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Top-down mass spectrometry on low-resolution instruments: Characterization of phosphopantetheinylated carrier domains in polyketide and non-ribosomal biosynthetic pathways

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Abstract—Mass spectrometry (MS) is an important tool for studying non-ribosomal peptide, polyketide, and fatty acid biosynthesis. Here we describe a new approach using multi-stage tandem MS on a common ion trap instrument to obtain high-resolution measurements of the masses of substrates and intermediates bound to phosphopantetheinylated (*holo*) carrier proteins. In particular, we report the chemical formulas of 12 diagnostic MS^3 fragments of the phosphopantetheine moiety ejected from *holo* carrier proteins during MS^2. We demonstrate our method by observing the formation of *holo*-AcpC, a putative acyl carrier protein from *Streptococcus agalactiae*.
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The biosynthesis of polyketide synthase (PKS) and non-ribosomal peptide synthetase (NRPS) natural products, including the well-established therapeutic agents penicillin, vancomycin, and rapamycin, requires post-translational modification of carrier proteins by addition of a 4′-phosphopantetheine (PPant) arm. The sulfur at the free end of this moiety forms a thioester bond with substrates and intermediates at each step of the biosynthetic process. Because these substrates and intermediates increase the overall mass of the associated carrier proteins, the latter are ideal targets for 'top-down' characterization of PKS and NRPS pathways by mass spectrometry (MS).[1] A top-down approach was recently shown to facilitate the study of phosphopantetheinyl-tethered substrates.[2] Thermal activation by infrared multiphoton dissociation (IRMPD) or collision-induced dissociation (CID) causes the *holo* form of acyl and peptidyl carrier proteins, which are found in PKS and NRPS pathways, respectively, to consistently 'eject' their PPant arm, preserving the thioester linkage to the substrate (Fig. 1a). This 'PPant ejection assay' allows the mass of substrates loaded onto carrier proteins to be readily deduced from the mass of the corresponding PPant fragments.[3] Specifically, when no substrate is linked to the PPant arm, the fragment ejected from the carrier protein has chemical formula $C_{11}H_{21}N_2O_3S^+$, giving an MS^2 peak at *m/z* 261.1267. When an acyl or peptidyl substrate is bound to the PPant arm, the PPant peak in the MS^2 spectrum is shifted by an amount equal to the mass of the substrate less a water molecule.[4]

The PPant ejection assay can greatly facilitate the characterization of NRPS and PKS systems by mass spectrometry, but demands instruments capable of both high sensitivity and high accuracy. Indeed, PPant ejection was originally observed on a custom-built Fourier transform ion cyclotron resonance (FTICR)-MS that utilized an accumulation octopole to collect ions before transmission to the FTICR mass analyzer.[5] However, commercial FTICR-MS instruments may not produce peaks sufficiently intense for reliable detection of the ejected PPant species. Conversely, ion trap mass

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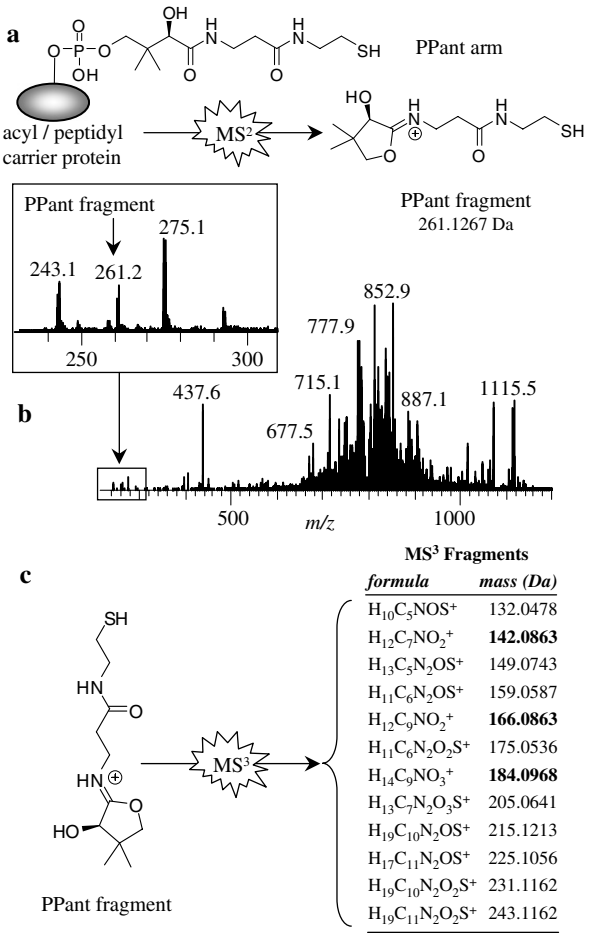

Figure 1. The MS^3 phosphopantetheine fragmentation method. (a) MS^2 experiments on *holo*-carrier proteins result in phosphopantetheine (PPant) ejection.[2] (b) MS^2 on the predicted 16+ charge state (*m*/*z* 796) of *holo*-AcpC produced a peak at *m*/*z* 261.2, which is consistent with the PPant fragment. (c) MS^3 on this PPant fragment gave 12 peaks, which may be useful to confirm the mass of a substrate linked to the PPant arm. The masses shown in bold should be substrate-independent, because the corresponding proposed formulas do not include the sulfur atom necessary to bind the substrates.

spectrometers, while typically more sensitive and capable of higher scan rates than FTICR instruments, do not always provide sufficient accuracy and signal-to-noise ratio to confirm the elemental composition of the PPant ejection peak.

These limitations became evident when we applied the PPant ejection assay on a Finnigan LTQ MS to characterize AcpC (Accession No. NP_735098), a putative 12.4-kDa acyl carrier protein encoded by the fourth open-reading frame in the *cyl* gene cluster of the bacterial pathogen *Streptococcus agalactiae*, or group B *Streptococcus* (GBS).[6] The *cyl* gene cluster of GBS is involved in the production of the β-hemolytic/cytolytic activity that contributes to the virulence of GBS, a major cause of infant mortality in industrialized countries. Remarkably, the structure of the final molecule produced by the *cyl* gene cluster is still unknown.[7] Because disruption of the *acpC* gene by transposon mutagenesis has suggested that AcpC is essential for hemolytic phenotype of GBS,[6] we decided to study this protein. To begin our investigation, we expressed an AcpC-His_6 fusion construct in *Escherichia coli* and enriched the protein by Ni–NTA affinity chromatography.[16] The resulting sample was subjected to nano-electrospray ionization (ESI) and collision-induced dissociation (CID) of the predicted 16+ charge state (*m*/*z* 796) in the linear ion trap, yielding a clearly discernible peak at *m*/*z* 261.2 (Fig. 1b).[17] This peak matches the mass of the MS^2 PPant fragment, suggesting that AcpC is a carrier protein and that AcpC can be phosphopantetheinylated by a PPant transferase (PPTase) naturally occurring in *E. coli*. However, the peak at *m*/*z* 261.2 was much weaker than the other peaks present in the MS^2 spectrum and the accuracy of the ion trap mass analyzer was not sufficient for unequivocal assignment of this peak to the PPant fragment.

We therefore investigated whether subjecting the putative MS^2 PPant fragment of *holo*-AcpC to another round of fragmentation would yield a more informative mass spectrum. Indeed, we observed a rich pattern of MS^3 peaks that appeared to be uniquely associated with the ejected PPant arm (Fig. 1c).[18] We reasoned that this fragmentation pattern could be generally exploited as a diagnostic tool to detect the presence of carrier proteins bearing the PPant arm and to determine the mass of any PPant-linked species. Hence, we developed an alternative top-down method for the unambiguous characterization of substrates and intermediates bound to carrier proteins of PKS and NRPS biosynthetic pathways.

The likely molecular formulas for 12 of the observed MS^3 PPant ions (Fig. 1c) indicate that nine of the fragments, whose calculated *m*/*z* values are 132.0, 149.1, 159.1, 175.1, 205.1, 215.1, 225.1, 231.1, and 243.1, contain the PPant thiol group, which bears the substrate during the biosynthesis of natural products. Loading a substrate onto the PPant arm would then be expected to shift these thiol-containing MS^3 fragments to higher masses, but should leave the mass of the other three MS^3 fragments unchanged at the calculated *m*/*z* values of 142.1, 166.1, and 184.1. The latter MS^3 ions provide a diagnostic pattern for detecting PPant ejection, while the nine thiol-containing MS^3 ions can be used to infer the mass of the loaded substrate. The *m*/*z* value of the MS^2 PPant peak resulting from fragmentation of a given carrier protein, as well as the *m*/*z* values of the MS^3 peaks resulting from fragmentation of the PPant fragment itself, can all be calculated a priori from the theoretical masses listed in Figure 1c and from the mass of the given substrate.[8]

To evaluate our approach and to optimize the instrumental settings, CouN5, a 11.8-kDa peptidyl carrier protein from the coumermycin A_1 NRPS biosynthetic pathway of *Streptomyces rishiriensis* DSM 40489,[9] was used as a model system, because CouN5 is known to produce an abundant MS^2 PPant fragment ion. *Holo*-CouN5 was prepared by incubation of *apo*-CouN5[19] with CoA and Sfp (Accession No. P39135), a 26.1-kDa promiscuous PPTase from the surfactin