Published in final edited form as:

Anal Chem. 2013 December 17; 85(24): 11952–11959. doi:10.1021/ac402850s.

Direct Detection of S-Palmitoylation by Mass Spectrometry

Yuhuan Ji^{1,2}, Nancy Leymarie¹, Dagmar J. Haeussler³, Marcus M. Bachschmid³, Catherine E. Costello^{1,2}, and Cheng Lin^{1,2,*}

¹Center for Biomedical Mass Spectrometry, Boston University School of Medicine, Boston, MA 02118

²Department of Biochemistry, Boston University School of Medicine, Boston, MA 02118

³Department of Medicine, Boston University School of Medicine, Boston, MA 02118

Abstract

Direct detection and quantification of protein/peptide palmitoylation by mass spectrometry (MS) is a challenging task because of the tendency of palmitoyl loss during sample preparation and tandem MS analysis. In addition, the large difference in hydrophobicity between the palmitoyl peptides and their unmodified counterparts could prevent their simultaneous analysis in a single liquid chromatography-MS experiment. Here, the stability of palmitoylation in several model palmitoyl peptides under different incubation and fragmentation conditions was investigated. It was found that the usual trypsin digestion protocol using dithiothreitol as the reducing agent in ammonium bicarbonate buffer could result in significant palmitoyl losses. Instead, it is recommended that sample preparation be performed in neutral Tris buffer with tris(2-carboxyethyl)phosphine as the reducing agent, conditions under which palmitoylation was largely preserved. For tandem MS analysis, collision-induced dissociation often led to facile palmitoyl loss, and electron capture dissociation frequently produced secondary side-chain losses remote from the backbone cleavage site, thus discouraging their use for accurate palmitoylation site determination. In contrast, the palmitoyl group was mostly preserved during electron transfer dissociation, which produced extensive inter-residue cleavage coverage, making it the ideal fragmentation method for palmitoyl peptide analysis. Finally, derivatization of the unmodified peptides with a perfluoroalkyl tag, *N*-[(3-perfluorooctyl)propyl] iodoacetamide, significantly increased their hydrophobicity, allowing them to be simultaneously analyzed with palmitoyl peptides for relative quantification of palmitoylation.

Introduction

S-Palmitoylation is a post-translational modification (PTM) which results from the attachment of a palmitoyl group to a cysteine residue through a thioester linkage. Palmitoylation is reversible and dynamic; the cycling between the palmitoylation and depalmitoylation states regulates several intracellular events such as protein trafficking, sorting, tethering, and protein-protein interaction.¹

The regulatory role of the palmitoylation/depalmitoylation cycle has been implicated in many studies. El-Husseini *et al.* showed that PSD-95, a major component of the postsynaptic density (PSD) at glutamatergic synapses, is important for AMPA receptor trafficking. Pharmacological interruption of the palmitoylation/depalmitoylation cycle of PSD-95 results in a change in the number of AMPA receptors at synapses, attenuating the synaptic signaling.² Wedegaertner *et al.* demonstrated that agonist activation of the β 2-

*To whom correspondence should be addressed: Phone: +1 (617) 638 6705, Fax: +1 (617) 638 6761, chenglin@bu.edu.

adrenergic receptor induces increased palmitate turnover on Gαs.³ The depalmitoylated Gαs loses its function in signal transduction after its release from the plasma membrane to the cytosol.⁴ Robinson *et al.* found that bradykinin, a G protein-coupled receptor ligand, induces depalmitoylation and translocation of endothelial nitric oxide synthase 3 (eNOS). They proposed that this regulation may be important for NO-related signaling pathway in endothelial cells.⁵ Burgoyne *et al.* showed that bovine aortic endothelial cells suffered apoptosis under oxidative stress, coincident with decrease in H-Ras palmitoylation, abnormal intracellular distribution of H-Ras, and reduced survival signaling from H-Ras. They further proposed that direct competition from oxidative PTMs of the C-terminal reactive cysteines prevents H-Ras palmitoylation, leading to endothelial dysfunction.⁶

In order to better understand the role of palmitoylation in biological systems, it is necessary to develop analytical methods that can localize palmitoylation sites as well as quantitatively measure the change in protein palmitoylation under various conditions. In early days, during decades of research, radioactive labeling was used for detection of palmitoylation. Although radioactive labeling is effective for quantitation, the procedure is tedious, potentially toxic and time consuming.⁷ Moreover, it is incapable of specifying the palmitoylation sites, unless mutations are made,⁸ or antibodies that target regions of interest are used in conjunction with enzymatic digestion.⁹ A mass spectrometry (MS) compatible method for palmitoylation analysis has recently been developed, which utilizes the acyl-biotinyl exchange (ABE) chemistry to irreversibly replace the thioester-linked palmitoyl modifications with stable biotin tags.¹⁰ This is achieved by blockage of all free thiol groups with *N*-ethylmaleimide (NEM) followed by removal of the palmitoyl group from cysteines *via* hydroxylamine-induced hydrolysis, and labeling of the newly freed thiol groups with biotin. The biotinylated proteins are affinity purified by streptavidin-agarose beads, enzymatically digested, and subjected to MS analysis. One advantage of the ABE approach is the achievement of palmitoyl peptide/protein enrichment from complex samples. However, false positives could arise due to incomplete blockage of free cysteines, and false negatives could occur due to inadequate thioester hydrolysis by hydroxylamine, or inefficient biotin-labeling. Further, palmitoylation is not the only form of thioester linkages, and the ABE method could lead to false assignments of palmitoylation when the cysteine is occupied by some other modification.

An alternative approach utilizing metabolic labeling with the palmitic acid analogue, 17-octadecynoic acid (17-ODYA), and click chemistry has shown great promise in large-scale profiling of protein palmitoylation using cell lines.¹¹ Click chemistry can be used to attach different functional groups to the palmitoyl mimic, allowing enrichment and detection of palmitoyl proteins using both MS and non-MS methods. In conjunction with mass spectrometry, the metabolic labeling approach can be used for identification of the palmitoylation sites, and characterization of the palmitoylation dynamics.¹² However, it is difficult and costly to extend its use to study palmitoylation in large organisms. Moreover, relative quantification of the protein palmitoylation remains challenging, as the unpalmitoylated proteins are not retained. As discussed below, care must also be taken to minimize loss of palmitoyl groups during multiple reaction and sample cleanup steps.

Direct detection of *S*-acylation by MS has also been reported.^{13–18} This usually starts with the purification of the proteins of interest, followed by reductive alkylation, and tryptic digestion either in-gel or in-solution. MS analysis, typically using a matrix-assisted laser desorption/ionization-time-of-flight (MALDI-TOF) or a MALDI-TOF/TOF instrument, can then be performed for palmitoyl peptide identification. Such an approach is well suited for discovery of novel palmitoylation sites, but could be problematic in quantitative studies, as palmitoyl loss may occur during sample preparation, because of the labile thioester linkage. In particular, controversy exists over the stability of palmitoylation when dithiothreitol

(DTT) is used as the reducing agent.^{18–20} Additionally, palmitoyl groups may be lost, through prompt or metastable fragmentation, during tandem MS analysis, leading to uncertainty in palmitoylation site determination. Finally, the large difference in the hydrophobicity of the palmitoylated and unpalmitoylated peptides makes relative quantification a challenging task.

Here, the stability of palmitoylation in several palmitoyl peptide standards under various experimental conditions was investigated in order to establish a sample preparation protocol that retains palmitoylation for MS analysis. The potential of a derivatization strategy for relative quantification of palmitoyl peptides and their unmodified counterparts was also explored. Lastly, fragmentation behaviors of palmitoyl peptides under several dissociation modes were studied to evaluate their applicability for characterization of palmitoyl peptides.

Experimental

Preparation of Palmitoyl Peptides

The palmitoylation reaction was performed as previously reported²¹ with some modifications. Each peptide standard (200 μg) was allowed to react with 1 μL of palmitoyl chloride in 10 μL of 100% TFA for 10 min at room temperature. The resulting mixture was dried under a nitrogen flow. Purification of the products was performed on an Agilent 1200 series HPLC system (Agilent Technologies, Santa Clara, CA) using a Vydac 214ms5215 column (C4, 5 μm , 300 $\text{\AA}$, 2.1 mm ID $\times$ 150 mm). Mobile phase A consisted of 95:5 water/ACN with 0.1% TFA and mobile phase B consisted of 85:10:5 ACN/IPA/water with 0.1% TFA. The sample was suspended in 400 μL of 30% B (singly palmitoylated peptides) or 40% B (doubly palmitoylated peptides), sonicated for 1 min, centrifuged for 10 min at 21,000 RCF. The supernatant was collected and centrifuged for another 10 min before HPLC injection. A linear gradient of 30–100% B (singly palmitoylated peptides) or 40–100% B (doubly palmitoylated peptides) over 20 min was employed with a flow rate of 0.3 mL/min. UV detection was performed at 214 nm. The palmitoyl peptide fractions were collected, aliquoted, and dried. The amount of palmitoyl peptide in each aliquot was determined by a micro BCA protein assay kit. Aliquots were frozen at $-80\text{ }^{\circ}\text{C}$ for later use.

Stability Test of Palmitoyl Peptides

Aliquots of HPLC-purified palmitoyl peptides were incubated in 100 mM ABC (pH 8.0), 50 mM Tris (pH 7.4), or 50 mM AA buffer (pH 4.0), with or without the presence of DTT or TCEP in different concentrations, at either $37\text{ }^{\circ}\text{C}$ or $55\text{ }^{\circ}\text{C}$. At several time points, a 0.5- μL aliquot was taken and diluted in 5 μL of 50% ACN/0.1% TFA. The diluted sample (0.5 μL) was co-crystallized with 0.5 μL of DHB (10 $\mu\text{g}/\mu\text{L}$ in 50% ACN/0.1% TFA) on a steel MALDI target plate, and later analyzed on either a Reflex IV MALDI-TOF mass spectrometer (Bruker Daltonics, Billerica, MA) or an ultrafleXtreme MALDI-TOF/TOF mass spectrometer (Bruker Daltonics, Bremen, Germany) with 25–50% laser power. For each spectrum, 200–2000 laser shots were accumulated. The MALDI-TOF mass spectra were analyzed using the FlexAnalysis 3.4 software.

Preparation of IAM- and FIAM-Labeled Peptides

Alkylation with IAM was performed according to the manufacturer's protocol.²² FIAM-labeling was performed following the protocol as described previously²³ with slight modifications. The peptide standard was first dissolved in 50 mM Tris buffer (pH 7.4) with 0.5 mM TCEP and kept at $37\text{ }^{\circ}\text{C}$ for 30 min; the resulting solution was incubated with equal amount of 10 mM FIAM dissolved in THF at $37\text{ }^{\circ}\text{C}$ for 30 min in the dark. The mixture was dried under a nitrogen flow. Purification was performed using a linear gradient of 30–100% B over 20 min at a flow rate of 0.3 mL/min. The amount of IAM- and FIAM-labeled

peptides in each fraction was determined using a micro BCA protein assay kit. Aliquots were frozen at -80°C for later use.

HPLC Separation

HPLC behavior of IAM-labeled, FIAM-labeled and palmitoyl peptides was investigated on an Agilent 1200 series HPLC system using a Vydac 214ms5215 column with the same solvent system as described above. The samples were introduced at 20% B and analyzed with a linear gradient of 20–100% B over 20 min at a flow rate of 0.3 mL/min. Peaks were detected by the UV absorption at 214 nm, and fractions were collected and analyzed by MALDI-TOF MS for identification.

LC-MS Quantification

Quantification was achieved by LC-MS on an LTQ-Orbitrap XL instrument (Thermo Fisher Scientific, San Jose, CA) equipped with a nanoACQUITY UPLC (Waters, Milford, MA) and a Triversa Nanomate system (Advion Biosystems, Inc., Ithaca, NY). A Waters BEH300 C4 column ($1.7\ \mu\text{m}$, $150\ \mu\text{m ID} \times 100\ \text{mm}$) was used for separation. Mobile phase A consisted of 95:5 water/ACN with 0.1% FA and mobile phase B consisted of 95:5 ACN/water with 0.1% FA. Palmitoyl peptides and FIAM-labeled peptides were combined at different ratios and introduced at 40% B with a flow rate of $0.5\ \mu\text{L/min}$. The gradient was held at 40% B for 20 min, followed by a ramp to 100% B over 30 min. It was then held at 100% B for 5 min, followed by a ramp to 40% B over 2 min, and maintained at 40% B for 23 min for column re-equilibration. All mass spectra were acquired in the Orbitrap and analyzed by the Xcalibur software.

Tandem MS Analyses

Off-line tandem MS analyses were performed on a 12-T solariX hybrid Qh-FTICR mass spectrometer (Bruker Daltonics, Bremen, Germany). HPLC-purified palmitoyl peptides were dissolved in 50:50 water/ACN with 0.1% FA to a concentration of $1\ \text{pmol}/\mu\text{L}$ and directly infused into the mass spectrometer. Precursor ions of interest were isolated by the front-end quadrupole and fragmented by different dissociation methods. Collision-induced dissociation (CID) was performed with the collision voltage set to 7 to 20 V; electron capture dissociation (ECD) was achieved with irradiation of $\sim 1.5\text{-eV}$ electrons for 50 ms; electron transfer dissociation (ETD) was performed with a 400-ms reagent accumulation and a 50 to 100-ms reaction time. A 1-s transient was acquired for each scan and each spectrum was the result of summing 100 transients. Fluoranthene anions were used as the reagent for ETD.

On-line LC-MS/MS analyses were performed on the LTQ-Orbitrap XL mass spectrometer. Data-dependent acquisition was performed by switching between one MS scan ($r = 60,000$ at m/z 400) and three MS/MS events ($r = 7,500$). The three most abundant ions with charge state ≥ 2 were isolated with a window of $\pm 3\ m/z$ for CID, high-energy CID (HCD), and ETD (with fluoranthene anions). The normalized collision energy was set at 35% for CID, and 30% for HCD. ETD reaction time was set at 80 ms with supplemental activation set at 15.

Results and Discussion

Stability of Palmitoyl Peptides

Synthetic peptides PDFRIAFQELLCLR, MGCVQCKDKEA, and ARAWCQVAQKF were chosen as model systems because their sequences contain palmitoylation motifs from proteins that are known to undergo *in vivo* palmitoylation: beta-2 adrenergic receptor,⁸ tyrosine-protein kinase Fyn,²⁴ and glutamate decarboxylase 2, respectively.²⁵ The chosen sequences do not contain serine, threonine, or tyrosine residues, which are targets of O-

palmitoylation that could complicate the analysis. The *in vitro* palmitoylation was performed in 100% TFA, under which condition all basic groups, including the N-terminal amine and the side chains of the lysine and arginine residues, were protonated and not palmitoylated. Tandem MS analysis showed that palmitoylation occurred at the cysteine and tryptophan residues, producing PDFRIAFQELL_{C_{palm}}LR, MGC_{palm}VQC_{palm}KDKEA, and ARAW_{palm}C_{palm}QVAQKF. Tryptophan palmitoylation likely occurred at the C2 position (Supplemental Scheme S1) via an electrophilic substitution reaction. The stability of palmitoylation in various buffers was investigated by incubating HPLC-purified palmitoyl peptides at 37 °C for 1 hr, 3 hr, 6 hr, and 16 hr. The MALDI-TOF mass spectra (Supplemental Figure S1) show that 6-hr incubation of MGC_{palm}VQC_{palm}KDKEA in the standard tryptic digestion buffer (100 mM ABC, pH 8.0) at 37 °C already led to significant palmitoyl loss, whereas all three palmitoyl peptides were stable after overnight incubation in either the neutral buffer (50 mM Tris, pH 7.4) or the acidic buffer (50 mM AA, pH 4.0). Further investigation revealed that even incubation in the pH 7.4 ABC buffer resulted in complete depalmitoylation within 16 hours, and this was attributed to the gradual pH increase of the ABC buffer (Supplemental Figure S2), presumably because CO₂ is more volatile than NH₃. Since most proteases used in proteomics research attain their highest enzymatic activities at or near physiological pH, Tris buffer was used in the following studies.

To test the effect of the reducing agents on the palmitoyl stability, palmitoyl peptide standards were incubated in Tris buffer in the presence of DTT or TCEP at 37 °C or at 55 °C for 30 min and 1 hr. The results are summarized in Figures 1 and S3, which show the relative abundances of the palmitoyl peptides and their depalmitoylated forms as a function of the incubation time and temperature, with DTT or TCEP. DTT, a reducing agent commonly used for reduction of disulfide bonds and other reversible oxidative modifications of cysteines, accelerated the depalmitoylation process for all three peptides, but the other widely-used reducing reagent, TCEP, did not cause appreciable loss of palmitoylation. Further, the depalmitoylation rate was significantly increased at higher temperature, as one would expect for any reaction with an activation barrier.

The mechanisms for disulfide bond reduction by DTT and by TCEP are illustrated in Supplemental Scheme S2. For DTT, disulfide reduction proceeds *via* sequential thiol-disulfide exchange reactions, where one of the two disulfide sulfur atoms is attacked by a thiolate group of DTT, releasing one cysteine residue and creating a mixed disulfide species; the subsequent nucleophilic attack by the remaining thiolate of DTT releases the other cysteine and forms cyclic oxidized DTT. Although thiol-disulfide exchanges are reversible, disulfide bond reduction by DTT is quite unidirectional, as the byproduct, the oxidized DTT, is highly stable due to its 6-membered ring structure. DTT is reactive only at pH >7 since the negatively charged thiolate is the only reactive form. Furthermore, DTT must be used in at least 20-fold molar excess to the disulfide bond to ensure complete reduction.²⁶ However, thiolate is a strong nucleophile that can also attack the carbonyl of the thioester linkage in *S*-palmitoylated peptides, resulting in disruption of the palmitoylation, as observed here.

The reactive group on TCEP is the free electron pair on the phosphine group, which can attack the disulfide bond, releasing one cysteine and forming an intermediate phosphorus-sulfur linkage. The subsequent nucleophilic attack to the positively charged phosphorus by the oxygen of a water molecule releases the second cysteine, and forms a phosphine oxide. This reaction is irreversible because of the formation of a strong phosphorus-oxygen double bond in the oxidized TCEP²⁷ and proceeds at nearly stoichiometric ratio. TCEP is a potent reducing agent, and works under a broad pH range since phosphine is a very weak base and retains its nucleophilic character even in acidic solutions. In addition, phosphine does not react with a thioester, making TCEP an ideal reducing agent for studying *S*-palmitoylation.

Analysis of Palmitoyl Peptides by RP-HPLC

Several HPLC methods have been developed to analyze very hydrophobic peptides²⁸ and peptides with lipid modifications.^{29,30} Due to their dramatic difference in hydrophobicity, it is challenging to analyze palmitoyl peptides and their unmodified counterparts in a single HPLC run. While it is possible to analyze lipid modified peptides on a C18 column, it requires the use of strong organic solvent systems. For example, Gustafsson *et al.* reported that SP-C, a 35-residue palmitoyl peptide, can be separated on a C18 column by using 60~75% methanol/ethanol as the initial solvent followed by elution with isopropanol,³⁰ under which conditions most unmodified peptides cannot be retained. Meanwhile, C4 columns retain palmitoyl peptides through weaker interactions, allowing separation and elution of palmitoyl peptides using mild organic solvents such as acetonitrile. However, C4 columns are not suitable for analysis of unmodified peptides which are usually not retained. Figure 2a shows the chromatogram of a mixture of the three palmitoyl peptide standards and their IAM-labeled counterparts, acquired on a C4 column with the gradient program shown in the inset. Although all three palmitoyl peptides were retained and well separated, two of the three IAM-labeled peptides flowed through with the initial solvent. One way to overcome this difficulty is to increase the hydrophobicity of unmodified peptides through derivatization. FIAM, with its structure shown in Supplemental Scheme S3, is a cysteine alkylation reagent with a hydrophobic perfluoroalkyl moiety, that our laboratory has demonstrated to be useful for enrichment of native cysteine-containing peptides and the RP-HPLC analysis of the mixtures of these products with their irreversibly modified analogs.²³ For the quantitative studies undertaken herein, we rationalized that this selective hydrophobic FIAM labeling of the cysteine thiol group in the unmodified peptides would allow them to be analyzed simultaneously with the palmitoyl peptides. Figure 2b shows the chromatogram of a mixture of the palmitoyl peptides and their FIAM-labeled counterparts, acquired on a C4 column using the same gradient program as shown in Figure 2a. All six peptides were retained on the C4 column in the initial phase, and were well resolved by gradient elution with mild organic solvent.

Relative Quantification of Palmitoyl Peptides by On-line LC-MS

Site-specific quantification of protein palmitoylation can be achieved by utilizing either the ABE chemistry or metabolic labeling. However, these methods are not suitable for relative quantification of palmitoyl peptides with respect to their unpalmitoylated forms, as the unpalmitoylated peptides are usually not retained under the HPLC conditions employed. As demonstrated above, with FIAM-labeling, all the cysteine-containing peptides, both with and without palmitoylation, can be analyzed in a single LC run. Here, an on-line LC-MS study of palmitoyl and FIAM-derivatized peptide mixtures was performed on an LTQ-Orbitrap XL mass spectrometer using the Waters BEH300 C4 column to explore the potential of using extracted ion chromatograms (EICs) for the relative quantification of peptide palmitoylation.

Figure 3a shows the total ion chromatogram of a mixture of palmitoyl and FIAM-labeled peptide standards containing equal amounts of each peptide. The six peptides were well separated from one another and eluted between 40% and 100% B. Note that the differences in the peak areas observed for these peptides likely results from differences in their ionization efficiencies. LC-MS analyses were then performed on mixtures of these peptides at seven different ratios (FIAM/palm = 10:1, 5:1, 2:1, 1:1, 1:2, 1:5, and 1:10). The ion abundance of each peptide was calculated as the sum of the charge-state normalized integrated peak areas of all observed charge states in their respective EICs using the Xcalibur Quan browser. The calibration curve for relative quantification was generated by plotting the ratio of the calculated ion abundance as a function of the ratio of the amount of peptides injected. As shown in Figures 3b–d, for all three pairs of palmitoyl and FIAM-

labeled peptides, a satisfying linear correlation exists between these two ratios, thus establishing the validity of relative quantification based on the integrated EIC peak areas of the palmitoyl and FIAM-derivatized peptides. It is important to note that all samples must be analyzed under the same LC conditions as those used to generate the calibration curve. A change in the column, solvent system, and/or gradient program would lead to changes in the solvent composition during elution of peptides of interest, and consequently, their ionization efficiencies.

Tandem MS Analysis of Palmitoyl Peptides

Tandem mass spectrometry is a powerful tool for identification and localization of PTMs, and has been applied to characterize lipid-modified peptides.^{23, 31–33} In general, the thioether linkage is stable under low-energy CID conditions, as illustrated by the retention of the perfluoroalkyl group in most CID fragment ions from the FIAM-labeled peptides.²³ Hoffman and Kast reported a CID study of peptides with various lipid modifications, including *N*-myristoylation, farnesylation, and *S*-palmitoylation.³¹ Whereas *N*-myristoylation was fairly stable under CID, *S*-palmitoylation was labile upon collisional activation, producing abundant $b - 238$ ions as the result of the thioester bond breakage either before or after the backbone amide cleavage. On the other hand, labile modifications are often preserved under ECD, which is considered non-ergodic and directional towards the backbone *N*- C_α bond cleavage. Guan studied the CID and ECD fragmentation behaviors of ghrelin,³² a peptide with *O*-acylation, and found that ECD produced far more extensive backbone fragmentation without breaking the ester bond, while the ester-linked octanoyl group was lost in many CID fragments. Kaczorowska *et al.* recently studied the CID and ECD fragmentation behaviors of *S*-dipalmitoylated peptides, and found that both CID and ECD could provide structural information on the peptide sequence and the modification.³³ However, in the *S*-dipalmitoylated peptides reported in this study, the palmitoyl groups were not directly attached to the cysteine thiol group, but were connected *via* ester linkages to a glycerol, which was linked to the cysteine via a thioether bond. Since the thioester linkage is more labile than the ester linkage, we felt that it would be important to study the fragmentation behaviors of *S*-acylated peptides under different dissociation conditions. A systematic investigation of the fragmentation behaviors of *S*-palmitoyl peptides was performed here with CID, ECD, and ETD as the dissociation modes. Tandem mass spectra were acquired either on-line on the LTQ-Orbitrap instrument (CID, HCD, and ETD), or off-line on the hybrid Qh-FTICR instrument (CID, ECD, and ETD). Representative tandem mass spectra of the three palmitoyl peptide standards and their corresponding cleavage maps are shown in Figures 4, 5, and S4, respectively.

For the triply charged peptide, PDFRIAFQELLC_{palm}LR, low-energy CID spectra acquired on both instruments provided complete inter-residue cleavage coverage, generating abundant *b*- and *y*-type ions, as well as a few *a*-ions (Figure 4a, b). Some *y*-ions, labeled with an asterisk (*y**), were also observed with loss of the palmitoyl group ($C_{16}H_{30}O$, 238.23 Da). In general, *y** ions were in much lower abundance than normal *y*-ions, and should not have a significant impact on palmitoylation site determination. Similar sequence coverage was obtained by HCD (Figure 4c). However, with its higher energy input, HCD also resulted in increased palmitoyl losses from many *y*-ions. Nonetheless, the palmitoylation site could still be localized to the cysteine residue based on the presence of a high-abundance y_3 ion with the palmitoyl group attached (m/z 629.44) and absence of any smaller palmitoyl group-carrying *y*-ions.

The ECD spectrum (Figure 4d) of the same precursor ion was characterized by extensive *c*- and *z*- ion series, providing complete inter-residue cleavage coverage. Surprisingly, ECD also produced many *z* ions with loss of a palmitoyl group, labeled as *z** ions, some of which

were in very high abundance. Considering that insignificant palmitoyl loss was observed in the CID spectra of this peptide, formation of these z^* ions was unlikely an ergodic process. It has been previously reported that the alpha carbon radical formed upon N-C $_{\alpha}$ bond cleavage in ECD can initiate further backbone and/or side-chain cleavages, with or without radical migration.^{34–36} Supplemental Scheme S4A illustrates a possible mechanism for the z^* ion formation, in which the backbone alpha carbon radical abstracts a hydrogen from the alpha carbon of the palmitoyl group, and the subsequent radical-induced alpha cleavage releases a tetradecylketene (C₁₆H₃₀O, 238.23), leaving the radical on the sulfur atom of the cysteine residue. Palmitoyl loss here was driven by the stability of the sulfur-centered radical formed, whereas in the case of *O*-acylation, no acyl loss was observed because it would have required the unfavorable formation of an oxygen-centered radical.³² The ECD spectrum also contains another series of ions, labeled as $z^{\S}$ ions, which correspond to z ions with partial loss of the cysteine side-chain (C₁₅H₃₁COS[•], 271.21 Da). The $z^{\S}$ ions were likely formed as the result of radical induced alpha cleavage following radical migration to the alpha carbon of the cysteine residue (Supplemental Scheme S4B). Loss of the C₁₅H₃₁COS[•] group was so energetically favored, once the radical was formed at the alpha carbon of the cysteine residue, that neither the z_3 nor the z_3^* ion was observed in the ECD spectrum. The N-C $_{\alpha}$ bond cleavage N-terminal to the palmitoylated cysteine residue was always followed by the partial cysteine side chain loss, leading to the formation of an abundant $z_3^{\S}$ ion. Overall, we found that ECD generated too many site-nonspecific side chain loss product ions as a result of radical migration to be useful for palmitoylation site determination.

Similar to ECD, ETD of the triply charged PDFRIAFQELLC_{palm}LR precursor also produced extensive *c*- and *z*- ion series, with complete inter-residue cleavage coverage. However, unlike ECD, no palmitoyl loss was observed in any of the z ions formed. Moreover, only one $z^{\S}$ ion was detected: a $z_3^{\S}$ ion. Note that the $z_3^{\S}$ ion is essentially the w_3 ion, and its formation did not require radical migration. It appears that radical migration was suppressed in ETD, either due to its lower energy input, or because of radical stabilization by collisional cooling. The palmitoylation site could be confidently assigned to the cysteine residue based on the mass difference between the c_{11} and c_{12} ions. The absence of the z_3 ion and the presence of the w_3 ion provided additional evidence for cysteine palmitoylation.

The tandem MS fragmentation behavior of the doubly palmitoylated peptide, MGC_{palm}VQC_{palm}KDKEA, was similar to that of the PDFRIAFQELLC_{palm}LR peptide, but also showed some distinct differences. For the triply charged MGC_{palm}VQC_{palm}KDKEA precursor ion, low-energy CID (Figure 5a) produced only a limited number of *b*- and *y*- type ions, and the palmitoylation site at the second cysteine residue could not be determined because no backbone cleavage at either side of that cysteine residue was observed. Although its HCD spectrum (Figure 5b) was more informative, and provided 100% cleavage coverage, most palmitoyl-containing fragment ions also underwent extensive palmitoyl loss during the fragmentation process, including some with loss of two palmitoyl groups, *e.g.* the y_{10}^{**} ion, thus preventing reliable palmitoylation site localization. For this peptide, unlike in the previous case, ECD (Figure 5c) produced complete cleavage coverage with minimum palmitoyl loss. Note that the PDFRIAFQELLC_{palm}LR peptide contains polar residues (D, E, and R) near both termini, allowing it to adopt a folded gas-phase conformation, in which the palmitoyl-cysteine residue was close to several potential backbone cleavage sites. Upon the N-C $_{\alpha}$ bond cleavage by ECD, the radical could migrate from the initial C $_{\alpha}$ position to the spatially adjacent palmitoyl-cysteine residue even if it was distant in sequence, initiating further side chain losses and formation of $z^{\S}$ and z^* ions. Such spatial proximity may not be present in the MGC_{palm}VQC_{palm}KDKEA peptide, because all of its polar residues (K, D, and E) were located on one side of the palmitoyl-cysteine residues, and the two bulky hydrophobic palmitoyl-cysteine side chains further prevented formation of a compact structure. Consequently, radical migration was not prevalent here, resulting in only two low-

abundance z_7^8 and z_8^8 ions. As in the previous case, z ions with an N-terminal cysteine, z_6 and z_9 , were not observed, instead, cleavages N-terminal to the two cysteine residues led to the formation of the z_6^8 (w_6) and z_9^8 (w_9) ions, which could be used to confirm the two cysteine palmitoylation sites. The ETD spectrum of $\text{MGC}_{\text{palm}}\text{VQC}_{\text{palm}}\text{KDKEA}$ (Figure 5d) was very similar to its ECD spectrum, producing a complete series of N- C_α bond cleavages, with no palmitoyl loss. Partial cysteine side chain loss was only observed for z ions with an N-terminal cysteine. Interestingly, two c ions with partial cysteine side chain loss, c_9^8 and c_{10}^8 , were also observed. As c ions are even electron species, radical migration was unlikely to play a role here. Since both c_9^8 and c_{10}^8 ions were singly charged ions produced from triply charged precursor ions with all three potential protonation sites residing within the first nine residues, these two ions were likely formed as a result of two electron transfer processes, with one accounting for the cysteine side chain loss, and the other leading to the backbone N- C_α bond cleavage.

The general features of the tandem mass spectra (Figure S4) of the triply charged peptide, $\text{ARAW}_{\text{palm}}\text{C}_{\text{palm}}\text{QVAQKF}$, resemble those of the other two peptides. Abundant palmitoyl losses were observed in its low-energy CID (Figure S4a) and HCD spectra (Figure S4b). For ECD (Figure S4c), in addition to the palmitoyl loss and cysteine side chain loss, abundant tryptophan side chain loss remote from the ECD cleavage site was observed, in agreement with findings from a previous study.³⁷ Again, the ETD spectrum (Figure S4d) was the most informative with minimal secondary side chain losses, except for the characteristic cysteine side chain loss at the cysteine cleavage site.

Conclusions

S-palmitoylation is a labile modification both in solution and in the gas phase. In order to minimize palmitoyl loss during proteomic sample preparation, it is recommended that palmitoyl (and other acylated) proteins and peptides be processed under neutral or slightly acidic conditions and at room temperature. Use of DTT should be avoided; instead, TCEP is the preferred disulfide reducing agent for palmitoyl protein analysis. The drastic difference in hydrophobicity between palmitoyl peptides and their unpalmitoylated forms can be reduced by derivatization of free cysteine residues with a perfluoroalkyl tag, enabling relative quantification of palmitoylation by LC-MS. CID of *S*-palmitoylated peptides can lead to facile loss of palmitoyl groups, whereas ECD can result in extensive radical migration and secondary side chain loss driven by the formation of stable sulfur-centered radicals. ETD appeared to be the best fragmentation method for tandem MS analysis of palmitoyl peptides because it produced extensive backbone fragmentation with minimum palmitoyl loss. In summary, this study presents a comprehensive strategy, including sample preparation, LC-MS and tandem MS analysis, for direct detection and quantification of *S*-palmitoyl peptides.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

This research is supported by the NIH grants P41 RR10888/GM104603, P01 HL068758, R37 HL104017, S10 RR015942, S10 RR025082, S10 RR020946, S10 OD010724, and NIH/NHLBI contract HHSN268201000031C.

References

1. Salaun C, Greaves J, Chamberlain LH. J Cell Biol. 191:1229–38. [PubMed: 21187327]

2. El-Husseini AED, Schnell E, Dakoji S, Sweeney N, Zhou Q, Prange O, Gauthier-Campbell C, Aguilera-Moreno A, Nicoll RA, Bredt DS. *Cell*. 2002; 108:849–863. [PubMed: 11955437]
3. Wedegaertner PB, Bourne HR, von Zastrow M. *Mol Biol Cell*. 1996; 7:1225–33. [PubMed: 8856666]
4. Thiagarajan MM, Bigras E, Van Tol HHM, Hébert TE, Evanko DS, Wedegaertner PB. *Biochemistry*. 2002; 41:9470–9484. [PubMed: 12135370]
5. Robinson LJ, Busconi L, Michel T. *J Biol Chem*. 1995; 270:995–998. [PubMed: 7530714]
6. Burgoyne JR, Haeussler DJ, Kumar V, Ji Y, Pimental DR, Zee RS, Costello CE, Lin C, McComb ME, Cohen RA, Bachschmid MM. *FASEB J*. 2012; 26:832–841. [PubMed: 22085642]
7. Li L, Dong L, Xia L, Li T, Zhong H. *J Chromatogr B*. 2011; 879:1316–1324.
8. O'Dowd BF, Hnatowich M, Caron MG, Lefkowitz RJ, Bouvier M. *J Biol Chem*. 1989; 264:7564–7569. [PubMed: 2540197]
9. Linder ME, Middleton P, Hepler JR, Taussig R, Gilman AG, Mumby SM. *Proc Natl Acad Sci USA*. 1993; 90:3675–3679. [PubMed: 8475115]
10. Wan J, Roth AF, Bailey AO, Davis NG. *Nat Protoc*. 2007; 2:1573–84. [PubMed: 17585299]
11. Martin BR, Cravatt BF. *Nat Methods*. 2009; 6:135–138. [PubMed: 19137006]
12. Martin BR, Wang C, Adibekian A, Tully SE, Cravatt BF. *Nat Methods*. 2012; 9:84–89. [PubMed: 22056678]
13. Hensel J, Hintz M, Karas M, Linder D, Stahl B, Geyer R. *Eur J Biochem*. 1995; 232:373–380. [PubMed: 7556184]
14. Liang X, Lu Y, Neubert TA, Resh MD. *J Biol Chem*. 2002; 277:33032–33040. [PubMed: 12105219]
15. Bizzozero OA, Malkoski SP, Mobarak C, Bixler HA, Evans JE. *J Neurochem*. 2002; 81:636–645. [PubMed: 12065672]
16. Roos M, Soskic V, Poznanovic S, Godovac-Zimmermann J. *J Biol Chem*. 1998; 273:924–931. [PubMed: 9422751]
17. Kordyukova LV, Serebryakova MV, Baratova LA, Veit M. *J Virol*. 2008; 82:9288–9292. [PubMed: 18596092]
18. Xue L, Gollapalli DR, Maiti P, Jahng WJ, Rando RR. *Cell*. 2004; 117:761–771. [PubMed: 15186777]
19. Tu Y, Wang J, Ross EM. *Science*. 1997; 278:1132–1135. [PubMed: 9353196]
20. McClure M, DeLucas LJ, Wilson L, Ray M, Rowe SM, Wu X, Dai Q, Hong JS, Sorscher EJ, Kappes JC, Barnes S. *Protein Eng Des Sel*. 2012; 25:7–14. [PubMed: 22119790]
21. Yousefi-Salakdeh E, Johansson J, Ströberg R. *Biochem J*. 1999; 343:557–562. [PubMed: 10527933]
22. <http://www.piercenet.com/instructions/2162099.pdf>.
23. Ying W, Perlman DH, Li L, Théberge R, Costello CE, McComb ME. *Rapid Commun Mass Spectrom*. 2009; 23:4019–4030. [PubMed: 19924777]
24. Koegl M, Zlatkine P, Ley SC, Courtneidge SA, Magee AI. *Biochem J*. 1994; 303:749–753. [PubMed: 7980442]
25. Soghomonian JJ, Martin DL. *Trends Pharmacol Sci*. 1998; 19:500–505. [PubMed: 9871412]
26. Rügg UT, Rudinger J. *Methods Enzymol*. 1977; 47:111–116. [PubMed: 927167]
27. Burns JA, Butler JC, Moran J, Whitesides GM. *J Org Chem*. 1991; 56:2648–2650.
28. Grandier-Vazeille X, Tetaert D. *J Chromatogr A*. 1984; 296:301–308.
29. Kwok Y, Sung WC, Lin ALH, Liu HH, Chou FAH, Hsieh SSY, Leng CH, Chong P. *Proteomics*. 2011; 11:2620–2627. [PubMed: 21630453]
30. Gustafsson M, Curstedt T, Jönvall H, Johansson J. *Biochem J*. 1997; 326:799–806. [PubMed: 9307030]
31. Hoffman MD, Kast J. *J Mass Spectrom*. 2006; 41:229–241. [PubMed: 16421873]
32. Guan Z. *J Am Soc Mass Spectrom*. 2002; 13:1443–1447. [PubMed: 12484464]
33. Kaczorowska M, Cooper H. *J Am Soc Mass Spectrom*. 2013; 24:1224–1227. [PubMed: 23728547]

34. Leymarie N, Costello CE, O'Connor PB. J Am Chem Soc. 2003; 125:8949–8958. [PubMed: 12862492]
35. Li X, Lin C, Han L, Costello C, O'Connor P. J Am Soc Mass Spectrom. 2010; 21:646–656. [PubMed: 20171118]
36. Moore BN, Ly T, Julian RR. J Am Chem Soc. 2011; 133:6997–7006. [PubMed: 21495634]
37. Fung YME, Chan TWD. J Am Soc Mass Spectrom. 2005; 16:1523–1535. [PubMed: 16023365]

Figure 1.

Stability of three palmitoyl peptides, as represented by the relative abundance of the palmitoyl peptides and their various depalmitoylated forms after 30 min or 60 min of incubation in Tris buffer (50 mM, pH 7.4) at 37 °C or at 55 °C, in the presence or absence of DTT or TCEP.

Figure 2.

(a) Chromatogram of a mixture of three palmitoyl peptides and their IAM-labeled counterparts; (b) Chromatogram of a mixture of three palmitoyl peptides and their FIAM-labeled counterparts.

Figure 3.

Relative quantification by UPLC-MS analysis on an LTQ-Orbitrap XL mass spectrometer. (a) The total ion chromatogram of a mixture of palmitoyl and FIAM-labeled peptides; (b), (c), and (d) the relative quantification calibration curves of the PDFRIAFQELL_{FIAM}LR and PDFRIAFQELL_{C_{palm}}LR peptide pair, the MGC_{FIAM}VQC_{FIAM}KDKEA and MGC_{palm}VQC_{palm}KDKEA peptide pair, and the ARAWC_{FIAM}QVAQKF and ARAW_{palm}C_{palm}QVAQKF peptide pair, respectively.

Figure 4.

Tandem MS spectra of PDFRIAFQELLCPalmLR: (a) CID on solariX, (b) CID on Orbitrap, (c) HCD on Orbitrap, (d) ECD on solariX, and (e) ETD on solariX. * indicates loss of $C_{16}H_{30}O$ (238.23 Da); § indicates loss of $C_{15}H_{31}COS^+$ (271.21 Da).

Figure 5. Tandem MS spectra of MGC_{palm}VQC_{palm}KDKEA: (a) CID on Orbitrap, (b) HCD on Orbitrap, (c) ECD on solariX, (d) ETD on Orbitrap. * indicates loss of C₁₆H₃₀O (238.23 Da); § indicates loss of C₁₅H₃₁COS* (271.21 Da)