

PROTEOMICS METABOLOMICS GENOMICS INFORMATICS
 GLY ILE VAL CYS GLU GLN ALA SER LEU ASP ARG
 CYS VAL LYS PRO LYS PHE TYR THR LEU HIS LYS

Noncovalently Associated Complexes: Apomyoglobin and Myoglobin Studies Using the HPLC-Chip/MS System Coupled with ESI-TOF Mass Spectrometry

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Abstract

Various techniques have been employed to analyze protein complexes governing biological processes. Many traditional methods used to study noncovalent protein interactions require large amounts of sample, are relatively slow, and can be nonspecific. Because of its sensitivity, speed, and selectivity, electrospray ionization/mass spectrometry (ESI/MS) has recently been utilized to study noncovalently associated complexes. In this paper, an Agilent 6210 Time-of-Flight mass spectrometer equipped with an HPLC-Chip MS interface and Infusion Chip was used to analyze the myoglobin noncovalently associated complex. The analysis was sensitive, completed in less than five minutes, and provided exact mass information that confirmed the identity of the complex. The ESI/MS approach allows for efficient, accurate analysis and thus makes this method a more effective and sensitive alternative to traditional methods for the study of protein complexes.



Introduction

Proteins generally do not act individually in biological processes; most cellular events are controlled by large complexes comprising multiple proteins. Signaling and regulation, protein biosynthesis, immune response, enzyme catalysis, and other similar mechanisms all involve non-covalent interactions between proteins and other molecules. Examples of macromolecular interactions include protein-protein, protein-ligand, and protein-DNA complexes.

Traditional methods used to study noncovalent protein interactions include ultracentrifugation, calorimetry, and various types of spectroscopy. Many of these methods require large amounts of sample, are relatively slow, and can be nonspecific.

Because of its sensitivity, speed, and selectivity, electrospray ionization/mass spectrometry (ESI/MS) has recently been utilized to study noncovalently associated complexes (1, 2). In general, picomoles or less of a protein complex are required for analysis by ESI/MS and analysis times using infusion for sample introduction are only a few minutes. The low-energy ESI process is well suited to study protein complexes because in many cases the energy associated with the ionic interactions between macromolecules is higher than the dissociation energy of the complex. However, care must be taken to ensure the protein complex does not denature in the solvents used for ESI. In addition, other instrument parameters such as temperature, voltage, and pressure must be controlled to avoid fragmentation of the complex.

High resolution, accurate mass, and high mass scanning are critical features for the identification of macromolecular assemblies, as well as the detection of dissociated complexes. Time-of-Flight (TOF) mass spectrometers possess all of these capabilities, with >12,000 resolution, <2 ppm mass accuracy, and highly efficient mass scanning. Therefore TOF mass analyzers have become the instruments of choice for studying noncovalent associations.

This application note explores the use of the Agilent Infusion Chip coupled to the Agilent 6210 TOF MS to analyze the noncovalently associated complex of myoglobin. The HPLC-Chip/TOF system demonstrates the mass accuracy and resolution necessary for the analysis of protein complexes. In addition, the design of the Infusion chip provides an extremely stable nanospray and significantly improves the ease of use and reliability of nanoflow ESI.

Experimental

Sample preparation

Horse heart myoglobin was obtained from Sigma-Aldrich, Inc. A stock solution of 8 mg/ml of horse heart myoglobin was made in 100 mM ammonium acetate, pH 6.8. A 1:100 dilution of the stock solution was made in 50% acetonitrile (ACN), 0.1% formic acid; these conditions denatured the myoglobin and disrupted any solution-phase, noncovalent interactions. A similar dilution was made in 25 mM ammonium acetate, pH 6.8; these conditions maintained any solution-phase, noncovalent interactions. The final protein concentration for the samples was ~4 picomoles/ μ l.

Analysis

All experiments were performed using an Agilent 6210 TOF LC/MS system equipped with an Agilent HPLC-Chip MS interface (Figure 1), microwell plate sampler, nanoflow pump, and capillary pump. An Agilent Infusion Chip (Product No. G4240-62002) was used for sample introduction. The Infusion chip is a microfluidic device that shares many common features with the Protein ID Chip: a grounded polyimide emitter with a dual electrode and orthogonal orientation (3). These features of the Infusion chip provide an extremely stable nanospray. Because of its plug-and-play design, the Infusion chip significantly improves the ease of use and reliability of nanoflow ESI. The chip can be connected to either a microwell plate autosampler (flow injection) or an external syringe pump (infusion). For these experiments each sample was infused into the TOF at 600 nL/min using a syringe pump.

TOF MS conditions

Ionization mode: positive ESI

ESI voltage: 1650 V

Drying gas flow: 4.0 L/min

Drying gas temperature: 350°C

Fragmentor voltage: 275–300 V

Mass range: 400–3200 Daltons

Scan speed: 1 scan/second (averaging 10,000 transients per scan)

Scan time: each sample was scanned for ~2.5 minutes

Internal reference ion mass calibration: On

Results and Discussion

For this study, the protein complex of horse heart myoglobin was analyzed using the Agilent HPLC-Chip MS system interfaced to an Agilent 6210 TOF MS. Myoglobin is the resident oxygen-carrying molecule of cardiac and skeletal muscle. The myoglobin complex consists of the apomyoglobin protein and Heme b. The capacity of myoglobin to bind oxygen depends on the presence of Heme b, a nonpolypeptide prosthetic group consisting of one Fe atom bound to the center of a heterocyclic organic ring (empirical formula $C_{34}H_{32}N_4O_4Fe$; $[M+H]^+ = 616.1767$). Heme is involved in the reversible binding of oxygen in myoglobin; the Fe atom at the center of the Heme group can bind one molecule of oxygen.

First, the capacity of the HPLC-Chip/TOF system was assessed for its ability to detect and identify apomyoglobin and Heme b.



Figure 1. The Agilent 6210 TOF LC/MS system equipped with an Agilent HPLC-Chip MS interface. The interface allows easy changeover from HPLC to infusion mode by simply switching Agilent chips.

The mass spectrum of horse heart myoglobin in 50% ACN (solution conditions that cause denaturation and disrupt noncovalent interactions) is shown in Figure 2. The spectrum showed a multiply charged ion envelope for apomyoglobin. The charge state distribution in this spectrum was typical for a denatured protein; it had a large number of sites than could be protonated and therefore contained a large number of highly charged ion species. The maximum entropy deconvolution of the spectrum identified the average molecular weight of the protein as 16,951.46 Daltons with an error of -1.18 ppm

(Figure 3). An additional ion at $m/z = 616.1768$ was also observed in the spectrum. Further analysis using the TOF data editor associated the $m/z = 616.1768$ ion with the empirical formula of Heme b ($C_{34}H_{32}N_4O_4Fe$), with a mass accuracy of 0.0866 ppm (Figure 4). These data strongly support that there was no association of Heme b with the apomyoglobin protein.

Next, intact myoglobin was characterized using the HPLC-Chip/TOF system. The spectrum of myoglobin in 25 mM ammonium acetate (solution conditions that do not denature

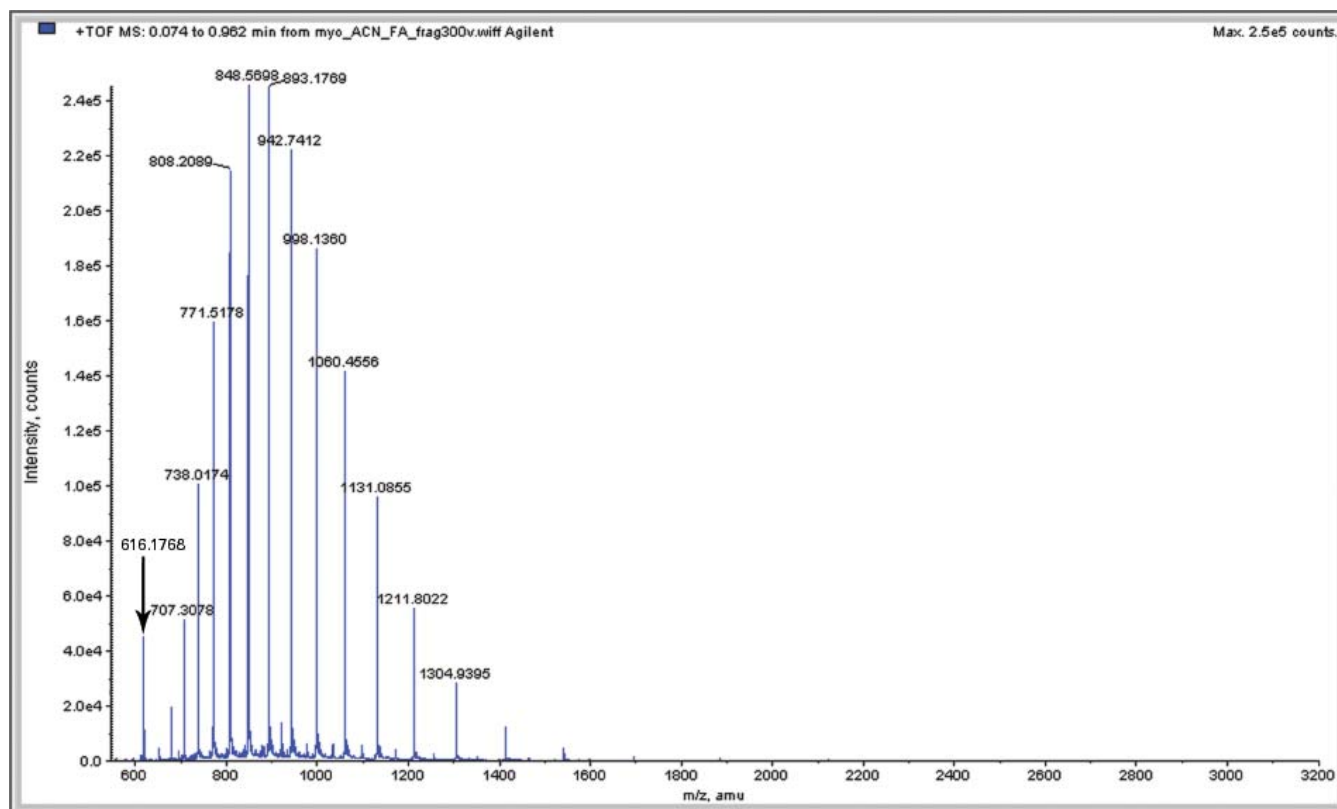


Figure 2. ESI TOF mass spectrum of horse heart myoglobin in 50% ACN and 0.1% formic acid infused at 600 nL/min via an Infusion Chip. The fragmentor setting was 300 V. Arrow denotes the ion corresponding to Heme b.

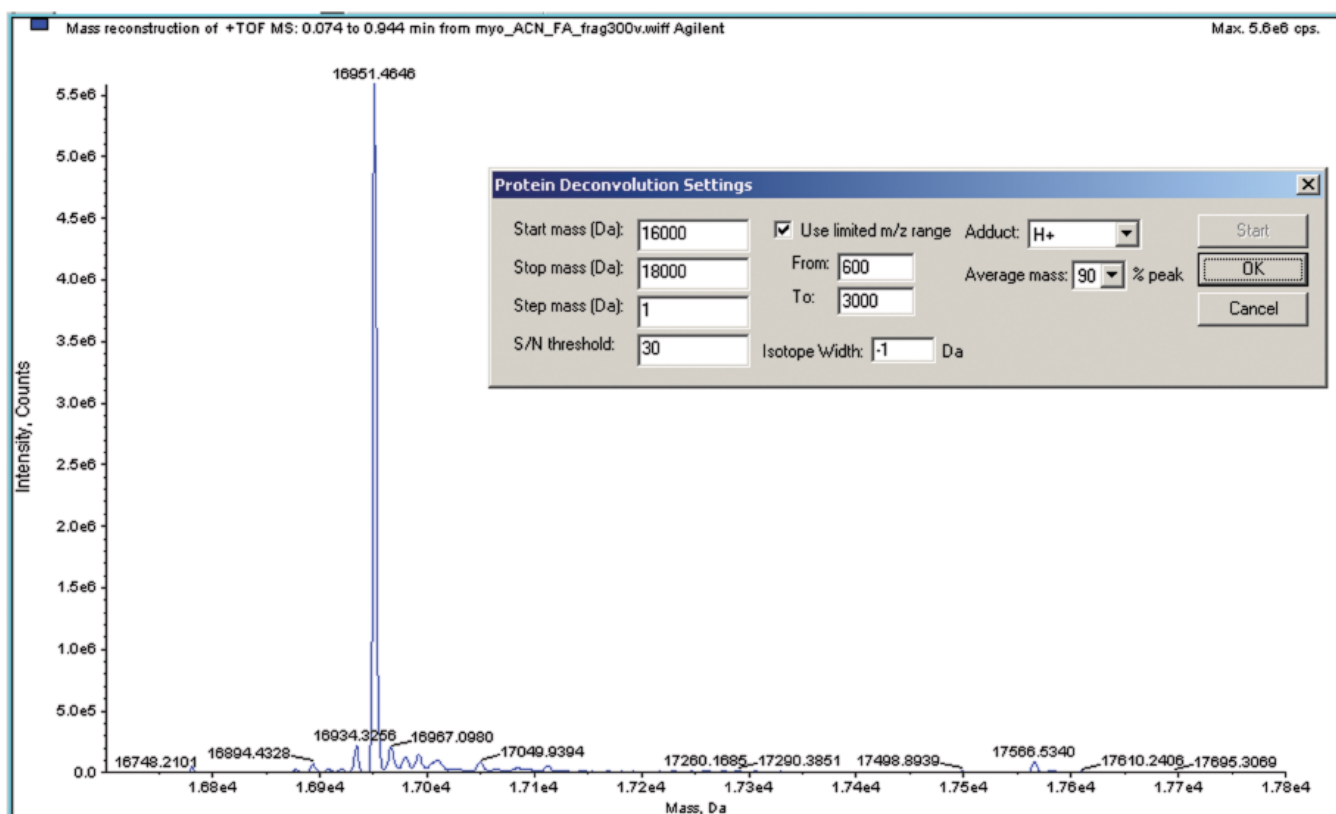


Figure 3. Maximum entropy deconvolution of apomyoglobin. Deconvolution settings are shown. The calculated average mass of apomyoglobin was 16,951.48 Da. The mass error was -1.18 ppm.

Calculators

Elemental Composition | Hypermass | Elemental Targeting | Mass Property | Isotopic Distribution

Input parameters:

Target m/z: 616.1768 amu

Tolerance: 3 ppm

Result type: Elemental

Max num of results: 100

Min DBE: -0.5 Max DBE: 25

Electron state: OddAndEven

Num of charges: +1

☐ Add water ☐ Add proton

Set elements and limits

Calculate Show isotopic Export to file Help

	Formula ...	Calculated m/z (amu)	mDa Error	ppm Error	DBE
1	C ₃₄ H ₃₂ N ₄ O ₄ Fe	616.1767	0.0534	0.0866	21
2	C ₁₂ H ₃₀ N ₁₁ O ₁₈	616.1764	0.3197	0.5189	3.5
3	C ₁₁ H ₂₄ N ₁₈ O ₁₃	616.1764	0.3251	0.5276	9
4	C ₁₀ H ₁₈ N ₂₅ O ₈	616.1764	0.3305	0.5364	14.5
5	C ₉ H ₁₂ N ₃₂ O ₃	616.1764	0.3359	0.5451	20
6	C ₃ H ₂₈ N ₂₄ O ₁₀ Fe	616.1764	0.3855	0.6257	2
7	C ₂ H ₂₂ N ₃₁ O ₅ Fe	616.1764	0.3909	0.6344	7.5
8	C ₁₆ H ₁₆ N ₃₈ Fe	616.1764	0.3963	0.6432	13
9	C ₁₈ H ₂₂ N ₂₃ Fe	616.1772	-0.4437	-0.7202	19.5
10	C ₁₉ H ₂₈ N ₁₆ O ₅ Fe	616.1772	-0.4491	-0.7289	14
11	C ₂₀ H ₃₄ N ₉ O ₁₀ Fe	616.1772	-0.4545	-0.7376	8.5
12	C ₂₁ H ₄₀ N ₂ O ₁₅ Fe	616.1772	-0.4599	-0.7464	3
13	C ₂₇ H ₂₄ N ₁₀ O ₈	616.1773	-0.5095	-0.827	21
14	C ₂₈ H ₃₀ N ₃ O ₁₃	616.1773	-0.5149	-0.8357	15.5
15	C ₂₆ H ₂₈ N ₆ O ₁₂	616.1759	0.8277	1.3433	16
16	C ₂₅ H ₂₂ N ₁₃ O ₇	616.1759	0.8331	1.352	21.5
17	C ₁₉ H ₃₈ N ₅ O ₁₄ Fe	616.1759	0.8827	1.4326	3.5
18	C ₁₈ H ₃₂ N ₁₂ O ₉ Fe	616.1759	0.8881	1.4413	9

Figure 4. Data editor display of empirical formulas at target m/z 616.1768. Heme b (C₃₄H₃₂N₄O₄Fe) was identified with a mass error of 0.0866 ppm.

the protein and favor noncovalent interactions) is shown in Figure 5. The protein charge envelope shifted dramatically to one containing lower charge states (i.e., high mass range). This shift reflected the conformation differences between the native conformation and denatured protein. The native conformation restricted protonation of internal sites in the molecule and produced a lower charge distribution (i.e., higher mass) compared to the “open structure” of the denatured

molecule. Figure 6 shows the maximum entropy deconvolution of the myoglobin noncovalent complex spectrum and the calculated average mass of 17,566.82 Da, with an error of -7.40 ppm. The calculated mass corresponded closely to the sum of the calculated masses of apomyoglobin and Heme b. Further evidence for a noncovalent interaction was indicated by the absence of the Heme b ion at m/z 616.1767.

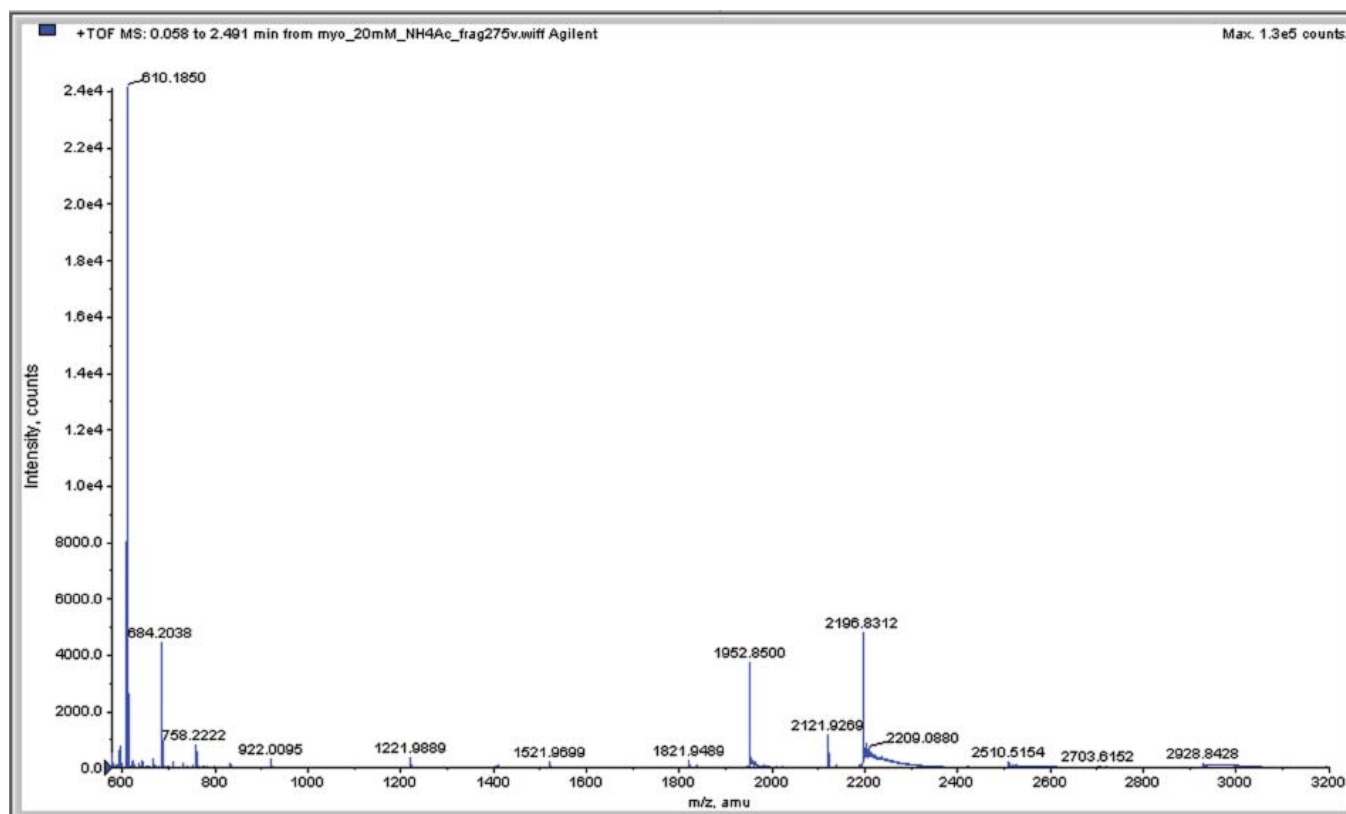


Figure 5. ESI TOF mass spectrum of horse heart myoglobin in 25 mM ammonium acetate, pH 6.8, infused at 600 nL/min via an Infusion Chip. The fragmentor setting was 275 V. The native conformation of myoglobin restricted protonation of internal sites in the molecule and produced a lower charge distribution (i.e., high mass range). The m/z 616.1767 ion corresponding to Heme b is absent.

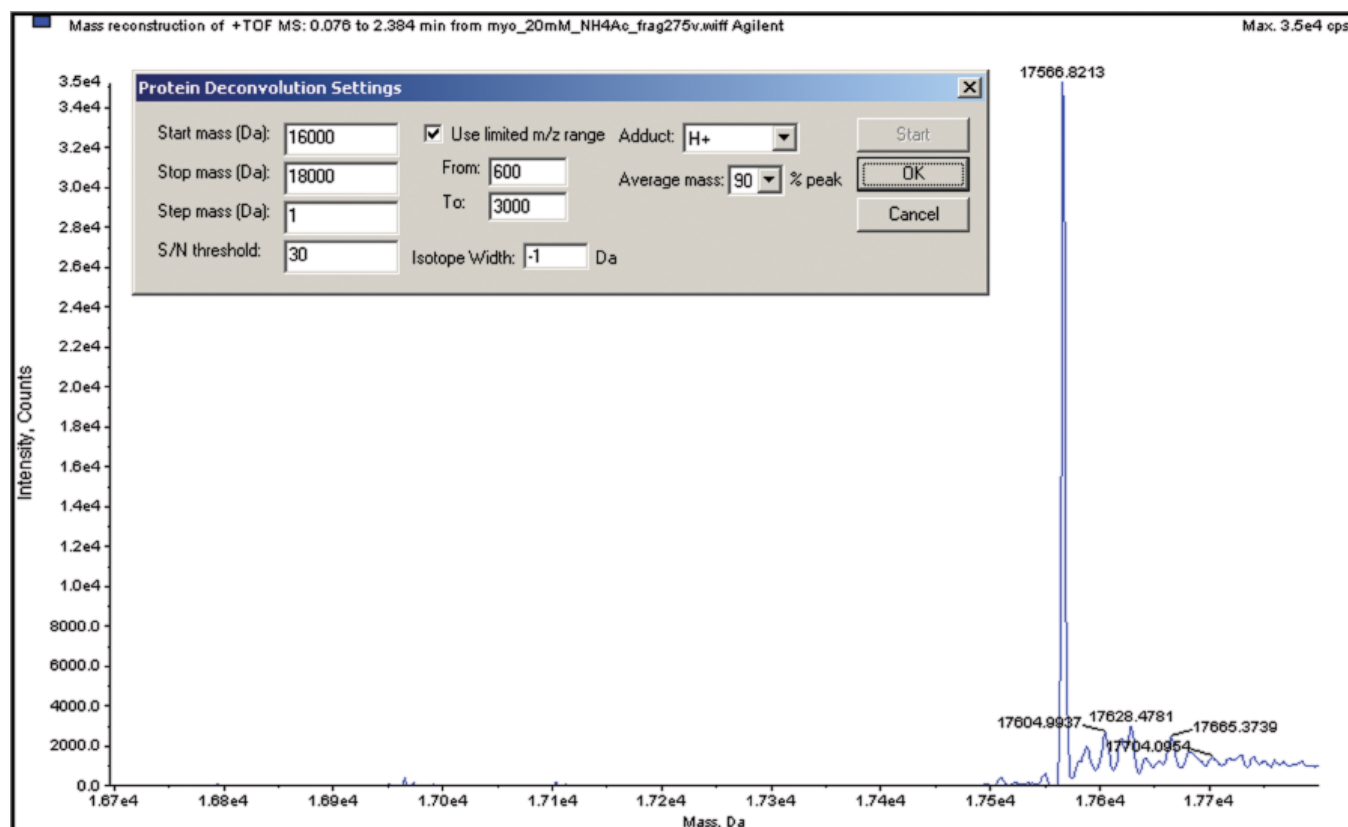


Figure 6. Maximum entropy deconvolution of myoglobin. Deconvolution settings are shown. The calculated average mass of myoglobin was 17,566.95 Da. The mass error was -7.40 ppm.

Conclusions

Effective analysis of noncovalent protein interactions requires sensitivity, speed, and selectivity. In this study, the Agilent 6210 TOF/MS coupled with the Infusion Chip was used to analyze noncovalently associated protein complexes. The analysis was sensitive, completed in less than five minutes, and provided exact mass information that confirmed the identity of the complex. The results of this study demonstrate that an ESI/MS approach is a powerful method for characterizing noncovalently associated protein complexes. The ESI/MS approach allows for efficient, accurate analysis and thus makes this method a more effective and sensitive alternative to traditional methods for the study of protein complexes.

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