Characterization of a New Electrophoretically Silent Hemoglobin Variant

Hb Saale OR $\alpha_2\beta_2$ 84(EF8)Thr → Ala$	extsuperscript{a}$

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A new abnormal hemoglobin was detected in a young German anemic patient by cation-exchange high performance liquid chromatography (HPLC). Using a combination of electrospray mass spectrometry, HPLC, direct sequencing, and family screening with polymerase chain reaction/restriction digestion approach, we have characterized this hemoglobin variant as resulting from a Thr → Ala replacement at $\beta$84(EF8). It could be separated neither by electrophoresis nor by isoelectric focusing. Hb Saale is slightly unstable, exhibiting a moderate tendency to auto-oxidize. Functional properties and the heterotropic interactions are similar to those of Hb A.

Recently we had the opportunity to analyze blood samples from a 3-year-old German girl who participated in a screening program for hemoglobinopathies in anemic infants. She was found to be heterozygous for a new hemoglobin variant, designated hemoglobin Saale (Hb Saale) after the name of the river crossing the city where the propositus lived.

In this paper we describe the characterization of this abnormal Hb using a series of protein chemistry and molecular biology approaches. Hematological findings on the propositus and her relatives are also presented.

EXPERIMENTAL PROCEDURES

Blood Samples—Samples collected both into tubes containing EDTA and not containing any anticoagulant were obtained from the propositus and nine family members. Informed consent was obtained prior to collection.

Hematological and Hemoglobin Analyses—Hematologic data were obtained with automated cell counters, while other routine parameters were determined by standard methods. The red cell lysates were examined by electrophoresis on agarose gel at pH 8.7 and 6.0, by isoelectric focusing (IEF), and by different stability tests performed as reported previously (1). Various erythrocyte enzymes were quantified by the procedure described by Beutler (2, 3). The oxygen affinity of the whole blood was determined as reported previously (4). The abnormal hemoglobin was quantified by cation-exchange high performance liquid chromatography (HPLC) and by reverse-phase HPLC (5), which was also used to purify hemoglobin chains.

Functional Studies—The hemolysate was stripped of anions by passage through a mixed ion-exchanger column. The purified Hb Saale and Hb A fractions were isolated by PolyCAT A HPLC (using bis-Tris-KCN buffer) and concentrated by ultrafiltration using 10-kDa cut-off membranes.

Oxygen binding properties of the hemolysate were measured by a continuous method using the Hemox analyzer (TCS, Southampton, PA) at 25 °C in 50 mM bis-Tris buffer to which 50 μM Na-EDTA and catalase (20 μg/ml) were added to limit metHb formation (6). The Hb concentration was 60–70 μM on a heme basis. The hemoglobin content was calculated from the optical spectrum recorded at the end of the oxygen equilibrium measurements. $P_{50}$ and $n_{50}$ values were calculated by linear regression from the Hill equation for oxygen saturation levels between 40 and 60%. The magnitude of the DPG effects was calculated as a $\Delta \log P_{50} = [1 \text{ mM DPG}]$. For all conditions, the data for the hemolysate were compared with data for stripped Hb A obtained under identical conditions.

The kinetics of carbon monoxide recombination, after photodissociation by 10-ns pulses at 532 nm, were measured as described previously (7). Experiments were made at 25 °C, pH 7, for 60 μM (on a heme basis) samples.

The rate of auto-oxidation for the hemolysate was measured by adsorption spectrophotometry (SLM-Amino DW2000) at 37 °C under air in 20 mM potassium phosphate buffer, pH 7.0 (hemoglobin concentration was 40 μM on heme basis).

DNA Sequencing of $\beta$ Gene—Genomic DNA was isolated from the white cells using disposable columns (8). Sequencing of the $\beta$-hemoglobin gene (from 130 bp upstream of the cap site to position 109 in intron 2 and from nucleotide 640 in intron 2 to 190 bp downstream of the termination codon) was performed by the dyeoxy-chain termination method (9) as described previously (8).

Protein Structural Analyses—The procedures described previously (8) were used. These include electrospray mass spectrometry (ESMS) analyses of crude hemolysate and purified $\beta$ chains, liquid chromatography mass spectrometry (LC-MS) analysis of $\beta$ chains digested with endoproteinase Lys-C, and amino acid analysis of selected fractions using Edman degradation. Inclusion bodies observed within the erythrocytes of the affected subjects were isolated by the method of Fessas et al. (10). The elucidation of the nature of the inclusions followed the methodology described above.

The three-dimensional structure of the deoxygenated Hb A (T state) was determined using the VISP program (22) on a Silicon Graphics 4D25G work station. The Hb A crystallographic coordinates were taken from the file 3HHB (Protein Data Bank, Rutgers University, New Brunswick, NJ) reported by Fermi et al. (11).

RESULTS

The Family—The propositus, a 3-year-old German girl, was anemic with a hemoglobin of 9.6 g/dl. She showed at this age a normal physical and intellectual development. The routine Hb electrophoresis was normal, but further examination with cat-

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ion-exchange HPLC showed that the patient was a heterozygous carrier of an abnormal hemoglobin (Hb X) eluting close to normal Hb A. Of the eight other members of the family examined, three were carriers of the same Hb variant (Hb X). Hb X represented between 37.5 and 40.0% of the red blood cell total Hb content. Hematological and biochemical parameters of the propositus, together with those of the examined members of the family, are listed in Table I.

Erythrocyte enzyme levels were within the normal range, except for the grandfather (I-2) who was found to have a glucose-phosphate-isomerase (GPI) deficiency. The GPI activity of I-2 was 17.2 units/g Hb, which represents about 55% of the normal mean. It showed a residual activity of 62.6% after 2-h incubation at 45 °C (data not shown), indicating that the patient is a heterozygous carrier for the deficiency. None of the descendants of I-2 inherited the GPI deficiency.

In affected subjects peripheral blood smears stained with brilliant cresol blue showed red cells carrying Heinz bodies, which ranged from 22 to 32% (normal value: <10%), but the spleen was not palpable, and they had never required treatment for jaundice. The serum concentration of the indicators of jaundice. The serum concentration of the indicators of the results obtained by reverse-phase HPLC was digested with Lys-C, and the resulting peptide mixture was analyzed by LC-MS. Fig. 3 illustrates the separation of Lys-C digest peptides using LC-MS mode, and the selected peptides are listed in Table II. All peaks appeared at a retention time and with a mass expected for a wild-type β chain except two peaks, 8 and 9, that eluted within 50.5 and 51.0 min (Fig. 3). As elucidated in Table II, peak 9 is heterogeneous, consisting of two peptides, oxidized T3-4 and T10. Peak 8 displayed a mass of 4217.42 Da, which is 30 Da lower than the peptide of the wild-type βT10-T11-12 (T10 and T11-12 are linked by a disulfide bond). This peptide was therefore suspected to be carrying the abnormal residue affecting the β chain. It was collected and submitted to 30 cycles of Edman degradation. As expected for T10 and T11-12, each of the 13 first cycles yielded two different phenylthiohydantoin-derivatives. All phenylthiohydantoin-derivatives detected were consistent with those predicted for T10 and T11-12 from the wild-type β chain (Table II), with the exception on one released at the second cycle. Histidine and alanine were observed instead of histidine and threonine. This indicates clearly that a mutation affects position 2 of the peptide T10, involving the substitution of alanine for threonine. Thus, the site of mutation was localized at residue 84. Indeed, this mutation induces a difference in mass of minus 30 Da, as observed by mass measurements in the crude hemolyte (Fig. 2).

The DNA analysis of the propositus revealed heterozygosity for A to G transition in codon 84 (ACC to GCC) in the second exon of the β-globin gene that substitutes alanine for threonine (Fig. 4). This missense (Thr84 → Ala) was also examined by a restriction endonuclease assay, because the change of A to G on the nucleotide level creates a new recognition site for the Hhal restriction enzyme. The digestion of a 782-bp PCR product from mutant allele results in a 179- and 603-bp-long fragments. It was used for the rapid detection of ACC→GCC change at codon 84 in the DNA samples from three additional members of the family. No other mutant was found. Fig. 5 shows the relative position of Thr84 in the three-dimensional structure of Hb A in T state.

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**Table I**

| Pedigree no. | I-1 | I-2 | I-3 | I-4 | I-5 | I-6 | I-7 | I-8 | I-9 |
|-------------|-----|-----|-----|-----|-----|-----|-----|-----|-----|
| Sex/age     | F/63| M/63| F/50| M/55| M/28| M/28| M/24| F/5 | F/7 |
| Hb (g/dl)   | 14.5| 16.9| 14.4| 13.9| 11.2| 15.3| 13.3| 10.5| 13.7|
| PVC (liter/liter) | 0.433 | 0.499 | 0.431 | 0.413 | 0.343 | 0.461 | 0.391 | 0.315 | 0.408 |
| Red blood cell (10^12/liter) | 4.66 | 5.18 | 4.69 | 4.00 | 4.03 | 5.10 | 4.58 | 3.87 | 4.85 |
| MCV (fl)    | 92.1| 96.3| 91.9| 88.4| 84.8| 90.2| 85.3| 81.4| 83.8|
| MCHC (g/dl) | 31.1| 32.6| 30.7| 29.1| 27.9| 30.0| 29.1| 27.1| 28.1|
| Reticulocytes (%) | 0.75 | 1.26 | 0.78 | 1.11 | 0.87 | 0.97 | 0.83 | 0.70 | 1.08 |
| Heinz bodies (%) | 22 | 32 | 6 | 32 | 31 | 5 |
| EPO (mIU/ml) | φ | φ | φ | φ | 8.0 | 9.0 | 10.9 | 8.0 | 6.0 | 9.0 |
| Hb X (%)    | 42.4 | 45.2 | 39.8 | 40.9 |
| Hb A2 (%)   | 2.2 | 2.1 | 2.3 | 2.3 | 2.9 | 2.2 | 2.4 | 2.3 | 2.5 |
| P50 (mm Hg) | 27.6 | 29.7 | 26.3 | 34.4 | 31.2 | 27.6 | 30 | 33 | 25.9 |

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**Notes:**
- Whole blood.
- Heterozygous for the GPI deficiency.
the presence of 1 mM DPG or at pH 6.5 are also identical to those of Hb A in the same experimental conditions. These data indicate that Hb Saale and Hb A display undistinguishable O₂ binding properties, including the DPG and Bohr effects (Table III).

It was not possible to record the oxygen equilibrium curve of the purified Hb Saale samples due to the large methemoglobin content. We were unable to avoid appreciable formation of methemoglobin during the purification procedures. Nevertheless, the recombination traces of CO after photodissociation were the same for the purified Hb Saale and Hb A samples (Fig. 6), confirming similar ligand binding properties for these two hemoglobins.

At 37 °C, the oxidation rate of the hemolysate in air was slightly increased compared with that of Hb A solution (by a factor of 1.5), indicating that Hb Saale exhibits a small propensity to auto-oxidize faster than Hb A. This finding is consistent with the moderate instability found for this Hb variant by heat stability (data not shown).

**DISCUSSION**

Hb Saale is the second Hb variant resulting from a mutation at ß84(EF8), but the replacement of threonine by alanine at this position, as well as at others positions had never been reported in human hemoglobin before (12). The inverse has been reported in variants such as Hb Mantes-La-Jolie (a79(EF8)Ala → Thr (13)), Hb Mosella (a111(G18)Ala → Thr (13)), and Hb F-Baskent (Ay128(H6)Ala → Thr (14)). Mutant hemoglobins with nearly the same surface charge as Hb A due to neutral substitutions are difficult to detect by classical techniques based on electrophoretic or chromatographic behavior of Hb variants. Therefore, the separation of Hb Saale from Hb A by cation-exchange HPLC is rather unexpected. In addition, the substitution of alanine for threonine at residue 84 is not consistent with the relative reverse-phase elution time based on hydrophobicity (15) of ßSaale. The amount (37–47%) of variant found in the blood may be due to the incomplete separation of Hb A and Hb Saale by cation-exchange HPLC (Fig. 1). There is no special function attributable to residue 84 in the helical notation (16, 17). However, Hb Saale contrasts with Hb Kofu.
Hb Saale, a Slightly Unstable Hb with a Moderate Auto-oxidation

Fig. 3. Signal monitored by UV absorption at 214 nm (a) and base peak intensity (BPI) of the spectrum (b). Peak 8 (elution time = 50.5 min) yielded a mass spectrum showing a mass of 30 Da lower than the peptide \( \beta \text{T}10-\text{T}11-\text{T}12 \) of the wild type.

Fig. 4. Direct nucleotide sequencing of the sense strand showing the A to G substitution in codon 84.

Fig. 5. Three-dimensional structure of the Thr\(^{84} \) site of Hb A in the T state obtained by using the VISP program (academic gift courtesy of E. De Castro and J. S. Edelstein, University of Geneva, Switzerland) with a silicon Graphics 4D25G work station.

The slightly increased oxidation rate displayed by the hemolysate containing Hb Saale indicates that the substitution of Thr for Ala at \( \beta^{84} \) position could induce some small structural modification near the heme pocket, even if this residue is located at the surface of the hemoglobin molecule (Fig. 5). An

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**TABLE II**

| Peak no. (ET) | Experimental mass (Da) | Interpretation | Theoretical mass (Da) |
|--------------|------------------------|----------------|-----------------------|
| 1 (22.9)     | 952.13 ± 0.47          | T1: V\(^1\)HLTPEEK\(^*\) | 952.08               |
| 2 (28.9)     | 1149.56 ± 0.15         | T13: E\(^{121}\)FPPPVGAAKYK\(^{122}\) | 1378.55 |
|              | 1378.09 ± 0.42         | T14: V\(^{135}\)VAGVANALAHK\(^{144}\) | 1378.55 |
| 3 (31.4)     | 1449.84 ± 0.19         | T14–15: V\(^{135}\)VAGVANALAHKYH\(^{144}\) | 1449.88 |
| 4 (32.2)     | 932.04 ± 0.37          | T2: S\(^{2}\)AYTALWKG\(^{27}\) | 932.09               |
| 5 (37.2)     | 1797.90 ± 0.50         | T8–9: K\(^{16}\)VLGAFSDGLAHLDNLK\(^{42}\) | 1798.07 |
| 6 (37.7)     | 1798.23 ± 0.11         | T8–9: K\(^{16}\)VLGAFSDGLAHLDNLK\(^{42}\) | 1798.07 |
| 7 (38.1)     | 1669.91 ± 0.49         | T9: V\(^{16}\)LGAFSDFGLHLDNLK\(^{42}\) | 1669.90 |
| 8 (50.5)     | 4217.42 ± 0.39         | T10: G\(^{20}\)FATLSELHC DK\(^{40}\) linked by disulfide bond to \( \text{T}11–12: \) | 4247.93–30 |
|              | 4627.67 ± 0.33         | L\(^{20}\)HVDPEENFRLLGNVLVCLAHHPK\(^{120}\) with mass default of 30 Da | 4628.20 |
| 9 (51.0)     | 1149.56 ± 0.15         | T10 linked by disulfide bond to \( \text{T}11–12–13: \) | 5590.45–30 |
|              | 4611.02 ± 0.48         | L\(^{20}\)HVDPEENFRLLGNVLVCLAHHPK\(^{120}\) with mass default of 30 Da | 4612.20 |

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(\( \beta^{84} \text{(EF}8 \) Thr \( \rightarrow \) Ile (18)) in several characteristics. Hb Kofu is readily detected by IEF, while Hb Saale is not. The slight decrease of pH with regard to Thr \( \rightarrow \) Ala substitution seems to reflect a conformational change that probably is responsible for the moderate instability and for the chromatographic behavior of Hb Saale. The mutation in the case of Hb Kofu was associated neither with hemoglobin instability nor with the abnormality of the oxygen affinity. Both the oxygen equilibrium studies on the hemolysate containing 40% of Hb Saale and the CO recombination kinetics after flash photodissociation on the purified Hb Saale sample indicate identical functional properties compared with normal Hb A. Also, the heterotropic effects, evaluated on the hemolysate, are similar to those of Hb A. These results are consistent with those described for Hb Kofu (18).
alanyl in position β84 cannot make the hydrogen bond formed by the atom OG1 of Thrβ84 with the main chain carbonyl of asparagine β80. This may alter the interaction energies within the coil EF8, but it is hard to predict what effect this might have on the oxidation rate of the heme. It is worth mentioning that the same situation formed in Hb Saale is present in normal human α-Hb chains, where an Ala is formed in position EF8 (α79), characterized by faster auto-oxidation rates compared with normal β chains (19).

The presence of inclusion bodies in red cells of the peripheral blood from Hb Saale carriers may be due to the propensity of this variant to auto-oxidize. The presence of Heinz bodies in red cells from patients with unstable hemoglobin variants has been demonstrated in several occasions (20, 21). Severe unstable Hb syndromes are associated with variable hemolysis and a relative high reticulocyte count. The disorder in the Hb Saale carriers bears no resemblance to these conditions. Indeed, clinical and laboratory data gave no indication for increased hemolysis and for an ineffective erythropoiesis. In addition, de-

TABLE III
Oxygen binding properties of purified Hb A/Hb Saale hemolysate

| Experimental conditions | Hemolysate | Hb A/ Hb Saale | P50 | n50 |
|-------------------------|------------|---------------|-----|-----|
| pH 7.2                  | 5.1        | 2.7           | 5.3 | 2.7 |
| pH 7.2 + DPG = 1 mM     | 16.5       | 2.8           | 17.6| 2.5 |
| pH 6.5                  | 11.3       | 2.6           | 11.2| 2.4 |

Heterotropic effects of purified HbA/Hb Saale hemolysate

Bohr effect (ΔlogP50/ΔpH) -0.49
DPG effect (ΔlogP50 ± 1 mM DPG) +0.51

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