Mass Spectrometry

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Ultraviolet Photodissociation: Developments towards Applications for Mass-Spectrometry-Based Proteomics

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lasers · mass spectrometry · phosphorylation · radicals · sequence determination

Unraveling of all of the information contained in proteomes poses a tremendous chemical challenge, which is balanced by the promise of potentially transformational knowledge. Mass spectrometry offers an unprecedented arsenal of tools for diverse proteomic investigations. Recently, it was demonstrated that ultraviolet light can be utilized to initiate unique and potentially useful fragmentations in peptides and proteins. Either nonspecific dissociation or highly specific dissociation at engineered chromophoric sites is possible following photon absorption. The level of specificity and control over fragmentation in these experiments is greater than with other fragmentation methods. Novel techniques made possible by this technology are poised to make substantial contributions to the field of proteomics.

1. Introduction

Mass spectrometers are increasingly powerful devices. Improvements in resolution,[1] sensitivity,[2] dynamic range,[3] and ionization sources[4,5] have enabled dramatic contributions to the study of proteins[6] and other biological molecules.[7] However, regardless of instrument performance, mass spectrometers are fundamentally restricted to the measurement of mass/charge and relative abundance. Although the molecular weight of an intact molecule is frequently a very useful piece of data, modern mass spectrometers can typically be utilized to obtain much more information. For example, fragmentation is frequently employed to examine covalent bond structure in both large and small molecules.[8] Noncovalent interactions can also be explored to determine quaternary structure in proteins[9] or examine solution-phase tertiary structure.[10] Exchange reactions can be implemented to explore both gas- and solution-phase behavior.[11–13] Ion mobility (in various guises) can be used with mass spectrometry for rapid separations[14,15] or for examining intact gas-phase structure.[16] Imaging experiments can be utilized to determine spatial relationships between compounds in complex samples, such as tissues.[17] Therefore, although only mass/charge and relative abundance are ever measured, the knowledge that can be obtained by mass spectrometry is quite diverse.

Among the many technologies coupled to mass spectrometers, it is not surprising to find that lasers are commonly employed. Lasers of varying sizes, powers, and wavelengths have been implemented in numerous ion sources and used for ionization, dissociation, and even spectroscopy.[18] Although the combination of ultraviolet (UV) lasers with mass spectrometers has a substantial history,[19–21] including several elegant experiments employing multiple lasers,[22,23] the method has become more common only recently as a means for generating novel dissociation pathways. Advances in both laser and MS technology are driving the union. This emerging technology complements the more established methods that employ low-energy photons.[24]

One area in particular in which MS continues to play a major role is the field of proteomics, as detailed in several reviews.[25–27] The technical and chemical challenges associated with this field are strongly connected to the fact that proteins are highly diverse and complicated molecules, as are their surroundings. The relative abundances of proteins span at least 10 orders of magnitude.[28] The 20 naturally occurring side chains introduce substantial chemical diversity, but this diversity is dwarfed by the challenges resulting from post-translational modifications (over 200 known modifications cannot be predicted from genomic information).[29] When all of these isoforms are included, the number of different molecules to be evaluated is staggering. The sheer numbers require methodology that can be scaled to high-throughput formats. When all of these considerations are taken into account, no single technique or methodology is sufficient.

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Rather, a multipronged approach is preferable. Recent developments in high energy photodissociation mass spectrometry have enabled new approaches to several challenges in proteomics, as detailed herein.

2. Implementation of Photodissociation

In the simplest possible terms, photodissociation (PD) MS requires that ions and photons be located in the same place at the same time to enable absorption of light by the ions. This requirement is most easily fulfilled with "trapping" instruments, in which ions are stored in a particular place for some period of time. Thus, the difficulty in timing the laser pulse is relaxed considerably. Linear-ion-trap and ion-cyclotron-resonance mass spectrometers are particularly well suited for photodissociation experiments owing to geometrical properties which facilitate the introduction of a laser pulse (Figure 1). PD can be implemented in these instruments by the simple addition of a quartz window, without modifying the mass spectrometer itself. The use of 3D ion traps is also possible; however, access holes are then required. These holes are typically drilled through the ring electrode of the ion trap itself. Ions that are not trapped can also be dissociated, but the timing requirements for such experiments are much more stringent, because the laser must fire at the precise moment that ions are passing the accessible region. Similarly, ions must be grouped sufficiently for substantial overlap to occur between the total ion population and the laser pulse.

Laser wavelengths and power vary. Light of wavelength 157, 193, 266, or 355 nm is commonly used (see Table 1). The use of light of wavelength 157 nm requires vacuum coupling between the laser and the mass spectrometer to avoid absorption by air. Another consideration related to many trapping instruments is the amount of time required to fill the trap. Many instruments have an automatic feedback control, which varies the trapping time such that the ideal number of ions is introduced regardless of variations in ion current from the source. As a result, the trap fill time may vary continuously. Ideally, the laser used for PD should be capable of firing at a discontinuous repetition rate that can be matched to variations in trap fill time. Most Nd:YAG lasers (YAG = yttrium aluminum garnet) do not operate well under these conditions; however, there are exceptions among recently developed lasers.

Table 1: Relevant energies for UV lasers.

Laser	λ [nm]	E [kJ mol^{-1}]	E [eV]
F_2 excimer	157	762	7.9
ArF excimer	193	620	6.4
Nd:YAG (4th harmonic)	266	450	4.7
Nd:YAG (3rd harmonic)	355	337	3.5

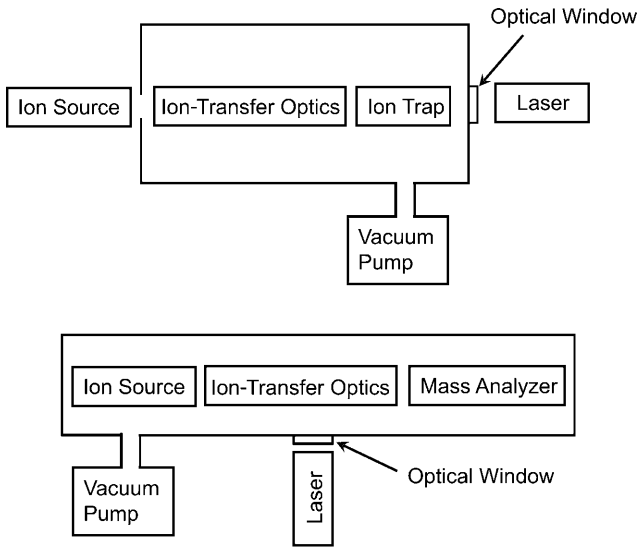

Figure 1. Generic instrument diagrams for trapping and "on-the-fly" photodissociation experiments.

In addition to instrumentation, PD in the ultraviolet region requires that the ion have a suitable chromophore. Experiments conducted at 193 nm, and particularly at 157 nm, avoid this limitation as result of the density of absorptions at these wavelengths. At 266 nm, only tryptophan and tyrosine exhibit some absorption. Peptides that do not contain these residues will not absorb to a significant extent. Essentially no absorption will occur at 355 nm without an appended chromophore. There are advantages to working in both regions. Shorter wavelengths obviate the need to attach chromophores, but longer wavelengths enable experiments in which the location and nature of energy deposition can be controlled rigorously. Potential fragmentation pathways discussed herein are shown schematically in Figure 2.

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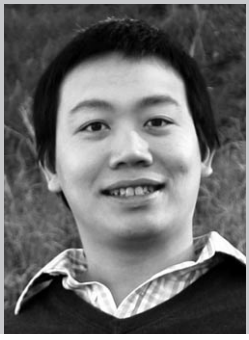

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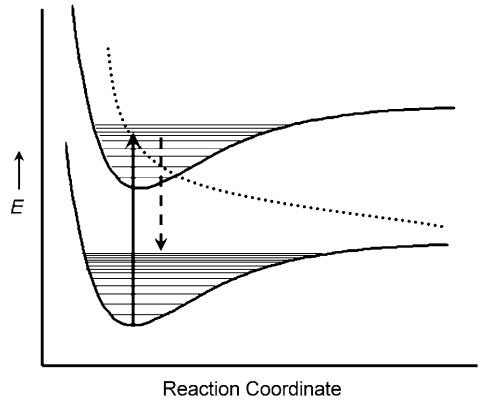


Figure 2. Simplified diagram of energy absorption and fragmentation pathways in PD. Absorption (solid line) of photon energy leads to excitation to a higher electronic state. Coupling to an antibonding state (dotted line) leads to direct dissociation. Otherwise, statistical redistribution of the energy into vibrational modes occurs; this process can be followed by unimolecular dissociation in the ground electronic state.

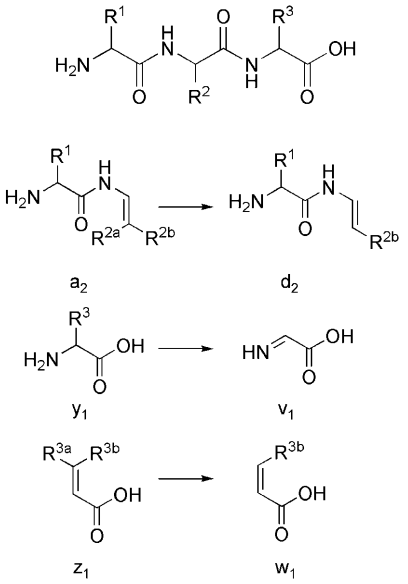
3. Applications in Proteomics

As mentioned in the Introduction, proteomics poses many experimental challenges. UVPD offers several advantages over other fragmentation techniques. First, energy deposition occurs on a timescale that is orders of magnitude faster than that of other methods. Even low-energy PD methods, such as infrared multiphoton dissociation (IRMPD), typically take place on the millisecond timescale.[24,30] UVPD can easily occur on the nanosecond to microsecond timescale. Direct dissociations occur much faster. This speed is advantageous any time a method is scaled up for a high-throughput application, because more data will be acquired in less time. For example, on an ion-trap instrument, an additional 1800 peaks could be examined during a 1 h liquid-chromatography (LC) separation. This speed is even more advantageous in certain instrumental configurations, such as TOF–TOF, in which the time required for fragmentation can be substantial relative to the total time for a scan. Gains in efficiency in this case could be of an order of magnitude or more. A second advantage afforded by some UVPD experiments is control over the introduction of energy. In other words, the specificity of photon/chromophore interactions can be used to dictate the location and amount of energy introduced into a molecule. This control enables directed dissociation experiments that are not possible with other methods.

3.1. Alternative Sequencing Methods

3.1.1. Direct Bond Absorption

Short-wavelength lasers are capable of exciting the peptide backbone directly, which obviates the need for suitable side chains or appended chromophores.[31–35] Fragmentation can then take place by at least two possible mechanisms. The first involves internal conversion of the energy into vibrational modes, followed by fragmentation in the ground electronic state. This process should lead to fragmentation resembling that observed with collision-induced dissociation (CID), IRMPD, or other methods of dissociation under statistical control. Interestingly, PD with short-wavelength lasers does not lead to such fragmentation, and many fragments that are not typically observed by CID are generated. In particular, an extensive range of x- or a-series ions are produced, depending on the location of the charge in the peptide. For relevant structures and standard nomenclature, see Scheme 1.[36,37] Reilly and co-workers attributed the origin of these nonstatistical products to direct



Scheme 1. Standard fragmentation nomenclature and corresponding structures.[36,37]

or prompt dissociation, which occurs in the excited state. Initially, it was thought that this dissociation may occur only at 157 nm,[38] but other experiments have demonstrated similar fragmentation at 193 nm.[34,19] The timescale for observation is critical in these experiments. Unique fragmentation dominates at short observation times (microseconds), whereas fragmentation observed after several milliseconds more closely resembles CID. The v-, w-, and d-type ions observed in these experiments can also be used to extract side-chain information. PD can discriminate readily between leucine and isoleucine as a result of side-chain losses which are not typically observed by CID or other statistical fragmentation methods (Figure 3). To sum up, UVPD reveals useful sequence information that is complementary to or additional to that obtained by other methods. This approach was recently reviewed.[39]

3.1.2. Noncovalent Chromophore Attachment

Wavelengths longer than 193 nm are not absorbed well by peptides and proteins owing to the paucity of chromophores at these wavelengths. Although this issue can be circumvented by the covalent attachment of a suitable chromophore, such

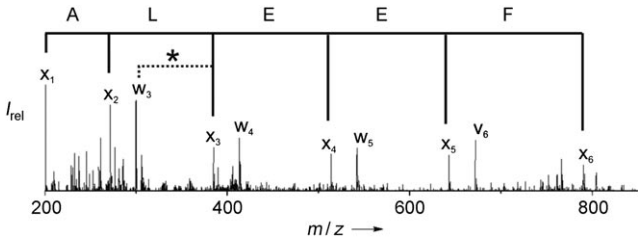


Figure 3. PD spectrum acquired at 157 nm demonstrating complete sequence coverage in the x ion series. Leucine is differentiated from the isobaric amino acid isoleucine by the characteristic mass shift between x_3 and w_3 (*). Adapted from reference [31] and reprinted with permission.

an approach is undesirable because of the additional chemical transformations that must be carried out. An alternative solution is to attach chromophores through noncovalent interactions. In this case, it is only necessary to add the relevant molecules to the sample solution prior to analysis. Two examples of such molecules are shown in Figure 4. This

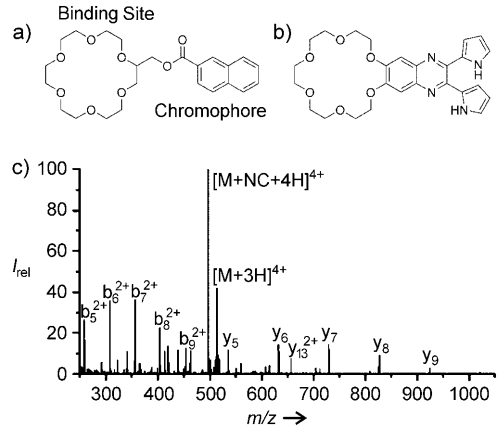


Figure 4. The noncovalent attachment of a UV chromophore, for example, with a) naphthoyl [18]crown-6 ether (NC) or b) [18]crown-6 dipyrrolylquinoxaline, facilitates fast deposition of energy into the peptide. c) The resulting fragmentation is identical to collision-induced dissociation; however, UVPD was shown to occur on a much shorter timescale. Adapted from references [40] and [41] with permission.

experiment is quite counterintuitive in that energy must be absorbed by the chromophore, converted into vibrational energy, and then transferred to the biomolecule prior to fragmentation of the noncovalent union. Of course, the amount of energy transferred must then be sufficient to break covalent bonds! Nevertheless, this process has been shown to be effective by two research groups with several different molecules.[40,41] A sample spectrum showing abundant fragmentation is shown in Figure 4c. One key appears to be a multidentate interaction at the noncovalent junction. This interaction decreases the likelihood of the simultaneous excitation of all noncovalent bonds prior to significant energy transfer.

3.1.3. Femtosecond Fragmentation

Recent experiments conducted at 800 nm with a femtosecond titanium sapphire laser are also of interest for this Minireview.[42] Although it is clear that 800 nm is not in the UV portion of the electromagnetic spectrum, the fragmentation observed in these experiments is much more similar to that of UVPD[23] than it is to that of IRMPD. Furthermore, as there are no peptide chromophores in this region of the spectrum, nonlinear absorption is probably occurring. Also, the relevant energetics dictate that several photons must be absorbed. These experiments generate a substantial amount of directly observable photoionized peptide and fragments derived from this species (Figure 5). The photoionized peptide is hydrogen-deficient and (by mass) identical to the

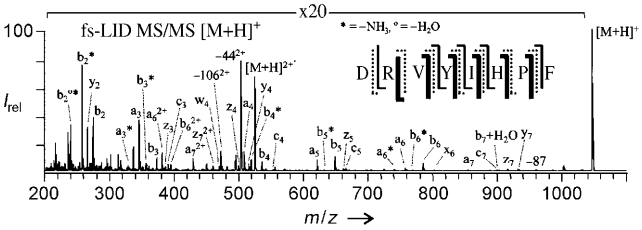


Figure 5. Femtosecond laser induced dissociation mass spectrometry (fs-LID MS/MS) of angiotensin II with 800 nm photons. The backbone fragmentation observed is consistent with dissociation of the $[M+H]^{2+\cdot}$ species and other high-energy fragmentation processes. Adapted from reference [42] with permission.

peptide radicals discussed in Section 3.3. Not surprisingly, many of the products observed in these experiments are also similar to those described in Section 3.3. Other fragments similar to those generated by short-wavelength UVPD, as described in Section 3.1.1, are also observed (such as w- and a-series ions). The results suggest that several processes may occur in these experiments, but further results will be needed for an understanding of the exact mechanisms involved. Nevertheless, it is clear that a high-intensity pulse of short duration can be used to elicit fragmentation, as observed in previous experiments with femtosecond pulses.[23]

3.2. Selective Fragmentation

3.2.1. Appended-Chromophore Suicide

One advantage of UVPD is that a paucity of appropriate chromophores exists at certain wavelengths. For example, absorption by native peptides and proteins is minimal at 355 nm. Wilson and Brodbelt cleverly used this property to direct fragmentation in modified peptides.[43] These experiments rely on derivatization of one end of the peptide with a chromophore that absorbs at 355 nm. Internal conversion of the initial photoexcitation energy into vibrational energy leads to fragmentation of the peptide. The key to these experiments lies in the fact that fragments which do not contain the chromophore cease to absorb further energy and are therefore detected readily. On the other hand, fragments

containing the chromophore continue to absorb photons and are fragmented further, to a point at which they are essentially not detected. In this manner, a series of ions originating from the side of the peptide opposite to the chromophore is generated and can be evaluated readily without the presence of complicating complementary pairs (Figure 6). Selective fragmentation is thus used to eliminate unwanted ions and is only possible as a result of the high specificity of the photon/chromophore interaction. Similar results were observed at 266 nm, although in this case the possibility for interference by native chromophore absorption exists.[44]

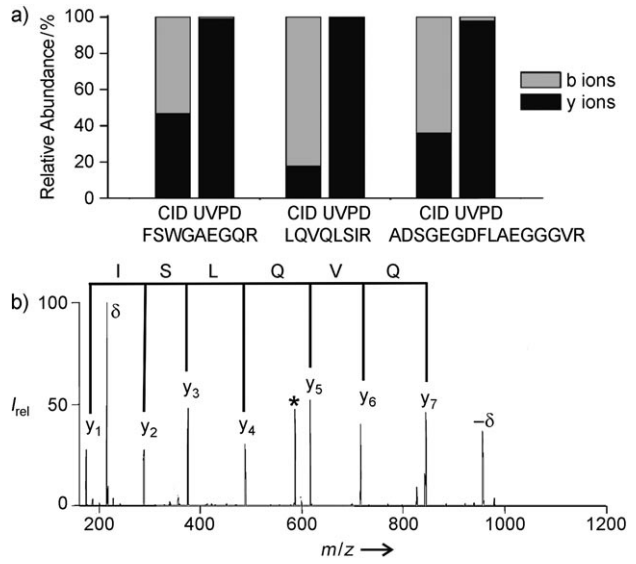


Figure 6. UVPD (at 355 nm) of peptides modified with a UV chromophore covalently attached to the N terminus leads to selective degradation of the b-sequence ions. a) A comparison of the fragment ions produced by CID and UVPD is shown for three representative peptides. b) UVPD of the modified peptide, LQVQLSIR. Importantly, the presence of only a single type of fragment ion enables de novo sequencing. Adapted from reference [43] with permission. δ = 7-amino-4-methyl-coumarin-3-acetic acid; * precursor ion.

3.2.2. Disulfide Annihilation

Disulfide bonds are critical in terms of structure formation and stability in proteins and peptides; however, they are a nuisance in terms of analysis by MS. Disulfide bonds form cyclic elements within proteins. These elements complicate linear sequence analysis. The typical method for dealing with this issue is to reduce and alkylate all disulfide bonds, but this approach is undesirable owing to the need for additional solution-phase chemistry and the potential heterogeneity that can result from incomplete alkylation. Zubarev and coworkers introduced a method in which light of wavelength 157 nm is used for the selective fragmentation of disulfide bonds, which absorb strongly at this wavelength.[45] Both intra- and intermolecular disulfide bonds can be broken. This cleavage frequently yields the largest peaks in the spectrum. The method requires far less time than that needed for solution-phase approaches. Its primary limitation is that absorption by the peptide backbone still occurs to yield competitive fragments. Ultimately, this undesired fragmentation will limit sensitivity, particularly for larger systems. However, in the case of smaller molecules, this method can lead to efficient fragmentation and full sequence identification (Figure 7).

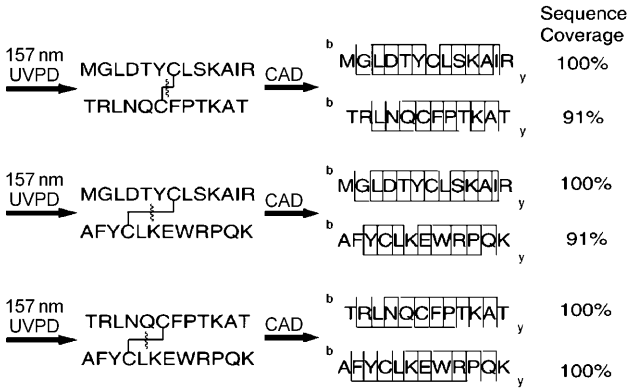


Figure 7. Disulfide linkages decrease fragmentation efficiency and are typically reduced in solution prior to mass spectrometry. UVPD has been shown to fragment disulfide bonds selectively. Nearly full sequence coverage was observed for three peptides containing disulfide linkages with a combination of UVPD and collisionally activated dissociation (CAD). Adapted from reference [45] with permission.

3.3. Radical Initiation

Substantial interest has been generated by methods in which electrons are used for fragmentation; that is, electron capture dissociation (ECD)[46] and electron transfer dissociation (ETD).[47] Although the exact mechanism that precedes fragmentation by these methods is still under investigation,[48,49] it is clear that radical chemistry is involved, because both methods generate odd-electron species. The challenge with electron-generated radicals with respect to selectivity is that neither the electron nor the energy released from capturing it can be directed to a particular bond. For this and other reasons, alternate methods have been explored for radical generation.[50,51] UVPD has been demonstrated to yield abundant peptide or protein radicals in a highly controllable fashion.

The photochemistry utilized to generate radicals by PD is substantially different from that employed in the standard UVPD experiment. Several bond types, including C–I and C–S bonds, are susceptible to direct fragmentation following the absorption of a UV photon.[52,53] Homolytic fragmentation in the excited state occurs to yield radical species directly (dotted curve in Figure 2). Although iodine-containing species can absorb UV light directly, the fragmentation efficiency is greatly enhanced for both C–I and C–S bonds by direct coupling to an appropriate chromophore. The energy absorbed by the chromophore is coupled efficiently into the dissociative state. In this manner, energy can be delivered effectively to a particular site to generate the radical at that location. C–I bonds can be introduced readily into proteins and peptides by the iodination of tyrosine. C–S bonds can be

introduced at cysteine or phosphorylated serine or threonine residues. Both processes are described in greater detail below.

3.3.1. Whole Protein Radicals

The iodination of proteins and peptides in solution occurs preferentially at tyrosine residues, and is further selective in whole proteins for the most exposed residues.[54] Therefore, careful control of the reaction can yield abundant monoiodo-substituted protein, in which the iodine substituent resides mainly at a single tyrosine residue. Subsequent photoactivation with a 266 nm photon yields a radical at the tyrosine residue in substantial yield (Figure 8a,b).[55] Collisional activation of this radical product leads to fragmentation primarily in the vicinity of the radical (Figure 8a,c). Fragmentation at the tyrosine residue and proximate proline residues was observed to be particularly abundant. This level of controllable selectivity in directing the fragmentation of a whole protein had not been demonstrated previously. It was further demonstrated that the time required to analyze proteomics data can be decreased significantly (by several orders of magnitude) if fragmentation positions are controlled and the number of fragments limited in this manner. Efficiency is improved, because the information content of the data is increased when the results are predictable. Such an approach remains hypothetical, as it has not been applied to a high-throughput experiment, but offers the promise of substantial advantages over current methods.

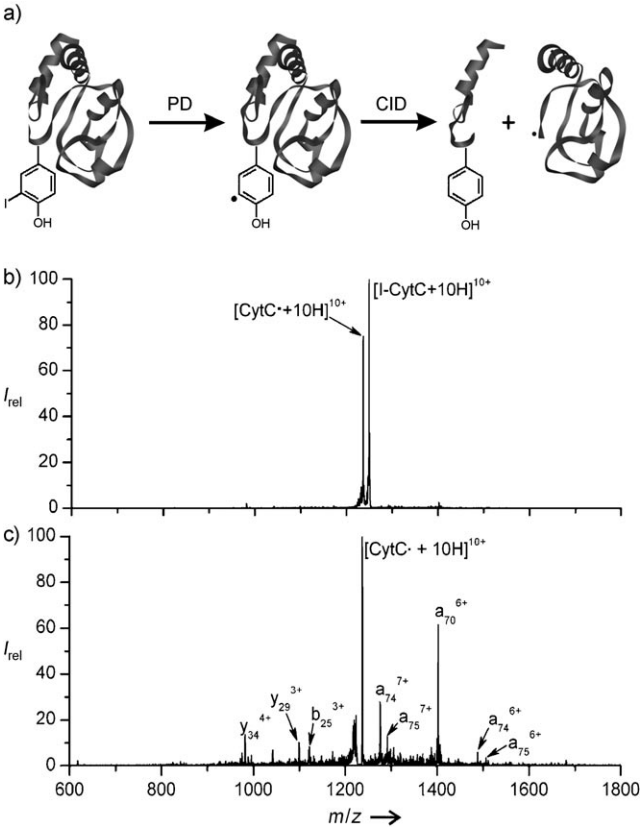


Figure 8. a) Illustration of the concept of selective fragmentation in a whole protein. b) UVPD of iodine-labeled proteins generates a radical on the side chain of tyrosine residues site specifically. c) CID of the radical product leads to backbone fragmentation in proximity to the modified tyrosine residue (Tyr74). CytC = cytochrome *c*.

3.3.2. Identification of Phosphorylation

The identification of posttranslational modifications (PTMs) remains an important challenge in all proteomics experiments.[29] Even if PTMs are not targeted specifically in an experiment, their presence can still complicate data evaluation and must be accounted for. As demonstrated above, the specificity of the photon/chromophore interaction can provide a control over energy deposition that is not available with other fragmentation methods. With respect to PTMs, phosphorylation at threonine and serine residues provides an excellent and specific handle for derivatization.[56] By eliminating the phosphate and introducing a chromophore containing a C–S bond, a photoinitiated radical can be placed in precisely the required location for the fragmentation of the peptide backbone. In other words, photoactivation of the modified peptide leads to a single fragmentation at the modified site.[57] This experiment offers not only tremendous simplicity for the interpretation of the results, but also fundamental sensitivity enhancements due to the greatly diminished number of peaks generated. For example, in a 20 residue peptide, around 40 fragments would be expected from ECD/ETD, whereas the photodissociation experiment would yield only two or three. Sample spectra illustrating this difference are shown for a smaller peptide in Figure 9. The relevant information in Figure 9b is contained in the largest peak in the spectrum, and virtually all of the fragment intensity identifies the site of phosphorylation. Therefore, the gas-phase chemistry of UVPD is at least an order of magnitude more sensitive than traditional approaches. It has also been demonstrated that the identification of phosphorylation sites by this method is much more reliable than by the more traditional CID methodology.

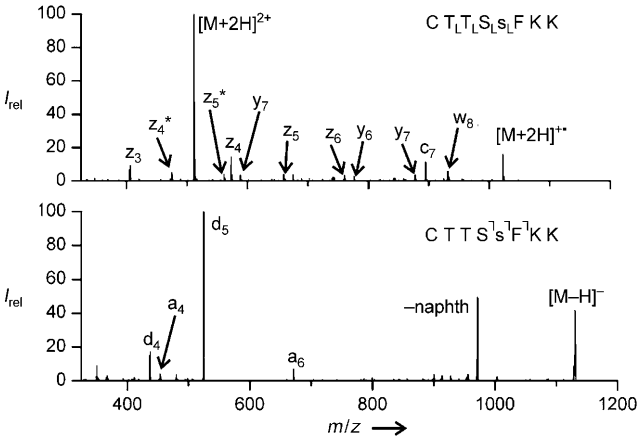


Figure 9. ECD (top) and radical directed dissociation (RDD; bottom) spectra of the phosphopeptide CTTSsFKK reveal that both techniques identify the phosphorylation site correctly. However, RDD is superior in fragmentation efficiency (in terms of both yield and selectivity), which becomes important for phosphopeptides present at low concentrations. The bottom spectrum was adapted from reference [57] with permission. * Loss of H_3PO_4.

By using a very different approach, Dugourd and coworkers demonstrated that UVPD can be used to generate radical anions.[58] In these experiments, laser irradiation of a dianionic phosphopeptide with light of wavelength 262 nm led to electron detachment. Further collisional activation of the anionic radical peptide produced a series of a- and x-type fragments and led to side-chain losses, including loss of the labile phosphate group (Figure 10). However, the phosphate group was retained in most of the backbone fragments, which enabled identification of the phosphorylation site. Although this approach is nonspecific, the introduction of a radical appears to lower backbone-fragmentation thresholds and make these processes comparable energetically to the loss of the phosphate group.

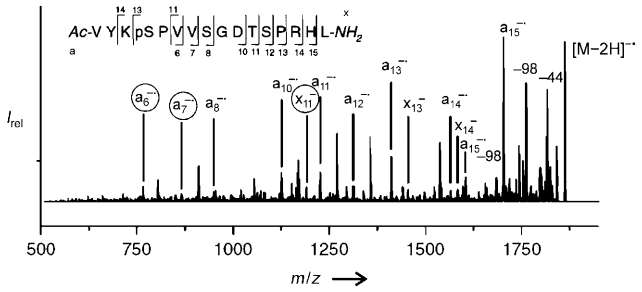


Figure 10. UVPD of a triply charged phosphopeptide anion detaches an electron to produce a doubly charged radical species. Further collisional activation yields backbone fragments that retain the phosphate group. These fragments enable identification of the phosphorylation site. Key fragment ions that identify Ser4 as the phosphorylation site are circled. Adapted from reference [58] with permission.

3.3.3. Noncovalent Radical Delivery

Ideally, routes to generate radical peptides should not require derivatization in solution. A similar approach to that described in Section 3.1.2 can be implemented to obtain radical peptides and proteins, but only in combination with PD.[59] A photolabile radical precursor, such as iodonaphthyl, is coupled to a noncovalent delivery agent, such as [18]crown-6. Following photoactivation of the radical, subsequent transfer of the radical to the complexed peptide through hydrogen-atom migration can occur. Following collisional activation, the radical-delivery agent is removed, and the isolated peptide radical is obtained. Similar attempts to use this methodology in conjunction with CID activation of the initial radical precursor failed. In this case, the radical is generated under statistical control; that is, the noncovalent bonds will typically rupture prior to generation of the radical. An interesting result that emerged from the examination of a body of these hydrogen-deficient peptide radicals is that such species yield a-type ions preferentially following backbone fragmentation at sites with enhanced β-radical stability (Figure 11). Notably, all aromatic residues, including histidine, are among the favorable sites for a-type fragmentation. The enhanced presence of these ions in other PD experiments may signal the occurrence of photoionization to generate hydrogen-deficient radicals.

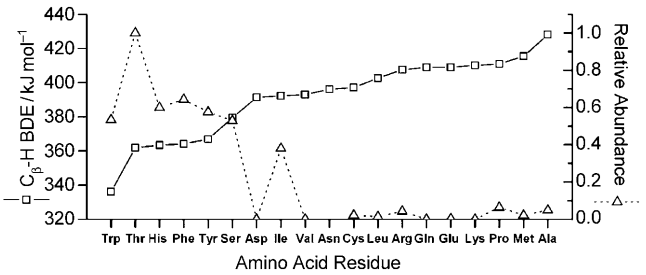


Figure 11. A comparison of calculated C_β–H bond-dissociation energies (squares, left *y* axis) and radical-induced backbone fragmentation (triangles, right *y* axis) shows that higher backbone fragmentation correlates with a lower C_β–H bond-dissociation energy (BDE). Radical-induced backbone dissociation is most likely to occur at residues containing aromatic or alcohol side chains.

4. Summary and Outlook

Advances in technology in both mass spectrometry and lasers will continue to open new avenues for research in which these technologies are combined. In particular, the release of linear ion traps, which are both inexpensive and well-adapted for coupling to lasers, promises to promote further development in this area. The control and speed afforded by UVPD has enabled unique experiments that are not possible with other methods, and it is likely that experiments based on this technique will continue to be designed and implemented.

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