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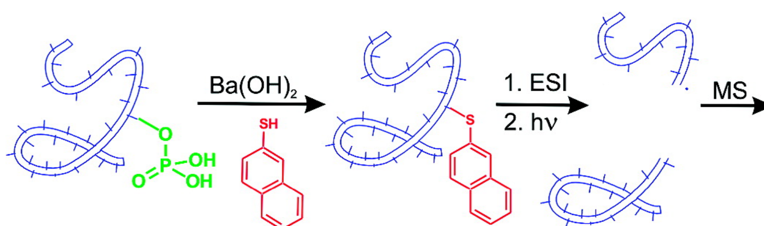

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Site-Specific Radical Directed Dissociation of Peptides at Phosphorylated Residues

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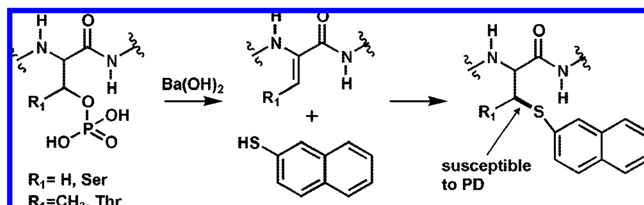
Phosphorylation plays a critical role in biochemical signaling pathways and is the most common post-translational modification (PTM) of the side chains of proteins.[1] Identification of sites of phosphorylation is essential.[2] The most straightforward (although previously unrealized) approach to obtain this information is to selectively fragment proteins or peptides that are phosphorylated at the site of the modification, revealing both the presence and location of the PTM. Unfortunately, directed fragmentation of specific bonds in large molecules is a difficult task. We have recently reported that site specific radicals generated on tyrosine residues lead to highly localized fragmentations in experiments with whole proteins.[3] Herein we report an extension of this method that allows for precise radical directed dissociation that is residue specific and occurs only at phosphorylated residues in peptides.

Direct dissociation following absorption of an ultraviolet photon is one of the few methods for breaking bonds while circumventing intramolecular vibrational energy redistribution (IVR).[4,5] This type of photodissociation (PD) can be used to generate a radical in a specific location on a large biomolecule without heating the entire molecule.[3] Previous work on small molecules has demonstrated that carbon−sulfur bonds are susceptible to direct PD, yielding a radical homolytically.[6,7] To harness this chemistry at phosphorylated residues, a suitable carbon−sulfur bond must first be introduced. Fortunately, there is well established Michael-Addition chemistry that can accomplish this in a selective fashion.[8,9] For peptides with phosphorylated serine or threonine (not tyrosine), the addition of a strong base leads to elimination of the phosphate which can be followed by addition of a suitable thiol. In this fashion, a phosphorylated residue can be used as a point of insertion for a chromophore capable of absorbing in the ultraviolet that is also linked through a carbon−sulfur bond. Peptides were modified accordingly by the specific transformation shown in Scheme 1. A napthyl based chromophore was implemented because previous experiments have demonstrated good absorbance at 266 nm in the gas phase.

Scheme 1



The instrumentation has been described in detail elsewhere.[3] Briefly, an LTQ linear ion trap was modified to allow an ~4 mJ pulse from a 266 nm YAG to intersect the trapped ion cloud. Ions can be isolated, photodissociated, and detected in a manner entirely analogous to much more common collision induced dissociation (CID) experiments. The PD spectrum for deprotonated KEAPPA-PPEsP (with the naphthyl modified serine denoted by lower case s) is shown in Figure 1a. There are two large fragments of interest.

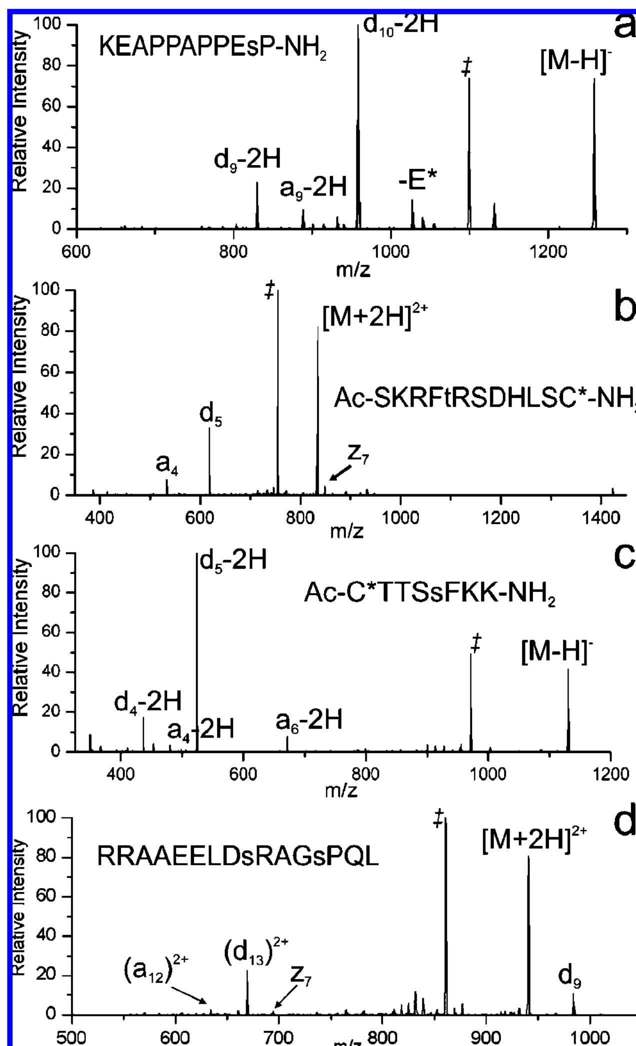


Figure 1. PD spectra for several peptides modified according to Scheme 1. Selective fragmentation yields the largest backbone fragment C-terminal to the site of phosphorylation in each case. Peptide sequences are given for each plot, with lower case letters indicating sites of modification. ∗ refers to oxidized cysteine. ‡ corresponds to loss of the naphthylsulfide.

The carbon−sulfur bond connecting the chromophore to the peptide is homolytically cleaved and yields a major product ion (denoted ‡). The base peak in the spectrum results from fragmentation of the backbone to produce a d_{10} fragment at the modified serine residue. The a_9 fragment is also observed at much lower intensity as are several fragments corresponding to loss of all or part of the glutamic acid side chain. The only observed backbone cleavages are flanking the two sides of the (originally) phosphorylated serine residue. The additional minor side chain losses occur at the residue adjacent to serine, suggesting only fragmentation in immediate proximity to the initially produced radical is observable. Threonine

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containing peptides yield very similar results,[10] indicating that the additional methyl group in threonine does not interfere with selective backbone fragmentation. Additionally, the process does not appear to be very sensitive to the charge state. In fact, similar results are obtained for a variety of charge states, including anions.[10] This suggests that the charge does not play a large role in the resulting chemistry, either directly or indirectly, which contrasts sharply with most gas phase fragmentation experiments.[11]

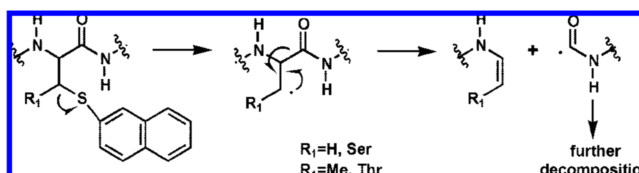
Peptides frequently contain multiple serine or threonine residues. SKRFtRSDHLSC* (C* = oxidized cysteine) has four potential sites for phosphorylation. The results for this peptide are shown in Figure 1b. Phosphorylation at Thr5 is unambiguously revealed by PD of the modified peptide, which yields a large d_5 ion and a much less abundant a_4 ion. These two fragments once again flank the sides of the residue that was originally phosphorylated.

The difficulty is further increased with the peptide that is subjected to PD in Figure 1c, C*TTSsFKK. There are four sequentially continuous possible sites for phosphorylation in this peptide. The phosphorylation site is again easily identified by a large d-type fragment corresponding to cleavage at Ser5, the modified residue. Additional minor a-type fragments flanking both sides of the residue are observed at Ser4 and Phe6. The cleavage at Phe6 is interesting because backbone fragmentation C-terminal to the modified residue is not typically observed. This may relate to the facility with which the β-hydrogen can be abstracted from aromatic residues,[3] which is known to lead to a-type fragments.[12] Further experiments will be required to pinpoint the cause of this cleavage; however, it does not interfere with interpretation of the results because the d_5 fragment is by far the major product. It is further interesting to note that for both SKRFtRSDHLSC* and C*TTSsFKK phosphorylation site identification using typical CID data followed by automated data analysis yields ambiguous or simply incorrect results.[10]

Another complexity that is frequently encountered with phosphorylation is multiple modifications on the same peptide.[13] This challenge is addressed in Figure 1d, where the results for RRAAEELDsRAGsPQL are shown. Addition of a single naphthyl group yields both d_9 and d_{13} ions following PD, suggesting that the single naphthyl is distributed between both sites. The double naphthyl derivative yields similar results, but additional peaks are observed due to the presence of two naphthyls.[10]

In each case above, the primary backbone fragmentations occur at the phosphorylated sites. The mechanism which yields this exclusivity merits further discussion. As noted previously, cleavage of the carbon−sulfur bond occurs directly by photodissociation; however, the remaining fragmentations are not expected products of direct photodissociation. Rather, these reactions are most likely radical directed dissociations that occur after formation of the radical and are facilitated, in part, by the remaining energy. The 266 nm laser provides ~115 kcal/mol of energy, while the carbon−sulfur bond dissociation energy is estimated to require ~71 kcal/mol;[6,14] some of the difference will be converted into vibrational energy in the peptide. Further examination reveals that backbone fragmentation occurs promptly following generation of the radical. For example, reisolation of the radical ion resulting from photodissociation of the carbon−sulfur bond, followed by CID, does not yield the d_{10} fragment in a measurable quantity.[10] Instead, glutamic acid side chain losses are almost exclusively observed. These results suggest that backbone dissociation must occur immediately following or simultaneously with generation of the radical species, or the active radical is rapidly lost to side reactions. This interpretation is in agreement with the predicted high reactivity for the initially formed carbon radical.[15]

Scheme 2



We propose that fragmentation occurs according to the pathway shown in Scheme 2. Photodissociation leads to loss of the naphthylsulfide radical, generating a primary or secondary radical in the β-position. A purely electronic rearrangement then leads to backbone fragmentation generating a stable d-type fragment with an unstable radical $x+1$ complement. The x-type ions are never observed, although the x fragment can decompose to a z-type ion by loss of isocyanic acid. The corresponding z-ions are observed (see Figure 1b); however, the z-ions are always minor peaks because they can degrade further by several pathways involving sequential radical migrations.

Site directed fragmentation at phosphorylated residues has not been previously observed. Herein, we have demonstrated that phosphorylated serine and threonine can be easily and unambiguously identified by selective photofragmentation chemistry. The directed dissociation is further shown to be insensitive to charge state, yielding results for negatively or positively charged peptides. This offers certain advantages compared to ECD and ETD, which are promising methods but suffer due to the requirement for at least two positive charges.[16,17] Data analysis for the present approach is also tremendously simplified relative to other methods because highly specific information is obtained. In more general terms, the present work illustrates a generic method for eliciting site specific fragmentation in large molecules, a task for which there are many potential uses.

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Supporting Information Available: Experimental details and additional spectra. This material is available free of charge via the Internet at http://pubs.acs.org.

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