Characterization of the Elusive Disulfide Bridge Forming Human Hb Variant: Hb Ta-Li β83 (EF7)Gly → Cys by Electrospray Mass Spectrometry

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An electrospray mass spectrometric approach to the identification of a human hemoglobin (Hb) variant involving a Cys residue incorporation is presented. In Hb Ta-Li (β83Gly → Cys), Cys83 forms inter-molecular disulfide bridges. Routine analysis of the denatured Hb showed the presence of a minor β chain variant whose mass apparently was 1 Da less than the expected mass difference of 46 Da for a Gly → Cys substitution. Reduction of the globin chains with dithiothreitol gave an intense monomer with the expected mass difference for the Gly → Cys substitution. After reprocessing the original raw data from the denatured Hb and taking into account the possibility of dimer formation, a component was revealed whose mass was consistent with a disulfide linked dimer of Ta-Li β globins. The mutation was localized to peptide βT10 by analysis of a tryptic digest. Tandem mass spectrometry and DNA sequencing confirmed the Gly → Cys substitution occurred at residue 83 of the β chain. Problems encountered in identifying the components in mixtures of monomers and dimers are discussed. (J Am Soc Mass Spectrom 2002, 13, 187–191) © 2002 American Society for Mass Spectrometry

Mass spectrometry has become widely used in the investigation of Hb variants since it was first used by Wada et al. as a novel method for the identification of variant Hb in 1981 [1–10]. Electrospray (ES) in combination with tandem mass spectrometry (MS/MS) is a commonly used technique for the identification of Hb variants [2–10]. Shackleton and Witkowska [3] and more recently Wild et al. [4] have elaborated the application of ES mass spectrometry to the identification of Hb variants. In our laboratory ES mass spectrometry is used as a secondary as well as a final tool in the identification process following screening of the blood samples by HPLC and/or isoelectric focusing [5, 6]. Initially, denatured blood samples are analyzed by ES mass spectrometry in order to determine accurately the molecular masses of the proteins present. Software based on maximum entropy (MaxEnt) is generally used to process the raw data [7]. This software automatically deconvolutes the original multiply charged data, and allows the mass of the aberrant globin chain in a heterozygote to be determined to a fraction of a Dalton, provided that its mass differs by more than 6 Da from the normal chain [3]. In routine work, the deconvolution parameters are usually chosen on the assumption that only monomeric globin chains are likely to be present in order to minimize the processing time (~30 s). ES mass spectrometry has been used not only to identify Hb variants but also to characterize post-translational modifications of mutant amino acids in some Hb variants [8–10].

Only 13 cases out of over 800 Hb variants described to date involve an amino acid replacement by a cysteinyl residue [11]. Besides being an uncommon substitution, a possible explanation for the low number of Cys containing Hb variants could be that almost all of the

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possible single base substitutions generate neutral to neutral amino acid exchange (Gly → Cys, Phe → Cys, Ser → Cys, Trp → Cys, Tyr → Cys) which tend to escape detection by charge based separation techniques. In several cases the newly occurring Cys residue forms an inter-molecular or intra-molecular disulfide (–S–S–) bridge. In Hb Pôrto Alegre (β9Ser → Cys), the first described variant involving Cys, the Cys residue is present on the external surface of the Hb tetramer and therefore forms inter-molecular –S–S– bridges [12]. Hb Rainier (β145Tyr → Cys), the most extensively studied Cys containing variant, involves the internal $\alpha_1\beta_2$ contact of the Hb molecule and forms intra-molecular –S–S– bridges with β93Cys [13]. On the other hand, in Hb Nunobiki (α141Arg → Cys) where the variant substitution is at the C-terminal end (internal $\alpha_1\beta_2$ contact), no intra- or inter-molecular –S–S– bridges were observed [14].

The variant reported here is also a Cys containing variant known to polymerize in vitro via –S–S– bonds between the variant β chains. This communication describes the mass spectrometric approach to the characterization of this enigmatic and rare Hb variant. A drawback of employing the deconvolution technique is discussed.

Experimental

Isoelectric Focusing and HPLC

Abnormal Hb fractions were found during routine diabetic screening (i.e., quantification of glycated Hbs) by ion exchange HPLC of blood samples from two subjects of Swedish origin living in the same geographical region. Isoelectric focusing and anion exchange HPLC were performed as previously described [15].

Mass Spectrometry

All ES experiments were performed on a Quattro Ultima triple quadrupole mass spectrometer (Micromass, Altrincham, UK). A stock solution of 50-fold diluted whole blood was prepared in water. A mass spectrometric sample was made by 10-fold dilution of the stock solution with solvent A (1:1 acetonitrile:water containing 0.2% formic acid) and desalted as previously described [4]. The sample was introduced into the ES ion source at a flow rate of 8 μL/min. The needle-to-skimmer (capillary voltage) potential was 3.0 kV. The declustering potential (cone voltage) was set to 75 V. Data were acquired over an *m/z* range 930–1180 and accumulated for 2 min. The raw data were calibrated internally against the normal α chain and deconvoluted onto a true mass scale using the MaxEnt based program supplied with the mass spectrometer. Proportions of the variants were estimated as described previously [5].

For the tryptic digests, data were acquired by scanning from *m/z* 150 to 2100 at 8 s per scan for 3 min. The cone ramp gradient was set from 15 V at *m/z* 150 to 200 V at *m/z* 2000. The *m/z* scale was internally calibrated from normal α and β chain tryptic peptides present in the digests.

Dithiothreitol (DTT) Reduction and S-Carboxyamidomethylation

Reduction was effected by adding 10 μL of 100 mM DTT and 120 μL of acetonitrile to 120 μL of Hb stock solution. S-Carboxyamidomethylation of Cys residues in globin chains was performed by mixing all of the DTT reduced Hb solution with 10 μL of 1 M aqueous ammonium bicarbonate pH 8.0 and 12 μL of 200 mM aqueous iodoacetamide solution. The mixture was incubated for 15 min at room temperature. A working sample solution was prepared by mixing 20 μL of the reduced sample with 180 μL of solvent A and analyzed on the mass spectrometer to confirm the appropriate derivatization. The remainder of the derivatized Hb sample was subjected to tryptic digestions after removing the excess reagents using a 10 kDa cut off centrifuge filter (Microcon YM-10, Millipore Corporation, Bedford MA).

Tryptic Digestion

Trypsinization (Sigma T-8642) was performed on both carboxyamidomethylated (CAM) and on denatured Hb stock solutions in 50 mM ammonium bicarbonate buffer solution at pH 8.0 [4]. After 30 min at 37 °C, a 20 μL aliquot of the digest was withdrawn and diluted 10-fold with solvent A. The remainder was left to digest for 12 h. An aliquot of the 12 h digest solution was reduced with 10 mM DTT followed by dilution with solvent A for mass analysis of peptides and subsequent MS/MS experiments.

Tandem Mass Spectrometry

The precursor ion for the peptide of interest was selected and subjected to collision induced dissociation with methane as the collision gas. The cone voltage was set at 80 V. The "collision energy" was set at 50 V. The product ions were analyzed on the final quadrupole of the instrument. Data were acquired over the *m/z* range 50–2000 for 10 min. The *m/z* scale was calibrated against the $Na_{n+1}I_n$ ions from a separate introduction of sodium iodide (1 mg/mL in 1:1 2-propanol:water).

Results and Discussion

Isoelectric Focusing and HPLC

Routine quantification of glycated Hb by ion exchange HPLC of the fresh (less than two weeks old) blood samples revealed the presence of an abnormal Hb. The electrophoretic study showed that the abnormal Hb had a lower isoelectric point than normal Hb with mobility comparable to Hb Fukuyama (β77His → Tyr). This

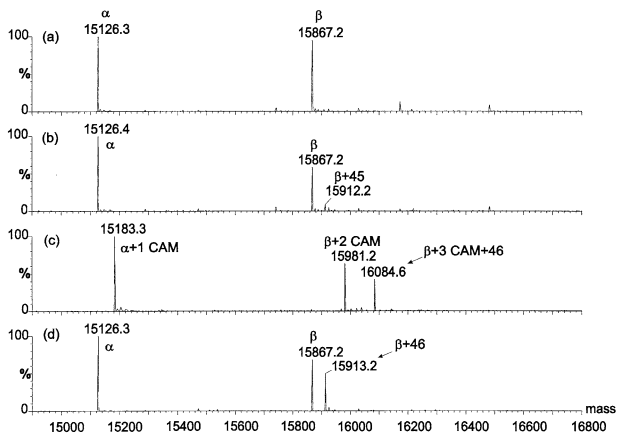

Figure 1. Deconvoluted ES mass spectra from (**a**) denatured normal blood, (**b**) denatured blood of a heterozygote carrying Hb Ta-Li, (**c**) blood of a heterozygote carrying Hb Ta-Li after DTT reduction and carboxyamidomethylation (CAM), and (**d**) blood of a heterozygote carrying Hb Ta-Li after DTT reduction. CAM adds 57.05 Da per free Cys.

suggested that a negative charge is gained in the amino acid exchange event, i.e., neutral to acidic amino acid or basic to neutral amino acid exchange. The proportions of the variants determined by anion exchange HPLC were 32 and 39% of the total Hb. Both subjects showed no hematological or clinical manifestations. A structural study of the Hb variant was performed by ES mass spectrometry of blood samples from both subjects and data from one of them are presented below.

Mass Spectrometry

The initial ES mass spectrum of the denatured intact globins following routine MaxEnt processing showed a normal α chain (15,126.4 Da), and a variant β chain apparently 45 Da heavier than the mass of the normal β chain (15,867.2 Da) (Figure 1b). The proportions of the variant ranged from 26% to well below 15% of the total β chains, and were low compared to those observed by HPLC (32% of total Hb) and decreased as the samples aged. Since there are not any single amino acid substitutions that result in a 45 Da change, a mass measurement error ($\pm$1 Da) was considered. There are two possible amino acid substitutions that will yield a +44 Da mass shift, i.e., Ala $\rightarrow$ Asp and Cys $\rightarrow$ Phe and only one (Gly $\rightarrow$ Cys) for a +46 Da change. Because the Cys $\rightarrow$ Phe and Gly $\rightarrow$ Cys substitutions are neutral to neutral amino substitutions and do not agree with the isoelectric focusing pattern, these substitutions were considered unlikely. Believing Ala $\rightarrow$ Asp to be the most likely substitution, we set about analyzing the tryptic fragments. However, prior to digestion, reduction followed by CAM derivatisation of the Cys residues was performed. Normal Hb from an adult carries one Cys in each α chain and two Cys residues in each β chain. Analysis of the reaction products by ES mass spectrometry showed the presence of an extra Cys residue in the β chain indicating that the variant involves an amino acid replacement by a Cys residue (Figure 1c). The observed mass difference between the variant and the derivatized normal β chain was equivalent to 1 CAM +46 Da. There is only one possible amino acid substitution that gives +46 Da change, i.e., Gly $\rightarrow$ Cys. Subsequent analysis of the DTT-reduced intact globin chains also displayed the +46 Da increase (Figure 1d). The proportion of the variant was 37% of total β chains as judged from the CAM intact chains.

Mass spectra of a 30 min tryptic digest displayed (data not shown) an abnormal βT10–βT11 fragment suggesting that the variant lies in this fragment. The presence of this fragment after the short digestion time shows the inefficiency of trypsin to cleave at its target residue (β95Lys) when occurring C-terminal to an acidic residue (β94Asp). On analysis of the 12 h digests, peptides reduced with DTT showed peaks at the expected *m/z* of the variant βT10 peptide (Figure 2). The intensity of the normal $\beta T10^{2+}$ peptide ion (*m/z* 711.4) in the variant sample (Figure 2b) was lower than in the control sample (Figure 2a), while a peak at *m/z* 734.4, 23 Th heavier than the normal $\beta T10^{2+}$ peptide appeared. This implies the mutation is β83 (Gly $\rightarrow$ Cys) since there is only one Gly residue in the normal tryptic βT10 peptide (GTFATLSELHCDK), corresponding to residue 83 in the normal β chain. Subsequent MS/MS analysis of the doubly protonated variant and normal peptides showed the same *m/z* values for members of the complete y ion series, while *m/z* values for b_2–b_4 ions and beyond were increased by 46 Th (Figures 3a and b). These observations corroborate the mutation β83 (Gly $\rightarrow$ Cys). When Gly at this position is replaced by Cys a +46 Da change occurs. This has previously been reported in a Chinese family as Hb Ta-Li (β83Gly $\rightarrow$ Cys) [16]. Although Gly $\rightarrow$ Cys is a neutral to neutral amino acid substitution, the isoelectric focusing results indicated it to be an anodal variant (gain in negative charge) from normal Hb. This may be due to the oxidation of thiol group of reactive Cys to form cysteine-sulfinic acid

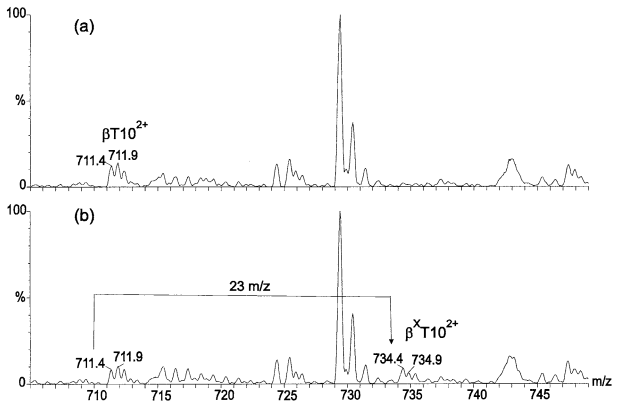

Figure 2. Part of ES mass spectra of tryptic digests from (**a**) a diluted normal blood sample and (**b**) a mixture of Hbs from a heterozygous carrier of Hb Ta-Li showing the 23 Th shift in $\beta T10^{2+}$ peptide ion.

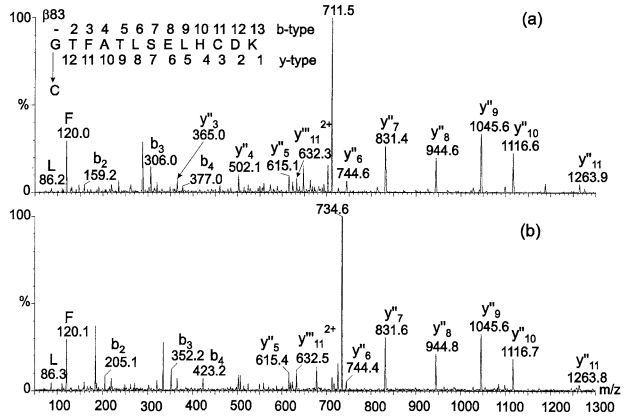

Figure 3. Tandem mass spectra of (**a**) normal $\beta T10^{2+}$ peptide ion and (**b**) variant $\beta T10^{2+}$ peptide ion. Shown in the inset is the amino acid sequence of the βT10 peptide.

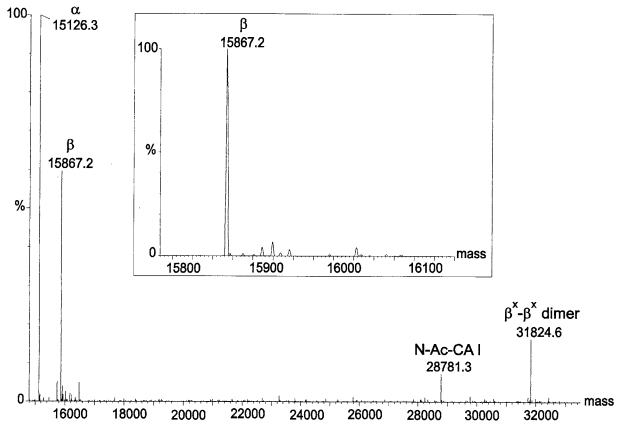

Figure 5. Deconvoluted ES mass spectrum from a mixture of Hbs from a heterozygous carrier of Hb Ta-Li showing the dimeric nature of Ta-Li globins. Also encompassed in this figure is the mass of N-acetylated carbonic anhydrase I (28,781.3 Da). The inset shows essentially no monomeric Ta-Li β globins.

(Cys-SO_2H) or cysteine-sulfonic acid (Cys-SO_3H), however there was no conclusive evidence for this in the mass spectra of the sample analyzed.

An earlier study has also shown that the variant polymerizes in vitro through inter-molecular –S–S– bridges [16]. Decreasing proportions of the variant in the unmodified blood sample in consecutive runs over a period of 120 days indicated rapid in vitro polymerization. Dimers were evident in the present study on 20 weeks old sample as shown by a series of multiply charged ions (D) in the raw data obtained from the denatured blood sample (Figure 4a). The D ions with odd numbers of charges can only arise from the dimer, and together with the D ions with even numbers of charges, form a fairly regular multiply charged series. In other words, the evenly charged D ions do not appear to be significantly more intense than the oddly charged D ions, implying the variant is mainly present as the dimer. Note that the evenly charged D ions could also arise from the monomer, since these species differ in *m/z* by only 1 part in 15,913, well beyond the resolving capabilities of the technique (6 Da in 16,000 Da [3]). After the sample was reduced with DTT (Figure 4b), the D ions with odd numbers of charges disappeared concomitantly with a marked increase in the intensity of ions very close in *m/z* to the D ions with even numbers of charges, implying conversion of dimer to monomer. After reprocessing the raw data used to generate Figure 1a by MaxEnt so as to accommodate the possible presence of dimers (output mass range of 14,800–33,500 Da), the Ta-Li dimer was observed (Figure 5),with essentially no monomer (Figure 5 inset). Note that the MaxEnt deconvoluted spectrum exhibits negligible levels of artifactual dimers from the intense normal α- and β-chains, and also shows a component of mass 28,781.3 Da, attributable to carbonic anhydrase I (N-acetylated sequence mass 28,781.1 Da), adding credibility to faithful deconvolution of the original data. In the strictest sense, the output mass range parameter in the MaxEnt software should encompass all the components in the *m/z* spectrum. Otherwise the program will generate artifacts. In routine analysis of denatured Hbs, we generally assume that only monomeric chains are present in order to limit the processing time to ~30 s, which increases to ~7 min when dimers are assumed to be present. When only monomers are assumed to be present in a spectrum containing dimers, minor artifacts produced by components such as carbonic anhydrase can generally be ignored, but the software generates an artefactual monomer. The apparent monomeric variant β chain at 15,912.2 Da (Figure 1b) is an artifact caused by operating the deconvolution software with a limited output mass range (14,800–16,800 Da) and appears at half the mass of the dimer (31,824.6/2 Da). The mass difference of 1 Da per chain from the expected mass change is explained by inter-molecular –S–S– bond

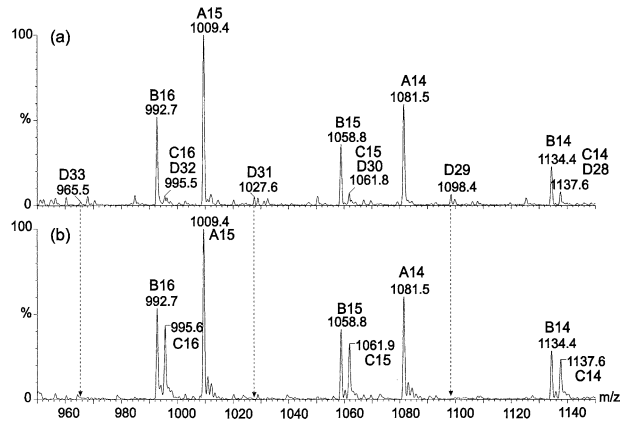

Figure 4. Part of ES mass spectra from (**a**) unmodified blood of a heterozygote carrying Hb Ta-Li showing the presence of peaks corresponding to the dimer; (**b**) DTT reduced mixture of Hbs from a heterozygous carrier of Hb Ta-Li. The mass spectra show the multiply charged ions of normal α (A), normal β (B), monomeric Ta-Li (C), and dimeric Ta-Li (D) globins. The data from (**a**) were deconvoluted to generate the spectra in Figure 1(**b**) and Figure 5, while the data from (**b**) were used to generate Figure 1(**d**).

formation where each variant β subunit loses one hydrogen atom from the newly introduced Cys residue. If there had been an intra-molecular –S–S– bridge, the expected mass difference per chain would be 2 Da, which has been observed by others in the monomer Hb Rainier [13]. This Ta-Li example illustrates how limiting the deconvolution output mass range can give misleading results when there are dimers in the sample, a situation that can be remedied by reprocessing the data to accommodate dimers or by reducing the sample. Nevertheless, distinguishing the components in monomer–dimer mixtures by mass spectrometry remains problematical, particularly when present at relatively low levels as in this case, and recourse to the original data should be made along the lines of Figure 4 in order to increase confidence in the interpretation.

This is the first case of Hb Ta-Li (β83Gly → Cys) reported in a Caucasian patient. DNA sequencing revealed a G → T base substitution in the codon corresponding to Gly (GGC) → Cys (TGC).

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