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ORIGINAL ARTICLE

Direct analysis of α- and β-chains of hemoglobins from mammalian blood samples by nanoESI mass spectrometry during in-capillary proteolytic digestion

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Abstract α- and β-chains of hemoglobins derived from several species were analyzed directly from diluted blood samples by simultaneous in-capillary proteolytic digestion and nanoESI MS and MS/MS analysis. Starting from fresh or frozen and thawed blood samples, sequence coverages of >80% were usually obtained. Only 2 h after resuspension of a dried blood spot, human origin could be demonstrated from data obtained by in-capillary tryptic digestion, nanoESI mass spectrometric analysis, and data base search. A fast and facile differentiation of closely related species by hemoglobin-derived proteolytic "marker peptides" was demonstrated for Asian (*Elephas maximus*) and African elephants (*Loxodonta africana*). Finally, amino acid sequences deduced from collision-induced dissociation experiments during in-capillary proteolytic digestion of the corresponding blood samples allowed de novo sequencing of previously unknown sequences of hemoglobin chains of the Patagonian cavy (*Dolichotum patagona*) and the Persian gazelle (*Gazella subgutturosa subgutturosa*). 100% of the α-chain sequences and more than 85% of the β-chain sequences were covered for both the species. Additionally, sequence data derived from tandem MS experiments obtained with the Q-Tof analyzer were confirmed by high resolution Fourier-transform ion cyclotron resonance mass spectrometric experiments. Accurate protein mass determination of the intact hemoglobin chains directly from the corresponding blood samples by use of a Fourier-transform ion cyclotron resonance mass spectrometer corroborated the deduced sequences of the respective α-chains. The present study demonstrates that in-capillary digestion allows fast characterization and/or sequencing of hemoglobin chains directly from blood samples.

Keywords Hemoglobin · In-capillary digestion · NanoESI MS · FT ICR MS · De novo sequencing

Introduction

Hemoglobins (Hbs) are frequently found in many groups of organisms comprising prokaryotes, fungi, plants and animals; they exhibit a high diversity of functions such as catalysis of redox reactions or intracellular oxygen transport (Hardison 1998). Hbs represent the most abundant proteins found in the erythrocytes of vertebrates, serving as oxygen carriers between tissues in the body (Eaton et al. 1999). In mammals, Hbs are usually found as heterotetrameric complexes in which two pairs of α- and β-subunits, each of them containing a heme group, are arranged in a tetrahedral geometry.

Deciphering of the primary sequence of Hb chains from different species gives rise to a deep insight into the evolutionary tree of hemoglobin. Hbs exhibit a high degree of polymorphism not only between different species but also within the same organism (Kleinschmidt and Sgouros 1987; Hardison 1998; Wajcman and Kiger 2002; Griffith and Kaltashov 2006). In humans, more than 1,000 Hb mutations have been reported so far. Thus,

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hemoglobinopathies represent the most frequently occurring inherited disorder, which is found in approximately 7% of the world population exhibiting a remarkable regional specificity of globin gene mutations (Hardison et al. 2002; Patrinos et al. 2004; cf also http://globin.cse.psu.edu).

Classical approaches for structural characterization of Hbs involve paper chromatography and analysis of amino acids after hydrolysis of the resulting peptides (Pauling et al. 1949; Ingram 1959). Further developments resulted in the application of peptide mapping by two-dimensional paper chromatography during the 1970s and HPLC-based methods in the early 1980s (Brennan 2008). The advent of novel desorption and ionization methods for larger (bio)molecules allowed the introduction of mass spectrometry-based methods in hemoglobin analysis. The first examples on elucidation of the primary structure of human hemoglobin variants by use of field desorption and fast atom bombardment mass spectrometry were reported by Wada et al. (1981, 1983). Since then, mass spectrometry has established as a versatile method for structural characterization of Hb and especially its polymorphism in humans (Wada 2002; Zanella-Cleon et al. 2009; Griffith and Kaltashov 2006; Shimizu et al. 2006; Kleinert et al. 2008; Wajcman et al. 2001). Matrix-assisted laser desorption ionization (MALDI) and laser-induced liquid beam desorption and ionization (LILBID) have been used for desorption and ionization of separated globin α- and β-chains from blood samples or other specimens (Lin et al. 2007; Houston and Reilly 1997) or the intact tetrameric hemoglobin complex (Charvat and Abel 2007). Alternatively, electrospray ionization (ESI) has been demonstrated to represent a perfect tool for the generation of gaseous intact hemoglobin-derived ions (Wild et al. 2001; Davison et al. 2008). ESI provides very mild ionization conditions, and under native conditions, tertiary and quaternary structures of globin chains and oligomeric (non-covalent) complexes are retained in the gas phase (Simmons et al. 2004; Griffith and Kaltashov 2003, 2007; Boys et al. 2007). A comprehensive mass spectrometric characterization of the primary sequence of hemoglobin variants is usually achieved by de novo sequencing of either intact globin chains (Rai et al. 2002) or globin-derived peptides (Green and Williams 2007; Griffith and Kaltashov 2008). The latter approach is referred as "bottom-up" sequencing and it usually comprises either enzymatic or chemical degradation of the protein into peptides. Subsequently, the resulting peptides are structurally characterized with respect to their amino acid sequence mainly by use of low-energy collision-induced dissociation (CID). Tandem MS-based strategies are now widely being used for analysis of the primary structure of hemoglobins as well as for identification of their mutations and modifications in both humans and other vertebrates. In a very recent example, the potential of a combination of top-down and bottom-up MS/MS experiments applied to the phenotypic analysis of Atlantic cod hemoglobin chains has been demonstrated (Griffith and Kaltashov 2008).

We have recently developed a method for direct identification and/or sequencing of proteins by performing proteolytic digestion in the electrospray capillary and simultaneous analysis of the resulting peptides by nanoESI MS and MS/MS (Pohlentz et al. 2005). This strategy has been shown to yield high sequence coverages since no peptides are lost in the course of purification steps. Here, we demonstrate the applicability of this method to the analysis of hemoglobins (Hbs) from various species directly from diluted blood samples.

Materials and methods

Materials

Human blood samples were collected from volunteers from our laboratory. Animal blood samples were kindly provided by Dr. Michael Flügger (Tierpark Hagenbeck, Hamburg, Germany). Trypsin, chymotrypsin, and pepsin were purchased from Roche Diagnostics GmbH (Mannheim, Germany). All the solvents used were of HPLC grade purity.

Methods

Preparation of blood samples

2 µL of fresh or frozen and thawed blood were diluted in 80 µL of 50% methanol to yield a corresponding stock solution. These stock solutions were diluted five- to tenfold to a total volume of 20 µL containing final concentrations of 10–20% methanol, 10 mM NH_4HCO_3, and 0.01–0.02 µg/µL trypsin (or chymotrypsin). For pepsin digestions, ammonium hydrogen carbonate was replaced by 5–10 mM ammonium acetate (pH 3.6).

A dried blood droplet corresponding estimately to 2–5 µL of blood was resuspended in 40 µL of 50% methanol. Undissolved material was removed by centrifugation. Proteolytic digestions were performed as described above.

For mass determination of the intact Hb chains, the corresponding blood samples were diluted 1:4,000 in 50% methanol, containing 1% formic acid.

Mass spectrometry

Nanoelectrospray mass spectrometry experiments were carried out using a quadrupole time-of-flight (Q-TOF) mass spectrometer (Micromass, Manchester, UK) in the positive

ion mode [ESI(+)]. A Z-spray atmospheric pressure ionization (API) source was used with the source temperature set to 80°C and a desolvation gas (N_2) flow rate of 75 L/h. Proteolytic digestion mixtures were transferred to the electrospray capillary and data acquisition was started immediately.

For low-energy CID, peptide precursor ions were selected in the quadrupole analyzer and fragmented in the collision cell by use of a collision gas (Ar) pressure of 2.5×10^{-5} mbar and collision energies of 20–40 eV (E_{lab}).

NanoESI FT-ICR MS experiments were performed by use of a Bruker Apex II Fourier Transform Ion-Cyclotron Resonance mass spectrometer (FT-ICR MS) equipped with a 9.4 T actively shielded magnet. Gas-phase ions were generated from in-capillary tryptic digestion solutions or diluted blood sample prepared as described above by nanoESI in the positive ion mode using an Apollo ion source. Typical source conditions were: capillary voltage of −650 V and a capillary exit voltage of 60 V. The electrospray-generated ions were accumulated for 0.5 s in the hexapole located after the second skimmer of the ion source and then transferred into the ICR cell. The ions were trapped inside the Infinity™ ICR cell by application of a "sidekick", the trapping voltages were set to +0.9 V at both the trapping electrodes. All mass spectra were acquired in the broadband mode with 512 kword data points. The time-domain signals were zerofilled once and apodized by a quadratic sine bell function prior to Fourier transformation. For all the spectra, 64 scans were accumulated. The spectra were calibrated internally by use of the characteristic peptide ions generated by auto-digestion of trypsin.

Results and discussion

Identification of human hemoglobin

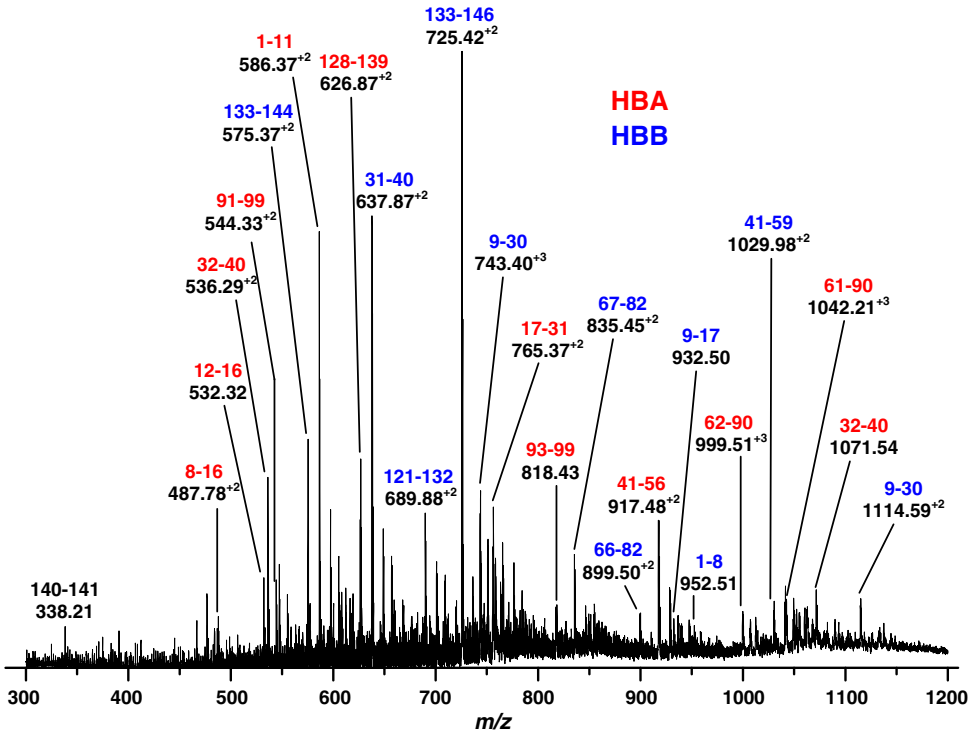
Starting from diluted blood samples adjusted to 10 mM NH_4HCO_3 and 10% methanol, the data acquisition was started immediately after protease addition and subsequent transfer of the mixture to the electrospray capillary. Figure 1 shows a typical nanoESI mass spectrum obtained from a human hemolysate during in-capillary tryptic digestion after 30 min as a representative example. All detectable peptide ions were identified by comparison of the experimentally obtained masses with those calculated from the known sequences of the hemoglobin α- and β-chains. Peptide maps obtained during in-capillary digestions of blood samples in general covered more than 80% of the hemoglobin sequences.

Alternatively, a dried blood droplet was resuspended in 50% methanol. Undissolved particles were removed by centrifugation and the clear supernatant was subjected to in-capillary tryptic digestion. The resulting nanoESI mass spectrum exhibits an essentially identical peptide map as compared to the spectrum displayed in Fig. 1. Ionic species corresponding to 38 different peptide ions could be detected. A data base search by MS-Fit with "The protein prospector" (Clauser et al. 1999), using the experimentally obtained peptide map yielded a match of 21 peptides to HbA from man, chimpanzee, or bonobo (identical HbA sequences) and 14 of the 17 remaining peptides to HbB from man, chimpanzee, bonobo, and gorilla (identical HbB sequences). The MOWSE scores were 9.97×10^7 (HbA) and 1.05×10^5 (HbB) and the sequence coverages were 74 and 78% for α- and β-chain, respectively. The verification of the blood droplet originating (most probably) from man starting from the initial resuspension took less than 2 h. Notably, no purification step of the hemoglobin or globin chain, respectively, was necessary prior to mass spectrometric analysis of the blood samples in contrast to previous protocols (Nakanishi et al. 1995).

Differentiation of closely related species

The potential of in-capillary digestion for rapid hemoglobin analysis was further probed by differentiation of closely related mammalian species. As an example, we analyzed blood samples from Asian (*Elephas maximus*) and African elephants (*Loxodonta africana*). Their Hbs differ by three amino acids in the α-chain and by one amino acid in the β-chain, giving rise to diagnostic "marker peptides" in the peptide maps (Kleinschmidt et al. 1986). Evaluation of the data obtained from the nanoESI MS spectra of tryptic in-capillary digestions of the respective elephant hemolysates renders 100% sequence coverage for both α- and β-globin chains of *Loxodonta africana*, while sequence coverages of 98 and 85% were obtained for the α- and β-globin chains of *Elephas maximus*. Mass scale expansions displaying the *m/z* region for the expected diagnostic peptide ions are shown in Fig. 2.

The exchange of α_{49}S (Asian) to α_{49}G (African) gives rise to doubly charged peptide molecular ions at *m/z* 910.44 (Asian) and *m/z* 895.40 (African), respectively. Accordingly, the alteration of β_{52}D (Asian) to β_{52}E (African) leads to the corresponding triply charged peptide molecular ions at *m/z* 706.97 (Asian) and *m/z* 711.63 (African). Each particular position of the mutations within the amino acid sequence were confirmed by low-energy CID experiments for all relevant peptide ions indicating the presence of the predicted alterations (data not shown). Our results show that closely related species can be fast and easily differentiated by in-capillary digestion of their hemolysates, which is particularly interesting if obvious anatomical



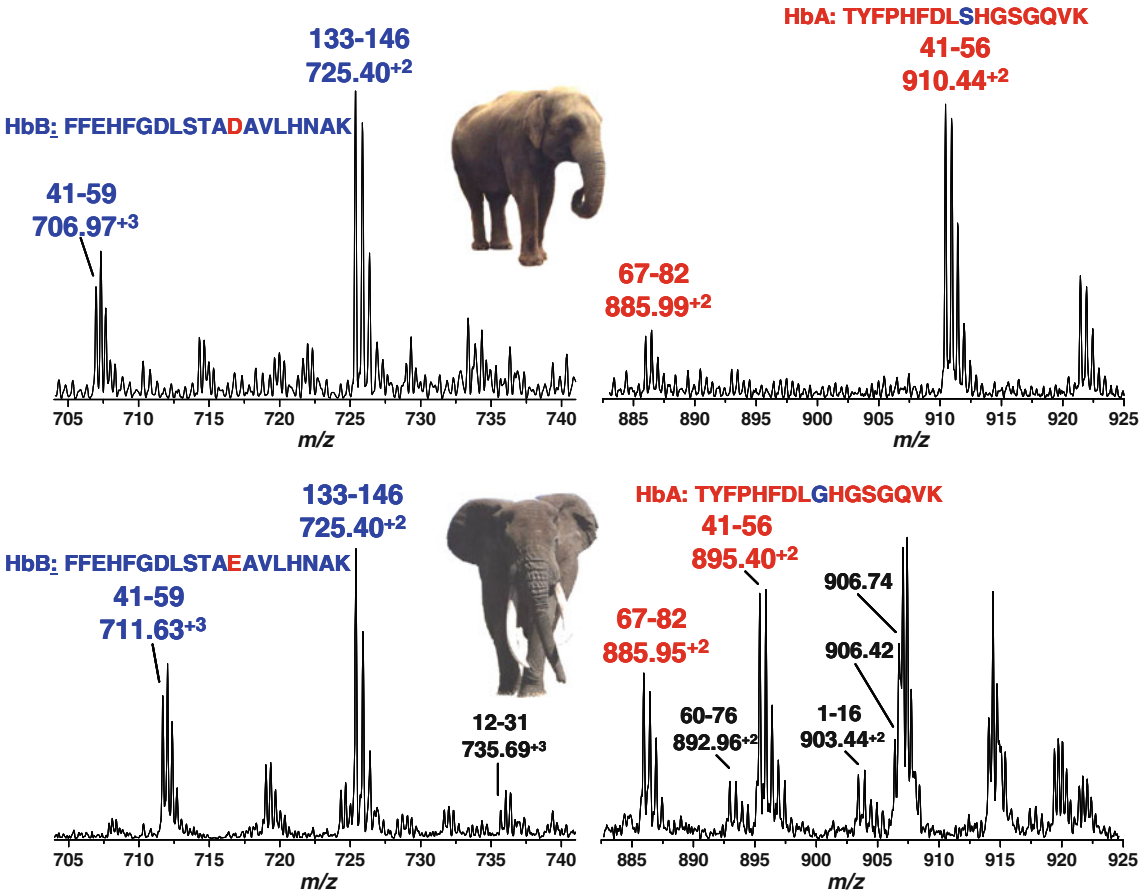


Fig. 1 NanoESI mass spectrum of tryptic peptides obtained by in-capillary digestion of a hemolysate from human. *Light gray* peptides derived from α globin chain, *dark gray* peptides derived from β globin chain

Fig. 2 Mass scale expansion of nanoESI mass spectra obtained during tryptic in-capillary digestions of blood samples from an Asian (*top*) and an African elephant (*bottom*)

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(a) HbA

    VLSAADKGNV KSVWDKIGGH AAEYGAEALN RMFASFPTTK TYFHHIDLSP GSAAIKAHGK
  1 VLSAADKNNV KTTWDKIGGH AAEYVAEGLT RMFTSFPTTK TYFHHIDVSP GSGDIKAHGK  60

    KVADALTTAV GHLDDLPSAL SALSDLHAHK LRVDPVNFKF LSHCLLVTLA AHLGPDFTPH
 61 KVADALTTAV GHLDDLPTAL STLSDVHAHK LRVDPVNFKF LNHCLLVTLA AHLGADFTPS 120

    AAASLDKFLA SVSTVLTSKY R
121 IHASLDKFFA SVSTVLTSKY R

    HbB

    VNLTAEEKAA VMGLWAKVNV EEIGGEALGR LLTVYPWTQR FFEKFGDLSS ASAIMSNPHV
  1 VHLTAAEKSA ILDLWGKVNV GEIGAEALGR LLVVYPWTQR FFEKFGDLSS ASAIMSNAHV  60

    KAHGAKVLAS FSEGLKHLDN LKGTFAK                   LLGNMI LIALAHHHAA
 61 KSHGAKVLAS FSEGLKHLQD LKGTFAKLSE LHCDKLHVDP ENFRLLGNMI VIALAHHHPS 120

    DFPPSTQAAF HKVTAGVANA LAHKYH
121 EFTPCTQAAF QKVTAGVANA LAHKYH

(b) HbA

    VLSAADKSNV KAAWGKVGGN AGAYGAEALE RMFLSFPTTK TYFPHFDLSH GSAQVKGHGE
  1 VLSAADKSNV KAAWGKVGGN AGAYGAEALE RMFLSFPTTK TYFPHFDLSH GSAQVKGHGE  60

    KVAAALTKAV GHLDDLPGTL SDLSDLHAHK LRVDPVNFKL LSHSLLVTLA SHHPNDFTPA
 61 KVAAALTKAV GHLDDLPGTL SDLSDLHAHK LRVDPVNFKL LSHSLLVTLA CHLPNDFTPA 120

    VHASLDKFLA NVSTVLTSKY R
121 VHASLDKFLA NVSTVLTSKY R

    HbB

    MLTAEEKAAV TGFWGKVNVD EAGAEALGRL LVVYPWTQRF FEHFGDLSSA DAVMNNPKVK
  1 MLTAEEKAAV TGFWGKVKVD EVGAEALGRL LVVYPWTQRF FEHFGDLSNA DAVMNNPKVK  60

    AHGKKVLDSF SNGLKHLDDL KGNF           LHVDPE NFRLLGNVLV VVLARRHGTE
 61 AHGKKVLDSF SNGMKHLDDL KGTFAQLSEL HCDKLHVDPE NFRLLGNVLV VVLARHHGNE 120

    FTPVLQADFQ KVVTGVANAL AHRYH
121 FTPVLQADFQ KVVAGVANAL AHKYH
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Fig. 3 Sequences of hemoglobins of the Patagonian cavy (*Dolichotum patagona*) (**a**) and the Persian gazelle (*Gazella subgutturosa subgutturosa*) (**b**) deduced from nanoESI CID spectra of peptide molecular ions obtained during in-capillary proteolytic digestions of the corresponding blood samples. The deduced sequences were aligned to the corresponding HbA and HbB sequences of the closest relatives Guinea pig (Patagonian cavy) and sheep (Persian gazelle), respectively

differences, for practical reasons, cannot be taken into consideration.

De novo sequencing of Hbs

Blood samples derived from the non-annotated species Patagonian cavy (*Dolichotum patagona*) and Persian gazelle (*Gazella subgutturosa subgutturosa*) were separately subjected to in-capillary digestions using trypsin, chymotrypsin, or pepsin as described in "Methods". The proteolytic peptide precursor ions were fragmented by CID and de novo sequenced. 100% of the α- and more than 85% of the *β*-Hb chain sequences could be covered for both species. In Fig. 3, the deduced sequences are aligned to the corresponding database (BLAST, Altschul et al. 1997) derived sequences of closely related species [Guinea pig for the Patagonian cavy (Fig. 3a) and sheep for the Persian gazelle (Fig. 3b)].

Furthermore, peptide mixtures obtained from tryptic in-capillary digestions of different hemolysates were submitted to high resolution nanoESI Fourier-transform ion-cyclotron resonance mass spectrometry (FT-ICR MS). Owing to the high mass accuracy of the instrument, the resulting peptides were readily identified according to their

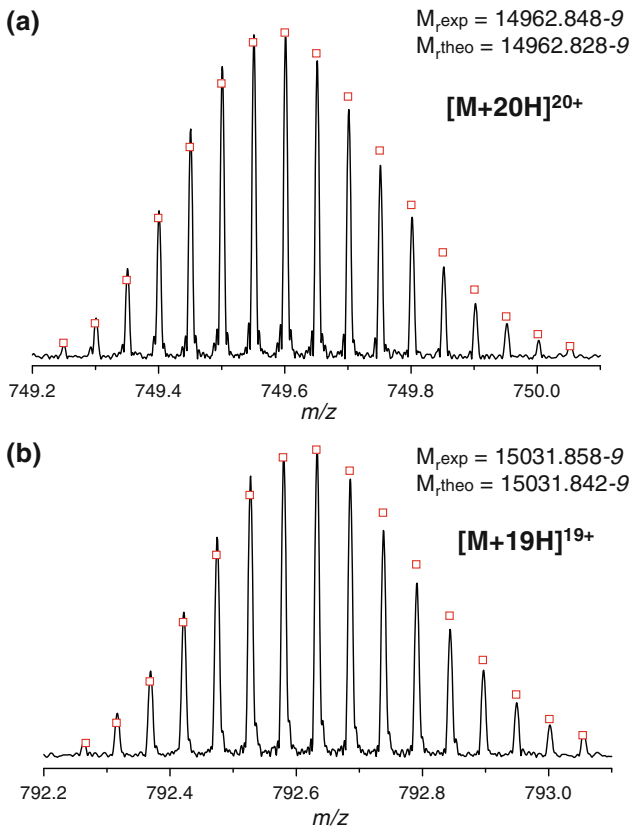

Fig. 4 Mass scale expansions (+) nanoESI-FT-ICR mass spectra obtained from the corresponding diluted blood samples depicting the indicated charge states of intact HbAs of the Patagonian cavy (**a**) and Persian gazelle (**b**). The *rectangles* represent the theoretically abundant distribution of the different isotopomeric species derived from the elemental composition of the protein. The exact masses were determined by comparing the experimentally obtained isotopic envelopes (ion charge state $20H^+$, the most abundant isotopomer) to the theoretical isotopic distributions. The mass of HbA of Patagonian cavy was determined to be 14962.848–*9* Da (theoretical value 14962.828–*9*), while it is 15031.858–*9* Da (theoretical value 15031.842–*9*) for the Persian gazelle [the mass values are followed by the value of the mass difference (in units of 1.0034 Da) between the most abundant isotopic peak and the monoisotopic peak; so "–*9*" indicates that the most abundant isotopomer contains 9 ^{13}C atoms]

exact mass, thereby confirming the novel primary sequences of α- and *β*-globin chains of *Dolichotum patagona* and *Gazella subgutturosa subgutturosa* (cf. Supplementary material Tables S1 and S2). Alternatively, the molecular weights of the intact Hb chains were determined by FT-ICR mass spectrometric analysis of the respective diluted blood samples.

Molecular weights of full-size HbA of Patagonian cavy and Persian gazelle were determined by mass deconvolution (Senko et al. 1995) of the centroid distribution in a Gaussian shaped envelope with base line separation of isotopically resolved multiprotonated species (Fig. 4).

The resulting values were in excellent agreement with the theoretical values calculated from the respective amino acid sequences. The experimentally determined HbA molecular weights 14962.848–*9* (Patagonian cavy) and 15031.858–*9* (Persian gazelle) fit perfectly to the calculated values 14962.828–*9* and 15031.842–*9*, respectively (cf. Fig. 4), thus corroborating the sequences deduced from the nanoESI Q-Tof CID spectra. The accuracy of the experimentally determined molecular masses was typically better than 1.5 ppm.

Conclusion

The generally high sequence coverages obtained from in-capillary digestion MS analyses, combined with the fact that hemoglobin is by far the most abundant protein in blood, allow direct studies on Hbs from blood samples. The fast and easy species identification starting from dried blood droplets may be of forensic interest. Also, the presented strategy might have some potential as a rapid method for studying different hemoglobin phenotypes in humans, which still represents an integral field in the diagnosis of hemoglobinopathies (Wajcman and Riou 2009). The determination of modified hemoglobin species, e.g., glycated hemoglobin A1c (HbA1c), representing an important marker for long-term assesment of the glycemic state in patients with diabetes (Larsen et al. 1990), is not feasible by use of the in-capillary digestion method presented in this study due to the low concentrations of glycated species (∼2% in healthy male adults) (Desmeules et al. 2010). Recently, it has been demonstrated that selective enrichment of glycated peptides is a prerequisite for their detection by use of mass spectrometry (Takátsy et al. 2009). However, it has been shown already that posttranslationally glycosylated proteins are amenable to mass spectrometric analysis by use of in-capillary digest (Henning et al. 2007).

The application of CID experiments during in-capillary digestions enables de novo sequencing for phenotypic analysis of Hbs derived from non-annotated species. The method worked perfectly for the HbAs of the species under investigation, however, to date, we were not able to close the gaps in the corresponding *β*-chains. The latter observation can be explained by the presence of unmodified cystein residues with the respective sequence segments exhibiting rather high reactivities with respect to redox processes. Thus, reactions with other species present in the studied blood samples might occur leading to various unintended reaction products of low abundance. However, the results obtained so far are encouraging to further analyze hemoglobins from other species.

In the future, we aim at expanding the in-capillary digestion strategy—after removal of the red blood cells—to serum proteins of various species.

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