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Charge Manipulation for Improved Mass Determination of High-mass Species and Mixture Components by Electrospray Mass Spectrometry

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The manipulation of the charge states of high-mass ions can facilitate mass determination in electrospray (ES) mass spectrometry. Specifically, the reduction of charge (which leads to ions of higher mass-to-charge ratios) can significantly reduce peak overlap. Signals associated with various charge states of high-mass ions are more easily resolved at low charge states and chemical noise tends to be significantly lower at high mass-to-charge ratios than in the normal mass-to-charge window typically associated with electrospray. Algorithms that transform ES mass spectra to zero-charge spectra are most likely to yield unambiguous results when charge states are clearly resolved and when signal-to-noise ratios are relatively high. Charge manipulation can enhance the value of the transformation algorithms in cases in which compromises their utility. Such situations include ES mass spectra of high-mass species that yield charge states that are not baseline resolved, mixtures with many components and mixtures in which the signals from major components overwhelm signals from minor components. Examples of improved mass determination are illustrated for proteins using ion–ion chemistry as the means for charge state manipulation and the quadrupole ion trap as the mass analyzer. © **1998 John Wiley & Sons, Ltd.**

KEYWORDS: electrospray mass spectrometry; multiply charged ions; ion–ion reactions, quadrupole ion trap; transformation algorithms

INTRODUCTION

Multiple charging is a hallmark of electrospray (ES) ionization of high-mass polymers, including biopolymers.[1–4] The determination of polymer mass from the spectrum of multiply charged ions is facilitated by the coherence of charge states. Several procedures have been described that permit the transformation of the normal mass-to-charge spectrum to a zero-charge spectrum.[5–9] Algorithms that convert the experimentally derived mass spectrum (i.e. mass-to-charge spectrum) to a zero-charge mass spectrum take advantage of the precision improvement in mass determination afforded by multiple peaks derived from the same molecule[5] and simplify the appearance of spectra derived from mixtures. The various algorithms for transforming ES mass spectra to zero-charge spectra vary in computational complexity, susceptibility to artifact peaks, assumptions regarding peak shape, mass differences arising from the charge-bearing species (e.g. protons, ammonium cations and alkali metal cations) and so forth. Regardless of which algorithm is used, transformation to the zero-charge spectrum is most likely to yield reliable results when individual charge states are well resolved and when signal-to-noise ratios are high. This paper illustrates how the manipulation of the initial charge state distribution formed by ES ionization can yield improved algorithm performance in cases in which charge states are not baseline resolved and when there are relatively high levels of chemical noise.

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EXPERIMENTAL

Materials and electrospray conditions

The proteins chicken conalbumin, rabbit muscle phosphorylase b, tuna heart cytochrome *c*, bovine heart cytochrome *c* and horse heart cytochrome *c* were obtained from Sigma Chemical (St Louis, MO, USA). The molecular masses of these species, as indicated on the labels supplied by the vendor, are 76 244, 138 973, 12 027, 12 230 and 12 359 Da, respectively. Perfluoro-1, 3-dimethylcyclohexane (PDCH) was purchased from Aldrich (Milwaukee, WI, USA). Solutions for ES were prepared by dissolving the sample in water and then diluting with methanol to give a concentration of 2 μM

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in at least 1:1 (v/v) methanol–water. Acetic acid was added at 1% (v/v) to all protein solutions. All solutions were infused at rates of 0.6–0.9 μl min^{-1} through a 50 μm i.d. fused-silica needle. Electrical contact was made at a metal fitting upstream of the fused silica with an applied positive potential of 2.5–3.0 kV.

Mass spectrometry

All ion–ion reaction experiments were performed using a Finnigan ion trap mass spectrometer modified for ES, with ion injection through an end-cap electrode, and glow discharge, with ion injection through a hole in the ring electrode.[10] All experiments were controlled by ICMS software provided by N. Yates and the University of Florida. Cations were injected into the ion trap over a period of 0.075–0.2 s. The radiofrequency (r.f.) sine-wave amplitude applied to the ring electrode during ion injection was 1150–1380 V zero-to-peak. In all cases, helium was admitted into the vacuum system to a total pressure of 1 mTorr (1 Torr = 133.3 Pa) with a background pressure in the instrument of 2×10^{-5} Torr without the addition of helium. Negative ions were formed by sampling the headspace vapors of PDCH into the glow discharge operated at 800 mTorr. Negative ion accumulation times were typically 10–20 ms and negative ion/positive ion mutual storage times were 100 ms for the chicken conalbumin experiment and 350 ms for the phosphorylase b experiment.

Mass/charge analysis was effected via resonance ejection[11,12] to yield a mass/charge range as high as ~85 000 ($q_{eject} = 0.0077$) using resonance ejection amplitudes of 2–4 V peak-to-peak. In all cases, the rate of change of the amplitude of the r.f. voltage applied to the ring electrode was the standard rate supplied by the ITMS electronics (128 V ms^{-1}). The mass/charge scale for the ion–ion reaction spectra was calibrated using ion–ion reaction data generated from solutions of the individual standards. In the case of the cytochrome *c* mixture, ES mass spectra were acquired using a Perkin-Elmer (PE) Sciex API 165 quadrupole mass analyzer calibrated with a solution of polypropylene glycol. The measured masses of tuna, bovine and horse cytochrome *c* were 12 029, 12 232 and 12 357 Da, respectively. In this work, the mass/charge ratios of the various charge states of the standards were based either on the molecular masses provided by the manufacturer (conalbumin and phosphorylase b) or were measured using the PE Sciex API 165 instrument (cytochrome *c*), as mentioned above, and could be used to determine a correction for the mass scale provided by the ion trap data system. Ion–ion reaction times were adjusted to provide for prominent signals from at least four charge states. The spectra shown here were typically the result of an average of 50–100 individual scans.

Data files acquired with the modified ITMS system were converted to Macintosh text format so that the files could be manipulated by PE Sciex BioMultiView 1.2 software running on the data system for a PE Sciex API 165 mass spectrometer. The data were then converted to zero-charge spectra using the BioSpec Reconstruct algorithm provided with the BioMultiView software. The BioSpec Reconstruct algorithm computes the most probable spectrum based on Bayesian statistics.[13]

RESULTS AND DISCUSSION

Algorithms that transform normal ES mass spectra to zero-charge spectra yield fewer artifact peaks and are more likely to identify components of low abundance in spectra of mixtures when charge states are well resolved and when signal-to-noise ratios are relatively high. ES typically yields ions within a relatively narrow mass-to-charge range ($m/z < 2500$), which makes it compatible with analyzers with relatively modest upper mass-to-charge limits. However, the multiple charging phenomenon allows a potentially wide range of analyte masses to fall within a relatively narrow mass-to-charge range. As analyte mass increases, the envelope of peaks for each charge state increases in width owing to the increasingly broad isotopic distribution. Furthermore, in the case of proteins the likelihood of heterogeneity and glycosylation also tends to increase with protein size. The absolute number of charges also increases with mass, leading to a decrease in spacing between charge states. Therefore, high-mass analytes and mixtures of analytes can lead to poor peak definition in mass-to-charge space. Compounding the situation further is the fact that most chemical noise also appears in the same ES mass-to-charge window. The resulting spectral congestion can therefore challenge the capability of an algorithm to yield a reliable zero-charge spectrum.

An obvious strategy to reduce peak overlap is to reduce the charge states associated with the multiply charged ions. This approach reduces charge state overlap and reduces chemical noise both by spreading it out over the mass-to-charge scale and by moving high-mass species to relatively high regions of mass-to-charge, where chemical noise associated with singly charged species is minimal. Such an approach requires a mass analyzer with a mass-to-charge range that can accommodate higher mass-to-charge ions formed via charge state reduction and requires a means for charge state reduction.

Charge state manipulation can be effected to some extent via adjustment of either solution conditions[14] or vacuum/atmosphere interface conditions.[15,16] These are the most widely available approaches but they may compromise ionization and/or transmission efficiency. Another approach is to effect charge state reduction via gas-phase ion chemistry. For example, ion–molecule proton transfer chemistry has been used to reduce charge states of multiply charged ions to facilitate charge state determination.[17–20] Similarly, negative ions have also been used to reduce the charges of multiply protonated proteins both external to[21,22] and within a mass spectrometer.[10,23–27] An advantage of effecting charge state reduction within the mass spectrometer is that the ionization and transmission steps can be optimized independently of charge state manipulation. The use of negative ions for charge state reduction, at least in the quadrupole ion trap, has clear advantages over

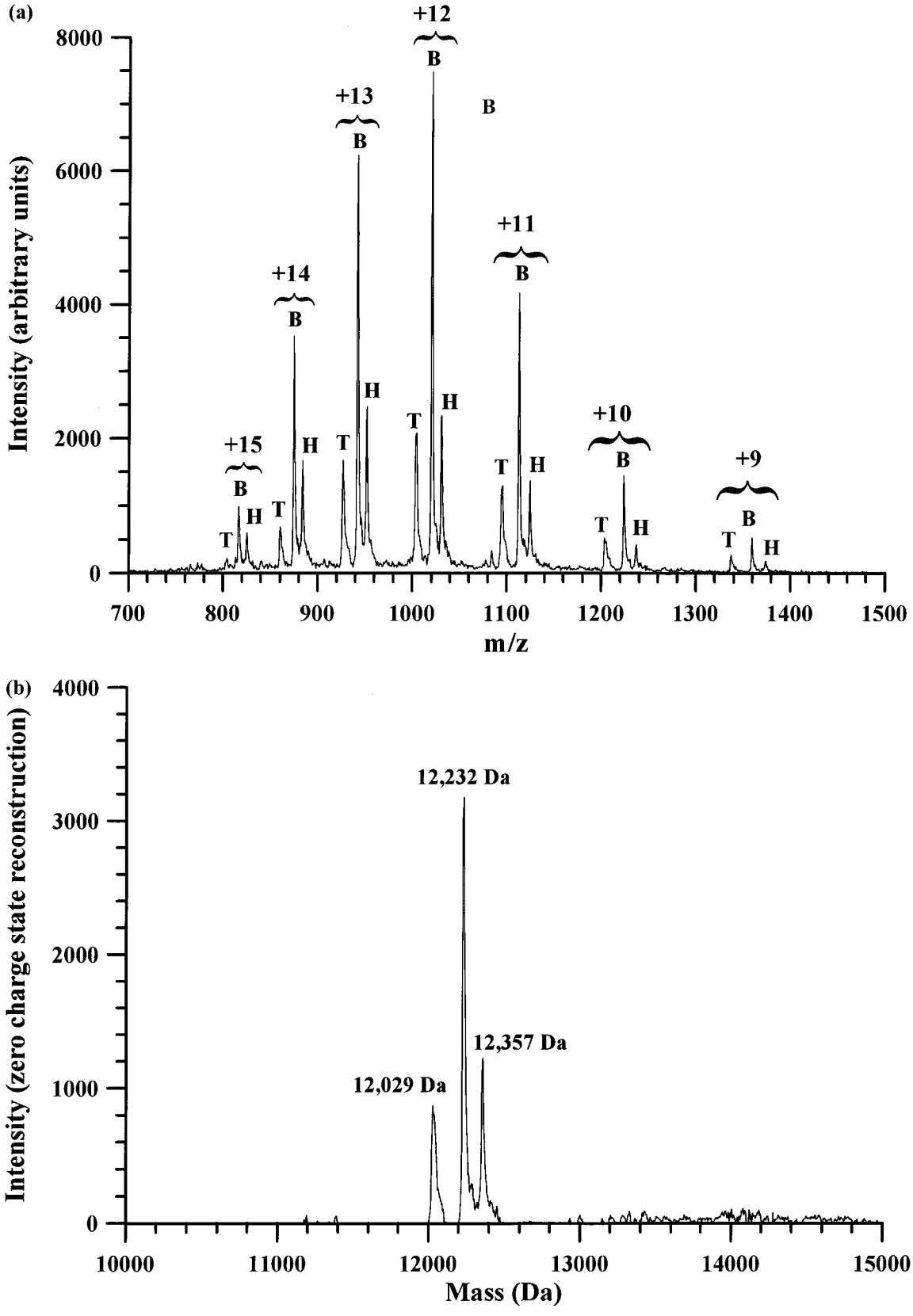

Figure 1. (a) ES mass spectrum of a mixture of three types of cytochrome *c* [tuna (T), bovine (B) and horse (H)] and (b) the transformed (i.e. zero-charge) spectrum.

the use of strong neutral gaseous bases. These include greater universality, more predictable kinetics,[27] lack of clustering (using suitable negative ions)[24] and greater control over the admission and removal of the charge-reducing agents (negative ions versus strong neutral bases). However, while cases are illustrated here showing the desirability of charge state reduction for mass determination using ion–ion reactions in the Paul

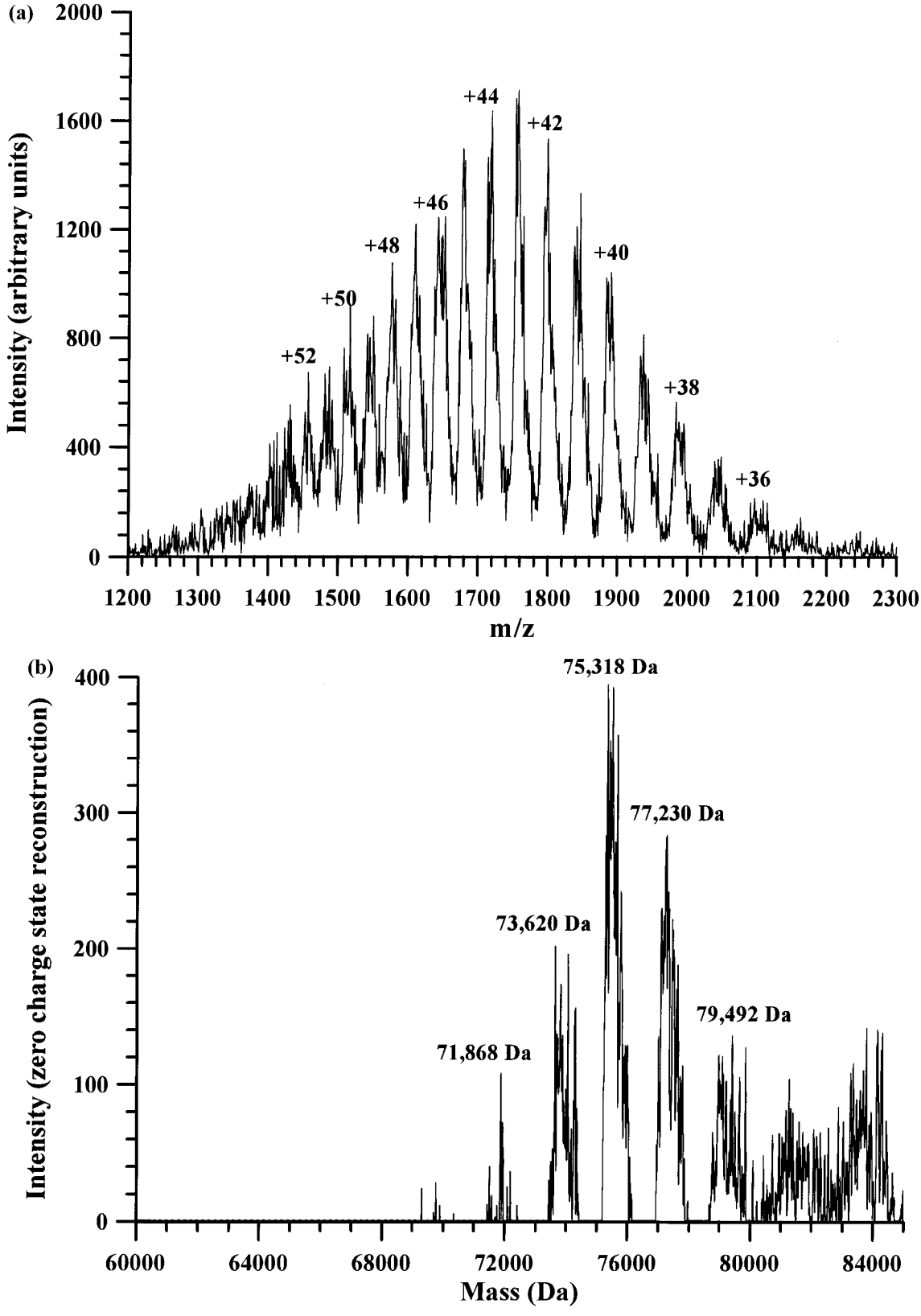

Figure 2. (a) ES mass spectrum of chicken conalbumin and (b) the transformed spectrum.

trap, any of the approaches to charge state reduction might also be considered.

Figure 1(a) shows the ES mass spectrum of a mixture of three types of cytochrome *c* (tuna, bovine and horse) and Fig. 1(b) shows the transformed spectrum. The spectrum in Fig. 1(a) is illustrative of one in which the transformation algorithm is effective in yielding an unambiguous zero-charge spectrum. That is, the various

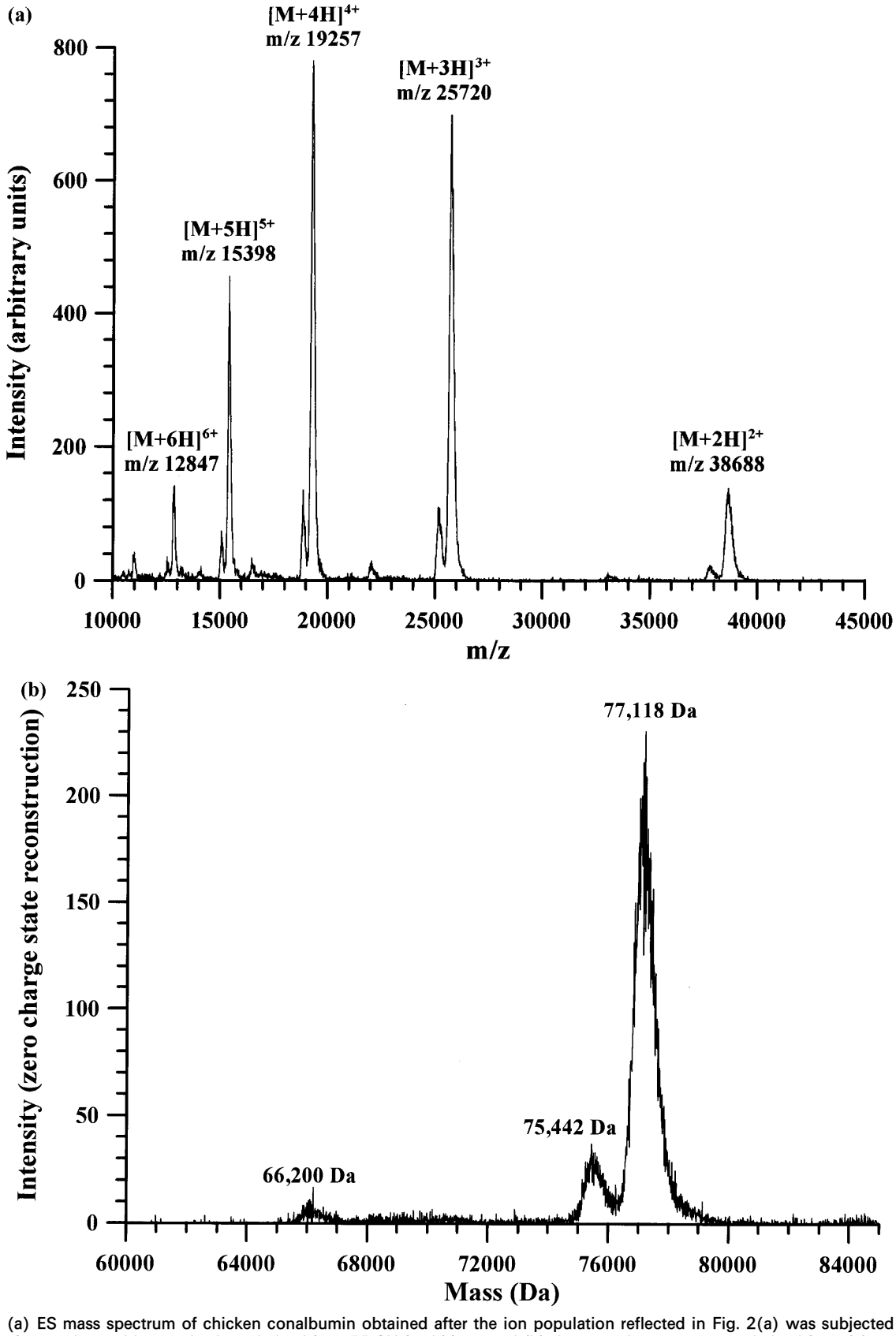

Figure 3. (a) ES mass spectrum of chicken conalbumin obtained after the ion population reflected in Fig. 2(a) was subjected to ion–ion proton transfer reactions with negative ions derived from PDCH for 100 ms and (b) the zero-charge spectrum derived from (a).

peaks associated with the multiply charged ions are well resolved and signal-to-noise ratios are relatively high. There is little or no benefit to be gained in this case by reducing charge states from the 8+ to 15+ range shown in Fig. 1(a) to any lower range of charge states (data not shown). The example provided in Figs 2 and 3, however, illustrates a situation in which transformation of the ES mass spectrum yields ambiguous results.

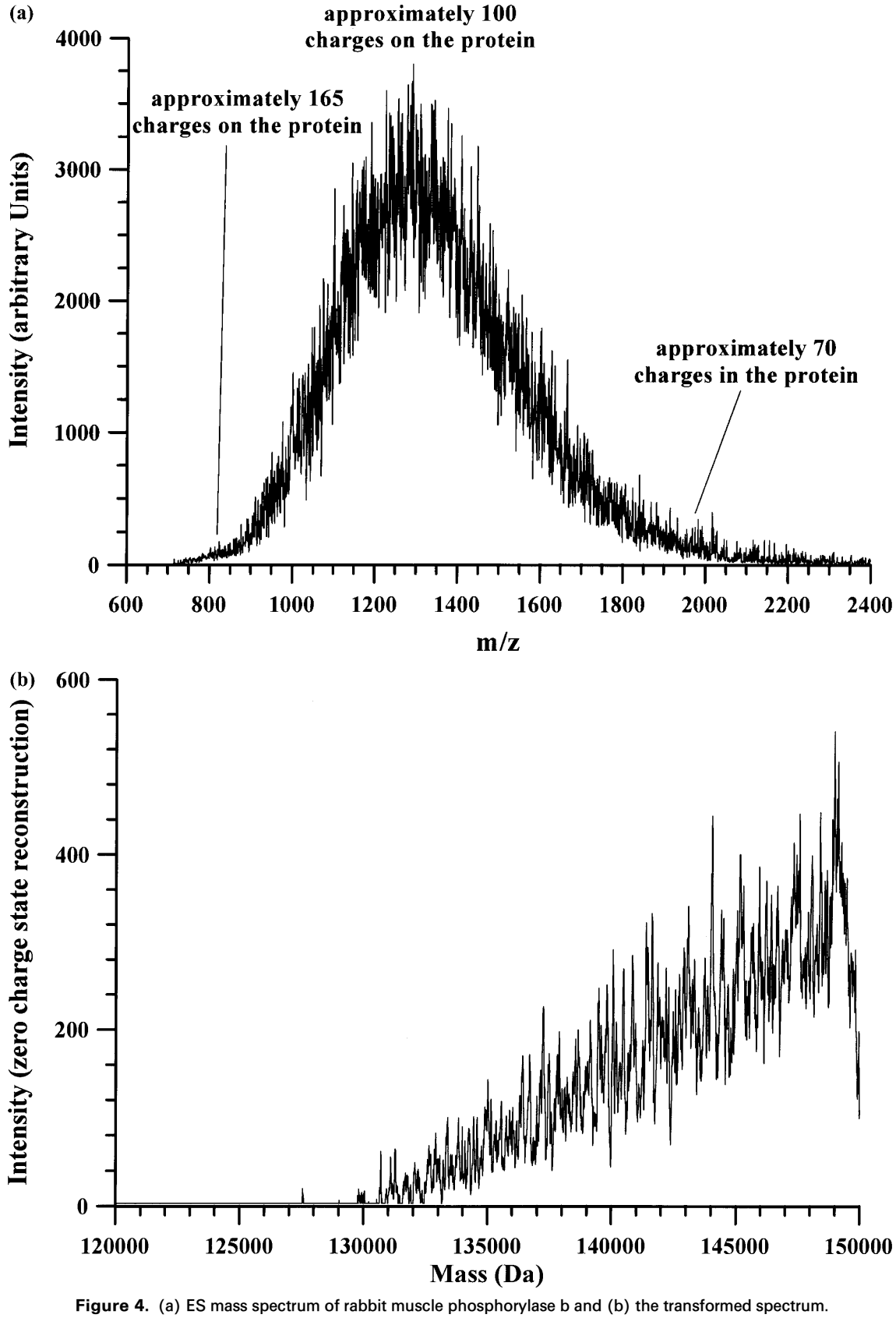

Figure 4. (a) ES mass spectrum of rabbit muscle phosphorylase b and (b) the transformed spectrum.

Figure 2(a) shows the normal ES mass spectrum of chicken conalbumin and Fig. 2(b) shows the transformed spectrum. The transformed spectrum obtained after 25 cycles, where further changes in the spectrum are not discernible, shows as many as seven 'peaks' at regular spacing. There is no obvious basis on which to select one of these peaks as that representing the true molecular mass. The appearance of multiple regularly

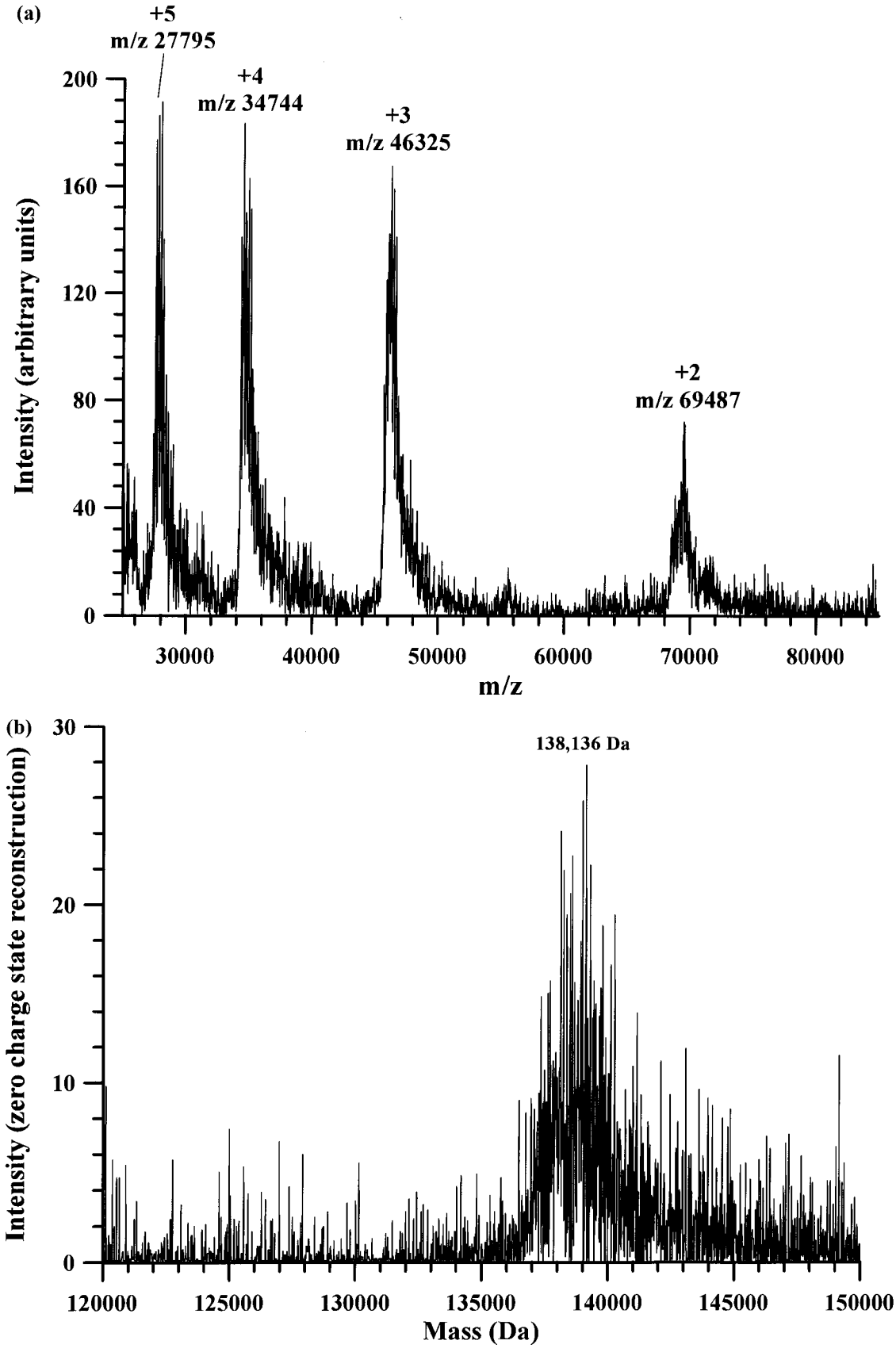

Figure 5. (a) ES mass spectrum obtained after the ions that give rise to the signal in Fig. 4(a) were allowed to undergo ion–ion proton transfer reactions for 340 ms and (b) the zero-charge spectrum derived from (a).

spaced peaks in the transformed spectra obtained with the ion trap instrument used in this study is most common for proteins in the 40–100 kDa range. This is the protein size range that tends to yield closely spaced charge states, many of which are not baseline resolved. The multiple peaks appearing in the transformed spectrum are analogous to the multiple candidate masses that can arise from the uncertainty in assigning charge states when making manual interpretations of spectra with closely spaced, poorly defined peaks.

Figure 3a shows the spectrum of conalbumin obtained after the ion population reflected in Fig. 2(a) was subjected to ion–ion proton transfer reactions with negative ions derived from PDCH for 100 ms. Note that it was necessary to extend the mass-to-charge range of the ion trap to slightly beyond 40000 to analyze the $(M + 2H)^{2+}$ ion of conalbumin. Figure 3(a) shows a charge state range of 2+ to 6+, in contrast to the range of 36+ to 52+ shown in Fig. 2(a). In the case of Fig. 3(a), charge states can easily be assigned with confidence with manual interpretation of the spectrum. Likewise, the transformed spectrum of Fig. 3(b) yields far less ambiguous information than that of Fig. 2(b). Also apparent in both spectra in Fig. 3 are minor components in the spectra that are not clearly apparent in the data in Fig. 2. For example, a peak on the low mass-to-charge side of each of the conalbumin ion signals is clearly observed for all charge states in Fig. 3(a) and the species giving rise to these ions is also reflected in the transformed spectrum. At least one other component yielding much lower ion abundance than either conalbumin or its low-mass neighbor is also reflected in Fig. 3(a) and a small transformed peak appears at roughly 66 kDa in Fig. 3(b) that corresponds to this component. Clearly, the data in Fig. 3 provide more reliable mass assignments than the data in Fig. 2 and also exhibit minor components present in the original ion population that are not apparent, but must be present, in the original ES mass spectrum. This improved performance can be attributed to the better resolved and defined peaks following charge state manipulation and the lower noise levels in the data acquired after the ion–ion reactions. Lower relative noise levels are likely to be due to the fact that the chemical noise arising from low-mass species does not follow the high-mass ions as they increase in mass-to-charge as a result of ion–ion chemistry and because the noise is spread over a much wider range of mass-to-charge than is the case in the normal ES mass spectrum.

As protein mass and the degree of protein heterogeneity and post-translational modification increase, difficulty in making reliable mass measurements from the normal ES mass spectrum (or identifying minor components) also increases. The upper limit to mass and/or protein complexity is mass analyzer dependent. For the ion trap used in this work, heterogeneous proteins of mass of the order of 100 kDa or greater can yield poorly resolved charge states. An example is given in Fig. 4(a), which shows the ES mass spectrum of rabbit muscle phosphorylase b as a very broad poorly resolved peak. Transformation of this broad ion signal is shown in Fig. 4(b). The algorithm outputs a sloping baseline with no indication of convergence to a single protein component. Figure 5(a) shows the ES mass spectrum obtained after the ions that give rise to the signal in Fig. 4(a) were allowed to undergo ion–ion proton transfer reactions for 340 ms. (These data were acquired before a realignment of the glow discharge source was made. This realignment significantly improved the rate at which the ion trap could be filled with negative ions. This is the reason why the reaction time used to acquire the data in Fig. 5(a) was longer than that used to acquire the data in Fig. 3(a). The data in Fig. 3(a) were acquired after the realignment of the glow discharge ion source.) The most intense signals show the expected mass-to-charge relationships for ions of 2+ to 5+. Upon transformation, a single peak dominates the spectrum, consistent with the expected mass of rabbit muscle phosphorylase b. This example clearly shows how charge state reduction can benefit the mass determination of high-mass species formed with relatively high charge via electrospray.

CONCLUSIONS

The manipulation of the charge states of multiply charged ions derived from ES can allow for improved mass determination in cases in which the normal ES mass spectrum is too noisy and/or congested, provided the upper mass-to-charge limit of the mass analyzer can accommodate high-mass ions of low charge. Situations like this arise for relatively high-mass proteins that are formed at high charge states, when chemical noise is high and when mixtures are too complex for reliable transformation to zero-charge spectra. Charge state reduction can lead to better defined peak shapes, more reliable charge state assignment and reduction in the relative level of chemical noise. Chemical noise tends to be lower at high mass-to-charge levels both because there are likely to be fewer background species at high mass-to-charge ratio and because chemical noise is spread over a wider mass-to-charge range. Algorithms that transform ES mass spectra to zero-charge spectra have been shown to be useful in deconvoluting ES mass spectra of mixtures. The use of such algorithms in conjunction with charge state manipulation can enhance the value of the algorithms for high-mass species and for mixtures in which the signals arising from small components are buried under the signals arising from the major component(s). The use of negative ions to reduce the charges of multiply charged cations is particularly convenient and useful in the Paul trap. Fortunately, the mass-to-charge range of Paul traps typically used in chemical research can be extended to >60 000 using resonance ejection.

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