Anal. Chem. **2002,** *74,* 2097−2102

Electrospray Tandem Mass Spectrometry of Intact β-Chain Hemoglobin Variants

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In this report, we present data to illustrate how human hemoglobin (Hb) variants can be identified by electrospray tandem mass spectrometry (MS/MS) of the intact Hb chains following the one-step dilution of whole blood. MS/MS spectra were recorded on a series of intact β-chain human Hb variants. The resultant spectra were interpreted, and using the information gleaned from the fragmentation patterns of known variants, two unknown β-chain variants were characterized solely by this mass spectrometric method. Fragment ions that serve to identify β-chain variants were identified. The fragmentation patterns of the intact β-chain $[M + 18H]^{18+}$ ions showed classical facile cleavages adjacent to acidic residues and N-terminal to proline residues, with Thr50-Pro51 being the most prominent cleavage site. Abundant product ions were formed by peptide bond cleavage in the regions close to the termini of the β chain, the central region being less well-represented in the MS/MS spectra. Nearly 50% of the β-chain primary structure could be determined by MS/MS of the intact chain. However, analysis of the Hb variants where mutations have occurred in the inner region (residues 58−111) of the β globin proved to be difficult and required mass spectrometric analysis of their tryptic peptides for a complete identification.

Electrospray (ES) mass spectrometry has been widely used in characterizing hemoglobin (Hb) variants.[1−7] The usual approach to the characterization of Hb variants by mass spectrometry involves three stages: (1) mass spectral screening of whole blood, (2) peptide mass mapping of proteolytically cleaved abnormal globin chains identified in the initial screening step, and (3) tandem mass spectrometry (MS/MS) analysis of variant peptides. This procedure not only involves an enzymatic cleavage,[1−7] but also may require separation of the peptides by chromatography prior to mass spectrometric analysis,[1−3] thus increasing the time for diagnosis. However, with the advent of ES, it has become possible to perform MS/MS studies on large biomolecules.[8,9] Although the MS/MS data from large molecules are complex, and may contain ambiguities as a result of difficulty in assigning the charge states to the product ions generated from multiply charged precursors, specific structural studies can benefit from this approach. The generation of series of product ions with similar charge states and a progression of charge states for a given product ion are established phenomenon observed in MS/MS spectra of large polypeptides and proteins.[8,9] In the early 1990s Shackleton and co-workers applied MS/MS to the study of intact Hb β-chain variants following a chromatographic separation step.[10] They were able to determine the partial sequence (29%) of the intact β globin, mainly covering the ends of the polypeptide chain, and therefore concluded that diagnosis of variants contained within these limits was possible. Later, Hb Chicago (α136Leu→Met) was characterized solely by MS/MS within a clinical diagnostic setting.[11] Recently, a slightly higher degree of structural information (34%) of the total sequence of a normal β globin chain was determined using ion−ion interaction ion-trap collision activation dissociation mass spectrometry.[12] In the present report we show that Hb variants can be characterized by conventional MS/MS of the intact β chain. This analysis can be performed directly on diluted whole blood without any requirement for globin separation.

The dominating adult human Hb consists of two α and two β globin chains. An α chain is made up of 141 amino acids (M_r = 15 126.4), and the β chain consists of 146 amino acids (M_r = 15 867.2). In the presence of variants in the heterozygous state, the α chain variants are expressed in lower proportion (about 25%

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10.1021/ac011068f CCC: $22.00
Published on Web 03/21/2002

of total Hb), as compared to β-chain variants (about 50% of total Hb), since the α-globin genes are duplicated. It is, therefore, not surprising that the number of β-chain variants found so far is almost double the number of α-chain variants.[13] The majority of the Hb variants are due to single base mutations at the gene level and correspond to single amino acid substitutions at the protein level.

In this study, fragment ions important for the identification of β-chain variants in a single MS/MS experiment have been established. We present MS/MS data of a normal Hb β chain and examples of Hb variants. Compound heterozygous Hb SC and a heterozygous Hb Tacoma were diagnosed within the clinical settings solely by MS/MS of the intact globin chains.

EXPERIMENTAL SECTION

Samples. Frozen whole blood samples collected in potassium ethylenediamine tetraacetic acid containing vacutainers were used. For MS/MS experiments on the intact globins, a stock solution of the whole blood was prepared in distilled water (1:50 v/v) and diluted 1:2500 in 50% methanol containing 0.2% formic acid. Where necessary, tryptic digestion and mass analysis of the peptides was performed as has been described previously.[5] Each sample had previously been analyzed by HPLC and isoelectric focusing (IEF)[14] as part of an independent study.

Instrumentation. Mass spectrometry experiments were performed in the positive ion mode using nano-ES on a quadrupole (Q) time-of-flight (TOF) instrument (Q-TOF, Micromass, Manchester, U.K.) equipped with a Z-spray source. Initially, ES mass spectra were recorded and deconvoluted onto a true mass scale using maximum entropy software.[15] Mass differences between the variants and the normal β chain were determined. In MS/MS experiments, normal or variant β globin $[M + 18H]^{18+}$ ions were selected using the quadrupole of the Q-TOF instrument and transmitted to the collision cell. Argon gas was introduced to the collision cell located after the quadrupole but before the TOF analyzer, and product ions formed by collision-induced dissociation were analyzed by the orthogonal TOF analyzer. MS/MS spectra from normal and variant β chains were examined for differences in fragmentation pattern that correspond to the mass difference observed in the deconvoluted mass spectra.

RESULTS AND DISCUSSION

Initially, it was necessary to determine the general fragmentation pattern for β-chain $[M + 18H]^{18+}$ ions. This was achieved by recording MS/MS spectra of the normal β chain. The $[M + 18H]^{18+}$ ion was selected for MS/MS experiments, because it fragments to give a wide range of b and y fragment ions as a consequence of the availability of mobile protons[16] necessary to catalyze amide bond cleavage. At higher charge states, it has been suggested that proton mobility is reduced due to large columbic repulsion resulting in limited fragmentation.[17]

The nomenclature used to describe product ions generated in MS/MS experiments follows the conventions proposed by Roepstroff and Fohlman[18] and modified by Biemann.[19] The product ions belong mainly to either the b or the y ion series in accord with the general characteristics of product ion spectra generated at low collision energy.[20] A Thomson (Th) unit is used to describe the mass-to-charge ratio, as proposed by Cooks and Rockwood.[21] The normal β globin with 18 protons fragment predominantly at the Thr50−Pro51 amide bond producing complementary y^{n+}_{96} and b^{m+}_{50} ions. This was similarly observed by Shackleton and coworkers,[10] who further discussed this fragmentation in relation to the effect the Pro residue has on protein confirmation.

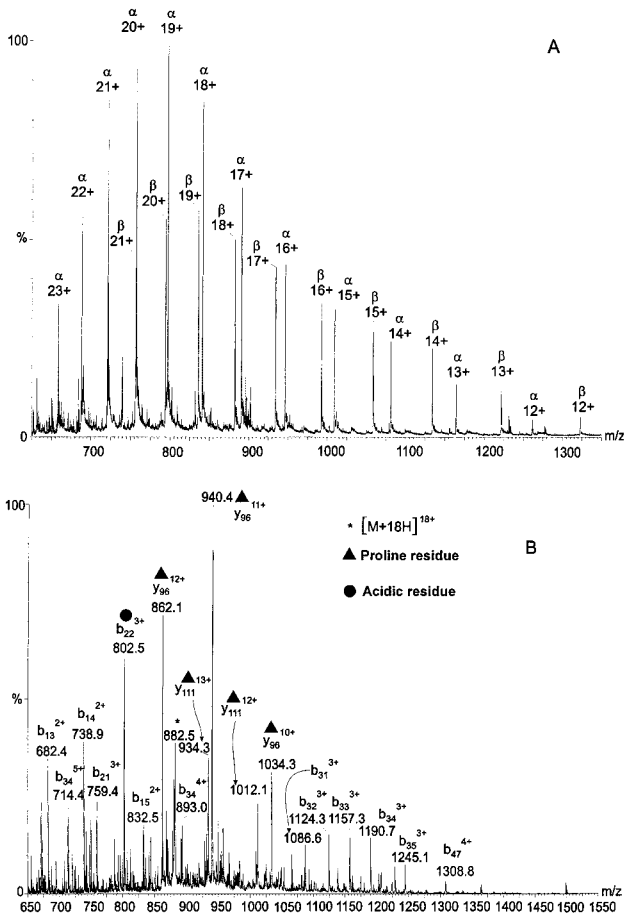

Figure 1. ES mass spectra of normal human Hb. (A) Raw data from the initial mass scan and (B) MS/MS spectrum of the $[M + 18H]^{18+}$ ion of the normal human β chain. ▲, cleavage N-terminal to Pro; ●, cleavage adjacent to acidic residue.

Normal β Chain. Analysis of a blood sample containing normal Hb by ES mass spectrometry gave a spectrum showing multiply protonated forms ($23H^+$ to $12H^+$) of the α- and β-globin chains (Figure 1A). Fragmentation of the $[M + 18H]^{18+}$ ion from the normal β chain generated a series of multiply charged b- and y-type ions, with prominent ions resulting from facile cleavage sites, i.e., adjacent to acidic residues and Xxx−Pro sites (Figure 1B). The most abundant product ions are y_{96}^{11+} and y_{96}^{12+}, which

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Table 1. Register of Reproducibly Observed b-Type and y-Type Ions in the MS/MS Spectra of the $[M + 18H]^{18+}$ Ion of the β Chain of Human Hb

m/*z*	b ion	charge	*m*/*z*	y ion	charge
237.1	2	1	156.1	1	1
350.2	3	1	584.3	4	1
451.3	4	1	655.3	5	1
677.4	6	1	768.4	6	1
806.4	7	1	839.5	7	1
934.5	8	1	477.3	8	2
1092.6	10	1	512.8	9	2
546.8	10	2	1180.6	11	1
596.4	11	2	590.8	11	2
646.9	12	2	626.3	12	2
682.4	13	2	675.9	13	2
738.9	14	2	725.4	14	2
832	15	2	623.8	17	3
555	15	3	647.4	18	3
574	16	3	671.1	19	3
1031.1	19	2	713.7	20	3
687.7	19	3	779.1	22	3
1080.6	20	2	811.6	23	3
720.8	20	3	608.8	23	4
1138.1	21	2	845.2	24	3
759.1	21	3	634.1	24	4
569.6	21	4	926.1	34	4
1202.7	22	2	740.9	34	5
802.1	22	3	761.4	35	5
601.8	22	4	781.5	36	5
835.1	23	3	878	89	11
626.6	23	4	723.9	94	14
854.2	24	3	1025.6	95	10
640.8	24	4	931.5	95	11
916.2	26	3	854.2	95	12
687.4	26	4	788.5	95	13
977.6	28	3	1149.1	96	9
1048.6	30	3	1034.3	96	10
786.7	30	4	940.4	96	11
1086.3	31	3	862.1	96	12
814.9	31	4	1044.5	97	10
1124	32	3	949.5	97	11
1157	33	3	870.5	97	12
868	33	4	957.6	98	11
1190.7	34	3	983.6	101	11
893.3	34	4	1104.1	111	11
714.8	34	5	1012.2	111	12
1245.1	35	3	934.5	111	13
747.5	35	5	947	112	13
1100.6	40	4	879.5	112	14
1137.7	41	4	954.1	113	13
1174.9	42	4	1009	120	13
1207.1	43	4	1067.7	138	14
965.9	43	5	1102.2	142	14
1229	44	4			
1265.3	45	4			
1308.8	47	4			

arise from cleavage of the Thr50−Pro51 amide bond. The most abundant b ion is b_{22}^{3+}, which results from cleavage at the Glu22−Val23 site. Series of product ions with the same charge, e.g., b_{32}^{3+}−b_{35}^{3+}, and progressions of product ions in different charge states, e.g., b_{34}^{3+}, b_{34}^{4+}, and b_{34}^{5+}, were also noted. Product ions reproducibly observed in MS/MS spectra of the $[M + 18H]^{18+}$ ion of β chains are listed in Table 1. It should be noted that spectral interpretation of ions as large as those derived from intact Hb chains is complicated by the fact that in some cases, *m*/*z* values for different fragment ions overlap. Using the MS/MS data of the normal β chain, the spectra of the following variants were interpreted.

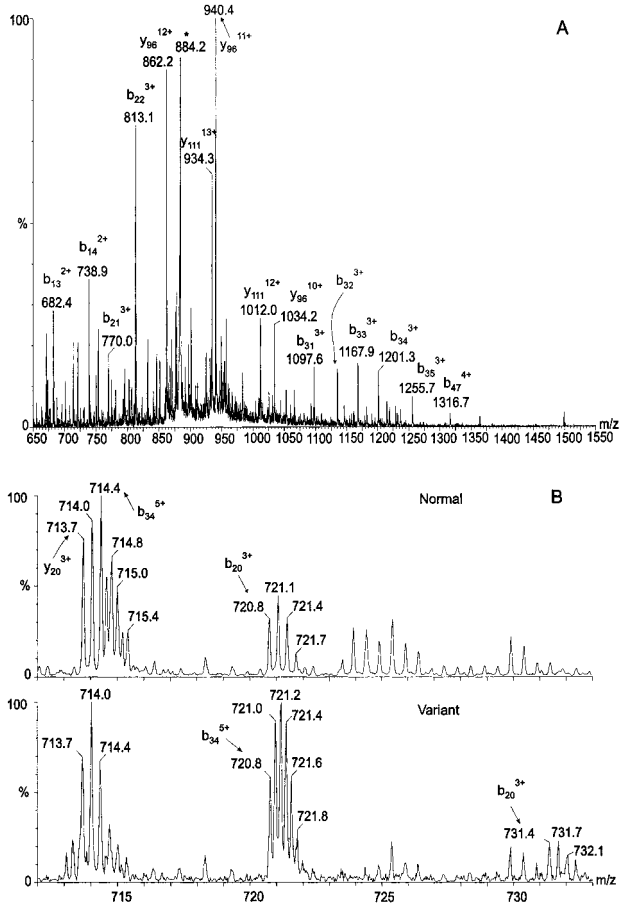

Figure 2. (A) MS/MS spectrum of the $[M + 18H]^{18+}$ ion of the intact β chain from Hb Olympia (β20Val→Met) and (B) *m*/*z* range 710−733 shown on an expanded scale for normal (upper panel) and variant Hb (lower panel). The shift of 10.6 Th is evident in the b_{20}^{3+} ions, and 6.4 Th in the b_{34}^{5+} ions.

Hb Olympia (β20Val→Met). The deconvoluted mass spectrum of the intact β chain reveals that the variant is 32 Da heavier than the normal β chain. This mass difference can only correspond to a Val→Met substitution, assuming a single-point mutation. The MS/MS spectrum of the variant $[M + 18H]^{18+}$ ion showed series of multiply charged b ions (b_{13}^{2+}−b_{15}^{2+}, b_{21}^{3+}, b_{22}^{3+}, b_{31}^{3+}−b_{35}^{3+}, and b_{47}^{4+}) and y ions (y_{95}^{11+}−y_{98}^{11+}, y_{101}^{11+}, and y_{110}^{12+}−y_{113}^{12+}) (Figure 2A). On comparing the MS/MS spectra of the variant and normal, the presence of similar *m*/*z* values for b_{13}^{2+}−b_{15}^{2+} rules out the substitution being in the first 15 residues of the β chain. Equivalent *m*/*z* values for multiply charged y_{111} ions in both spectra indicate the absence of the modification in the last 111 residues. However shifts in *m*/*z* values for b_{21}^{3+}, b_{22}^{3+}, b_{31}^{3+}−b_{35}^{3+}, and b_{47}^{4+} ions of the variant β chain suggests the substitution has occurred before residue 21 of the β chain. The data, thus, direct the location of the variant to residues between 15 and 21. There are only two Val residues in this stretch of amino acids located at positions 18 and 20 of the β chain. On close examination of the MS/MS spectrum, the b_{20}^{3+} ion in the variant spectrum is revealed to be shifted by 10.6 Th (Figure 2B). An equivalent *m*/*z* shift was observed for triply charged b ions greater than b_{20}^{3+}, but this *m*/*z* shift was not observed for b_{19}^{3+} ions. This confirms the substitution Val→Met at residue 20 (Hb Olympia, β20 Val→Met). Diagnosis for this case had been reached previously by mass spectrometry analysis of the tryptic digest.[6]

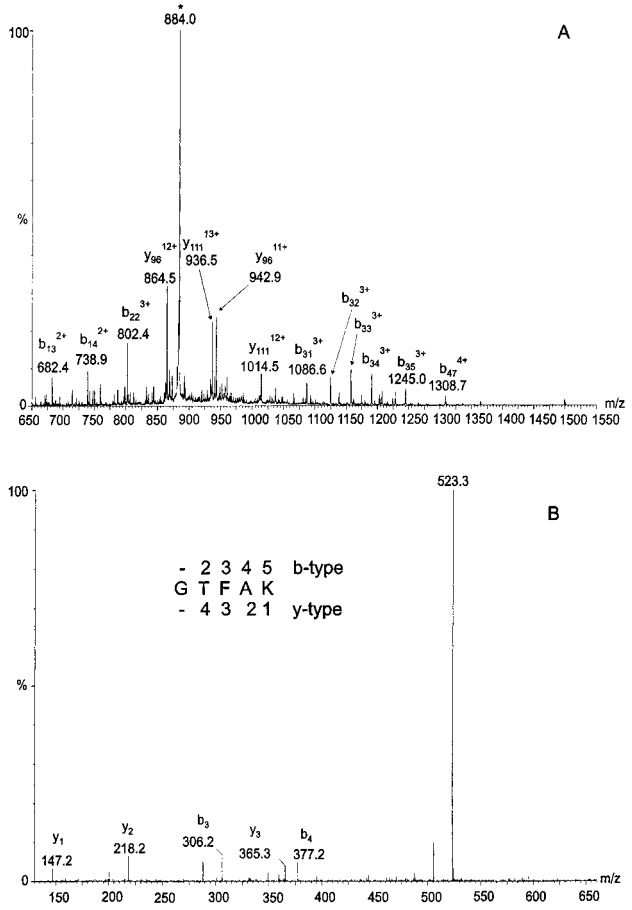

Figure 3. MS/MS spectra (A) of the $[M + 18H]^{18+}$ ion from Hb D-Ibadan (β87Thr→Lys) and (B) the $[M + H]^+$ ion of the variant peptide (GTFAK) generated by tryptic digestion of Hb D-Ibadan.

Hb D-Ibadan (β87Thr→Lys). The initial ES mass spectrum revealed a 27 Da mass shift for the variant. This mass change corresponds to a Thr→Lys or a Ser→Asn substitution. The MS/MS spectrum showed multiply charged y ions (y_{96}^{12+}, y_{96}^{11+} and y_{111}^{13+}, y_{111}^{12+}) shifted in m/z from the normal (cf. Figures 1A and 3A) but b_n ions ($n \leq 47$) were not observed to change in m/z. This indicates that the amino acid substitution is not within the first 47 residues but lies within the last 96 residues. On closer analysis of the MS/MS spectrum, the m/z values were not found to shift for the y_{36}^{5+} ion (or y_n ($n < 36$)), but the peak corresponding to y_{89}^{11+} was shifted from the normal position. The information obtained from these y ion peaks directs the location of the substitution to between residues 58 and 110 of the polypeptide chain. Since there are two Thr residues in this region at positions 84 and 87, and two Ser residues at positions 72 and 89 of the β chain, it was necessary to perform a tryptic digestion followed by ES analysis to pinpoint the mutation. The replacement of Thr by Lys will generate two new tryptic peptides. The ES mass spectrum of the tryptic digest demonstrated the appearance of a new peptide at m/z 523.3 corresponding to β83−87 (sequence GTFAK). Subsequent MS/MS analysis of this peptide confirmed this amino acid sequence (Figure 3B) and the mutation to be β87Thr→Lys (Hb-D Ibadan). This was in agreement with the DNA sequencing result.

Compound Heterozygous Hb SC (β6Glu→Val and β6Glu→Lys). Previous HPLC and IEF analysis of this sample suggested it to be Hb SC. Hb SC is a compound heterozygous Hb variant characterized by the mutations β6Glu→Val (Hb S) and β6Glu→Lys (Hb C) in two different β-globin chains. The β6Glu→Val substitution was indicated by the deconvoluted mass spectrum that showed a mass of 15837 Da for one of the variants, i.e., a −30 Da mass shift from the normal value of 15867 Da. The presence of the β6Glu→Lys substitution in the second variant (M_r = 15 866) was suggested by a −29 Da mass difference between the two Hb β chains present. If the second chain was a normal β globin, rather than the Glu→Lys variant, this mass difference would be −30 Da. Thus, the second variant must have a mass 1 Da lower than the normal β chain. Significantly, there is no single amino acid substitution from a single base mutation that results in a 29 Da mass change. The mass spectrum thus suggests the presence of compound heterozygous Hb SC (Figure 4A). The MS/MS spectrum of the suspected Hb S chain showed a series of multiply charged b_n ions (for $n \geq 14$) at m/z values consistent with a −30 Da shift, but the observed peaks for the multiply charged y ions y_{89}, y_{96} and y_{111} did not shift from the normal values (Figure 4B). On closer examination of peaks corresponding to b_n ions ($n < 14$), a peak corresponding to the singly charged variant b_6 ion (m/z 647.4) 30 Th lower than the normal b_6 ion (m/z 677.4) was observed (Figure 4C). Confirmation of the β6Glu→Val (Hb S) substitution was made on the peptide level by MS/MS after tryptic digestion.

MS/MS analysis of the intact suspected Hb C chain did not show m/z changes for the major y ions (y_{23}, y_{36}, y_{96}, and y_{111}) but noticeable mass shifts for some b_n^{3+} ions (for $n \geq 21$) were observed. The data led us to believe that the amino acid substitution lies before residue 21 of the β chain (Figure 4D). On close examination of the MS/MS spectrum, a peak for a singly charged b_6 ion at m/z 676.4 was observed, which is one m/z unit lower than the peak for the normal b_6 ion (m/z 677.4), as shown in Figure 4E. The substitution Glu→Lys at residue 6 was confirmed by analysis of the tryptic fragments where peaks for the new peptide corresponding to β1−6 (sequence VHLTPK) was evident. Subsequent MS/MS of the doubly protonated variant peptide (m/z 347.7) confirmed the amino acid sequence (Figure 4F).

Anodal β Variant. In a previous investigation, IEF showed this variant to be slightly anodal, that is, the substitution results in a gain of negative charge. The ES spectrum showed the presence of a β variant 69 Da lower in mass than the normal β chain (Figure 5A). The only possible substitution that can result in a −69 Da mass shift is Arg→Ser. The MS/MS spectrum of the intact variant β chain showed m/z values of b ions b_{13}^{2+}, b_{14}^{2+}, b_{21}^{3+}−b_{23}^{3+}, and b_{20}^{2+}−b_{23}^{2+} and prominent y ions y_{96}^{10+}, y_{96}^{11+}, y_{96}^{12+}, y_{98}^{11+}, y_{111}^{12+}, and y_{111}^{13+} to be identical to those from a normal β chain (cf. Figures 1B and 5B). However, the m/z values for b_{32}^{3+}−b_{34}^{3+} ions were shifted by −23 Th from the respective product ions in the normal β chain. A close inspection of the MS/MS spectrum of the variant revealed the m/z value for b_{29}^{3+} to be unchanged, but the m/z for b_{30}^{4+} in the variant β chain (m/z 769.4) to be shifted by −17.3 Th from the normal b_{30}^{4+} ion (m/z 786.7) (Figure 5C). Thus, the variant substitution is localized at residue 30, and this corresponds to Hb Tacoma (β30Arg→Ser).[13]

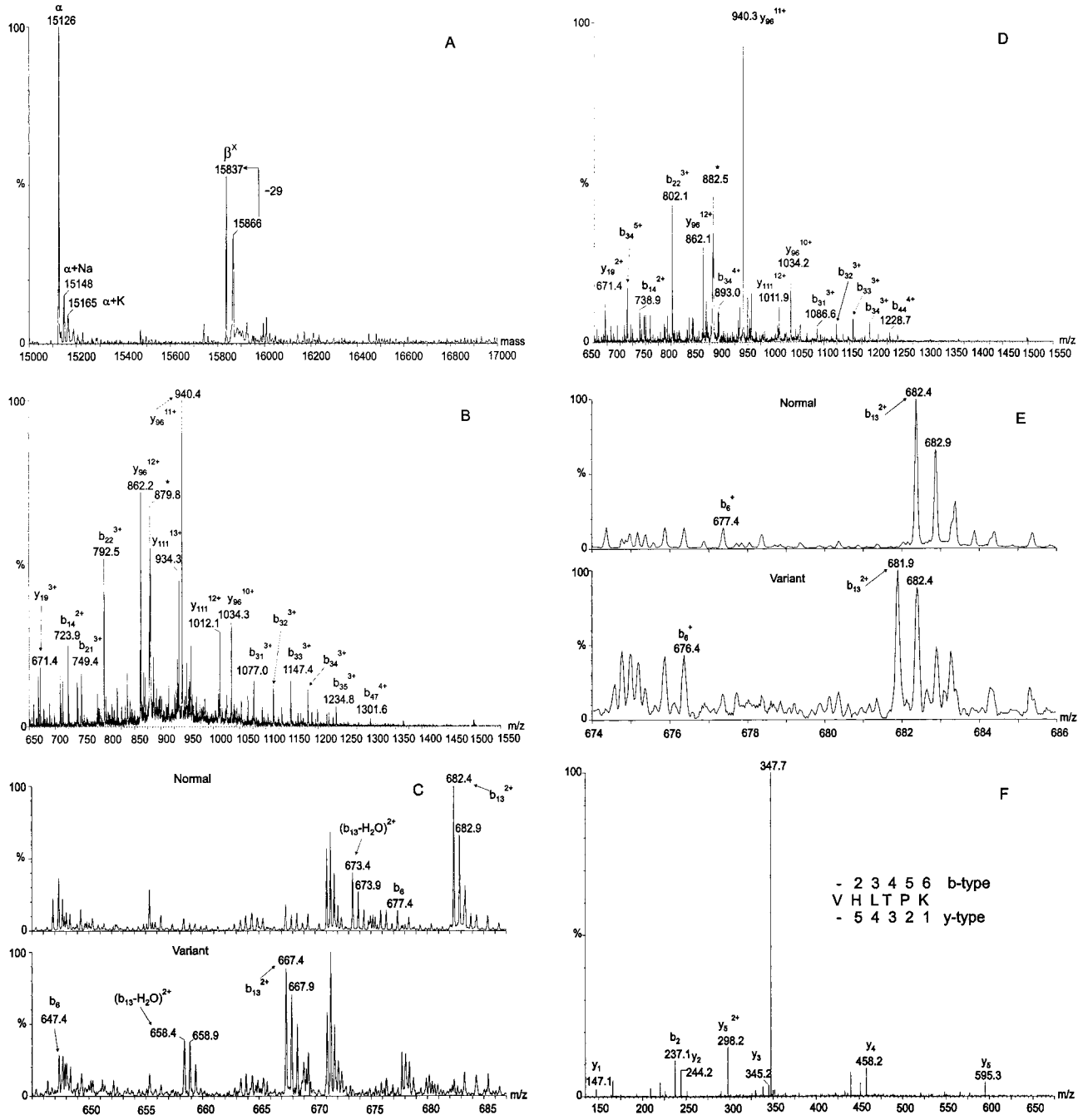

Figure 4. ES spectra for compound heterozygous Hb SC (β6Glu→Val and β6Glu→Lys). (A) Deconvoluted mass spectrum. (B) Product ion spectrum for the $[M + 18H]^{18+}$ ion of the intact Hb S chain. (C) Expanded view of the MS/MS spectrum showing the 30 Th shift in b_6 ions. The upper panel shows normal Hb; the lower panel, Hb S. (D) Product ion spectrum for the $[M + 18H]^{18+}$ of the intact Hb C chain. (E) Expanded view of the MS/MS spectrum showing 1 Th shift in b_6 and 0.5 Th shift in the b_{13}^{2+} ions. The normal Hb is shown in the upper panel, and the Hb C in the lower panel. (F) Product ion spectrum for the variant peptide $[M + 2H]^{2+}$ ion (VHLTPK) (*m*/*z* 347.7).

CONCLUSIONS

MS/MS on the intact chain can be performed in a single step following dilution of whole blood with no requirement for globin separation. The above study shows that MS/MS of intact abnormal β chains can give an unequivocal diagnosis (see Scheme 1). The MS/MS data shows that diagnosis of β-chain Hb variants can be made for substitutions lying within 40−50 residues from either termini of the polypeptide chain. In the absence of a definitive diagnosis by MS/MS of the intact protein, the information obtained from these spectra helps an investigator to focus on a specific region of the protein during subsequent analysis of a peptide digest. Some commonly occurring Hb variants, such as Hb C (β6Glu→Lys), Hb E (β26Glu→Lys), Hb D-Los Angeles or Hb D-Punjab (β121Glu→Gln), and Hb O-Arab (β121Glu→Lys), have similar mobilities on routine electrophoresis[22] but differ in mass from the normal β chain by 1 Da. These variants are

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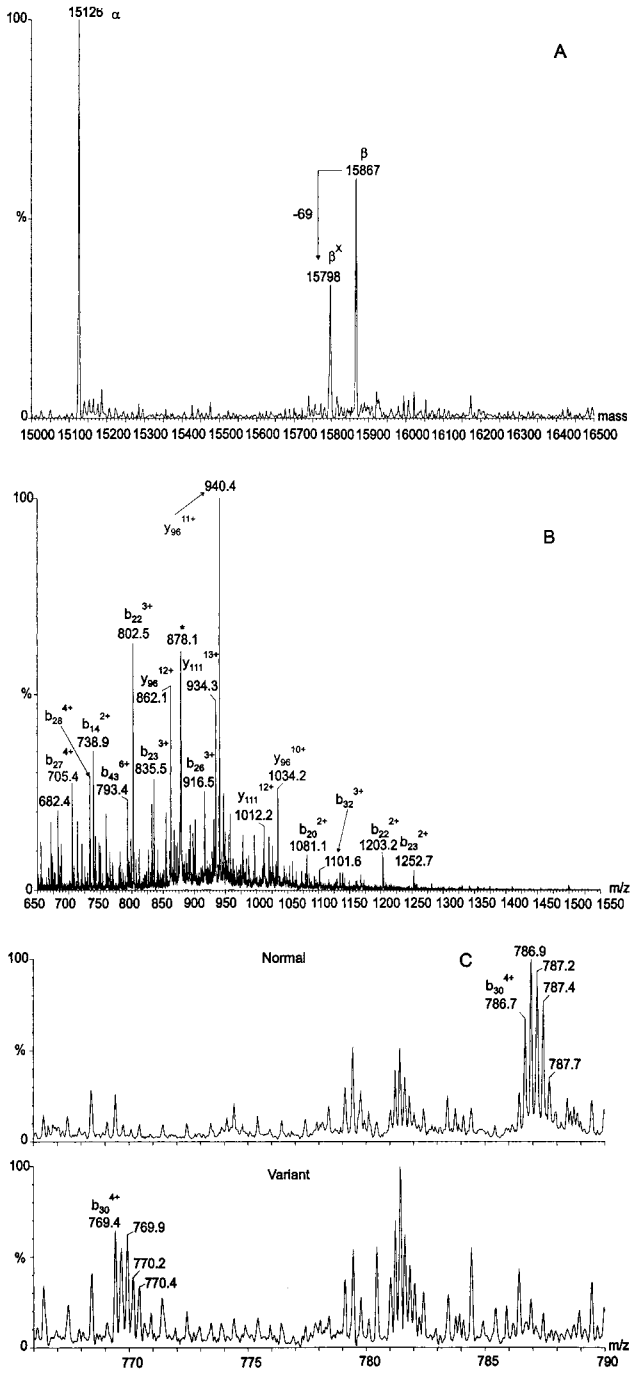

Figure 5. ES spectra for the anodal β-chain variant (A) deconvoluted mass spectrum, (B) product ion mass spectrum for the $[M + 18H]^{18+}$ intact β chain, and (C) expanded view of the MS/MS spectrum showing 17.3 Th shift in the b_{30}^{4+} ions. Normal Hb is shown in the upper panel, and the variant in the lower panel.

difficult to identify on the intact globin level unless the variant is separated from the normal Hb or a mass spectrometer of ultrahigh resolution (i.e., Fourier transform) is used.[23−25] However, if any of these variants is suspected to coexist with another variant, a conclusive diagnosis can be reached solely by MS/MS of the intact β chains, provided the chains are separated by more than 11 Da.

With the increasing availability of medium-high-resolution ES tandem mass spectrometers, the data presented in Table 1 will aid in the rapid diagnosis of Hb variants using ES-MS/MS. The data are also of use for investigators of Hb variants using low resolution instruments, i.e., triple quadrupole mass spectrometers. The information incorporated in Table 1 can be easily introduced into a computer algorithm that can be used to identify Hb variants. With the falling cost of Q-TOF-like instruments, automated direct analysis of Hb variants in a clinical setting becomes a real possibility.

Scheme 1. Amino Acid Sequence of the Normal Hb β Chain.[a]

Δ-69 = R→S

Δ-30 = T→A: W→R: S→G: M→T: E→V

Δ-1 = D→N: E→Q: N→I: E→K

Δ+27 = S→N: T→K

Δ+32 = V→M

[a] Mass shifts observed in the Hb β variants studied are indicated along with the region of the chain in which they are localized by MS/MS of the intact chain. The possible amino acid changes corresponding to such shifts are tabulated.

ACKNOWLEDGMENT

This work was supported by the Swedish Medical Research Council Grant no. 03X-12551, Karolinska Institutet, Stockholm.

Received for review October 9, 2001. Accepted January 29, 2002.

AC011068F