International Journal of Mass Spectrometry 278 (2008) 114–121

Contents lists available at ScienceDirect

International Journal of Mass Spectrometry

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

Phenotypic analysis of Atlantic cod hemoglobin chains using a combination of top-down and bottom-up mass spectrometric approaches

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ARTICLE INFO

Article history:
Received 7 February 2008
Received in revised form 24 April 2008
Accepted 25 April 2008
Available online 3 May 2008

Keywords:
Atlantic cod
Hemoglobin
Amino acid sequence
Polymorphism
Mass spectrometry

ABSTRACT

Amino acid sequence information of both α- and β-chains of hemoglobin collected from Atlantic cod was obtained using a combination of mass spectrometry-based top-down and bottom-up approaches. Although complete sequencing is a very challenging undertaking due to the high degree of phenotypic variation, we were able to obtain between 38%, 28%, 46%, and 18% sequence coverage for the α-1-globin, α-2-globin, β-1-globin, and β-2-globin, respectively. The results of this work provide some evidence that the hemoglobin of Atlantic cod caught off the New England shoreline in the United States show genotypic/phenotypic variation from the hemoglobin of the same species caught off the coast of Norway. Though full coverage of the sequences was not accomplished, this work demonstrates the importance of further study to catalog the phenotypic variation of the American populations of Atlantic cod.

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1. Introduction

The primary sequences of hemoglobin (Hb) chains have been used for many years as an indicator of the evolution and divergence of various organisms [1]. It has been shown that a phylogenetic tree that is constructed based on the homological comparisons of the α- and β-globin chains from various species almost exactly coincides with the evolutionary "Tree of Life" itself. As is the case with many other proteins, Hb displays a large degree of polymorphism even within the same organism. Comprehensive analysis of these polymorphisms using a number of biochemical and serological methods offers a convenient way of identifying distinct genetic populations or stocks of a species. It also allows one to study underlying genetic mechanisms that result in individual variation and ultimately race differentiation in many organisms [2]. Genetic variations based on Hb polymorphism have been widely demonstrated in mammals, especially in humans [3]. In the case of humans the numbers of these polymorphs or Hb variants that have been discovered and characterized is ever increasing [4] with approximately 1000 different mutant alleles having been characterized at the molecular level, where there is great geographic regional specificity for these mutations [5,6]. A variety of pathological conditions are associated with Hb polymorphism in humans [4].

Although genetic polymorphism in fish has been noticed over four decades ago [7], it was never subjected to the same scrutiny as Hb polymorphism in mammals. Indeed, very few variations have ever been documented, and most unlike the case found in humans, the significance of these biochemical genetic variations in fish is often unclear [8]. Polymorphism in Atlantic cod (*Gadus morhua*) Hb is characterized by three different and common genotypes called Hb-I(1/1), Hb-I(1/2) and Hb-I(2/2) [9]. It has been demonstrated that the frequency of the two alleles Hb-I(1) and Hb-I(2) shows a north–south cline along the Norwegian coast [10] as well as the North American East coastline [11]. It has also been shown that oxygen affinity of Atlantic cod Hbs depends on the phenotype/polymorph present [8]. Though the regional distribution of the various variant Atlantic cod Hb along the Norwegian coast have been extensively studied, to date not much work has gone into similar studies along the North American coast.

Mass spectrometry (MS) has been used extensively in the past to study various aspects of Hb structure, including both higher order organization [12–16] and the analyses of mutations/polymorphism in Hb [3]. Wada et al. first applied mass spectrometry to study Hb variants over two decades ago [17] and

Abbreviations: Hb, hemoglobin; MS, mass spectrometry; ESI, electrospray ionization; MALDI, matrix-assisted laser desorption/ionization; FT ICR MS, Fourier transform ion cyclotron resonance mass spectrometer/spectrometry; CID, collision-induced dissociation.

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doi:10.1016/j.ijms.2008.04.020

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(A) Alpha_1   MSLSSKDKAT VKLFWGRMSG KAELIGADAL SRMLAVYPQT KTYFSHWKSL SPGSPDVKKH 60
    Alpha_2   MSLSSKQKAT VKDFFSKMST RSDDIGAEAL SRLVAVYPQT KSYFSHWKDA SPGSAPVRKH 60
              ******:*** ** *:.:**  ::: ***:** **::****** *:******.  ****. *:**

    Alpha_1   GKTIMMGIGD AVTKMDDLER GLLTLSELHA FKLRVDPTNF KLLSLNILVV MAIMFPDDFT 120
    Alpha_2   GITTMGGVYD AVGKIDDLKG GLLSLSELHA FMLRVDPVNF KLLAHCMLVC MSMIFPEEFT 120
              * * * *: * ** *:***:  ***:****** * *****.** ***:  :**  *:::**::**

    Alpha_1   PMAHLAVDKL FCGRALALAE KYR 143
    Alpha_2   PQVHVAVDKF LAQLALALAE KYR 143
              * .*:****: :.  ****** ***

(B) Beta      MVEWTDSERA IINSIFSNLD YEEIGRKSLC RCLIVYPWTQ RYFGGFGNLY NAETILCNPL 60
    Beta_2    MVEWTDEERT IINDIFSTLD YEEIGRKSLC RCLIVYPWTQ RYFGAFGNLY NAETIMANPL 60
    Beta_1    MVEWTAAERR HVEAVWSKID IDVCGPLALQ RCLIVYPWTQ RYFGEFGDLS TDAAIVGNPK 60
              *****  **   :: ::*.:*  :  *  :*  ********** **** **:*  .  :*: **

    Beta      IAAHGTKILH GLDRALKNMD DIKNTYAELS LLHSDKLHVD PDNFRLLADC LTGVIAAKMV 120
    Beta_2    IAAHGTKILH GLDRALKNMD DIKNTYAELS LLHSDKLHVD PDNFRLLADC LTVVIAAKMG 120
    Beta_1    VAAHGVVALT GLRTALDHMD EIKSTYAALS VLHSEKLHVD PDNFRLLCEC LTIVVAGKMG 120
              :****.  *  **  **.:** :**.*** ** :***:***** *******.:* ** *:*.**

    Beta      PAFTVDTQVG WQKFRSFVVS ALGREYH 147
    Beta_2    PAFTVDTQVA VQKFLSVVVS ALGRQYH 147
    Beta_1    KKLSPEMQAA WQKYLCAVVS ALGRQYH 147
                :: : *..  **: . *** ****:**
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Fig. 1. Alignments of all published sequences for *Gadus morhua* (Atlantic cod) hemoglobin chains. Alignment was performed using T-Coffee, a multiple sequence program available for use free of charge on the World Wide Web (http://www.ebi.ac.uk/t-coffee/).

today MS still remains at the forefront of Hb primary sequence analysis. Despite all of these efforts, sequences for a number of the Atlantic cod Hb variant chains remained unpublished until very recently [18]. Verde et al. used DNA sequencing as well as MALDI-TOF mass spectrometry (for confirmatory purposes) to provide and compare the sequences of other Atlantic cod Hb chains. Analysis showed that the N-termini of the α-globin chains were acetylated. The calculated average masses for the isolated *G. morhua* globin chains (including N-terminal acetylation of the α-globins) are 15948.8, 15826.4, 16713.2, 16326.0, and 16635.1 for the α-1-globin (Swiss-PROT ID# P84609), α-2-globin (O42425), β-globin (O13077), β-1-globin (P84610), and the β-2-globin (P84611), respectively. Aligned sequences for these globins are presented in Fig. 1.

Tandem MS offers an elegant and efficient way of polypeptide sequencing by either combining enzymatic cleavage in solution with collision-induced dissociation (CID) of proteolytic fragments in the gas phase, or inducing fragmentation of the entire protein in the gas phase and totally bypassing the proteolytic step in solution. The former (traditional) approach is known as a bottom-up sequencing, while the latter is usually referred as a top-down sequencing. Top-down sequencing is usually carried out using high-end mass spectrometric equipment due to the very demanding requirements on mass resolution, accuracy and the fragmentation efficiency. One approach to circumvent this though has been demonstrated by McLuckey and co-workers where ion–ion proton-transfer reactions in a simple quadrupole ion trap mass spectrometer was employed to dramatically simplify mass spectral analysis [19–21]. The traditional, more commonly used (bottom-up) approach to protein sequencing allows the sequence information on larger proteins to be obtained using relatively inexpensive mass spectrometers. This is achieved by first breaking down the protein into smaller parts by using either proteolytic or chemical digestion.

Current work shows the application of mass spectrometry to the phenotypic analysis of *G. morhua* (Atlantic cod) Hb from specimens collected along the northeastern American shoreline at Glouchester, MA. A combination of the top-down and bottom-up approaches to protein sequencing is employed.

2. Experimental

2.1. Materials

G. morhua (Atlantic cod) Hb was generously donated by Prof. Herbert Hultin (University of Massachusetts Marine Station, Glouchester, MA). Proteomics-grade TPCK-treated trypsin was purchased from Sigma Chemical Co. (St. Louis, MO). All other chemicals, buffers, and solvents were of analytical grade or higher.

2.2. Proteolytic digestion

Atlantic cod hemolysate was digested with trypsin in 100 mM ammonium bicarbonate, pH 8.5 using approximately a 1:25 molar ratio of enzyme to Hb tetramer. Incubation was carried out at 37 °C for approximately 40 h.

2.3. Liquid chromatography

Globin chain separation was performed by reversed-phase HPLC. 20 μL of Atlantic cod Hb was applied on a C18 analytical column (Polaris C18-A, 4.6 × 250 mm, 3 μm). Solvent A consisted of 5% acetonitrile, ACN in 0.1% trifluoroacetic acid, TFA; and Solvent B consisted of 95% ACN in 0.1% TFA. Globin separation was obtained using an elution profile that was isocratic at 45% B for 15 min, increased from 45 to 50% B in 30 min, and was isocratic at 50% B for an additional 15 min. The flow rate used was 1 mL/min. Absorbance was measured at 214 nm. Peak fractions were collected and pooled for a large number of injections. The identities of the representative peaks for the α- and the β-chains were confirmed by electrospray ionization mass spectrometry (ESI-MS) measurements. The pooled fractions were frozen in liquid nitrogen and lyophilized to dryness.

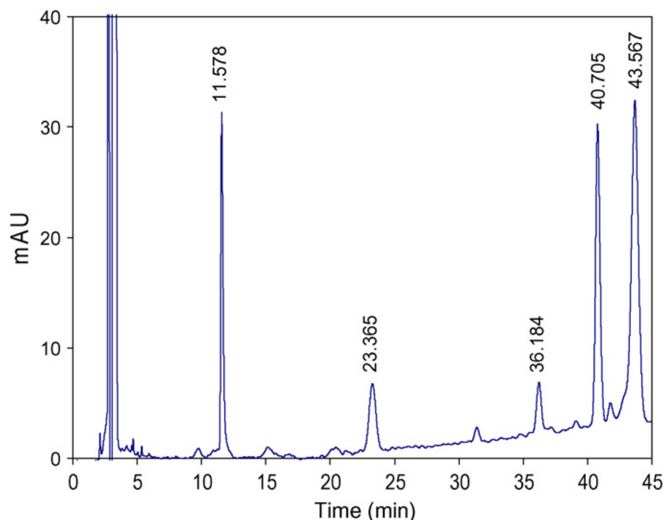

Fig. 2. Reversed-phase HPLC chromatogram for the isolation of the chains of Atlantic cod hemoglobin from untreated hemolysate.

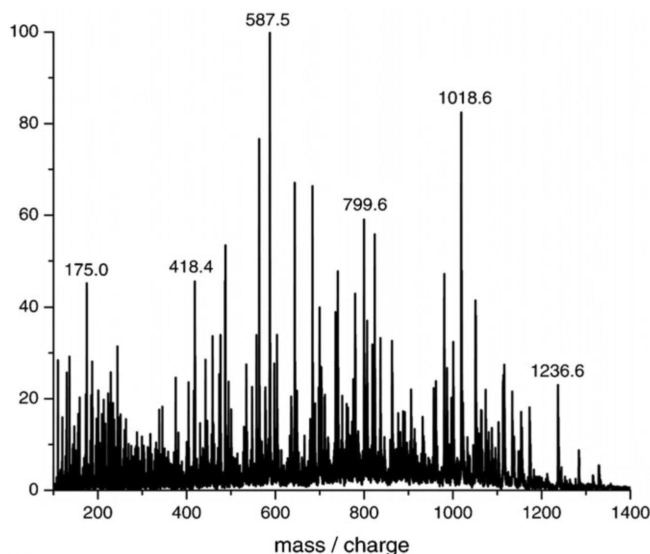

Fig. 4. ESI mass spectrum of the tryptic digest of Atlantic cod hemolysate recorded on a QIT mass spectrometer.

2.4. Mass spectrometry

Two different mass spectrometers were used in this project: Esquire-LC quadrupole ion trap (QIT) mass spectrometer equipped with a standard ESI source (Bruker Daltonics, Billerica, MA) for bottom-up analysis of the tryptic digest; and 4.7 T Apex III Fourier transform ion cyclotron resonance (FT ICR) mass spectrometer equipped with a standard ESI source (Bruker Daltonics, Billerica, MA) for top-down analysis of the isolated globin chains.

For the top-down analysis of the isolated globin chains, all samples were prepared at an approximate concentration of 10 μM in a solution containing 50% methanol and 3% glacial acetic acid in water. The entire charge state distribution for the globin species was isolated for fragmentation by collision-induced dissociation and top-down sequence analysis. Tandem mass spectra were recorded in the m/z range 650–1750.

For the bottom-up sequencing of the tryptic peptides, the digest mixture was diluted to a concentration of approximately 10 μM in a solution containing 50% methanol and 3% glacial acetic acid in water. After a mass spectrum representative of the digest mixture of tryptic peptides was acquired, each peptide was isolated, fragmented by collision-induced dissociation and tandem mass spectra recorded in the m/z range 100–2200.

3. Results

For the chromatographic separation and isolation of *G. morhua* globin chains, untreated hemolysate was injected directly into the liquid chromatograph. Baseline separation of the α-globin and the β-globin chains was achieved (Fig. 2) with the β-globin eluting first after 40.7 min and the α-globin eluting at 43.6 min. Baseline separation by well over 1 min facilitated the collection of each globin with no contamination from any other protein species. Fractions around 40.7 and 43.6 min were pooled and analyzed directly by ESI-MS. Fig. 3A and B shows the ESI mass spectra for these pooled LC fractions containing the α- and β-globin species, respectively. Deconvolution of the mass spectra provided an average mass of 15829.8 for the α-globin and 16664.5 for the β-globin, which do not correspond with any of the calculated masses of the published sequences (including N-

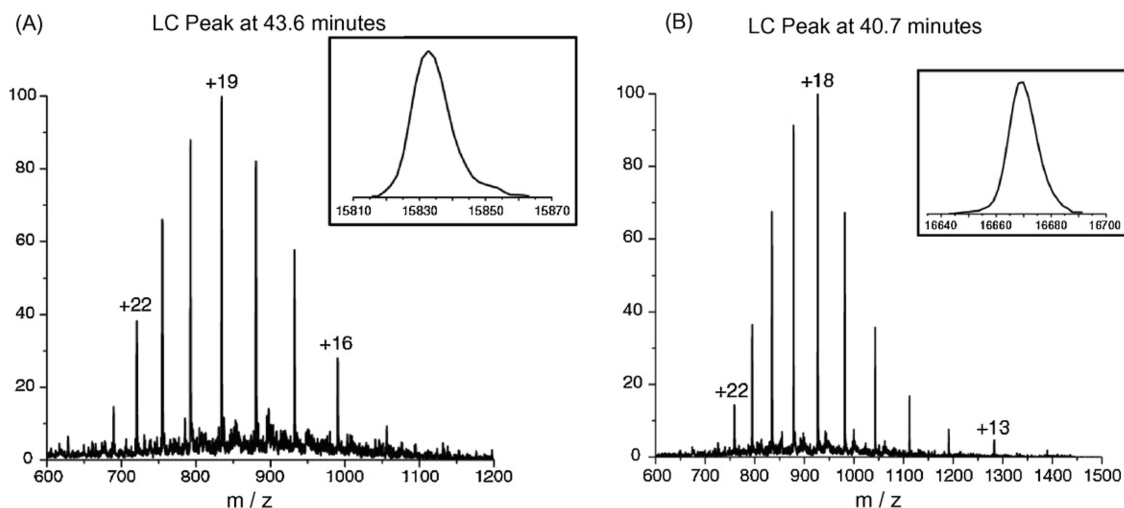

Fig. 3. Electrospray ionization (ESI) mass spectra showing the isolation and mass measurement of the HPLC fractions containing the (A) β-2 and (B) α-1 globin chains, which eluted at 40.7 and 43.6 min, respectively. Insets show the deconvolution of these mass spectra to represent the neutral mass of each protein species.

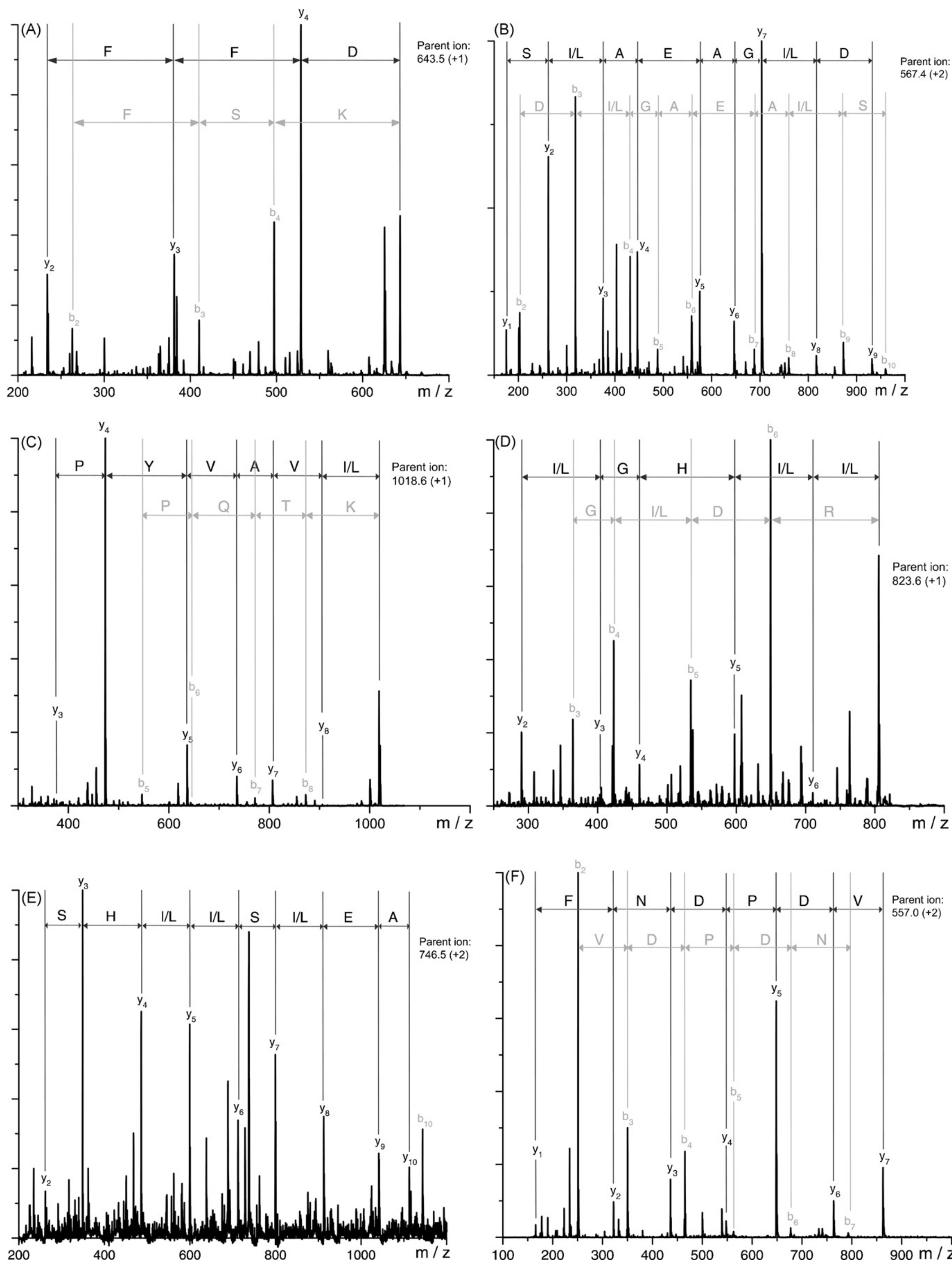

Fig. 5. Tandem mass spectra for representative peptides from the tryptic digest of Atlantic cod hemolysate. b- and y-type fragment ions used to confirm the sequence identities for the peptides are labeled along with the determined sequence for: (A) α-2, 13–17; (B) α-2, 22–32; (C) α-2, 33–41; (D) β-2, 68–74; (E) β-2, 84–96; (F) β-1, 97–105; (G) α-2, 49–58; (H) α-2, 75–79; (I) β-2, 135–144; (J) β-1, 2–9. In J, the peptide was found lacking the N-terminal methionine residue present in the published sequence.

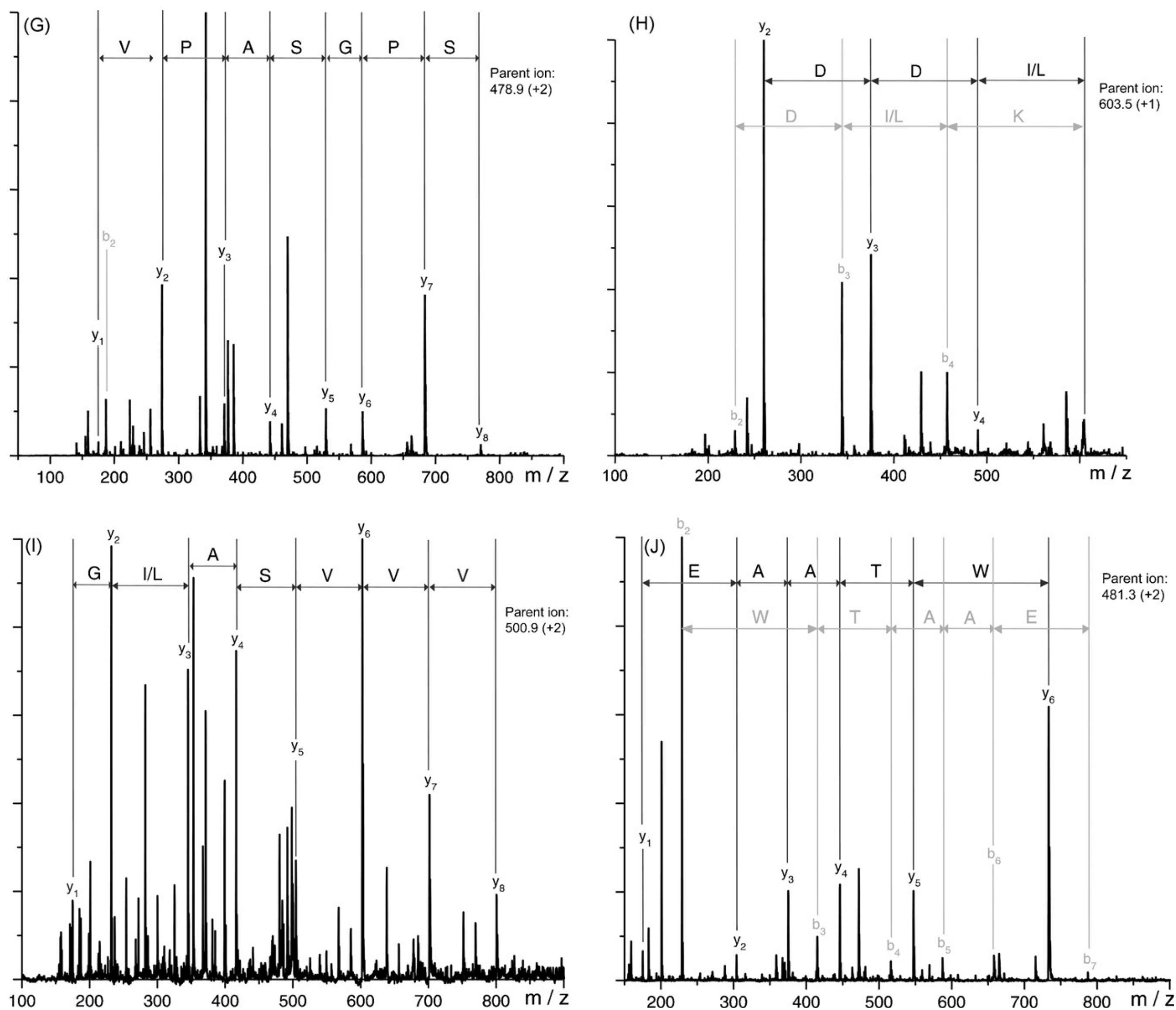

Fig. 5. (*Continued*).

terminal acetylation of the α-globins), which are 15948.8, 15826.4, 16713.2, 16326.0, and 16635.1 for the α-1-globin (Swiss-PROT ID# P84609), α-2-globin (O42425), β-globin (O13077), β-1-globin (P84610), and the β-2-globin (P84611), respectively. All published aligned sequences for *G. morhua* hemoglobin chains are provided in Fig. 1.

The ESI mass spectrum of the hemolysate digested with trypsin is shown in Fig. 4. Based on mass measurement alone, a number of the peptide peaks in the mass spectrum were identified by comparison with the masses of the tryptic peptides that were calculated from the published sequences of the α- and β-chains and confirmed by tandem mass spectrometry. Collision-induced dissociation fragmentation mass spectra for some of these peptides are provided in Fig. 5A through J. Sequences for these peptides are listed in Table 1 along with their positions in the published sequences of the globins. The tandem mass spectrum of a +2 charge state peptide at *m/z* 481.3 (Fig. 5J) was identified as the N-terminal peptide of the β-1-globin (indicated by "*" in Table 1). It was shown to not contain the N-terminal methionine residue that was expected from the published sequences. The α-1-globin peptide at sequence position 63–74, which contains two methionine residues was identified to contain 0, 1, and 2 methionine oxidations providing masses 619.0 (+2), 627.0 (+2), 635.0 (+2), respectively. (Tandem mass spectra for these peptides are provided in Supplementary information.) Peptide mapping from the tryptic digest of the hemolysate showed that the Atlantic cod hemolysate was composed of α-1, α-2, β-1, and β-2-globins. β-globin chains were not found to be present though two peptides 'ILHGLDR' and 'NTYAELSLLHSDK' are consistent with its sequence at positions 68–74 and 84–96, respectively. These peptides are also present in the β-2-globin sequence and have been assigned to this protein because no other peptides were found with sequences corresponding to β-globin.

Analysis of the isolated α- and β-chains using the top-down approach was carried out by inducing fragmentation of the protein by CID in the ICR cell of the FT ICR MS. Data representative of the β- and α-chains are shown in Figs. 6 and 7, respectively. In the tandem mass spectrum for the β-2-globin (Fig. 6), singly charged fragment ions form a "ladder", whose sequence, 'FLSVVVSALGR' covers the C-terminal segment β133-144. This confirms that this polypeptide, with HPLC elution time of 40.7 min was in fact the β-2-globin, as this peptide sequence was not present in any of the other Atlantic cod globins. In the data for the globin with HPLC elution

Table 1
Table summarizing the peptides identified from the bottom-up approach (both by mass measurement and tandem mass spectrometry)

No.	Globin chain	Sequence position	$M_{meas.}$ z)	Monoisotopic $M_{meas.}$ (z)	Peptide sequence
1	α-l	9–12	418.3 (+1)	418.3 (+1)	ATVK
2	α-2	13–17	643.5 (+1)	643.3 (+1)	DFFSK
3	α-1	22–32	558.5 (+2)	558.3 (+2)	AELIGADALSR
4	α-2	22–32	567.4 (+2)	567.3 (+2)	SDDIGAEALSR
5	α-2	33–41	1018.6 (+1)	1018.6 (+1)	LVAVYPQTK
6	α-2	49–58	478.9 (+2)	478.7 (+2)	DASPGSAPVR
7	α-1	63–74	619.0 (+2)	618.8 (+2)	TIMMGIGDAVTK
8	α-1	63–74	627.0 (+2)	626.8 (+2)	TIM_{ox}MGIGDAVTK
9	α-1	63–74	635.0 (+2)	635.3 (+2)	TI$M_{ox}M_{ox}$GIGDAVTK
10	α-2	75–79	603.5 (+1)	603.3 (+1)	IDDLK
11	α-1	75–80	397.8 (+2)	397.7 (+2)	M_{ox}DDLER
12	α-1	81–92	665.0 (+2)	664.9 (+2)	GLLTLSELHAFK
13	β-1	2–9	481.3 (+2)	481.7 (+2)	*VEWTAAER
14	β-2	10–26	667.7 (+3)	667.0 (+3)	TIINDIFSTLDYEEIGR
15	β-2	27–31	606.6 (+1)	606.3 (+1)	KSLCR
16	β-2, β	68–74	412.3 (+2)	412.2 (+2)	ILHGLDR
17	β-1	74–83	587.0 (+2)	586.8 (+2)	TALDHMDEIK
18	β-2	78–83	735.5 (+1)	735.3 (+1)	NMDDIK
19	β-2, β	84–96	746.5 (+2)	745.8 (+2)	NTYAELSLLHSDK
20	β, β-1, β-2	97–105	557.0 (+2)	556.8 (+2)	LHVDPDNFR
21	β-2	135–144	500.9 (+2)	500.8 (+2)	LSVVVSALGR

Griffith and Kaltashov, Table 1. Measured as well as calculated masses of the peptide sequences, along with their globin chains and sequence positions are provided.

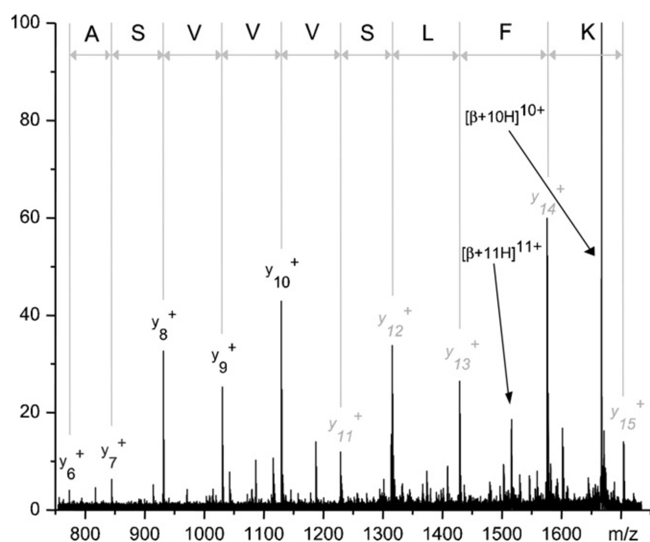

Fig. 6. Top-down approach on the β-2-globin using SORI-CAD fragmentation on a FT ICR mass spectrometer. The tandem mass spectrum shows a ladder of +1 charge state fragment ions that provides the sequence for residues 133–141.

time 43.6 min (Fig. 7A), which confirmed that this globin was the α-1-globin, a ladder of +10 charge state fragment ions covering the segment α117–124 'ILVVMAIM' was found. Intact (unfragmented) multiply charged protein ions in the mass spectrum are indicated by '*'.

4. Discussion

There are quite a number of variants of human Hb that have been discovered and characterized at the molecular level [4,22]. Mass spectrometry has played pivotal roles in both the identification and the analysis of these variants. Likewise, similar approaches to the ones used for the characterization of human Hb variants will be effective in the mutation and phenotypic analysis of the *G. morhua* (Atlantic cod) Hb. Atlantic cod hemoglobin shows a high degree of multiplicity, having three different hemoglobins. This is a trait that is found in a number of other acanthomorphic fish [18]. Amino acid sequences have been often used to investigate the evolutionary history of many globins [1], including those of the polar fish like the Atlantic cod [18]. It has been previously demonstrated by studying the genetic variation and functional properties of Atlantic cod hemoglobins, that oxygen affinity varies considerably between phenotypes [8]. There is indeed a functional relationship that exists between these biochemical genetic variants and their physiological capacity, including growth, feeding behavior, and the fundamental ability to survive. Salvanes and Hart [23] were able to show that the most successful fish were usually among the first to feed and tended to possess hemoglobin genotype *Hb-I(2/2)*, which has also been shown to possess the highest oxygen affinity at various temperatures (between 10 and 20 °C). By understanding how this genetic variation, and its subsequent phenotypic diversity controls the function of Atlantic cod hemoglobins and even the basic survival of the fish, we may be able to discern the sequence determinants for such phenomena as tetramer dissociation, oxygen affinity, allostery, and cooperativity in oxygen binding. Exploitation of this knowledge may be valuable in manipulating human hemoglobin sequences for production of more effective hemoglobin-based oxygen carrier solutions (HBOC) for use as blood transfusion products.

The ESI mass spectrum of the HPLC-purified isolated globins (Fig. 3) provides the molecular weight information on the predominant globin species present. The globin chain that eluted at 40.705 min was found to be the β-1-globin, while the globin chain with elution time 43.6 min was found to be the α-1 globin chain. The α-1-globin and the β-2-globin were determined to have a molecular weight of 15829.8 Da and 16664.5, respectively, which do not correspond with any of the calculated masses of the published sequences (including N-terminal acetylation of the α-globins) are 15948.8, 15826.4, 16713.2, 16326.0, and 16635.1 for the α-1-globin (Swiss-PROT ID# P84609), α-2-globin (O42425), β-globin (O13077), β-1-globin (P84610), and the β-2-globin (P84611), respectively (Fig. 1). This discrepancy between the masses determined and the calculated masses could not be explained based on mass measurement alone as it is almost impossible to predict any single amino acid residue substitution, insertion or deletion without direct sequence evidence from mass spectrometry sequencing experiments. The masses measured for the globin species also largely differ from those

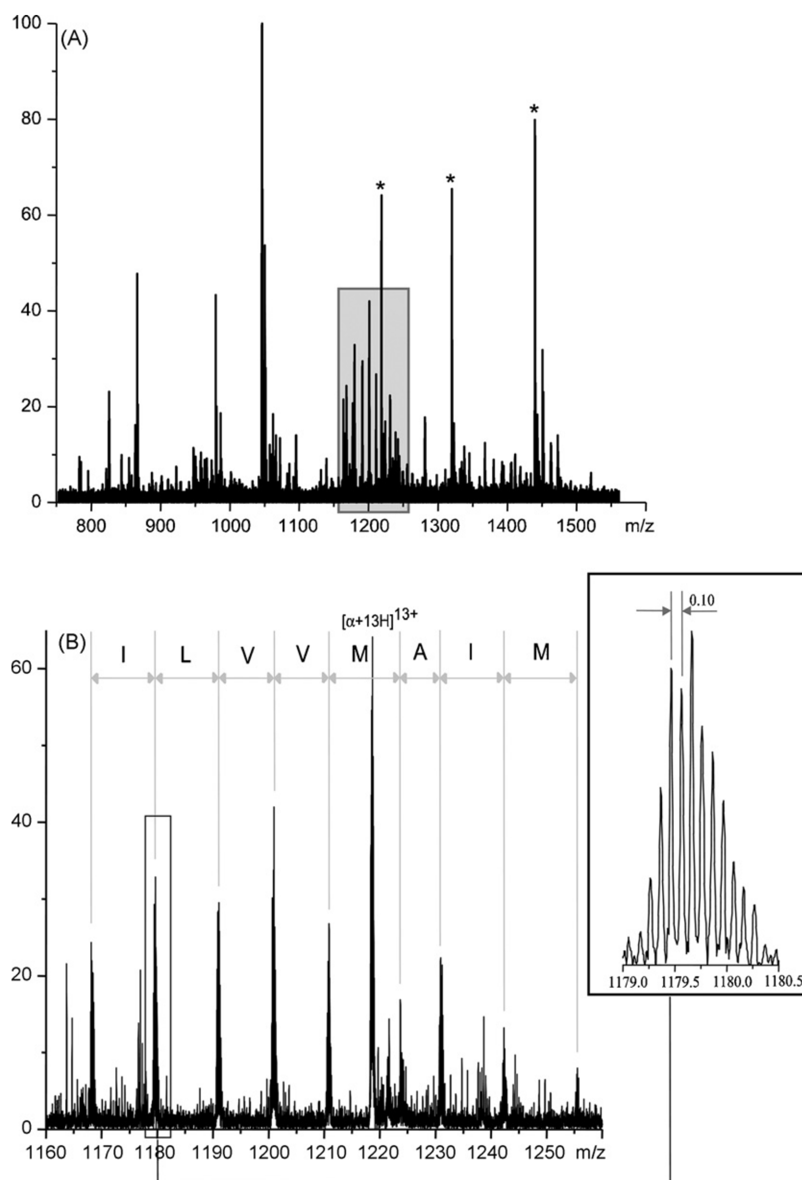

Fig. 7. (A) Top-down approach on the α-1-globin using SORI-CAD fragmentation on a FT ICR mass spectrometer. Intact, unfragmented protein ions are indicated with "*". The grey box in the tandem mass spectrum shows a ladder of +10 charge state fragment ions that provides the sequence for residues 107–114. For better visualization, a zoom of this ladder of +10 charge state ions (B) has been provided. The inset shows a zoom of one of the +10 charge state ions and demonstrates the high resolution that can be achieved with FTMS technology.

published Verdi et al.: 15,848, 15,693, 16,189, and 16,476 for the α-1, α-2, β-1, and β-2 globins, respectively, from their MALDI MS measurements [18].

The traditional (bottom-up) approach to protein sequencing was achieved by first breaking down the protein into smaller parts by using proteolytic digestion with trypsin. The ESI mass spectrum of the fish hemoglobin digested with trypsin is shown in Fig. 4. Based on mass measurement alone and comparison with the published sequences, a number of peptides can be identified. The identities of this peptide were confirmed by tandem mass spectrometric sequencing (Fig. 5A through J) using a quadrupole ion trap mass spectrometer (QIT MS). Indicated in the spectrum are the series of b and y fragment ions, which correspond to the calculated fragment ions for the peptide. A summary of information on these peptides identified by the bottom-up approach is provided in Table 1. Based on the peptides identified, the Atlantic cod specimen, which was caught off the Northeastern American shoreline was phenotyped as possessing the α-1, α-2, β-1, and β-2-globins. Atlantic cod hemoglobins are currently classified as Hb1, which is a heterotetramer composed of two α-1-globins and two β-1-globins; Hb2, which is a heterotetramer composed of two α-2-globins and two β-2-globins; and Hb3, which is a heterotetramer composed of two α-1-globins and two β-2-globins. Not enough information is available to definitively classify this Atlantic cod specimen by its major and minor hemoglobin components, but it is likely that both Hb1 and Hb3 are present.

The top-down sequencing approach involved chromatographically isolating each globin chain and fragmenting them by CAD in the ICR cell of the FTMS. In the chromatographic separation shown in Fig. 2, the β-chain eluted at 40.7 min, while the α-chain eluted at 43.6 min. Other, as yet unidentified, peaks present in the chromatogram are most likely due to the fact that the sample analyzed was a hemolysate, containing a number of other protein species in addition to the major hemoglobin component.

Fig. 6 illustrates the use of this top-down approach to obtaining sequence information for the β-2-globin by mass spectrometry. This fragment ion spectrum was acquired using sustained off-resonance irradiation collision-induced dissociation (SORI-CID) as a means of inducing protein ion dissociation in the gas phase. Although the β-globin molecular ions may generate hundreds of fragments upon dissociation within a relatively narrow m/z region, the superior resolving power of FT ICR MS [24] allows most of these fragment ions to be resolved based on the differences in their charge states and/or accurate masses. For example, a series of the singly charged fragment ions form a “ladder”, whose sequence covers a C-terminal segment β133-141 : KFLSVVVSA. Likewise, a similar top-down approach for the α-chain (Fig. 7A) produces a ladder of +10 charge state ions (grey box) from which the sequence ‘ILVVMAIM’ (residues 107–114) can be determined. A zoom of this region is provided in Fig. 7B for clearer visualization. The inset in Fig. 7B, which shows an expanded region around one of the +10 charge state fragment ions demonstrates the high resolving power provided by FTMS technology. Unfortunately the vast majority of the peaks in the mass spectra could not be assigned, which indicated that there were other mutations or genetic variation present in these globin chains, resulting in fragment ions having masses very different to those expected from the sequences.

5. Conclusions

Although only a small amount of data was shown here, the inability to fully match the MS and MS/MS data to any of the published hemoglobin sequences for fish of the same species caught off the coast of Norway indicates that there is some genetic/phenotypic variation when compared to the hemoglobins collected from the Atlantic Cod studied here, which was caught off the Northeastern American shoreline. A combination of both the top-down and bottom-up sequencing approaches was able to determine that α-1, α-2, β-1, and β-2 globins were present in the hemolysate extracted. As the calculated molecular weights of the corrected sequences still differ from the experimentally determined ones, it is quite clear that there is still yet more genetic and phenotypic variation in these fish and that not all of the mutations have as yet been identified. Complete sequence coverage may only be possible with strategies combining chromatographic separation of the peptides of the digested Hb with tandem MS and *de novo* peptide sequencing.

Acknowledgements

This work was supported by a grant from the National Science Foundation (CHE-0406302). *G. morhua* (Atlantic cod) Hb was generously donated by Prof. Herbert Hultin (University of Massachusetts Marine Station, Glouchester, MA). We would also like to thank Dr. Joshua K. Hoerner for assistance with FT ICR MS experiments and Dr. Stephen J. Eyles (Department of Polymer Science and Engineering, University of Massachusetts-Amherst) for many helpful discussions and advice.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ijms.2008.04.020.

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