International Journal of Mass Spectrometry 300 (2011) 130–142

Contents lists available at ScienceDirect

International Journal of Mass Spectrometry

journal homepage: www.elsevier.com/locate/ijms

Top-down analysis of small plasma proteins using an LTQ-Orbitrap. Potential for mass spectrometry-based clinical assays for transthyretin and hemoglobin

Roger Théberge, Giuseppe Infusini, Weiwei Tong, Mark E. McComb, Catherine E. Costello*

Center for Biomedical Mass Spectrometry, Boston University School of Medicine, Boston, MA 02118, USA

ARTICLE INFO

Article history:
Received 20 April 2010
Received in revised form 10 August 2010
Accepted 17 August 2010
Available online 24 August 2010

Keywords:
Top-down
Protein
Variant
Transthyretin
Hemoglobin
Post-translational modification

ABSTRACT

Transthyretin (TTR) amyloidosis and hemoglobinopathies are the archetypes of molecular diseases where point mutation characterization is diagnostically critical. We have developed a top-down analytical platform for variant and/or modified protein sequencing and are examining the feasibility of using this platform for the analysis of hemoglobin/TTR patient samples and evaluating the potential clinical applications. The platform is based on a commercial high resolution hybrid orbitrap mass spectrometer (LTQ-Orbitrap™) with automated sample introduction; automated data analysis is performed by our own software algorithm (BUPID top-down).

The analytical strategy consists of iterative data capture, first recording a mass profile of the protein(s). The presence of a variant is revealed by a mass shift consistent with the amino acid substitution. Nozzle-skimmer dissociation (NSD) of the protein(s) yields a wide variety of sequence-defining fragment ions. The fragment ion containing the amino acid substitution or modification can be identified by searching for a peak exhibiting the mass shift observed in the protein mass profile. This fragment ion can then be selected for MS/MS analysis in the ion trap to yield sequence information permitting the identification of the variant. Substantial sequence coverage has been obtained in this manner. This strategy allows for a stepwise MS/MS analysis of the protein structure. The sequence information obtained can be supplemented with whole protein NSD fragmentation and MS/MS analysis of specific protein charge states. The analyses of variant forms of TTR and hemoglobin are presented to illustrate the potential of the method.

1. Introduction

Current approaches to protein structure determination by mass spectrometry center on so-called bottom-up and top-down approaches. Bottom-up methods [1,2] utilize proteolytic digestions to break down the protein into constituent peptides that can be readily analyzed by LC–MS/MS whereby separation and dissociation of the peptides provides structural information for many peptides in a single experiment. The disadvantages associated with the bottom-up approach are: labor intensive and time consuming sample preparation, together with less than 100% sequence coverage, as well as the potential for introduction of artifacts during digestion. Furthermore, molecular weight information lost through proteolysis hinders the search for post-translational modifications and information on the relationship(s) among multiple post-translational modifications (PTMs) to a single protein is usually sacrificed.

For the top-down approach, proteins are directly introduced into the mass spectrometer and individual components can be mass-selected and dissociated in the instrument, yielding product ions containing structural information. Top-down [3,4] is also more efficient in its use of mass spectrometer system time because separations can be performed off-line if necessary. The methodology of top-down analysis circumvents the drawbacks listed above by analyzing intact protein molecular weights and fragments ions directly within the mass spectrometer. Although bottom-up remains the workhorse of proteomics, the characterization of point mutations by LC–MS/MS can be made difficult by the higher false positive rate that is observed when sequence variations are allowed in the database search.

Peptide mapping was demonstrated to be useful for hemoglobin variant determination by fast atom bombardment mass spectrometry [5]. The use of targeted analysis for common hemoglobin variants through multiple reaction monitoring (MRM) acquisition mode MS of whole blood tryptic digests was proposed recently as a population screening methodology [6]. The idea of generating primary structural information from intact proteins through MS/MS on a Fourier transform ion cyclotron resonance (FT-ICR) mass spectrometer [7,8] originated soon after the introduction electrospray ionization mass spectrometry. McLuckey et al. [9] have adapted

* Corresponding author at: Center for Biomedical Mass Spectrometry, Department of Biochemistry, Boston University School of Medicine, 670 Albany Street, Room 511, Boston, MA 02118-2646, USA. Tel.: +1 61 7 638 6490; fax: +1 61 7 638 6491.
E-mail address: cecmsms@bu.edu (C.E. Costello).

1387-3806/$ – see front matter © 2010 Elsevier B.V. All rights reserved.
doi:10.1016/j.ijms.2010.08.012

ion traps for the purpose of top-down protein sequencing. Pseudo MS^3 analyses using quadrupole orthogonal time-of-flight (Q-o-TOF) instruments were reported by Thevis et al. [10] for top-down determinations on transferrins. A triple quadrupole instrument was used by Witkowska et al. to characterize a genetic mutation that gave rise to a variant human α-globin [11]. Whilst a variety of instrumental configurations have thus been used for the top-down analysis of proteins, the high resolution, mass accuracy and MS^n capacity of FTICR MS [12,13], as well as the availability of multiple activation techniques, including infrared multiphoton dissociation (IRMPD) [14], electron capture dissociation (ECD) [15], electron transfer dissociation (ETD) [16], and sustained off-resonance collisionally activated dissociation (SORI-CAD) [17], continue to favor these instruments for top-down analysis.

The recent introduction of the LTQ-Orbitrap mass spectrometer [18] offers many of the high resolution, mass accuracy and MS^n advantages of FTICR-MS at a more affordable price. Macek et al. [19] demonstrated the ability of the LTQ-Orbitrap to generate top-down data using protein standards ranging from 10 to 25 kDa. However, their tandem analysis was performed exclusively within the linear ion trap portion of the system. The multistage MS/MS capability of the system was not investigated. The LTQ-Orbitrap has similarly been used in a top-down mode to characterize recombinant monoclonal antibodies [20,21].

Whilst these examples highlight the potential of the LTQ-Orbitrap for the top-down analysis of proteins, a comprehensive approach that fully exploits the high performance capabilities of this hybrid mass spectrometer has not been presented. This approach could be particularly useful in the case of small and easily isolated plasma proteins of clinical relevance such as transthyretin and hemoglobins, where characterization of amino acid substitutions that can define genetic point mutations integral to the pathogenesis of the diseases is necessary for precise diagnosis of hereditary transthyretin amyloidosis [22] and hemoglobinopathies [23], respectively. The use of mass spectrometry to characterize variants of these proteins has traditionally relied on bottom-up approaches [5,24–26]. A protocol that combines intact protein molecular weight profiling by FT-ICR MS with DNA sequencing has been implemented for the detection and characterization of TTR variants [27,28] in a clinical setting. The relatively small size, ease of isolation in abundant quantities, and absence of structural features refractory to collisional activation (*e.g.*, no intramolecular disulfide bonds) make TTR and hemoglobins ideal candidates for top-down analysis. However, to our knowledge, transthyretin-related proteins have only been analyzed using top-down in our published study of TTR fragments ranging from 7 to 10 kDa that were extracted from amyloid fibrils and sequenced with a Q-o-TOF MS [29].

Top-down MS/MS has been used for hemoglobins; indeed, they were among the first proteins to be analyzed in this manner [7,8,30]. Nevertheless, top-down MS/MS has only been applied to hemoglobin variant characterization in very few instances. The potential of top-down MS/MS for the characterization of hemoglobinopathies was recognized in 1996 but the complexity of spectra, unresolved charge state ambiguities and the lack of suitable software tools for spectra interpretation were cited as obstacles for the routine application of the method. In their review on the use of mass spectrometry in the hemoglobinopathy field published at this time, these researchers pointed out the desirability of developing top-down methods for the sequencing of intact hemoglobin chains [31].

The preliminary study whose results are reported herein aimed to explore the applicability of a simple top-down method to TTR and hemoglobin variant characterization. By combining automated sample introduction, LTQ-Orbitrap and customized software algorithms written in-house, an analytical platform was assembled and tested. The results presented in this preliminary study highlight the feasibility of this approach for characterizing variants of transthyretin and hemoglobins. The potential for this method to be applied in a clinical setting seems promising. Given that the LTQ-Orbitrap system is becoming quite widely available, it is important to raise the awareness of the clinical community to the potential of these instruments for straightforward top-down analyses.

2. Experimental

2.1. Samples

Transthyretin was obtained by immunoprecipitation from human serum, using the method outlined by Lim et al. [32] with the difference that a smaller volume of serum was used (50 μL *vs.* 200 μL). Hemoglobin was obtained from whole blood by diluting 1 μL of whole blood in 500 μL of ESI buffer. Use of de-identified patient samples was approved by BUSM. All samples were analyzed in ESI buffer composed of 50% water, 50% acetonitrile containing 0.2% formic acid. Sample concentration was approximately 1 pmol/μL.

2.2. Mass spectrometry

The samples were introduced into a "Classic" LTQ-Orbitrap mass spectrometer (Thermo-Fisher Corp., Waltham, MA) using a TriVersa NanoMate system (Advion Biosciences, Ithaca, NY). A low-flow ESI chip was used as a static emitter to deliver the sample at 20–40 nL/min. The LTQ-Orbitrap MS was operated in the nanospray positive-ion mode. The top-down analyses were performed in a number of different activation modes in order to explore ion distribution and completeness of information on sequence and PTMs. Nozzle-skimmer dissociation was produced by varying the "source fragmentation" voltage between 60 and 100 V. For MS/MS analysis, peaks with the m/z values of interest were selected within an isolation window width of m/z 4–8. Helium was employed as the collision gas for CID in the LTQ; analyses were performed at normalized collision energies of 25–35%. HCD (higher energy C-trap dissociation) was also evaluated, using energies in the range of 15–23%. For all experiments, the data acquisition parameters were similar to default values (activation $q = 0.25$, FT automatic gain control (AGC) 2×10^5 for the MS and 1×10^5 for the MS^n mode. All mass spectra measured for ions detected in the Orbitrap were recorded with 60,000 resolution @ m/z 400. Mass spectra were generated by averaging scans over a period of 1–2 min. Fragment ion mass spectra were generated by averaging scans over a period of 2–4 min, each scan including three microscans.

2.3. Data analysis

Fragment ion mass spectra were deconvoluted using the Xtract software from Thermo Fisher Scientific. The resulting fragment mass lists were analyzed using customized software algorithms written in-house, including BUPID top-down [33] (Boston University Protein Identifier). BUPID is capable of identifying b-, y-, c-, and z-ions as well as internal ions arising from the relevant activation techniques. This software will shortly be made available to the community for download.

3. Results and discussion

3.1. General comments

The basic analytical strategy consists in gathering a maximum of structural information by obtaining molecular weight data on the

intact protein(s) and then dissociating the molecular species to generate complementary ion pairs that have sufficient abundances and appropriate masses for subsequent further stages of tandem MS. In theory, given that the LTQ-Orbitrap offers three potential sites for ion activation (the nozzle-skimmer region, the linear ion trap (LTQ) and C-trap), multiple sequential MS^n experiments should be feasible (see supplementary information). However, this scenario is not fully realizable in practice due to the significant losses in ion abundance that occur during each MS/MS step. For the Orbitrap Discovery, we have found the most practical approach to be the use of nozzle-skimmer dissociation (NSD) to yield a rich array of MS^2 fragment ions that can be subsequently sampled by mass selection and collision-induced decomposition in the LTQ, followed by high resolution, accurate mass determinations of the fragments in the Orbitrap. Although the C-trap can be used for MS/MS with HCD, our results have so far demonstrated a significant loss in signal intensity when using this activation method. (Upgrade to Velos-type optics may partially or fully address this loss of sensitivity. Furthermore, although ETD can also be carried out on the LTQ-Orbitrap [34], this option had not yet been installed on our instrument when the reported analyses were carried out. It will be evaluated in future experiments.)

The sequence information obtained by NSD followed by LTQ-CID of the resulting fragment ions can be extended or confirmed by LTQ-CID of mass-selected individual charge states of the intact protein. For the two proteins that are the subject of this study, only minimal sample preparation was needed to achieve adequate sensitivity for clinically relevant sample amounts in analyses based on nozzle-skimmer dissociation. TTR can easily be isolated in a pure state by immunoprecipitation from serum; hemoglobins consist of a simple mixture of two proteins that can both be clearly observed by ESI-MS upon 500-fold dilution of whole blood.

A preliminary mass profile of each sample was first generated to determine the precise mass shift that could be attributed to an amino acid substitution or post-translational modification (PTM). This information enabled identification of the NSD-generated fragment(s) that bear an amino acid substitution or PTM. The relevant fragment ion(s) could, individually, be selected for MS^3 analysis to provide more detailed structural information. In the application of this method, it should be noted that, even in the absence of an apparent mass shift the NSD spectrum should always be acquired, given the possibility of variant mass shifts too small to be noted in the mass profile or of double sequence variations that could compensate one another, thus giving rise to small net mass shifts. The inspection of sequence-defining complementary ion pairs should assure the detection of all mass shifts, even those that are small or serve to compensate one another.

In development of the method, the next step was to mass-select specific intact protein charge states to map out the fragmentation of the analyte(s). It is of paramount importance to ascertain the origin of the NSD fragment ions if NSD/LTQ-CID MS/MS is to be used for sequence analysis. In the case of hereditary disorders, unless the sample donor is a homozygote, both wild-type and variant proteins can be detected. Furthermore, extensive PTMs are usually present in TTR and these give rise to multiple isoforms of both the wild-type and variant protein [24]. The fragmentation behavior of each of the major post-translationally modified forms was investigated to assure that these modifications could be determined. The hemoglobin samples, although much less subject to post-translational modification, consist of both α and β chains and this system offers its own challenges in the implementation of the NSD/LTQ-CID MS/MS analytical scheme.

It is well known that the fragmentation of proteins varies over the different charge state isoforms selected for top-down analysis (charge state-dependent dissociation behavior) and Mekecha et al. [35] have used this observation to generate extensive α-hemoglobin sequence coverage by recording the fragmentation of the full range of the protein charge states from 4+ to 20+ using a modified ion trap. However, this approach is not consistent with the methodology for higher throughput analysis of variant sequences developed herein for use in a clinical setting.

3.2. Top-down analysis of transthyretin

As noted above, there is no previous report of a top-down analysis of transthyretin, apart from our published study of TTR fragments that ranged from 7 to 10 kDa that were extracted from amyloid fibrils. Of the two analytical challenges considered in this paper, the characterization of TTR variants presents the simpler case, despite the presence of extensive PTMs. Fig. 1 shows the nano-ESI mass spectrum of a wild-type TTR sample; the inset provides an expansion of the region that includes the 14+ charge state of the molecular-weight related species. The TTR post-translational modifications overwhelmingly center on Cys 10, the only cysteine residue of the protein, and generate the *S*-thiolated (cysteine, CysGly and glutathione) and *S*-sulfonated forms [24,32]. The ratio between the two most abundant post-translationally modified forms, *S*-cysteinylated and *S*-sulfonated TTR, can vary widely from sample to sample. A higher abundance of *S*-sulfonation with respect to *S*-cysteinylation has been speculated to reflect disease states [36,37]. The *S*-CysGly and *S*-glutathionylated forms of TTR are present in low abundance. The unmodified form of the protein typically represents less than 15% of the total TTR-related isoforms observed in the mass spectra of TTR immunoprecipitated from human serum. One factor of note is the difference in the stabilities of these post-translational modifications under collisional activation conditions typically employed for NSD and CID. The *S*-thiolated forms are quite stable and generally remain intact. On the other hand, the modification in *S*-sulfonated TTR is very labile; the *S*-sulfonate group on a cysteine residue is easily cleaved [38,39] and the released protein appears in the NSD mass spectrum as unmodified TTR. However although this post-translational modification is lost as a result of the activation method, the capability of the method to obtain the complete amino acid sequence information for variant characterization is not adversely affected.

As discussed above, the most common approach to top-down MS/MS analysis of proteins is to mass select individual charge states of intact protein ions for MS/MS. The LTQ-CID MS/MS spectrum of the most abundant charge state, $[M+14H]^{14+}$ m/z 992.43, of a post-translationally modified wild-type TTR is shown in Fig. 2. This ion corresponds to the *S*-cysteinylated form of TTR. This mode of fragmentation does not yield extensive sequence coverage. The core section of the sequence and the N-terminal region are not very well covered. Fragmentation is dominated by the sequence-defining complementary ion pair b_{42} and y_{85}, as well as a series of low mass y-ions describing the C-terminus that cover positions 117–125 and tend to be ubiquitous features of top-down spectra of TTR-derived proteins using CID [29]. The high abundance of the C-terminal fragments is well illustrated by the expansion factor (*X*3) necessary to visualize the other fragments in Fig. 2. Higher collision energies favor the appearance of this ion series above all others.

More complete initial coverage can be obtained by interrogating several different charge states (data not shown). It should be noted that the use of higher skimmer potentials in NSD can lead to proton stripping. This can be advantageous for the top-down approach, as it gives the analyst access to lower charge states in significant abundance, enabling further probing of the charge state-dependent dissociation behavior. For example, an abundant TTR^{8+} charge state is generated at higher skimmer voltages and can

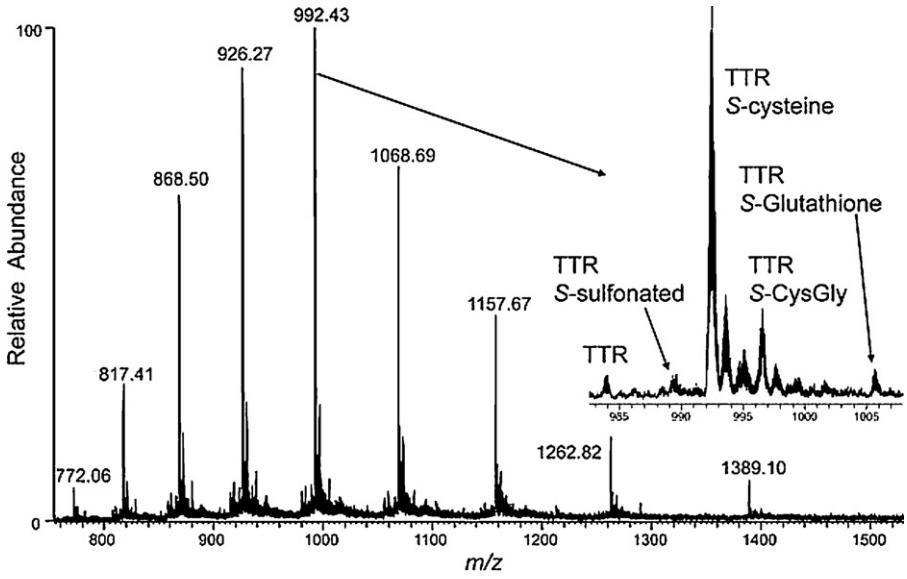

Fig. 1. Nano-ESI mass spectrum of a wild-type TTR immunoprecipitated from serum. The inset provides an expansion of the region that includes the 14+ charge state (m/z 980–1010) and the various post-translationally modified forms of TTR.

be subjected to MS/MS. Dissociation of this charge state showed suppression of the extensive C-terminal low mass fragmentation which, as noted above, tends to dominate the MS/MS spectrum of most charge states of the TTR isoforms and thus yielded broader sequence coverage (supplementary data). This observation can probably rationalized on the basis of the proton mobility fragmentation model as the TTR sequence contains 10 basic sites. Presumably, in the lower charge states, a proton is less likely to be in a position to initiate fragmentation of the C-terminal region than in more highly charged isoform of the protein. In other words, charge might be sequestered away from the C-terminal activation site in the TTR^{8+} isoform. Important questions remain to be explored in the study of protein CID mechanisms [40,41] but it is beyond the scope of this paper to address them.

MS^3 analysis of the very abundant b_{42}/y_{85} pair generated by CID of a selected charge state is possible and could be used for variant analysis. Nevertheless, despite the potential illustrated by precursor ion selection and fragmentation in the LTQ, our further investigations indicated that nozzle-skimmer dissociation offered greater advantages, in terms of both experimental simplicity and observed sensitivity, as the first step in the top-down analysis of TTR. The fact that the immunoprecipitation step used as the isolation procedure for transthyretin yields the protein in a very pure state, whereby no interference is present upon MS analysis, allows

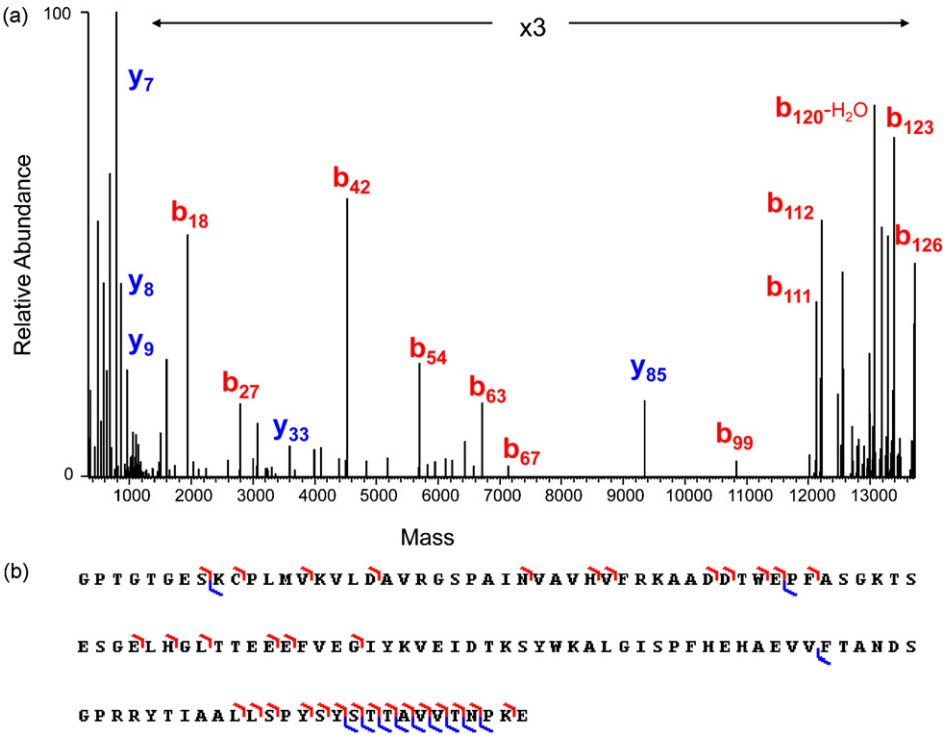

Fig. 2. (a) Deconvoluted LTQ-CID MS^2 spectrum of m/z 992.43 corresponding to the $[M+14H]^{14+}$ of *S*-cysteinylated wild type TTR. (b) Since the *S*-cysteinyl post-translational modification is eliminated immediately upon CID, the sequence coverage generated by BUPID corresponds to unmodified TTR.

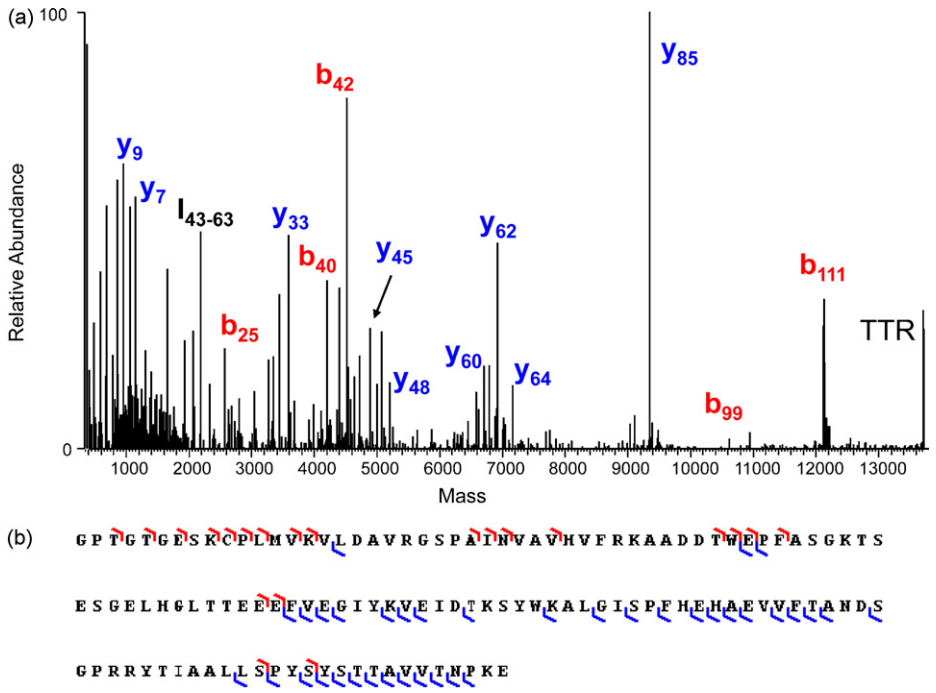

Fig. 3. (a) Deconvoluted NSD MS^2 spectrum of wild type TTR immunoprecipitated from serum. (b) Sequence coverage generated by BUPID.

the direct use of NSD. A representative NSD MS^2 fragment ion spectrum of immunoprecipitated TTR is presented in Fig. 3.

The mass spectrum of this sample indicated that *S*-cysteinylation was the most abundant post-translational modification. The most notable feature of this spectrum is the presence of the sequence-defining complementary ion pair, b_{42}/y_{85}. These two fragment ions capture the complete 127-amino acid TTR sequence. The genesis of this pair can easily be rationalized as it arises *via* the highly favored cleavage of a glutamic acid/proline (Glu42-Pro43) peptide bond. Of note is the diminished abundance of the low mass y-ion series, as compared to that found in the LTQ-CID of the $[M+14H]^{14+}$ m/z 992.43 shown in Fig. 2. For this type of experiment, a lower fragmentation voltage could be selected in order to favor the formation of an abundant b_{42}/y_{85} complementary ion pair for subsequent LTQ-CID MS^3. The presence of a relatively abundant internal ion $I_{43–63}$, resulting from cleavages of both the Glu42-Pro43 and Glu63-Phe64 bonds probably results from secondary fragmentation of the y_{85} fragment ion. Such internal ions can arise from secondary cleavages of b- or y-ions. These species can be identified by BUPID. (Although they were not needed for interpretation of the data presented herein, failure to properly assign them could introduce ambiguity to the sequence analysis.) At this point, the reliability of the assignment using b- and y-ions is the most important factor to be considered and, for that reason, we have chosen not to discuss internal fragment ions save for exceptional cases. As indicated in the spectrum shown in Fig. 3, the $[y_{85}+9H]^{9+}$ fragment ion (m/z 1039.861) is produced in high abundance even at relatively low "fragmentation" potential. The MS^3 spectrum of this NSD-generated fragment ion was produced by CID of the selected precursor in the LTQ and mass analysis of the products in the Orbitrap, which yielded a substantial set of accurate mass values for the b-, y- and internal fragments; the most abundant of these are labeled on the spectrum shown in Fig. 4a. The coverage obtained in this manner was specific and extensive, as indicated in the scheme that is presented above the spectrum.

For TTR, we have determined that the $[y_{33}+3H]^{3+}$ (m/z 1198.279 for wild-type TTR) and $[y_{45}+4H]^{4+}$ (m/z 1224.619 for wild-type TTR) fragment ions also yield detailed structural information on the C-terminal portion of the protein. Only positions 100–110 did not exhibit definitive fragments in any of the modes utilized to gather sequence information on this region of the protein. Using an FT-ICR instrument, we have recently determined that coverage of this region of the sequence can be improved by the use of electron transfer dissociation (ETD) or electron capture dissociation (ECD). These additional approaches will be explored further and the results will be reported in a subsequent contribution.

Obtaining an informative MS^3 spectrum for the cysteinylated $[b_{42}+5H]^{5+}$ fragment ion observed at m/z 906.060 (Fig. 4b) was more challenging, given the heterogeneity introduced by the Cys10-based PTMs [24] that distributed the signal over multiple species, and the lower absolute yield of this fragment ion. The coverage obtained in the MS^3 spectrum for the cysteinylated $[b_{42}+5H]^{5+}$ fragment ion was extensive, with a conspicuous b_{18}/y_{24} ion pair resulting from a cleavage on the C-terminal side of an aspartic acid residue.

Although the abundance of fragment ions could be moderately increased by varying the skimmer potential, the abundance of the b_{42} species remained lower than that of the y_{85} ion at any given value of this parameter. Nevertheless, these factors did not significantly hinder obtaining N-terminal sequence coverage from MS^3 of the $[b_{42}+5H]^{5+}$ ion, even though they decreased the numbers of ions available for that purpose.

For an unknown or partially known protein, subjecting each member of a complementary ion pair, such as this b_{42}/y_{85} example, to further tandem MS^n analysis can quickly yield substantial sequence information. As shown here in the case of variant sample analysis, the full sequence of the wild-type protein has already been established and, thus, the selection of the proper member of the complementary ion pair for further dissociation depends solely on the determination of which of the two fragments exhibits the same mass shift as that observed in the previously recorded mass profile of the intact protein sample. The variant fragment ion can then be mass selected for MS^3 analysis to obtain sequence information.

The usefulness of the approach presented here can be appreciated if one considers the case of a Cis double mutation of Ser6

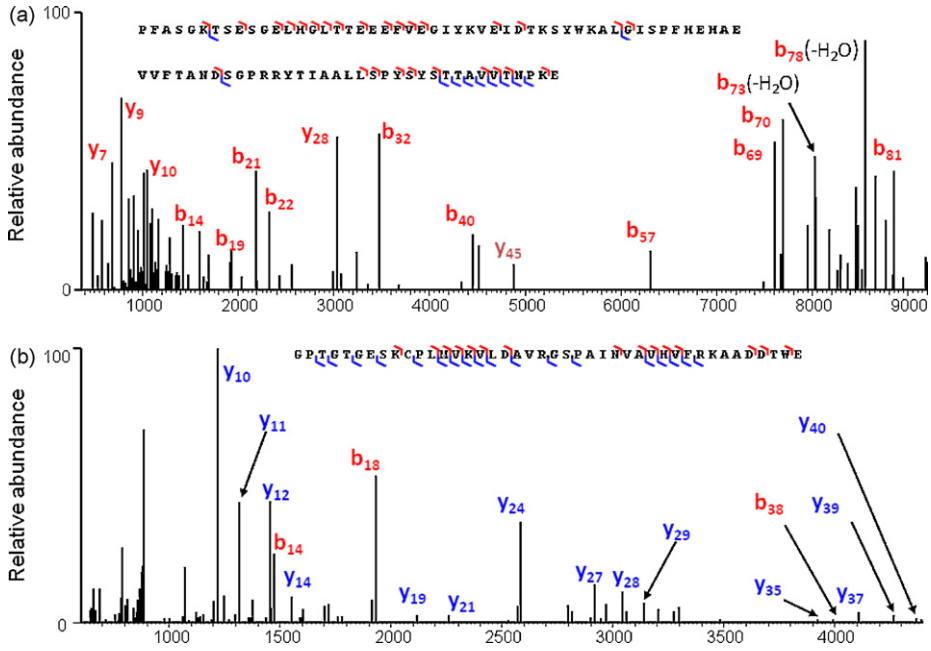

Fig. 4. (a) Deconvoluted LTQ-CID MS^3 spectrum of the $[y_{85}+9H]^{9+}$ fragment ion, containing residues 43–127, generated from the NSD of wild-type TTR immunoprecipitated from serum. The sequence coverage generated by BUPID is shown above the spectrum. (b) Deconvoluted LTQ-CID MS^3 spectrum of the cysteinylated $[b_{42}+5H]^{5+}$ fragment ion at *m*/*z* 906.060, containing residues 1–42, generated from the NSD of wild type TTR immunoprecipitated from serum. The sequence coverage generated by BUPID is shown above the spectrum.

and Ala30 reported to have been detected by the combination of molecular weight profiling by mass spectrometry and DNA analysis [28]. Using the method we describe here, mass spectrometry alone would be sufficient to detect this combination of changes in the protein structure. Examination of the b_{42}/y_{85} complementary ion pair in the expected NSD spectrum would reveal the presence of a +2-Da shift in the $b_{42}{}^{5+}$ ion. No single amino acid substitution can give rise to a mass shift of +2 Da, so this data would indicate that a double mutation is likely present. Hence, the NSD data can increase the ability of the analyst to accurately measure small mass shifts due to single or multiple sequence variations. The MS^3 analysis would include LTQ-CID of the of the $b_{42}{}^{5+}$ ion, providing the added structural information needed to fully define the variant positions.

The approach described above was applied to a TTR sample immunoprecipitated from patient serum. The nanospray mass spectrum indicated the presence of an unknown variant with a +30-Da mass shift (Fig. 5). The NSD spectrum showed a peak having a 5+ charge at *m*/*z* 912.546 that corresponded to a shift of +30 Da for the cysteinylated b_{42} fragment of the variant *vs.* that of the cysteinylated wild-type protein, and thus indicated that the variant was present within the first 42 positions of the sequence (Fig. 6a).

No ion corresponding to $(y_{85}+30)$ Da was observed. The position of the mutation could actually be narrowed somewhat by careful examination of the NSD data, following the b-ion series until the

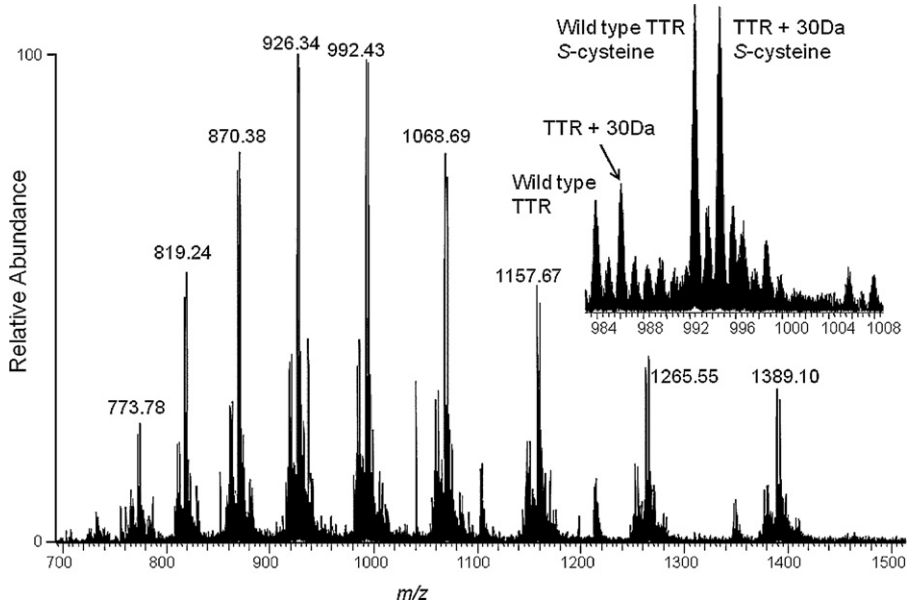

Fig. 5. Nano-ESI mass spectrum of an unknown TTR variant immunoprecipitated from serum exhibiting a +30-Da mass shift. The inset provides an expansion of the region that includes the 14+ charge state (*m*/*z* 980–1010) showing the peaks representing the wild-type and variant TTR appearing mostly in their *S*-cysteinylated form.

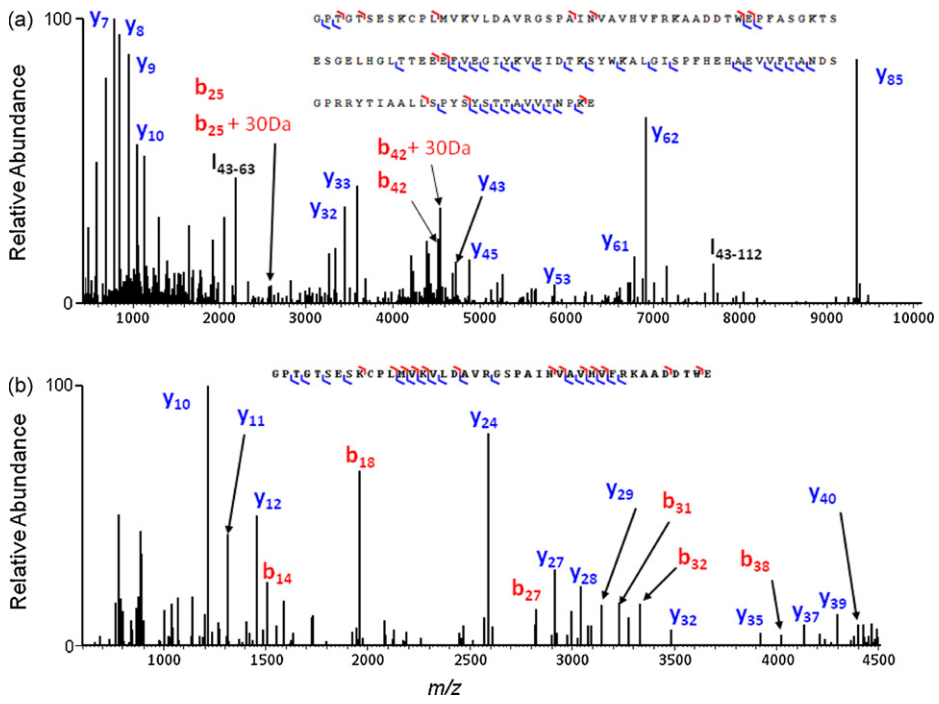

Fig. 6. (a) Deconvoluted NSD[2] mass spectrum of an unknown TTR variant immunoprecipitated from serum. The cysteinylated $[b_{42}+5H]^{5+}$ fragment is accompanied by a +30-Da component corresponding to the mass shift observed in the mass profile of the intact protein, indicating the presence of the variant amino acid in that region of the sequence. A similar b-ion pair is observed for b_{25}. (b) Deconvoluted LTQ-CID MS^3 spectrum of the cysteinylated $[b_{42}+5H+30\text{-Da}]^{5+}$ fragment ion at m/z 912.546 containing residues 1–42, generated from the NSD of an unknown TTR variant immunoprecipitated from serum. The sequence coverage generated by BUPID for the Gly6Ser variant of TTR is shown in both cases.

+30-Da mass shift was no longer observed. In this case, a b_{25} and (b_{25} + 30)-Da pair was observed, indicating that the amino acid substitution is in the first 25 amino acid positions of the sequence. The isotopic cluster at m/z 912.546, corresponding to the cysteinylated ($[b_{42}+5H+30\ Da]^{5+}$) fragment ion, was mass selected for CID-MS/MS in the LTQ (Fig. 6b). The fragmentation was compared to that obtained for the corresponding peak in the NSD spectrum of wild-type TTR. There was no change observed in the b-ion series, *i.e.*, the +30-Da mass shift was present in the smallest observed b-ion (b_9) and all higher members of this series, confirming that the amino acid substitution was present within the first nine positions of the sequence. The complementary y-ion series did not exhibit a +30-Da mass shift until y_{37}, indicating that the amino acid substitution was on Gly6 or Glu7 (see Table 1). A +30-Da mass shift can only result from one of five possible amino acid substitutions (Gly → Ser, Ala → Thr, Thr → Met, Val → Glu, Arg → Trp), so this sequence change must be Gly6 → Ser6The Gly6Ser variant is an established non-amyloidogenic polymorphism present in about 12% of humans [42]. Although this data is diagnostic for the presence of a non-symptomatic variant rather than an instance of the genetic TTR amyloid disease (familial amyloidotic polyneuropathy), this example demonstrates the simplicity of the approach. In essence, our results indicate that the NSD MS^2 spectrum, the subsequent MS^3 CID of the appropriate NSD-generated fragment(s), and possibly the MS^2 spectrum of the $[M+8H]^{8+}$ intact protein charge isoform should be analyzed together to generate maximum sequence coverage after obtaining a mass profile of the protein.

Table 1
Neutral masses of the y-ion series obtained from the deconvolution of the LTQ-CID MS^3 spectra of the $b_{42}{}^{5+}$ fragment ion of the wild type and Gly6Ser variant.

y-Ion series	Wild type	Gly6Ser variant
y_{40}	4368.174	4398.182
y_{39}	4267.128	4297.131
y_{37}	4109.069	4139.093
y_{35}	3922.993	3922.989
y_{32}	3485.853	3485.844
y_{30}	3275.718	3275.716
y_{29}	3144.677	3144.675
y_{28}	3045.611	3045.607
y_{27}	2917.516	2917.513
y_{26}	2818.446	2818.444
y_{24}	2590.337	2590.334
y_{21}	2264.131	2264.131
y_{13}	1554.755	1554.754
y_{12}	1455.686	1455.686
y_{11}	1318.628	1318.626
y_{10}	1219.562	1219.558
y_9	1072.492	1072.493

3.3. Top-down analysis of hemoglobins

Normal adult hemoglobins are composed of tetramers, each containing four globin chains. In normal adults, the most common components are two alpha (α) and two beta (β) chains. The α (M_r 15126.4) and β (M_r 15867.24) chains consist of 141 and 146 amino acids, respectively. The fact that the circulating hemoglobin tetramer consists of more than one protein complicates the challenge of elaborating a simple top-down methodology. The option of separating the α and β chains from each other before MS analysis defeats the purpose of designing a simple approach, as two key assets of mass spectrometry are its speed and selectivity. Introducing a separation step would negate speed and ignore selectivity. Our goal in the experiments reported herein was to establish that the direct top-down analysis of hemoglobin from whole blood can provide rapid extensive sequence coverage with minimal sample preparation and without chromatographic separation.

The LTQ-CID fragmentation obtained by dissociation of the abundant charge states for the α (15+) and β (17+) chains provides a wealth of information and appears to be more extensive

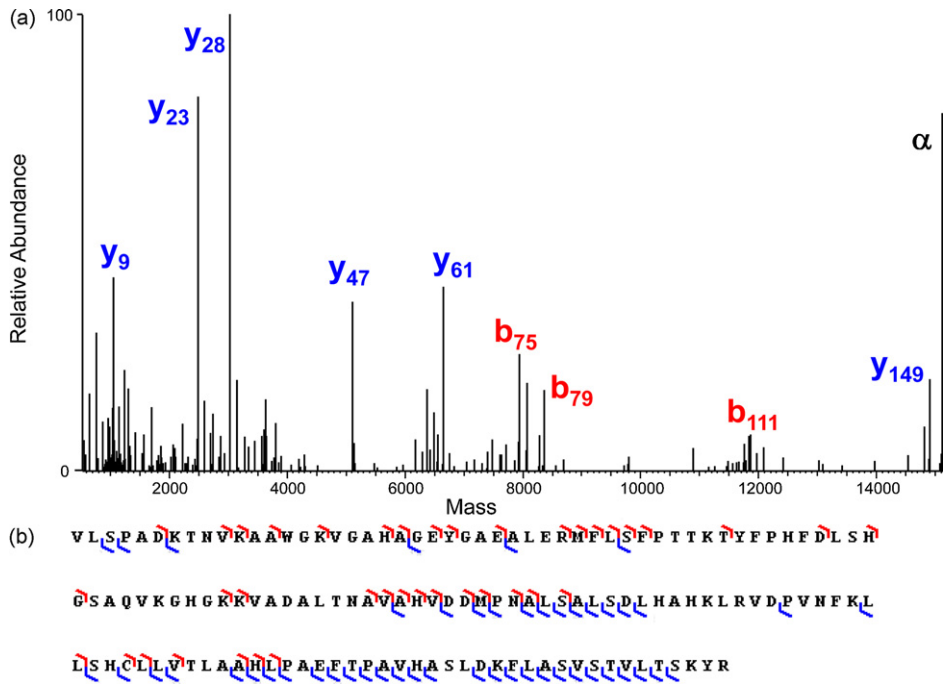

Fig. 7. (a) Deconvoluted LTQ-CID MS^3 spectrum of $[M+15H]^{15+}$ m/z 1009.408 from hemoglobin α chain in diluted whole blood. (b) Sequence coverage generated by BUPID.

than that obtained for TTR. The fragmentation of intact hemoglobin chains under collisional activation conditions has been described [8,35,43,44] and our results are in broad agreement with reported observations. The fragmentation exhibited by the α chain is particularly rich and plentiful, as exemplified by the MS/MS spectrum of $[M+15H]^{15+}$ m/z 1009.408 (Fig. 7). The most abundant product ions are derived from facile cleavage at sites on the C-terminal side of acidic residues (b_{75}) and the N-terminal side of Pro (y_{28}, y_{47}). The fragment ion b_{75} results from the Asp75-Met76 cleavage C-terminal to aspartic acid. Interestingly, the y_{66} fragment which forms a complementary ion pair with b_{75} is reported [35] to be abundant but is only observed with low signal intensity in this spectrum, with b_{61} being the closest-in-mass abundant fragment. The most dominant fragmentation channel is the cleavage at Leu113-Pro114 that gives rise to the y_{28} fragment. Similarly, the complementary ion in this pair, b_{113}, is only observed at low signal intensity, with b_{111} being the closest-in-mass abundant fragment. Many observed peaks could be assigned to products resulting from fragmentation in the "core" region of the protein and good overall sequence coverage was obtained, as shown in Fig. 7b.

In the LTQ-CID MS^2 data obtained for dissociation of the $[M+17H]^{17+}$ of the β chain that was observed at m/z 934.324 (Fig. 8), the main feature of the MS/MS spectrum is the $[y_{96}+11H]^{11+}$ fragment arising from the facile cleavage of the Thr50-Pro51 peptide bond. The high mass y-ions y_{98} and y_{111} form complementary ion pairs with the b_{50} and b_{35} ions, respectively. Of note is the presence of a long series of b-ions, from b_4 to b_{49}, describing most of the N-terminal sequence of the β chain, with the series from b_{30} to b_{36} being particularly prominent. These general observations are in qualitative agreement with those of Schaaff et al. [44]. The core sequence information obtained from the LTQ-CID MS^2 spectrum of the $[M+17H]^{17+}$ of the β chain is less extensive than was found for dissociation of the intact α-chain $[M+15H]^{15+}$ ion (Fig. 8b). These preliminary LTQ-CID MS^2 spectra provided a preview of what could be expected from nozzle-skimmer dissociation of the α and β chains, and established the provenance of the fragment ions generated by NSD.

The NSD spectrum of diluted whole blood is shown in Fig. 9. Many fragment ions can be observed. It proved possible to select a number of fragment ions from each chain that could be used to implement the strategy presented above for TTR, whereby MS^3 analysis of complementary ion pairs is used to obtain complete sequence information.

All the candidate fragment ion assignments were confirmed by MS/MS. In the case of the β chain, the prominent $[y_{96}+11H]^{11+}$ m/z 940.42 and $[b_{47}+5H]^{5+}$ m/z 1047.56, covering 143 of 146 amino acid positions, were determined to be suitable, as both fragment ions have reasonable abundance and are amenable to further MS^n analysis (*i.e.*, they provide good sequence coverage/information). The $[y_{96}+11H]^{11+}$ fragment ion could be efficiently generated in high abundance by NSD of the diluted blood sample and mass selected to undergo MS^3 in the LTQ (Fig. 10a). The sequence coverage thus obtained (Fig. 11b) provided information from the "core region" not available from the LTQ-CID mass spectrum obtained for the 17^+ charge state of the β chain. This region of the β-chain sequence (residues 58–111) has been reported to be difficult to sequence using CID of the intact β chain [45], but the approach presented here offers a simple and elegant solution to this problem.

The NSD spectrum exhibited peaks that could be assigned as α-chain fragments $[b_{75}+9H]^{9+}$ and $[y_{61}+8H]^{8+}$ at m/z 884.148 and 832.424, respectively, covering 136 of the 141 amino acids. While these pairs are not as complementary as of the pair selected for sequence analysis of TTR, they nevertheless cover close to 95% of the sequence. To some extent, the abundance of each of the chosen fragment ions can be increased by varying the fragmentation potential (by raising the skimmer voltage). To date, we have observed that implementation of the strategy using NSD to generate fragment ions which can be subsequently submitted to MS^3 analysis is much more difficult for hemoglobins than for TTR.

This state of affairs is likely due to the fact that isolating and performing MS^3 on some of the NSD-generated fragments is made more difficult when the peaks of interest have relatively low abundances and/or many other components are detected in the spectrum. This increases the possibility that NSD-generated frag-

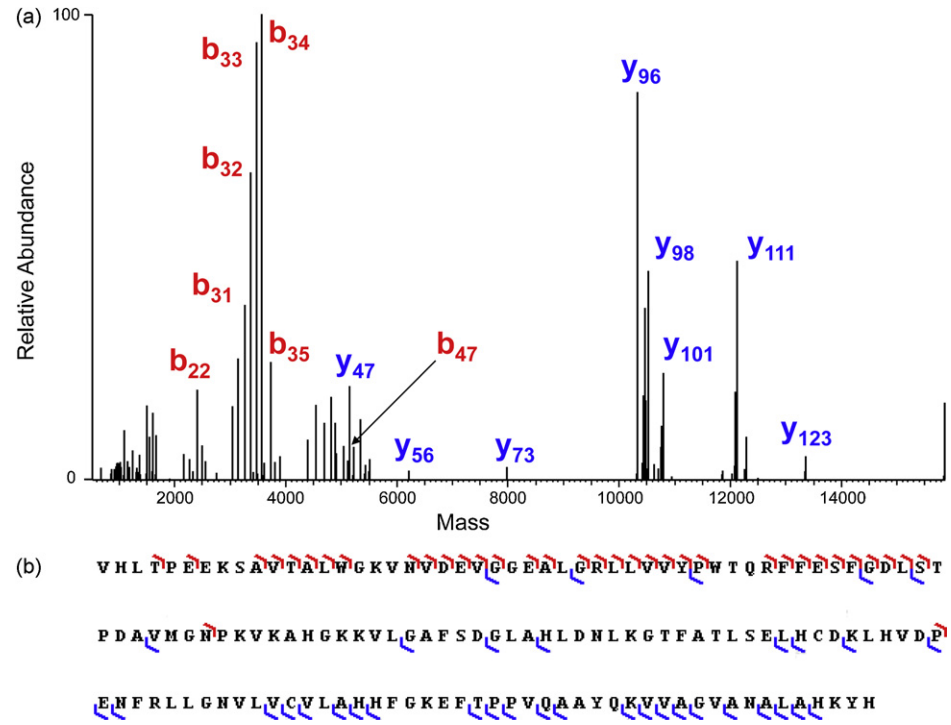

Fig. 8. (a) Deconvoluted LTQ-CID MS^2 spectrum obtained for the $[M+17H]^{17+}$ of the hemoglobin β chain in diluted whole blood that was observed at m/z 934.324. (b) Sequence coverage generated by BUPID.

ment ions corresponding to the variant will appear at an m/z values that are overlapped by interfering fragment ions. Furthermore, the presence of two proteins instead of one complicates the NSD fragment ion spectrum by simply increasing the number of fragments. Such overlap is not a problem when the intact protein charge states are chosen for MS^2 analysis. The peaks due to wild-type and variants can be mass-selected together, without hindering the data interpretation, since all the sequence information obtained is common to wild-type and variant, except for those fragments that contain the amino acid substitution. In fact, it may not be necessary to apply, in all cases, the strategy based on the MS^3 analysis of NSD-generated fragment ions that was found necessary and proven successful for TTR. Contrary to TTR, the intact protein MS/MS of some charge states of the intact α chain (particularly 14+ and 15+) show that it undergoes CID readily, yielding rich fragmentation and extensive sequence coverage. This is less true for the β chain where the "core" of the sequence was reported to be difficult to determine when CID was performed on the intact β chain [45]. This report is

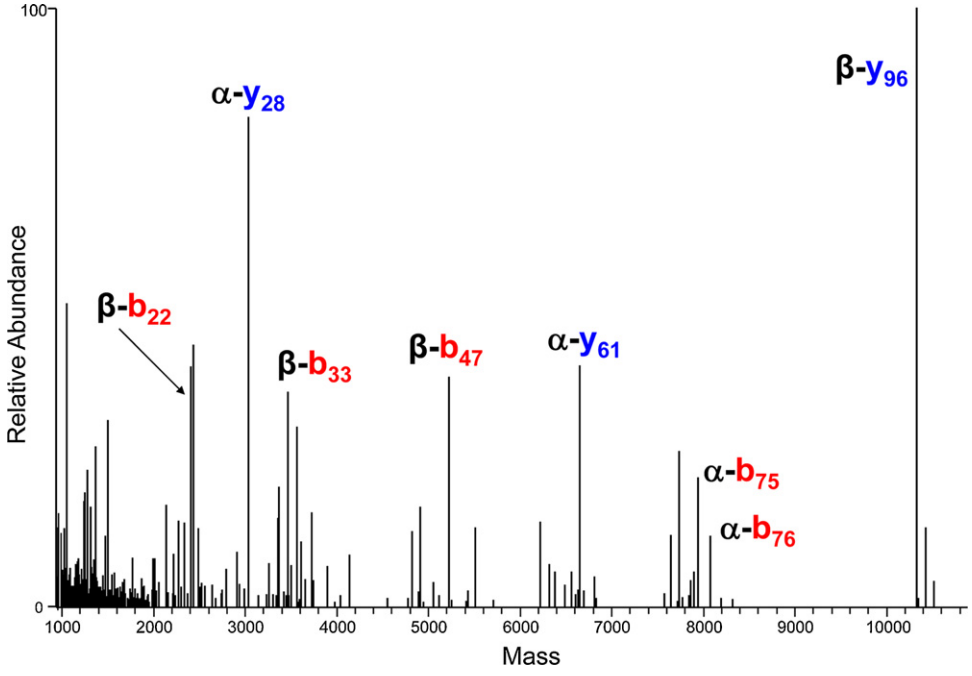

Fig. 9. Deconvoluted NSD MS^2 spectrum of diluted whole blood. Most of the abundant peaks can be assigned as fragments of α or β hemoglobin. The key fragment ions constituting complementary ion pairs that could be used for subsequent LTQ-CID MS^3 are labeled.

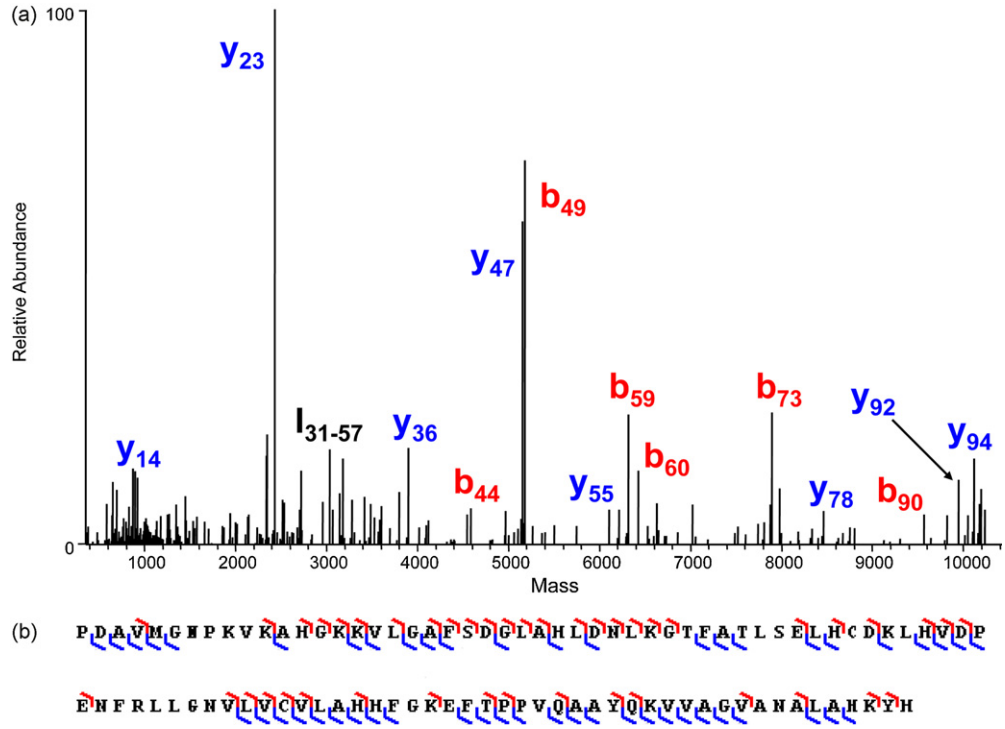

Fig. 10. (a) Deconvoluted LTQ-CID MS^3 spectrum of the Hbβ $[y_{96}+11H]^{11+}$ fragment at *m/z* 940.42, containing residues 51–146, generated by NSD of diluted whole blood. (b) Sequence coverage generated by BUPID.

consistent with our own observations. However, we have found that use of the NSD-generated β-y_{96} for MS^3 can resolve this issue. Hence, the best strategy in the case where the hemoglobin mass profile exhibits a β-chain variant, is to obtain the NSD spectrum and ascertain if the amino acid substitution is present within the y_{96} portion of the sequence. If so, the LTQ-CID MS^3 spectrum of the variant y_{96} can be acquired. If not, MS/MS of intact protein from one of the charge states 14+ to 17+ can be used to gain sequence information.

On the basis of the results presented here, we can summarize the top-down strategy for the analysis of hemoglobin variants as follows. First, a mass profile is obtained. If an α-chain variant is present, then LTQ-CID of the 15+ or 14+ charge state of the intact protein variant should be obtained. If a β-chain variant is found

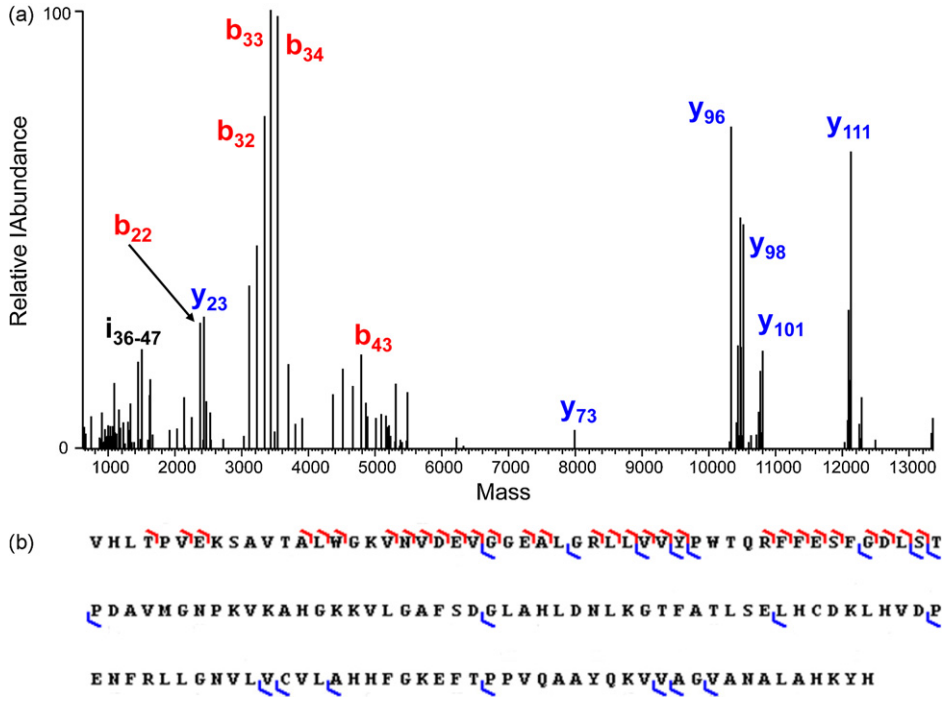

Fig. 11. (a) Deconvoluted LTQ-CID MS^2 spectrum obtained for the $[M+17H]^{17+}$ ion observed at *m/z* 932.632 that corresponds to the hemoglobin β-chain variant exhibiting a −30-Da mass shift. (b) Sequence coverage generated by BUPID.

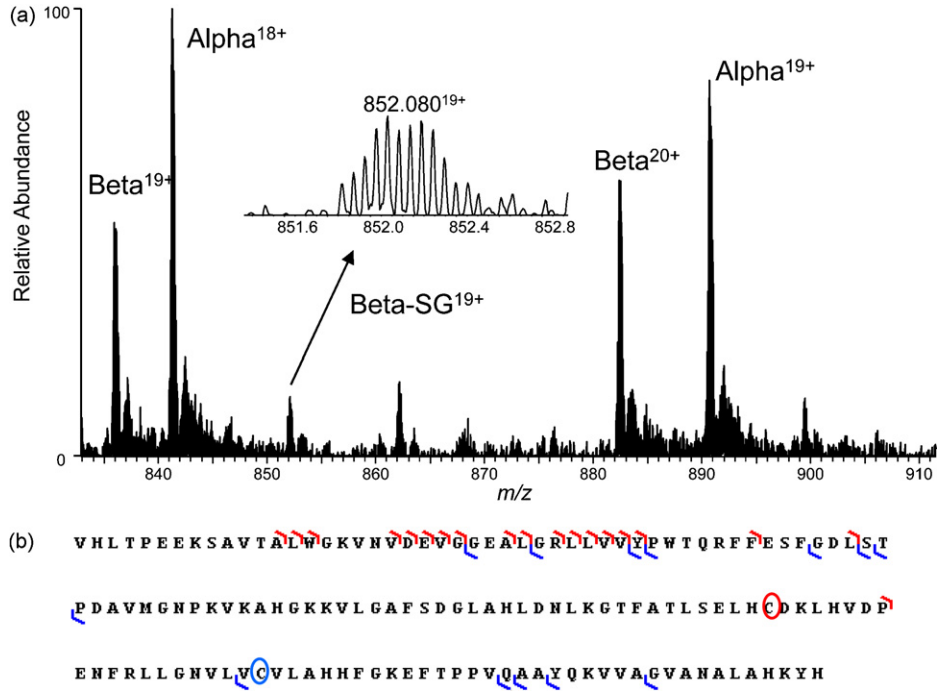

Fig. 12. (a) Nanospray mass spectrum showing the expanded region containing the ion corresponding to $[M+19H]^{19+}$ of the S-glutathionylated hemoglobin β chain (m/z 852.080^{19+}). (b) Sequence coverage generated by BUPID top-down from the LTQ-CID MS^2 of m/z 852.080^{19+}. The potential sites of glutathionylation, Cys93 and Cys112, are marked with circles. The top-down MS/MS results indicated that Cys 93 is the modified residue.

to be present, the NSD profile is acquired. If a variant β-chain y_{96} is detected, it can be analyzed by LTQ-CID MS^3. Otherwise, NSD fragmentation and MS^2 of the intact β-chain variant charge state (14+ to 17+) serves to characterize the variant.

The results obtained for a sickle cell variant (Hbβ Glu6 → Val) illustrate the amount of information that can be extracted from the data. The mass profile of diluted whole blood known to contain a variant was obtained and an apparent β-chain variant with a −30-Da shift was observed (data not shown). The NSD spectrum did not exhibit a β-y_{96} variant peak; this result suggested that the amino acid substitution was present within positions 1–50. The β b-ion series revealed the presence of b-ion pairs (*e.g.*, b_{13}, b_{22} and b_{33}) separated in each case by 30 Da. As noted earlier, the N-terminal region of the β-chain sequence may be determined by using MS^2 of the intact protein charge states 14+ to 17+. In the MS/MS spectrum of the variant $[M+17H]^{17+}$ (Fig. 11), the −30-Da shift of the b-ions was observed down to b_6, whereas the b_4 mass corresponded to that of the wild-type sequence (Table 2). This data indicated that the amino acid substitution was at Pro5 or Glu6. In this case, the only possible amino acid substitution yielding a −30-Da shift is Glu6 → Val6.

Table 2
List of neutral masses from the b-ion series of the deconvoluted LTQ-CID MS^2 mass spectra of the $[M+17H]^{17+}$ of the wild type and Glu6Val hemoglobin β chain. This data indicated that the amino acid substitution was at Pro5 or Glu6. The only possible amino acid substitution yielding a −30 Da shift is a Glu-to-Val substitution.

b-Ion series	Wild type	Glu6Val variant	Sequence
b_4	450.259	450.259	VHLT
b_6	676.341	646.362	VHLTP(E → V)
b_{13}	1362.683	1332.738	VHLTP(E → V)EKSAVTA
b_{14}	1475.771	1445.822	VHLTP(E → V)EKSAVTAL
b_{15}	1661.848	1631.902	VHLTP(E → V)EKSAVTALW
b_{20}	2159.135	2129.198	VHLTP(E → V)EKSAVTALWGKVNV
b_{21}	2274.159	2244.224	VHLTP(E → V)EKSAVTALWGKVNVD
b_{22}	2403.200	2373.267	VHLTP(E → V)EKSAVTALWGKVNVDE

An added feature of the top-down analysis of hemoglobins is the ability to characterize PTMs. The mass profile of a hemoglobin sample was recorded and the deconvoluted data indicated the presence of a minor component (10% abundance relative to the base peak series) corresponding to the molecular weight of the β-chain +305 Da. This mass shift is usually indicative of *S*-glutathionylation at cysteine, a modification that is believed to be an indicator of oxidative stress [46]. The low abundance $[M+19H]^{19+}$ peak at m/z 852.083 (Fig. 12a) (corresponding to glutathionylated β-chain) was subjected to LTQ-CID. The fragmentation information obtained (Fig. 12b) from this experiment was incomplete but was sufficient to place the modification on Cys93, and to exclude modification at Cys112. This method of locating the post-translational modification is considerably less cumbersome than the bottom-up approach.

4. Conclusions

The combination of automated sample introduction, the high resolution and high mass accuracy of the hybrid LTQ-Orbitrap and customized software algorithms written in-house (BUPID top-down) provides an integrated analytical platform amenable to the analysis of TTR and hemoglobin variants and their post-translational modifications. The method easily lend itself to automation for use in clinical laboratories. Two limitations should be noted: (1) the method achieves high sequence coverage but does not provide specific information for every amino acid position. (2) Variants that produce isobaric molecular (and primary fragment ions) cannot be detected by measurement of the molecular weights of the proteins (and/or their primary fragments) and therefore will be fully characterized only through MS^3or higher stage measurements. However, TTR and hemoglobin variant characterizations by MS in a clinical context are usually preceded by more conventional tests [47]. These tests and the clinical definition of a phenotype may narrow the options that need to be considered during the MS analysis and may lead one to carry out MS^n analy-

sis in the absence of a detectable mass shift. The ease of use and simplicity of the method and its ability to yield substantial structural information without labor-intensive and time-consuming sample preparation are remarkable. Furthermore, the instrument time is efficiently used, given that lengthy chromatographic separations are avoided. The data necessary for top-down analysis can be acquired in about 5 min. A typical LCMS experiment will run 30–60 min, plus the time required for re-equilibration of the chromatographic system. The results shown here indicate that CID techniques are very effective when judiciously used. Nevertheless, the use of ETD or ECD could generate data complementary to that obtained by NSD of the proteins of interest in these studies. Our preliminary FT-ICR data indicates that this approach is feasible.

The top-down approach described herein is ideal for small, abundant, easily isolated and clinically relevant proteins such as transthyretin and hemoglobins. The LTQ-Orbitrap offers a convenient means of generating top-down data in a routine manner and its capabilities in this domain are ready to be more fully exploited.

Acknowledgements

This research is supported by NIH-NCRR grants P41 RR010888 and S10 RR020946 and by NIH-NHLBI contract N01 HV028178. Transthyretin samples were supplied by the BUSM Amyloid Treatment and Research Program. Samples containing variant hemoglobins were provided by the BUSM Sickle Cell Center.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ijms.2010.08.012.

References

[1] R. Aebersold, M. Mann, Mass spectrometry-based proteomics, Nature 422 (2003) 198–207.
[2] B.T. Chait, Mass spectrometry: bottom-up or top-down? Science 314 (2006) 65–66.
[3] N.L. Kelleher, H.Y. Lin, G.A. Valaskovic, D.J. Aaserud, E.K. Fridriksson, F.W. McLafferty, Top down versus bottom up protein characterization by tandem high resolution mass spectrometry, J. Am. Chem. Soc. 121 (1999) 806–812.
[4] N. Siuti, N.L. Kelleher, Decoding protein modifications using top-down mass spectrometry, Nat. Methods 4 (2007) 817–821.
[5] Y. Wada, T. Matsuo, I.A. Papayannopoulos, C.E. Costello, K. Biemann, Fast atom bombardment and tandem mass spectrometry for the characterization of hemoglobin variants including a new variant, Int. J. Mass Spectrom. Ion Processes 122 (1992) 219–229.
[6] Y.A. Daniel, C. Turner, R.M. Haynes, B.J. Hunt, R. Neil Dalton, Rapid and specific detection of clinically significant haemoglobinopathies using electrospray mass spectrometry–mass spectrometry, Br. J. Haematol. 130 (2005) 635–643.
[7] J.A. Loo, C.G. Edmonds, R.D. Smith, Primary sequence information from intact proteins by electrospray ionization tandem mass spectrometry, Science 248 (1990) 201–204.
[8] K.J. Lightwahl, J.A. Loo, C.G. Edmonds, R.D. Smith, H.E. Witkowska, C.H.L. Shackleton, C.S.C. Wu, Collisionally activated dissociation and tandem mass-spectrometry of intact hemoglobin beta-chain variant proteins with electrospray ionization, Biol. Mass Spectrom. 22 (1993) 112–120.
[9] G.E. Reid, S.A. McLuckey, 'Top down' protein characterization via tandem mass spectrometry, J. Mass Spectrom. 37 (2002) 663–675.
[10] M. Thevis, R.R.O. Loo, J.A. Loo, Mass spectrometric characterization of transferrins and their fragments derived by reduction of disulfide bonds, J. Am. Soc. Mass Spectrom. 14 (2003) 635–647.
[11] H.E. Witkowska, B.N. Green, M. Morris, C.H.L. Shackleton, Intact protein electrospray ionization tandem mass spectrometry can be the sole technique used for confirming the structure of a variant hemoglobin, J. Mass Spectrom. 30 (1995) S111–S115.
[12] J.A. Loo, J.P. Quinn, S.I. Ryu, K.D. Henry, M.W. Senko, F.W. McLafferty, High-resolution tandem mass spectrometry of large biomolecules, Proc. Natl. Acad. Sci. U.S.A. 89 (1992) 286–289.
[13] F.W. McLafferty, High-resolution tandem FT mass-spectrometry above 10-kDa, Acc. Chem. Res. 27 (1994) 379–386.
[14] D.P. Little, J.P. Speir, M.W. Senko, P.B. O'Connor, F.W. McLafferty, Infrared multiphoton dissociation of large multiply-charged ions for biomolecule sequencing, Anal. Chem. 66 (1994) 2809–2815.
[15] R.A. Zubarev, N.L. Kelleher, F.W. McLafferty, Electron capture dissociation of multiply charged protein cations. A nonergodic process, J. Am. Chem. Soc. 120 (1998) 3265–3266.
[16] J.E.P. Syka, J.J. Coon, M.J. Schroeder, J. Shabanowitz, D.F. Hunt, Peptide and protein sequence analysis by electron transfer dissociation mass spectrometry, Proc. Natl. Acad. Sci. U.S.A. 101 (2004) 9528–9533.
[17] J.W. Gauthier, T.R. Trautman, D.B. Jacobson, Sustained off-resonance irradiation for collision-activated dissociationiinvolving Fourier-transform mass spectrometry—collision-activated dissociation technique that emulates infrared multiphoton dissociation, Anal. Chim. Acta 246 (1991) 211–225.
[18] M. Hardman, A.A. Makarov, Interfacing the orbitrap mass analyzer to an electrospray ion source, Anal. Chem. 75 (2003) 1699–1705.
[19] B. Macek, L.F. Waanders, J.V. Olsen, M. Mann, Top-down protein sequencing and MS3 on a hybrid linear quadrupole ion trap-orbitrap mass spectrometer, Mol. Cell. Proteomics 5 (2006) 949–958.
[20] Z.Q. Zhang, B. Shah, Characterization of variable regions of monoclonal antibodies by top-down mass spectrometry, Anal. Chem. 79 (2007) 5723–5729.
[21] P.V. Bondarenko, T.P. Second, V. Zabrouskov, A.A. Makarov, Z.Q. Zhang, Mass measurement and top-down HPLC/MS analysis of intact monoclonal antibodies on a hybrid Linear Quadrupole Ion Trap-Orbitrap mass spectrometer, J. Am. Soc. Mass Spectrom. 20 (2009) 1415–1424.
[22] M.D. Benson, T. Uemichi, Transthyretin amyloidosis, Amyloid-Int. J. Exp. Clin. Invest. 3 (1996) 44–56.
[23] http://globin.cse.psu.edu.
[24] R. Theberge, L. Connors, M. Skinner, J. Skare, C.E. Costello, Characterization of transthyretin mutants from serum using immunoprecipitation, HPLC/electrospray ionization and matrix-assisted laser desorption/ionization mass spectrometry, Anal. Chem. 71 (1999) 452–459.
[25] C.H.L. Shackleton, A.M. Falick, B.N. Green, H.E. Witkowska, Electrospray mass spectrometry in the clinical diagnosis of variant hemoglobins, J. Chromatogr.-Biomed. Appl. 562 (1991) 175–190.
[26] A. Shimizu, T. Nakanishi, A. Miyazaki, Detection and characterization of variant and modified structures of proteins in blood and tissues by mass spectrometry, Mass Spectrom. Rev. 25 (2006) 686–712.
[27] H.R. Bergen, S.R. Zeldenrust, M.L. Butz, D.S. Snow, P.J. Dyck, P.J.B. Dyck, C.J. Klein, J.F. O'Brien, S.N. Thibodeau, D.C. Muddiman, Identification of transthyretin variants by sequential proteomic and genomic analysis, Clin. Chem. 50 (2004) 1544–1552.
[28] A.I. Nepomuceno, C.J. Mason, D.C. Muddiman, H.R. Bergen, S.R. Zeldenrust, Detection of genetic variants of transthyretin by liquid chromatography-dual electrospray ionization Fourier-transform ion-cyclotron-resonance mass spectrometry, Clin. Chem. 50 (2004) 1535–1543.
[29] J.S. Kingsbury, R. Theberge, J.A. Karbassi, A. Lim, C.E. Costello, L.H. Connors, Detailed structural analysis of amyloidogenic wild-type transthyretin using a novel purification strategy and mass spectrometry, Anal. Chem. 79 (2007) 1990–1998.
[30] G.E. Reid, J. Wu, P.A. Chrisman, J.M. Wells, S.A. McLuckey, Charge-state-dependent sequence analysis of protonated ubiquitin ions via ion trap tandem mass spectrometry, Anal. Chem. 73 (2001) 3274–3281.
[31] C.H.L. Shackleton, H.E. Witkowska, Characterizing abnormal hemoglobin by MS, Anal. Chem. 68 (1996) A29–A33.
[32] A. Lim, T. Prokaeva, M.E. McComb, P.B. O'Connor, R. Theberge, L.H. Connors, M. Skinner, C.E. Costello, Characterization of transthyretin variants in familial transthyretin amyloidosis by mass spectrometric peptide mapping and DNA sequence analysis, Anal. Chem. 74 (2002) 741–751.
[33] R.T.W. Tong, G. Infusini, W. Cui, D.H. Perlman, C. Lin, M.E. Mccomb, C.E. Costello, BUPID-top-down: database search and assignment of top-down MS/MS data, in: Proceedings of the 57th American Society Conference on Mass Spectrometry and Allied Topics, Philadelphia, PA, 2009.
[34] G.C. McAlister, W.T. Berggren, J. Griep-Raming, S. Horning, A. Makarov, D. Phanstiel, G. Stafford, D.L. Swaney, J.E.P. Syka, V. Zabrouskov, J.J. Coon, A proteomics grade electron transfer dissociation-enabled hybrid linear ion trap-orbitrap mass spectrometer, J. Proteome Res. 7 (2008) 3127–3136.
[35] T.T. Mekecha, R. Amunugama, S.A. McLuckey, Ion trap collision-induced dissociation of human hemoglobin alpha-chain cations, J. Am. Soc. Mass Spectrom. 17 (2006) 923–931.
[36] G.L. Nelsestuen, Y. Zhang, M.B. Martinez, N.S. Key, B. Jilma, M. Verneris, A. Sinaiko, R.S. Kasthuri, Plasma protein profiling: unique and stable features of individuals, Proteomics 5 (2005) 4012–4024.
[37] U.A. Kiernan, D. Nedelkov, R.W. Nelson, Multiplexed mass spectrometric immunoassay in biomarker research: a novel approach to the determination of a myocardial infarct, J. Proteome Res. 5 (2006) 2928–2934.
[38] M.J. Raftery, Selective detection of thiosulfate-containing peptides using tandem mass spectrometry, Rapid Commun. Mass Spectrom. 19 (2005) 674–682.
[39] R. Theberge, Y. Jiang, M.E. McComb, T. Prokaeva, L.H. Connors, M. Skinner, D.C. Seldin, C.E. Costello, Detection and Characterization of Immunoglobulin Light Chain Post-translational Modifications Using LC/MS–MS/MS, CRC Press-Taylor & Francis Group, Boca Raton, 2008.
[40] H. Zhai, X.M. Han, K. Breuker, F.W. McLafferty, Consecutive ion activation for top down mass spectrometry: improved protein sequencing by nozzle-skimmer dissociation, Anal. Chem. 77 (2005) 5777–5784.
[41] E.R. Badman, C.S. Hoaglund-Hyzer, D.E. Clemmer, Dissociation of different conformations of ubiquitin ions, J. Am. Soc. Mass Spectrom. 13 (2002) 719–723.
[42] D.R. Jacobson, I.L. Alves, M.J. Saraiva, S.N. Thibodeau, J.N. Buxbaum, Transthyretin Ser-6 gene frequency in individuals without amyloidosis, Hum. Genet. 95 (1995) 308–312.

[43] R. Bakhtiar, Q. Wu, S.A. Hofstadler, R.D. Smith, Charge-state specific facile gas-phase cleavage of Asp-75 Met-76 peptide bond in the alpha-chain of human apohemoglobin probed by electrospray-ionization mass spectrometry, Biol. Mass Spectrom. 23 (1994) 707–710.
[44] T.G. Schaaff, B.J. Cargile, J.L. Stephenson, S.A. McLuckey, Ion trap collisional activation of the (M+2H)(2+)–(M+17H)(17+) ions of human hemoglobin beta-chain, Anal. Chem. 72 (2000) 899–907.
[45] D.K. Rai, B. Landin, G. Alvelius, W.J. Griffiths, Electrospray tandem mass spectrometry of intact beta-chain hemoglobin variants, Anal. Chem. 74 (2002) 2097–2102.
[46] T. Niwa, Protein glutathionylation and oxidative stress, J. Chromatogr. B 855 (2007) 59–65.
[47] B.J. Wild, B.J. Bain, Detection and quantitation of normal and variant haemoglobins: an analytical review, Ann. Clin. Biochem. 41 (2004) 355–369.