# HEMOGLOBIN CHAPEL HILL OR $\alpha_2{}^{74}$ Asp→Gly$_{\beta_2}$

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Received 7 April 1976

## 1. Introduction

While evaluating a middle-aged woman for persistant, low-grade erythrocytosis known to be present for many years, a new hemoglobin variant was discovered. This communication describes the structural characterization and some functional analyses of this mutant hemoglobin.

### 2. Materials and methods

Blood samples were collected in Vacutainers using EDTA or ACD as anticoagulant and were shipped air mail, special delivery, from Chapel Hill to Augusta, Ga. Hematological analyses were made by standard methods [1]. Red cell hemolysates were prepared by mixing 1 volume of saline-washed packed red cells, 1 volume of distilled water and 0.2 volume of carbon tetrachloride. Cellular debris was removed by centrifugation.

#### 2.1. *Hemoglobin studies*

Starch gel electrophoresis of red cell hemolysate used a Tris-EDTA-borate buffer, pH 9.0 [2]. Fetal hemoglobin ($F_{AD}$) was determined by the method of Betke et al. [3]. The hemoglobin variant was quantitated by column chromatography using DEAE-Sephadex [4,5]. The same procedure was used for the isolation of larger amounts of the variant. The abnormal hemoglobin was studied by hybridization with canine hemoglobin to define the variant chain [6].

#### 2.2. *Structural analyses*

Globin was prepared by the method of Anson and Mirsky [7], and was separated into its component chains by chromatography on carboxymethyl cellulose [8]. The $\alpha^X$ was isolated by freeze-drying after removal of urea and salts by gel filtration on 2.5 × 70 cm columns of Sephadex G-25 equilibrated with 0.5% (v/v) formic acid and water. About 130 mg of the α chain was digested with trypsin (Worthington Biochem. Corp.; TPCK trypsin) at room temperature for 6 h at pH 9.0 in a pH stat (Radiometer) with addition of enzyme at zero time and after 30 min in an enzyme to protein ratio of 1 to 100. The pH of the digest was lowered to 6.5, and the insoluble core isolated by centrifugation. The soluble tryptic peptides were separated on a 0.9 × 60 cm column of chromobead type P (Technicon Instruments) at 37°C using pyridine–acetic acid volatile buffers [9,10]. Isolated peptides were hydrolyzed with 6 M HCl under reduced pressure at 110°C for 24 h. Amino acid analyses were made with a Spinco model 121 automated amino acid analyzer equipped with high sensitivity cuvettes and an Infotronics integrator (Columbia Scientific Industries, Austin, Texas).

The core was oxidized with performic acid [11] and digested for 22 h with chymotrypsin at room temperature in a pH stat at pH 9.0. Enzyme was added at zero time and at 8 h in an enzyme to protein ratio of 1 to 50. After the pH was adjusted to 2.5, the digest was lyophilized, and the chymotryptic peptides were separated on a similar column of chromobead resin type P as used for the separation of the soluble tryptic peptides.

Peptide T-9 was digested with thermolysin; 1.8 μmol was dissolved in 6 ml of 0.25 M trimethylamine and the pH was adjusted to 8.0 with 0.2 M acetic acid. Approx. 150 μl of a thermolysin preparation which contained 1 mg enzyme/ml was added. Digestion took place for 6 h at 37°C. The thermolytic fragments were isolated by lyophilization and separated by Dowex 1-X2 chromatography [12]. Sequential degradation of T-9 by the Edman phenylthiohydantoin procedure followed the method of Schroeder [13].

#### 2.3. *Functional analyses*

The whole blood oxygen affinity was measured using the IL-517 system of the Instrumentation Laboratory, Inc. (Lexington, Mass., USA). Hb-Chapel Hill and Hb-A were isolated by DEAE-Sephadex chromatography and were dialyzed against 0.1 M bis-Tris buffer of desired pH. The oxygen affinity of these isolated hemoglobins was measured by the method of Benesch et al. [14,15].

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## 3. Results

### 3.1. *Case history*

The patient is a 45 year old Caucasian female who had an elevated hematocrit for at least fifteen years. Throughout this time her hematological parameters including white blood cell count, differential, platelet count, reticulocyte index, and red cell morphology have been completely normal. On physical examination, the patient demonstrates striking plethora of the face and palms. There is no detectable splenomegaly. Hematological data at the time of this study included: Hgb: 15.5 g/dl; PCV: 0.473 1/1; RBC: 5.17 × $10^{12}$/1; MCV: 92 fl; MCH: 32.3 pg; MCHC: 34.5 g/dl; 2,3-DPG: 16.3 μmol/gHb (normal control: 16.9 μmol/gHb); ATP: 4.8 μmol/gHb (normal control: 4.6 μmol/gHb). There is no evidence of shortened red cell survival. The red cell mass has varied between 31 and 35 cc per kg body weight on several determinations, values slightly above normal for our laboratory. Intravenous pyelography was normal, and an evaluation of her pulmonary function including arterial blood gas analysis, chest roentgenogram, and spirometry have also been within normal limits.

### 3.2. *Hemoglobin analyses*

Starch gel electrophoresis at pH 9.0 showed the presence of a major variant (Hb-X) with a mobility similar to that of Hb-S and a minor variant (Hb-$X_2$) which moves slower than Hb-$A_2$. DEAE-Sephadex chromatography gave the following quantitative data: Hb-$X_2$: 0.7%; Hb-$A_2$: 2.5%; Hb-X: 23.5%; Hb-A: 73.3%. The level of Hb-F (Hb-$F_{AD}$) was normal, namely 0.8%. Hybridization of the variant with canine hemoglobin confirmed the presence of an abnormal α chain in this hemoglobin type.

### 3.3. *Structural analyses*

Approx. 1400 mg of pure Hb-X was available; chain separation on columns of CM-cellulose provided over 400 mg of the abnormal α chain. The soluble tryptic peptides from a 6 h digest of 130 mg $\alpha^X$ chain were isolated by cation exchange chromatography, and amino acid analyses showed that all peptides (T-1, T-2, T-3, T-4, T-5, T-6, T-7, T-8, T-10, T-11, T-13, and T-14) except T-9 had the expected composition. Data from the analysis of T-9 are listed in table 1; it appears that one of the four aspartyl residues or, less likely, one of the two asparaginyl residues of this peptide is replaced by a glycyl residue (positions 64, 74, 75, and 85 are each occupied by aspartyl residue, and positions 68 and 78 by an asparaginyl residue).

Sequential analyses determined the first seven residues of peptide T-9 and excluded Asp in position 64 and Asn in position 68 from being replaced. Chromatography of the thermolytic digest on T-9 on a column of Dowex 1-X2 resulted in the recovery of four fragments. The amino acid composition of these peptides are listed in table 1. It appears that fragment

Table 1
Amino acid composition of αT-9 and some of its thermolytic fragments[a]

| Amino Acid | T-9 | Fragment[b] 1 | Fragment[b] 2 | Fragment[b] 3 | Fragment[b] 4 |
|---|---|---|---|---|---|
| Lysine | 1.02(1) | | | | 1.10(1) |
| Histidine | 2.76(3) | | 1.00(1) | | 1.96(2) |
| Aspartic acid | 4.81(6) | 1.00(1) | 1.06(2) | 0.90(1) | |
| Threonine | 1.17(1) | | 0.69(1) | | |
| Serine | 1.87(2) | | | 1.80(2) | |
| Proline | 1.12(1) | | | | |
| Glycine | 1.23(0) | | 0.83(0) | | |
| Alanine | 6.67(7) | 1.15(1) | 2.93(3) | 2.00(2) | 1.00(1) |
| Valine | 3.10(3) | 0.85(1) | 2.24(2) | | |
| Methionine | 0.60(1) | | | | |
| Leucine | 3.84(4) | | 1.00(1) | 1.93(2) | 0.90(1) |
| Residues in chain | 62–90 | 62–64 | 65–74 | 79–85 | 86–90 |

[a]Numbers between parentheses are the expected values based on analyses of the α chain of Hb-A.
[b]See Fig.1.

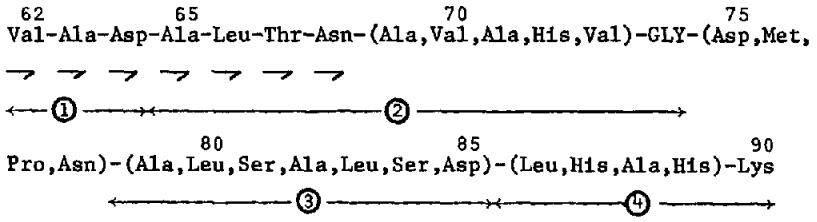


Fig.1. The sequence of T-9 of the α chain of Hb-Chapel Hill. The numbers above the residues indicate the positions in the intact chain, and the numbers in circles refer to thermolytic fragments which are also listed in table 1.

1 originates from positions 62–64, fragment 2 from positions 65–74, fragment 3 positions 79–85, and fragment 4 from positions 86–90; the fragment from positions 75–78 which contains a methionyl residue was not recovered (fig.1). The data show the expected compositions except for fragment 2 which contains one glycyl residue and one aspartyl residue (no glycyl residue, one asparaginyl residue in position 68, and one aspartyl residue in position 74 are present in the corresponding segment of the α chain of Hb-A). This information together with the sequence data of the amino terminus of the T-9 peptide indicate an Asp→Gly substitution in position 74 (fig.1). Such a replacement has not been observed before; therefore, the variant is named Hb-Chapel Hill after the place of medical evaluation of the patient. The chymotryptic peptides which were isolated from a digest of the oxidized core of the $\alpha^X$ chain (residues 99–139, inclusive) by cation exchange chromatography had the expected amino acid compositions, indicating the absence of an additional substitution.

### 3.4. *Functional analyses*

Oxygen equilibrium data for whole blood and isolated hemoglobin components are listed in table 2. The decrease in $P_{50}$ value for whole blood was small but fell outside the range of values found for normal adults (24.5–28.5 mm Hg) in our laboratories. This slight increase in oxygen affinity is also observed when data on isolated Hb-Chapel Hill and Hb-A are compared. The Bohr effect and the subunit interaction of the two hemoglobins are probably not different.

## 4. Comments

The substitution in Hb-Chapel Hill, $\alpha_2{}^{74\ \mathrm{Asp}\rightarrow\mathrm{Gly}}\beta_2$, involves a replacement of an aspartyl residue in position EF3, i.e. the third position of the interhelical EF segment. This aspartyl residue is not involved in contacts between chains nor in the contact with the heme group. Its counterpart in the β chain, i.e. aspartyl residue in position 79, forms a salt bond to lysyl

Table 2
Results of some oxygen equilibrium analyses

| Material | Temp. | pH | $P_{50}$ | W |
|---|---|---|---|---|
| Whole blood - Patient[a] | 37°C | 7.42 | 23.0 | 2.1 |
| Whole blood - Patient[b] | 37°C | 7.39 | 27.5 | 2.3 |
| pure Hb-Chapel Hill[b] | 26°C | 6.53 | 2.3 | 1.4 |
| | 26°C | 7.00 | 1.4 | 1.3 |
| | 26°C | 7.51 | 0.9 | 1.6 |
| pure Hb-A[b] | 26°C | 6.52 | 4.2 | 1.7 |
| | 26°C | 7.29 | 2.2 | 1.3 |
| | 26°C | 7.43 | 1.5 | 1.3 |

[a]The levels of 2,3-DPG were determined by the method of Grisolia et al. [16] and were 16.3 and 16.9 μmol/gHb, respectively.
[b]Isolated from red cell hemolysate of the propositus by DEAE-Sephadex chromatography [4,5].

residue in position 8 of the same chain when the hemoglobin is deoxygenated. However, no such bond has been reported for the Asp α-74 residue [17,18]. It is, therefore, unclear why the replacement of this residue by a glycyl residue would affect the oxygen binding properties of the hemoglobin molecule; the differences that have been observed are small but appear to indicate a slight increase in oxygen affinity.

Two other variants have been observed that involve substitutions of the same aspartyl residue; these are Hb-Mahidol also known as Hb-G-Taichung and Hb-G-Thailand in which the substitution concerns a histidyl residue [19,20], and Hb-G-Pest in which the aspartyl residue is replaced by an asparaginyl residue [21]. These two variants do not exhibit significant change in their functional properties. It appears, therefore, rather doubtful that the presence of some 25 percent of Hb-Chapel Hill with its slight increase in oxygen binding properties would result in a striking erythrocytosis but could perhaps cause a slight increase in total red cell mass as was observed in our patient.

**Acknowledgement**

This research was in part supported by US Public Health Service Research Grants HL-05168 and HL-15158.

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