

Expression and Properties of Recombinant HbA₂ ($\alpha_2\delta_2$) and Hybrids Containing δ - β Sequences

Kenji Inagaki,¹ Junko Inagaki,¹ Antoine Dumoulin,² Julio Cesar Padovan,³ Brian T. Chait,³ Anthony Popowicz,³ Lois R. Manning,⁴ and James M. Manning^{4,5}

Received November 27, 2000

Hemoglobin A₂ ($\alpha_2\delta_2$), which is present at low concentration (1–2%) in the circulating red cells of normal individuals, has two important features that merit its study, i.e., it inhibits polymerization of sickle HbS and its elevated concentration in some thalassemias is a useful clinical diagnostic. However, reports on its functional properties regarding O₂ binding are conflicting. We have attempted to resolve these discrepancies by expressing, for the first time, recombinant hemoglobin A₂ and systematically studying its functional properties. The construct expressing HbA₂ contains only α and δ genes so that the extensive purification required to isolate natural HbA₂ is circumvented. Although natural hemoglobin A₂ is expressed at low levels *in vivo*, the amount of recombinant $\alpha_2\delta_2$ expressed in yeast is similar to that found for adult hemoglobin A and for fetal hemoglobin F when the $\alpha + \beta$ or the $\alpha + \gamma$ genes, respectively, are present on the construct. Recombinant HbA₂ is stable, i.e., not easily oxidized, and it is a cooperative functional hemoglobin with tetramer–dimer dissociation properties like those of adult HbA. However, its intrinsic oxygen affinity and response to the allosteric regulators chloride and 2,3-diphosphoglycerate are lower than the corresponding properties for adult hemoglobin. Molecular modeling studies which attempt to understand these properties of HbA₂ are described.

KEY WORDS: Hemoglobin; tetramer strength; delta gene; allosteric regulator.

1. INTRODUCTION

Hemoglobin A₂, $\alpha_2\delta_2$, is a naturally occurring hemoglobin expressed at low concentrations in normal individuals but at elevated levels in some patients with thalassemia, for whom it is used as a diagnostic. Its δ -chain differs from the β -chain of normal adult HbA at 10 of 146 total amino acids. HbA₂ is nearly as efficient as HbF ($\alpha_2\gamma_2$) in inhibiting the *in vitro* polymerization of HbS because it interferes with essential contacts between

deoxy HbS tetramers. In contrast to HbF, however, no known pharmacological agent induces δ -chain expression such as hydroxyurea does for γ -chain synthesis, forming the basis for a current therapy for sickle cell anemia. Nevertheless, it would be extremely informative to know the details of its antipolymerization effects.

Oxygen binding of hemoglobins that coexist with HbS in the red cell is linked to the abnormal polymerization of HbS since low oxygen tension favors the latter process, whereas increased oxygen tension has the opposite effect. The reports in the literature (Huisman *et al.*, 1962; Eddison *et al.*, 1964; Santa, 1969; Bunn and Briehl, 1970; De Bruin and Jansen, 1973) over the past 40 years regarding the *in vitro* oxygen-binding properties of hemoglobin A₂ are conflicting, with some indicating properties very similar to those of HbA and others showing much different values. Some possible reasons for these discrepancies include a less stable tetrameric structure or a more readily oxidizable heme. None of these has been systematically investigated or excluded, perhaps

¹ Okayama University, Okayama, Japan.

² Valigene, Paris, France 92086.

³ Mass Spectrometry Laboratory, Computing Services, Rockefeller University, New York, New York 10021.

⁴ Department of Biology, Northeastern University, Boston, Massachusetts 02115.

⁵ To whom correspondence should be addressed at Department of Biology, Mugar 414, Northeastern University, 360 Huntington Avenue, Boston, Massachusetts 02115. e-mail: jmmanning@lynx.neu.edu

due to the very low concentration of HbA₂ in human blood, requiring extensive and careful purification to remove the vast excess of adult HbA. We have sought to circumvent this difficulty by expressing hemoglobin A₂ using only the α and δ genes, thus avoiding extensive purification, so that its properties could be studied rapidly and systematically. In order to achieve this goal, we first constructed the human δ -gene from the β -gene by substitution of their 10 amino acid differences and we linked this construct to the human α -gene in the yeast system described previously (Dumoulin *et al.*, 1997, 1998). Tetramers containing partial hybrid δ - β subunits and α subunits have also been expressed in order to determine the contributions of various segments of the δ -subunit to the O₂-binding properties of HbA₂ and to the response to the regulatory molecules chloride and 2,3-diphosphoglycerate (2,3-DPG). The expressed HbA₂ as well as the partial α_2 (δ - β)₂ hybrids have been subjected to rigorous characterization by mass spectrometry to give reliable conclusions on the properties of these hemoglobins. A recombinant Hb containing the delta gene in addition to the Glu6 \rightarrow Val sickle substitution has been expressed in order to address the question of the effect of HbA₂ on HbS polymerization (Adachi *et al.*, 1996).

2. EXPERIMENTAL PROCEDURES

2.1. Materials

Restriction enzymes, alkaline phosphatase, and T4 DNA ligase were from New England Biolabs (Beverly, MA). DNA polymerases used for PCR were Taq polymerase (Amersham), Vent DNA polymerase (New England Biolabs), and cloned Pfu polymerase (Stratagene). The deoxynucleotide set (dNTP) was from Amersham. The oligonucleotides containing the mutations were synthesized by Operon Technologies (Alameda, CA) or by the Protein Sequencing Facility at the Rockefeller University (New York, NY). 2,3-DPG was purchased from Sigma (St. Louis, MO). The GeneClean kit was from Bio 101 (Vista, CA). Qiagen plasmid midi kit was from Qiagen Inc. (Santa Clarita, CA). *Escherichia coli* XLI-Blue was used for cloning manipulations, and *Saccharomyces cerevisiae* GSY112 Cir^o was used for expression. Growth conditions in a 20-L New Brunswick Fermentor BioFlo IV, and the plasmids used in cloning, pGS189 $\alpha\beta$, pGS189 α , and pGS389, have been described previously (Dumoulin *et al.*, 1997, 1998; Li *et al.*, 1999; Martin de Llanò *et al.*, 1993; Martin de Llanò and Manning, 1994; Yanase *et al.*, 1994).

2.2. Construction and Purification of Recombinant Hemoglobins

Ten amino acids of the β -globin subunit were serially replaced in four steps by the corresponding amino acids of the δ -globin subunit (Table I). The mutagenesis procedure was a two-step amplification method adapted from the splicing by overlap extension method described previously (Dumoulin *et al.*, 1997). Since the 10 codons mutated were clustered in four regions of the β -globin gene, we designed four pairs of oligonucleotides overlapping each of these regions. One pair of oligonucleotides, each flanking one extremity of the β -coding sequence and containing an *Xho*I restriction site, were also used in the amplification reactions (Dumoulin *et al.*, 1998). The size of the final polymerase chain reaction (PCR) product comprising the complete β -globin expression cassette was determined on an agarose gel, purified using GeneClean III and digested with the *Xho*I restriction enzyme. The resulting *Xho*I fragment was inserted into pGS190 α (Dumoulin *et al.*, 1997). Both α and β cassettes were extracted together from pGS190 $\alpha\beta$ plasmid with a *Not*I digestion and ligated into the *E. coli*/yeast shuttle plasmid pGS389. After expression in yeast in a 15-L culture in a New Brunswick Bio Flo IV fermentor, each recombinant hemoglobin was purified to homogeneity using methods described previously (Dumoulin *et al.*, 1997, 1998).

2.3. Characterization of Mutants

Each recombinant Hb was pure, as ascertained by isoelectric focusing, where each migrated as a single band in the expected position based on the nature of the substitutions. The spectrum of each recombinant Hb in the visible and the ultraviolet ranges was normal and showed no evidence of the oxidized (ferric) form. Circular dichroism spectra were taken on a Jasco J-715 instrument. The strategy used to characterize the recombinant hemoglobins by mass spectrometry has been described elsewhere (Li *et al.*, 1999). Molecular masses of the intact Hb subunits were measured by ES/MS using a Finnigan-MAT TSQ-700 electrospray-triple quadrupole mass spectrometer (Thermo-Quest, San Jose, CA). Fifty-nanomolar hemoglobin subunits in water-methanol-acetic acid (49:50:1, v/v/v) were infused at 1 μ l min⁻¹ through a 50- μ m (I.D.) fused silica capillary into the ion source of the mass spectrometer and electrosprayed at +3.8 kV. Desolvation of protein ions was accomplished by maintaining the heated capillary at 180°C. One hundred spectral scans were acquired at a rate of 1 scan/sec and averaged to produce the final spectrum.

For peptide analysis, 250 pmol of recombinant hemoglobin was diluted in 100 mM aqueous ammonium bicarbonate solution, pH 8.2, 2 mM calcium chloride, and incubated for 5 min at 37°C. Modified L-1 tosylamido-2-phenylethyl chloromethyl ketone-trypsin (Boehringer Mannheim) was added at zero time and at 4 hr (final substrate-to-enzyme ratio at 20:1, w/w), for 8 hr total at 37°C with occasional stirring. The final reaction volume was 50 μ l and final hemoglobin concentration was 5 μ M. The reaction was stopped by adding 50 μ l of water-methanol-acetic acid (49:49:2, v/v/v). Aliquots of 1 μ l were mixed with 19 μ l of saturated solutions of α -cyano-4-hydroxy-cinnamic acid (4hcca) in either water-acetonitrile (1:1, v/v) or water-acetonitrile-TFA (1:2, v/v, 0.1% TFA). Aliquots of 0.5 μ l of the latter solutions were deposited onto a gold-coated sample plate and allowed to air-dry. The dried spots were washed with 0.5 μ l of cold 0.1% aqueous TFA solution prior to analysis on a matrix-assisted laser desorption/ionization time-of-flight mass spectrometer (model STR Voyager, PerSeptive, Perkin Elmer, Boston, MA) equipped with delayed extraction and ion reflection.

For mass spectrometric fragmentation of the peptides containing mutated residues, the hemoglobin digest was diluted to 0.05 μ M in water-methanol-acetic acid (49:50:1, v/v/v) and infused at 1 μ l min⁻¹ through a 50- μ m (I.D.) fused silica capillary into the source of a Finnigan-MAT LCQ electrospray-ion trap mass spectrometer (ThermoQuest, San Jose, CA) and electrosprayed at 2.8 kV. Desolvation of peptide ions was accomplished by maintaining the heated capillary at 125°C. For each peptide precursor ion selected, mass spectrometric fragmentation yielded a series of b- and y-type ions.

2.4. Tetramer-Dimer Strength and Oxygen-Binding Properties

The tetramer-dimer dissociation constant (K_d) of HbA₂ was not reported in any of the previous studies. It was measured by a rapid and precise small-zone gel filtration method on a Superose-12 column of an FPLC system (L. R. Manning *et al.*, 1996, 1999). Different concentrations of liganded Hb in 100 μ l were applied and eluted with 150 mM Tris-Ac buffer, pH 7.5, at a flow rate of 0.4 ml/min. The Hb concentrations used depended on the K_d value for a particular Hb and ranged from 0.01 to 0.10 μ M for HbF and from 0.10 to 3.0 μ M for HbA, for example. The concentration of each Hb was determined by amino acid analysis. The eluent was monitored at 405 nm and the calculations were performed using Graft.

Oxygen-binding curves of hemoglobin were determined at 37°C on a modified Hem-O-Scan instrument (American Instrument Co., Silver Springs, MD) as de-

scribed previously (Dumoulin *et al.*, 1997, 1998; Li *et al.*, 1999; Martin de Llano *et al.*, 1993; Martin de Llano *et al.*, 1994; Yanase *et al.*, 1994). Before the measurements, the buffer was changed to 50 mM bis-Tris-Ac, pH 7.5, by passage through a Sephadex G-25 column (Pharmacia). Hemoglobin was converted from the CO to the oxy form, and the sample was concentrated using a Centricon Microcon ultrafiltration device (10,000 molecular weight cutoff, Amicon, Beverly, MA) to a final concentration of 0.6–1.0 mM for measurements of O₂ binding with a precision of ± 1 mm Hg. The measurements were carried out without or with a fivefold molar excess of 2,3-DPG or 0.2 or 0.3 M chloride. Hill coefficients were calculated from the slopes of the Hill plots.

3. RESULTS AND DISCUSSION

3.1. Yields of Recombinant Hemoglobin A₂ and Hybrid δ - β Mutants in Yeast Expression System

The completely substituted recombinant HbA₂ and the δ - β hybrid mutant hemoglobins, whose sequence substitutions are shown in Table I, were expressed in the yeast system. In each instance, the yields of purified recombinant HbA₂ and of the hybrids were comparable to those found for recombinant HbA ($\alpha_2\beta_2$) (ca. 8–10 mg of purified Hb per 15-L fermentation) and did not show the decreased synthesis exhibited by HbA₂ *in vivo*.

3.2. Characterization of Recombinant Hemoglobins

Each recombinant Hb, purified by CM-52 chromatography and subsequently by FPLC as described

Table I. Amino Acid Substitutions in Delta versus Beta Subunits

Residue number	Complete mutant (HbA ₂)		Mutant #1 ^a	Mutant #2 ^a	
	β -Subunit	δ -Subunit			
9	Ser	Thr	↓		
12	Thr	Asn			
22	Glu	Ala		↓	
50	Thr	Ser			
86	Ala	Ser			
87	Thr	Gln			
116	His	Arg			
117	His	Asn			
125	Pro	Gln			↓
126	Val	Met			

^a Remaining positions are those of the β -subunit. A third mutant Hb containing the substitutions in mutants 1 and 2 was also expressed. Substitutions at residues 50, 86, and 87 were present only in the complete mutant (HbA₂).

previously (Dumoulin *et al.*, 1997, 1998; Li *et al.*, 1999; Martin de Llano *et al.*, 1993; Martin de Llano and Manning, 1994; Yanase *et al.*, 1994), showed a single band by isoelectric focusing (IEF) in the expected position. Further characterization of the multisubstituted hemoglobins was accomplished by mass spectrometric analysis of both the intact subunits and the tryptic fragments. These results for the complete recombinant HbA₂ (Fig. 1) showed conclusively that the amino acid replacements shown in Table I were achieved; its α subunit had a mass of 15,127.1 (theory 15,126.3) and its δ subunit had a mass of 15,924.4 (theory: 15,924.3). The masses of the tryptic peptides were in complete accord with the correct substitutions. The mass spectrometric analyses of the δ - β mutants 1, 2, and 1 + 2 were also in agreement with predicted values (Figs. 2–4).

3.3. Spectral Properties

The purified recombinant HbA₂ had a normal absorption spectrum in both the visible and ultraviolet regions and there was no evidence that it readily oxidized to ferric Hb even during the conversion of the CO form to the O₂ form prior to the functional oxygen-binding studies. Analysis of the circular dichroism spectrum of purified recombinant HbA₂ in the far-ultraviolet region and in the heme-absorbing region did not indicate any folding or heme-binding abnormalities, respectively. Both absorption spectra and circular dichroism corresponded to those HbA.

3.4. Tetramer–Dimer Dissociation

Earlier results on the tetramer strength of fetal hemoglobin (HbF) showed that it dissociates into dimers much less than does adult hemoglobin (HbA) (Dumoulin *et al.*, 1997, 1998; J. M. Manning *et al.*, 1999). The tetramer–dimer dissociation constant of recombinant HbA₂ shown in Fig. 5 is 0.77 μ M, which is practically identical to the 0.68 μ M value we reported for HbA (Dumoulin *et al.*, 1997; J. M. Manning *et al.*, 1999) under the same conditions. The three δ - β hybrid tetramers that were constructed and expressed had nearly the same K_d values as the completely substituted HbA₂ (Table II). Therefore, neither the substitutions themselves nor the sites at which they were made had a deleterious effect on tetramer strength. The results show that there is nothing intrinsically problematic with $\alpha_2\delta_2$ with respect to its ability to form a tetramer. This result has not been reported previously.

3.5. Functional Properties

There have been at least five reports during the last four decades on the oxygen affinity of natural HbA₂ and the effects of allosteric regulator 2,3-DPG on this hemoglobin (Huisman *et al.*, 1962; Eddison *et al.*, 1964; Santa, 1969; Bunn and Briehl, 1970; De Bruin and Jansen, 1973). Some of these reports show that HbA₂ has an oxygen affinity identical to that of HbA, but the others indicate an increased oxygen affinity. There are several possible reasons for these diverse conclusions given the occurrence of very small amounts of naturally occurring HbA₂ in human blood and the difficulty in obtaining it in a pure form without oxidation of the heme moiety and the attendant effects on oxygen affinity. The results (Table III and Fig. 6) show that the purified recombinant HbA₂, which was rapidly purified to homogeneity in two steps without encumbrances from large amounts of adult HbA, has a higher oxygen affinity than adult HbA and a lower response to chloride and to 2,3-DPG than does HbA. The partially substituted mutants (1, 2, and 1 + 2) have the same properties.

3.6. Location of Substitutions

In an effort to understand the reasons for the increased oxygen affinity of HbA₂ relative to HbA and its decreased response to allosteric regulators we have examined the locations of some of the substitutions in HbA₂. The crystal structure of HbA₂ has not been solved, so molecular modeling has been employed. Two of the substitutions in mutant 1 are in the A helix of the δ -subunit, and in mutant 2 all four substitutions are in the G and H helices, where many of the packing contacts of the rigid dimer interface between α - and δ -subunits monomers occur. Other results we have reported using this approach of partial sequence substitutions between various hemoglobins (Dumoulin *et al.*, 1997, 1998) have shown that this region of the tetramer contributes marginally to the functional properties of the tetramer, although such a conclusion is tempered by the experimental difficulty in studying this region of the tetramer due to its very low dissociation properties. We showed that the properties of the $\alpha_1\beta_1$ interface could be evaluated indirectly by determining how substitutions in this region affected the allosteric $\alpha_1\beta_2$ interface whose movements are closely linked with both O₂ binding and the DPG response, and this is the approach we have taken in this communication. The V126- β to M126- δ substitution in the H-helix (Fig. 7a) would be expected to generate unfavorable contacts with the L14 residue in the A-helix (Fig. 7b). These close contacts, within 1.62 Å, would force

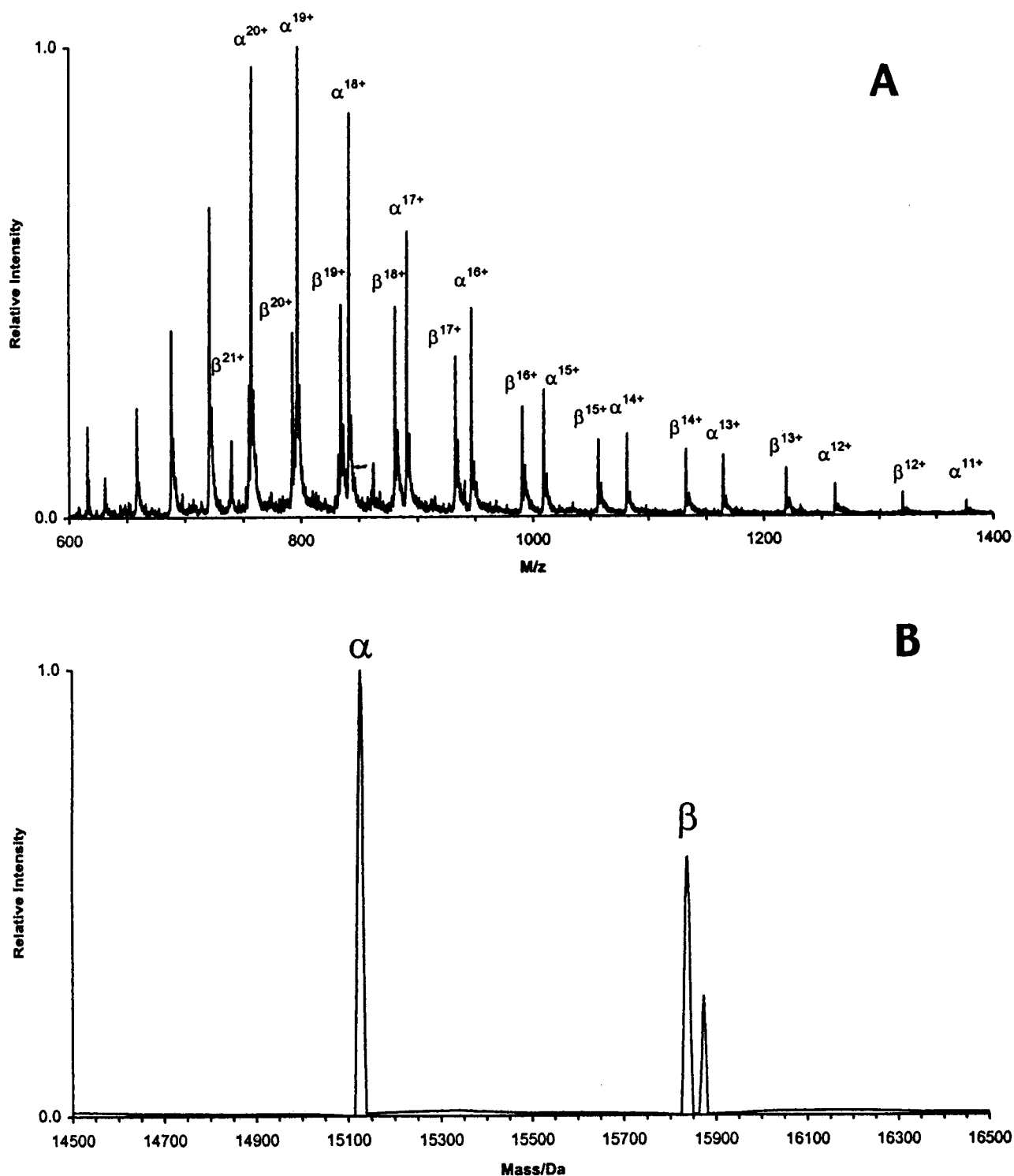


Fig. 1. ESI/MS spectrum of recombinant hemoglobin A₂ subunits. Fifty nM hemoglobin solution in water–methanol–acetic acid (49:50:1, v/v/v) was infused at 1.0 $\mu\text{l}/\text{min}$ and electrosprayed at +3 kV ($I < 0.3 \mu\text{A}$); a heated capillary was kept at 165°C to assist desolvation; 100 m/z spectra were collected with a sampling rate of 25 points per m/z unit and averaged to produce the spectrum shown in panel A. The transformed mass spectrum is shown in panel B: chain α (15,127.1 \pm 0.7) Da ($\Delta = +0.8$, expected 15,126 Da) and chain δ (15,924.4 \pm 0.5) Da ($\Delta = +0.1$ expected 15,924.3 Da) (average \pm STD, $\pi = 10$). Adduction (possibly K^+ ions) to the δ chain is also observed.

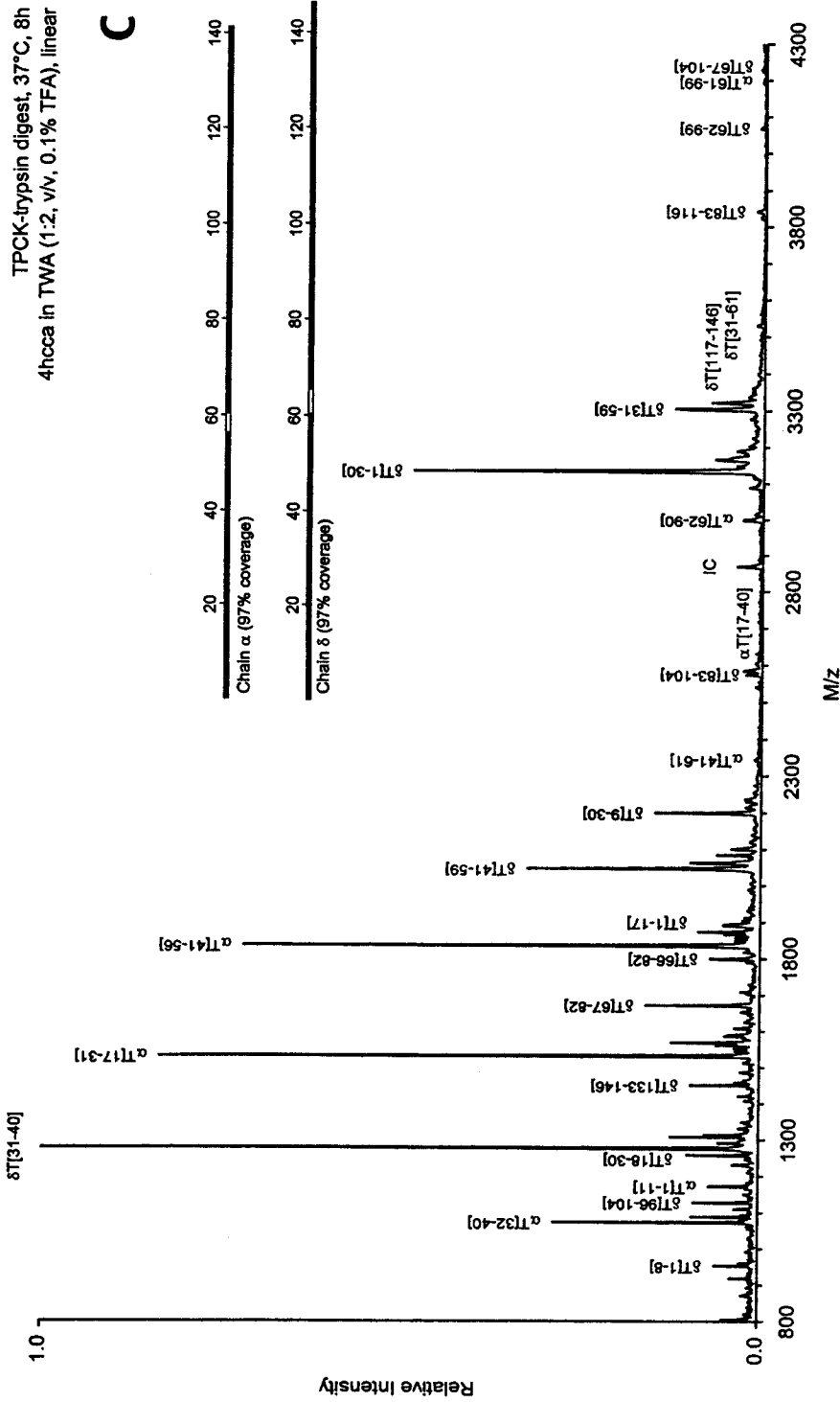


Fig. 1. Continued. Panel C shows the MALDI-TOF/MS spectrum of recombinant hemoglobin A₂ peptides. Protein (250 pmol or 5 μ M in hemoglobin) was digested with 800 ng of Boehringer Mannheim modified TPCK-trypsin (*S/E* = 20, w/w) in a final volume of 50 μ l of 100 mM aqueous ammonium bicarbonate buffer, pH 8.2, 2 mM calcium chloride, for 8 hr at 37°C (400 ng of trypsin added at 0 and 4 hr). Reaction was stopped by dilution to 100 μ l (5 μ M in peptides) with methanol-acetic acid (98:2, v/v). An aliquot of 1 μ l of the peptide mixture was mixed with 1 μ l of insulin (2 μ M, I.C.) and 18 μ l of a saturated solution of α -cyano-4-hydroxy-cinnamic acid (4hcca) in water-acetonitrile (1:1, v/v) (WA). Average of 200 individual shots collected in linear mode. Coverage for each polypeptide chain is also shown.

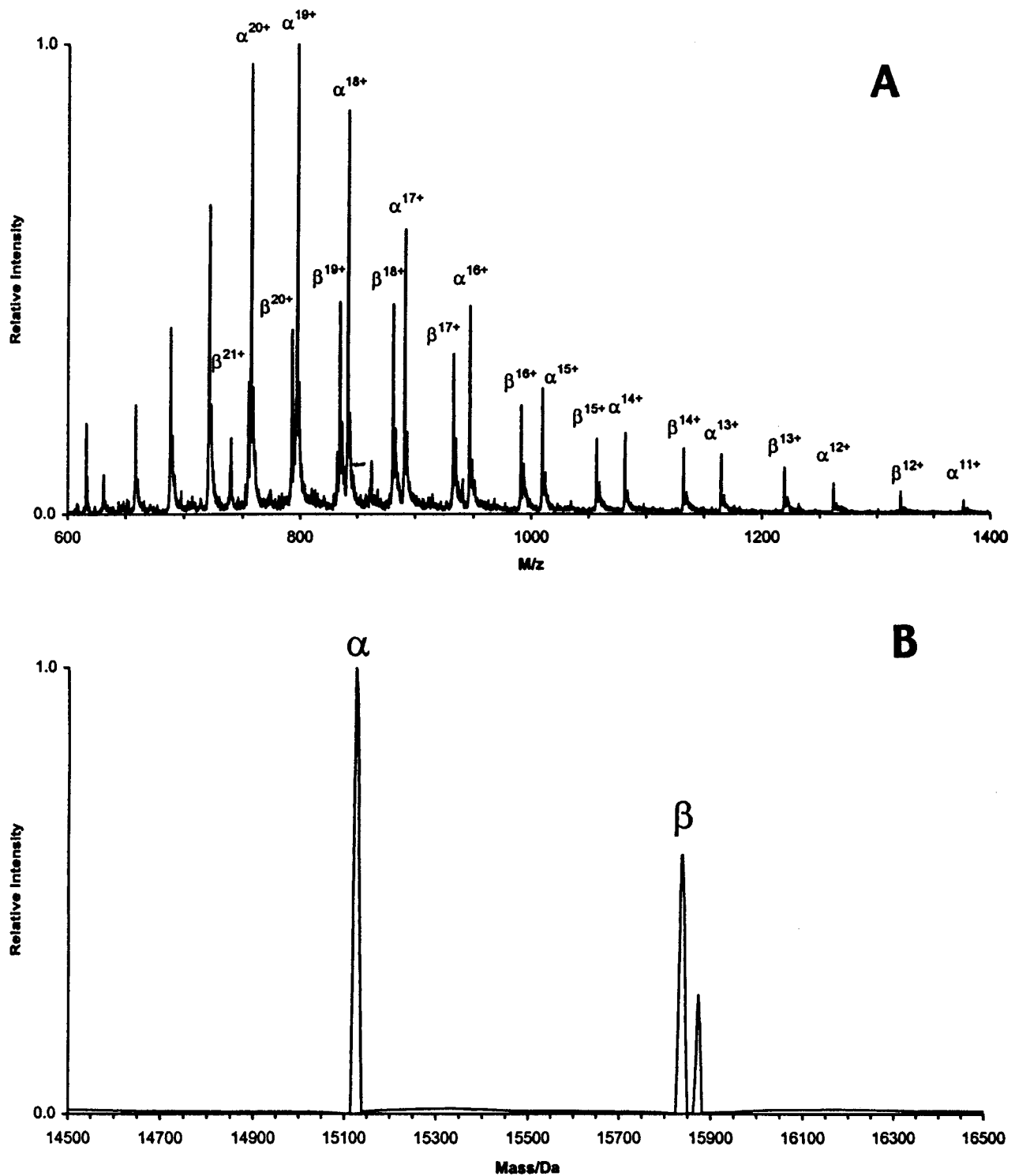


Fig. 2. ESI/MS spectrum of mutant 1 containing substitutions S9 β T, T12 β N, E22 β A. The details are in the legend to Fig. 1. The data were averaged to produce the spectrum shown in panel A. The transformed mass spectrum is shown in panel B: chain α (15,126.1 \pm 0.5) Da (Δ = +0.1, expected 15,126.3 Da) and chain β (15,836.8 \pm 0.5) Da (Δ = +0.6, expected 15,836.2 Da) (average \pm STD, π = 10). Adduct formation (possibly K⁺ ions) with the β chain is also observed.

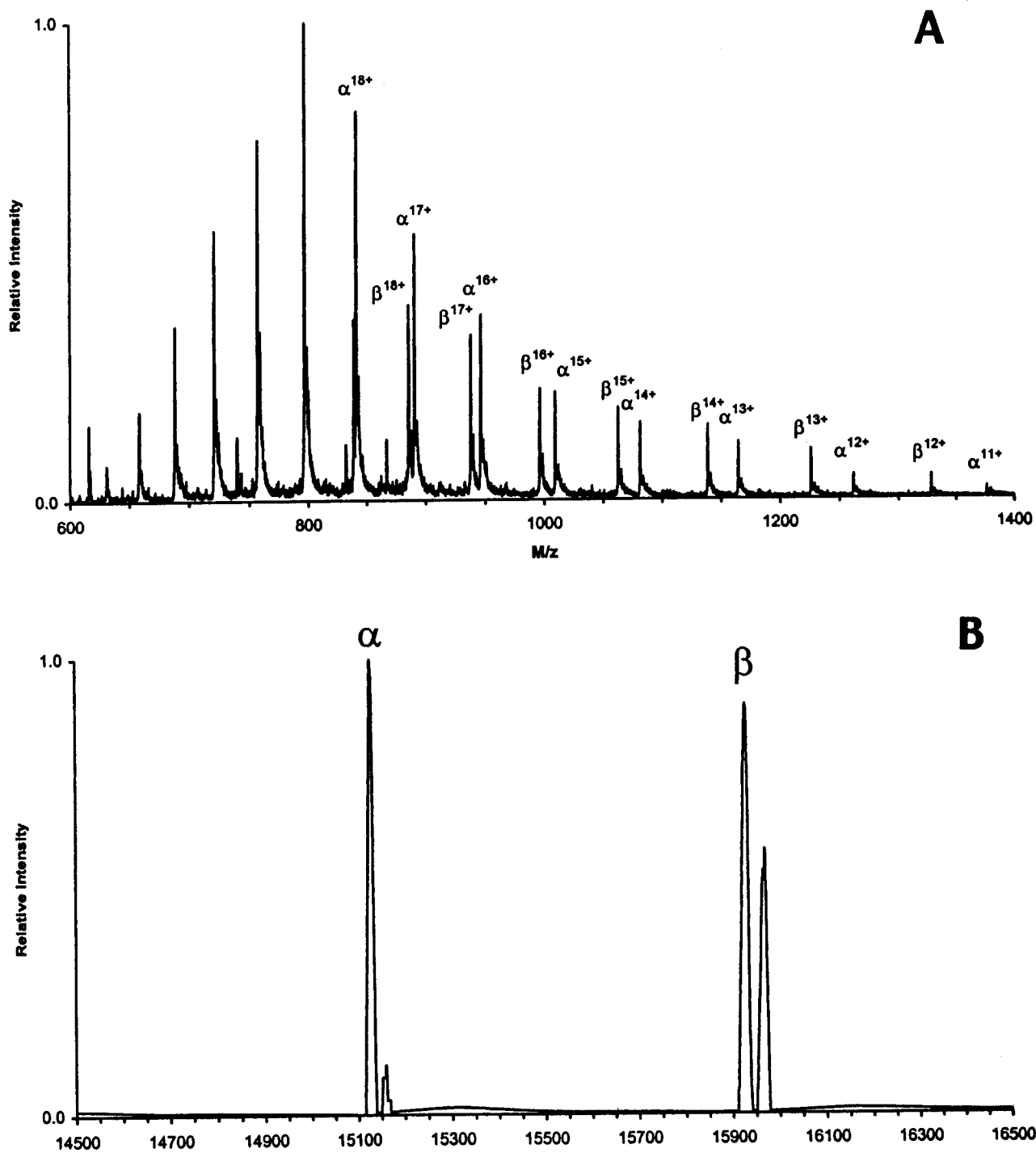


Fig. 3. ESIMS spectrum of mutant 2. The details are given in the legend to Fig. 1. The data were averaged to produce the spectrum shown in panel A. The transformed mass spectrum is given in panel B: chain α ($15,126.1 \pm 0.7$ Da ($\Delta = 0.2$, expected 15,126.3 Da) and chain β ($15,925.4 \pm 0.4$ Da ($\Delta = 0.9$, expected 15,926.3 Da) (average \pm STD, $\pi = 10$).

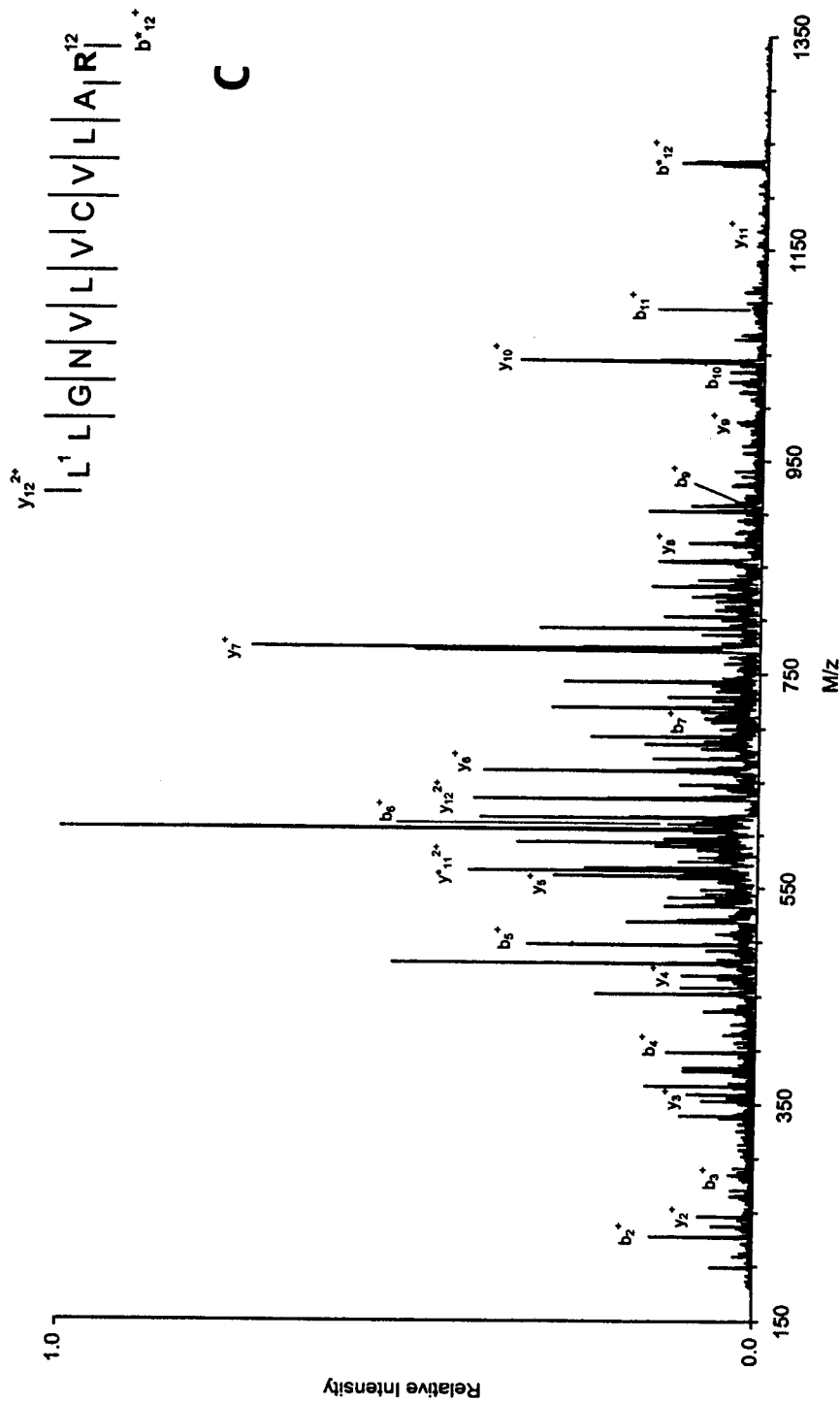


Fig. 3. Continued. Panel C shows the ESI-ion trap/MS spectrum of tryptic peptide β T (105–116) from mutant 2. The substituted H116R is in bold.

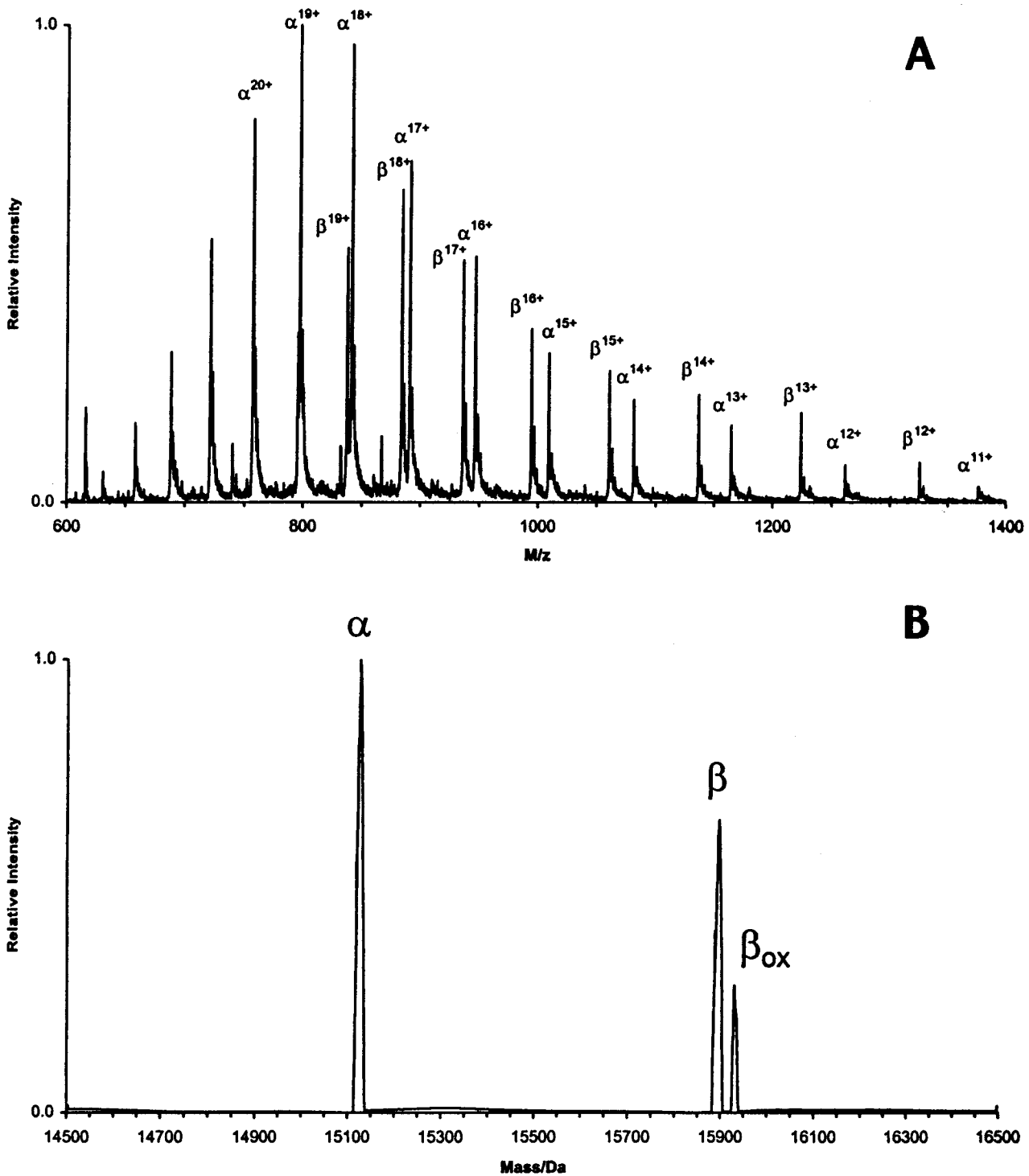


Fig. 4. ESIMS spectrum of mutants 1 + 2 containing substitutions S9 β T, T12 β N, E22 β A, H116 β R, H117 β N, P125 β Q, V126 β M. The data were averaged to produce the spectrum shown in panel A. The transformed mass spectrum is shown in panel B: chain α (15,125.0 \pm 0.4) Da (Δ = 0.3, expected 15,126.3 Da) and chain β (15,894.7 \pm 0.7) Da (Δ = 0.5, expected 15,895.3 Da) (average \pm STD, π = 10).

Hemoglobin (CB99-002-004)
 TPCK-trypsin digest, 37°C, 8h
 4hcca in WA (1:1, v/v), linear

C

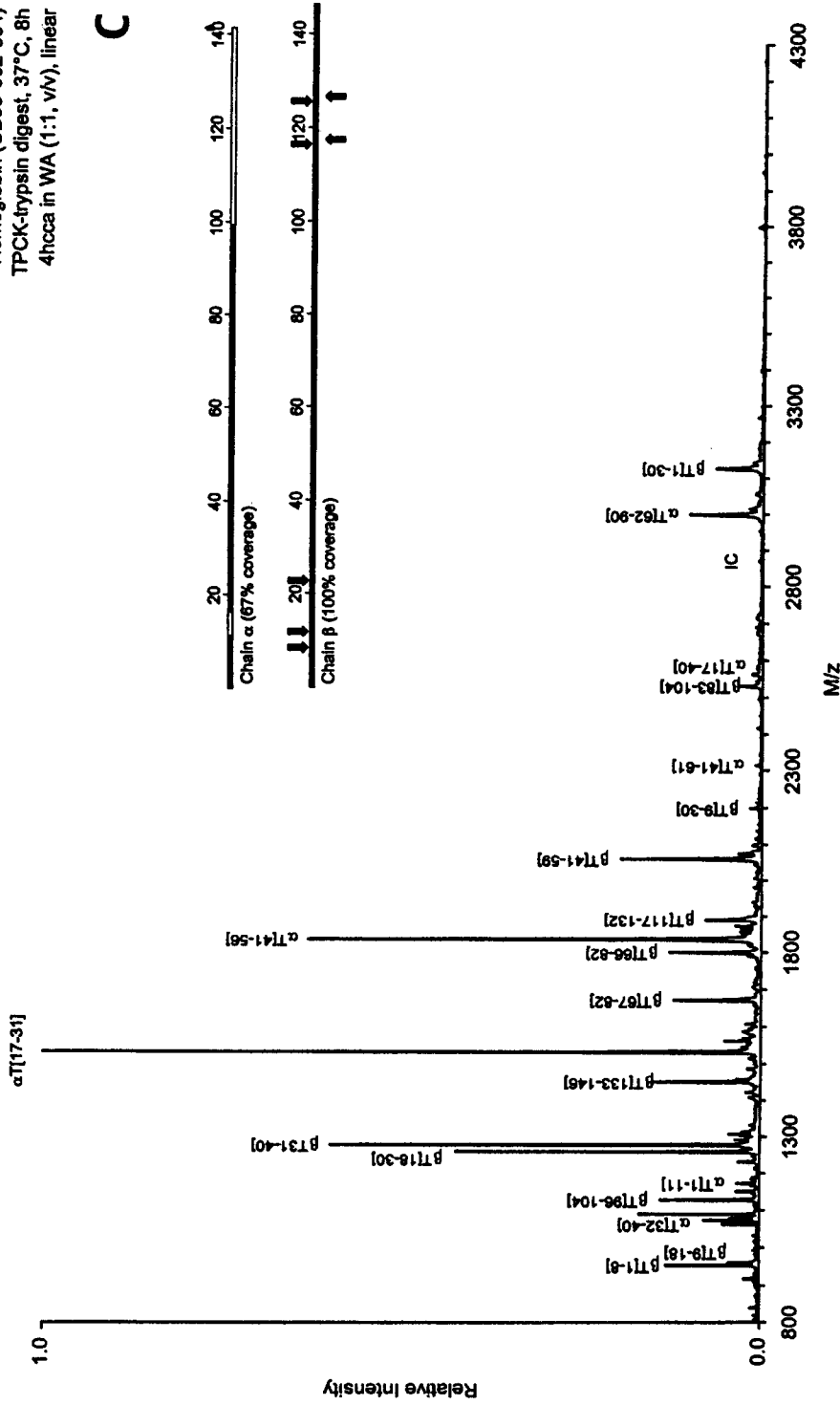


Fig. 4. Continued. Panel C shows the MALDI-TOF/MS spectrum of tryptic peptides of mutant 2 containing substitutions S9βT, T12βN, E22βA, H116βR, H117βN, P125βQ, V126βM. Peptides containing the mutated residue(s) (arrows) are shown. Coverage for each polypeptide chain is also shown.

