Mass Spectrometric Characterization of a Series of Adenosylated Peptides Acting as Bisubstrate Analogs of Protein Kinases

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We are currently developing strategies to synthesize bisubstrate analogs as potential inhibitors of serine and tyrosine protein kinases; several such analogs have been synthesized. The initial target proteins were the cAMP dependent protein kinase (cAPK) and the Ca^{+2} /calmodulin dependent protein kinase (CaM kinase II). These bisubstrate analogs were based on either known peptide substrates such as kemptide, a seven amino acid peptide substrate of cAPK, or on inhibitory peptides such as a seventeen amino acid peptide encompassing the autoinhibitory domain of CaM kinase II. Peptides containing a single phosphoserine group were first synthesized and then adenosine 5'-monophosphate (AMP), adenosine 5'-diphosphate (ADP), or adenosine 5'-triphosphate (ATP) was coupled through the serine phosphate with prior activation by 1,1-carbonyldiimidazole using either a solution or solid phase reaction scheme. In this current study, we report the characterization of the bisubstrate analogs by liquid secondary ionization mass spectrometry (LSIMS), matrix-assisted laser desorption mass spectrometry (MALDI), and tandem mass spectrometry (MS/MS).

In the positive-ion mode, the LSIMS spectra of the bisubstrate analogs yielded a series of molecular ions containing mono-, di-, and trivalent cation adducts. Cation adducts were absent in the negative-ion mode where the dominant species were deprotonated molecular ions, $[\text{M} - \text{H}]^-$, making this latter technique more useful for confirming product identity and assessing purity. Analysis of these compounds by MALDI in both the positive- and negative-ion modes yielded molecular ions which also contained metal ion adducts, although they were limited primarily to Fe^{+2} adducts. Unlike LSIMS, the MALDI spectra showed no evidence for the elimination of the phosphoadenosine or other structural moieties. When these compounds were subjected to high energy collision-induced dissociation (CID), the dominant fragmentation pathways under positive-ion MS/MS conditions resulted from cleavage of the phosphate linkages to the adenosine moiety with charge retention on the peptide, although a major peak for 5'-deoxyadenosine was also seen at m/z 250. Charge retention in the negative-ion mode was most pronounced for ion fragments containing the highly acidic phosphate moieties and yielded phosphoadenosine related ions, for example, $(\text{AMP-H})^-$, $(\text{AMP-H-H}_2\text{O})^-$, $(\text{ADP-H})^-$, etc., as well as ions originating from the phosphate linker such as PO_3^- , H_2PO_4^- , HP_2O_6^- , $\text{H}_3\text{P}_2\text{O}_7^-$, and $\text{H}_2\text{P}_3\text{O}_5^-$. The largest phosphoadenosine ion in the negative-ion CID spectra for each bisubstrate analog, for example, m/z 426 $(\text{ADP-H})^-$, m/z 506 $(\text{ATP-H})^-$, or m/z 586 $(\text{AP}_n\text{-H})^-$, indicated that the desired covalent modification had been formed between the phosphoserine and AP_n moieties. (*J Am Soc Mass Spectrom* 1994, 5, 443-451)

In recent years, protein phosphorylation has emerged as a phenomenon closely linked to the control of cell growth in eukaryotic systems. Many of the proteins involved in the regulation of cellular proliferation are modulated by phosphorylation, including the enzymes that catalyze this process. Protein phosphorylation occurs by the catalytic transfer of the gamma phosphate of ATP to the hydroxyl of serine,

threonine, and tyrosine residues [1] or to the imidazole nitrogen of histidine residues [2] by specific protein kinases. It is thought that protein phosphorylation modulates protein activity by altering either the structure of the protein at the active site or the binding of protein substrates to the enzyme [3]. Protein phosphorylation is rapidly reversible and dephosphorylation is catalyzed by protein phosphatases that belong to several distinct classes [4, 5]. It is probably the rapid reversibility of this modification that makes protein phosphorylation so ideal for regulating cellular growth and proliferation.

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Many peptide growth factor receptors, including the receptors for epidermal growth factor (EGF) [6] and the insulin-like growth factor I (IGF-I) [7] possess cytoplasmic domains that are protein tyrosine kinases. Receptor signaling is usually initiated by binding of an external peptide ligand (EGF, IGF-I, insulin, etc.) to the extracellular domain, resulting in activation of the cytoplasmic kinase activity. This activation can result in a cascade of intracellular signaling events that govern processes such as cell growth and division. Inappropriate expression or mutation of these receptors can result in a kinase domain that is constitutively activated or upregulated, leading to an elevated level of protein phosphorylation and an increase in cellular proliferation [8]. It is not surprising then that growth factor receptors, including the receptors for IGF-I and EGF, have been directly implicated in numerous cancer processes [9-14].

In general, protein kinases are known to mediate phosphoryl transfer between ATP and target amino acid residues in endogenous cytoplasmic proteins; however, their mechanism of action is not well understood. It is generally assumed that protein kinases catalyze the transfer of the γ -phosphate of ATP coordinated to Mg^{+2} in a nucleophilic attack by a hydroxyl group of a serine, threonine, or tyrosine residue (for example, see Scheme I). Given the important link between tyrosine kinase activity and cancer, finding specific inhibitors of these enzymes may lead to new drugs and more effective cancer chemotherapies, and would also provide clues as to the mechanism of the enzymatic process.

Despite the lack of details of the mechanism and nature of the catalytic site for tyrosine kinases, some significant progress has been made towards the development of inhibitors. For example, several natural products isolated from plants [15] and fungal fermentation broths [16] have been shown to possess inhibitory activity against several growth factor receptors. To increase selectivity, efforts have been made to enhance the tyrosine mimetic character of these inhibitors using benzylidene compounds [17] and 4-hydroxycinnamamide derivatives [18, 19]. More recently, Kruse et al. [20] reported the synthesis of several

multisubstrate inhibitors that contained moieties resembling those of tyrosine and ATP.

One approach towards increasing selectivity among kinase inhibitors would be to capitalize on the unique substrate specificities of the individual protein kinases. Shoelson et al. [21], for example, synthesized substrate analogs where side-chain groups not susceptible to phosphorylation replaced the target tyrosine in peptide substrates, and these compounds were capable of selectively blocking the autophosphorylation activity of both the insulin and EGF receptors. Yuan et al. [22] have prepared gastrin analogs containing D- and L-tetrafluorotyrosine and K_i values of between 4 and 20 μM were reported against the insulin receptor. In our own work, we have sought to design bisubstrate analogs based on incorporating the ATP and peptide substrates into a single compound. However, studies by Hampton et al. [23] on the design of inhibitors of hexokinase and thymidine kinase have shown that the distance between the adenosine base and the second substrate needs to be varied in order to find the optimum distance in a multisubstrate inhibitor. One way to accomplish this for the protein kinases would be to vary the number of phosphates between the peptide substrate and the adenosine moiety (see Scheme II).

The bisubstrate analogs we have synthesized and characterized in this study consist of a seven amino acid peptide substrate "kemptide" (Leu-Arg-Arg-Ala-Ser-Leu-Gly), where the target serine has an ADP, ATP, or AP_4 moiety covalently attached to the hydroxyl group. Although cAMP dependent protein kinase is a serine specific kinase, it is a readily available and soluble enzyme, and is the only protein kinase whose three-dimensional structure has been determined [24]. This enzyme also shares considerable sequence homology in the catalytic domain with the tyrosine kinase family [25]. Therefore, insight gained into the mechanism of inhibition of this serine kinase should provide insight on how to develop potent and selective bisubstrate inhibitors of the tyrosine kinase family.

In this report, we describe the mass spectrometric characterization of these bisubstrate analogs. Mass

Scheme I

Scheme II

spectrometry was chosen as the tool of choice to characterize these analogs because it had been shown by several groups that this technique can be successfully used to analyze phosphopeptides, both in their identification and sequence assignment [26-30]. However, it was not known prior to this study to what extent the addition of adenosine and phosphodiester linkages to peptides would affect their mass spectrometric behavior, and whether or not mass spectrometry could provide the necessary structural data to identify and confirm the structures of these peptide analogs.

Experimental

Synthesis and Purification of Adenosylated Peptides

Adenosylated peptides were synthesized by either solution or solid phase based strategies. For both synthetic strategies, peptides were first prepared on an automated peptide synthesizer (Protein Technologies PS-3) using 9-fluorenylmethyloxycarbonyl (Fmoc) temporary protection and 2-(1H-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate as the activator [31]. The target serine was introduced without side chain protection. The N-terminus was protected with the acid labile *tert*-butoyloxycarbonyl (Boc) group or was permanently blocked by acetylation. Phosphorylation was carried out with di-*tert*-butyl-N,N-diisopropylphosphoramidite in the presence of 1H-tetrazole followed by oxidation with *tert*-butyl hydroperoxide. To release the phosphopeptide intermediate, the resin was treated with trifluoroacetic acid (TFA). The final adenosylated peptides were then prepared by coupling the free phosphopeptides with AMP, ADP, and/or ATP which had undergone prior activation with 1,1-carbonyldiimidazole.

Alternatively, a solid phase approach was used where the phosphorylating agent was replaced with di-allyl-N,N-diisopropylphosphoramidite (see Scheme III). The phosphate protecting group was then selectively removed by tetrakis(triphenylphosphine)palladium(0) and the appropriate activated adenosine phosphate coupled to the peptide still attached to the resin. Multiple washes of the resin-bound peptide product easily removed excess reagent. Lastly, side chain protecting groups were removed with TFA, which also served to liberate the adenosylated peptide

product(s) from the resin. Details of these synthetic procedures will be published elsewhere (Medzih-radszky and Gibson, manuscript in preparation).

LSIMS Analysis

Adenosylated peptides obtained from both the solution and solid-phase synthesis schemes were then separated from other components of the final reaction mixture by high-performance liquid chromatography (HPLC) (Vydac C₁₈ analytical column) and directly analyzed by liquid secondary ionization mass spectrometry (LSIMS). A Kratos MS50S mass spectrometer equipped with a cesium ion source [32] was used for all experiments. The peptide samples were dried under vacuum and then dissolved in H₂O containing 0.1% TFA. Approximately 1 μ l of glycerol/thioglycerol (1:1, v/v) was applied as the liquid matrix to the stainless steel probe tip along with a small aliquot of the peptide solution (~ 1 -2 μ g). In the positive-ion mode the matrix also contained 1% TFA. A Cs⁺ ion primary beam energy of 10 keV was used, and the secondary sample ions were accelerated to 6-8 keV. Scans were taken at 100 or 300 s per decade and recorded with a Gould ES-1000 electrostatic recorder. Mass assignments were made to an accuracy of better than ± 0.3 Da by manual calibration using Ultramark 1621 as an external reference. All experiments were carried out at a mass resolution of ~ 1500 -2000 (M/ Δ m).

MALDI Time-of Flight Mass Spectrometry

Peptide samples were prepared in a matrix of 2,5-dihydroxybenzoic acid (DHB) and fucose according to the method of Billeci and Stultz [33]. Peptide solutions were prepared at a concentration of ~ 100 μ g/ml, and a 5 μ l aliquot was mixed with an equal volume of 50 mM DHB (in 2% acetonitrile and 0.1% TFA) and 100 mM fucose (in 0.1% TFA). Approximately 0.7 μ l of the final matrix-peptide solution was then transferred to the well of the laser target and air dried for 30 min prior to analysis. All samples were analyzed using a Kratos Kompact matrix-assisted laser desorption mass spectrometry (MALDI) time-of-flight mass spectrometer (Manchester, England). Spectra were acquired in the linear mode at an acceleration voltage of 20 kV. Spectra were summed from 40 to 100 laser shots taken across the entire sample well (~ 3 mm). An external mass calibration was set up for the positive-ion mode using oxidized insulin B-chain, MH⁺ average 3496.9, and kemptide, MH⁺ average 772.9. For the negative-ion mode, oxidized insulin B-chain, [M - H]⁻ average 3494.9 and phosphokemptide, [M - H]⁻ average 850.9, were used as external mass standards. A laser power of 45% of maximum was used in the positive-ion mode and 55% of maximum in the negative-ion mode.

Scheme III

Tandem CID Mass Spectrometry

Selected phosphorylated and adenosylated peptides were analyzed by tandem mass spectrometry (MS/MS) using a four-sector mass spectrometer (Kratos Concept II HH, Kratos Analytical, Manchester, England) equipped with an array detector on MS-2 [34]. Spectra were taken in the positive- and negative-ion modes using a Cs^+ as the primary ion beam (15–18 keV). The isotopically pure ^{12}C -component of the parent molecular ion was then selected in MS-1 and activated in the collision cell between MS-1 and MS-2. The helium collision gas was used at a pressure sufficient to attenuate the parent ion beam to approximately one third of its initial value. Fragment ions resulting from decomposition of the collisionally activated parent ions were mass analyzed and detected in MS-2. The collision cell was floated at a potential of 2 kV, resulting in a collision energy of 6 keV. Mass calibrations were carried out with a Kratos Mach 3 data system with CsI as a reference compound. All samples were prepared as described above for the two-sector experiments using 1–2 μg of peptide. Peptide fragment ions are reported according to the standard nomenclature [35].

Results and Discussion

In our approach to develop specific bisubstrate inhibitors of protein kinases, we have taken advantage of known peptide substrates of specific protein kinases as the starting point in our molecular design. The first attempt at synthesizing a series of adenosylated peptides as bisubstrate analogs used "kemptide" as the base peptide. Kemptide is one of the smallest known substrates for cAPK ($K_m = 16 \mu\text{M}$) [36] and consists of only seven amino acids containing a single target serine, Leu-Arg-Arg-Ala-Ser-Leu-Gly. This peptide was first prepared as the phosphoserine derivative prior to coupling with AMP, ADP, and ATP by 1,1-carboxydimidazole. The final adenosylated peptides from both the solution phase and solid phase syntheses were purified by reverse phase HPLC before being extensively characterized by mass spectrometry.

LSIMS Analysis of the Kemptide Analogs

In the positive-ion mode, the LSIMS spectra of the kemptide-ATP product gave a complex molecular ion pattern consistent with the formation of a series of cationated molecular adducts, including those for Na^+ and/or Mg^{+2} , Al^{+3} , K^+ , Mn^{+2} , and/or Fe^{+2} (see Figure 1a). It is well known that ATP itself readily coordinates to divalent cations due to the presence of adjacent acidic phosphate groups, and this preferred binding appears to be preserved in these bisubstrate analogs as well. In contrast, the LSIMS spectra of this same kemptide-ATP product taken in the negative-ion mode (see Figure 1b) yielded a single major molecular ion species corresponding to the expected deprotonated molecules, $[\text{M} - \text{H}]^-$ at m/z 1259.7.

Figure 1. Molecular ion region of the LSIMS spectra of kemptide-ATP in the (a) positive-ion mode and (b) negative-ion mode. The expected masses of the molecular ions for kemptide-ATP are $[\text{M} + \text{H}]^+ = 1261.46$ and $[\text{M} - \text{H}]^- = 1259.45$. Metal ion adducts are indicated by the presumed valence states (Na^+ , Mg^{+2} , Al^{+3} , etc.) which require proton exchange for the di- and trivalent cations, for example, $[\text{M} - \text{H} + \text{Mg}^{+2}]^+$, $[\text{M} - 2\text{H} + \text{Al}^{+3}]^+$, etc. The ion cluster slightly up-mass from the Al^{+3} adduct is a metastable decomposition product.

In the LSIMS spectra of the other adenosylated peptides, the pattern of metal ion adducts initially observed in the positive-ion spectra of kemptide-ATP is also present (see Figure 2). However, in many of these adenosylated kemptide preparations Zn^{+2} adducts were also observed that tended to compete out the Fe^{+2} binding. We presume that the differences in the metal ion adducts (see Figures 1a and 2b, for example) are largely due to difference in the purification scheme, where exposure to trace metals in glassware and solvents would be responsible for the degree and extent of these metal adducts. However, it is also apparent from these spectra that the number and extent of metal ion adducts increases as the phosphate linker increases in size, suggesting that different affinities among the metal ions toward the bi-, tri-, and tetraphosphate linkers also contributes to the formation of these metal ion adducts. It is worth noting that the trivalent Al^{+3} adduct is either not present or of very low abundance in the positive-ion spectrum of kemptide-ADP (see Figure 2a). Because kemptide-ADP contains only two phosphates, it appears to have a diminished affinity for this trivalent metal. In the corresponding negative-ion spectra (data not shown) dominant deprotonated molecules were observed for both kemptide-ADP (m/z 1179.6) and kemptide- AP_4 (m/z 1339.7), similar to that shown in Figure 1b for kemptide-ATP. Although negative-ion LSIMS of these analogs yielded less complex mass spectra, the metal ion adducts present in the positive-ion LSIMS spectra were useful for identifying products in the reaction mixtures that contained the phosphodiester group.

MALDI Analysis

Two of the main disadvantages of using LSIMS to assay for product purity is the limited dynamic range due to high matrix background and the formation of

Figure 2. Molecular ion region of the positive-ion LSIMS spectra of (a) kemptide-ADP, (b) kemptide-ATP, and (c) kemptide-AP₄. Some variations in the nature and extent of the cation adducts were observed in different preparations of the adenosylated peptides. Compare the solution phase reaction product for kemptide-ATP (Figure 1a) with the solid phase product in panel b above.

fragment ions that can confuse or mask the presence of undesired products or contaminating reactants. In the LSIMS spectra of the adenosylated kemptide series, some low abundance peaks were observed corresponding to the unsubstituted phosphopeptide formed by cleavage between the first and second phosphate relative to the serine hydroxyl group, for example, neutral losses of AMP from kemptide-ADP, ADP from kemptide-ATP, and ATP from kemptide-AP₄ (data not shown). In order to find a technique that would minimize these problems, MALDI mass spectrometry was evaluated as an alternative to LSIMS.

The MALDI spectra for phosphokemptide, kemptide-ADP, kemptide-ATP, and kemptide-AP₄ are shown for the positive-ion mode in Figure 3. All these spectra contain very abundant molecular ion peaks for the appropriate species, but a clear pattern of increasing metal adducts, especially those containing Fe²⁺,

Figure 3. Positive-ion MALDI spectra of (a) phosphokemptide, (b) kemptide-ADP, (c) kemptide-ATP, and (d) kemptide-AP₄. Peaks labeled as Na⁺ and Fe²⁺ may also contain Mg²⁺ and Mn²⁺, respectively, as their masses would not be adequately resolved under the instrument resolution used to obtain these spectra. All spectra were background subtracted and smoothed. Samples are the same as used to obtain the spectra shown in Figure 2.

was apparent as the number of phosphates increased through the adenosylated peptide series. For example, phosphokemptide shows a dominant [M + H]⁺ ion at *m/z* 852.3 and a very small peak corresponding to an iron adduct, [M - H + Fe²⁺]⁺ at *m/z* 906.5. As one progresses through the adenosylated peptides with two, three, and four phosphates, the protonated molecular ions decrease in relative abundance to the Fe²⁺ (and/or Mn²⁺) adducts. This trend culminates in kemptide-AP₄ where one no longer observes a protonated molecule at all, but only adducts containing one or more iron atoms, for example, *m/z* 1394.7 [M - H + Fe²⁺]⁺ and 1448.3 [M - 3H + 2Fe²⁺]⁺.

In the negative-ion MALDI spectra of this same peptide series, a pattern very similar to that observed

in the positive-ion mode was found (see Figure 4). The dominant peaks for phosphokemptide and kemptide-ADP are the deprotonated molecular ions, but the abundance of the iron adducts increases as one proceeds through the series to kemptide-AP₄. Therefore, unlike negative-ion LSIMS, negative-ion MALDI does not appear to favor formation of the deprotonated molecules and no advantages are seen in sensitivity or in the absence of metal adducts when compared to the positive-ion mode.

Unlike the other MALDI spectra, kemptide-ADP also contained a low mass peak that indicated the presence of phosphokemptide. This was particularly evident from the positive-ion spectrum (m/z 852.2) but could also be observed at very low abundance in the negative-ion spectrum. None of the other spectra

indicated such a peak, and therefore it was unlikely phosphokemptide was formed via an initial fragmentation process that was unique to kemptide-ADP. To ensure that this was indeed a contaminant peptide, a capillary electrophoretic separation was carried out on this preparation which confirmed the presence of a small contaminating peak whose elution position and UV spectrum was consistent with phosphokemptide (data not shown).

Tandem CID Spectra of Kemptide Analogs

Although the LSIMS and MALDI spectra provided important molecular weight information as to the identity of the desired adenosylated peptide products, more detailed structural information was needed to determine the product identity unambiguously. For example, it is conceivable that the 1,1-carbonyldiimidazole activated AMP, ADP, and ATP moieties could couple to other functional groups in the phosphopeptide in the solution-phase synthesis scheme. These peptide products would have the same molecular weight as the desired product and from their corresponding MALDI or LSIMS spectral data they probably would not be distinguished from one another. (Negative-ion LSIMS spectra of the adenosylated kemptide peptides often contained limited fragmentation that ruled out modification at the N- and/or C-terminus. For example, in the negative-ion LSIMS spectrum of kemptide-ATP, fragment ions were seen for loss of N-terminal Leu (y_6 at m/z 1146) and C-terminal glycine (c_6 at m/z 1201).) Tandem mass spectrometry should be able to provide the necessary information to rule out these potential and unwanted side-reaction products as well as yield additional sequence information on the adenosylated peptides should it be needed.

Tandem mass spectra taken under collision-induced dissociation (CID) conditions produced a series of complex fragment ions in both the positive- and negative-ion modes that were largely dominated by ions originating from the phosphoadenosine groups. For example, in the low mass region of the positive-ion spectrum of kemptide-ATP shown in Figure 5a, the largest peak is the deoxyadenosine fragment at m/z 250. The major fragments, however, were formed by cleavages of the triphosphate linker between adenosine and serine hydroxyl group with charge retention on the peptide. The highest mass ion in this series was seen at m/z 1012, resulting from the neutral loss of adenosine to form kemptide-P₃, and is followed by the additional losses of the three phosphate groups yielding ions at m/z 932 (kemptide-P₂), 852 (kemptide-P), and 772 (kemptide). The base peak at m/z 754 is the dehydroalanine-containing peptide that is formed by β -elimination of the entire ATP group. The corresponding positive-ion CID spectrum of kemptide-ADP (see Figure 5b) and kemptide-AP₄ (spectrum not shown) contains many of the same features as those for kemptide-ATP.

Figure 4. Negative-ion MALDI spectra of (a) phosphokemptide, (b) kemptide-ADP, (c) kemptide-ATP, and (d) kemptide-AP₄. Peaks labeled as Fe⁺² may also contain Mn⁺², as their masses would not be adequately resolved under the instrument resolution used to obtain these spectra. All spectra were background subtracted and smoothed. Samples are the same as used to obtain the spectra shown in Figures 2 and 3.

Figure 5. Positive-ion tandem CID spectra of (a) kemptide-ATP and (b) kemptide-ADP. All standard peptide cleavages are labeled according to the conventional nomenclature [35]. All fragment ions are listed as their nominal masses. The unlabeled peaks in the regions just below the molecular ion (m/z 1250–1100) and below the abundant dehydroalanine peptide fragment m/z 754 (m/z 730–650) mostly originate from side-chain losses and contain little or no sequence information.

Despite the domination of fragments associated with the ATP (or ADP and AP_4) group, low abundant ions originating from the typical peptide backbone fragmentation processes were also present. These ions are exclusively N-terminal fragments (a, b, c, and d-ions) due to the presence of two arginines near the N-terminus. These sequence ions were not sufficient, however, to derive a complete peptide sequence or

Figure 6. Negative-ion tandem CID spectrum of kemptide-ATP. All standard peptide cleavages are labeled according to the conventional nomenclature [35], but modified to represent the analogous negative-ion fragments 2 Da lower in mass [28]. All fragments ions are listed as their nominal masses.

determine the position of the ATP (or ADP and AP_4) modification in any of the spectra.

The negative-ion spectrum of kemptide-ATP shown in Figure 6 is in many ways complementary to the positive-ion spectrum. Like the positive-ion CID spectrum, negative-ion fragments were also derived from the ATP moiety, but these ATP-related ions turned out to be more useful in confirming the presence of the ATP modification. For example, the most abundant ions were those corresponding to deprotonated ADP (m/z 426) and ATP (m/z 506), and their respective $-H_2O$ peaks at m/z 408 and 488. To a considerably lesser extent, peaks for deprotonated AMP (m/z 346) and its $-H_2O$ counterpart (m/z 328) were also seen (see Table 1). The two ATP peaks at m/z 506 and 488 were especially noteworthy as they provided the first clear evidence that the appropriate covalent linkage between phosphoserine and ADP had been formed:

Table 1. Relative ion abundances of key phosphoadenosine-related fragments in negative-ion CID of adenosylated kemptides

Compound	(AMP- H_3O) ⁻ m/z 328	(AMP-H) ⁻ m/z 346	(ADP- H_3O) ⁻ m/z 408	(ADP-H) ⁻ m/z 426	(ATP- H_3O) ⁻ m/z 488	(ATP-H) ⁻ m/z 506	(AP_4 - H_3O) ⁻ m/z 568	(AP_4 -H) ⁻ m/z 586
Kemptide-ADP	2	3	30 ^a	26	—	—	—	—
Kemptide-ATP	3	2	100	26	17	16	—	—
Kemptide- AP_4	0	0	7	10	100	10	2	2
Compound	PO_3^- m/z 79	$H_2PO_4^-$ m/z 97	$HP_2O_6^-$ m/z 159	$H_3P_2O_7^-$ m/z 177	$H_2P_3O_9^-$ m/z 239	$H_4P_3O_{10}^-$ m/z 257	$H_3P_4O_{12}^-$ m/z 319	$H_5P_4O_{13}^-$ m/z 337
Kemptide-ADP	100	24	45	5	—	—	—	—
Kemptide-ATP	12	< 1	22	7	4	1	—	—
Kemptide- AP_4	3	1	8	2	4	< 1	1	< 1

^a All ions are listed in relative ion abundances with the most abundant ion in each series shown in bold type.

Likewise, in the negative-ion tandem mass spectra of kemptide-ADP and kemptide-AP₄, the highest mass phosphoadenosine ions were those for ADP (m/z 408 and 426) and AP₄ (m/z 568 and 586), respectively (data not shown), although for kemptide-AP₄ these ions were near the limit of detection, and much larger ions were seen for loss of ATP at m/z 488 and 506 (see Table 1). The most likely explanation for the low abundance of AP₄ ions is the increasing number of pathways open to it for further phosphate elimination once these ions are formed. For example, the low mass region of the tandem mass spectra contained peaks originating from multiple remote bond cleavages of the internal polyphosphate linkers. In the tandem mass spectrum of kemptide-ATP these ions were observed at m/z 79, 159, 177, and 239 (and possibly m/z 257), and correspond to PO₃⁻, HP₂O₆⁻, H₃P₂O₇⁻, H₂P₃O₉⁻, and H₄P₃O₁₀⁻, respectively:

And as one might expect, kemptide-ADP contained only PO₃⁻, HP₂O₆⁻, and H₃P₂O₇⁻, while kemptide-AP₄ contained all those observed in the spectra of kemptide-ATP as well as an additional ion at m/z 319 corresponding to H₃P₄O₁₂⁻.

In the negative-ion spectrum of kemptide-ATP, the phosphate peak at m/z 239 (and possibly m/z 257) gave further support for the formation of a triphosphate linkage, as opposed to potential isomeric side-products containing unsubstituted phosphoserine and an ADP modification elsewhere on the peptide. It is also interesting to note that the ion corresponding to free phosphate at m/z 97 (H₂PO₄⁻) is also observed in the negative-ion tandem mass spectra of the adenosylated kemptides, although at lower abundance than in the corresponding phosphokemptide spectrum [28]. In contrast, the absence of peaks corresponding to neutral loss of HPO₃ and H₃PO₄ from the parent molecular ions of the adenosylated peptides under CID conditions is clearly different from phosphokemptide and may be indicative of the absence of an unsubstituted phosphoserine group, as this fragmentation is quite prominent for phosphopeptides [28, 30, 37]. A comparison of some of the major ions observed in the negative-ion tandem mass spectra of adenosylated and phosphoserine-containing peptides is summarized in Table 2.

Unfortunately, very little conventional peptide sequence ions were present in these negative-ion spectra. The absence or low abundance of these ions is likely the result of much more favorable fragmentation pathways available through the phosphoadenosine groups. Nevertheless, a few sequence ions could be identified in each spectrum that allowed for the sequence localization of the adenosylated residue to the C-terminal half of the peptide, -Ser-Leu-Gly-OH.

Table 2. Fragmentation of phosphoserine and ADP-serine in the negative-ion mode

Molecular Ion	Low Mass Ions	High Mass Ions
 -Ser(P)-	PO ₃ ⁻ m/z 79 H ₂ PO ₄ ⁻ m/z 97 HN=CH-CH ₂ -OPO ₃ H ⁻ m/z 138	 [M-H-H ₃ PO ₄] ⁻ [M-H-HPO ₃] ⁻
Molecular Ion	Low Mass Ions	High Mass Ions
 -Ser(ADP)-	PO ₃ ⁻ m/z 79 H ₂ PO ₄ ⁻ m/z 97 HP ₂ O ₆ ⁻ m/z 159 H ₃ P ₂ O ₇ ⁻ m/z 177 1.2 [HO-P(=O)(OH) ₂] m/z 346 and 328 (-H ₂ O) m/z 426 and 408 (-H ₂ O)	 [M-H-H ₃ PO ₄] ⁻ [M-H-HPO ₃] ⁻

Conclusions

In this study, mass spectrometry was used to identify and characterize a series of adenosylated peptides formed by covalent coupling of AMP, ADP, and ATP to phosphokemptide. LSIMS and MALDI mass spectrometry were used first to identify the desired products after separation by HPLC. In the LSIMS analysis, negative-ion spectra contained a single dominant deprotonated molecule for each adenosylated derivative and that afforded a good assessment of both peak identity and purity. In contrast, the positive-ion spectra yielded numerous metal ion adducts to the molecular ion that prevented an accurate determination of product purity. MALDI spectra in both the positive- and negative-ion modes yielded similar metal ion adducts, but these were limited primarily to Fe²⁺ adducts, and therefore were useful in determining peak identities. In addition, the much larger dynamic range of MALDI and the lack of observed fragments allowed even minor impurities to be detected.

To confirm unambiguously both the site of adenosylation and the precise nature of the covalent phosphoadenosine conjugate (i.e., ADP, ATP, or AP₄), MS/MS was essential. Under conditions of high energy CID the spectra obtained for the adenosylated kemptide series

contained ions that allowed for confirmation of the desired phosphoadenosine (AP_n) adduct, but not a complete sequence. The lack of peptide sequence ions can be rationalized by the preference for fragmentation of the AP_n moieties over backbone cleavages. The negative-ion CID spectrum was most useful in unambiguously confirming the presence of ADP, ATP, and/or AP_4 through β -elimination of the entire ADP, ATP, or AP_4 moiety and/or cleavage at the neighboring O-P bond. Moreover, in the low mass region of the negative-ion tandem mass spectra, evidence also was present that supported the nature of the AP_n conjugate by defining the nature of the phosphate linker, for example, bi-, tri-, or tetraphosphate. Our previous studies on sequencing phosphopeptides by MS/MS (including phosphokemptide) [28] has shown that complete sequences can be obtained under high energy CID conditions. Therefore, it would appear that sequencing the intermediate phosphopeptide prior to the coupling of the AP_n group would be the preferred strategy, especially if the sequence and/or position of the intermediate phosphoserine-containing peptide was in any way in doubt.

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