Enhanced Detection of Phosphopeptides in Matrix-Assisted Laser Desorption/Ionization Mass Spectrometry Using Ammonium Salts

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Matrix-assisted laser desorption/ionization mass spectrometry (MALDI MS) has been used successfully to detect phosphorylation sites in proteins. Applications may be limited by the low response of phosphopeptides compared to nonphosphorylated peptides in MALDI MS. The addition of ammonium salts to the matrix/analyte solution substantially enhances the signal for phosphopeptides. In examples shown for equimolar mixtures, the phosphorylated peptide peaks become the largest peaks in the spectrum upon ammonium ion addition. This can allow for the identification of phosphopeptides in an unfractionated proteolytic digestion mixture. Sufficient numbers of protonated phosphopeptides can be generated such that they can be subjected to postsource decay analysis, in order to confirm the number of phosphate groups present. The approach works well with the common MALDI matrices such as α-cyano-4-hydroxycinnamic acid and 2,5-dihydroxybenzoic acid, and with ammonium salts such as diammonium citrate and ammonium acetate. (J Am Soc Mass Spectrom 1999, 10, 35–44) © 1999 American Society for Mass Spectrometry

Protein phosphorylation is probably the single most common and important reversible intracellular signal transduction event [1]. Understanding the regulation of protein function by phosphorylation/dephosphorylation is a key objective in many areas of biomedical research, including studies involving cell cycle regulation, enzyme activation/deactivation, and protein–protein association. Phosphorylation serves other functions as well in protein chemistry. Protein-bound phosphate may serve to maintain the structure of an enzyme without regulating its activity [2]. Also, as in the classic cases of those found in egg yolk and milk, phosphoproteins serve as stores of phosphate and bound metal ions [2].

Direct analytical methods to identify phosphorylation sites of proteins of known sequence, at or below the picomole level, are critical to these research areas. Mass spectrometry has played an essential role in mapping posttranslational modifications of proteins such as phosphorylation [3]. Both matrix-assisted laser desorption/ionization mass spectrometry (MALDI MS) [1, 3–6] and electrospray ionization mass spectrometry (ESI MS) [3, 7, 8] have been used effectively, each with its strengths and limitations.

Suppose a protein P is multiply phosphorylated. One approach to locate the phosphorylation sites is to first subject P to a proteolytic digestion using, typically, trypsin. The set of degradation products $\{D_i\}$ may be analyzed in a "batch" method. That is, all of the products can be analyzed simultaneously using MALDI [4, 6]. If the mixture is complex, high-performance liquid chromatography (HPLC) can be used to fractionate the mixture. Fractions can be collected and each subjected to MALDI analysis [1, 6, 9], or the HPLC effluent can be analyzed directly using ESI MS. Elegant methods for locating phosphorylated fragments and determining the site of the modification using HPLC/ESI MS have been described in the literature [10–14]. If HPLC fractions are collected for subsequent MALDI analysis, incorporation of ^{32}P labeling can be used to indicate which D_i's are phosphopeptides [9]. Mass mapping techniques [6, 15] are commonly used to predict the mass-to-charge ratio values of the protonated proteolytic digestion products. If a peak is observed in a MALDI mass spectrum which is 80 Da higher than a predicted peak, it may indicate phosphorylation. If multiple phosphorylations are present, a mass shift of 80 for each will be observed. The 80 Da shift corresponds to addition of HPO_3 to the –OH group of a serine, threonine, or tyrosine residue. To confirm the presence of the phosphate group, a portion of the sample can be treated with an enzyme such as alkaline phosphatase [6]. If the peak observed truly represents a protonated phosphopeptide, its mass will decrease (by 80 u) upon enzymatic dephosphorylation.

In MALDI MS, an analyte peptide M will usually yield a peak representing $[M + H]^+$ ions. However, in the direct analysis of a mixture of several peptides, such

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as those encountered in digestions, peaks for all components are usually not observed—and certainly not with equal intensities for equally abundant components. One reason for this has been attributed to suppression effects [16, 17], in which the presence of one peptide inhibits the response for another. Thus, some form of fractionation would seem reasonable to use, although time consuming, prior to MALDI MS analysis of digestion mixtures. The situation is particularly problematic when phosphopeptides are present. Liao et al. [6] have shown that the desorption/ionization efficiencies for phosphopeptides in MALDI MS are approximately an order of magnitude lower than for the nonphosphorylated forms [5]. The situation is worse when more than one phosphate group is present [6]. These facts limit the utility of MALDI MS for the identification of phosphorylation sites. If one uses the trypsin/alkaline phosphatase combination, and a peptide containing three phosphates is encountered, for example, no signal may be detected for the intact analyte. When the phosphates are removed, the dephosphorylated form may be detected, but without knowing the mass-to-charge ratio values before and after dephosphorylation, the number of phosphorylation sites cannot be determined.

Why do phosphorylated peptides exhibit low response in MALDI? Presumably the presence of the phosphate group is exhibiting a direct influence. This group exists in solution in an anionic form. If the peptide skeleton is considered in its neutral form, each phosphate group can carry up to two charges. If a multiply charged analyte is trapped in the MALDI matrix crystals in ionic form, insufficient energy is available in the MALDI experiment for the direct desorption of multiply charged species. For most analytes, singly charged ions are predominantly formed [18]. A tetraphosphorylated protein fragment could hold a charge of -8; certainly the -8 form of the ion would not be generated in the gas phase in the MALDI experiment. If a low energy chemical pathway does not exist that allows the analyte to lower its charge, then no signal will be observed [18]. Similar considerations have been discussed in the analysis of multiply charged anionic [19] and cationic [20] analytes in the fast atom bombardment (FAB) MS experiment.

This paper discusses the use of matrix additives in the MALDI experiment to allow for improved detection of phosphopeptides. Oligonucleotides have been successfully analyzed in MALDI MS [21–24]; these analytes contain a high density of negatively charged phosphate groups, and can carry a substantial accumulated charge in solution. In this field of research, ammonium salts such as ammonium acetate or diammonium citrate are routinely added, and appear to lower the charge on the analyte [22–24], making detection possible. A basic model describing the influence of ammonium ions has been proposed [25]. Ammonium cations complex with negatively charged groups to form $(NH_4^+)_n$ (phosphate^{-n}). Either prior to or during the desorption/ionization (D/I) process, ammonia is lost, leaving the charged group in a neutral form containing protons as the counter ions, $(H^+)_n$ (phosphate^{-n}). It should be noted that ammonium ions have been implicated as being protonating (ionizing) agents in the MALDI experiment as well [24]. We will show here that such additives, when used correctly, dramatically improve the desorption/ionization efficiency for phosphopeptides in MALDI. The enhanced response in both positive and negative ion mode suggests that citrate ions scavenge alkali ions [23], to limit their access to phosphate groups, whereas the ammonium ions serve to ultimately generate the analyte in neutral form. With the phosphopeptide available in neutral form, it can be protonated or deprotonated as are most unmodified peptides in the MALDI experiment.

This paper demonstrates the utility of matrix additives with common MALDI matrices for the analysis of single and multiply phosphorylated peptides, both as pure analytes and in complex mixtures. Conditions are presented that can lead to the preferential detection of phosphorylated peptides in a digestion mixture. With multiply phosphorylated peptides, signals can be enhanced to levels that allow for MS/MS experiments to be performed.

Experimental

Materials

Bradykinin, β-casein, equine myoglobin, angiotensin I, and angiotensin III were purchased from Sigma Chemical (St. Louis, MO). The phosphopeptides LKRApSLG-amide and LKRApTLG-amide were purchased from the University of Michigan Protein and Carbohydrate Facility (Ann Arbor, MI). Peptides and proteins were used as supplied without further desalting or purification. Trypsin was purchased from Promega (Madison, WI).

A four component equimolar mixture of the peptides LKRApSLG-amide, LKRApTLG-amide, angiotensin III, and bradykinin was prepared in 1:1 acetonitrile(ACN)/water at a concentration of 1 pmol/μL (mixture I) and diluted to 500 and 100 fmol/μL. Another four component mixture of the peptides LKRApSLG-amide, KIGEGpTpYGVVYK, angiotensin I, and angiotensin III was prepared in 1:1 ACN/H_2O at a concentration of 1 pmol/μL (mixture II) and further diluted to 100 fmol/μL. A two component mixture of β-casein and equine myoglobin was prepared at a concentration of 10 pmol/μL in 1:1 ACN/H_2O (mixture III). The matrix used for mixtures I and II was a saturated solution of α-cyano-4-hydroxycinnamic acid (4-HCCA) (Sigma Chemical) in 1:1 ACN/H_2O. For mixture III, a saturated solution of 2,5-dihydroxybenzoic acid (DHB) (Sigma Chemical) was employed. In cases where diammonium citrate (J.T. Baker, Phillipsburg, NJ) or ammonium acetate (Sigma Chemical) was used as an additive, a 1 mM solution and a 25 mM solution, respectively, was prepared in 1:1 ACN/H_2O. In the text, citric acid will be

referred to as H_3Cit, so diammonium citrate will be designated as $(NH_4)_2HCit$. An incomplete tryptic digest of β-casein was performed in 40% ACN with a 20:1 excess of β-casein to trypsin. The digest was performed in a 60 mM NH_4HCO_3 (Sigma Chemical) buffer (pH = 8.2) and allowed to incubate at 40 °C for 24 h. The reaction was terminated using formic acid (J.T. Baker). A 10 mL 1:1 ACN/H_2O solution was saturated with 4-HCCA and the pH measured with an Accumet model 15 pH meter, yielding an average value of 2.45.

Mass Spectrometry

Linear MALDI mass spectra were recorded on a PerSeptive Biosystems (Framingham, MA) Voyager Elite delayed extraction time-of-flight reflectron mass spectrometer equipped with a nitrogen laser (337 nm, 3 ns pulse). For mixtures I and II and the tryptic digest, the accelerating voltage in the ion source was 20 kV, the delay time used was 50 ns, the grid voltage was set to 93.0% of the accelerating voltage, and the guide wire voltage was set to a magnitude of 0.25% of the accelerating voltage. For mixture III, the accelerating voltage in the ion source was 21.5 kV, the delay time used was 100 ns, the grid voltage was 92.0% of the accelerating voltage, and the guide wire voltage was set at 0.25%. Typically, 128–256 laser shots were averaged for each spectrum. The sample stage used was a gold sample plate capable of holding 100 targets. The postsource decay (PSD) experiment was performed in the reflectron mode with an accelerating voltage of 21 kV, delay time of 100 ns, grid voltage of 70.0%, and guide wire voltage of 0.15%. The timed ion selector was set at m/z 3124 and three spectra, with mirror voltage:accelerating voltage ratios of 1.00, 0.96, and 0.67, were stitched together to obtain the PSD spectrum.

Sample Preparation

Targets were prepared by mixing 1 μL of analyte and 1 μL of matrix on a sample stage and allowing them to air dry. When ammonium additives were used, 1 μL of the ammonium salt solution was also mixed with analyte and matrix solutions on the sample stage.

For the experiment involving a PVDF membrane (Millipore, Bedford, MA), 1 μL of the 1 pmol/μL mixture I was spotted onto the membrane and allowed to air dry. The saturated matrix was then introduced by the perfusion method described by Strahler et al. [26]. Two pieces of filter paper cut in rectangular strips were loaded into a shallow well and sealed on the bottom and all four sides. The filter paper was saturated with matrix. A piece of PVDF membrane carrying analyte, identical in size to the filter paper, was then placed on the filter paper stack such that the matrix solution could only evaporate through the membrane surface. The membrane containing matrix crystals was then introduced to the MALDI instrument by placing a fine conducting grid on top, as described previously [26]. When an ammonium salt was used, it was introduced again by the perfusion method—a mixture of saturated 4-HCCA and 25 mM diammonium citrate (1:1 ratio) was used to saturate the filter paper in this experiment.

Results and Discussion

We show here that the addition of $(NH_4)_2HCit$ to the MALDI matrix/analyte target solution just prior to crystal formation leads to enhanced detection of phosphopeptides. The enhancements shown were observed upon addition of other ammonium salts such as ammonium acetate, but the influence of the citrate salt is greater. Also, experiments were performed at a variety of matrix:additive:analyte ratios. Although the effects shown can be realized with addition of diammonium citrate using millimolar to micromolar concentrations, we believe that introduction of 1 μL of a 1 mM diammonium citrate solution to the MALDI target solution (1 μL matrix solution+1 μL analyte) works well when the matrix is 4-HCCA, whereas use of a 25 mM additive solution works well when the matrix is DHB. These are suggested ammonium salt concentrations for the matrices tested, and for the analysis of phosphopeptides in the femtomole to low picomole range. The fact that different concentrations are required for different matrices may be related to the varying matrix compound solubilities.

Figure 1a shows the positive ion MALDI spectrum of a 4-component peptide mixture (mixture I) using 4-HCCA as the matrix. Each component is present at a level of 500 fmol. Two components are clearly detected—the peak at m/z 917.5 represents protonated angiotensin III molecules, and the peak at m/z 1060.6 represents protonated bradykinin molecules. All mass-to-charge ratio values represent monoisotopic masses unless otherwise indicated. The other two components present are singly phosphorylated peptides, and exhibit substantially smaller signals. The peak for protonated LKRApSLG-amide is observed at m/z 823.5 and that for LKRApTLG-amide is at m/z 837.5; both are barely detectable above the noise. This example shows typical relative responses for singly phosphorylated peptides relative to nonphosphorylated peptides of similar mass. When the target crystals are grown with inclusion of $(NH_4)_2HCit$, the same sample yields the spectrum shown in Figure 1b. The increase in intensity and signal-to-noise ratio for the peaks representing the phosphorylated peptides is typical of our experience using this additive. The phosphate-containing peptides now yield the most intense peaks in the spectrum. Figure 1c shows the spectrum following addition of $(NH_4)_2HCit$, where each analyte is present at the 100 fmol level. Again, the phosphorylated components are clearly detectable, whereas they are not detectable when the additive is not used.

Although not the point of this paper, a comment should be made on the influence of this additive in negative ion mode. When analytes are negatively

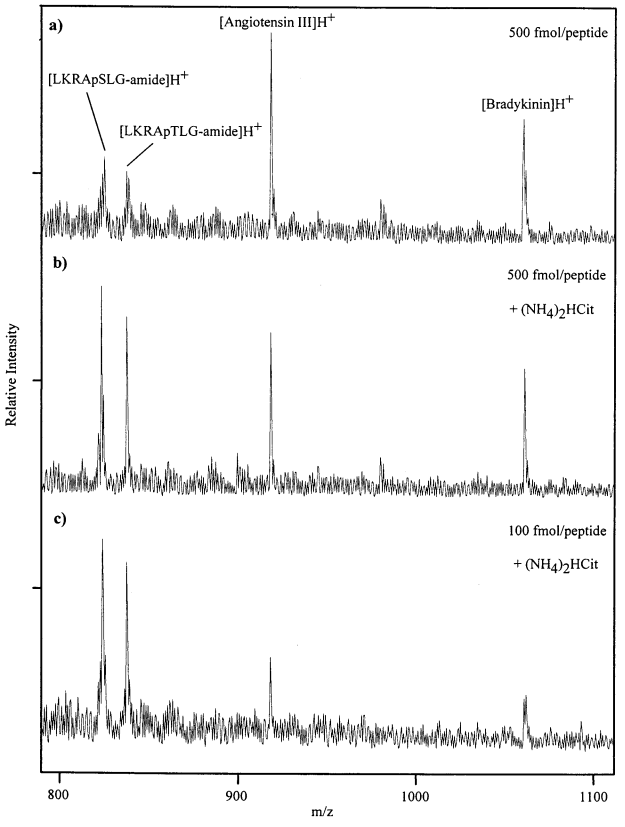

Figure 1. MALDI mass spectra of mixture I in (**a**) α-cyano-4-hydroxycinnamic acid and (**b**, **c**) α-cyano-4-hydroxycinnamic acid with addition of diammonium citrate.

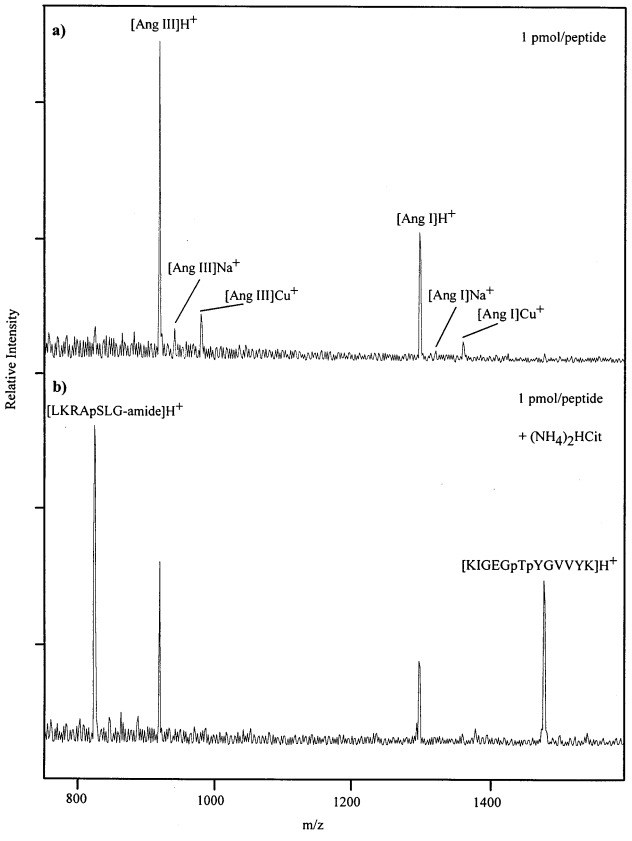

Figure 2. MALDI mass spectra of mixture II in (**a**) α-cyano-4-hydroxycinnamic acid and (**b**) α-cyano-4-hydroxycinnamic acid with addition of diammonium citrate.

charged in solution, mass spectrometrists frequently turn to negative ion modes of mass spectrometry analysis when desorption/ionization techniques such as FAB and MALDI are used. Small signals are observed for phosphorylated peptides in the negative ion mode of MALDI MS, however their intensities greatly increase when diammonium citrate is added, similar to that observed in positive ion mode. This suggests a common, neutral form of the analyte, as a starting point in the ionization chemistry, which can be protonated or deprotonated to yield cationic and anionic gas phase ions.

Multiply phosphorylated peptides exhibit low responses in MALDI, and the effect of diammonium citrate addition is also dramatic. This is shown using mixture II. Figure 2a shows the positive ion MALDI mass spectrum of a four component equimolar mixture of peptides (1 pmol/peptide). The peaks representing protonated angiotensin III (m/z 917.5) and angiotensin I (m/z 1296.7) are the only clearly visible peaks in the spectrum. For comparison with results from Figure 1, LKRApSLG-amide is a component (m/z 823.5). The fourth component is the diphosphorylated peptide KIGEGpTpYGVVYK (m/z 1474.7). Figure 2b shows the MALDI spectrum of the same mixture after the addition of diammonium citrate. The monophosphorylated and diphosphorylated peptide components are now detectable, and these peaks are the most intense. A comparison of pairs of spectra, with and without the additive, clearly indicates which components are phosphorylated. Also of note is the decrease in the relative intensities of the metal ion adducts. The peak representing protonated angiotensin III is accompanied by an $[M + Na]^+$ peak (m/z 939.5) and an $[M + Cu]^+$ peak (m/z 979.4) in Figure 2a. The 4-HCCA matrix has been cited as one that "seems to encourage copper attachment to some peptides" [27]. Metal ion adducts are also present for angiotensin III. The intensities of the metal ion adducts of these species, relative to the protonated forms, decrease upon ammonium ion addition. Similar effects have been observed in DNA fragment analysis using MALDI, when ammonium salts are added [22–24] or when ammonium-charged ion exchange beads are used [21]. Similar to the data in Figure 1, if the analysis of this mixture is performed at the 100 fmol level, the diphosphorylated peptide does not yield a discernible peak. However addition of $(NH_4)_2HCit$ results in a peak at m/z 1474.7 with a signal-to-noise (S/N) ratio greater than 6:1.

Membranes such as PVDF are frequently used in the analysis of proteins and peptides. Samples can be directly applied, and the membrane then washed to remove salts, buffers, contaminants, etc. Also, proteins

or digested protein fragments can be electroblotted from polyacrylamide gels onto membranes. MALDI mass spectra can be obtained directly from membranes [26, 28–31], and $(NH_4)_2HCit$ has the same effect. When the peptide mixture used to generate the data shown in Figure 1 was applied to a PVDF membrane, similar enhancements and displacement of metal ions were observed (data not shown). In work with membranes, a perfusion approach is used. The membrane is placed on a stack of wet filter paper containing the matrix solution. A mask prohibits solvent evaporation from anywhere except the membrane surface. As matrix and solvent pass through the membrane, solvent evaporates from the "top" side, where matrix crystals grow. This perfusion approach appears to increase analyte availability for incorporation into the matrix crystals. When ammonium salts are used, they can be introduced simultaneously with the matrix by this perfusion approach.

The influence of $(NH_4)_2HCit$ as a MALDI matrix additive is also observed when larger numbers of phosphates per peptide are encountered, and for species of higher mass. The pentaphosphorylated peptide β-casein has an average mass-to-charge ratio for $[M + H]^+$ of 23,984.3. Although it can be detected using MALDI [32], without the use of additives, the response is low. Also, its response can be suppressed by the presence of other high molecular weight analytes. For example, even at a level of 10 pmol, β-casein cannot be detected in the presence of an equimolar amount of equine myoglobin (avg. mass-to-charge ratio for $[M + H]^+$ 16,951.7). This is shown in Figure 3a, using DHB as the matrix, for mixture III. DHB was chosen because it gives good results for high mass peptides and proteins in our laboratory. When 1 μL of a 25 mM solution of an ammonium salt is added, the spectrum in Figure 3b is obtained. The pentaphosphorylated peptide is now clearly detectable. To obtain the data in Figure 3b, ammonium acetate was used. In many situations, the acetate is as effective as the citrate salt.

A comment should be made on changes in ion intensities when ammonium salts are added. The data shown here are typical of our experiences to date, in that the relative intensities of peaks representing phosphorylated peptides increase upon addition of the additive. In some cases, this represents a real increase in peak intensity. In other cases, the absolute intensities of all of the peaks may increase or decrease. We note that Woods et al. reported the observation that ammonium salts can influence the response for nonphosphorylated peptides in MALDI as well [33]. It is not our intention to suggest that absolute intensities for phosphorylated peaks alone will always increase upon addition of ammonium salts.

At this point a comment should be made on a common additive in MALDI, trifluoroacetic acid (TFA). TFA is frequently added to increase the solubility of polypeptides. TFA presumably changes the pH of the droplet from which crystals are formed, however it is also volatile. Acid hydrolysis is always possible when acidic solutions are used, and it is known that TFA reacts with amino groups of peptides [27], so it should be used with caution. However, studies which measured relative responses for phosphorylated and nonphosphorylated forms of peptides, showing lower D/I efficiencies for the phosphorylated forms, were performed using TFA [5, 6]. Although TFA was not used in the experiments presented here, it should be made clear that the addition of diammonium citrate or ammonium acetate to the matrix/analyte sample also works well in the presence of TFA.

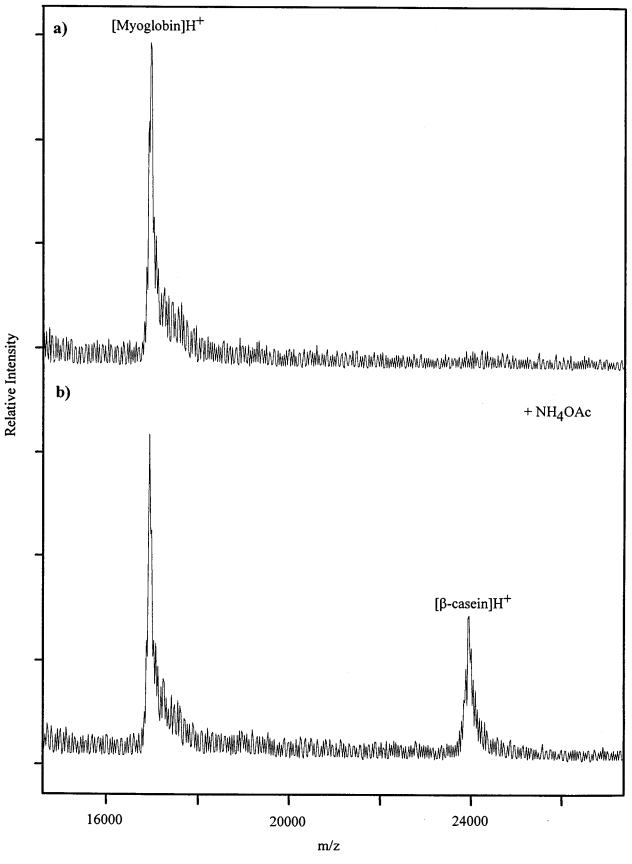

Figure 3. MALDI mass spectra of 10 pmol/component of mixture III in (**a**) 2,5-dihydroxybenzoic acid and (**b**) 2,5-dihydroxybenzoic acid with addition of ammonium acetate.

If the products of a proteolytic digestion of a phosphopeptide are analyzed as a mixture in a single MALDI experiment, the phosphorylated fragments yield small peaks [5, 6]. Addition of ammonium citrate will enhance their relative intensities, confirming their assignments as containing one or more phosphate groups. This is very helpful in extracting targeted information. The mass spectrometrist need not spend time interpreting peaks representing fragments which do not contain phosphorylation sites, when posttranslational modifications are the structural information desired. It is critical that this additive enhances the response for multiply phosphorylated polypeptides,

because phosphoserine resides frequently are found in chains [2]; thus, multiply phosphorylated tryptic fragments of large phosphoproteins are to be expected. For example, shown here is the sequence of β-casein [2, 6]. Its five phosphorylation sites are indicated (all serine residues), and sites of cleavage by trypsin are designated by slashes (\).

1 R\ELEELNVPG EIVEpSLpSpSpSE ESITR\
26 INK\K\I EK\FQpSEEQQQ TEDELQDK\IH
51 PFAQTQSLVY PFPGPIHNSL PQNIP
76 PLTQT PVVVPPFLQP EVMGVSK\VK\E
101 AMAPK\HK\EMP FPK\YPVEPFT ESQSL
126 TLTDV ENLHLPLPLL QSWMHQPHQP
151 LPPTVMFPPQ SVLSLSQSK\V LPVPQK
176 K\AVPY PQR\DMPIQAF LLYQEPVLGP
201 VR\GPFPIIV

This peptide was digested using trypsin at a level of 40 pmol, and the crude digest analyzed using MALDI, with DHB as the matrix. Each spectrum was obtained from 1/8 of the total digestion solution (corresponding to digestion products from 5 pmol of the protein). A relevant portion of the resulting mass spectrum, in which fragments containing approximately 25 residues are observed, is shown in Figure 4a. The largest peaks in the spectrum are (average values are listed for all) m/z 3723.5, which represents the protonated form of fragment {177–209}, and m/z 4485.4 representing {170–209}. Neither is phosphorylated. The minor peaks at m/z 3124 {1–25} and m/z 3479.4 {1–28} both represent peptides that contain the four phosphorylated serine residues at positions 15, 17, 18, and 19. Figure 4b shows the MALDI analysis of the same tryptic digest after the addition of $(NH_4)_2HCit$. Note the substantial increase in the peaks representing the phosphorylated components. They become the most intense peaks in the spectrum.

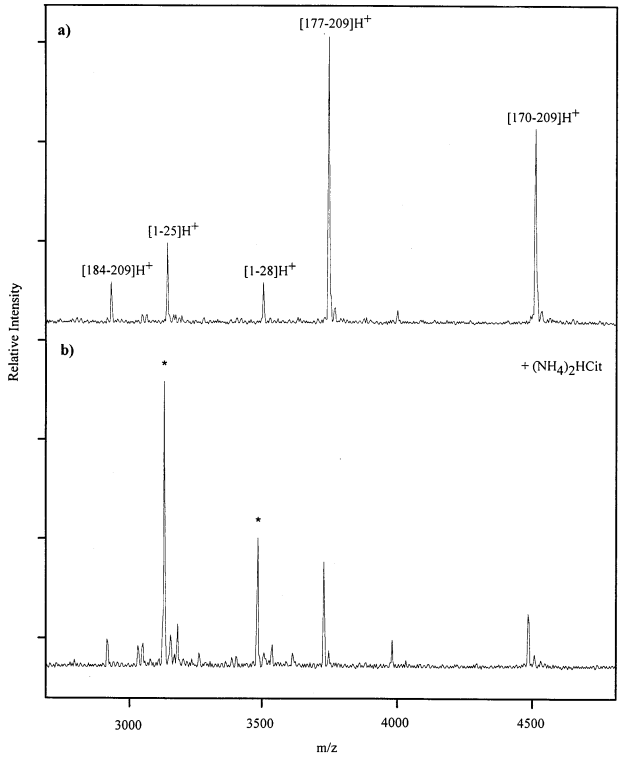

Figure 4. MALDI mass spectra of a tryptic digestion mixture from 5 pmol of β-casein in (**a**) 2,5-dihydroxybenzoic acid and (**b**) 2,5-dihydroxybenzoic acid with addition of diammonium citrate. The peaks marked by an asterisk represent phosphorylated components.

It should be noted that ammonium bicarbonate was used as a buffer in the digestion. This volatile buffer was removed by application of a vacuum to the sample for 12 h before the MALDI matrix was added. This allowed the spectrum in Figure 4a to be obtained. Then, additives were introduced to obtain the spectrum in Figure 4b. This raises an interesting observation. One approach following digestion is separation using HPLC, and analysis of the fractions using MALDI. If this is done to simplify the mixtures analyzed and to avoid possible suppression effects, the ammonium buffer salt is separated from the peptides, and the response for phosphopeptides is low. If the digestion products are analyzed directly, and ammonium salts are used as buffers, one may be more likely to detect the phosphorylated components because of the influence of ammonium ions.

An additional advantage of enhanced response, as displayed in Figure 4b, is that the phosphopeptide peaks are now sufficiently intense that PSD mass spectra [1, 34, 35] can be obtained with a good S/N ratio. Annan and Carr [1] have shown that the PSD spectra of singly modified peptides phosphorylated at serine and threonine eliminate H_3PO_4 (loss of 98 u), with a relatively minor loss of HPO_3 (−80 u). For phosphorylated tyrosine, loss of HPO_3 dominates. If the ions at m/z 3124, {1–25} H^+, in the linear spectrum of the unfractionated tryptic digest mixture are subjected to PSD analysis, the spectrum that results is shown in Figure 5. The spectrum shows four consecutive losses of phosphate groups, confirming its assignment.

The fragmentation processes leading to the peaks observed in Figure 5 warrant comment. Loss of multiple phosphates must be sequential, and possible scenarios for forming the observed ions are presented in Figure 6. The tetraphosphorylated precursor ion is at m/z 3124; all four phosphorylation sites are serine residues. Loss of 98 yields the peak at 3026. There is no peak corresponding to HPO_3 loss at m/z [3124 − 80], and there could be two reasons for this. The first may be that, when the internal energy content of the ion is high, the process leading to loss of H_3PO_4 has a higher rate than that leading to loss of HPO_3, so the former is observed exclusively. Alternatively, the ion with m/z 3044 may be formed but may dissociate completely, to undergo further eliminations. We suggest that the former explanation is correct. Analysis of the entire spectrum shows

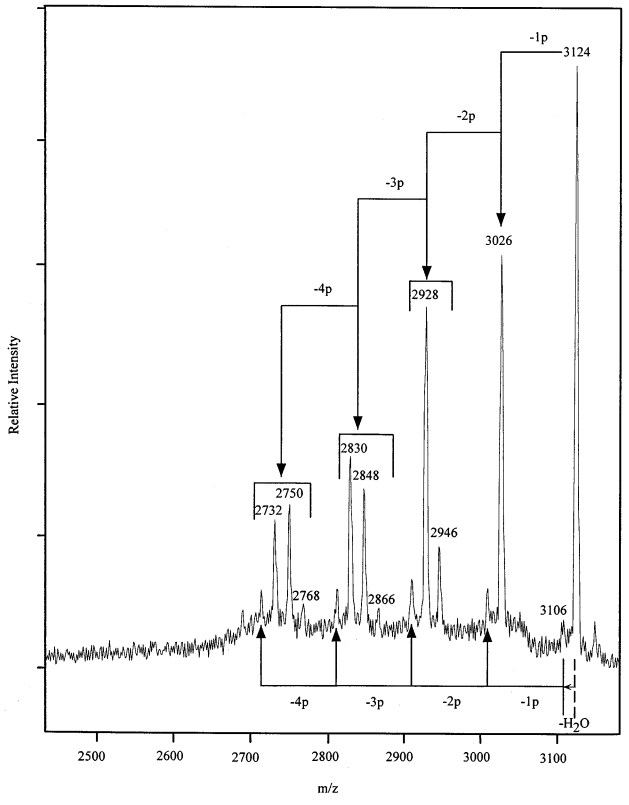

Figure 5. PSD spectrum of m/z 3124, a tryptic digestion product of β-casein containing four phosphate groups (p).

that phosphate elimination via loss of 98 dominates, which is consistent with Carr's observation for phosphoserine [1]. However, all four phosphates are obviously not lost as H_3PO_4. Three forms of the completely dephosphorylated ions are present. The peak at m/z 2732 corresponds to $[3124 - 4(98)]^+$, m/z 2749 corresponds to $[3124 - 3(98) - 80]^+$ and m/z 2768 to $[3124 - 2(98) - 2(80)]^+$. As successive eliminations occur, loss of 80 becomes more competitive with loss of 98. With each phosphate loss, internal energies drop and relative rates change. For the completely dephosphorylated forms, relative intensities and mass-to-charge ratio values suggest that 82% of the phosphates were eliminated as H_3PO_4 and 18% as HPO_3. Also of note, there is a small peak at m/z 3106, which corresponds to $[3124 - H_2O]^+$. This ion also appears to lose phosphates, and is responsible for the low intensity peaks at mass-to-charge ratio values below those of the $[3124 - n(98)]^+$ peaks, such as m/z 3008, $[(3124 - H_2O) - 98]^+$. In the context of the PSD experiments, we note a publication by Gorman et al. [36], introducing a mixture of 2,6-dihydroxyacetophenone and diammonium citrate as a MALDI matrix. This matrix was shown to decrease fragmentation because of phosphate loss for a phosphopeptide, relative to what is observed using α-cyano-4-hydroxycinnamic acid as the MALDI matrix. The ammonium salt may have been playing a key role in this experiment.

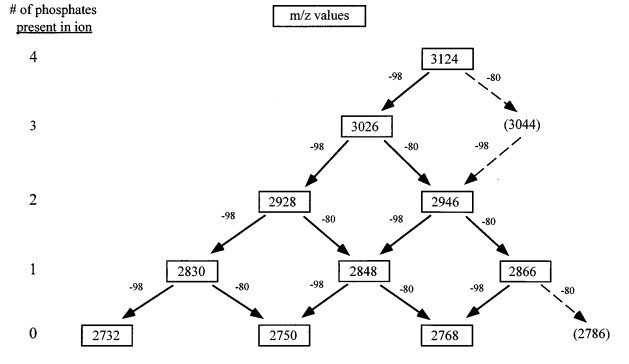

Figure 6. The mass-to-charge ratio values of observed fragment ions resulting from losses of 80 and 98 Da from the tetraphosphorylated peptide at m/z 3124. The mass-to-charge ratio values in parentheses represent peaks not detected. The loss of 98 Da corresponds to elimination of H_3PO_4; loss of 80 indicates loss of HPO_3.

Phosphate elimination occurs at a higher rate than skeletal bond cleavage, and the available internal energy is apparently used to eliminate multiple phosphate groups rather than from sequence-specific fragment ions. This has been observed previously for a triphosphorylated peptide by MALDI [1] and a pentaphosphorylated peptide using electrospray [37]. Thus, whereas the PSD spectrum indicates the number of phosphates present, it does not also yield the sequence. The sample should be treated with alkaline phosphatase, and the sequence determined from PSD analysis of the nonphosphorylated form. Alternatively, the sample may be treated with base resulting in β-elimination of H_3PO_4. This may aid in identifying the location of the phosphate group as well as obtaining sequence specific fragment ions [37].

Influence of $(NH_4)_2HCit$ on the MALDI Mass Spectra of Phosphopeptides: Mechanistic Considerations

To understand the role of diammonium citrate in this experiment, consider the order in which compounds precipitate from the matrix target solution as the volatile solvents evaporate. To do so, initial concentrations were defined, and the volume decreased in regular steps. At each step, new concentrations were computed, and the products of concentrations were computed to determine the volumes at which solubility limits were reached. This exercise contains a number of approximations and assumptions, and is meant to serve a simple purpose rather than to be a rigorous description of the system. In the experiment described, the initial solution has a volume of 3 μL, and is described in the sidebar of Figure 7. It contains matrix molecules, in which it is nearly saturated. It contains diammonium citrate. Assume that NaCl is present. To show the role that the additive can play, we will assume that NaCl is introduced with the matrix because it is a common impurity

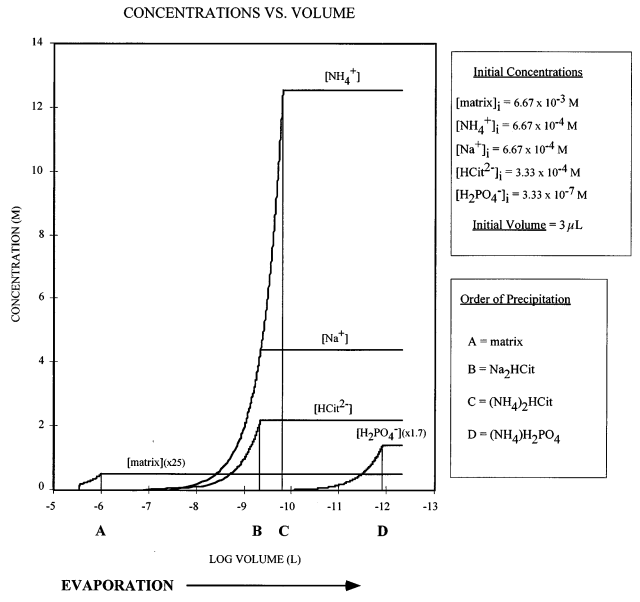

Figure 7. Plot of concentrations of ions vs. volume of an evaporating sample droplet containing matrix, a phosphopeptide, a sodium salt, and diammonium citrate. The volumes at which precipitation of each solid would occur is indicated by points A, B, C, and D.

in matrices and peptides [38]. The 4-HCCA matrix is provided from the manufacturer as 97% pure, so we will make the extreme assumption that the remaining 3% is NaCl, leading to the initial $[Na^+]$ of approximately 0.7 mM. There is also a phosphopeptide present. Information on the solubilities of phosphopeptides is not readily available, so for simplicity we will consider the phosphopeptide as a phosphate ion. A picomole of phosphopeptide in a solution of 3 μL results in a solution which is initially 0.3 μM in phosphopeptide (phosphate).

Figure 7 suggests how concentrations change as solvent evaporates and volume decreases. When the volume decreases by a factor of 3, the solution becomes saturated in matrix, and precipitation of matrix crystals begin. This is indicated as point A in Figure 7. Further reduction in volume should not yield increases in matrix concentration—it will remain constant and formation of solid matrix will continue. Of note is the pH of the solution from which other compounds will precipitate. The pH of a solution saturated in the matrix 4-HCCA was measured, yielding a value of 2.45. At this value, most of the phosphopeptide is expected to be in the form of $R–OPO_3H^-$; hence, the form of phosphate used in the calculation is $H_2PO_4^-$.

As solvent evaporates further, the species with the highest concentrations become Na^+, NH_4^+, and $HCit^{2-}$. For the initial concentrations used, and considering the K_{sp} [39] of Na_2HCit to be 43 (in water) and that for $(NH_4)_2HCit$ to be larger (345), Na_2HCit will precipitate first, at the point indicated by B in Figure 7. This process has two consequences. Once the point of saturation is reached, $[Na^+]$ and $[HCit^{2-}]$ will remain constant as further solvent evaporates. Also, it indicates that citrate is effective in "capturing" sodium ions and forming a precipitate. Thus, Na^+ ions can be effectively removed during the solvent evaporation process by the $HCit^{2-}$ ions. Further evaporation increases the ammonium ion concentration to the point where diammonium citrate precipitates, indicated by C in Figure 7, limiting further increases in ammonium ion concentration. The last ionic species in solution is the phosphate (phosphopeptide), which can precipitate in the high concentration of ammonium ions as the ammonium salt, which degrades at some later point in the experiment via ammonia loss as explained previously. In this calculation, concentrations are never achieved to allow precipitation of sodium phosphate, the phosphate only precipitates as the ammonium salt [K_{sp} $(NaH_2PO_4) = 26$, K_{sp} $(NH_4H_2PO_4) = 10$]. Although the calculation can be carried to very small solution volumes, there is a point where the system can no longer be considered as a solution—where there are an insufficient number of solvent molecules per solute molecule. Thus, the sample becomes a solid with partially hydrated species at a volume around points B/C. Thus, it is probably not the K_{sp} values of NaH_2PO_4 and $NH_4H_2PO_4$ that determine which cation is captured by the phosphate group; this is probably reflecting the relative availability/concentrations of the ammonium ions vs. sodium ions.

Obviously, the calculation resulting in Figure 7 is not rigorous—it was done to assist in considering possibilities. Concentrations, not activities, were used, and ionic strengths can become considerable during solvent evaporation. Equilibrium (aqueous) values are of limited use in such a system when volatile solvents are rapidly evaporating. The mixed solvent system presumably changes composition during evaporation. Trapping of analyte and salt molecules, in neutral and charged forms, in precipitating matrix crystals is not considered but obviously takes place [40–43].

In such a calculation, it is difficult to make general statements concerning the behavior of polypeptides/proteins. Solubilities of proteins in aqueous solutions vary enormously [44]. Solubilities can be enhanced with mixed solvents, acids, salts, and other solubilizing agents. Also, proteins can "salt out" at high salt concentrations, notably when ammonium salts are used [44, 45]. Ammonium sulfate has been used to help precipitate nonphosphorylated hydrophobic peptides prior to MALDI analysis [33]. Peptides frequently have solubilities on the order of tens of mg/mL. For example, angiotensin I, angiotensin III, and myoglobin have aqueous solubilities of 10, 25, and 20 mg/mL, respectively [46]. Phosphorylation leads to an increase in solubility in some cases [2, 47].

The calculation suggests a useful model. As solvent evaporates, matrix first begins to precipitate. If sodium ions are present in a sufficient amount, citrate ions can form sodium citrate. This will limit sodium ion concentration. The phosphopeptide can then interact with ammonium ions in the later stages of evaporation,

leading to the ultimate "neutralization" of the phosphate group(s). Also of note, ammonium acetate frequently was found to be as effective as ammonium citrate as a matrix additive. The K_{sp} for sodium acetate is smaller than that for ammonium acetate, analogous to the relationship for the citrate salts, so in such systems, acetate can limit the role of sodium ions as does citrate. Such calculations can be particularly instructive when ionic species are involved.

Conclusions

The use of diammonium citrate as an additive in the MALDI matrix assists in the analysis of phosphopeptides as it does for oligonucleotides, presumably by lowering the charge state of the analyte, allowing for desorption to occur. The approach is simple, has real utility, and can be used in a variety of ways. For example, suppose a valuable polypeptide sample is subjected to MALDI analysis for a molecular weight determination, and no peak is detected. It may be phosphorylated, and is exhibiting a poor response. In this case, the matrix/analyte target can be removed from the instrument, and 1–2 μL of the diammonium citrate solution added. It has been verified that the target will redissolve and new crystals will be formed, incorporating the additive. This may lead to successful detection, and would suggest that the analyte may be phosphorylated. If so, the additive increases the probability that sufficient numbers of ions will be available to perform further analyses, to determine if phosphate groups are present, and how many. The use of this simple additive will allow investigations of more highly phosphorylated polypeptides to establish their fragmentation patterns, and to establish the maximum number of phosphate groups that could be determined by PSD or CAD.

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