Infrared Multiphoton Dissociation and Electron-Induced Dissociation as Alternative MS/MS Strategies for Metabolite Identification

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A major challenge encountered in mass spectrometric metabolite analysis is the identification and structural characterization of metabolites. Fourier transform ion cyclotron resonance mass spectrometry is a valuable technique for metabolite structural determination because it provides accurate masses and allows for multiple MS/MS fragmentation strategies, including infrared multiphoton dissociation (IRMPD) and electron-induced dissociation (EID). Collision activated dissociation (CAD) is currently the most commonly used MS/MS technique for metabolite structural characterization. In contrast, IRMPD and EID have had very limited, if any, application for metabolite characterization. Here, we explore IRMPD and EID of phosphate-containing metabolites and compare the resulting fragmentation patterns to those of CAD. Our results show that CAD, IRMPD, and EID provide complementary structural information for phosphate-containing metabolites. Overall, CAD provided the most extensive fragmentation for smaller (<600 Da) phosphate-containing metabolites; however, IRMPD generated more extensive fragmentation for larger (>600 Da) phosphate-containing metabolites, particularly for species containing increased numbers of phosphate groups. EID generally provided complementary fragmentation to CAD and showed extensive fragmentation with relatively evenly abundant product ions, regardless of metabolite size. However, EID fragmentation efficiency is lower than those of CAD and IRMPD.

Metabolites play multiple key roles in living cells and their levels represent integrative information on cellular function and define the phenotype of a cell or tissue in response to genetic or environmental changes. mRNA levels do not always correlate with protein levels, and translated protein may or may not be enzymatically active. Therefore, changes observed in the transcriptome or proteome do not always directly correspond to phenotypic alterations. Thus, measurement of the metabolites synthesized by a biological system is often crucial to completely assess genetic function.1–6

Metabolite analysis includes identification and quantification of all intracellular and extracellular metabolites by different analytical techniques. Metabolites have wide variations in chemical (e.g., molecular weight, polarity, acidity) and physical (e.g., volatility) properties, rendering it difficult to analyze various metabolites simultaneously. Mass spectrometry (MS), nuclear magnetic resonance (NMR), and Fourier transform infrared (FT-IR) and Raman spectroscopies are all used for metabolite analysis.7–12 FT-IR and Raman spectroscopy allow rapid, nondestructive, and high-throughput analyses of a diverse range of sample types. These two techniques are typically used for metabolic profiling due to their holistic nature, although absorptions at specific wavelengths can be used for identification of unknown metabolites. However, the sensitivity and selectivity of FT-IR and Raman spectroscopy are not as high as for other methods.11 NMR spectroscopy also constitutes a rapid and nondestructive, high-throughput method that requires minimal sample preparation. Furthermore, chemical shifts can be assigned to specific metabolites. However, NMR requires millimolar to high micromolar concentrations of samples, and thus, its sensitivity is not as high as that of mass spectrometry. Another major weakness of NMR is its poor dynamic range (10^3), which results in detection of only the most abundant components.11,13 Mass spectrometry remains the most suitable technology for measurement of metabolites because of its wide dynamic range (10^4–10^10), good sensitivity (nM) and ability to detect a diverse number of molecular species.7 Gas chromatography/mass spectrometry (GC/MS) has been the most commonly used MS method for small molecule analysis. However, this technique often involves sample derivatization to improve volatility and is limited in terms of molecule size and type due to volatility and polarity constraints.

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Therefore, liquid chromatography/mass spectrometry (LC/MS) has become a popular alternative choice for metabolite analysis. This technique has advantages over GC/MS in that sample derivatization is generally not required, although it can be beneficial to improve chromatographic resolution and sensitivity, and more diverse chemical structures and increased molecular sizes can be observed.7,11,14

One major challenge encountered in MS-based metabolite research is the identification and characterization of the hundreds to thousands of detected metabolites. Currently, mass spectral libraries are not complete enough to allow identification of all unknown metabolites. For example, the Golm metabolome database (http://csbdb.mpimp-golm.mpg.de/csbdb/gmd/gmd.html) includes mostly plant metabolites and is limited to GC/MS data,15 and the National Institute of Standards and Technology (NIST) database (http://www.nist.gov/srd/nist1.htm) includes only electron ionization (EI)-MS data.16 The METLIN (METabolite LINk) database (http://metlin.scripps.edu/) includes an annotated list of known metabolite structural information with tandem mass spectra and LC/MS data, but the number of mass spectra is still not sufficient to cover the large number of metabolites typically detected.10 Nevertheless, successful metabolite identification has been reported by utilizing molecular weight matching with the MetaCyc, NIST, and the Kyoto Encyclopedia of Genes and Genomes (KEGG) databases.15,17,18 However, the existence of compounds with similar or identical molecular weights, i.e., isobars and isomers, can result in misidentification. In such cases, tandem mass spectrometry (MS/MS) involving collision activated dissociation (CAD) has been used to obtain structural information about metabolites, thereby aiding in their identification.8,18–21

Fourier transform ion cyclotron resonance (FTICR) mass spectrometry provides several advantages for metabolite analysis. First, the accurate mass capabilities of FTICR MS can reduce the number of possible metabolite candidates at a given nominal mass.22 Second, the ultrahigh resolution of FTICR MS allows analysis of highly complex metabolite mixtures.9 Third, an FTICR mass analyzer coupled with mass selective external ion accumulation provides improved dynamic range.23,24 Furthermore, FTICR MS offers MS/MS techniques other than CAD, such as electron-induced dissociation (EID)25,26 and infrared multiphoton dissociation (IRMPD),27,28 that can provide additional structural information, sometimes crucial to the determination or confirmation of metabolite structure.7 To identify and characterize an unknown metabolite, it is desirable to obtain as many bond cleavages as possible for increased confidence. For MS/MS of metabolites, negative mode ionization is generally preferred due to the increased ionization efficiency of many acidic and neutral metabolites. However, negative mode CAD often results in limited fragmentation, possibly due to the absence of a mobile proton.29 Thus, alternative fragmentation strategies (e.g., IRMPD and EID) appear promising to overcome this issue. Electron-induced dissociation, which involves irradiation of analyte ions with electrons, was first shown in 1979 by Cody and Freiser for radical cations.26

Phosphate-containing metabolites are important in life processes, such as phosphate metabolism and energy conversion. Phosphates are most commonly found in the form of nucleotides, cofactors, and phosphorylated carbohydrates. Phosphate-containing metabolites have been identified by accurate mass and/or CAD-based MS/MS, which provides limited structural information because it mainly results in cleavages at phosphate groups, or charge-directed fragmentation.18,19,33 IRMPD has been shown to be an efficient technique for fragmentation of nucleic acids and phosphoproteins due to strong phosphate absorption at the 10.6 μm wavelength typically used.25,35–38 However, IRMPD of phosphate-
containing metabolites has, to our knowledge, not previously been described. Here, we report the utility of both IRMPD and EID FTICR MS for characterization of phosphate-containing metabolites, including phosphorylated carbohydrates and nucleotides. These fragmentation techniques are also compared to CAD of the same species.

EXPERIMENTAL SECTION

Sample Preparation. Phosphorylated metabolites investigated in this work were purchased from Sigma-Aldrich (St. Louis, MO) and used without further purification. Negative mode electrospray solvent consisted of 1:1 (v/v) isopropanol/water (Fisher, Fair Lawn, NJ) with 10 mM ammonium acetate (Fisher). The final metabolite concentration was 1–20 μM.

Fourier Transform Ion Cyclotron Resonance Mass Spectrometry. All experiments were performed with a 7 T quadrupole (Q)-FTICR mass spectrometer20 (Bruker Daltonics, Billerica, MA) operating in the negative ion mode. Samples were infused via an external Apollo electrospray ion source at a flow rate of 60–70 μL/h, mass selectively externally accumulated23,24 (6 m/z isolation window) for 1–3 s, and captured in the ICR cell by dynamic trapping. The accumulation, ion transfer, and capture events were looped 2–3 times to improve precursor ion abundance.

CAD was performed in the external hexapole at a collision cell dc offset of 4–20 V with Ar as the collision gas. IRMPD was performed inside the ICR cell with a vertically mounted 25 W, 10.6 μm, CO2 laser (Syrnad, Mukilteo, WA). The laser beam was deflected by two mirrors for alignment through a hollow dispenser cathode to the center of the ICR cell. The beam entered the vacuum system through a BaF2 window. Photon irradiation was performed for 50–100 ms at 8.75–10 W laser power. An indirectly heated hollow dispenser cathode was used for electron generation.40 A heating current of 1.8 A was applied to a heater element located behind the cathode. For EID, performed inside the ICR cell, the cathode bias voltage was pulsed to 15–20 V for 6–8 s.

All mass spectra were acquired with XMASS software (version 6.1, Bruker Daltonics) in broadband mode from m/z 21 to 1000 with 512k data points and summed over 10–15 scans. Data processing was performed with the MIDAS analysis software.41 Calculated masses of precursor ions, [M – H]-, and product ions corresponding to either loss of the phosphate moiety, or adenine, were used for internal calibration. After calibration, assigned product ions were within 10 ppm with only a few exceptions. H2O losses were considered if 18 Da differences between adjacent peaks could not be explained by direct fragmentation of metabolites.

RESULTS

CAD, IRMPD, and EID of Phosphorylated Carbohydrates Involved in Glycolysis. Glucose-6-phosphate (G6P) is a glucose molecule phosphorylated at carbon number 6. Such phosphorylation occurs immediately following glucose entry into a cell. G6P participates in two important metabolic pathways: glycolysis and the pentose phosphate pathway. Other carbohydrates, such as fructose 1,6-bisphosphate (F1,6BP), phosphoenolpyruvate (PEP), and phosphoglycerate (PG), participating in glycolysis are also phosphorylated. Figure 1 shows CAD, IRMPD, and EID MS/MS spectra from deprotonated G6P, and observed bond cleavages are summarized in Scheme 1. From Figure 1 and Scheme 1, it is evident that sugar cross-ring cleavages are generated for this carbohydrate with all three fragmentation methods although they are most prevalent in CAD, which produced three cross-ring fragments (at m/z 139, 169, and 199) differing by 30 amu (i.e., CH2O). IRMPD resulted in similar product ions as CAD although cross-ring cleavage was less prominent, and the most abundant product ions were instead from cleavage at the phosphate group (m/z 97). The latter product ions are likely abundant in external CAD as well; however, due to the time-of-flight effect when transferring ions to the ICR cell, they are likely not trapped as efficiently as in IRMPD in which product ions are generated inside the ICR cell. By contrast, EID showed only limited fragmentation (two cross-ring fragments were detected) compared to the other two dissociation methods, and phosphate group cleavages were not observed. Other phosphorylated carbohydrates, including F1,6BP, PEP, and PG, involved in glycolysis were also investigated, and the only observed product ions in MS/MS (sustained off-resonance irradiation collision activated dissociation (SORI-CAD),42 IRMPD, and EID) spectra corresponded to phosphate group cleavages (data not shown). Only limited fragmentation was observed in IRMPD and EID of F1,6BP, PEP, and PG, likely due to low precursor ion abundances and the necessity to use in-cell precursor ion isolation due to interfering species with similar m/z values. The latter approach affects the position of the ion cloud in the cell and, consequently, can reduce the overlap between precursor ions with photons or electrons. For phosphorylated carbohydrates, CAD appears to be the most suitable MS/MS strategy although phosphate group cleavages are more apparent in IRMPD. Similar CAD fragmentation of small phosphorylated carbohydrates has been reported by Ferule et al.34

CAD, IRMPD, and EID of Adenosine 5′-Triphosphate (ATP). ATP is a multifunctional nucleotide with several biochemical roles. This molecule transports chemical energy within cells and is produced during photosynthesis and cellular respiration. ATP is also one of four nucleotides required for the synthesis of ribonucleic acids. The structure of this molecule consists of a purine base (adenine) attached to the 1′ carbon atom of a pentose (ribose), which also has three phosphate groups attached at its 5′ carbon (see Scheme 2).

CAD, IRMPD, and EID of deprotonated ATP were explored, and the results are shown in Figure 2 and in Scheme 2. From these data, it is evident that CAD and EID of ATP provide complementary structural information. Product ions at m/z 328, 371, and 426, originating from neutral loss of the two terminal phosphates (178 Da), adenine (135 Da), and terminal phosphate (80 Da), respectively, were observed only in CAD. Furthermore, the carbon–oxygen ester bond of adenosine was only cleaved in

EID (resulting in the product ion at \(m/z 257\)) and \(\text{H}_2\text{O}\) losses from secondary fragmentation (\(m/z 310\) and 353 in CAD and \(m/z 390\) in EID) were only observed in CAD and EID. Neutral loss of the terminal phosphate group (98 Da) was the dominant fragmentation channel in both CAD and IRMPD, whereas no fragmentation channel was particularly dominant in EID, which resulted in product ions of relatively even abundance. In summary, CAD provided most structural information for ATP although EID resulted in the additional carbon-oxygen bond cleavage of adenosine. However, the fragmentation efficiency of EID was much lower than that of CAD.

CAD, IRMPD, and EID of Adenosine 5'-Diphosphate-ribose (ADP-ribose). ADP-ribose has a structure similar to ATP, except that the terminal phosphate is replaced with ribose (see Scheme 3). This molecule has been proposed to be involved in cellular signaling leading to necrosis or apoptosis.\(^{43}\) Figure 3 shows CAD, IRMPD, and EID spectra of deprotonated ADP-ribose. Product ions at \(m/z = 291\) and 498, corresponding to cleavage of one phosphoester bond to lose diphosphate-ribose and ribose cross-ring cleavage, respectively, were only observed in CAD. The carbon-oxygen ester bond of adenosine was only fragmented (resulting in \(m/z 309\)) in EID, similar to the case of ATP (Figure 2 and Scheme 2). Neutral ribose ring loss, corresponding to \(m/z 426\), was observed in CAD and EID but not in IRMPD, whereas deprotonated adenine (\(m/z 134\)) was observed in IRMPD and EID but not in CAD. Product ions from secondary fragmentation were observed in EID and IRMPD but not in CAD. EID generated more extensive secondary fragments than IRMPD, including \(m/z 159\), 177, and 273, which were all unique to EID. In IRMPD, phosphoanhydride bond cleavage and phosphate loss dominated. Overall, for ADP-ribose, EID showed the most extensive fragmentation although relative product ion abundances were weak.

CAD, IRMPD, and EID of Nicotinamide Adenine Dinucleotide (NAD). Nicotinamide adenine dinucleotide (NAD) is an important cofactor found in cells. NAD, which is the oxidized form of NADH, is extensively used in glycolysis and the citric acid cycle of cellular respiration. The reducing potential stored in NADH can be converted to ATP through the electron transport chain or used for anabolic metabolism. In NAD, nicotinamide is attached at the 1' carbon of ribose (see Scheme 4).

In CAD of deprotonated NAD (Figure 4), only neutral nicotinamide loss (resulting in \(m/z 540\)) was observed, independent of the collision energy. By contrast, extensive fragmentation of NAD occurred in both IRMPD and EID. Phosphoanhydride bond

cleavages (to yield m/z 328 and 346) and loss of deprotonated adenine (m/z 134) dominated in IRMPD along with neutral nicotinamide loss (m/z 540). The same fragments were observed in EID, but once again, the latter technique showed relatively evenly abundant product ions although the fragmentation efficiency was lower than that of IRMPD.

**CAD, IRMPD, and EID of Nicotinic Acid-Adenine Dinucleotide Phosphate (NAADP).** Recent work suggests that NAADP constitutes one of the Ca²⁺ signaling messengers.⁴⁴,⁴⁵ The structure of NAADP is similar to NAD, but it contains nicotinic acid instead of nicotinamide and a phosphate group is attached to the 2′ carbon of the adenosine ribose (Scheme 5).

CAD of deprotonated NAADP (Figure 5) showed dominant neutral nicotinic acid loss (m/z 620) (independent of the collision energy), but IRMPD and EID both resulted in rich fragmentation patterns, similar to NAD. In IRMPD, secondary fragmentation was extensive: dominant product ions resulting from phosphate loss, cleavages of a phosphoester bond (m/z 159, 255, and 488), and cleavages of phosphoanhydride bonds (m/z 193, 291, 328, 408, and 426) were observed, along with combined loss of the nicotinic acid nucleoside and the adenine 2′ nucleotide (m/z 177). The EID spectrum was quite similar to IRMPD although fragmentation was not as efficient and more product ions were observed in IRMPD.

**DISCUSSION**

The MS/MS spectra shown represent experimental conditions that provided the highest fragmentation efficiency. In CAD, the collision energy was chosen to reduce the precursor ion abundance by ~50%. For NAD and NAADP, higher collision energy provided no further fragmentation, i.e., only nicotinic acid or nicotinamide neutral loss was observed independent of the energy.

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chosen. In IRMPD, abundances of lower m/z product ions increase with increased irradiation time or laser power due to increased secondary fragmentation. Irradiation conditions were chosen to yield similarly abundant product ions at lower and higher m/z values. In EID, electron energy plays an important role for the fragmentation outcome. The utilized cathode bias voltage range of −(15–20) V yielded similar EID spectra at the same irradiation time. Lower quality EID data were obtained within 2 V outside this range. However, lower electron energy generated no fragmentation, and higher electron energy resulted in significant depletion of precursors ions without detection of product ions. The detrimental effects of too high or low electron energy could be somewhat compensated for by adjusting the irradiation time (longer time for lower energy electrons and shorter time for high-energy electrons).

In the experiments reported above, CAD showed very limited fragmentation for NAD and NAADP with dominant nicotinic acid or nicotinamide neutral loss. By contrast, CAD provided more extensive fragmentation than IRMPD and EID for smaller molecules, including G6P, ATP, and ADP-ribose. However, EID provided quite extensive fragmentation for all metabolites, including structural information complementary to that obtained from CAD for smaller molecules. For smaller molecules, IRMPD provided somewhat less information than CAD, except for the phosphate loss (due to secondary fragmentation) observed for ADP-ribose. By contrast, IRMPD resulted in extensive fragmentation, similar to EID, for larger molecules, although the IRMPD fragmentation efficiency was higher than that in EID. The product ions being dominant in CAD were also dominant in IRMPD along with product ions generated by phosphate loss and phosphoester and/or phosphaanhydride bond cleavages. For the larger phosphate-containing metabolites, EID and IRMPD both showed extensive secondary fragmentation.

From these results, it appears that CAD is the best MS/MS strategy for small phosphorylated carbohydrates involved in glycolysis although EID can provide complementary structural information.

![Figure 3. CAD (15 V collision cell dc offset, 5 scans, top), IRMPD (10 W, 70 ms, 5 scans, middle), and EID (16 eV, 6 s, 5 scans, bottom) MS/MS spectra of adenosine diphosphate-ribose. The open circles indicate product ions providing complementary information compared to other fragmentation strategies. v_2 and v_3 indicate harmonic peaks. Electronic noise peaks are marked with asterisks.](image)

### Scheme 3. Structure and Observed MS/MS Fragmentation of Adenosine Diphosphate-ribose

![Scheme 3. Structure and Observed MS/MS Fragmentation of Adenosine Diphosphate-ribose](image)
information that can aid the identification of such metabolites. IRMPD appears superior to both CAD and EID for larger phosphate-containing metabolites due to its higher extent of fragmentation and higher fragmentation efficiency. Furthermore, characteristic product ions at $m/z$ 79, 97, 159, and 177 are indicative of phosphate moieties. Such ions, along with product ions generated by phosphoester and phosphoanhydride bond cleavages, are most prominent in IRMPD.

In CAD, precursor ions collide inelastically with inert gases and are vibrationally excited by the resulting energy transfer. However, this process does not always provide sufficient internal energy deposition to dissociate larger biomolecules, and it is known that the collisional activation efficiency decreases in a manner inversely proportional to the precursor ion mass. Thus, it is not surprising that CAD provided extensive structural information (including cross-ring cleavage) for smaller metabolites, such as G6P. The limited fragmentation observed in CAD of larger metabolites, such as NAD and NAADP, may also be explained through charge-directed fragmentation (both NAD and NAADP contain a fixed charge at the preferred cleavage site).

IRMPD generally showed similar fragmentation as CAD for smaller metabolites, however, IRMPD of NAD and NAADP was quite different from CAD. The efficiency of IRMPD is not diminished with ion mass because excitation by IR absorption is characterized as a slow heating method in which many excitation steps are involved. As stated in the introduction, phosphate-containing compounds can efficiently dissociate when they are activated by IR irradiation because the $P-O$ stretch ($9.6-11 \text{ } \mu m$) is in direct resonance with the $CO_2$ IR laser ($10.57-10.63 \text{ } \mu m$). In addition, the presence of aromatic phosphates (e.g., phosphotyrosine) results in a significantly greater dissociation rate than for aliphatic species, which is attributed to their higher absorptivity of IR photons. G6P contains only one phosphate group while the other metabolites investigated in this work contain at least one, two, or three.  

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two. NAD has one phosphate group whereas NAADP has three. Consequently, as the number of phosphate groups increased, more extensive fragmentation was observed in IRMPD. In addition, the aromaticity of NAADP and NAD may contribute to more extensive fragmentation although this effect should be smaller than for phosphotyrosine due to the larger distance between aromatic rings and phosphate groups in NAADP and NAD.

EID of phosphate-containing metabolites generally showed extensive fragmentation and provided complementary structural information compared to the other dissociation methods. Reilly and co-workers showed that 157 nm photons, corresponding to 8 eV, can deposit 3–4 eV more than required to cleave a peptide backbone, thereby generating secondary fragmentation. Thus, we speculate that the electron energy (15–20 eV) used in EID is
sufficient to generate secondary fragmentation of metabolites. Furthermore, the relatively long irradiation time (6–8 s) may also promote secondary fragmentation, although the precise fragmentation mechanism is unknown. However, the fragmentation efficiency of EID is lower than that of other fragmentation strategies. Efforts to increase EID fragmentation efficiency should be undertaken because it is apparent that EID can provide unique metabolite structural information, which could be crucial for metabolite identification.

CONCLUSION

CAD, IRMPD, and EID spectra of phosphate-containing metabolites provided valuable complementary structural information, which can aid in the identification of such compounds. In our experiments, CAD showed most extensive structural information for smaller phosphate-containing metabolites such as G6P and ATP. For larger phosphate-containing metabolites, CAD resulted in more limited fragmentation. However, IRMPD showed extensive fragmentation for larger phosphate-containing metabolites, including NAD and NAADP. The increased number of bond cleavages could be attributed to an increased number of phosphate groups and, possibly, to their aromatic character. EID generally provided extensive fragmentation regardless of metabolite size, although fragmentation efficiency is lower than those of other MS/MS strategies. Thus, CAD should be the preferred MS/MS strategy for smaller phosphate-containing metabolites. However, for larger phosphate-containing metabolites, particularly for species containing multiple phosphate groups, IRMPD is an advantageous strategy for metabolite structural identification. EID generally showed extensive fragmentation for all phosphate-containing metabolites and provided complementary structural information. Thus, EID could be used as a complementary MS/MS strategy to CAD and IRMPD to yield further structural confirmation.

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