

Facile Detection of Acyl and Peptidyl Intermediates on Thiotemplate Carrier Domains via Phosphopantetheinyl Elimination Reactions during Tandem Mass Spectrometry[†]

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ABSTRACT: With the emergence of drug resistance and the genomic revolution, there has been a renewed interest in the genes that are responsible for the generation of bioactive natural products. Secondary metabolites of one major class are biosynthesized at one or more sites by ultralarge enzymes that carry covalent intermediates on phosphopantetheine arms. Because such intermediates are difficult to characterize in vitro, we have developed a new approach for streamlined detection of substrates, intermediates, and products attached to a phosphopantetheinyl arm of the carrier site. During vibrational activation of gas-phase carrier domains, facile elimination occurs in benchtop and Fourier-transform mass spectrometers alike. Phosphopantetheinyl ejections quickly reduce >100 kDa megaenzymes to <1000 Da ions for structural assignment of intermediates at <0.007 Da mass accuracy without proteolytic digestion. This “top down” approach quickly illuminated diverse acyl intermediates on the carrier domains of the nonribosomal peptide synthetases (NRPSs) or polyketide synthases (PKSs) found in the biosynthetic pathways of prodigiosin, pyoluteorin, mycosubtilin, nikkomycin, enterobactin, gramicidin, and several proteins from the orphan *pksX* gene cluster from *Bacillus subtilis*. By focusing on just those regions undergoing covalent chemistry, the method delivered clean proof for the reversible dehydration of hydroxymethylglutaryl-*S*-PksL via incorporation of ²H or ¹⁸O from the buffer. The facile nature of this revised assay will allow diverse laboratories to spearhead their NRPS–PKS projects with benchtop mass spectrometers.

Approximately 50% of today's drugs and 75% of today's antimicrobials are derived from secondary metabolites (1, 2). Many of those secondary metabolites are of polyketide or nonribosomal peptide origin. With the emergence of resistance and the genomic revolution, there is a “renaissance” ongoing in the discovery of bioactive natural products and the characterization of the genes responsible for their

production (1). Nonribosomal peptide synthetases (NRPSs)¹ and polyketide synthases (PKSs) are large enzymes (often >>100 kDa) that biosynthesize their natural products (e.g., the antibiotics penicillin and vancomycin) via covalent intermediates on phosphopantetheine arms (3). Currently, even when NRPS and PKS proteins can be overproduced, their direct interrogation by mass spectrometry (MS) is difficult and time-consuming, with reports from relatively few laboratories appearing in the primary literature (4–6). Therefore, the NRPS and PKS community would greatly benefit if new MS-based methods for characterizing these proteins were developed and easier to implement. This paper describes such a method.

The purpose of this paper is fourfold. (1) It introduces a new and efficient method that utilizes a gas-phase elimination reaction that takes place during tandem mass spectrometry

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¹ Abbreviations: ESI/FT-ICRMS, electrospray ionization Fourier-transform ion cyclotron resonance mass spectrometry; MS, mass spectrometry; MS/MS, tandem mass spectrometry; PKS, polyketide synthase; NRPS, nonribosomal peptide synthetase; IRMPD, infrared multiphoton dissociation; CAD, collisionally activated dissociation; ACP, acyl carrier protein; 2ACP, tandem acyl carrier proteins; PCP, peptidyl carrier protein; HMG, hydroxymethylglutaryl; Acac, acetoacetyl; HPLC, high-pressure liquid chromatography; TFA, trifluoroacetic acid; ACN, acetonitrile.

(MS/MS) to quickly characterize substrates, intermediates, and products that are loaded onto the phosphopantetheinyl arm on carrier domains of NRPS and PKS systems. (2) The paper provides a mechanistic rationale for the phosphopantetheinyl eliminations that are utilized in this assay. (3) The paper demonstrates that the phosphopantetheinyl ejections can be performed not only on peptides but also on intact proteins. Tandem mass spectrometry on intact proteins is also known as the "top down" approach (7). (4) Finally, we show that the method is readily adapted to benchtop mass spectrometers, making it useful to researchers that do not have access to high-end MS instrumentation.

We provide support for the robustness and generality of this assay via 33 examples as this approach was used to characterize the loading of substrates, intermediates, and products of proteins involved in the biosynthesis of the antibiotic nikkomycin (8), the antifungal agent pyoluteorin (9, 10), the antibiotic prodigiosin (11, 12), the antibacterial agents gramicidin (13, 14), and mycosubtilin (15, 16), and the siderophore enterobactin (17, 18). Further, intermediates were detected via this gas-phase elimination on four proteins from the orphan *pksX* cluster of *Bacillus subtilis* that produces an unknown antibiotic (19–21). In addition, we show clear evidence for the rehydratase activity of PksH via stable incorporation of the isotope from the solvent, an experiment greatly streamlined by observing just that portion of the protein substrate undergoing chemistry. PksL (28 kDa), NikP1 (74 kDa), and GrsA (127 kDa) are used to show the extensibility of this assay format to top down experiments that circumvent the need for proteolysis before mass spectrometry.

RESULTS AND DISCUSSION

Using electrospray ionization Fourier-transform ion cyclotron resonance mass spectrometry (ESI/FT-ICRMS), thio-template-bound substrates and intermediates of NRPS and PKS systems have been visualized (9, 12, 16, 18, 19, 23, 24). The FT-ICRMS assay has recently been adapted into a tool for substrate screening, but the mass accuracy has been between 0.05 and 0.5 Da and required, in many cases, digestion and active site mapping prior to the analysis (25). Detection of key peptides carrying acyl intermediates by peptide mapping is one of the rate-limiting steps in the characterization of thio-template enzymes by mass spectrometry (23). Using tandem mass spectrometric methods, such as infrared multiphoton dissociation (IRMPD) (26) and collisionally activated dissociation (CAD) (27), we observed two reactions that result from the elimination of the phosphopantetheinyl functionality of carrier domains found on NRPSs and PKSs. As an example, subjecting the 12+ charge state of pyrrolyl-S-PltL to IRMPD resulted in two high-abundance 11+ fragment ions. These 11+ ions correspond to the apoprotein –18 Da or the apoprotein + 80 Da (Figure 1A), and their 1+ complementary ions were also observed. These ion pairs sum to the intact mass of the precursor ions and represent the dominant dissociation channels observed in the experiment. Thus, 12+ precursor ions of an intact protein generated primarily 11+ and 1+ fragment ions, along with minor product ions from amide bond cleavage. The fragmentation of pyrrolyl-S-PltL, as a result of IRMPD, creates two complementary ion pairs. The observed masses

for the eliminated products are 354.156 and 452.132 Da and agree with the calculated ions to within 0.007 Da (Figure 1B,C).

Similar results were obtained when monochloropyrrolyl-S-PltL, dichloropyrrolyl-S-PltL, and dibromopyrrolyl-S-PltL (9) were subjected to tandem mass spectrometry (Figure 1D,E). These results also show that this approach can be used for the direct visualization of species that have distinctive isotopic signatures without the need for protein expression in ^{13}C , ^{15}N doubly depleted media (9). As an example, the chloropyrrolyl-S-PltL and dichloropyrrolyl-S-PltL phosphopantetheinyl ejection ion show the A + 2 isotope that comes from ^{37}Cl (Figure 1D,E) which comprises 24% of the chlorine in nature. In addition, the ejection of phosphopantetheinyl from dibromopyrrolyl-S-PltL allowed the direct observation of the +2 and +4 isotopes that come from the incorporation of one and two ^{81}Br atoms when they were subjected to tandem mass spectrometry (Figure 2). The natural abundance of ^{81}Br is 49% of the total natural bromine isotopic content, while it is 51% for ^{79}Br . The isotopic pattern observed in the dibromopyrrolyl elimination reaction is quite similar to the predicted isotopic pattern (Figure 2).

There are two main mechanisms that can be invoked for the elimination of the phosphopantetheinyl functionality to generate a mass consistent with the apo form of the protein that has lost 18 Da and a charge. Both of these mechanisms give a product of identical mass and charge. The first mechanism involves a rearrangement in which the α -carbon of serine is deprotonated and a rearrangement takes place as shown in Figure 3A. The resulting protein ions have a mass shift from the apo form of the protein with a loss of 18 Da via the generation of a dehydroalanine and would experience a reduction in charge. In this paper, we refer to this 1+ ion product as PPant (Figure 3A). The second proposed mechanism is analogous to the elimination of a phosphate from a per-protonated phosphorylated serine or threonine during collisionally activated dissociation as recently proposed (28). During this elimination, an oxazolium-containing protein ion is produced that also has a mass shift of the apo peptide –18 Da (Figure 3B).

The second major type of phosphopantetheinyl elimination leaves a phosphate functionality on the protein but also involves the loss of one charge from an ejected 1+ ion. The mechanism that accounts for this is shown in Figure 3C. Following the elimination reaction, a phosphate anion is left behind and the carbonyl of an amide displaces the pantetheinylate moiety, forming a five-membered ring with a protonated imine that is positively charged (Figure 3C). In this paper, we abbreviate this ejected ion as Pant.

To demonstrate that this elimination reaction can also be used to observe small mass changes with high mass accuracy, the ACP domain from MycA (13.4 kDa) on the mycosubtilin pathway loaded with either acetoacetate or β -aminobutyrate that was generated using an in cis aminotransferase activity was investigated (16). These two species have a calculated mass difference of 1.032 Da. Following digestion and separation of the ACP domain, the latter was subjected to MS/MS using IRMPD. If the region below m/z 600 is examined, the ions of the phosphopantetheinyl functionality loaded with β -aminobutyrate gave +1 ion signals at m/z 346.180 and 444.154 (Figure 4B,D). The calculated theoretic-

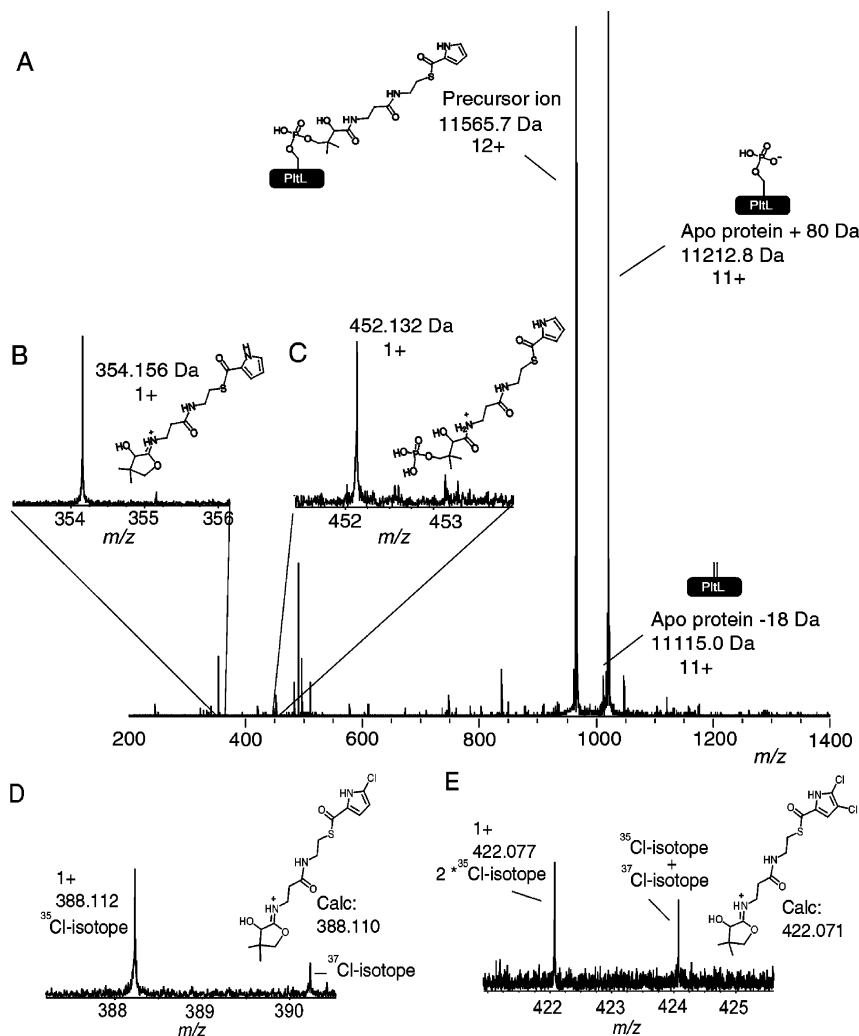


FIGURE 1: Phosphopantetheinyl elimination fragments observed during tandem mass spectrometry. (A) Pyrrolyl-S-PitL subjected to tandem mass spectrometric method IRMPD. (B) Enlargement of the mass spectral region that corresponds to the smaller +1 pantetheinyl fragment ion. (C) Enlargement of the mass spectral region that corresponds to the smaller +1 phosphopantetheinyl fragment ion. (D) Elimination fragment of monochloropyrrolyl-S-PitL. (E) Elimination fragment of dichloropyrrolyl-S-PitL. The protein-derived ions are reported as the neutral monoisotopic mass, while the PPant-eliminated ions are reported as the +1 monoisotopic mass. The remaining signals in the m/z 450–500 region are frequency spikes or amide backbone cleavage of the protein itself.

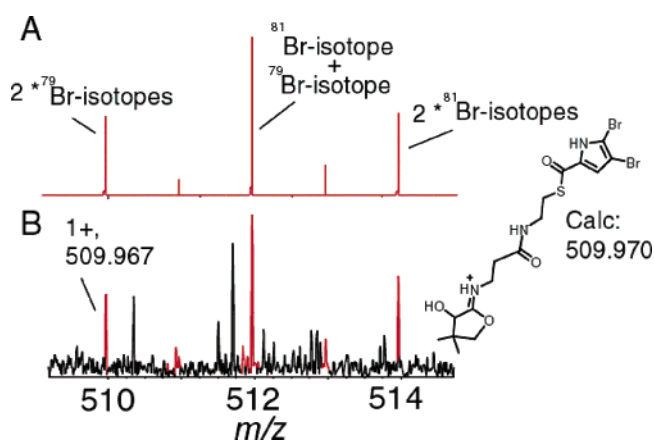


FIGURE 2: (A) Theoretical isotopic distribution of the elimination ion of dibromopyrrolyl-S-PitL. (B) Elimination ion of dibromopyrrolyl-S-PitL.

cal masses for the eliminated products ions are 346.180 and 444.157 Da and are within 0.003 Da (7 ppm) of the observed values. In the case of MycA loaded with acetoacetate, the

eliminated fragments that were observed correspond to mass values of 345.151 and 443.123 Da that are within 0.003 Da of the calculated masses (345.148 and 443.125 Da, respectively; Figure 4A,C). The mass difference between the acylated form and the calculated holo form was 84.021 Da. On the basis of the accurate mass search as described in the Experimental Procedures, the best molecular formula that could be assigned was $C_4H_4O_2$ and is in agreement with the addition of the acetoacetyl substituent on the phosphopantetheinyl functionality as it matched within 5 ppm. This confirms that sufficient mass accuracy is available for assignment of an empirical formula to the acylated species, particularly important for discovery applications where structural elucidation of bound species is required.

In this paper, the PPant and/or Pant ejection ions for 33 different acyl-S-carrier domains are provided, and they are displayed in Table 1 and Figure 5. In Table 1, the elimination fragments ions are shown for pyrrolyl-S-PigG, pyrrolyl- β -ketoacyl-S-PigH-ACP₁, and pyrrolyl- β -ketoacyl-S-PigH-2ACP (12), the ACP and PCP domains of the four-domain construct MycA loaded with acetoacetate, other β -ketoacids

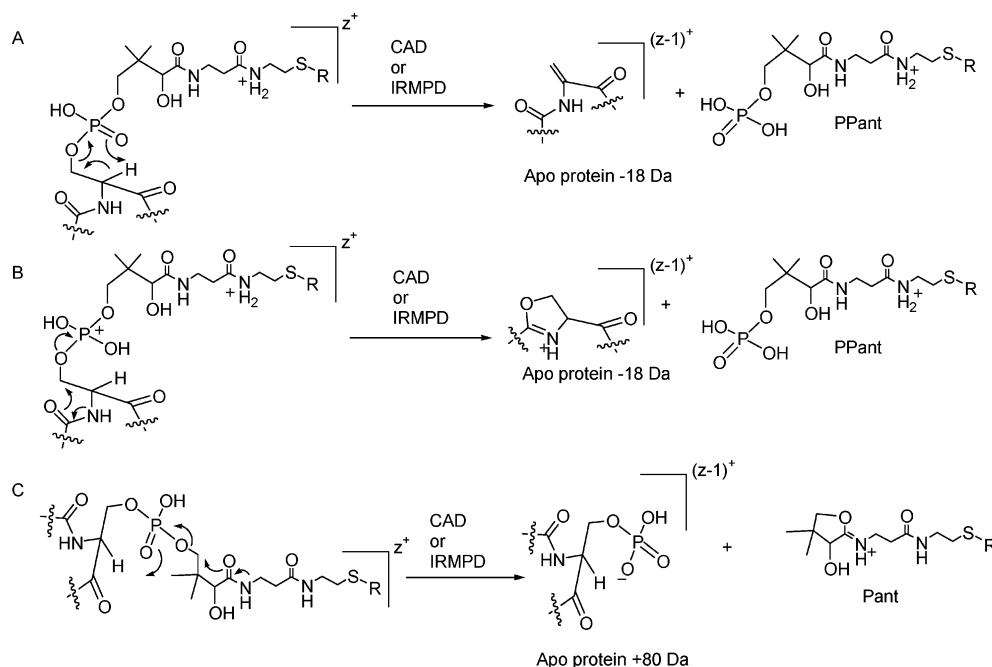


FIGURE 3: Different mechanistic proposals for the two major types of eliminations of the phosphopantetheinyl functionality during tandem mass spectrometry to generate the ejection ions with a charge of +1. R is H or an acyl substituent. z is the charge. In panels A and B, we show that the amide on the right side of the +1 ejection ion is protonated, and it is also possible that the other amide is protonated (37).

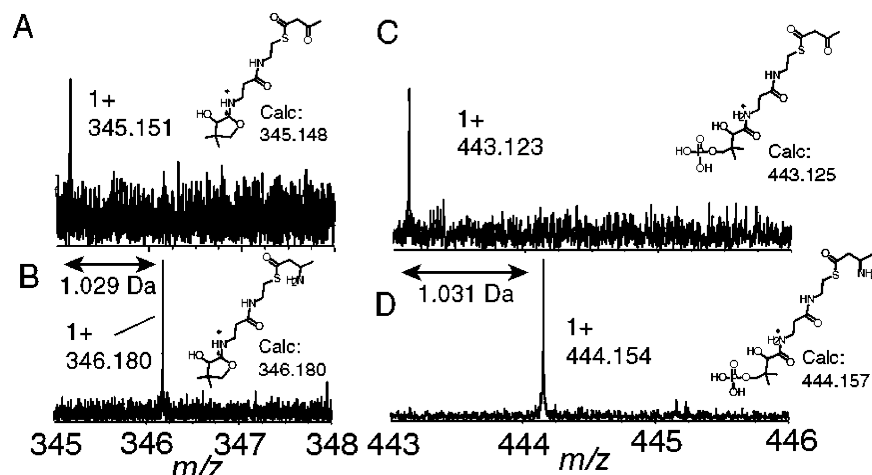


FIGURE 4: Phosphopantetheinyl fragment ions observed on ACP₂ of MycA upon incubation of acetoacetyl-S-MycA-ACP₂ with Gln. (A and C) Two phosphopantetheinyl elimination reactions of acetoacetyl-S-MycA-ACP₂. (B and D) Two phosphopantetheinyl elimination reactions of acetoacetyl-S-MycA-ACP₂ treated with glutamine to form β-aminobutyryl-S-MycA-ACP₂.

and the corresponding-β-aminobutyrate (16), pyrrolyl-S-PltL, monochloropyrrolyl-S-PltL, dichloropyrrolyl-S-PltL, dibromopyrrolyl-S-PltL (9), seryl-S-PksN (25), phenylacetyl-S-PksJ (29), acetoacetyl-S-AcpK, acetyl-S-AcpK, acetoacetyl-S-PksL-2ACP, hydroxymethylglutaryl-S-PksL-2ACP, dehydromethylglutaryl-S-PksL-2ACP, ²Δ-isoprenyl-S-PksL-2ACP (19), and dihydroxybenzoyl-S-EntB(ArCP) (17, 25). To determine if larger intact enzymes could be examined by the top down approach, the 74 kDa protein NikP1 on the nikkomycin biosynthetic pathway (13, 25) and the undigested 127 kDa phenylalanyl-S-GrsA or D5-deutero phenylalanyl-S-GrsA were analyzed (13, 14). In both these cases, one or both elimination products were observed with high mass accuracy. At this point, we do not know why in some of the cases one of the ejection products is observed but not the other, nor do we know what influences this. Below, we

highlight some of the experiments to emphasize the utility of this assay.

Recently, we were able to observe the loaded form of the undigested NikP1 by ESI-ICRFTMS by observing the increase in mass from 74356 ± 11 to 74498 ± 01 Da upon addition of histidine (13). This is a 142 ± 11 Da increase, in agreement with the expected mass increase of 137.059 Da for histidine. However, the mass increase is not very accurate because the protein did not have resolved isotopes. Performing IRMPD on the intact protein gave rise to a 496.164 Da fragment ion corresponding to the eliminated PPant that was within 2 ppm of the calculated mass. This ion is 137.060 Da larger than the calculated holo form (359.104 Da) and is within 0.001 Da of the calculated mass shift for histidine. A second intact construct that was analyzed in this fashion was the 127 kDa protein GrsA. Loading of

Table 1: Observed Masses of the Phosphopantetheinyl-Eliminated 1+ Fragment Ions

protein investigated	pathway	digestion method	fragmentation method	R ^a	elimination ion A ^b (calcd)	elimination ion B ^b (calcd)
PigH(ACPI)	prodigiosin	trypsin	OCAD	A	347.129 (347.128)	ND ^c
PigH(ACPI)	prodigiosin	trypsin	OCAD	B	396.161 (396.159)	ND ^c
PigH-2ACP	prodigiosin	not digested	IRMPD	A	347.130 (347.128)	ND ^c
PigH-2ACP	prodigiosin	not digested	IRMPD	B	396.161 (396.159)	ND ^c
PigG	prodigiosin	not digested	IRMPD	C	354.152 (354.149)	ND ^c
PltL	pyoluteorin	not digested	IRMPD	C	354.156 (354.149)	452.132 (452.126)
PltL	pyoluteorin	not digested	IRMPD	D	388.112 (388.110)	ND ^c
PltL	pyoluteorin	not digested	IRMPD	E	422.077 (422.071)	520.050 (520.048)
PltL	pyoluteorin	not digested	OCAD	F	509.967 (509.970)	ND ^c
MycA (ACP ₂)	mycosubtilin	trypsin	OCAD	G	345.151 (345.148)	443.124 (443.125)
MycA (ACP ₂)	mycosubtilin	trypsin	OCAD	H	346.180 (346.180)	444.154 (444.157)
MycA (ACP ₂)	mycosubtilin	trypsin	OCAD	I	ND ^c	445.150 (445.154)
MycA (PCP ₁)	mycosubtilin	trypsin	IRMPD	J	ND ^c	555.253 (555.251)
MycA (PCP ₁)	mycosubtilin	trypsin	IRMPD	K	ND ^c	557.283 (557.279)
MycA (PCP ₁)	mycosubtilin	trypsin	IRMPD	M	ND ^c	499.190 (499.188)
MycA (PCP ₁)	mycosubtilin	trypsin	IRMPD	N	ND ^c	501.219 (501.217)
MycA (PCP ₁)	mycosubtilin	trypsin	IRMPD	G	ND ^c	443.128 (443.125)
MycA (PCP ₁)	mycosubtilin	trypsin	IRMPD	K	ND ^c	445.155 (445.154)
AcpK	orphan	not digested	IRMPD	G	345.153 (345.148)	443.131 (443.125)
AcpK	orphan	not digested	IRMPD	N	ND ^c	401.119 (499.201)
PksN	orphan	trypsin	IRMPD	O	ND ^c	446.143 (446.136)
PksJ (A1)	orphan	trypsin	IRMPD	P	ND ^c	477.150 (477.146)
PksL-2ACP	orphan	not digested	IRMPD	G	345.151 (345.148)	443.127 (443.125) ^d
PksL-2ACP	orphan	not digested	IRMPD	Q	405.170 (405.170)	503.149 (503.146) ^d
PksL-2ACP	orphan	not digested	IRMPD	R	343.171 (343.170)	441.147 (441.146) ^d
PksL-2ACP	orphan	not digested	IRMPD	S	407.173 (407.174)	ND ^c
PksL-2ACP	orphan	not digested	IRMPD	T	406.176 (407.176)	ND ^c
PksL-2ACP	orphan	not digested	IRMPD	U	387.162 (387.159)	485.139 (485.136)
NikP1	nikkomycin	cnbr	OCAD	V	ND ^c	496.164 (496.163)
NikP1	nikkomycin	not digested	IRMPD	V	ND ^c	496.163 (496.163)
EntB	enterobactin	not digested	IRMPD	W	ND ^c	495.118 (495.120)
GrsA	gramicidin	not digested	IRMPD	X	408.198 (408.196)	506.179 (506.173)
GrsA	gramicidin	not digested	IRMPD	Y	413.231 (413.227)	511.212 (511.201)

^a The structures representing R on the elimination ions are shown in Figure 5. ^b Structures of Pant and PPant are shown in Figure 4. ^c Not detected. ^d Values from ref 19.

holo GrsA with phenylalanine resulted in a mass of 126 714 ± 15 Da, and the protein loaded with D5-deuterated phenylalanine had a mass of 126 726 ± 12 Da and therefore would require several measurements or digestion to determine with confidence which of the proteins was loaded with D0 or D5-phenylalanine. Subjecting the protein to IRMPD, however, showed the elimination fragments had a mass difference of 5.033 Da, in agreement with the 5.029 Da calculated difference between the two species (Figure 6, right). Such use of stable isotopes confirms the identity of a substrate or can assist in probing mechanisms.

In addition, this method can be used to investigate the incorporation of stable isotopes for mechanistic studies, even those using D₂O or H₂¹⁸O. This is demonstrated by a reaction of hydroxymethylglutaryl-S-PksL-2ACP (HMG-S-PksL-2ACP) with PksI or PksH. PksH and PksI are proteins from an orphan gene cluster in *B. subtilis* that are involved in the dehydration and decarboxylation of HMG-S-PksL-2ACP to produce ²Δ-isoprenyl-S-PksL-2ACP (scheme in Figure 7A) (16). PksH appears to be the dehydratase, while PksI catalyzes the decarboxylation. Similar findings were recently obtained with the PksH and PksI homologues on the curacin system (24). Incubation of HMG-S-PksL-2ACP with PksI resulted in no reaction. When HMG-S-PksL-2ACP was incubated with PksH, a partial dehydration product was observed. Because complete conversion to ²Δ-isoprenyl-S-PksL-2ACP was observed when both PksI and PksH were added, it was possible that the PksH reaction was reversible. To test this hypothesis, a racemic mixture of HMG-S-PksL-

2ACP was prepared by incubating hydroxymethylglutaryl-CoA with apo-PksL-2ACP and the phosphopantetheinyl-transferase Sfp. This was incubated with either PksI or PksH in 70% H₂¹⁸O or D₂O and subjected to mass spectrometry and IRMPD. If the reaction was reversible, incorporation of a deuterium and water from the buffer was anticipated (Figure 7B,C). Because a racemic mixture of HMG-S-CoA was used to prepare HMG-S-PksL-2ACP, it was anticipated that PksH would only function on one isomer, and therefore, a maximum of 50% of the total HMG-S-PksL-2ACP could serve as a substrate for PksH.

From the intact mass of HMG-S-PksL-2ACP (a 28 kDa species), it could not be unambiguously shown that partial ¹⁸O was incorporated even though we are using FT-ICRMS to detect the intact ions. Therefore, the ions were subjected to IRMPD, and the phosphopantetheinyl elimination reaction was analyzed. The elimination reaction in the presence of PksH in ¹⁸O buffer showed about 33% incorporation of ¹⁸O into HMG-S-PksL-2ACP (Figure 7D). We feel that quantifying the relative signals in this case is warranted, as both species should have similar ionization efficiencies and elimination reaction rates; however, if the substrates on the phosphopantetheinyl functionality differ dramatically, the relative ionization efficiencies must be established when quantification is desired. The observed mass of 407.173 Da agreed well with the predicted mass of 407.174 Da. Because PksH selectively dehydrates one of the two isomers of HMG-S-PksL-2ACP, it was anticipated that we could have a maximum of 35% incorporation of ¹⁸O; therefore, the

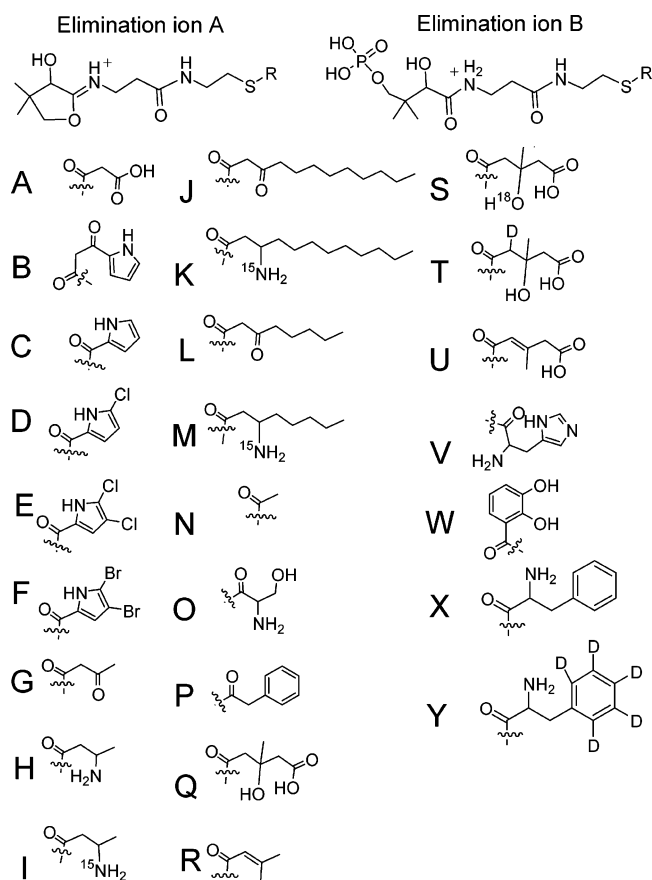


FIGURE 5: Structures of the R groups in Table 1 of the phosphopantetheinyl +1 fragment ions.

incorporation of ^{18}O appears to be nearly complete. Similarly, when the PksH reaction was repeated but this time in D_2O , an estimated 33% of the HMG-S-PksL-2ACP contained a deuterium as evidenced by a mass shift of 1.007 Da, again in agreement with the maximum of 35% deuterium incorporation that could be obtained for this system (Figure 7D). This supports the hypothesis that PksH catalyzes the dehydration as well as rehydration reaction while PksI does not recognize either of the isomers of HMG-S-PksL-2ACP as a substrate. On the other hand, when both PksI and PksH are added to HMG-S-PksL-2ACP, we see a loss of 62.002 Da (Table 1) (19). These results are in agreement with the reversible dehydration reaction catalyzed by PksH and decarboxylation catalyzed by PksI, results that cannot be obtained using the standard FT-ICRMS assay format with a 28 kDa protein, in particular when only partial conversion is observed, and provide an example of the type of studies that can be performed using this assay format.

In this paper, we describe the use primarily of a FT-ICRMS instrument because of its superior mass accuracy and it is the main instrument in our laboratory, but the assay is also applicable to non-FT-ICRMS instruments. In one of the original studies of NRPS systems by mass spectrometry, the elimination ions were observed using MS/MS of small peptides (<3 kDa) on a triple quadrupole instrument, but this process was not utilized as an assay to observe and characterize the acylated substrates loaded onto the phosphantetheine arm with high mass accuracy (30, 31). The results of these early observations provided an indication that this assay can be performed on smaller peptide fragments

from digests with low-resolution instruments but provided no indication that it could be accomplished with intact proteins rather than only digested samples. If this top down assay can be transferred to other less expensive and more common mass spectrometric instruments, it would make it a much more general assay to study NRPS and PKS biosynthetic pathways. To demonstrate the top down approach on a non-FTMS instrument, the phosphopantetheinyl ejection assay was repeated using a linear ion trap (LTQ) instrument, which is a very common benchtop MS instrument. Subjecting holo AcpK to trap isolation and CAD resulted in fairly abundant fragment ions at m/z 261.1 and 359.1. These are in agreement with the calculated masses of 261.127 and 359.104 Da, respectively, for the phosphopantetheinyl ejections (Figure 8C). Similarly, the phosphopantetheinyl ejection of the 28 kDa HMG-S-PksL-2ACP resulted in a new and very abundant peak at m/z 405.2, in agreement with the expected mass of 405.170 Da (Figure 8F). These two experiments establish that the LTQ, as a representative benchtop instrument, can be used to generate the eliminated ions from intact proteins as well.

To establish that the ion trap MS instrument could also be used to monitor small mass differences such as the 2 Da differences generated by the conversion of Acac-S-PksL-2ACP to isoprenyl-S-PksL-2ACP, HMG-S-PksL-2ACP was incubated with PksI, PksH, or both PksI and -H. When HMG-S-PksL-2ACP (prepared via the incubation of Ac-S-AcpK, PksG, and Acac-S-PksL-2ACP) was incubated with PksI, masses of 345.2 (Pant elimination of unreacted acetoacetyl) and 405.2 Da were observed (Figure 9A). Upon addition of PksH to HMG-S-PksL-2ACP, a new mass at 387.1 Da was observed (Figure 9B). The observation of the low level of steady-state dehydration using the LTQ showed a similar amount of the dehydrated species upon addition of PksH when compared to the FT-ICRMS results (Figure 9B, inset). This provides an indication that similar results are obtained using the LTQ when compared to those from FT-ICRMS. When both PksI and PksH were added, a new mass of 343.2 Da emerged that is 2 Da smaller than the eliminated ion from Acac-S-PksL-2ACP, consistent with the formation of an isoprenyl-S-PksL-2ACP product (Figure 9C). Panels D and E of Figure 9 are a direct comparison between the same phosphopanteteinyl ejection ion from HMG-S-PksL-2ACP as observed by the low-resolution LTQ instrument and the high-resolution FT-ICRMS experiment which shows that the FT-ICRMS has a significant advantage in resolution and mass accuracy. These comparisons do not invalidate the utility of the LTQ as a tool as there are very few instances where one might need to have a mass accuracy of ≤ 0.007 Da; in the majority of instances, a mass accuracy of 0.1 Da as obtained with the LTQ is sufficient. When a low-resolution instrument is used for this assay and the signal is in doubt, one could resort to stable isotope incorporation studies to confirm and solidify the identity of the signal.

In summary, the direct mass spectral visualization of substrates and intermediates via a gas-phase elimination presented in this paper will be very useful for studying NRPS and PKS but can also be readily extended to the study of fatty acid biosynthetic pathways. It gives the masses of the substrates at the phosphopantetheinylated active sites with very high mass accuracy and is useful for several reasons. The first major implication of this assay is that it immediately

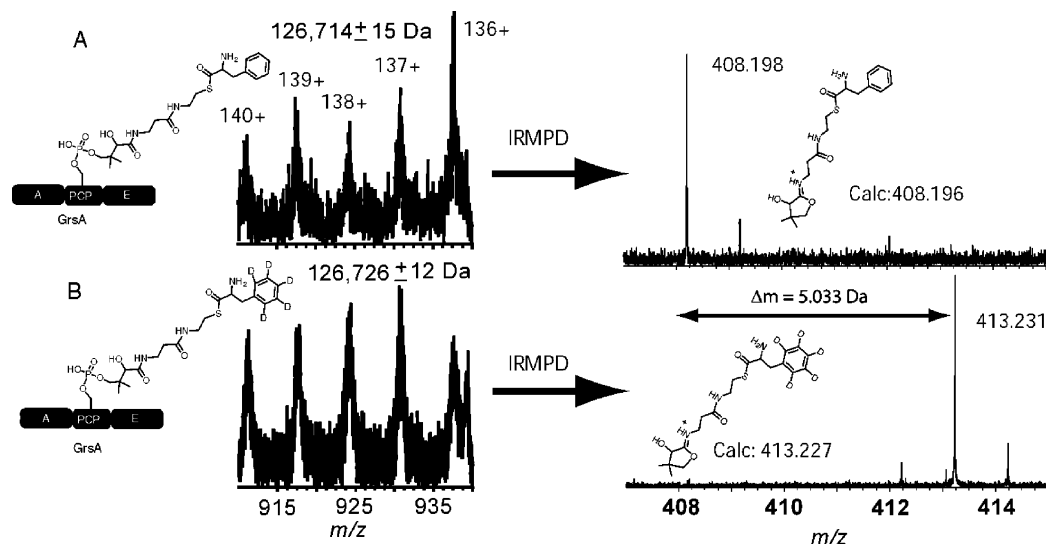


FIGURE 6: Phosphopantetheinyl elimination of D0-phenylalanyl-S-GrsA (A) compared to D5-phenylalanyl-S-GrsA (B). A denotes adenylation, PCP the peptidyl carrier domain, and E the epimerisation domain.

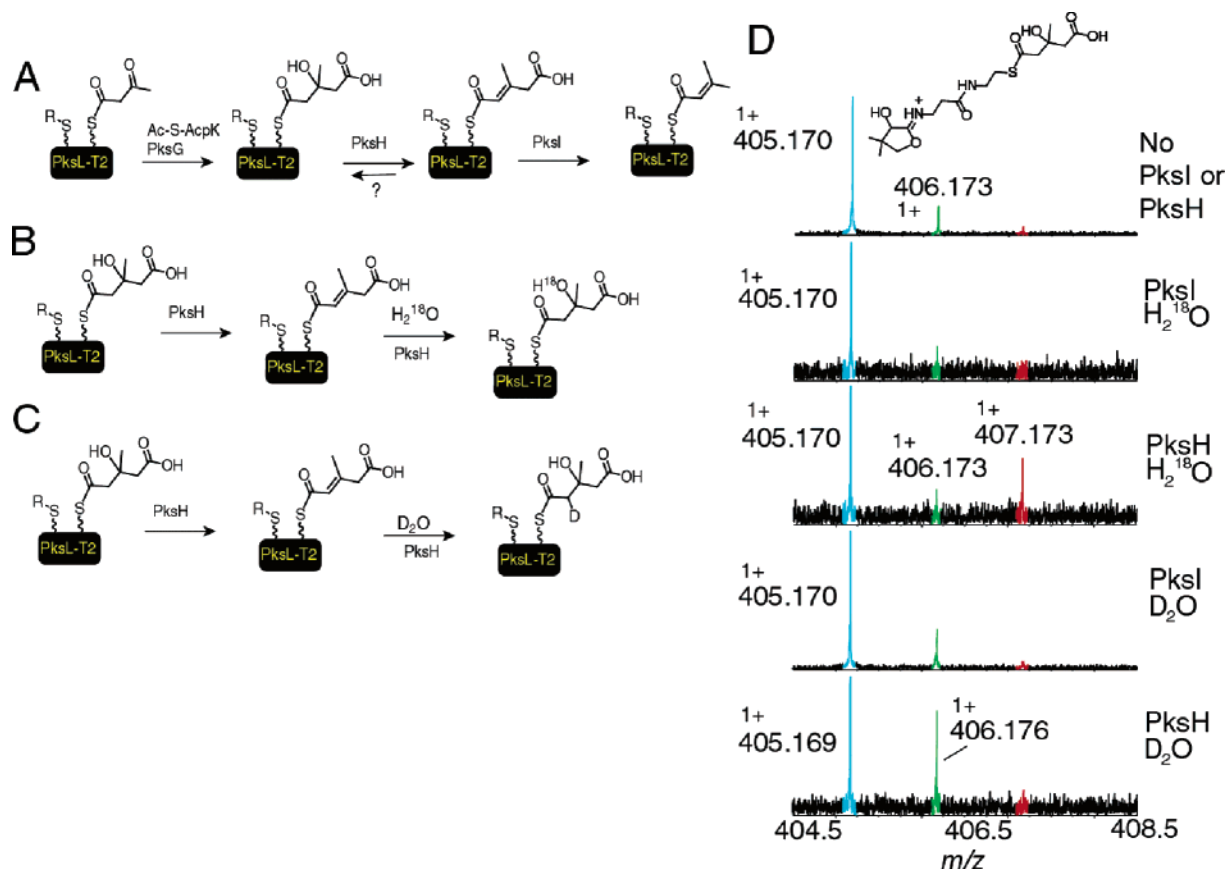


FIGURE 7: Rehydratase activity of PksH. (A) General overview of the conversion of Acac-S-PksL-2ACP to isoprenyl-S-PksL-2ACP as catalyzed by PksG, PksH, and PksI. For clarity, the chemistry on only one of the two carrier domains is shown. (B) Hypothesis for the hydroxyl exchange catalyzed by PksH in 70% H_2^{18}O . (C) Hypothesis for the deuterium exchange catalyzed by PksH in 70% D_2O . (D) Data showing the incorporation of ^{18}O and ^2H into the elimination fragment ion of HMG-S-PksL when the reaction was carried out in ^{18}O or ^2H buffers. PksI served as a negative control and demonstrates that the reaction was specific to PksH. The starting HMG-S-PksL-2ACP is generated from racemic HMG-CoA, and therefore, only 50% of this protein form could serve as a substrate.

confirms that the substrate or intermediate is linked to the phosphopantetheinyl functionality or not without the need for digestion. Such a confirmation cannot be accomplished by any other assay currently available to the NRPS and PKS community. It eliminates the possibility that the substrate or intermediate is somewhere else on the protein that may

be an in vitro artifact. Second, this phosphopantetheinyl ejection assay improves the speed at which activities of phosphopantetheinylated proteins can be characterized and in many cases will alleviate the need to hydrolyze the substrate or intermediate for characterization by HPLC or small molecule mass spectrometry. Third, if a substrate such

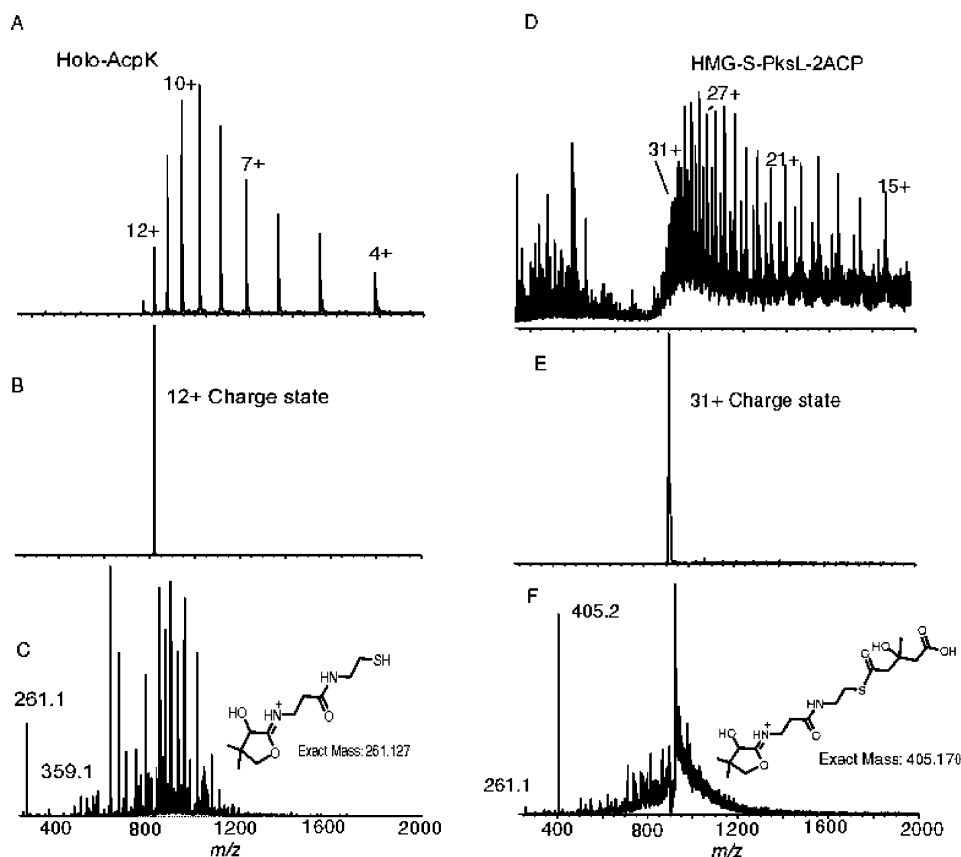


FIGURE 8: Phosphopantetheine ejection as observed with an ion trap instrument. (A) ESI/MS of holo AcpK (10.3 kDa). (B) Trap isolation of the +12 charge state. (C) CAD at 17% of the ions isolated in panel B. (D) Broadband spectrum of HMG-S-PksL-2ACP (28 kDa). (E) Trap isolation of the +31 charge state. (F) CAD at a normalized collision energy of 17% of the ions isolated in panel E. HMG-Pant is at m/z 405.2. Pant is at m/z 261.1.

as a halogen that has multiple isotopes is incorporated, the isotopic pattern can be directly verified. Fourth, it is possible to observe the direct incorporation of stable isotopes for biosynthetic studies. Finally, it is possible to confirm new activities such as the loading of phenylacetate on the first adenylation–thiolation module of PksJ. We project application of this MS/MS-based method to ever larger multidomain and even multimodular thiotemplate assembly lines of >200 kDa, and it is readily adapted to parent ion scanning (32, 33) and selected reaction monitoring for targeted detection of species at low levels (34).

EXPERIMENTAL PROCEDURES

Materials. The proteins SgcC2, SgcC1, SgcC4, Sfp, PigH, PigH-2ACP, PigJ, PigG, PigI, PigA, PltL, SsuE, PltA, GrsA, MycA (construct A4N7), AcpK, PksN, PksL-2ACP, PksI, PksH, NikP1, EntE, and EntB (ArCP) were obtained as described previously (9, 12, 16, 18, 19, 23). The first di-domain of PksJ with an N-terminal histidine tag was overproduced in *Escherichia coli* and also purified via nickel affinity chromatography (P. D. Straight, P. C. Dorrestein, N. L. Kelleher, and R. Kolter, unpublished results). Racemic HMG-CoA and all other chemicals were purchased from Sigma-Aldrich.

Digestion. When digestion was employed, it was achieved by cyanogen bromide or trypsin via protocols identical to those described previously (9, 12, 16, 18, 19, 23).

Purification of the Active Sites. Both the digested proteins and the undigested proteins (except GrsA) were subjected

to HPLC purification. The gradients used were water to acetonitrile gradients as described previously (9, 12, 16, 18, 19, 23). During the HPLC runs, the fractions containing the carrier domains were collected, frozen at -80°C , and lyophilized.

Preparation of GrsA Samples for Mass Spectrometry. For experiments involving the protein GrsA (PheATE), the holo protein was generated by incubating 500–600 μg of protein for 1 h in a reaction mixture containing 0.05 M Tris-HCl (pH 8), 0.01 M MgCl_2 , 0.002 M tris(2-carboxyethyl)-phosphine (pH 6), 200 μM coenzyme A, and 0.5 mg/mL Sfp (4 μL). After 1 h, the reaction mixture was supplemented with 0.01 M ATP and 0.01 M amino acid (L-phenylalanine or D5-L-phenylalanine) and allowed to incubate for an additional 30 min to generate loaded protein. Reaction mixtures were stored at -80°C until they were prepared for injection into the mass spectrometer. Immediately before being injected into the mass spectrometer, samples were prepared using C₄ ZipTips (Millipore). To prepare each sample, the ZipTip was washed five times with 10 μL of ACN and five times with 10 μL of a $\text{H}_2\text{O}/0.1\%$ TFA mixture. The sample was loaded onto the ZipTip by pipetting into the sample 10–15 times, each time injecting back into the reaction vial. The ZipTip was washed again eight times with 10 μL of a $\text{H}_2\text{O}/0.1\%$ TFA mixture, and the sample was eluted into 5 μL of a 78% ACN/2% acetic acid mixture. To prepare the sample for injection into the mass spectrometer, 25 μL of nanospray solution (49% MeOH, 49% H_2O , and 2% formic acid) was added to the eluted sample and the sample mixed thoroughly. The reported values of GrsA are

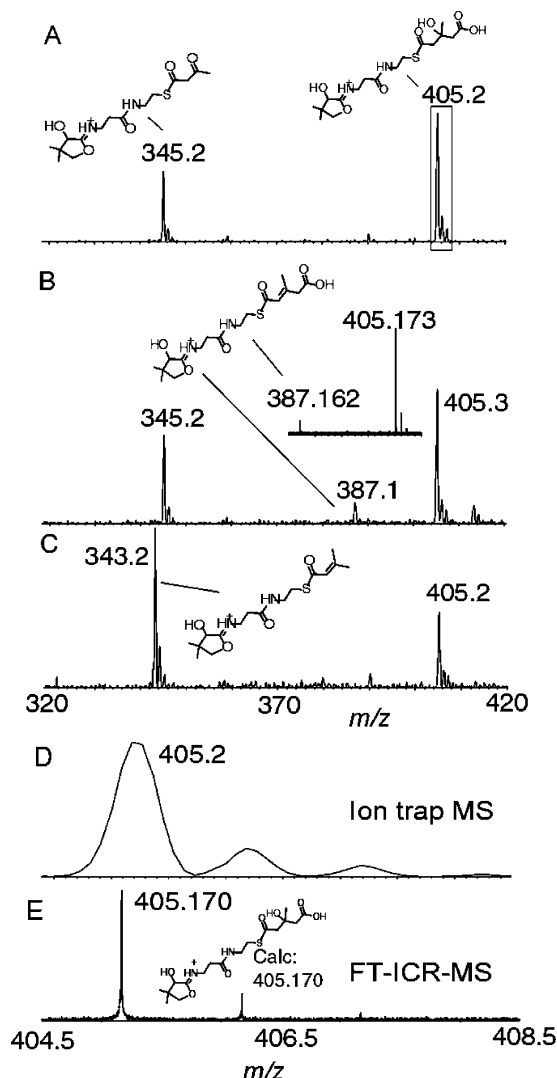


FIGURE 9: Low-resolution observation of the ejection ion for isoprenyl-S-PksL-2ACP as catalyzed by PksH and PksI. (A) Pant ejection of Acac-S-PksL-2ACP and HMG-S-PksL-2ACP following the incubation of Acac-S-PksL-2ACP with Ac-S-AcpK, PksG, and PksI. For comparison with FT-ICRMS data, the “boxed” data are enlarged in panel D. (B) Pant ejection of Acac-S-PksL-2ACP and HMG-S-PksL-2ACP following the incubation of Acac-S-PksL-2ACP with Ac-S-AcpK, PksG, PksH, and PksI. The inset shows the signal to show the similar level of dehydration observed by FT-ICRMS and using the LTQ. (C) Pant ejection of HMG-S-PksL-2ACP and isoprenyl-S-PksL-2ACP following the incubation of Acac-S-PksL-2ACP with Ac-S-AcpK, PksG, PksH, and PksI. (D) HMG-PksL-2ACP ejected ion enlarged from the boxed ion in panel A. (E) Same ion in panel D but generated and measured using FT-ICRMS. The normalized collision energy ranged from 15 to 30%.

the neutral most abundant masses, and the standard deviation is a result from the calculation of the mass at each different charge state.

FT-ICR Mass Spectrometry. The lyophilized fractions were resuspended in 50–400 μ L of electrospray solution (78% ACN and 0.1% acetic acid or 49% methanol and 1% formic acid). Usually, the 49% methanol, 1% formic acid solution was used with the larger protein/protein domains as this resulted in less unwanted fragmentation before the ions reached the cell. For mass spectrometric analysis, a custom 8.5 T ESI-FTMS mass spectrometer equipped with a front-end quadrupole was utilized (35). The samples were intro-

duced into the FTMS device using a NanoMate 100 instrument for automated nanospray (Advion Biosciences, Ithaca, NY). To introduce the samples, a back pressure of 0.45–0.8 psi and a voltage of 1.4–1.80 V were used. Typical instrument settings for the initial broadband analysis are as follows: 50–500 ms for ion accumulation per scan and 10–300 scans acquired per spectrum. While the majority of the time the quadrupole was not used to enhance the signal, when it was used, the accumulation times were changed from 50–500 to 500–7000 ms depending on signal intensity. For SWIFT excitation to isolate specific ions, the amplitude was set to 0.27 V_{p-p} and 30–50 waveform loops were used. For CAD, the ions were subjected to –10 to –40 V in the accumulation octupole (36). IRMPD was accomplished with a CO_2 laser by irradiation on axis with regard to the cell for 80–300 ms. The instrument was externally calibrated using ubiquitin [8560.65 Da monoisotopic M_r value (Sigma)] or on the electron capture dissociated fragment ions of ubiquitin. All the values described herein were determined manually. The masses for the PPant and Pant ejection products are reported as the ion masses. The FT-ICRMS settings were as follows with ranges indicated where appropriate: tube lens, 200–275 V; capillary heater, 3.5–4 A; quad filter, –20 V; skimmer off; capillary offset, 34 V; transfer of –10 to –80 V; transfer times, 1.00–1.25 ms; leak gas, $3.5\text{--}4.5 \times 10^{-5}$ Torr. Excitation was set to m/z 200–2000 or 400–2000 with a chirp rate of 6540 Hz/ μ s at a $V_{amplitude}$ of 0.45. For detection at low m/z values, the Nyquist frequency was set such that either m/z 204 or 408 was the cutoff. All data sets were collected as 512K or 1 Mb.

Generation of HMG-S-PksL-2ACP in ^{18}O or Deuterated Buffer. One hundred microliters of 50 mM Tris and 1 mM tris(2-carboxyethyl)phosphine (pH 7.6) was frozen and lyophilized. The resulting residue was redissolved in 100 μ L of D_2O or $H_2^{18}O$. To generate HMG-S-PksL-2ACP, a PksL-2ACP sample (75 μ L, 134 μ M) was incubated with racemic HMG-CoA (10 μ L, 3 mM), Sfp (3 μ L, 1.2 mg/mL), and $MgCl_2$ (1 μ L, 800 mM) for 30 min; 25 μ L of this solution was diluted with 70 μ L of the ^{18}O buffer or deuterated buffer described above.

^{18}O Exchange of HMG-S-PksL-2ACP by PksH. To HMG-S-PksL-2ACP in ^{18}O buffer was added 5 μ L of PksI (215 μ M), PksH (117 μ M), or both PksH and PksI, and the reaction was allowed to proceed for an additional 10 min before it was halted by the addition of 50 μ L of 10% formic acid. The samples were purified by HPLC before they were analyzed by mass spectrometry.

2H Exchange of HMG-S-PksL-2ACP by PksH. To HMG-S-PksL-2ACP in 2H buffer was added 5 μ L of PksI (215 μ M), PksH (117 μ M), or both PksH and PksI, and the reaction was allowed to proceed for an additional 10 min before it was halted by the addition of 50 μ L of 10% formic acid. The samples were purified by HPLC before they were analyzed by mass spectrometry.

LTQ MS Settings. The samples were introduced via direct infusion with a flow rate of 5 μ L/min, and the maximum intensity for the protein signal was obtained using the automatic tuning option on the LTQ instrument (Finnigan). After the maximum signal was obtained for a given sample, the ions of interest were isolated with an m/z 5 window, and the normalized collision energy for CAD was set to 15–30% to induce the phosphopantetheinyl ejections.

Empirical Formula Calculation for the Acyl Substituent. The calculated mass of the holo form (protonated, 261.127 Da) was subtracted from the observed mass of 345.148 Da. This mass of 84.021 Da represents the mass difference of the acylated species compared to the holo form of the protein. This mass value was imported into Xcalibur (Thermo-Finnigan), and the mass tolerance was set to 25 ppm. Only two empirical formulas were obtained that matched this mass difference. The first was $C_4H_4O_2$ and was within 4.99 ppm of the calculated mass of an acetoacetyl loaded form; the other was $C_2H_2NO_3$ for which the mass was within 20.97 ppm, but this chemically does not make sense.

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