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Hematology

Analysis of Minor Hemoglobins by Matrix-Assisted Laser Desorption/Ionization Time-of-Flight Mass Spectrometry

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Background: **Hemoglobin (Hb) heterogeneity arises mainly from posttranslational modifications of the globin chains, and cation-exchange chromatography reveals falsely increased concentrations of some minor Hbs in the presence of abnormal Hbs. Here we describe a method for identification of the globin chains and their posttranslational modifications contained in the Hb fractions.**

Methods: **We used cation-exchange HPLC (PolyCAT A column) for separation of Hb fractions and matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) for analysis of the separated globin chains. Globin chains were identified by their molecular masses. Posttranslational modifications of globin chains were identified by digestion of the proteins with endoproteinase V8 before MALDI-TOF MS of the resulting peptides.**

Results: **Analysis of the HbA_2 fractions of patients with HbS revealed 4 different globin chains. We found, in addition to the expected α- and δ-chains, the carbamylated α- and the β^S-chains. Additionally, we analyzed HbH, Hb Barts, HbA_{1b}, pre-HbA_{1c}, HbA_{1c}, HbF_1, HbF, HbA_{1d3a}, HbA_{1d3b}, HbA_2, and HbC_1 fractions from control and pathologic blood samples. We identified several posttranslational modifications of the globin chains, such as pyruvatization, glycation, acetylation, carbamylation, and acetaldehyde adduct formation.**

Conclusions: **The native and posttranslationally modified globin chains in minor and major Hbs are unambiguously identified by MALDI-TOF MS. A minor Hb containing the carbamylated α- and the β^S-chain elutes at the same time as normal HbA_2 ($\alpha_2\delta_2$) and thus leads to falsely increased HbA_2 values in patients with HbS when blood is analyzed with PolyCAT A chromatography.**

Major and minor hemoglobin (Hb)[3] components occur in erythrocytes. Examples of major Hb components are HbA, a tetramer consisting of 2 α- and 2 β-globin chains ($\alpha_2\beta_2$), which is predominant after childhood and during adult life, or HbF ($\alpha_2\gamma_2$), which is the main Hb during fetal development. HbF decreases rapidly after birth and becomes a minor Hb. During embryonic development, tetramers of ϵ- and ζ-chains in combination with α- and γ-chains are the predominant Hbs. Hb Gower 1 ($\zeta_2\epsilon_2$), Hb Gower 2 ($\alpha_2\epsilon_2$), and Hb Portland ($\zeta_2\gamma_2$) serve as oxygen carriers *(1)*. The minor Hb species, designated HbA_{1a} through HbA_{1e} according to their elution order in cation-exchange HPLC *(2)*, consist mainly of HbA_{1c} ($\alpha_2\beta_2^{glyc}$), HbA_{1d3}, and HbA_2 ($\alpha_2\delta_2$). HPLC analysis of hemolysates of healthy human erythrocytes shows that HbA_{1c} constitutes ~4.4%–5.2% of the total Hb *(3)*, whereas HbA_{1d3} and HbA_2 account for 3.5% *(4)* and 2%–3% *(5–8)*, respectively.

The heterogeneity of human Hb arises mainly from posttranslational modifications, principally glycation *(9)*. Glycated Hbs, which are referred to in order of their elution as HbA_{1a1}, HbA_{1a2}, HbA_{1b1} through HbA_{1b3}, and HbA_{1c}, result from the nonenzymatic attachment of glucose (in HbA_{1c}), fructose 1,6-diphosphate (in HbA_{1a1}), or

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[3] Nonstandard abbreviations: Hb, hemoglobin; MALDI-TOF MS, matrix-assisted laser desorption/ionization time-of-flight mass spectrometry; and TFA, trifluoroacetic acid.

glucose 6-phosphate (in HbA_{1a2}) *(9, 10)*. Furthermore, Hb can be glycated at multiple sites, including the amino terminus, as well as certain ϵ-amino groups *(9)*. There is no accurate information about the globin chains of HbA_{1d1}, HbA_{1d2}, and HbA_{1e} available in the literature. Possibly, HbA_{1d1} contains a globin chain modified by acetaldehyde *(11)*. On the other hand, the well-studied HbA_{1d3} is composed of at least 2 components, HbA_{1d3a} and HbA_{1d3b}, containing a carbamylated (urea adduct) α-chain and a glycated minor Hb *(4)*. Additionally, a novel Hb–glutathione adduct, which is increased in patients with diabetes and elutes in the HbA_{1d3} fraction, has been reported *(12, 13)*.

Acetylation and Hb adducts of aspirin, vitamin C, penicillin, and acetyl-CoA are additional posttranslational modifications *(14)*. Additionally, Hb adducts with pyruvic acid at the amino terminus of the β-chain (in HbA_{1b}) *(15)* or glycoinositolphospholipid at the carboxy terminus of the β-chain *(16)* are known.

The exact quantification of minor Hbs has important diagnostic implications. For example, HbA_{1c} is a marker for diabetes mellitus *(1, 17)*; HbA_{1d3a} and HbA_{1d3b} are increased in uremic and diabetic patients, respectively *(4)*; and the controversial influence of Hb–acetaldehyde adducts on HbA_{1d3} in female heavy drinkers has been reported *(4, 11)*. HbF is increased in hereditary persistence of fetal Hb, β-thalassemia intermedia, β-thalassemia major, and specific drug treatments *(18)*. Increased HbF is known to inhibit the polymerization of HbS, and the monitoring of HbF concentrations during the follow-up and treatment of patients with sickle cell anemia is mandatory. HbA_2 is increased in β-thalassemia and in megaloblastic anemia *(1)* but is decreased in α-thalassemia, iron deficiency, and sideroblastic anemia *(1, 18)*.

Quantification of minor Hbs requires precise and specific methods. A widely applied technique is cation-exchange HPLC, which, however, is known to give falsely increased HbA_2 values in HbS carriers *(5, 19–21)*. In HbD patients, increased *(22)* as well as decreased *(21, 23)* HbA_2 concentrations have been reported. Although incomplete separation of HbA_2 and HbD explains increased measured HbA_2 concentrations *(22)*, Suh et al. *(19)* suggested that coelution of HbS adducts, including glycated HbS, with HbA_2 also contributes to increased measured HbA_2 concentrations. Other common Hb analysis techniques include isoelectric focusing, reversed-phase HPLC, and capillary zone electrophoresis *(7, 14)*.

Since the development of soft ionization techniques, electrospray ionization and MALDI-TOF MS have provided precise, rapid, and reliable analysis of Hb components. These techniques are well suited to detect globin chain mutations and posttranslational modifications. To date, however, MALDI-TOF MS has seen remarkably little application to Hb analysis *(24, 25)* despite its advantages, such as high sensitivity and short analysis times. Because many of the β-chain variants, including HbC, D-Los Angeles, E, and O-Arab, exhibit mass shifts of only 1 Da, their analyses require high-resolution instruments.

In this study, we used MALDI-TOF MS to investigate globin chains constituting different minor Hbs. We also used this technique to elucidate the minor components in erythrocyte lysates responsible for falsely increased HbA_2 values in HbS patients.

Materials and Methods

BLOOD SAMPLES

We analyzed a collection of anonymized human blood specimens from a routine chromatography laboratory; therefore, no ethics approval was required. Blood samples had been collected with EDTA or heparin as anticoagulant, stored at 4 °C, and generally analyzed within 24 h. For the determination of HbA_2 values, we used fresh samples (Hb analysis with PolyCAT A HPLC is a routine analysis of our hospital). In most cases, analysis was performed within 24 h. For the isolation of different Hb fractions, we used some fresh and some previously collected samples (especially the blood samples from patients with rare diseases). HbA_2 fractions were isolated from fresh samples (in most cases not older than 24 h). We prepared hemolysates for chromatographic analysis by lysing washed erythrocytes in 1 mmol/L KCN.

Hb ANALYSIS WITH HPLC

We performed cation-exchange HPLC with a 200 × 4.6-mm (i.d.) PolyCAT A column (LCC Engineering & Trading GmbH) according to the method of Bissé et al. *(26)* on a Shimadzu LC-2010C instrument. The HPLC program included (*a*) a linear gradient from 15% to 65% solvent B in 30 min; (*b*) a linear gradient from 65% to 100% solvent B in 0.1 min; (*c*) an isocratic flow of 100% solvent B for 6 min; (*d*) a linear gradient from 100% to 15% solvent B in 0.1 min; and (*e*) an isocratic flow of 15% solvent B for 10 min [solvent A, 35 mmol/L Bis-Tris, 1.5 mmol/L KCN, 3 mmol/L NH_4CH_3COO (pH 6.67); solvent B, 35 mmol/L Bis-Tris, 1.5 mmol/L KCN, 17 mmol/L NH_4CH_3COO, 150 mmol/L $NaCH_3COO$ (pH 7.0)]. We injected 3 μL of sample, monitored peaks at 414 nm, and collected fractions of minor Hbs manually. We used WinSTAT® software for statistical calculations.

SAMPLE CONCENTRATION

We concentrated the isolated Hb fractions to a final volume of 50 μL with 5-kDa-cutoff Amicon® Ultra-4 Centrifugal Filter Devices (Millipore). Before mass spectrometric analysis, we further desalted 10 μL of isolated minor Hb samples with C18 ZipTips® (Millipore) and eluted them with 3 μL of 750 mL/L CH_3CN in 1 mL/L trifluoroacetic acid (TFA). We used 0.5 μL of the desalted sample for MALDI-TOF MS.

MALDI-TOF MS

To analyze globin chains, we used the autoflex® system (Bruker Daltonics®) for MALDI-TOF MS. The mass reso-

lution at 15 kDa was ~750 ($m/\Delta m$). Isolated HPLC fractions were analyzed by the overlayer method. We applied a thin layer of sinapinic acid (saturated solution in ethanol) to a ground-steel MALDI target. We then mixed equal volumes of protein sample and a saturated sinapinic acid solution (in 330 mL/L CH_3CN–1 mL/L TFA), applied 0.5 μL of the mixture to the thin layer, and dried the target at room temperature. To analyze the globin digests, we applied 0.5 μL of the sample directly to a 600-μm AnchorChip™ (Bruker Daltonics) target and added, on top of the analyte solution, 1.1 μL of a saturated matrix solution consisting of α-cyano-4-hydroxycinnamic acid (in 330 mL/L CH_3CN–1 mL/L TFA), diluted 1:10 in ethanol–acetone (67:33 by volume). After drying the target at room temperature, we performed on-target washing with 2 μL of 1 mL/L TFA.

PROTEIN DIGESTS

Globin chains were digested by adding 1 μg of endoproteinase Glu-C (Boehringer Mannheim) or 0.2 μg of trypsin (Promega) and 20 μL of digestion buffer (25 mmol/L NH_4HCO_3, pH 7.8) to the lyophilized sample. The solution was incubated overnight at room temperature (Glu-C digestion) or at 37 °C (trypsin digestion).

Results

PolyCAT A HPLC ANALYSIS OF LYSATES

A typical PolyCAT A chromatogram of the erythrocyte hemolysate prepared from blood of a healthy donor is shown in Fig. 1A. In addition to the major HbA, a series of minor Hbs are evident, of which the most intense are HbA_{1c}; HbA_{1d3} with its 2 components, HbA_{1d3a} and HbA_{1d3b}; and HbA_2. Other Hbs, such as HbA_{1a+b}, HbA_{1d1+2}, and HbA_{1e}, have lower intensities and are only partly resolved. Shown in Fig. 1B is a PolyCAT A chromatogram of the lysate from an individual heterozygous for HbS. Typically, an intense peak corresponding to HbS elutes at a retention time of 30 min. MALDI-TOF analysis of the isolated fraction of HbS revealed 2 globin chains, the α-chain at 15 127 Da and the β^S-chain at 15 838 Da (Fig. 1B, inset), whereas the typical α- and β-chains were detected in the HbA fraction (data not shown).

ANALYSIS OF MINOR Hbs BY MALDI-TOF MS

A selection of MALDI-TOF mass spectra of isolated fractions containing separated minor Hbs is shown in Fig. 2. The peaks are assigned according to the expected molecular masses of the globin chains. Table 1 summarizes the globin chains detected in the different fractions. As shown in Fig. 2A, the mass spectrum of a HbA_{1b} fraction isolated from a patient with glycogenose type I includes peaks that correspond to the α-chain (15 127 Da), the pyruvic acid adduct of the β-chain (15 938 Da), and the latter's decarboxylated form (15 894 Da). The mass spectra in panels B and C of Fig. 2 correspond to the globin chains in the pre-HbA_{1c} and the HbA_{1c} fractions, with glycated α- and β-chains at molecular masses of

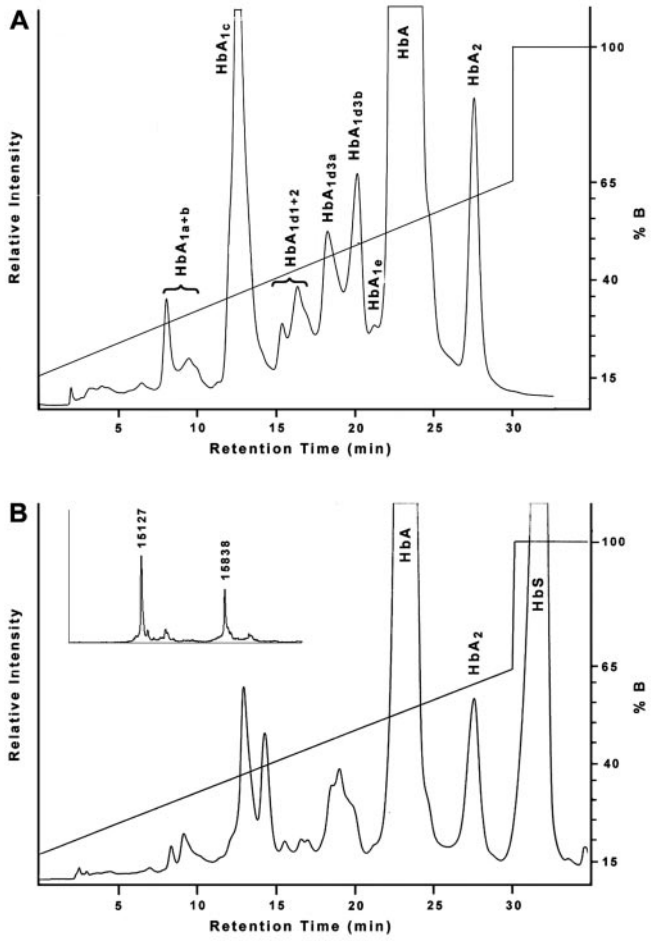

Fig. 1. PolyCAT A HPLC analysis of erythrocyte lysates from an adult control (*A*) and a patient with HbS (*B*).

(*A*), minor Hbs that are typically detectable in lysates from healthy adult controls. (*B*, *inset*), the isolated HbS fraction was investigated with MALDI-TOF MS. Shown are the α- and β^S-globin chains found in the isolated HbS fraction at 15 127 and 15 838 Da, respectively.

15 289 (Fig. 2B) and 16 030 Da (Fig. 2, B and C), respectively. Analysis of the HbC_1 fraction isolated from a patient with HbC revealed a very similar mass spectrum for HbA_{1c} (data not shown). HbC_1, a minor Hb eluting slightly before HbC, contains the characteristic α-chain and a glycated β^C-chain that differs by only 1 Da from the typical glycated β-chain, β^{glyc}.

Shown in Fig. 2D are the globin chains that produce the HbF_1 peak, a minor Hb in samples with high HbF content. The chains at 15 996 and 16 038 Da are assigned to the $^G\gamma$- and the acetylated $^G\gamma$-chains, respectively, and both peaks contain a shoulder representing the $^A\gamma$- and acetylated $^A\gamma$-chains, respectively. Finally, the mass spectra of the partly separated HbA_{1d3a} (Fig. 2E) and HbA_{1d3b} (Fig. 2F) peaks exhibit several different globin chains at 15 127 (α-chain), 15 868 (β-chain), 15 170, and 15 894 Da. From data reported in the literature, we concluded that the peak at 15 170 Da corresponds to the carbamylated α-chain (urea adduct), whereas the peak at 15 894 Da is consistent

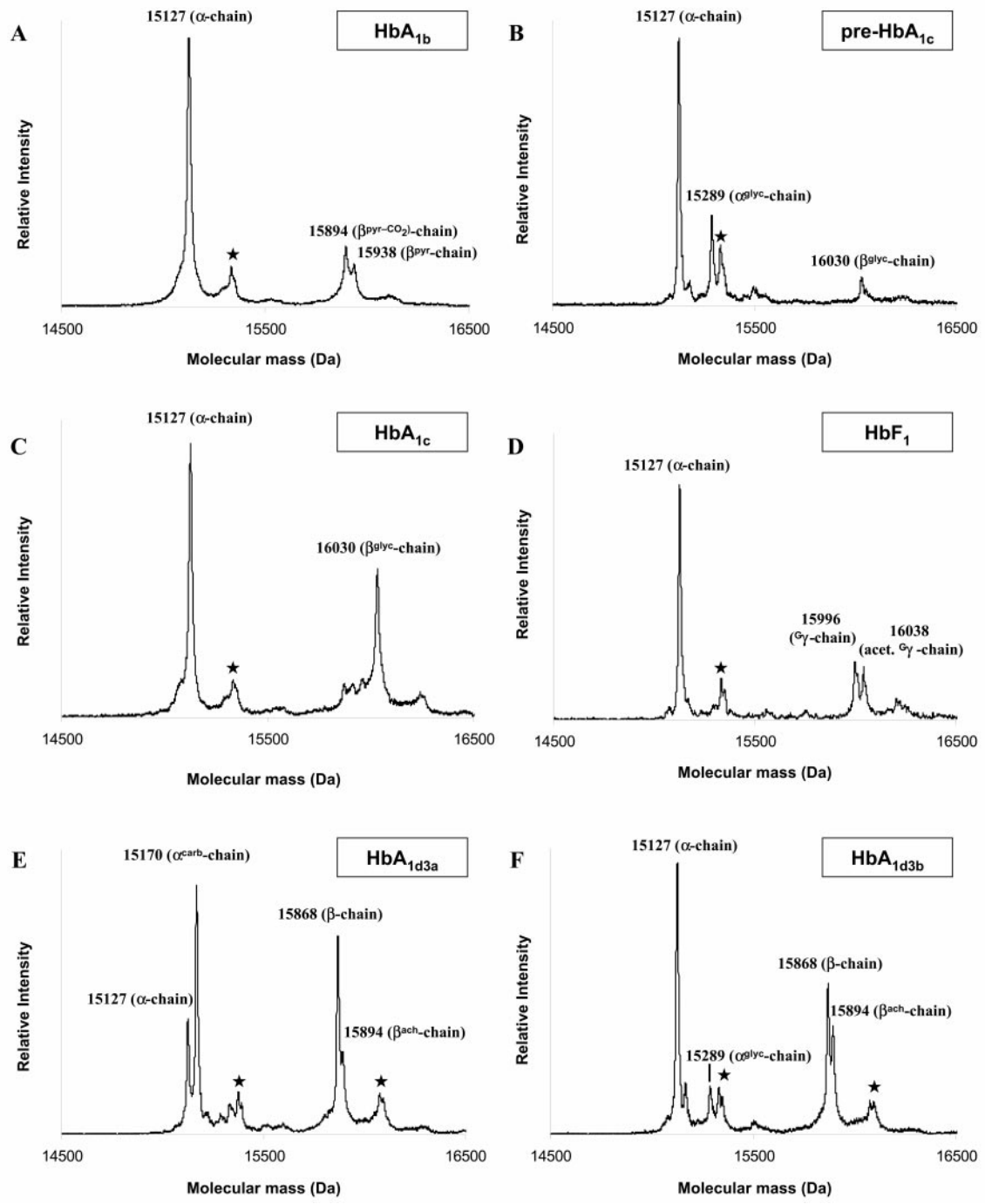

Fig. 2. Selection of minor Hbs analyzed by MALDI-TOF MS.

The HbA_{1b} fraction (*A*) was collected from a patient with glycogen storage disease type I; the pre-HbA_{1c} and HbA_{1c} fractions (*B* and *C*) are from a patient with diabetes; the HbF_1 fraction (*D*) is from a newborn; and the HbA_{1d3a} and HbA_{1d3b} (*E* and *F*) fractions are from a control sample. ⋆, peaks corresponding to matrix adducts.

with an acetaldehyde adduct of the β-chain. To confirm these assumptions, we digested the globin chains in the fractions with endoproteinase Glu-C or trypsin. After subsequent MALDI-TOF MS analyses of the peptides, we detected the urea adduct with the N-terminal peptide of the α-chain (data not shown). The tryptic digest of the peak at 15 894 Da, however, contained no acetaldehyde adduct with the N-terminal peptide of the β-chain. We conclude that a site other than the amino terminus must therefore form an adduct with acetaldehyde.

HbA_2 values determined with PolyCAT A HPLC

To investigate the reasons for increased HbA_2 concentrations in erythrocyte lysates of HbS carriers, we analyzed

Table 1. Major and minor Hbs and the detected globin chains.

Hb	Elution in PolyCAT A HPLC, % B	Globin chains detected by MALDI-TOF MS (mass in Da)	Sample origin
HbH	18	β (15 868)	α-Thalassemia
HbBarts	18	$^{G}\gamma$ (15 996); $^{A}\gamma$ (16 010)	α-Thalassemia
HbA_{1b}	27	α (15 127); β^{pyr} (15 938); $\beta^{pyr\text{-}CO_2}$ (15 894)	GSD-I[a]
pre-HbA_{1c}	30	α (15 127); α^{glyc} (15 289); β^{glyc} (16 030)	Diabetes
HbA_{1c}	35	α (15 127); β^{glyc} (16 030)	Diabetes
HbF_1	30	α (15 127); $^{G}\gamma$ (15 996); $^{A}\gamma$ (16 010, shoulder); acet. $^{G}\gamma$ (16 038); acet. $^{A}\gamma$ (16 052, shoulder)	Newborn
HbF	37	α (15 127); $^{G}\gamma$ (15 996); $^{A}\gamma$ (16 010)	Newborn
HbA_{1d3a}	45	α^{carb} (15 170); α (15 127); β (15 868); β^{ach} (15 894)	Control
		β-GSH (16 173)	GSD-I
HbA_{1d3b}	48	α (15 127); α^{glyc} (15 289); β (15 868); β^{ach} (15 894)	Control
HbA	52	α (15 127); β (15 868)	Control
HbA_2	60	α (15 127); δ (15 925)	Control
HbA_2	60	α (15 127); α^{carb} (15 170); δ (15 925); β^{S} (15 838)	HbS
HbS	100	α (15 127); β^{S} (15 838)	HbS
HbC_1	100	α (15 127); $\beta^{C,\,glyc}$ (16 030)[b]	HbC
HbC	100	α (15 127); β^{C} (15 868)[b]	HbC

[a] GSD-I, glycogen storage disease type I; pyr, pyruvic acid adduct; pyr-CO_2, decarboxylated pyruvic acid adduct; glyc, glycated; acet, acetylated; carb, carbamylated; ach, acetaldehyde adduct; GSH, glutathione.

[b] Because of limited instrument mass resolution, the expected mass shift of 1 Da, compared with the normal glycated β-chain, is not detectable with conventional MALDI-TOF instruments.

32 samples from different heterozygous HbS patients, 42 samples from homozygous HbS patients, and 200 samples from unaffected controls by cation-exchange HPLC (PolyCAT A). We also analyzed a second control group that included 47 samples from patients with β-thalassemia minor (Table 2). Compared with the control samples, the HbA_2 concentrations in HbS patients (homozygous and heterozygous) were statistically significantly increased ($P < 0.0001$, Student t-test). In homozygous and heterozygous HbS patients, the concentrations were 4.95% and 4.22% of total Hb, respectively. These concentrations are clearly increased relative to the concentration (2.68%) measured in the control samples. The HbA_2 concentration in homozygous HbS patients (4.95%) approached that of β-thalassemia patients (5.35%).

ELUCIDATION OF UNKNOWN MINOR Hb

We assumed that a minor Hb coeluted with HbA_2 and thus falsely increased HbA_2 values in patients with sickle cell anemia. We therefore isolated the HbA_2 fractions of 12 HbS samples (3 heterozygotes and 9 homozygotes) and 2 β-thalassemia samples. The MALDI-TOF mass spectra of the HbA_2 fractions with 2 intensive peaks at 15 127 and 15 925 Da correspond to the normal α- and δ-chains, respectively (Fig. 3). Both β-thalassemia samples gave similar spectra. On the other hand, the MALDI-TOF mass spectrum of the HbA_2 fraction isolated from a patient with HbS included 4 different globin chains with masses at 15 127, 15 170, 15 838, and 15 925 Da (Fig. 3B). All HbA_2 fractions isolated from 9 HbS samples gave similar MALDI-TOF mass spectra (data not shown). To exclude artifacts, we purified an isolated HbA_2 fraction of a heterozygous HbS carrier 3 times by cation-exchange chromatography, and MALDI-TOF analysis revealed the peak at 15 838 Da. As in Fig. 3A, the peaks at 15 127 and 15 925 Da were assigned to the normal α- and δ-chains. The peak at 15 838 Da, moreover, corresponds to the β^{S}-chain, whereas the peak at 15 170 Da is consistent with a posttranslationally modified α-chain. Considering the results obtained for the HbA_{1d3a} fraction, we assumed that carbamylation of the α-chain accounted for the mass shift of 43 Da compared with the normal α-chain. With digestion of the HbA_2 fraction by endoproteinase Glu-C and subsequent MALDI-TOF MS analysis of the resulting peptides, we confirmed this assumption (data not shown). The 15 170-Da globin chain corresponds to the N-terminal–carbamylated α-chain.

Table 2. HbA_2 concentrations in patients with HbS or β-thalassemia, and in control samples.

Samples	n	Mean (range), % of total Hb	CV, %
Controls	200	2.68 (2.0–3.5)	11
β-Thalassemia minor	47	5.35 (4.1–7.3)	13
HbS heterozygote	32	4.22 (3.6–5.1)	7.6
HbS homozygote	42	4.95 (3.1–7.0)	33

Discussion

Traditional methods for Hb analysis, such as chromatography and electrophoresis, reveal only symmetric Hb tetramers because of the rapid dissociation of tetramers to dimers relative to the separation time *(27)*. We confirmed this finding by analyzing a heterozygous HbS sample (Fig. 1B). MALDI-TOF analysis of the isolated HbA and HbS fractions revealed only globin chains of symmetric Hbs, $\alpha\beta$ (tetrameric structure, $\alpha_2\beta_2$) and $\alpha\beta^{S}$ ($\alpha_2\beta_2^{S}$), re-

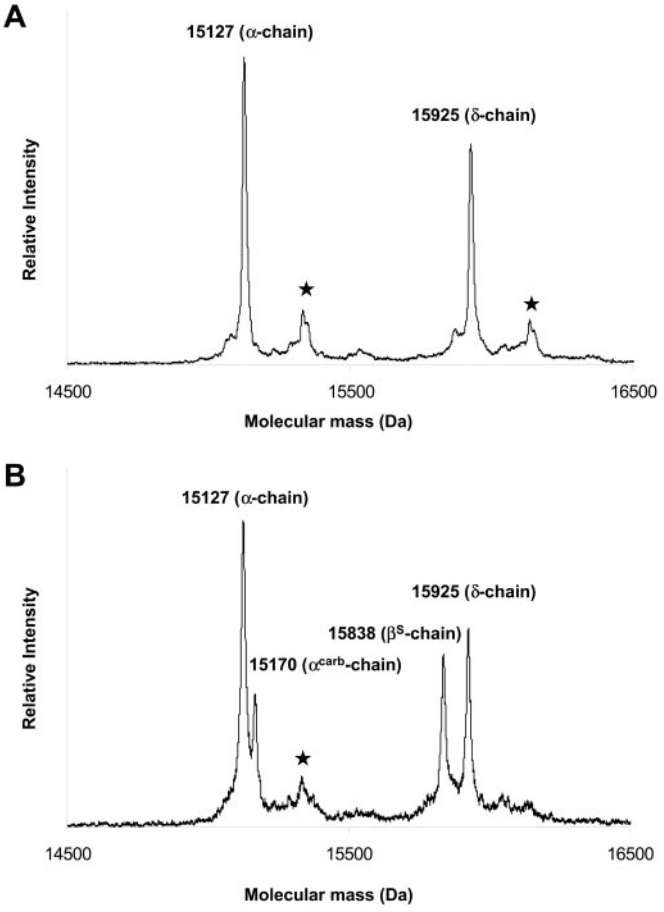

Fig. 3. MALDI-TOF mass spectra of isolated HbA_2 fraction from a β-thalassemia patient (*A*) and from a HbS patient (*B*).

The α^{carb}- and β^{S}-globin chains are found only in patients with HbS (homo- and heterozygotes). ⋆, peaks corresponding to matrix adducts.

spectively. We observed no peak for the expected asymmetric tetramer $\alpha_2\beta\beta^S$, leading us to conclude that the observed peaks in cation-exchange HPLC all correspond to dimers and not to tetramers. The signals in a PolyCAT A chromatogram therefore do not reflect the real tetrameric Hbs.

Use of MALDI-TOF MS to analyze desalted and concentrated minor Hb fractions allows measurement of the globin chains that build the tetrameric structure of the Hb. We applied our procedure to isolated Hbs detected in Hb samples from healthy individuals and from patients with various disorders that produce hemoglobin variants (Table 1). The mass spectrum of the HbA_{1b} fraction (Fig. 2A) isolated from a patient with glycogenose type I is characterized by 2 posttranslationally modified β-chains, of which the peak with mass 15 938 Da represents the pyruvated β-chain built by nonenzymatic condensation of pyruvic acid with the β-chain amino terminus *(1)*. Additionally, the decarboxylated form of the pyruvated β-chain (15 894 Da) is present. Pyruvate adducts correspond well with increased pyruvate concentrations in patients with glycogen storage disease type I.

The mass spectrum of a pre-HbA_{1c} fraction that elutes slightly before HbA_{1c} (at 30% solvent B), in a sample from a patient with diabetes, is shown in Fig. 2B. In addition to the normal α-chain, the glycated α- and β-chains are detectable at 15 289 and 16 030 Da, respectively. We conclude that pre-HbA_{1c} must contain a Hb with at least 1 glycated α-chain. On the other hand, the HbA_{1c} fraction (Fig. 2C), an important diabetes marker, contains the expected α-chain and β^{glyc}-chain, leading to the known $\alpha_2\beta_2^{glyc}$ structure. The analysis of a HbC_1 fraction revealed a mass spectrum very similar to that for a normal α-chain and a glycated β^C-chain, which differs by only 1 Da from glycated β-chain (data not shown). In diabetic patients with HbC, therefore, the minor HbC_1 peak represents a dimer $\alpha\beta^{C,\,glyc}$ and must be included in the quantification of glycated Hbs.

The mass spectrum of the HbF_1 fraction, an Hb that appears with high HbF concentrations, exhibits the acetylated $^G\gamma$- and $^A\gamma$-chains (shoulder of the peak; Fig. 2D). Acetylation as a posttranslational modification occurs frequently in human proteins. Native Hb, however, is not N-terminally acetylated (except for γ-globin) because the N-terminal valine of α-, β-, and δ-globins inhibits acetylation *(28)*. Both nearly identical γ-globin chains are expressed by the $^G\gamma$-globin and $^A\gamma$-globin genes. In humans at the age of 5 months, $^A\gamma$-globin chains become predominant *(8)*. Finally, HbF_1 and HbF ($\alpha_2\gamma_2$) are increased in patients with homozygous HbS on hydroxyurea treatment. This drug increases, by as yet unclear processes, the HbF concentration, which inhibits the polymerization of HbS, thereby reducing vaso-occlusive events *(29)*.

HbA_{1d3a} (Fig. 2E) and HbA_{1d3b} (Fig. 2F), which eluted at 45% and 48% solvent B, respectively, could not be separated completely. Of the 4 intense peaks detected in the HbA_{1d3a} fraction, the peak at 15 170 Da corresponds to the carbamylated α-chain, and the peak at 15 868 Da is consistent with the normal β-chain. We attribute the chains with masses of 15 127 and 15 894 Da to the partly overlapping HbA_{1d3b} fraction. HbA_{1d3a} is reported to be significantly higher in uremic than in nonuremic patients *(4)*. These observations correspond well with our findings of a carbamylated α-chain (adduct of urea to the amino terminus of the α-chain); on the other hand, the HbA_{1d3b} fraction exhibited 3 major chains, the normal α- and β-chains and a modified β-chain at 15 894 Da (Fig. 2F). The characteristic α- and β-chains in this fraction probably arise partly from overlapping of the highly intense HbA peak. HbA_{1d3} is reported to be increased in female heavy drinkers and in alcoholic individuals, but the increase is not statistically significant *(11)*. The first metabolite of ethanol, acetaldehyde, is known to form adducts with Hbs *(10)*. We therefore assume that the peak at mass 15 894 Da could be attributed to the β-chain adduct with acetaldehyde (β^{ach}), which corresponds well with the observed mass shift of 26 Da. Occasionally, in patients with diabetes, the glutathionylated β-chain at mass 16 173

Da is also detected (data not shown), a finding consistent with other reports *(4, 12, 13)* of increased glutathionylated β-chain concentrations that lead to increased HbA_{1d3b} concentrations. Additionally, a minor peak corresponding to a glycated α-chain (15 289 Da) was detected. Assuming a dimeric structure of PolyCAT A-separated compounds, we conclude that this fraction must contain at least 2 different Hbs, namely $\alpha\beta^{ach}$ and $\alpha^{glyc}\beta$.

The HbA_{1d} fraction is reported to increase slowly with hemolysate age *(30)*. Possibly, carbamylation plays a crucial role in Hb aging. Elucidation of the aging process, however, will require further studies.

HbBarts (γ_4) and HbH (β_4), 2 Hbs consisting of only one type of globin chain, are markers for α-thalassemia. These Hbs elute at 18% solvent B when PolyCAT A HPLC is used. Analysis of the isolated fraction of HbBarts revealed only 1 intense peak at 16 010 Da, corresponding to the γ-chain (data not shown).

We also tried to analyze other fractions containing minor Hbs, such as HbA_{1a}, HbA_{1d1+2}, and HbA_{1e}. Low concentrations and strong overlapping of intense Hbs, however, prevented successful analysis of these components. To identify these minor Hbs, a promising strategy is the analysis of disease samples that exhibit increased minor Hbs as observed in the HbA_{1b} peak isolated from a patient with glycogenose type 1.

We also used analysis of minor Hbs by MALDI-TOF MS to investigate HbA_2 in HbS carriers. It is well established that HbA_2 is increased in the presence of the β-chain variant HbS *(5, 19–21)* when analyzed by chromatographic methods. Additionally, HbA_2 is reported to inhibit polymerization of deoxy-sickle hemoglobin (HbS) in vitro *(31)*. In this study, we analyzed samples from HbS carriers and compared their HbA_2 values with the values for controls and confirmed β-thalassemia carriers. The HbA_2 concentrations of the HbS patients were clearly increased compared with those of the controls (Table 2) and agree with those of Shokrani et al. *(5)*. In 1996, Suh et al. *(19)* undertook a study to investigate the increased HbA_2 concentrations in greater detail. They assumed that coelution of HbS adducts, including glycated HbS with HbA_2 on HPLC, was responsible for the increased HbA_2 value. They were not able, however, to elucidate the supposed coeluting Hb component.

To investigate this proposed HbS adduct, we analyzed isolated HbA_2 fractions of HbS and β-thalassemia samples with MALDI-TOF MS. In the HbA_2 fraction of a patient with β-thalassemia (without HbS), the expected α- and δ-chains (Fig. 3A) were present. The different signal intensities of the 2 peaks resulted from distinct mass spectrometric signal responses and not from various amounts of the globin chains. In contrast to the spectrum of the β-thalassemia sample, we found in all investigated HbS samples additional globin chains at 15 170 and 15 838 Da (Fig. 3B). The 15 838-Da globin chain could easily be assigned to the variant β-chain, β^S. The presence of the β^S-chain in the HbA_2 fraction is explained by incomplete separation of an intense HbS peak and the HbA_2 peak when analyzed with cation-exchange HPLC. In heterozygous HbS samples, we also detected the peak at 15 838 Da, but we could separate the HbS peak and the HbA_2 peak completely. We therefore conclude that the variant β^S-chain is participating in coelution of the minor Hb with HbA_2.

The peak at 15 170 Da must be assigned to a posttranslationally modified α-chain. Considering the previously found globin chains in HbA_{1d3a}, we suspected that the modification is carbamylation of the α-chain. Mass spectrometric analysis of the peptides resulting from endoproteinase Glu-C digestion of the HbA_2 fraction confirmed this assumption (data not shown). From these results, we conclude that the increase in HbA_2 in patients with HbS cannot be attributed to the presence of glycated β^S, as is commonly accepted, but arises from coelution of the minor Hb $\alpha^{carb}\beta^S$ with HbA_2.

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