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# Parent Ion Scans of Large Molecules

**Gitte Neubauer and Matthias Mann***
Protein and Peptide Group, European Molecular Biology Laboratory (EMBL), Meyerhofstrasse 1, D-69012 Heidelberg, Germany

**The utility of parent ion scans for the selective detection of small molecules which yield indicative fragments in tandem mass spectrometry is well recognized. This work established the feasibility of parent (or precursor) ion experiments with large molecules such as proteins and oligonucleotides. Tandem mass spectrometry of proteins yields substantial and specific immonium ion fragmentation and fragments characteristic of post-translational modifications. Phosphoproteins are specifically detected by parent ion scans of the phospho group ($PO_3^-$) in the negative ion mode. The same ion allows mass measurements of oligonucleotides with improved signal-to-noise ratio. Scans for the parents of the oxonium ion of hexosamine distinguish glycoproteins from other proteins in mixtures. Parent ion scans of intact proteins is a novel concept which may find applications in the determination of post-translational modifications, in protein charting and in the study of the fragmentation behavior of large molecules.**

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## INTRODUCTION

Electrospray[1,2] and matrix-assisted laser desorption ionization (MALDI)[3,4] have made it possible to obtain routinely and sensitively the molecular masses of intact proteins.[5] Such molecular mass measurements, while an important and frequently indispensable part of protein characterization, often solve only part of the analytical problem of primary structure elucidation of a large biomolecule. In the analysis of post-translational modifications of proteins, for example, the next step is typically enzymatic digestion and measurement of the resulting peptides, so called 'peptide mapping.' However, peptide mapping takes time and effort and, more importantly, it is difficult to cover the protein sequence completely with measured peptides. An alternative and analytically appealing approach is to fragment the protein mass spectrometrically and thereby learn more about its primary structure than the mass alone provides. Smith and co-workers[6] first showed that it is possible to obtain sequence information from intact proteins. More recently, McLafferty and co-workers[7,8] developed a 'top down' approach to sequencing by trapping intact protein or oligonucleotide ions in an Fourier transform ion cyclotron resonance (FTMS-ICR) instrument accompanied by nozzle skimmer fragmentation or fragmentation of the trapped ions. Extensive sequence information can be obtained, sufficient to identify the protein in a database search and to map differences between expected and found primary structures.[9]

Our laboratory has recently developed the technique of parent ion scans of unseparated peptide mixtures.[10] The nanoelectrospray ion source (nanoES)[11,12] was used to spray a peptide mixture without chromatographic separation of its constituents. Parent ion scans for the immonium ions of isoleucine/leucine or the $Y_1''$ ions[13] of tryptic peptides then provide additional information on the components in the mixture. The immonium ion scans in particular are useful for locating peptides which are present in the 'normal' or $Q_1$ scan with a signal-to-noise ratio of $<1$ but which may, nevertheless, yield sequence information upon fragmentation. Parent ion scans of the phospho group and the oxonium ion of sugar modifications selectively and sensitively identify modified peptides in complex mixtures.

Here we investigated whether it is possible to perform parent ion scans on large molecules such as proteins and oligonucleotides. If feasible, such parent ion scans could be used to distinguish large molecules from chemical noise, to 'interrogate' intact proteins for the presence of phosphorylations or glycosylations and to distinguish modified from unmodified proteins. Parent ion scans of intact proteins could also be a useful tool in the study of the fragmentation behavior of large molecules.

## EXPERIMENTAL

### Protein and oligonucleotide samples

Human carbonic anhydrase I (EC 4.2.1.1), bovine RNAse B and bovine $\beta$-casein were obtained from Sigma (St Louis, MO, USA). The oligonucleotide was synthesized and purified in-house (R. Eritja, EMBL). Protein and oligonucleotide solutions had concentrations of 5–10 pmol $\mu l^{-1}$. Solutions were 50:40:10 (v/v) methanol–water–formic acid for positive-mode experiments and 50:45:5 (v/v) methanol–water–ammonia for negative-mode experiments.

* Correspondence to: M. Mann.

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### Mass spectrometry

The mass spectra were acquired on an API III triple-quadrupole instrument equipped with an upgraded collision cell[14] (Perkin-Elmer Sciex Instruments, Thornhill, Canada). The NanoES ion source used in all experiments reported here was developed in our group.[11,12] Quadrupoles 1 and 3 ($Q_1$ and $Q_3$) were set to unit resolution in all experiments, also in parent ion scans. Argon was used as the collision gas at a collision gas thickness (CGT) of (2.9–3.0) $\times 10^{14}$ molecules $cm^{-2}$ (CGT setting of 290–300). The instrumental settings for the collision energy (difference between R0 and R2 potentials) was 35 eV in all experiments, except where stated otherwise. Step widths were 0.1 Da for $Q_1$ scans and 0.2 Da for parent ion and fragment ion scans, except where noted otherwise. The dwell time was 1 ms for all scans, except for parent ion scans, where it was 3 ms. The parent ion mass scale was calibrated with an immonium ion scan of myoglobin. The difference in mass scale between $Q_1$ scans was found to be 0.08–0.1 Da at the same step width and resolution setting. This correction can be taken into account by using normal $Q_1$ calibration files but using a fractional mass for the charging agent (1.1 Da in the positive and 0.9 in the negative mode) in deconvolution of the multiply charged ions,[15] which has the effect of shifting the mass scale by the required amount. Needles were prepared as described[12] and were filled with 1 $\mu$l of protein solution unless noted otherwise. A new needle was used for each experiment.

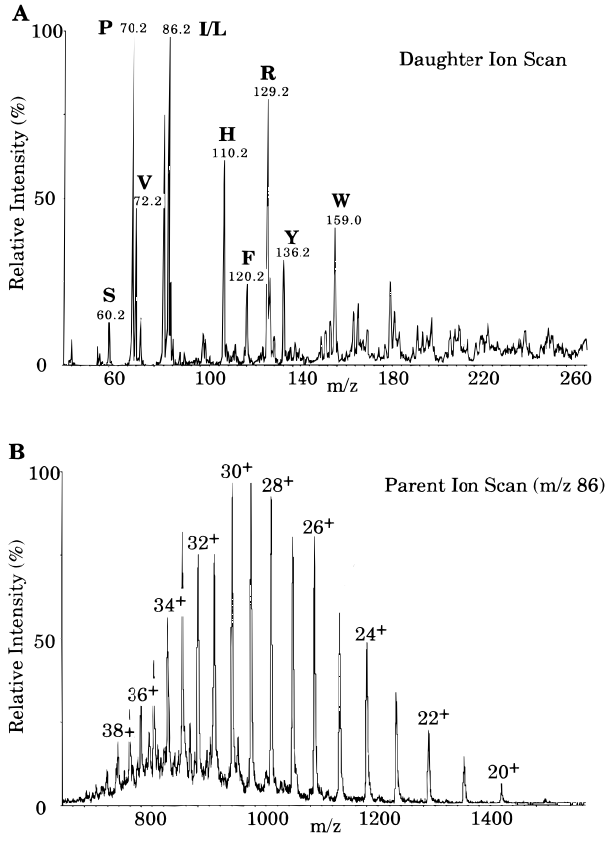


**Figure 1.** (A) Fragment ion spectrum of the 30 + charge state of carbonic anhydrase. Immonium ions are labeled. (B) Parent ion scan of the immonium ion of Ile/Leu (*m/z* 86) of carbonic anhydrase.

## RESULTS AND DISCUSSION

### Immonium ion scans of intact proteins

To establish whether large molecules give rise to specific low-mass fragmentation, the 30+ charge state of carbonic anhydrase was fragmented in the collision cell of the triple quadrupole and the low-mass region recorded. As shown in Fig. 1, ions characteristic of immonium ion fragmentation predominate the low-*m/z* part of the spectrum. The immonium ions of isoleucine/leucine (*m/z* 86) and proline (*m/z* 70) are the base peaks in this region of the spectrum. The overall low-*m/z* fragmentation pattern is very similar to peptide fragmentation spectra. However, unlike the case of peptide fragmentation spectra, the immonium ion peaks also form the base peaks of the total fragmentation spectrum.

Scans for the parents of the Ile/Leu immonium ion and for an adjacent *m/z* were recorded. The parent ion scan of *m/z* 86 reproduced a multiply charged envelope, as expected [Fig. 1(B)]. The scan of *m/z* 90 produced only electronic noise and no chemical noise (1–2 counts per measurement point; data not shown) demonstrating the specificity of the parent ion scan technique even for proteins. The resolutions of the $Q_1$ scan and parent ion scans were similar, as is our general observation (M. Wilm *et al.*, unpublished data). The ratio in counts per second between the added multiply charged ion abundance in the $Q_1$ mass spectrum and in the parent mass spectrum is about 10:1. This ratio depends on the collision energy but is generally in the range 10–100:1.

Fragment ion scans for Ile/Leu immonium ions were performed at different collision energies. The absolute intensity of the immonium ion peak increased with increasing collision energy, as did the relative intensity of the immonium ion peak compared with the high-*m/z*, sequence specific fragmentation of the protein (data not shown). Note that, as the collision energy is different for each charge state, the parent ion scan does not produce the same distribution of multiply charged ions as the $Q_1$ scan. In general, higher charge states will be represented more strongly as the immonium ion becomes more abundant with increasing collision energy.

From the above results, it appears that immonium ion formation is a substantial fragmentation pathway even for large molecules. This is surprising in view of the commonly held notion that immonium ions are formed by independent fragmentations at either side of the amino acid in question.[16] Such fragmentations should become less likely as the molecule becomes larger. (Of course, owing to multiple charging, the collision energy is much higher at the same instrumental settings.)

### Parent ion scans of glycosylated proteins

Glycosylated molecules can be specifically detected in complex mixtures by parent ion scans of the oxonimum ion of HexNac, *m/z* 204, in the positive mode.[10,17] To

test whether proteins would specifically fragment to lose this diagnostic ion, parent ion scans of RNAse B, a glycoprotein and carbonic anhydrase, a non-glycosylated protein, were performed. The parent ion scans of RNAse B but not of carbonic anhydrase led to the typical envelope of multiply charged ions (data not shown). We then tested whether the scan for the oxonium ion could be used to detect selectively a modified protein in the presence of an unmodified protein. In Fig. 2, the spectrum of a mixture of carbonic anhydrase and RNAse B is shown. The parent ion scan for *m/z* 204 on the mixture detects only RNAse B, not carbonic anhydrase [Fig. 2(B)]. To our knowledge, this is the first time that intact proteins have directly been 'interrogated' by tandem mass spectrometry for the presence of post-translational modifications.

As in the case of peptides,[10] the *m/z* 204 scan was found to be less discriminating than the phospho ion scan (see below). This is due to the larger number of combinations leading to mass 204 compared with 79 and the fact that the negative ion fragmentation yields less low-mass fragmentation.

The abundance of oxonium ion loss depends on the collision energy, as it does for immonium ions, but is in the range of abundance of the most abundant immonium ions. The oxonium ion is relatively stronger at low collision energy (about 1:1 abundance ratio) and decreases to about 1:10 compared with immonium ions at high collision energies. This finding suggests that the formation of the oxonium ion may require less energy than the formation of immonium ions.

The signal intensity of the oxonium ion is much larger than any single sequence-specific fragmentation of the protein. This raises an important question for the gas-phase mapping of modified proteins. While it can be readily imagined that a protein can be mapped in the gas phase, i.e. in a fragmentation experiment in the ICR cell of an FTMS instrument, a necessary precondition for assigning the location of the modification is that the modification does not easily cleave off before backbone cleavage occurs. In a previous investigation we found it necessary to subdigest a 2.7 kDa peptide to ascertain the location of glycosylation through tandem mass spectrometric fragments bearing the modification.[18,19] This and other anecdotal evidence, in addition to the data discussed above, suggest that cleavage of the carbohydrate moiety from the protein backbone may occur relatively readily. Such behavior is also plausible from steric considerations, i.e. the relatively large volume filled by even relatively small carbohydrate modifications.

## Parent ion scans of phosphorylated large molecules

In our laboratory, parent ion scans of the phospho group ($PO_3^-$, *m/z* 79) in the negative ion mode have become an indispensable tool to detect phosphorylation in complex peptide mixtures.[10,20] To determine whether intact phosphoproteins also specifically fragment to lose the phospho group, parent ion scans of β-casein, a phosphoprotein and carbonic anhydrase, which is not phosphorylated, were performed. As in the case of glycosylation, only the modified protein was detected by the parent ion technique (data not shown). Figure 3 shows the *m/z* 79 parent ion spectrum of β-casein. The signal intensity was a factor of ten lower in the parent ion mode compared with the $Q_1$ mode. Owing to the absence of immonium ion fragmentation in the negative ion mode, the *m/z* 79 scan is especially selective.

In Fig. 3(A), a bimodal charge distribution presumably caused by a folded and denatured protein conformation[21,22] can be seen. This is not the case in the parent ion scan in Fig. 3(B). In this and other examples we have noticed that the parent ion scans apparently discriminate against the folded conformation, even

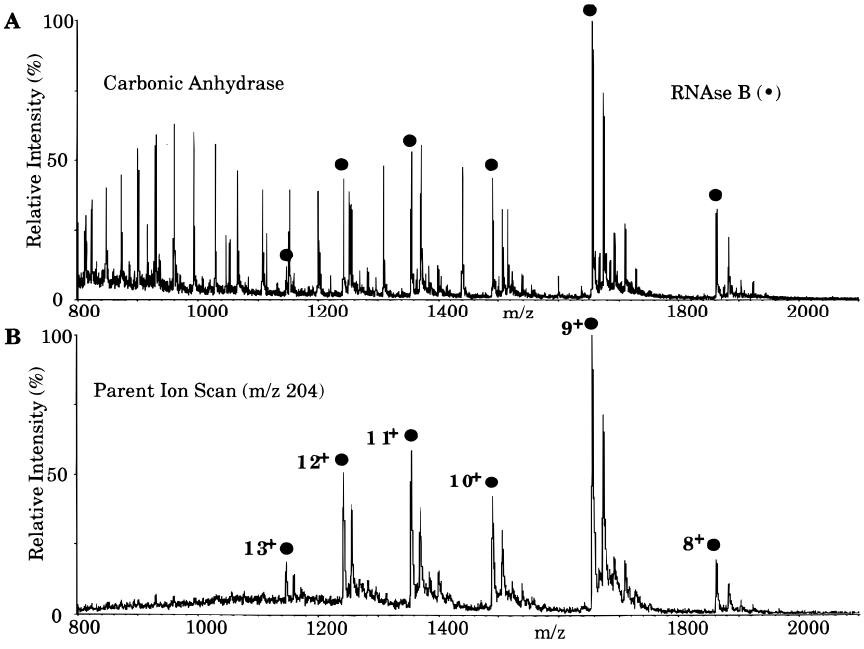


**Figure 2.** (A) Spectrum of the mixture of carbonic anhydrase and RNAse B in a 1:1 ratio. The major glycoform of RNAse B is marked by bullets. (B) Parent ion scan of the oxonium ion of HexNac (*m/z* 204).

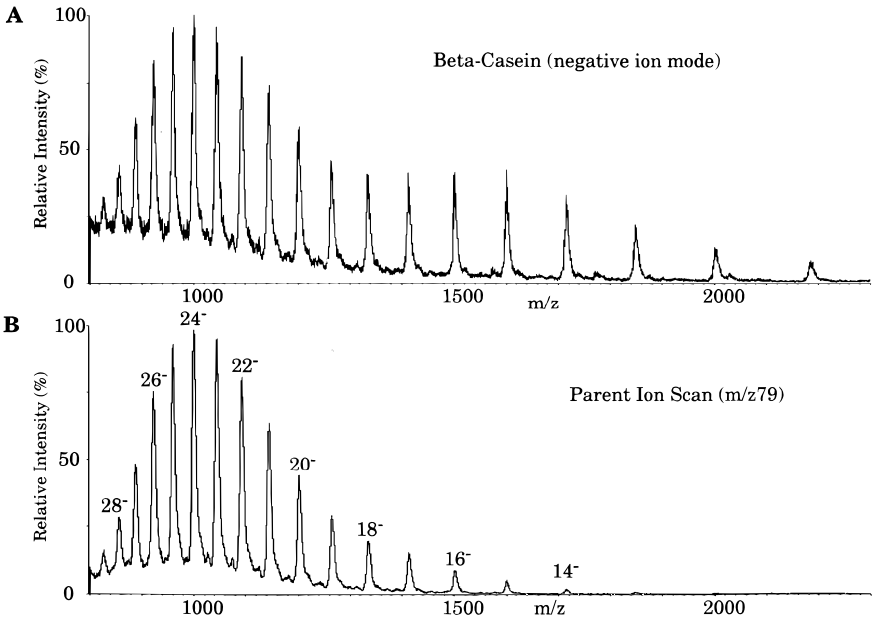


**Figure 3.** (A) Spectrum of $\beta$-casein in the negative ion mode. (B) Parent ion scan for the phospho group (*m/z* 79).

taking into account that the collision energy of these lower charge state species is less than in the denatured state (see above). This behavior may be caused by the different collision cross-sections of denatured and folded protein states.

As a practical consideration, we note that it is generally more difficult to spray proteins in the negative than the positive ion mode. This may limit the routine use of the parent ion scanning technique for assessing the phosphorylation state of a protein.

Oligonucleotide molecules also contain the phospho group and therefore a parent ion scan experiment was performed on a synthetic oligonucleotide molecule. Because of their polarity, oligonucleotides easily associate with counter ions (e.g. $Na^+$), making analysis more difficult. Salt adducts can be removed by repeated steps of HPLC purification or precipitation, but this makes the analysis more time consuming. A scan for the phospho group in the negative mode should improve the signal-to-noise ratio of the spectrum. This is demonstrated in Fig. 4. The upper trace ($Q_1$ spectrum) could not be used for mass assignment owing to excessive chemical noise. In contrast, the lower spectrum contains the parents of the phospho ion group and masses can easily be assigned to the different multiple charged peaks. This feature has turned out to be useful for routine analysis and is now frequently used for the molecular mass determination of oligonuleotides and the selective detection of oligonucleotide-containing constituents in complex mixtures in our laboratory. Since much less pretreatment is necessary, we expect this technique to be widely applicable in laboratories which are concerned with quality control of synthesized oligonucleotides.

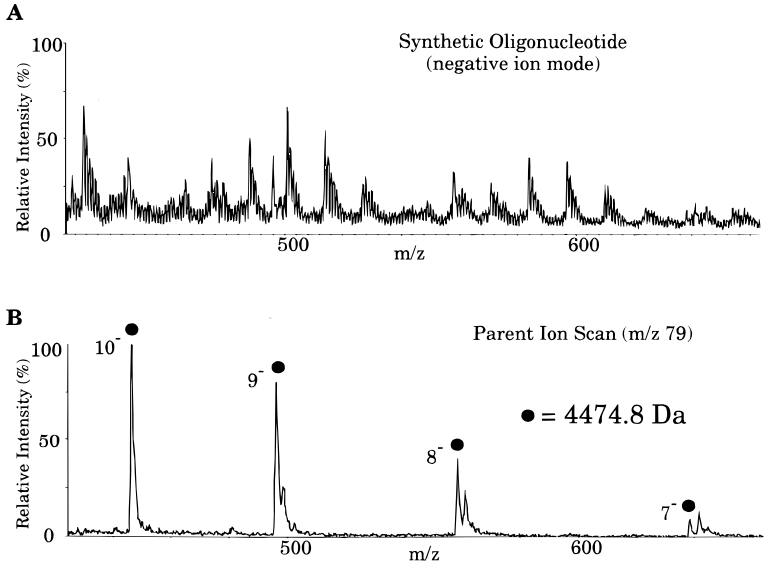


**Figure 4.** (A) Spectrum of a synthetic oligonucleotide (a modified 14mer) in the presence of contaminants from chemical synthesis and in negative ion mode. (B) Parent ion scan of the phospho group (*m/z* 79).

## CONCLUSION

We have conclusively shown that large molecules give rise to specific low molecular mass fragments which can be used for parent ion scans. Substantial immonium ion fragmentation was observed which allows the specific detection of proteins. Chemical noise can be reduced, as demonstrated on a synthetic oligonucleotide. The two most frequent protein modifications, phosphorylation and glycosylation, can be assessed directly on the intact protein via parent scans of the oxonium ion at $m/z$ 204 and the phospho group at $m/z$ 79 in the negative mode, respectively. The results obtained here also raise some provocative questions with regard to the order of fragmentation of large, modified proteins. We expect that parent ions scans of large molecules will become a useful addition to the toolkit of the mass spectrometrist.

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