Detection of a Thalassemic $\alpha$-Chain Variant (Hemoglobin Groene Hart) by Reversed-Phase Liquid Chromatography

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BACKGROUND: Hemoglobin (Hb) Groene Hart [$\alpha_{119}$ (H2)Pro→Ser ($\alpha1$)], also known as Hb Bernalda, is a nondeletional $\alpha$-thalassemic Hb variant that is frequent in southern Italy and North Africa. This variant is not supposed to be produced in the erythrocytes of carriers. The $\alpha$-thalassemic behavior of this variant has been explained as an impaired interaction between the $\alpha$-globin chain and the $\alpha$-Hb-stabilizing protein.

METHODS: To separate globin chains, we developed a modified reversed-phase liquid chromatography (RPLC) procedure that uses acetonitrile–water solvents containing up to 3 mL/L trifluoroacetic acid. After RPLC, we characterized the isolated globin chains by electrospray ionization (ESI) mass spectrometry (MS) and analyzed their tryptic peptides with matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) MS and nano-LC–ESI–MS/MS.

RESULTS: RPLC detected an abnormal peak with a retention time substantially greater than that of the wild-type $\alpha^A$-globin chain. We identified this variant as Hb Groene Hart and found it in the hemolysates of 11 unrelated patients (1 homozygote, 9 heterozygotes, and 1 heterozygote associated with the $\alpha^3.7$ deletion). These patients possessed abnormal hematologic features suggesting an $\alpha$-thalassemia phenotype. Molecular modeling suggested that the increase in hydrophobicity was due to opening of the GH interhelical segment following replacement of amino acid residue 119 with a nonhelix breaker residue.

CONCLUSIONS: This method allows the detection of Hb variants at low concentrations, and adjusting the composition of the organic solvents enables the method to identify Hb variants with large changes in hydrophobicity.

Approximately 900 human hemoglobin (Hb)$^6$ variants involving the $\alpha$ and $\beta$ chains have been described (1). A few $\alpha$-chain variants cause changes in the 3-dimensional structure of the Hb complex that impair the formation of the Hb $\alpha\beta$ dimer and/or tetramer (1–3), leading to the formation of highly unstable free $\alpha$-Hb species. When present in low amounts, such species degrade rapidly; at higher concentrations, they precipitate within erythrocytes and erythrocyte precursors to form insoluble inclusions and cause substantial oxidative stress to erythrocyte membranes (Heinz bodies). Free $\beta$-globin is concurrently synthesized normally and accumulates within the erythrocyte, possibly forming Hb H ($\beta_4$). This imbalance between $\alpha$- and $\beta$-globins is typical of $\alpha$-thalassemia. Assembly of the Hb $\alpha\beta$ dimer depends on the presence of a chaperone protein, the $\alpha$-Hb–stabilizing protein (AHSP), which forms a heterodimer with $\alpha$-Hb in erythroid precursors (4). Interactions between AHSP and $\alpha$-Hb involve the last 2 $\alpha$-helices (G and H) of the $\alpha$ chain (5, 6). Mutations in these helices impair the formation of the Hb $\alpha\beta$ dimer and thus decrease the availability of $\alpha$ chains for assembling the Hb complex, thereby increasing the pool of free $\alpha$ chains.

Mutations at position $\alpha_{119}$ (H2)Pro have recently been demonstrated to impair the AHSP/$\alpha$-Hb interaction (7). Under such conditions, the mutated $\alpha$-globin chain is rapidly proteolyzed. In previous investigations of patients carrying the Groene Hart mutation...
The apparent absence of the protein was in agreement with electrophoretic methods, liquid chromatography (LC), or electrospray ionization mass spectrometry (ESI MS). The mutation was identified by DNA analysis. This apparent absence of the protein was in agreement with the borderline \( \alpha \)-thalassemic phenotype found in these carriers. Hb Groene Hart was thus hypothesized not to be present as an Hb species in vivo.

In the present study, we developed a reversed-phase LC (RPLC) method to investigate whether or not Hb Groene Hart occurs as an Hb species in vivo. We isolated the \( \alpha \) \text{Groene Hart} globin chain by preparative RPLC, confirmed its identity via DNA sequencing, and fully characterized this globin chain by MS.

**Materials and Methods**

**Patients and Hematologic Procedures**
The unrelated patients included in this study were 1 homozygous patient (\( \alpha \alpha \) \text{Groene Hart}/\( \alpha \alpha \) \text{Groene Hart}), 1 Hb Groene Hart heterozygote associated with a heterozygous \( \alpha \)-thalassemia 3.7-kb deletion (\( -\alpha^{3.7} \)/\( \alpha \) \text{Groene Hart}), and 9 heterozygotes (\( \alpha \) /\( \alpha \) \text{Groene Hart}). Blood samples were drawn into collection tubes containing EDTA, and hematologic data were collected via standard methods. Hemolysates were analyzed by isoelectric focusing on polyacrylamide gels (11, 12), ion-exchange LC (Variant I™, Beta-Thalassemia Short Program; Bio-Rad Laboratories) (11, 12), and RPLC of globin chains.

We used the hematologic results to check for the presence of a thalassemic phenotype and then investigated patients with borderline \( \alpha \)-thalassemic erythrocyte data when we detected an abnormal peak that eluted at a high percentage of solvent B in the RPLC analysis (see below). DNA analysis was used to confirm the presence of the Hb Groene Hart mutation.

**RPLC of Globin Chains**
RPLC analyses were performed on a C4 Uptisphere column (4.6 \( \times \) 250 mm, 5-\( \mu \)m particle size, 30-nm mean pore size; Interchim). We eluted globin chains with a 2-solvent system [solvent A, 100 mL/L acetonitrile and 3 mL/L trifluoroacetic acid (TFA) in water; solvent B, 70 mL/L acetonitrile and 3 mL/L TFA in water] and a 3-step RPLC elution program consisting of a linear gradient of 50%–70% solvent B in 80 min, a linear gradient of 70%–90% solvent B in 10 min, and reequilibration with 50% solvent B for 10 min. Elution conditions were modified for some experiments, as is described later. The flow rate was 1 mL/min, and eluate absorbance was monitored at 220 nm. Samples were prepared from hemolysates adjusted to an Hb concentration of 40 g/L and were diluted further with water (75 \( \mu \)L hemolysate plus 925 \( \mu \)L water). We injected 20 \( \mu \)L of sample into the column for each assay. The same conditions were used in preparative RPLC runs, with the exception that the 20-\( \mu \)L injection volume was drawn from a sample dilution consisting of 225 \( \mu \)L hemolysate and 775 \( \mu \)L water.
calibration was carried out with the singly charged monoisotopic peaks produced with a peptide mixture from the Sequazyme™ Peptide Mass Standards Kit (Applied Biosystems).

**NANO-LC-ESI-MS/MS ANALYSIS OF TRYPIC PEPTIDES**

Experiments were performed on a Q-STAR® XL instrument (Applied Biosystems) equipped with a nanospray source equipped with a distal coated SilicaTip™ emitter (cat. no. FS150-20-10-D-20; New Objective) set at 2300 V. The instrument’s Information Dependent Acquisition (IDA) mode allowed peptide ions within a survey scan range of m/z 400–2000 to be analyzed for subsequent fragmentation. Tandem MS spectra were acquired in the m/z 65–2000 range for +2 to +4 charged ions. The collision energy was automatically set by the software (Analyst 1.1) and was related to the mass and charge of the precursor ion. The MS and MS/MS data were recalibrated with internal reference ions from a tryptic autolysis peptide at m/z 842.510 [M+H]+ and m/z 421.759 [M+2H]2+. We injected 5 µL of the acidified solution and separated the tryptic peptides with a Dionex UltiMate NanoLC system equipped with a Dionex C18 PepMap Micro Precolumn (300 µm × 5 mm, 5-µm particle size, 10-nm pore size) and a Dionex C18 PepMap nano-column (75 µm × 150 mm, 3-µm particle size, 10-nm pore size). We used a 2-solvent chromatography system (solvent A, 1 mL/L formic acid and 50 mL/L acetonitrile in water; solvent B, 0.8 mL/L formic acid and 800 mL/L acetonitrile in water) and separated the tryptic peptides with a 60-min linear gradient of 0%–50% solvent B at a flow rate of approximately 200 nL/min.

**DNA ANALYSIS**

Genomic DNA was extracted from whole blood as previously described (11, 12). The sequence of the Hb Groene Hart mutation was identified via PCR of the HBA17 (hemoglobin, alpha 1) and HBA2 (hemoglobin, alpha 2) genes and direct DNA sequencing of the PCR products on a LI-COR 4200 sequencer (LI-COR Biosciences). The primers used for amplifying the α-globin genes have previously been described (13). In addition, we screened for common α-thalassemic deletions by gap multiplex PCR (14).

**MOLECULAR MODELING**

The structure of the mutated Hb was drawn with the aid of Swiss-PdbViewer (15). The reference model was the deoxy-Hb structure (pdb 3hbb file).

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**Results**

**HB-AND DNA ANALYSES**

Isoelectric focusing and ion-exchange LC did not detect the presence of an Hb variant; however, we did observe an abnormal peak with RPLC. DNA sequencing revealed the mutation CCT→TCT (Pro→Ser) in codon 119 of the third exon of the HBA1 gene. Gap multiplex PCR analysis of 1 patient revealed an association with a heterozygous −α3.7 deletion.

In the homozygous case involving a 26-year-old woman of Algerian origin, the αGroene Hart-globin chain represented 19% of the α-globin chains. With an RPLC gradient of 50%–68% solvent B over 80 min, the retention time for the αGroene Hart-globin chain was 83.53 min, compared with 53.38 min for the α chain. This patient exhibited hypochromia and microcytosis as well as nonpathologic Hb A2, and F concentrations that strongly suggested α-thalassemia (erythrocyte count, 5.44 × 1012/L; Hb, 121 g/L; mean corpuscular hemoglobin, 22.3 pg; mean corpuscular volume, 72 fl; Hb A2, 3.3%; Hb F, 0.5%); however, the results of a search for a common α-thalassemic deletion were negative.

The compound heterozygote was a 4-year-old girl who showed values for hematologic variables that were similarly abnormal (erythrocyte count, 5.20 × 1012/L; Hb, 114 g/L; mean corpuscular hemoglobin, 21.9 pg; mean corpuscular volume, 67 fl; Hb A2, 2.5%; Hb F, 0.6%). Her parents were originally from Tunisia. With the elution conditions given in Materials and Methods (50%–70% solvent B), the αGroene Hart-globin chain had a retention time of 75.47 min, compared with 49.82 min for the α chain (Fig. 1). The αGroene Hart-globin chain constituted 17.8% of the α-globin chains. Gap multiplex PCR analysis revealed a heterozygous −α3.7 deletion. The Hb Groene Hart mutation in this patient was located on the intact HBA1 gene (genotype, −α3.7/ααGroene Hart), as previously described (16). The hematologic and biochemical data for the 9 unrelated heterozygotes (3 from Algeria, 2 from Morocco, 3 from Tunisia, and 1 of undetermined origin) are summarized in Table 1. We detected no Hb H (β4) in any of these patients.

**MS STUDIES**

**ESI MS ANALYSIS OF GLOBIN CHAINS**

The ESI MS analysis of the globin chains showed a mean (SD) molecular mass of 15 116.8 (1.4) Da for the αGroene Hart chain and 15 126.3 Da for the wild-type α chain (Fig. 2). The experimentally obtained shift in mass was −9.5 Da. The expected theoretical mass shift was −10.0 Da.

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2 Human genes: HBA1, hemoglobin, alpha 1; HBA2, hemoglobin, alpha 2.
MALDI-TOF MS ANALYSIS OF TRYPTIC PEPTIDES

The results of MALDI-TOF MS analyses of hydrogen peroxide–treated tryptic digests for the α\textsuperscript{Groene Hart}–globin and wild-type α-globin chains are shown in Fig. 1 of the Data Supplement that accompanies the online version of this article (http://www.clinchem.org/content/vol?/issue?). According to the ExPASy Molecular Biology Server \((17)\), the theoretical masses for the protonated wild-type α\textsuperscript{T-12} peptide and the Hb Groene Hart α\textsuperscript{T-12} peptide are 2967.61 Da and 2957.59 Da, respectively. Under these experimental conditions, the cysteinyl residue of the α\textsuperscript{T-12} peptide was oxidized to a cysteic acid residue, yielding a mass difference of +47.985 Da (see Fig. 2 in the online Data Supplement); thus, the theoretical masses for the wild-type and mutated α\textsuperscript{T-12} protonated peptides are 3015.59 Da and 3005.57 Da, respectively.

NANO-LC–ESI–MS/MS ANALYSIS OF TRYPTIC PEPTIDES

The MS/MS experiments with the mutated α\textsuperscript{T-12} peptide (3004.60 Da; \(m/z = 752.17\); charge = +4) showed a characteristic series of y daughter ions that allowed localization of the Pro–Ser substitution to position 119 (see Figs. 2 and 3 and Tables 1 and 2 in the online Data Supplement). These experiments confirmed the oxidation of the cysteine residue at position 104.

Table 1. Hematologic and biochemical data for patients heterozygous for Hb Groene Hart.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Median</th>
<th>Range (n = 9)</th>
<th>Reference interval</th>
</tr>
</thead>
<tbody>
<tr>
<td>Erythrocytes, (\times 10^{12}/\text{L})</td>
<td>4.74</td>
<td>4.08–5.68</td>
<td>3.80–5.80</td>
</tr>
<tr>
<td>Hb, g/L</td>
<td>121</td>
<td>90–164</td>
<td>115–145</td>
</tr>
<tr>
<td>MCV, fl</td>
<td>73</td>
<td>66.4–81.7</td>
<td>80–100</td>
</tr>
<tr>
<td>MCH, pg</td>
<td>24.7</td>
<td>22–29.1</td>
<td>27–32</td>
</tr>
<tr>
<td>RDW, %</td>
<td>13.5</td>
<td>13–19.1</td>
<td>11–14</td>
</tr>
<tr>
<td>Reticulocytes, %</td>
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<td>0.6–1.2</td>
<td>0.50–1.50</td>
</tr>
<tr>
<td>Hb A\textsubscript{2}, %</td>
<td>3</td>
<td>2.4–3.2</td>
<td>&lt;3.5</td>
</tr>
<tr>
<td>Hb F, %</td>
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<td>0.1–1.4</td>
<td>&lt;2</td>
</tr>
<tr>
<td>(\alpha\textsuperscript{Groene Hart}, %)</td>
<td>5.9</td>
<td>4.7–6.5</td>
<td>—</td>
</tr>
</tbody>
</table>

*MCV, mean corpuscular volume; MCH, mean corpuscular hemoglobin; RDW, red cell distribution width.

Discussion

We have shown that Hb Groene Hart is actually produced at a low concentration in affected individuals. In the first description of Hb Groene Hart, Harteveld et al. \((8)\) reported detecting no abnormal native or denatured fraction with electrophoresis, isoelectric focusing, ion-exchange HPLC, denaturing PAGE, and ESI MS. These investigators suggested that the mutated gene product was an abnormal monomer unsuitable for assembly into a normal Hb tetramer and that it was quickly catabolized by the ubiquitin-related proteolytic pathway \((18)\). The mutation occurred in the heterozygous state in 2 Moroccan patients and was accom
panied by typical α-thalassemic features with a balanced or slightly increased β/α ratio in globin chain synthesis. The mutation had also been found in a Tunisian child in association with the α-thalassemic – α3.7 deletion, and except for the expected presence of Hb Barts (γδ) at birth, no abnormal Hb was detected with routine analytical techniques (9). More recently, Giordano et al. (10) were the first to describe a case of Hb Groene Hart in the homozygous state, in a Moroccan female. They confirmed that a thalassemia phenotype was associated with this abnormal Hb variant. In addition, this Hb variant has frequently been found in southern Italy, where it is known as Hb Bernalda (1).

Hb Groene Hart is a routine finding in reference laboratories during the screening of large populations of North African origin for suspected α-thalassemia. The hematologic indication is usually present, although this feature varies among individuals (see Results). After the common deletions have been excluded with gap multiplex PCR analysis, sequencing of the α-globin genes reveals the presence of point mutations, and multiplex ligation-dependent probe amplification may or may not show the presence of large deletions (19). When a common α-thalassemic deletion is found, the genes coding α chains are not routinely sequenced to search for additional point mutations. In such cases, the method we have described may represent a protein-level diagnostic alternative to molecular analysis.

The discovery of AHSP and its properties has altered our understanding of Hb assembly (4–7). AHSP stabilizes α-Hb during Hb synthesis by acting as a chaperone to protect free α-Hb chains from oxidative stress and by facilitating their folding (20, 21). In vitro experiments with recombinant Hb proteins with mutations at amino acid residue 199(H2)Pro of the α-globin chain [Hb Groene Hart (→Ser) and Hb Diamant (→Leu)] have revealed impaired interactions between α-Hb and AHSP (22). The RPLC system used in our study clearly detected these 2 Hb variants: We observed a slight 3.17-min increase in the retention time for the αDiamant−globin chain relative to that of the αGroene Hart−globin chain (data not shown).

The integrity of helix H and the GH corner of the α-globin chain appears to be critical for the interaction between α-Hb and AHSP. With the exception of a few amphibians, a Pro residue occupies position H2 in the α-chain sequences of more than 280 animal species (23). The existence of a difference between the αGroene Hart− and αDiamant−globin chains in the AHSP−association constant could explain the observed differences in the clinical and biochemical phenotypes of these 2 Hbs (7). Molecular modeling with the SwissPdbViewer suggests that replacing the Pro residue at

Fig. 2. ESI MS analysis of the αGroene Hart globin chain. The mean (SD) experimental mass calculated after deconvolution is 15 116.8 (1.4) Da.
the GH corner accounts for the drastic increase in hydrophobicity, which is similar to that observed with Hb Nouakchott [\(\alpha114(GH2)\text{Pro}\rightarrow\text{Leu}\)] (24). In these Hb variants, replacing the Pro residue with a nonhelix breaker residue may facilitate the opening of the GH corner, thereby exposing a series of residues with hydrophobic side chains (Fig. 3). It is possible that replacing a Pro residue at position 114 or 119 with a Leu residue would retain some of these hydrophobic contacts in the cellular environment. This hypothesis would explain the absence of a thalassemic phenotype in individuals with Hb Diamant and Hb Nouakchott.

Our demonstration of the presence of Hb Groene Hart in the hemolysate ruled out the previously suggested possibility of rapid postsynthesis degradation (8), and we detected no posttranslational modifications. The failure to detect this Hb in earlier studies can be attributed to its considerably increased hydrophobicity, which makes it undetectable with conventional methods. The presence of the \(\alpha_{\text{Groene Hart}}\)-globin chain can be detected consistently with the modified RPLC method we have described.

A few technical points need to be emphasized. The highly increased hydrophobicity of the \(\alpha_{\text{Groene Hart}}\)-globin chain relative to that of the wild-type \(\alpha\)-globin chain required the use of long gradients that reached higher organic solvent concentrations. Increased TFA concentrations in the elution solvents decrease the retention times of these very hydrophobic species and allow their recovery. TFA is a known anionic ion-pairing agent and is effective at higher concentrations (>1 mL/L) for separating peptides (25–27).

When a DNA study reveals a point mutation causing a single amino acid substitution in a patient with thalassemic hematologic features, we recommend that a careful Hb study be performed to estimate the relative concentration of the variant. Furthermore, when the mutant Hb cannot be isolated with conventional methods because of a low concentration or high hydrophobicity, we recommend RPLC analysis. Although one may choose among chromatographic techniques that have already been described (28, 29), the new LC method we have described appears to be highly efficient for isolating hydrophobic molecules.
Characterization of Hb Groene Hart by Mass Spectrometry

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