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A TOF mass spectrometer for the study of noncovalent complexes

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ABSTRACT

A time-of-flight (TOF) mass spectrometer intended particularly for the study of noncovalent complexes and large proteins is described. In this device, a heated capillary couples an electrospray or nanospray source to an RF-only quadrupole ion guide. The ion guide provides collisional cooling, and injects the ion beam into a TOF mass spectrometer. 16 kV TOF acceleration potential improves transmission and detection efficiency, and also increases the m/z range for a given extraction frequency. A two-stage reflector, together with collisional cooling in the ion guide, provides resolving power up to 18,000 (FWHM) for small molecules, and somewhat improved resolution for larger ones, although the widths of the noncovalent peaks are mainly determined by their intrinsic spreads. Examples of m/z spectra obtained with this instrument in our laboratory are presented.

1. Introduction

The active forms of many proteins and enzymes are noncovalent complexes. They are involved in such processes as enzyme–substrate interaction, receptor–ligand binding, formation of oligomeric proteins, and the formation of cellular structures. Noncovalent interactions between biomolecules thus play a central role in molecular recognition processes, and in the formation of molecular quaternary structure.

The gentle nature of electrospray ionization has made it possible to study such interactions by mass spectrometry [1–9]. However, the earliest measurements on noncovalent complexes were carried out on quadrupole mass spectrometers [10,11]. Since many protonation sites in large noncovalent complexes are shielded, the corresponding ions often have large values of m/z, so the limited m/z range of quadrupole instruments (usually 4000 or less for most commercial instruments), restricted these measurements to relatively small complexes. Thus it was not usually possible by this technique to observe complexes of high m/z, or even to know that they were present.

For this reason, time-of-flight (TOF) mass spectrometers are a natural choice for noncovalent measurements, since they have a theoretically unlimited m/z range, apart from detection difficulties. The first such measurements were carried out in our laboratory [12,13]. However, these and some subsequent measurements were made on a TOF mass spectrometer with only ~4 kV accelerating voltage [12]. That limited both transmission and detection sensitivity.

In order to overcome these problems, we have built a new ESI-TOF mass spectrometer intended specifically for measurements on large proteins and noncovalent complexes. This effort was aided significantly by the development of a MALDI-TOF instrument at MDS-Sciex [14] (constructed specifically for MALDI applications, rather than electrospray) and many parts from the TOF section of that prototype were used in this one, but the necessary electrospray and nanospray sources were constructed in our own laboratory.

2. Experimental method

2.1. The TOF mass spectrometer

The instrument, shown in Fig. 1, is similar in principle to the one constructed in our laboratory previously [12], but with higher accelerating voltage in the TOF section, as well as significant improvements adopted from the newer Sciex design [14], including a long sleeved quadrupole for better ion transmission, and a two-stage mirror for improved resolution [15,16]. The two-stage

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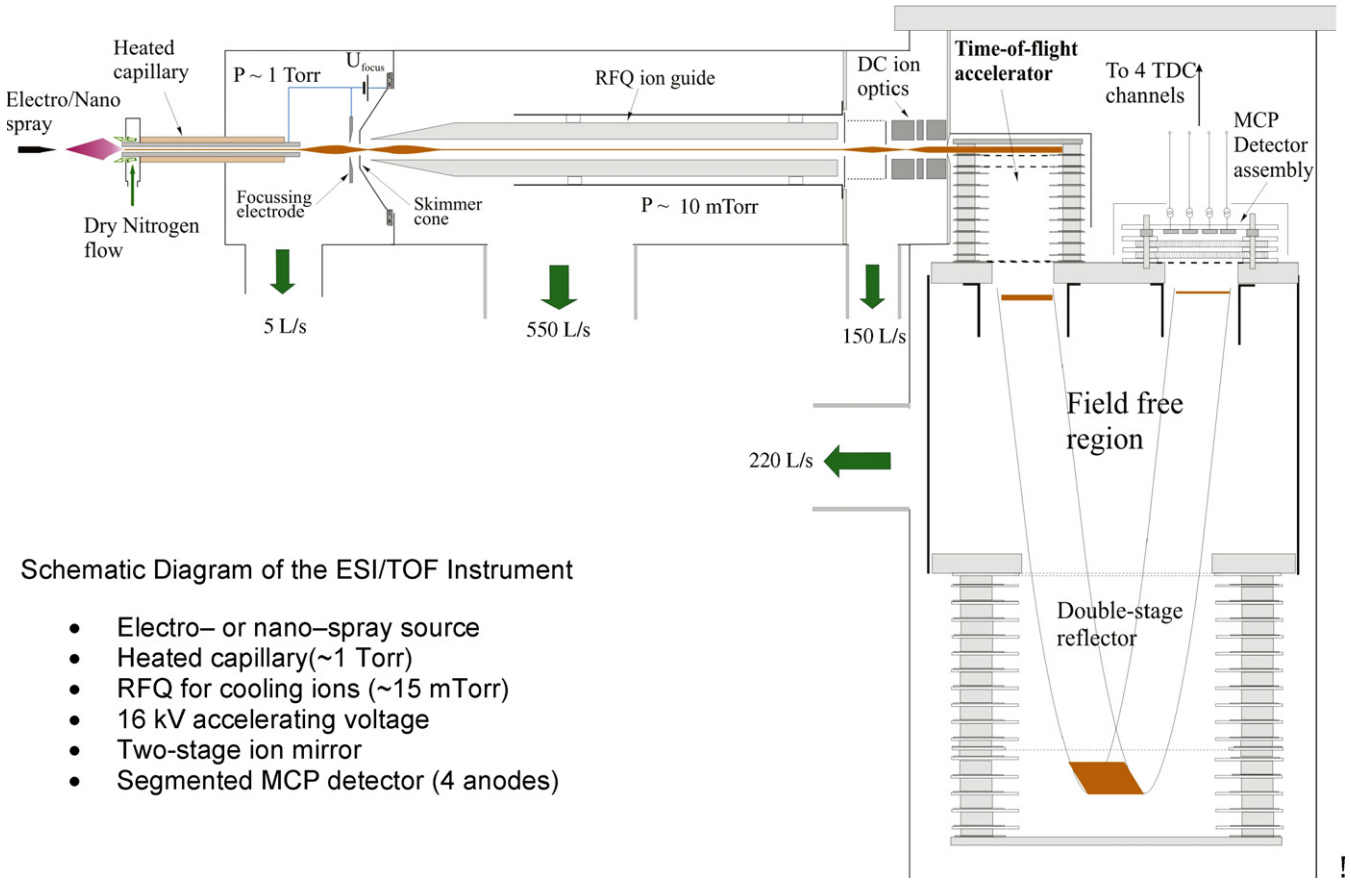

Fig. 1. Schematic diagram of the instrument.

mirror does not improve the resolution much for large noncovalent complexes themselves, since their widths are largely determined by their intrinsic spreads, but it does give considerable improvement for smaller molecules, and makes calibration (typically by Substance P, $m/z \sim 1348$ Da) easier.

A notable difference from the Sciex design, of course, is the replacement of the MALDI source assembly by an electrospray or nanospray source, built in our laboratory. These ion sources operate with standard Proxeon or New Objective emitters. The emitters inject ions into a heated capillary (0.4 mm ID × 11 cm long), which provides efficient desolvation of the charged clusters and droplets formed in electrospray. This is followed by a long RF-only quadrupole, which in this case acts simply as an ion guide to couple the ion beam to the TOF analyzer, and to provide collisional cooling of the ion beam [17,18]. We have taken advantage of a detailed discussion of the effects of gas pressure in such a device [18] to optimize its performance over a wide m/z range (up to m/z ~40,000 at 3.5 kHz in our experiments, but up to 70,000 at Sciex [14]). It should be noted, however, that this quadrupole is not designed to permit selection of a given parent ion mass (unlike some other recent instruments [4–8]) so it does not provide MS/MS measurements. For this reason, we have described here a number of applications in order to illustrate the capabilities of such an instrument, *without* parent ion selection.

The TOF analyzer itself has an acceleration voltage of 16 kV, somewhat lower than the 20 kV in the Sciex MALDI instrument [14], but much larger than the 4 kV in our original instrument [12], or even the 9.6 kV in a more recent design [4], assisting both transmission and detection of the ions. A double stage reflector [15,16] provides resolving power up to ~18,000 FWHM for small molecules, thus yielding considerable improvement in line width for them, and making calibration more convenient. It also provides somewhat improved resolution for larger molecules, although the resolution for noncovalent complexes is mainly determined by their intrinsic widths.

2.2. Materials and sample preparation

Substance P and bovine serum albumin (BSA) were purchased from Sigma–Aldrich Canada, Oakville, ON. All other proteins were prepared at the University of Manitoba and are described in detail in the section on practical applications. Pure proteins were buffer-exchanged into ammonium acetate (99.999%, Sigma–Aldrich Canada, Oakville, ON) or ammonium bicarbonate (Fisher Scientific, Ottawa, ON), as described elsewhere [19]. To determine exact masses, proteins were denatured in 1–2% acetic acid, 50% methanol to about 5–10 μM and analyzed by electrospray ionization. The solution was pumped from a 26 gauge needle connected by teflon tubing to a 25 μL Hamilton syringe pumped at 0.4 μL/min by a "Genie" pump (Kent Scientific Corp, Torrington, CT). Noncovalent complexes were diluted to 1 μM and analyzed by electrospray or nanospray ionization, using the concentration of buffer required to keep the protein in solution. For nanospray, 2–3 μL of sample were loaded into a New Objective PicoTip™ (New Objective Inc., Woburn, MA) held in a custom-made holder designed to fit into the electrospray bracket. Back pressure was applied with a hand-pump to about 5 psi. More details of the spray conditions can be found in the figure captions.

3. Results

3.1. Instrument design

As remarked above, the quadrupole and time-of-flight sections of the new instrument are based on Sciex prototype parts. A schematic representation of the instrument is shown in Fig. 1.

The mass spectrometer is composed of four main regions: the interface region, pumped down to about 1 Torr by an Edwards E2M30 rotary vane vacuum pump, the quadrupole region, pumped down to about 10 mTorr by a Varian 551 turbomolecular pump (550 L/s), the ion optics region, kept at a pressure of about 10^{-5} Torr, and the time-of-flight region, pumped down to $\sim 10^{-7}$ Torr. In the last two regions the vacuum is achieved by a dual inlet Leybold TW 220/150 turbomolecular pump. Both turbomolecular pumps are backed by a Varian DS 602 rotary vane vacuum pump. Pressures are maintained by a balance between the size of the orifice between the regions and the pumping speeds.

3.2. The heated capillary interface region

The ions are formed by the spraying of an appropriate solution from the tip of an electrospray or nanospray needle, maintained at high voltage (kV range) with respect to a heated stainless steel capillary [20]. This capillary (0.4 mm ID, 110 mm long, $T \sim 100\,^{\circ}C$) appears to be particularly suitable for analysis of large noncovalent protein complexes and high molecular weight proteins, since it aids the efficient desolvation of the charged clusters and droplets formed in electrospray or nanospray ionization. To further aid the desolvation process and to help keep the capillary clean, we introduce a counter-flow of pure dry nitrogen gas, or in some cases, SF_6 (see below).

After leaving the capillary, the ions expand as a supersonic jet in a region kept at a pressure of about 1.3 Torr. A focusing electrode, with an electrical connection to the capillary, is installed 5 mm downstream at the entrance to the quadrupole region. An important parameter is the so-called declustering voltage; i.e., the voltage between the capillary and the skimmer, which may be set to a value up to ~300 V for nitrogen counter-flow. This voltage has a considerable influence on the spectrum because it can increase desolvation, although it also induces fragmentation. Thus the value of the declustering voltage is chosen as a compromise between the need for desolvation and the amount of fragmentation desired. Such ability to control ion fragmentation in the capillary–skimmer interval provides a valuable tool for investigation of the complex stoichiometry, and the removal of the need to allow for MS/MS measurements makes the compromise easier to achieve [21,22].

The optional use of SF_6 has several advantages. As Bagal et al. point out [23], it helps to stabilize noncovalent complexes. It is also considerably more efficient in removing adducts and dissociating complexes because of its larger mass; a typical dissociation pattern at 250 V declustering voltage resembles the pattern at 300 V with nitrogen. Finally, it permits the use of larger declustering voltages than nitrogen (up to 400 V compared with <300 V), because of its superior insulating properties [24].

3.3. The quadrupole region

In our case, the quadrupole simply acts as an ion guide to deliver the ions that enter the section to the ion optics in the TOF section, and to improve the properties of the ion beam by collisional focusing. The impact of parameters such as pressure and length in the performance of a focusing quadrupole over a broad range of m/z has been discussed by Chernushevich and Thomson [18], who note that ions of high m/z in their native conformation possess longer stopping distances than denatured ions, so longer quadrupole rods are required to achieve full focusing at a given pressure. One solution to this problem, which we have adopted, is to increase the pressure to fit the ion path within the existing quadrupole length [18]. Such a pressure increase favors the transmission of ions of high m/z, while somewhat reducing the transmission of low m/z ions. Also we have imposed the additional requirement of a low working pressure (1×10^{-7} Torr) in the TOF section.

The design of the quadrupole used in our instrument takes into account all of these factors and constitutes a compromise solution. Its design is somewhat simplified by the absence of mass-selection, which produces additional dispersion [21,22], as well as having to operate the quadrupole at low frequency in order to select ions of high m/z [4,5,7]. Our resulting quadrupole has a length of 23 cm and operates at a pressure around 10 mTorr [14,18], which is not usually changed, although it is possible to adjust it to observe very large assemblies. The insertion of a sleeve around the quadrupole [18] allows for a range of pressures in the ion guide area, as well as the preservation of a good vacuum in the region outside.

The quadrupole is operated with an applied voltage of 1.7 MHz frequency and 1000 V amplitude. This sinusoidal signal is generated by a DS 335 function generator (Stanford Research Systems, Sunnyvale, CA) that serves as input for an ENI 240 L broad-band RF amplifier (MKS ENI Products, Rochester, NY). The amplified output is delivered to a coil assembly for the generation of two signals with opposite phases that are applied to the quadrupole rods.

3.4. Ion optics and TOF sections

After leaving the quadrupole, the ions enter the ion optics region, an intermediate stage of differential pumping, in which the ion beam is optimized in terms of shape and energy before it is injected into the extraction section of the TOF region.

Once in the extraction section, the ion beam is accelerated by an electric field produced by simultaneous positive and negative square pulses of 1.6 kV amplitude that are applied to the "Push" (upper electrode) and "Pull" (lower electrode). In this way they are extracted into the next part of the TOF region, the acceleration section.

Compared to our earlier instrument with 4 kV acceleration [12], the 16 kV TOF acceleration potential improves transmission and detection efficiency of heavy species, and also increases the overall m/z range for a given extraction frequency. For example, an extraction rate of 3.5 kHz in this design allows one to acquire ions with m/z values up to 40,000. The double-stage reflector design and collisional cooling of the ions in the ion guide provide high resolving power for the calibrating ions (up to ~18,000 FWHM or better for Substance P).

The detector contains a matched pair of microchannel plates (Photonis, Bordeaux, France) in a chevron configuration. Four anodes collect the electron current after amplification by the microchannel plates. The signals from the anodes are directed to four independent ion-counting channels, and a multichannel time-to-digital converter (TDC × 8; Ionwerks, Houston, TX) records the arrival time of each ion. This use of independent ion-counting channels improves the dynamic range of the ion-counting system by a factor of nearly 4, since the counting rate in each channel is reduced correspondingly.

The acceleration and mirror DC voltages, as well as the DC voltages needed for extraction pulse formation are provided by computer controlled high voltage power supplies (Spellmann High Voltage, Hauppage, NY). The DC voltages in the skimmer, quadrupole and ion optics are generated by a standard Sciex power supply module, controlled from a computer through digital analog converter modules. All the software for power supply and pulse

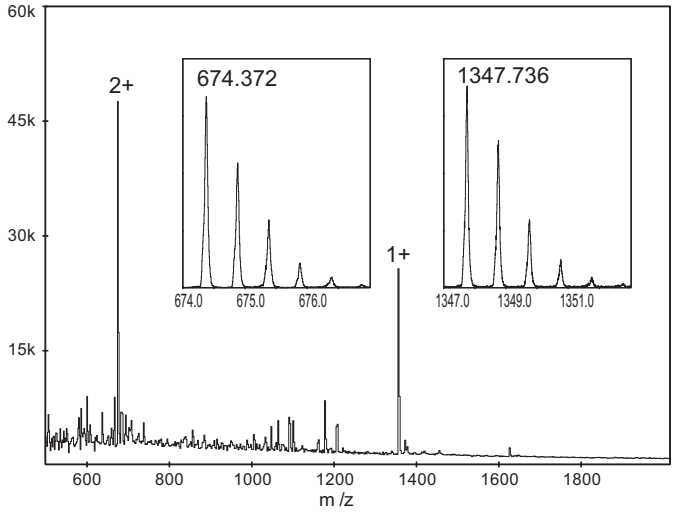

Fig. 2. A typical spectrum of Substance P, used as our calibration standard; electrospray ionization with 100 V declustering voltage. Inserts show the monoisotopic distributions of the doubly charged ion, at *m*/*z* 674, and the singly charged ion, at *m*/*z* 1347. Resolution is close to 18,000 for the singly charged ion, which is helpful for calibration.

generation control, as well as for mass acquisition, was developed in our own laboratory.

4. Mass spectrometry data

4.1. Resolving power

The instrument is calibrated with the singly and doubly charged ions of substance P; as shown in Fig. 2, these are the only two major ions in the spectrum. The two-stage mirror improves the resolution for these molecules (to nearly 18,000 for the monoisotopic $(M+H)^+$ ion). Resolution of larger ions is not as good, because the higher charge states create overlapping monoisotopic distributions. However, charge states from about 20+ to 60+ are clearly defined for a denatured protein, such as citrate synthase, shown in Fig. 3. In this case, the measured mass of the protein is

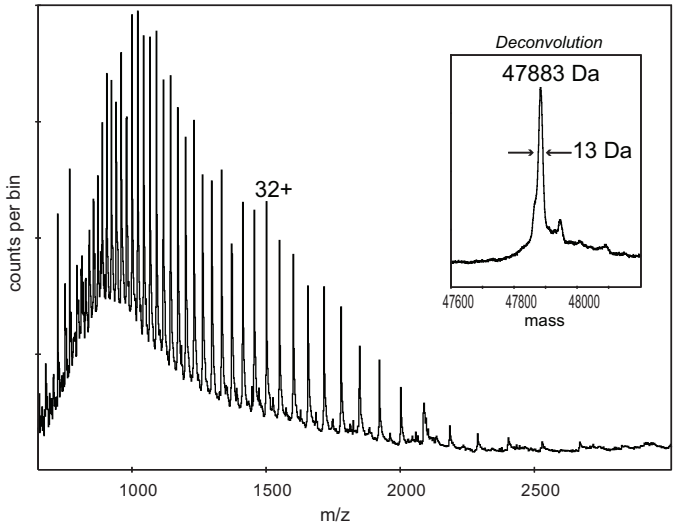

Fig. 3. Characteristic spectrum of denatured *Escherichia coli* citrate synthase; electrospray ionization of 8 μM protein with 120 V declustering voltage. The inset shows deconvolution of the 25+ to 50+ ions. The expected mass from the protein sequence is 47,885 Da.

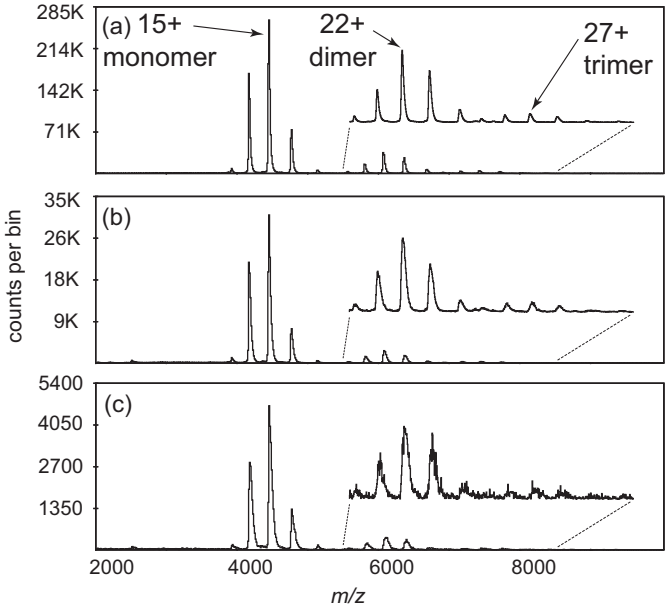

Fig. 4. Parts of the nanospray spectra of BSA prepared in 20 mM ammonium acetate; nanospray ionization with 150 V declustering voltage. Spectra were acquired for 1 min from ten-fold dilutions of protein: (a) 2.5 μM, (b) 0.25 μM, and (c) 0.025 μM.

47,883 Da, with the FWHM of 13 Da yielding a resolving power ~4000, a value normally adequate for determining amino acid replacements or post-translational modifications, as long as they create a mass difference of at least 5 Da. Proteins in their natural state do not have large spreads in their charge distributions, since charge is limited to the surface area of the folded protein or protein complex. Because of this, the width of the charge distribution is relatively small (<10 charges); see Figs. 4–8. However, a complication arises from buffer and salt adducts that may be retained inside a folded protein in a nonspecific manner, resulting in peak widths broader than expected from a "bare" protein. Sometimes an increase in voltage can strip off the excess mass to a certain extent, with the disadvantage that this may disrupt the complex (see Figs. 6–8).

4.2. Sensitivity

Normally, in electrospray ionization, there is a relationship between the protein concentration and the amount of time needed for acquisition of a suitable spectrum. The problem is illustrated by the series of spectra of bovine serum albumin (BSA), shown in Fig. 4. At 2 μM protein, there are three distinct ion envelopes (monomer, dimer and trimer). At 0.2 μM protein, the ions from the trimer are no longer as distinct, and after further dilution of the protein, the only measurable ions left are those of the monomer. Note that the conditions in this case take advantage of the excellent ionization exhibited by BSA.

4.3. Dynamic range

This mass spectrometer yields reliable *m*/*z* measurements *both* for small, singly charged monoisotopic ions, such as substance P (Fig. 2) and heme (Fig. 7), and for large multiply-charged ions such as those from citrate synthase pentamer at *m*/*z* 16,000 (Fig. 6), and proteolytic remains of the catalase HPII tetramer at *m*/*z* 12,000 (Fig. 8).

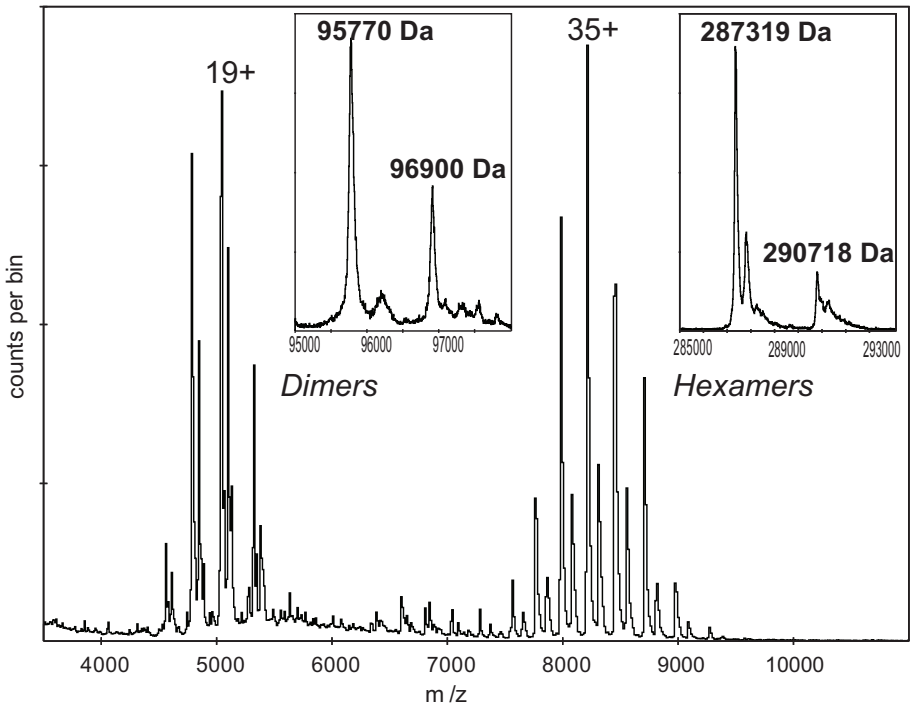

Fig. 5. Part of the nanospray spectrum from a mixture of two forms of wild type *E. coli* citrate synthase prepared in 20 mM ammonium bicarbonate, acquired at 280 V declustering voltage. The sample was made by mixing approximately equimolar mixtures of protein prepared in "natural" and ^{15}N-enriched media. The insets show deconvolutions of the 18+ to 20+ dimer ions (centred at *m*/*z* 5000) and the 33+ to 37+ hexamer ions (centred at *m*/*z* 8500).

4.4. Some practical examples

The examples below have been compiled from ongoing research projects, and represent illustrations of the usefulness of the mass spectrometer in the characterization of proteins. As remarked above, they are designated particularly to illustrate some of the applications that can be covered *without* parent ion selection.

1. *Citrate synthase (CS)* is an important metabolic enzyme that converts oxaloacetate and acetyl-CoA into citrate and free CoA, thus

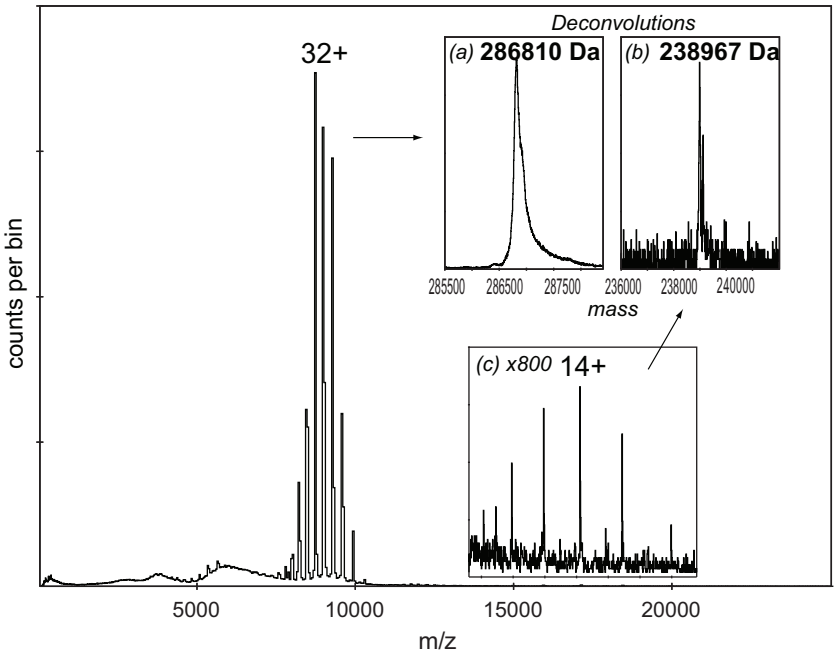

Fig. 6. Electrospray ionization of *E. coli* citrate synthase protein with two mutations, Arg119Leu and Arg319Leu; the protein was in 5 mM ammonium bicarbonate buffer. The spray was at 3 kV with 330 V declustering voltage and 98 V skimmer voltage (to limit discharge between the focus and skimmer plates). (a) Deconvolution of the major charge envelope at *m*/*z* 9000 (expected mass 6 × 47,799 = 286,794 Da) and (b) deconvolution of a lesser charge envelope at *m*/*z* 16,000, shown in an expanded scale in (c). The measured mass of 238,967 Da agrees well with that expected for a pentamer – not a natural form of normal citrate synthase. Under these voltage conditions, the wild type protein (Fig. 4) would have shown a large ion envelope for monomers, and no pentamers.

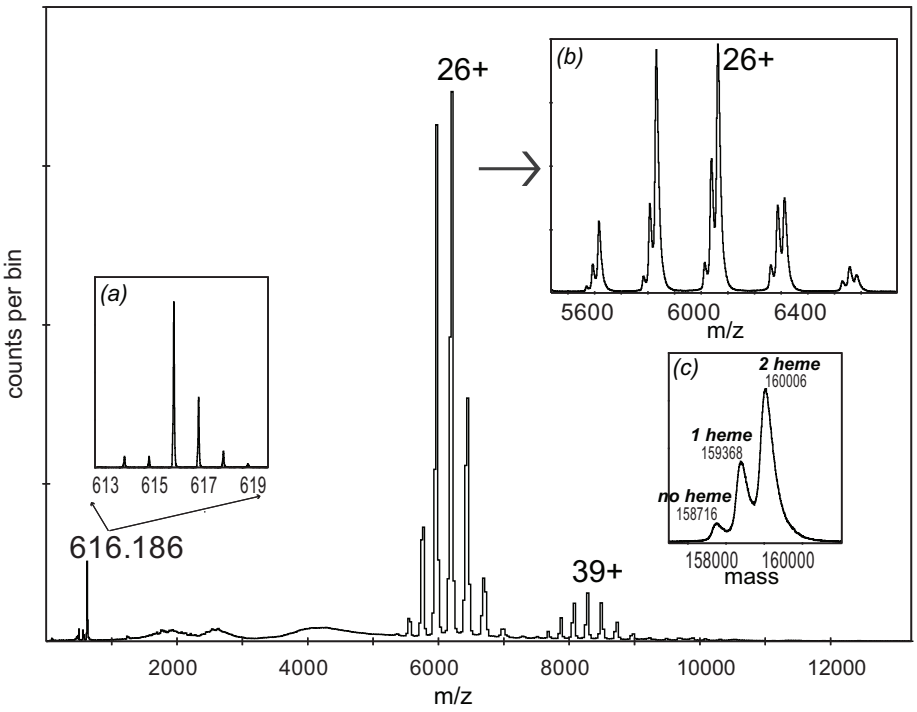

Fig. 7. Nanospray spectrum of wild-type catalase-peroxidase KatG from *Burkholderia pseudomallei*; the protein was 1 mM in 50 mM ammonium acetate. A declustering voltage of 280 V removes heme from the dimeric protein. (a) Monoisotopic ion of the released heme, (b) expansion of the ion envelope for the dimer showing the triplet ions, and (c) deconvolution of the 24+ to 28+ ions of the dimer showing three species with mass difference of heme. A small amount of tetramer is clearly visible at *m*/*z* 8000.

introducing two carbons into the TCA cycle. In most organisms the enzyme is a homodimer, but in gram-negative bacteria, such as *Escherichia coli*, the enzyme is a hexamer. The crystal structure of *E. coli* CS shows three identical dimer units arranged around a central axis [25]. However, the protein exhibits a dimer–hexamer equilibrium in solution that has been thoroughly investigated by electrospray ionization using our older time-of-flight instrument [26]. This equilibrium is sensitive to the concentration of protein, the presence of the specific inhibitor NADH, and to the concentration of the ammonium bicarbonate buffer, which mimics the natural activator, KCl [27].

Wild type and mutant proteins are expressed from a cloned version of the *glt*A gene, and purified from cell extracts as described previously [28]. Each pure protein sample is buffer-exchanged into 20 mM ammonium bicarbonate buffer using an Ultrafree50 unit (Amicon). An aliquot is denatured with 2% acetic acid, 50% methanol in order to measure the exact mass. A typical spectrum for the denatured wild-type enzyme is shown in Fig. 3. The expected mass is 47,885 Da per subunit and the measured mass of 47,883 Da (13 Da FWHM) is in good agreement with this value.

For the folded proteins, we are interested in the dynamics of the dimer–hexamer equilibrium, so we have used two "identical" proteins where one was prepared in normal media, and the other was prepared in minimal media enriched with $^{15}NH_4Cl$. A control run is illustrated in Fig. 5, where there was a slight excess of the unlabeled sample, but clear discrimination between the two kinds of protein, for both dimer and hexamer species. The protein has 578 nitrogen atoms per subunit, giving expected masses of 95,770 and 287,310 Da for natural abundance (99.634% ^{14}N), and 96,918 and 290,754 Da for 100% ^{15}N. A more detailed analysis of the incorporation rate has been reported [29].

Some of the mutants of citrate synthase have quite different properties from the wild type enzyme, and considerable information about their stability can be found by mass spectrometry analysis. One particularly stable mutant protein, with two mutations of Arg to Leu (expected mass 47,799 Da monomer, 286,794 Da hexamer) is shown as an illustration of the use of extreme voltage where the wild type enzyme would have had ions from the monomer (Fig. 6). In this case, there are not even prominent ions from the dimer, but rather a small but distinct ion envelope at *m*/*z* 16,000 with a mass consistent with loss of one subunit.

2. *Catalases and catalase-peroxidases* are the front line of protection from the cellular damage that may be caused by H_2O_2 or its degradation products. Any given bacterium may have both kinds [30,31]. Their subunit structure varies from monomer to tetramer, with distinct structural features. Two very different enzymes are presented below.
   (a) Catalase-peroxidase from *Burkholderia pseudomallei* is naturally a dimer with one heme per subunit [32]. Unlike citrate synthase, the protein does not denature in the presence of acid, possibly because of a covalent modification at the active site [33]. When the protein is prepared in 50 mM ammonium acetate buffer, and gently ionized with 150 V declustering voltage, the spectrum has one ion envelope (not shown). However, at voltages just above normal tolerance, as shown in Fig. 7, the folded protein loses the noncovalent heme b, easily identified by the monoisotopic ion distribution. On close inspection of the ions near *m*/*z* 6000, the folded dimer is made up of triplet ions, whose mass differences correspond to loss of one or two heme molecules. The expected mass of the dimer without heme is $2 \times 79{,}315 = 158{,}630$ Da. We assume that the extra mass comes from associated buffer salts or water.

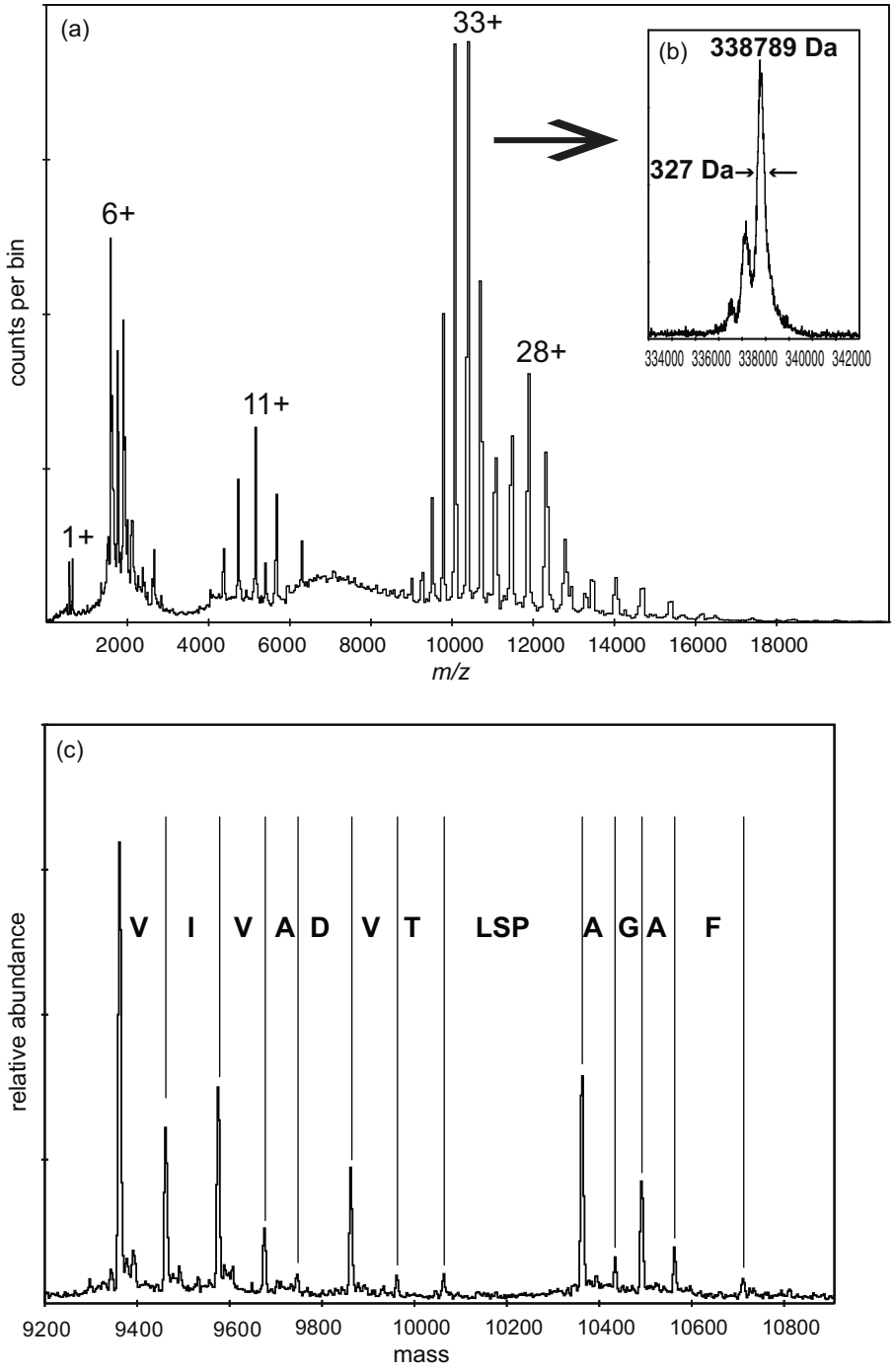

Fig. 8. (a) Electrospray spectrum of wild type *E. coli* catalase HPII acquired under very harsh ionization conditions. Protein was 5 μM in 5 mM ammonium acetate, with carrier gas SF_6 and 280 V declustering voltage. There is one major charge envelope, the 31+ to 36+ ions of a folded tetramer. This has mass 338,789 Da, FWHM 327 Da, as shown in the insert (b). Only this charge envelope is seen under gentler ionization. The smaller species has lost one heme, marked as 1+ at *m*/*z* 632. The ions near *m*/*z* 5000 belong to a species of 56,090 Da (FWHM 67 Da). Ions of 5+ to 7+ at *m*/*z* 2000 and those centred at *m*/*z* 12,000 add up to the complete tetramer. (c) Deconvolution of the 5+ and 6+ ions results in a series of masses with intervals that match amino acids. This sequence can be fitted from Phe654 (10,703 Da) to Pro667 (9361 Da), and indicates loss of the protein C-terminus.

(b) Catalase HPII from *E. coli* is a large homotetramer that is highly resistant to proteolysis compared to other catalases [34,35]. A truncated version of the protein, with losses at both N- and C- termini (mass change from 338 to 223 kDa) remains folded and can be crystallized [36]. Here the exact sites of proteolysis were determined using incomplete denaturation of the protein sample [36]. Another kind of proteolysis, probably from long term storage, is illustrated in Fig. 8 [37]. In this case, a "normal" folded control HPII protein was subjected to extreme voltage conditions. Under ideal conditions (280 V with N_2 as the carrier gas), we would see only the ion envelope at *m*/*z* 10,000. The singly charged ions at low *m*/*z* are from heme (with and without the iron centre). Those at *m*/*z* 2000 and at *m*/*z* 12,000 are parts of the tetramer. The former ions show an unusual pattern after deconvolution (Fig. 8c) and can be assigned to the region of the protein from Phe654 to Pro667 – an exposed loop of the C-terminus [37]. This type of experiment is greatly

facilitated by the versatility of the instrument described here.

5. Conclusions

The results of this study demonstrate that the ESI Q-TOF mass spectrometer described above is a powerful tool for characterizing noncovalently associated protein complexes.

The instrument shows a resolution of 18,000 for $m/z \sim 1348$ and it is possible to observe high m/z compounds without having resolution losses in the low m/z area. A sensitivity of about 2 fmol has been demonstrated for a BSA sample and the accuracy for noncovalent compounds is in the range of 30–40 ppm, or better.

There is room for improvement regarding thermal stability. Drifts of peak position have sometimes been a problem, particularly in the summer, when sometimes the room air conditioning has been inadequate. Ideally, the instrument should be enclosed in a cover equipped with fans and a thermal feedback system that would allow keeping the instrument temperature as constant as possible. This would avoid the occasional need to repeat a measurement because of peak drifts.

The characteristics and operational parameters of the long sleeved quadrupole have been successful in focusing compounds with very high m/z, while preserving the transmission and resolution over the whole m/z range.

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