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Ion trap tandem mass spectrometry of intact GTP-binding protein γ-subunits

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Abstract

Tandem mass spectrometry (MS), including both MS^2 and MS^3 product ion scans, has been applied for the structural characterization of intact γ-subunits of heterotrimeric GTP-binding proteins (G-Proteins). Intact γ-subunits were ionized by electrospray ionization and fragmented by collision-induced dissociation in a quadrupole ion trap instrument. Extensive fragmentation was observed for the multiply charged precursor ions of these 7–8 kDa proteins. Spectral interpretation was facilitated by loss of the labile isoprenyl modification and fragmentation at C-terminal proline residues. General rules for interpretation of product ion spectra were developed for determination of sequence and posttranslational modification. Second generation product ion scans were shown to be valuable for assisting in spectral interpretation. Analysis of charge state effects on product ion spectra indicated nearly identical fragmentation patterns with differing charges on resulting product ions and provided confirmation of product ion structure assignments. This approach can be used to identify specific γ-subunits and to determine the structures of posttranslational modifications without the need for proteolytic peptide mapping. (Int J Mass Spectrom 212 (2001) 377–388) © 2001 Elsevier Science B.V.

Keywords: Collision-induced dissociation; Electrospray; Nanospray; Isoprenylation

1. Introduction

Since the pioneering studies of Kruger [1,2] and others [3,4] using tandem mass spectrometry (MS/MS) for structure elucidation of small molecules it has been clear that this approach for ion structure determination would be a powerful tool in the arsenal of organic chemists. With the introduction of ionization techniques such as electrospray ionization (ESI) [5] and matrix-assisted laser desorption ionization (MALDI) [6], MS/MS has become an essential tool for biochemists for the characterization of biomolecules including peptides, proteins, and DNA. The sensitivity, throughput, and specificity of the MS/MS approach for peptide sequencing makes this method the quintessential method for large-scale protein identification required in the proteome era [7,8]. Because of the advantages of peptide sequencing by MS/MS, thousands of peptides can be sequenced in a single experiment leading to the identification of thousands of proteins in a sample in a single experiment [9]. An additional significant feature of protein structure determination by MS/MS is the ability to identify sites and structures of posttranslational modifications [10].

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Dedicated to R. Graham Cooks on the occasion of his sixtieth birthday.

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One of the limitations of protein structure determination by tandem mass spectrometry is the need to cleave the parent protein into smaller peptidic fragments prior to sequencing due to the mass range limitation for obtaining interpretable sequence information. The mass limitation has been demonstrated to be 2 000–2 500 Da for singly charged ions [11] and is due, in part, to the inadequacy of internal energy deposition in collision induced dissociation (CID) of large precursor ions [12]. In fact, Rice–Ramsperger–Kassel–Marcus theory predicts that ergodic fragmentation (fragmentation after randomization of the internal energy among molecular degrees of freedom) requires large amounts of internal energy to be deposited in order to observe large molecule fragmentation on the instrumental timescale [13]. Typically, the proteolytic enzyme trypsin is used to produce peptide fragments within the mass limitation of CID. Peptides fragment predictably [10,14] and this has resulted in a number of peptide sequence interpretation algorithms based on interpretation of MS/MS data [15,16]. In order to identify a protein by a short amino acid sequence, a digest prior to the MS/MS experiment is required; however, to identify posttranslational modifications, each tryptic peptide must be analyzed. This may require a high-resolution peptide separation prior to mass spectrometric analysis.

Due to the practical and theoretical limits to protein sequencing by tandem mass spectrometry, a number of investigators have explored the fragmentation of intact proteins to produce structurally informative product ions. Their results suggest that fragmentation of large proteins can be facilitated by intramolecular coulombic repulsion in multiply charged precursor ions [17] and by nonergodic fragmentation [18]. Several strategies have been employed to produce sequence information on intact parent proteins with molecular weights of up to 150 kDa [19]. These approaches include electrospray ionization coupled with CID in triple quadrupole instruments [20], Fourier transform mass spectrometry (FTMS) instruments [21,22], and quadrupole ion traps [23,24], and most recently electron capture dissociation (ECD) and activated ion ECD in an FTMS instrument [18,25]. An advantage of the FTMS instrument is the high resolving power allowing charge state determination of highly charged product ions. Ion-ion reactions have also been used in quadrupole ion traps to facilitate interpretation of complex MS/MS data by converting multiply charged product ions to primarily singly charged ions thereby removing ambiguities of assignments [22,23]. From this previous CID work on large proteins, it is clear that specific fragmentations are favored including: cleavage N-terminal to proline and C-terminal to acidic residues [26,27].

The focus of this work is on structural characterization of heterotrimeric GTP-binding protein γ-subunits by ESI-MS^n. G-proteins are important, ubiquitous signaling molecules that transduce extracellular signals to intracellular effector molecules [28]. The heterotrimers consist of α-, β-, and γ-subunits of 40, 38, and 8 kDa, respectively. There are 12 γ-subunit genes in the human genome having predicted molecular weights in the range of 7–8.5 kDa. The γ-subunits are modified by C-terminal truncation of three amino acids followed by carboxymethylation and isoprenylation with either a farnesyl group or a geranylgeranyl group [29]. The N-terminus is differentially modified by methionine cleavage and/or acetylation. The diversity of cell stimuli that are transduced by G-proteins and the diversity of cellular responses are impressive and suggest an important role for the diversity of G-protein structure. We have identified over 40 distinct forms of bovine brain γ-subunits based on differentially modified forms of six amino acid sequences [30,31]. We report here on a tandem mass spectrometry strategy for structure determination of G-protein γ-subunits. Intact γ-subunit ions generated by ESI are probed with MS^2 and MS^3 scans and the resulting spectral patterns are interpreted to provide sequence identification and posttranslational modification information.

2. Methods

Bovine brain heterotrimeric G-proteins were isolated from by the method of Sternweis and Robishaw [32] as modified by Kohnken and Hildebrandt [33]. The method of Cook et al. [34] was used to fractionate

γ-subunits from purified heterotrimeric G-proteins. Briefly, γ-subunits were isolated by reversed-phase high-performance liquid chromatography using a 220 × 4.6 mm aquapore phenyl column (Brownlee) with a gradient of 95 to 60% solvent A over 10 min, followed by 60 to 54% solvent A over 65 min, followed by 54% solvent A to 100% solvent B over 5 min, operated at a flow rate of 1 mL/min. Solvent A is 10% acetonitrile, 0.1% trifluoroacetic acid (TFA) and solvent B is 75% acetonitrile, 25% isopropanol, 0.095% TFA. Fractions were collected at one minute intervals and speed-vacuum dried. Dried fractions were suspended in 5–10 μL of methanol/water/1-propanol/acetic acid (50/26/20/4, v/v/v/v).

Nanospray ionization was accomplished by loading a sample into a pulled borosilicate glass capillary that was pulled with a Sutter Instruments laser puller (Model P-2000) to a tip internal diameter of approximately 5 μm. Capillary action supplied 1–2 μL of sample to the tip for analysis. High voltage, 1–2 kV, was applied to the solution by insertion of a wire into the blunt end of the capillary. The capillary was placed on a custom nanospray manipulator made for the Finnigan LCQ Classic. Spray times varied between 5–30 min. One exception was for the product ion scan of the $[M + 5H]^{5+}$ ion of the γ_2 subunit which was acquired during an on-line liquid chromatography/MS/MS experiment under conditions developed for γ-subunit separation [34]

Mass spectra and MS^n spectra were acquired using a Finnigan LCQ Classic. Precursor ions were selected with a 2.5 Da window and fragmented with normalized collision energy of 45%. Default ion activation time (30 msec.) and q values (0.25) were employed. Typically 30–60 MS/MS scans were acquired and averaged to produce a product ion spectrum. Interpretation of product ion spectra was accomplished with the assistance of the Sherpa Lite 4.0 software [35].

3. Results

G-protein γ-subunits are readily ionized by both MALDI [30] and ESI-MS [29]. Fig. 1 shows the ESI mass spectra for isolated γ_2- and γ_7-subunits demonstrating efficient charging of proteins up to the 10+ charge state. The deconvoluted molecular weight spectra are shown in the inserts. The calculated molecular weights based on predicted amino acid sequence and known posttranslational modification are 7 750.1 and 7 409.7 Da for γ_2 and γ_7, respectively. Signals marked by asterisks are due to loss of the isoprenyl group (geranylgeranyl) from the intact protein most likely from collisions in the nozzle/skimmer region of the ion source and indicate the lability of the thioether linkage [36]. These mass spectra also indicate the purity of the isolated protein and the array of precursor ions to be interrogated by tandem mass spectrometry.

The $[M + 7H]^{7+}$ ion for the γ_7-subunit (*m/z* 1 059.6) was isolated and subjected to CID in the ion trap. Abundant fragmentation was observed in the product ion spectrum (Fig. 2) obtained under default CID conditions in the LCQ instrument. The general appearance of this product ion spectrum is typical of all γ-subunits studies to date and allows some rules to be developed regarding interpretation of such data. A prominent ion observed in all product ion spectra for G-protein γ-subunits is loss of the isoprenyl group. For G-proteins isolated from brain the isoprenyl group is a C_{20} group termed a geranylgeranyl (gg) group (MW 272 Da). Although this loss can occur from the selected $[M + nH]^{n+}$ molecular ion, typically this loss is accompanied by a loss of a proton and is observed as the $[M+(n - 1)H - gg]^{(n - 1)+}$ ion. The most abundant product ion in the spectrum corresponds to the b_{45}^{5+} ion due to cleavage at the labile Asp–Pro bond [29]. The complementary C-terminal y ions are also typically observed. In the case of the γ_7-subunit the y_{19}^{2+} ion is observed at 1 163.6 (predicted, 1 164.0). Since all but one of the γ-subunit sequences contain a C-terminal Asp–Pro bond, cleavage at this site is typically a dominant signal in product ion spectra. An additional series of y ions are observed in γ-subunit product ion spectra corresponding to cleavage N-terminal to proline residues. In the case of the γ_7-subunit, these products are observed as y_4 (*m/z* 761.6) and y_{13} (*m/z* 1 746.9 and 874.3). This information is enough to uniquely identify the γ-sub-

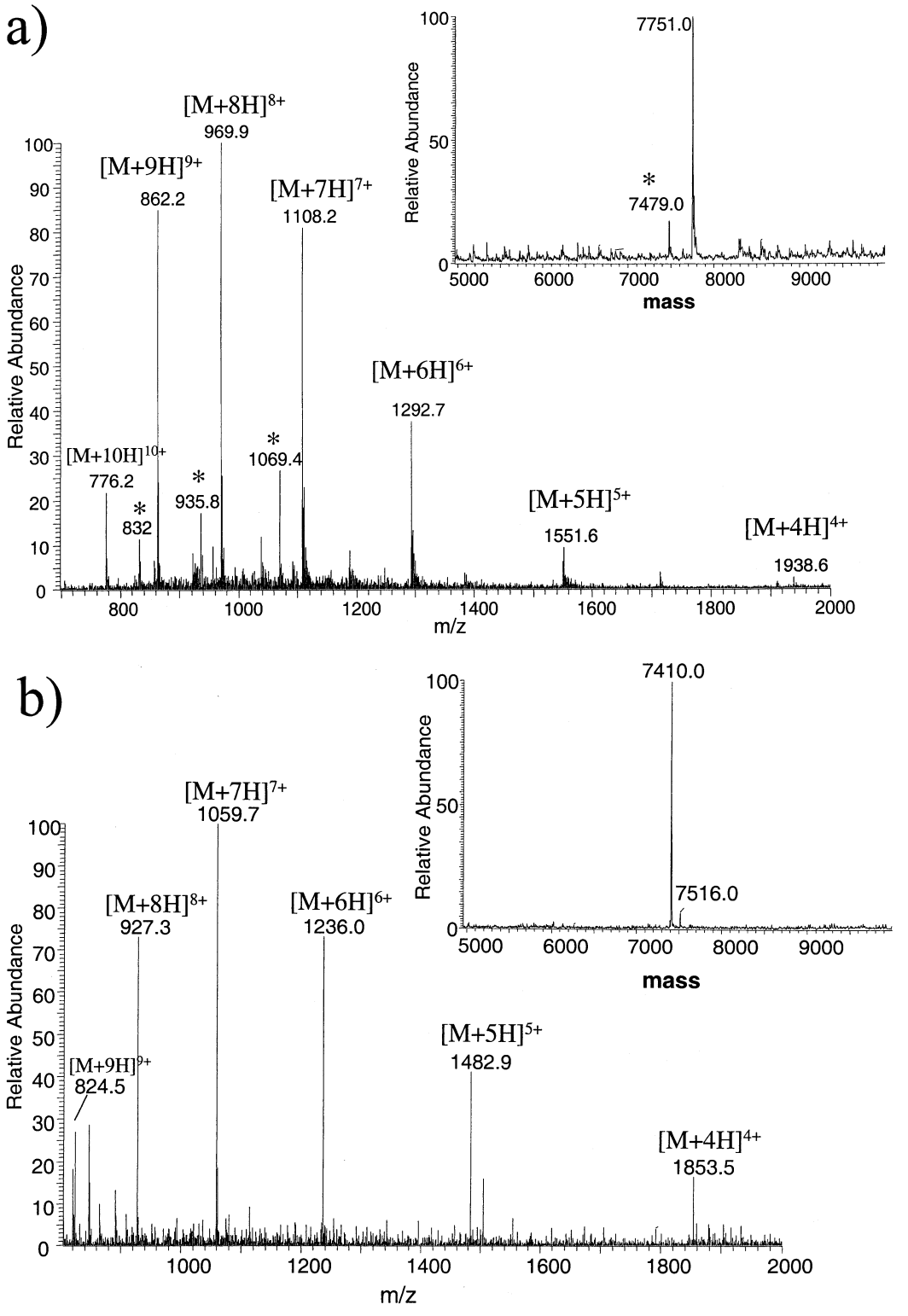

Fig. 1. ESI mass spectra of (a) γ_2-subunit and (b) γ_7-subunit acquired with a custom nanospray source on an LCQ ion trap instrument. Asterisks indicate loss of geranylgeranyl from intact γ-subunits. Inserts display deconvoluted spectra indicating calculated molecular weights.

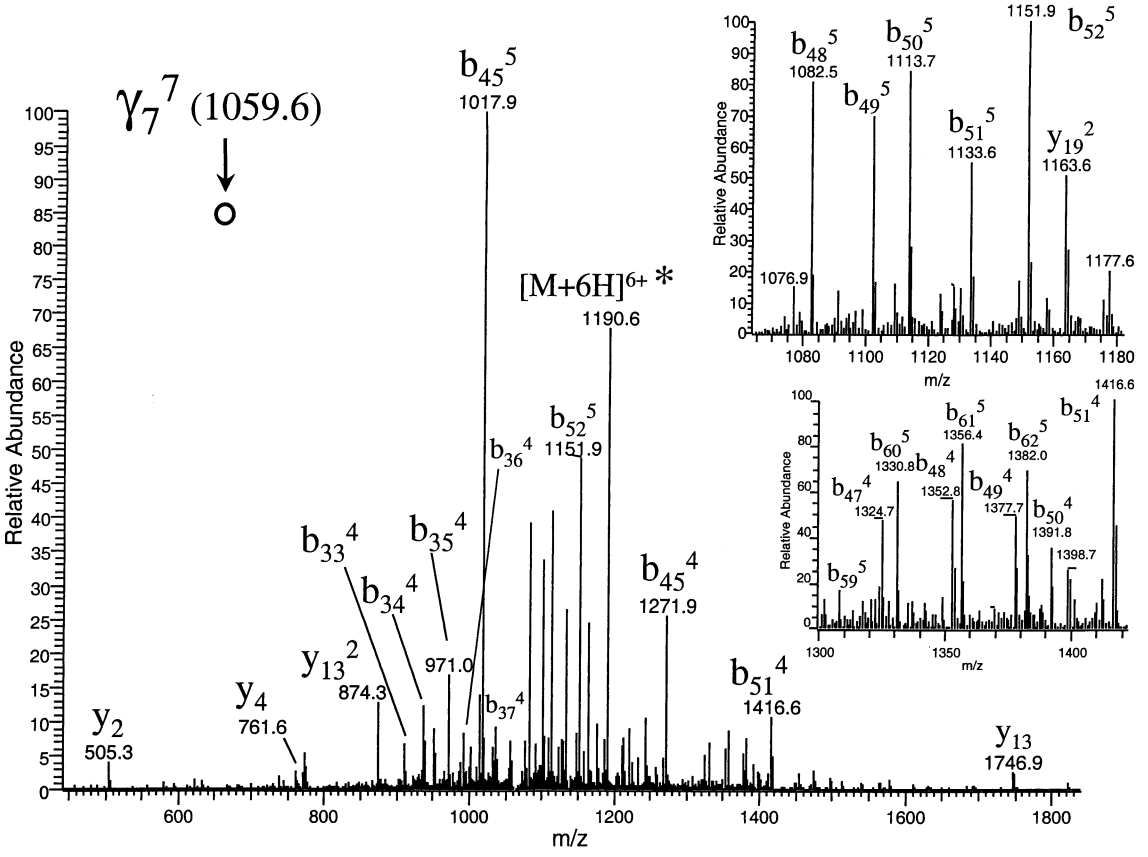

Fig. 2. Product ion spectrum of the $[M + 7H]^{7+}$ ion, *m/z* 1 059.6, from bovine γ_7. Superscripts indicate ion charge states and the asterisks indicate loss of the geranylgeranyl group. The arrow and the open circle under the selected *m/z* indicate a product ion scan to analyze all product (fragment) ions of the selected ion. Product ions are labeled according to the Biemann nomenclature [10].

unit isoform in a sample. Additional information is provided in the low abundance, but reproducible, ions representing series of b ions having two charge states, 4+ and 5+. The assignment of these ions can be challenging due to the fact that both mass and charge are unknown. Assignment is based on the predicted masses from the known sequence and the *m/z* difference between two adjacent ions in a series. That is, the *m/z* difference between two adjacent signals can be multiplied by a series of charge states until an amino acid residue mass is produced. Once a candidate charge state is calculated, this same charge state should be applied to adjacent signals and produce a series of amino acid residue masses that are consistent with the predicted protein sequence. If a question of assignment arises, an advantage of the ion trapping instrument is the ability to carry out MS^3 experiments to further interrogate the structure of product ions in an MS/MS experiment.

An MS^3 experiment was carried out to examine the structure of the predicted y_{13}^{2+} ion at *m/z* 874.3. The resulting second-generation product ion spectrum is shown in Fig. 3(a) and confirms the assignment. A series of singly charged y ions are observed as well as loss of the geranylgeranyl group. In addition, a series of ions is generated corresponding to ions containing the N-terminus of the y_{13} ion. These ions at *m/z* 986.3, 1 114.4, and 1 242.5 are formally assigned as internal ions from the intact γ_7-sequence and are labeled according to the Biemann nomenclature [10]. As

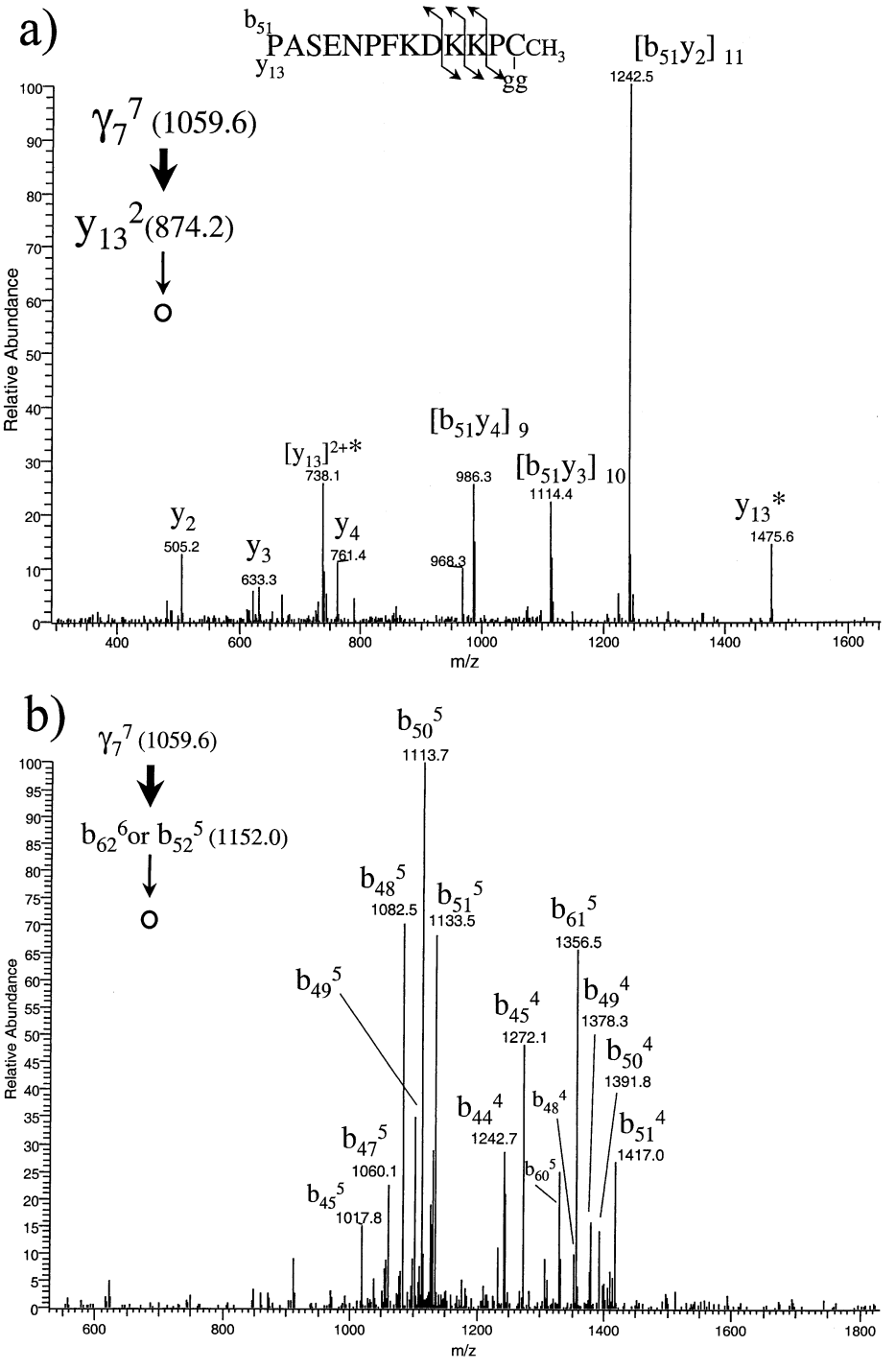

Fig. 3. Second generation product ion (MS^3) scans of (a) *m/z* 874.2, and (b) *m/z* 1 152.0, both originated from the $[M + 7H]^{7+}$ ion, *m/z* 1 059.6, of γ_7. The asterisk indicates loss of the geranylgeranyl group. The thick arrow indicates a fixed *m/z* difference (1 059.6 → 874.2) and (1059.6 → 1 152.0). The thin arrow and open circle indicate a second-generation product ion scan to analyze for all fragments of *m/z* 874.2 and 1 059.6, respectively. The sequence of the y_{13} ion is shown at the top of the figure.

observed in the product ion spectrum of the intact γ_7-subunit, cleavage N-terminal to a proline residue is favored to produce the dominant $[b_{51}y_2]_{11}$ ion as well as the y_2 ion.

A second MS^3 experiment was carried out on a highly charged b ion at *m/z* 1 152.0. This signal in the product ion spectrum could be assigned to either a b_{52}^{5+} ion (predicted *m/z*, 1 152.9) or a b_{62}^{6+} ion (predicted *m/z*, 1 151.8). Because no adjacent signals could be assigned to a 6+ series the assignment was based on the observed 5+ series in the product ion spectrum in Fig. 2 and was labeled as a b_{52}^{5+} ion. The MS^3 data in Fig. 3(b) suggest that at least some of the 1 152.0 signal is derived from a b_{62}^{6+} product ion as suggested by the presence of the b_{60}^{5+} and the b_{61}^{5+} signals. Because of the predominance of the 5+ series at 1 082.5, 1 113.7, and 1 133.5, it is likely that a significant portion of the *m/z* 1 152 signal arises from the b_{52}^{5+} product ion. Although these signals could also arise from the b_{62}^{6+} product ion, one would also expect to see the 6+ series between *m/z* 1 000 and 1 152 and these ions are present at very low abundance.

The effect of precursor ion charge state on resulting fragmentation was examined for the γ_2-subunit as shown in Fig. 4. The general rules established previously for γ-subunit fragmentation apply to each charge state and allow for interpretation. Interestingly, very similar product ions are generated from precursor ions of different charge states with the difference being the charge states of the product ions. The abundant loss of the geranylgeranyl group is observed in each spectrum with different charges depending on the precursor ion charge state. The ions in common between product ion spectra: y_{14} at *m/z* 994, y_{14}* at *m/z* 1 715, y_{16} at *m/z* 1 092, and y_{20} at *m/z* 1 304, represent cleavage at C-terminal proline residues. Each product ion spectrum, regardless of precursor ion charge state has a series of low abundance b ions. Having a series of product ions of different charge states can assist in assigning charge states as with simple ESI mass spectra. For example, a series of b ions from b_{28}–b_{47} (except $b_{40,41}$) are observed in the 3+ charge state originating from the $[M + 5H]^{5+}$ ion and a series of b ions from b_{35}–b_{51} (except $b_{41,44-46}$) are observed in the 4+ charge state originating from the $[M + 7H]^{7+}$ ion. Careful examination of each product ion spectrum reveals these b ion series that provide a significant amount of sequence coverage (summarized in Fig. 5). Unique product ions are observed in several spectra including a series of triply charged y ions (y_{33-36}) at *m/z* 1 362.0, 1 385.8, 1 410.9, and 1 433.8, respectively in Fig. 4(b), and triply charged y ions ($y_{21,24*}$) in Fig. 4(d).

An additional use of the MS^3 second-generation product ion scan in spectral interpretation is shown in Fig. 6 where a product ion from the $[M + 8H]^{8+}$ ion of the γ_2-subunit at *m/z* 1 069.5 which has lost the geranylgeranyl group was selected for further fragmentation. The second generation product ions which have changed mass compared to the first generation product ions in Fig. 4(d) represent y ions because only C-terminal y ions would be affected by the selected reaction, i.e. the loss of a geranylgeranyl group. Those ions which have not changed mass can be interpreted as N-terminal containing b ions. The only exception to this interpretation is those y ions in the product ion spectrum that have lost the isoprenyl group.

All of the observed fragmentation patterns are summarized on the sequences shown in Fig. 5. Note that a significant amount of sequence information is available even when only one charge state is examined as was the case for the γ_7-subunit in this study. Clearly more information is obtained when multiple charge states can be interrogated as demonstrated for the γ_2-subunit.

4. Discussion

The results described previously demonstrate that sequence interpretation and positive identification of G-protein γ-subunits can be accomplished by tandem mass spectrometry of intact proteins without the need for proteolytic digestion. Indeed, extensive structural information can be obtained including C-terminal sequence and posttranslational modification, based on the effective fragmentation of these proteins in the ion trap instrument. The presence and identity of the isoprenyl modification can readily be determined by its facile loss in product ion spectra, as well as in the

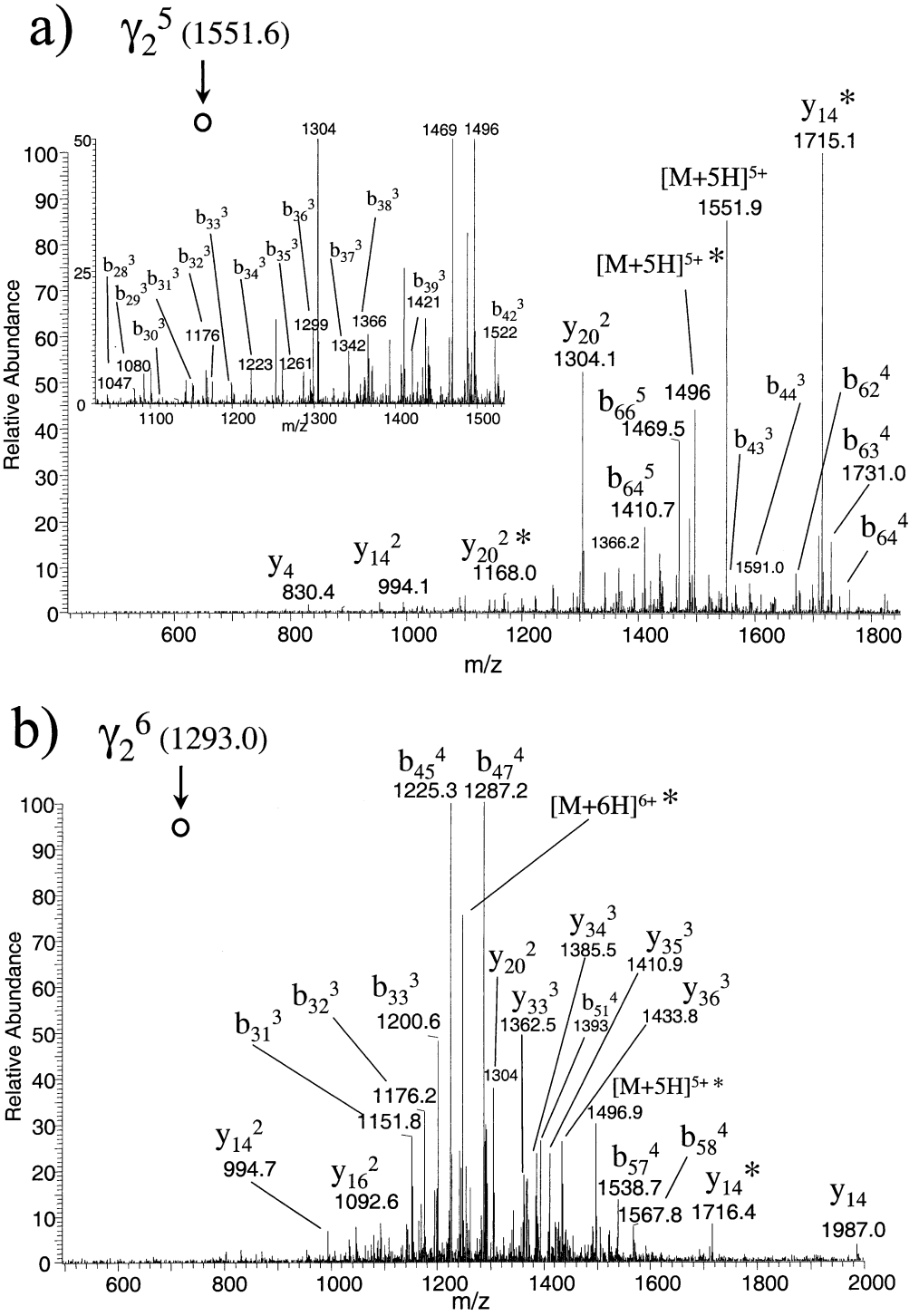

Fig. 4. Product ion spectra of (a) the $[M + 5H]^{5+}$ ion, *m/z* 1 551.6; (b) the $[M + 6H]^{6+}$ ion, *m/z* 1 293.0; (c) the $[M + 7H]^{7+}$ ion, *m/z* 1 108.0; and (d) the $[M + 8H]^{8+}$ ion, *m/z* 970.0 from γ_2. The asterisk indicates loss of the geranylgeranyl group. The insert displays a series of b_n^{3+} product ions formed from the $[M + 5H]^{5+}$ ion.

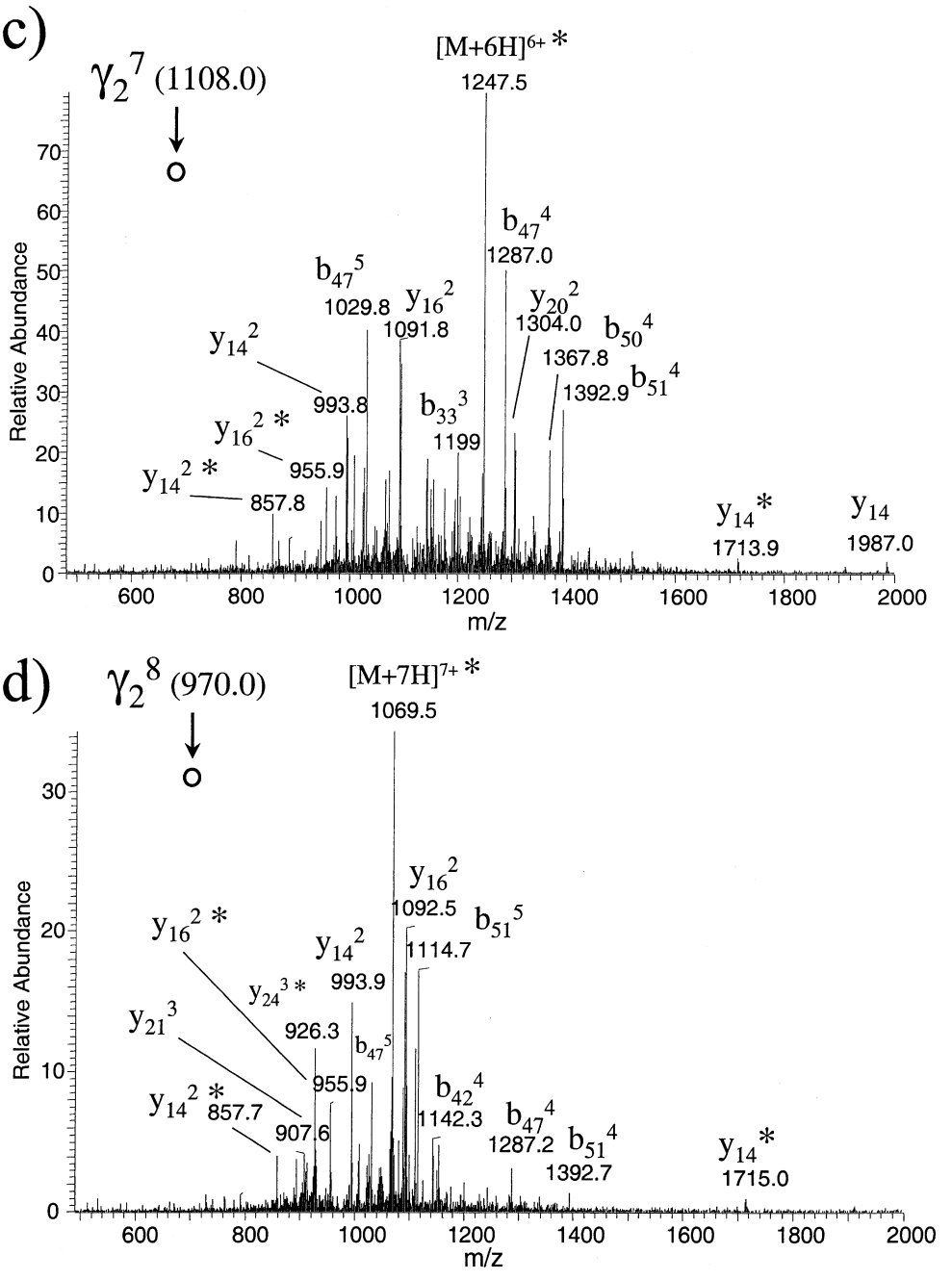

Fig. 4. (*continued*)

ESI mass spectra. This feature can be used to distinguish geranylgeranyl modification from farnesyl modification found in the retinal G-protein, transducin [37]. Furthermore, the facile loss of the isoprenyl group could be used, with appropriate instrumentation, in a neutral loss scan to identify all isoprenylated proteins in a mixture. However, due to the typical loss of a proton and therefore charge state difference

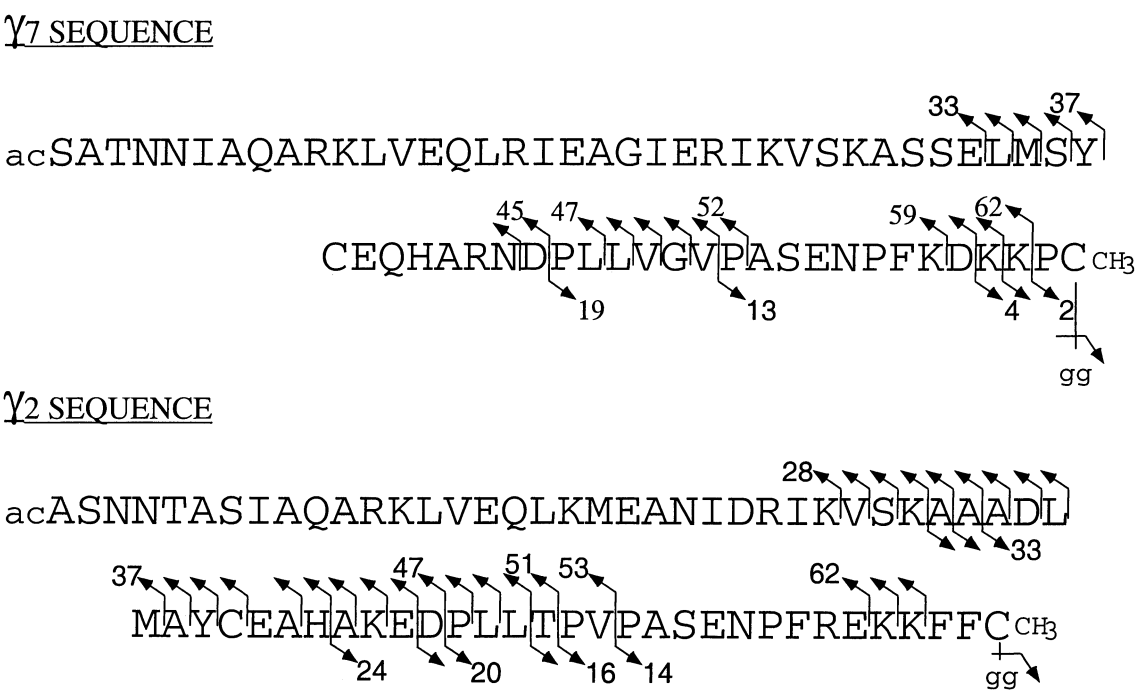

Fig. 5. Sequences of bovine γ_7- and γ_2-subunits indicating the fragments observed by tandem mass spectrometry.

between intact protein and product ion corresponding to loss of the isoprenyl group, a linear functional relationship scan [38] could be programmed and carried out to identify isoprenylated proteins.

Analysis of spectral fragmentation revealed several rules for interpreting product ion spectra of G-protein γ-subunits that are consistent with those presented for other intact protein analyses [23,26]. As mentioned previously, facile loss of the isoprenyl group is always observed, typically accompanied by loss of a proton. Facile cleavage at the N-terminus of proline residues, particularly at the C-terminus of the protein is observed. The resulting complementary product ions are observed as small y ions or large multiply charged b ions. Since all of the bovine γ-subunits have unique C-terminal sequences and therefore unique proline cleavage fragments, observation of these masses alone can be used to identify the parent γ-subunit isoform. Lastly, high mass, highly charged b ion series are observed from C-terminal cleavage. With these rules in mind, product ion spectra of G-protein γ-subunits have been interpreted to reveal alternative sequences [39] and differentially modified forms [29].

The data in Figs. 3 and 5 reveal the power of the MS^3 capability of the ion trapping instrument. This capability allows for more simple interpretation of complex product ion spectra and confirmation of product ion assignments.

5. Conclusion

Ions larger than previously reported mass limits can be effectively fragmented and sequenced by the CID experiment in an ion trap instrument. The specific examples in this work reveal patterns of fragmentation that can be applied to spectral interpretation of similar proteins. Furthermore, the general rules for fragmentation observed in this work are consistent with those derived from other larger protein fragmentation patterns and will serve as a guide for interpretation of product ion spectra generated from such

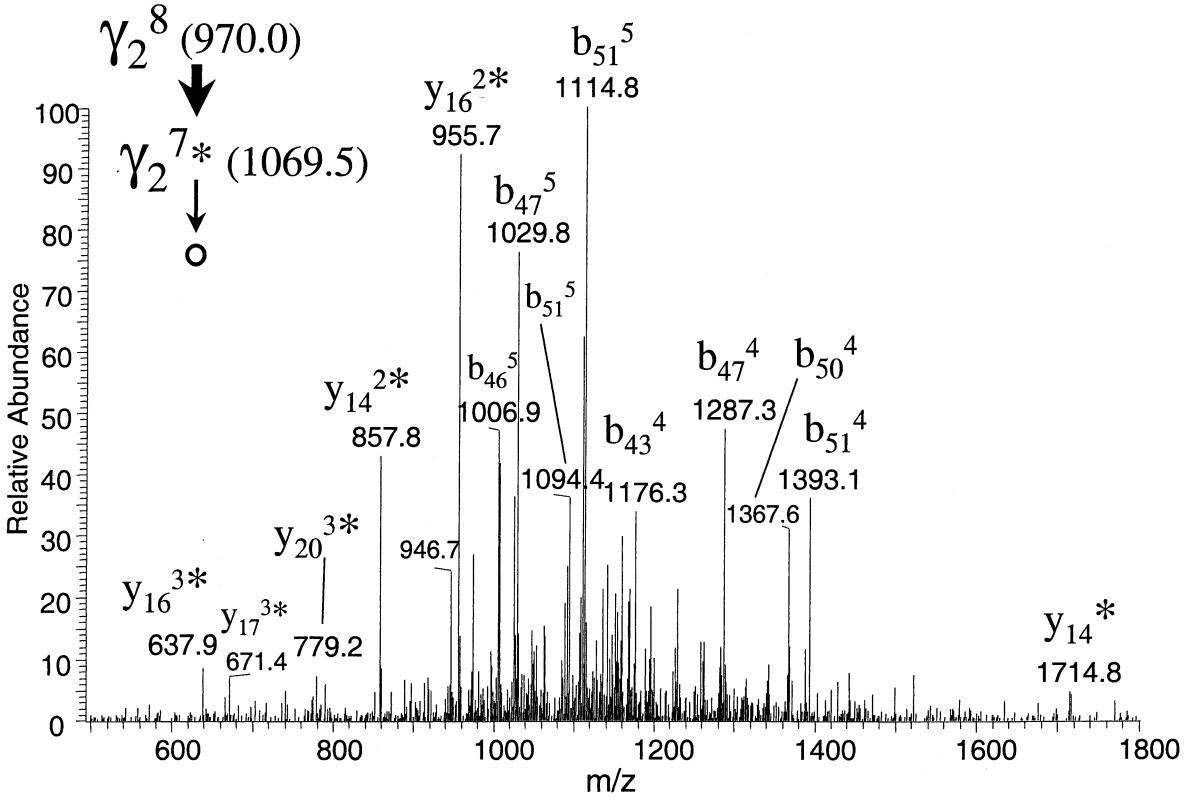

Fig. 6. Second generation product ion (MS^3) spectrum of the selected $[M + 7H]^{7+}$* fragment ion, *m/z* 1 069.5 originating from the $[M + 8H]^{8+}$ ion, *m/z* 970.0, from γ_2. The asterisk indicates loss of the geranylgeranyl group. The thick arrow indicates a fixed *m/z* difference (970.0 → 1 069.5). The thin arrow and open circle indicate a second-generation product ion scan to analyze for all fragments of *m/z* 1 069.5.

proteins. As the sensitivity of tandem mass spectrometers continues to increase, product ion sequencing of intact proteins could replace sequencing of proteolytic peptides and the necessary reconstruction of the intact sequence required by this approach [40].

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