Ohio/β-chain total ratio of 0.49, a value that closely approximates the percentage of Hb Ohio in the hemolysate.

No evidence of hemoglobin instability was detected by treating the blood with heat and isopropanol,\(^{3,6}\) nor were there unusual intraerythrocytic inclusion bodies before or after treatment with BCB.

**RESULTS**

**Electrophoretic Studies and Hemoglobin Stability**

The electrophoretic patterns are illustrated in Fig. 1. On cellulose acetate in Tris-EDTA-borate (TEB) buffer (pH 8.5), the hemolysate resolves into approximately equal amounts of HbA and a fraction moving between HbA and HbJ Baltimore, and slightly anodically to HbK Woolwich. In citrate agar electrophoresis (pH 6.0), the fraction is again slightly anodic to HbK Woolwich. The respective β-globin chains, (β Ohio and β K Woolwich) move similarly on globin electrophoresis at pH 8.9 and with only slight differences at pH 6.0. Analysis of the hemolysate by column chromatography\(^{2,6}\) gave the following values: 49.8% HbA, 47.7% Hb Ohio, and 2.5% HbA\(_2\). Hemoglobin biosynthesis studies\(^{2,4}\) revealed a balanced α/non-α synthetic ratio of 1.19 and a β Ohio/β-chain total ratio of 0.49, a value that closely approximates the percentage of Hb Ohio in the hemolysate.

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**Structural Studies**

The abnormal β-chain from Hb Ohio was isolated from a CM-cellulose-urea column as a discrete peak eluting in a position representing the gain of one net negative charge. Chemical modification of the abnormal β-chain and analysis of the tryptic peptides by peptide mapping revealed a pattern of peptides similar in distribution to those of Hb Hope (β136 Gly→Asp). This observation suggested that the amino acid substitution in Hb Ohio involved a single charge change located in the same βT-14 peptide, which is altered in Hb Hope.

Examination of the tryptic peptide map indicated that abnormal βT-14 and the uncleaved peptide βT-14, 15 of Hb Ohio were well isolated and less cathodic than the normal peptides. The amino acid composition of abnormal βT-14 (eluted from the peptide map) indicated the loss of one residue of alanine with a corresponding gain of one residue of aspartic acid (Table 2).

Further analysis of the tryptic peptides by column

### Table 1. Hematologic Data on Patients With Hb Ohio

<table>
<thead>
<tr>
<th>Family Member</th>
<th>Age (yr)</th>
<th>Hb (g/dl)</th>
<th>RBC (x 10(^{12})/liter)</th>
<th>PCV</th>
<th>MCV (fl)</th>
<th>MCH (pg)</th>
<th>MCHC (g/dl)</th>
<th>WBC (10(^3)/liter)</th>
<th>Hb Type (%)</th>
<th>HbF (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Father</td>
<td>68</td>
<td>19.8</td>
<td>6.5</td>
<td>58.1</td>
<td>89</td>
<td>30.4</td>
<td>34.0</td>
<td>6.3</td>
<td>A/Ohio</td>
<td>0.6</td>
</tr>
<tr>
<td>Brother</td>
<td>43</td>
<td>19.3</td>
<td>6.6</td>
<td>55.4</td>
<td>85</td>
<td>29.7</td>
<td>34.9</td>
<td>5.9</td>
<td>A/Ohio</td>
<td>0.6</td>
</tr>
<tr>
<td>Propositus</td>
<td>40</td>
<td>18.6</td>
<td>6.1</td>
<td>54.4</td>
<td>88</td>
<td>29.5</td>
<td>34.2</td>
<td>5.9</td>
<td>A/Ohio</td>
<td>0.8</td>
</tr>
</tbody>
</table>

### Table 2. Amino Acid Composition of Peptides From Hb Ohio

<table>
<thead>
<tr>
<th>Amino Acid</th>
<th>Fingerprint βT-14</th>
<th>Ion-Exchange βT-4 and βT-14</th>
<th>Expected βT-4</th>
<th>Expected βT-14</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lys</td>
<td>0.8</td>
<td>0.9</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>His</td>
<td>0.6</td>
<td>0.8</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Arg</td>
<td>0.9</td>
<td></td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>Asp</td>
<td>1.8</td>
<td></td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Thr</td>
<td>1.0</td>
<td></td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>Glu (Gln)</td>
<td>1.1</td>
<td></td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>Pro</td>
<td>0.9</td>
<td></td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>Gly</td>
<td>1.4</td>
<td>0.9</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>Ala</td>
<td>2.9</td>
<td>2.6</td>
<td>4</td>
<td></td>
</tr>
<tr>
<td>Val</td>
<td>1.8</td>
<td>2.9</td>
<td>2</td>
<td>3</td>
</tr>
<tr>
<td>Leu</td>
<td>1.0</td>
<td>2.9</td>
<td>2</td>
<td>1</td>
</tr>
<tr>
<td>Tyr</td>
<td>0.9</td>
<td></td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>Trp</td>
<td>0.9</td>
<td></td>
<td>1</td>
<td></td>
</tr>
</tbody>
</table>

The amino acid values are expressed as molar ratios.

The short hydrolysis time (24 hr) did not allow for complete cleavage of the Val-Val sequence (133-134); therefore the value for valine is low.

Tryptophan was not determined.
Table 3. Sequence Analysis of Mixed Peptides \( \beta T-4 \) and \( \beta T-14 \) from Hb Ohio

<table>
<thead>
<tr>
<th>Cycle</th>
<th>Residue No.</th>
<th>Identification</th>
<th>nmole</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>31 133</td>
<td>Leu* Val*</td>
<td>-</td>
</tr>
<tr>
<td>2</td>
<td>32 134</td>
<td>Leu Val</td>
<td>466.1 314.6</td>
</tr>
<tr>
<td>3</td>
<td>33 156</td>
<td>Val Ala</td>
<td>502.1 321.7</td>
</tr>
<tr>
<td>4</td>
<td>34 136</td>
<td>Val Gly</td>
<td>480.6 266.0</td>
</tr>
<tr>
<td>5</td>
<td>35 137</td>
<td>Tyr Val</td>
<td>334.2 233.1</td>
</tr>
<tr>
<td>6</td>
<td>36 138</td>
<td>Pro Ala</td>
<td>289.8 213.8</td>
</tr>
<tr>
<td>7</td>
<td>37 139</td>
<td>Trp Asn</td>
<td>117.5 157.5</td>
</tr>
<tr>
<td>8</td>
<td>38 140</td>
<td>Thr Ala</td>
<td>319.3 189.9</td>
</tr>
<tr>
<td>9</td>
<td>39 141</td>
<td>Gin Leu</td>
<td>118.7 114.2</td>
</tr>
<tr>
<td>10</td>
<td>40 142</td>
<td>Arg ASP</td>
<td>77.7</td>
</tr>
<tr>
<td>11</td>
<td>143</td>
<td>His</td>
<td>62.8</td>
</tr>
<tr>
<td>12</td>
<td>144</td>
<td>Lys</td>
<td>-</td>
</tr>
</tbody>
</table>

*Modified with 4-sulfophenylnitrosothiocyanate and not determined.

Initial sample of \( \beta T-14 \) and \( \beta T-4 \) were 700 and 950 nmole, respectively.

Asn, Gin, and His were quantitated by high performance liquid chromatography.

Asp, Tyr, and Trp were quantitated by on-column silylation on a gas chromatograph.

The results indicate the two sequences that were obtained for mixed peptides \( \beta T-4 \) (residues 31-40, Leu-Leu ... Arg) and \( \beta T-14 \) (residues 133-144, Val-Val ... Lys).

Oxygen Binding Studies

The oxygen equilibrium curve of the proband's red cell suspension measured under physiologic conditions (37°C, pH 7.4) revealed only one-half the oxygen transport capacity \([P_02] = 100\) mm Hg - \([P_02] = 40\) mm Hg) found in normal cells (Fig. 3). The erythrocyte DPG content at the time of these measurements was 15.4 μmole DPG/g Hb for the proband and 11.6 μmole DPG/g Hb for the control. The normal range is 12.8 ± 2.3.

Purified fractions of Hb Ohio and HbA used for oxygen affinity studies were obtained by DEAE-Sephadex chromatography: each showed a single band on electrophoresis. The methemoglobin content of samples subjected to oxygen equilibrium studies was no more than 6% at the end of the equilibrium measurement.

Fig. 2. Chromatographic separation of an aminoethylated tryptic digest of the \( \beta \)-chain from Hb Ohio. A linear gradient of pyridine acetate (0.2 M, pH 3.1 - 2.0 M, pH 5.0) was used to elute the peptides from a column (0.9 x 23 cm) of Aminex A-5 at 52°C and at a flow rate of 30 ml/hr. Ten percent of the effluent was removed and reacted with ninhydrin to allow for automated monitoring at 570 nm.

Fig. 3. Oxygen equilibrium curve of a red cell suspension in isotonic phosphate buffer (pH 7.4, 37°C). Extrapolation from these curves indicates that the functional capacity for delivery of oxygen by the red cells containing Hb Ohio is one-half that of normal cells.
The pH dependence of oxygen binding or alkaline Bohr effect (ΔlogP50/ΔpH between pH 7.0 and 8.0) was significantly lower for Hb Ohio (−0.38) than for normal HbA (−0.58). The Hill plots for isolated Hb Ohio and HbA at pHs of 6.5, 7.4, and 9.0 are represented in Fig. 4. Significant differences between the oxygen binding of Hb Ohio and HbA were found at low degrees of oxygen saturation with a shift of Hb Ohio towards a high affinity state. The amplitude of the differences decreased with increase of pH. This observation implies that a part of the difference between the deoxy structure of Hb Ohio and HbA contributes to the decrease of Bohr effect in Hb Ohio. On the other hand, because the Hill plots of Hb Ohio and HbA approach one another at the upper asymptotes, their oxy (R) conformational structures would appear to be similar. Consistent with these observations, the cooperativity of oxygen binding (Hill coefficient) over the pH range examined was found to be somewhat less for Hb Ohio (2.5–1.8) than for HbA (2.8–2.3).

Chloride, DPG, and inositol hexaphosphate binding studies, which will be reported elsewhere, indicate that the anions had significantly less effect on the oxygen affinity of Hb Ohio than on HbA.

**DISCUSSION**

The four electrophoretic methods used in the preliminary characterization of Hb Ohio are those recommended by the International Committee for Standardization in Hematology for the presumptive identification of abnormal hemoglobins. Of more than 200 variant hemoglobins that have been studied in our laboratories, only one, Hb K Woolwich (β132 Lys→Asn) might be confused with Hb Ohio in this system. Moreover, the increased oxygen affinity of Hb Ohio and its electrophoretic behavior on citrate agar, suggested that the alteration in the β-chain might be located in the H helix. This suggestion was helpful for determining the area in which the efforts involved in structural analysis were focused.

The increased oxygen affinity and erythrocytosis associated with Hb Ohio (β142 Ala→Asp) are apparently due to a decrease in the stability of the deoxy or T molecular conformation. The substitution of a charged aspartyl residue for the smaller alanyl residue at position β142 (H2O) could alter the conformation of the C terminal region of the polypeptide chain. In deoxy Hb Ohio, the increased volume of aspartic acid as compared to alanine (127.2 versus 89.4 Å³), and the introduction of a charge, could lead to a displacement of the H helix in relation to the G helix. The resulting alteration in the conformation of the C terminal region of the molecule could decrease the stability of the intrachain salt bridge between the imidazole group of β146 His and the aspartyl residue β94 (FG1). This salt bridge contributes about one-half of the alkaline Bohr effect and is also involved in stabilizing the deoxy quaternary structure of hemoglobin. The perturbation of this salt bridge could account for the observed reduction in the alkaline Bohr effect as well as the increased oxygen affinity in Hb Ohio. However, since the subunit cooperativity is still largely preserved, the interchain salt bridge formed in deoxy hemoglobin between the α carboxyl group of the βC terminal histidine and the ε amino group of α40 Lys (C6) is probably still preserved in Hb Ohio.

The functional properties of Hb Ohio can be compared to those of Hb S Travis (β6 Val, β142 Val), in which one of the two substitutions in the β-chain involves the replacement of alanine at β142 with valine. Hemoglobin S Travis, like Hb Ohio, exhibits high oxygen affinity, a decreased affinity for anions, and a slightly decreased cooperativity; however, the Bohr effect is unchanged.

A reduced conformational change in the C terminal region of the β-chain of Hb S Travis as compared to Hb Ohio probably accounts for the normal Bohr effect. Since the volume of valine (141.1 Å³) is greater than that of either alanine or aspartic acid, it appears that the charge on aspartic acid at the β142 site is more important than size in contributing to the perturbation of the salt bridge between β146 His and β94 Asp. A definitive explanation regarding the actual conformational changes in Hb Ohio and Hb S Travis,
and the relationship of these changes to functional properties, will come from x-ray crystallographic studies in progress.

It is interesting to note that almost all of the dozen known variants with alternations between residues β143–146, exhibit high oxygen affinity, attesting to the essential role that the C terminal region of the β-chain plays in the stabilization of the deoxy conformation.²⁸

In the patient with Hb Ohio, the compensatory erythrocytosis is perhaps inadequate, and insufficient delivery of oxygen to the brain may produce the headache, dizziness, and bifrontal pounding of which the propositus complained.

Further oxygen equilibrium, kinetic, and x-ray crystallographic studies on Hb Ohio are in progress and will be reported elsewhere.

ACKNOWLEDGMENT

The authors thank Dr. Rosalie M. Baine, B. Hightower, D. L. Jue, and M. H. Johnson for assistance, and Dr. William A. Graham of the East Liverpool City Hospital, East Liverpool, Ohio, and Dr. Robert G. Gagliano, Las Vegas, Nevada for providing clinical information on the patient.

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