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Baseline Resolution of Isobaric Phosphorylated and Sulfated Peptides and Nucleotides by Electrospray Ionization FTICR MS: Another Step toward Mass Spectrometry-Based Proteomics

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Electrospray ionization broadband FTICR mass spectrometry at a mass resolving power, $m/\Delta m_{50\%} \geq 400\,000$ has achieved the first direct mass spectral resolution of phosphorylated and sulfated peptides (or nucleotides) of the same nominal mass. The elemental composition difference in each case is PH versus S (9.5 mDa), requiring a minimum mass resolving power ($(m_2 - m_1)/m_1$) of 118 000 (C terminal amidated cholecystekinin fragment 26−33 (CCK-8), DY(PO_3H_2)MGWMDF-NH_2 versus DY(SO_3H)MGWMDF-NH_2) or 65 400 (adenosine triphosphate vs 3-phosphoadenosine 5′-phosphosulfate). The isobaric mass doublets were detected in broadband mode ($400 < m/z < 1400$) in the presence of dozens of other species. It is therefore now possible to distinguish phosphorylated from sulfated peptides, even when both species are present at the same time in a protein digest.

Mass-based proteomics, namely, the identification of proteins based on determination of the masses of peptides in a condensed-phase (solution, gel, or immobilized) enzymatic digest, gas-phase fragmentation experiment(s), or both, is rapidly gaining acceptance for determining protein relative abundances as well as the number and sites of posttranslational modifications.[1−4] Such methods may be enhanced by any of a host of methods employing isotopically labeled amino acids, typically based on enrichment of heavy isotopes of H, C, or O.[5−10] Conversely, depletion of ^{13}C and ^{15}N can greatly simplify the mass spectrum of an intact protein,[11] to the point that even intact proteins may be identified from their accurate masses and on-line gas-phase fragmentation.[12] "Top down" sequencing, in which a protein ion's amino acid sequence may be determined almost completely by extensive gas-phase fragmentation to produce charged complementary fragments, has been significantly advanced by the advent of the electron capture dissociation technique.[13]

Until recently, most such methods have been based on mass measurement accuracy to within ∼1 Da. However, it has long been recognized that the number of possible amino acid sequence candidates drops rapidly with increasing mass measurement accuracy.[14−23] If only a single, well-resolved peptide is present at a given nominal mass, then it is relatively easy to distinguish, for example. glutamine from lysine (differing in elemental composition by CH_4 versus O, corresponding to a mass difference of 36 mDa),

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as in the electrospray (ESI) orthogonal time-of-flight mass spectra of separate samples of the two peptides, MICHAELQARAS (1328.6329 Da) and MICHAELKARAS (1328.6693 Da).[24] An order of magnitude higher mass resolving power is needed to resolve other possible pairs of (separated) peptides of different elemental composition—e.g., (Met + Leu) versus (Pro + Phe), differing in elemental composition by SH_4 versus C_3 (3.4 mDa). Yet another order of magnitude higher resolving power is needed to separate that same mass difference if both peptides are present simultaneously, as in the ESI Fourier transform ion cyclotron resonance (FTICR) mass spectrum of ALANGMARSHALL versus ALANGPARSHALF (each ~1323 Da neutral mass).[25] The current record for mass resolution of two peptides present in the *same* sample is a (nonroutine) ESI FTICR *narrowband* mass spectrum of RVMRGMR and RSHRGHR, of neutral monoisotopic mass, ~904 Da, differing in elemental composition by N_4O versus S_2H_8 (0.000 45 Da), which is less than one electron's mass (0.000 55 Da)![26] However, baseline resolution of 3−10 mDa is relatively routinely achievable in *broadband* ($200 < m/z < 1200$ Da) ESI FTICR MS.

The mass difference between phosphorylation and sulfation (PH, 31.9816 Da and S, 31.9721 Da) is 9.5 mDa—i.e., within the previously demonstrated capability of ESI broadband FTICR MS. Here, we present the first experimental baseline resolution of two peptides (and two nucleotides), each pair differing by the presence of a phosphate versus a sulfate group. We next stop to consider the relevance of such a measurement.

Phosphorylation and sulfation of a peptide or protein serve very different biological purposes. Biological reversible phosphorylation regulates protein function. For instance, phosphorylation inactivates glycogen synthase.[27−29] More generally, kinases regulate other proteins in so-called "signaling pathways" by attaching phosphate group(s) to them, as in the pathway leading from the initial binding of insulin to its membrane-bound receptor to the ultimate conversion of glycogen to glucose.[27,30−32] Biological sulfation, on the other hand, is a permanent modification critical to the function of the modified protein, as in cholecystokinin-8-sulfate (CCK8-S), which has been implicated in inducing satiety as well as in schizophrenia.[33−35] Finally, artificial sulfation facilitates low-energy charge site-initiated fragmentation of matrix-assisted laser desorbed/ionized[36] or electrosprayed[37] peptides, by producing extensive cleavage to yield y-type fragment ions.

Several methods exist for determining protein sulfation. For example, a cell culture may be incubated with $^{35}SO_4^{2-}$, the cells then lysed and the proteins analyzed for label incorporation.[38] Although sensitive, that method requires specialized training and facilities to handle the radioactive compounds. Alternatively, tyrosine residues may be labeled with ^{125}I. Electrophilic iodine generated with chloramine T reacts with the tyrosine aromatic ring, but sulfated tyrosine rings react much slower.[39−41] While having the advantages of sensitivity and no cellular incubation period, the ^{125}I method is kinetically based and is sensitive to protein concentration, temperature, and other control variables. The kinetic method may also fail to differentiate phosphorylated from sulfated tyrosine, because phosphorylated tyrosines also react slower than unaltered tyrosines. An alkaline hydrolysis assay can differentiate phosphorylated and sulfated peptides based on radioisotope labeling,[42,43] but uses $^{35}SO_4^{2-}$ and $^{32}PO_4^{3-}$, necessitating proper facilities for handling radioisotopes.

Chromatographic separation has also been used to separate and speciate phosphoproteins and peptides. Ion metal affinity chromatography (IMAC) relies on specific interactions between metal ions covalently bound to a ligand on a chromatographic stationary phase and various side chains to separate specific proteins and peptides in a mixture.[44] The metal ion−side chain interaction causes specific protein(s) to adhere to the column. The protein can be eluted from the column by adding an excess of another ligand to displace the metal ion−side chain bond after all others have passed through the column. Metal ion affinity columns for phosphoproteins may employ a Ga^{3+} chelation complex or an Fe^{3+} chelate.[45−47] IMAC columns have also been used as a purification step prior to mass spectral analysis, in both liquid chromatography/mass spectrometry and liquid chromatography/tandem mass spectrometry methods (LC/MS and LC/MS/MS).[47,48] This specific interaction is very useful for purifying a single component type from a mixture, but may not be as useful for analyzing an entire mixture, as only the phosphoproteins are retained by the column. Moreover, there have been no reports of a sulfate-specific column, thereby limiting the approach only to phosphoproteins and peptides.

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The difficulties of the above methods may be avoided by use of mass spectrometry. Mass spectrometry has been used previously for determining phosphorylations and sulfations on side chains,[49−54] but not when both modifications are present simultaneously. For example, Wolfender and co-workers combined MALDI, ESI, and tandem mass spectrometry to identify a sulfated tyrosine in conotoxin.[55] The number of phosphates on a peptide may be determined by comparing mass spectra obtained before and after collisional dissociation or infrared multiphoton dissociation (IRMPD). High-energy collision-induced dissociation of negative ions results in a fragment ion of 79 Da (PO_3^-) for a phosphorylated peptide or protein versus a fragment of 80 Da (SO_3^-) for a sulfated protein.[56] Low-energy collision-induced dissociation of negative ions yields the fragment ions, $H_2PO_4^-$ for a phosphorylated peptide and HSO_4^- and SO_4^- for a sulfated peptide. Thus, for low-energy fragmentation of negative ions, high resolution and mass accuracy are required to distinguish the isobaric product isobaric ions, $H_2PO_4^-$ and HSO_4^-. Low-energy fragmentation of positive ions, as by IRMPD, can result in a mass loss of 98 Da per phosphate (H_3PO_4), thereby establishing the presence and number of phosphorylated amino acids.[57] Low-energy collision-induced dissociation positive ion MS/MS experiments show a loss of SO_3 for sulfated peptides, allowing for differentiation of the two modifications by tandem mass spectrometry.[58] MS/MS can thus differentiate phosphorylation from sulfation but at the cost of an additional experiment requiring higher signal-to-noise ratio for the original mass spectrum.

Finally, if phosphorylation and sulfation occurred at different amino acid side chains, then one might hope to distinguish them by gas-phase cleavage of peptide bonds without detaching, for example, the phosphate group.[59,60] However, sulfation and phosphorylation both occur at tyrosine residues. In any case, we shall show that mass measurement can distinguish the two directly.

Other biomolecules that exhibit the PH versus S mass difference are the nucleoside cofactors responsible for protein sulfation. Adenosine triphosphate (neutral composition, $C_{10}H_{16}N_5O_{13}P_3$) is a cofactor for making the precursor sulfating cofactor, 3′-phosphoadenosine 5′-phosphosulfate (neutral composition, $C_{10}H_{15}N_5O_{13}P_2S$).[61−64] Although these two molecules are distinguishable by their different MS/MS product ion distributions, high mass resolution is still needed to be sure that PAPS is not 3′-phosphoadenosine 5′-diphosphate.[65−68]

Table 1. Theoretical and Experimental Masses for Individual Phosphorylated or Sulfated Nucleotide and Peptide Samples[a]

ion	predicted mass	measured mass	error (ppm)
External Calibration			
$[CCK\text{-}8/SO_3H - 2H]^{2-}$	1140.3362	1140.3315[b]	4.1
$[CCK\text{-}8/PO_3H_2 - 2H]^{2-}$	1140.3457	1140.3440[b]	1.5
$[CCK\text{-}8/SO_3H - 2H]^{2-}$	1140.3362	1140.3465[c]	9.2
$[CCK\text{-}8/PO_3H_2 - 2H]^{2-}$	1140.3457	1140.3562[c]	9.2
$[ATP + 3Na]^+$	573.94884	573.95031[d]	2.6
$[PAPS + 3Na]^+$	573.93933	573.94204[d]	4.7
$[ATP + 3Na]^+$	573.94884	573.95554[e]	12
$[PAPS + 3Na]^+$	573.93933	573.94614[e]	12
Internal Calibration			
$[CCK8/SO_3H\text{-}2H]^{2-}$	1140.3362	1140.3358[b]	0.32
$[CCK8/PO_3H_2{-}2H]^{2-}$	1140.3457	1140.3454[b]	0.24
$[CCK8/SO_3H\text{-}2H]^{2-}$	1140.3362	1140.3361[c]	0.03
$[CCK8/PO_3H_2{-}2H]^{2-}$	1140.3457	1140.3458[c]	0.05
$[ATP + 3Na]^+$	573.94884	573.94875[d]	0.16
$[PAPS + 3Na]^+$	573.93933	573.93969[d]	0.62
$[ATP + 3Na]^+$	573.94884	573.94912[e]	0.49
$[PAPS + 3Na]^+$	573.93933	573.93978[e]	0.80

[a] Mass errors are listed for external and internal calibration. As a sample theoretical calculation, the ATP monoisotopic ion mass is computed from the neutral elemental composition, $C_{10}H_{14}N_5O_{13}P_3Na_3$, minus the mass of one electron (0.000 55 Da). [b] Sulfated or phosphorylated CCK8 alone. [c] Sulfated and phosphorylated CCK8 mixture. [d] ATP or PAPS alone. [e] ATP and PAPS mixture.

EXPERIMENTAL METHODS

Sample Preparation. Sulfated CCK-8 fragment 26−33 amide (DY[SO_3H]MGWMDF-NH_2), adenosine 5′ triphosphate sodium salt (ATP), and 3′-phosphoadenosine 5′-phosphosulfate lithium salt (PAPS) were purchased from Sigma Chemical (St. Louis, MO) and used without further purification. Phosphorylated CCK-8 fragment 26−33 amide (DY(PO_3H_2)MGWMDF-NH_2) was synthesized by solid-phase methods at the Biological Analysis and Synthesis Service (BASS) laboratory at Florida State University. F-moc phosphotyrosine was purchased from Nova Biochem (San Francisco, CA) for synthesis of the phosphorylated analogue of CCK-8 fragment 26−33. Sodium trifluoromethanesulfonate and poly(ethylene glycol) biscarboxymethyl ether (PEGBCME) were purchased from Aldrich Chemical and used without purification. HPLC grade water and acetonitrile were purchased from Fisher Scientific (Savannah, GA) and were used without further purification.

The peptides were dissolved in 1:1 v/v water/acetonitrile with 5 μM added sodium triflate to effect ionization. CCK-8 phospho-

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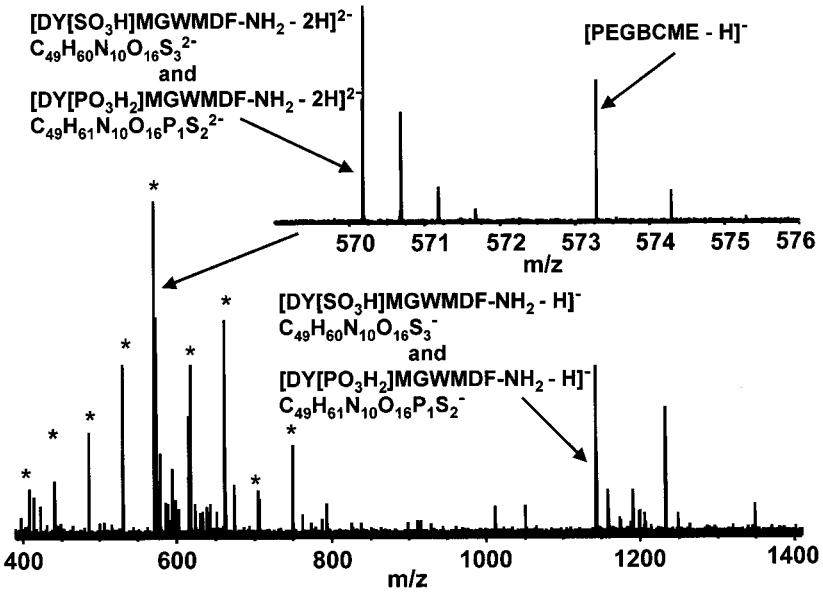

Figure 1. Broadband negative ion ESI FTICR mass spectrum of a mixture of PEGBCME (internal mass calibrant,peaks marked with *) and phosphorylated and sulfated CCK-8 amidated fragment 27−33. The peptides are present in equimolar quantity, and the spectrum was collected in broadband mode as an 8 Mword time domain transient. The *m*/*z* scale-expanded inset shows a calibrant ion in proximity to the isotopic distribution of the two peptides, but does not visually resolve the peptide isobars.

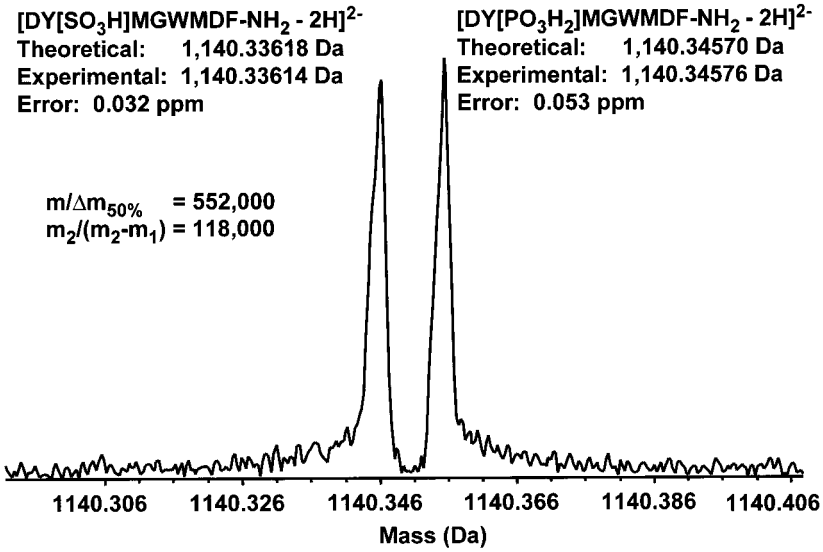

Figure 2. Mass scale expansion near *m*/*z* 570 in the broadband negative ion ESI FTICR mass spectrum of Figure 1. The high mass accuracy and resolution unambiguously identify each peptide. A full analysis of mass accuracy is given in Table 1 and associated text.

rylated and sulfated peptides were dissolved to a final concentration of 10 μM and were sprayed in negative ion mode at an infusion rate of 500 nL/min. The electrospray ionization conditions were as follows: tube lens −0.370 kV, emitter potential −3.5 kV, accumulation and transport octapole frequency set to 1.5 MHz, and amplitude 166 V_{p-p}.

For internal mass calibration, PEGBCME was added to the above solution and its concentration adjusted until the signal magnitudes of both the peptides and polymer were similar. The final concentration of PEGBCME was 5 μM.

ATP and PAPS were dissolved in 1:10 (v/v) water/acetonitrile with 5 μM added sodium triflate to effect cationization. Samples were prepared to a final concentration of 10 μM and were infused at a rate of 500 nL/min. Synthetic PAPS is moderately unstable in solution at room temperature; so a lower proportion of water was used for the electrosprayed solution. Samples were also kept in an ice/$CaCl_2$ bath to prevent degradation. Positive-ion ESI FTICR mass spectra were collected with a tube lens voltage of +0.370 kV and an emitter voltage of +3.5 kV. The external accumulation octapole was set to 1.5 MHz and 166 V_{p-p} amplitude. Mass spectra for nucleosides were acquired as for the peptides (see below).

Electrospray Ionization High-Field FTICR Mass Spectrometry. ESI FTICR mass spectra were acquired with a home-built 9.4-T FTICR instrument.[69,70] Data were collected with a

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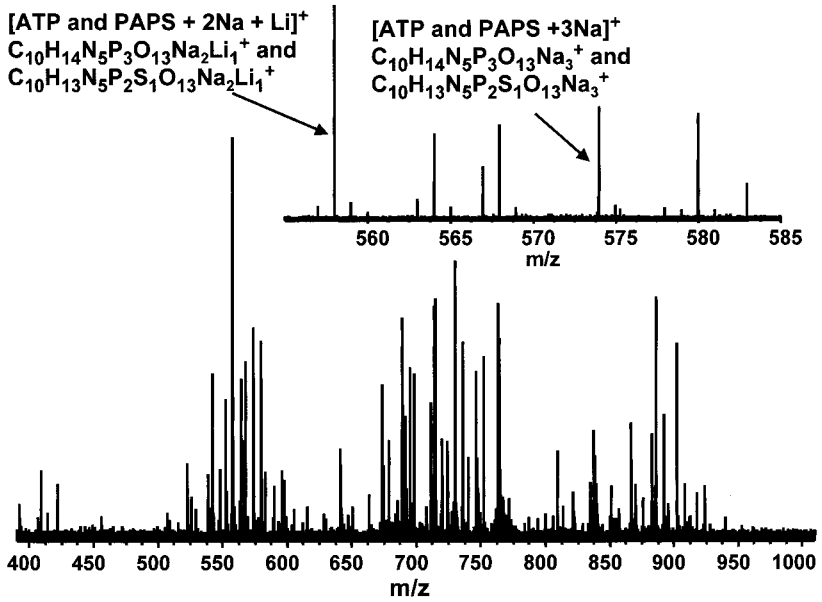

Figure 3. Broadband positive ion ESI FTICR mass spectrum of a mixture of ATP and PAPS with added sodium triflate. The ions observed are predominantly sodium and lithium adducts, dimers, and hydrolysis product dimers. The *m/z* scale expansion (inset) shows that ATP and PAPS both form sodium and lithium ion adducts.

MIDAS 160 data station.[71,72] Ions for analysis were accumulated externally for 20 s in an octopole ion trap. Despite the long accumulation period, only minor amounts (~0.5% of parent peak height) of desulfated or dephosphorylated peptide were observed, likely arising from solution-phase hydrolysis (since no fragments of PEGBCME resulted from external ion storage.) After accumulation, the ions were transported to an open cylindrical ICR cell (background pressure $\sim 5 \times 10^{-10}$ Torr) and were axially cooled as follows.[73] The initial ion population was confined by a 2-V trapping potential for 1 s. The trapping potential was then lowered from 2.0 to 0.75 V in five 1-s intervals. Because so many Penning-trapped ions are lost during the axial cooling process,[73] it is desirable to start with a large number of ions initially—hence the long external accumulation period. Axial cooling results in higher mass resolving power, as well as eliminating shot-to-shot shift in ICR frequency for efficient time domain signal averaging without significant peak broadening.

After axial cooling, the ions were chirp excited and the time domain signal was acquired in direct mode. Time domain transients were collected as 64 coadded 8 Mword files; each transient lasted 3 min. A Mword here is defined for a 16-bit ADC; thus, an 8 Mword file is 32 MB of data. The accumulated time domain transient was truncated to 4 Mword, zero-filled once, Hamming apodized, subjected to fast Fourier transformation and magnitude computation, and then frequency-to-m/z converted.[74] The theoretical masses and isotope distributions for the peptides, nucleotides, and PEGBCME were calculated with Isopro 3.1.[75]

RESULTS AND DISCUSSION

Phosphorylation versus Sulfation in an Isolated Peptide or Nucleotide. In a complex proteolytic digest or other biological peptide mixture, it is possible that two nominally isobaric peptides could be phosphorylated or sulfated at a given tyrosine residue. We therefore synthesized a phosphorylated peptide whose sulfated equivalent is also available. The peptide is the C-terminal amidated cholecystokinin fragment, fragment 26−33 (CCK8-S): DY(PO_3H_2)-MGWMDF-NH_2, monoisotopic (neutral) mass, 1142.36025, or DY-(SO_3H)MGWMDF-NH_2, monoisotopic mass, 1142.35073 Da. The phosphorylated and sulfated nucleotides, ATP and PAPS, are commercially available.

The peptides were analyzed by negative-ion ESI FTICR MS analysis, whereas the nucleotides were observed as positive ions. Negative-ion analysis is essential for the sulfated peptide, because (a) the sulfate moiety can be lost in positive-ion mode,[76] and (b) the unsulfated molecule is biologically inactive.[33,77,78]

As noted above, the mass difference between PH versus S is 0.0095 Da: i.e., ~15 ppm at 574 Da (ATP) or ~8 ppm at 1140 Da. Each peptide and nucleoside was first examined individually. ESI FTICR MS, internally calibrated with PEGBCME, easily identifies which is which, based on the sub-ppm mass accuracy in Table 1.

Phosphorylated versus Sulfated Peptide in a Mixture. Figure 1 shows the negative-ion ESI FTICR mass spectrum of an equimolar mixture of phosphorylated and sulfated peptides. An expansion of the $569 < m/z < 576$ region (Figure 1, inset) shows the proximity of the signals from the peptides (not visually resolved from each other) and the calibrant polymer (peaks marked with *). The polymer concentration is easily adjusted to yield a signal-to-noise ratio similar to that of the analytes, ensuring high mass accuracy. The unit spacing between the internal

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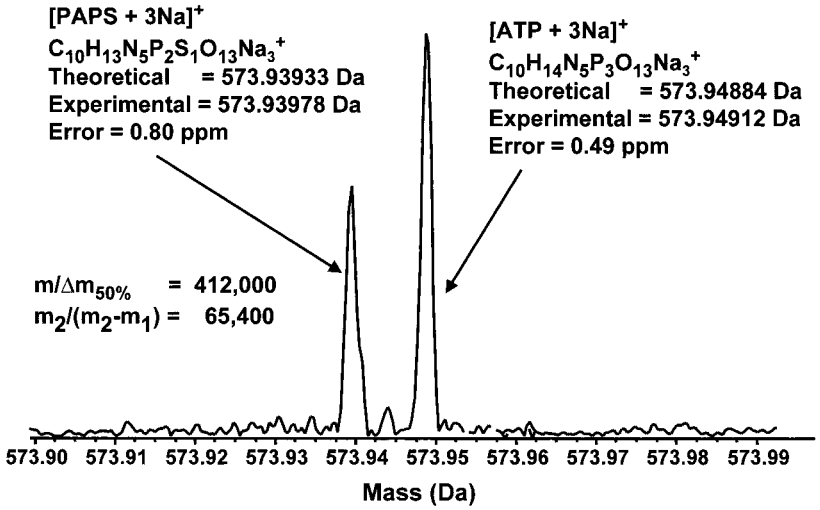

Figure 4. Mass scale expansion near *m*/*z* 573 in the broadband positive ion ESI FTICR mass spectrum of a mixture of ATP, PAPS, and PEGBCME with added sodium triflate. The ATP and PAPS sodium adduct ions are cleanly resolved from one another and identified with high mass accuracy. The peak between PAPS and ATP is noise and did not appear in other spectra of ATP, PAPS, and the peptides. Although the *m*/*z* ratios of the ions are similar to those in Figure 2, the mass resolving power is lower due to the lower charge state (1+ vs 2+).[79] A full analysis of mass accuracy is given in Table 1 and associated text.

calibrant m/z spectral peaks (m/z ~573, ~574) shows that it is singly charged, whereas the half-integer spacing between the peptide peaks (m/z >570) shows that it is doubly charged.

A highly mass scale-expanded segment for the monoisotopic peptide ions (Figure 2) baseline-resolves the phosphorylated and sulfated peptides. The observed peak separation (0.0096 Da) is in excellent agreement with the theoretical separation (0.0095 Da). The mass resolving power for either peak, $m/\Delta m_{50\%} = 552\,000$ was achieved by processing the entire 8 Mword digitized time domain signal. Although this experiment requires somewhat more effort (i.e., axial cooling prior to detection) than typical broadband FTICR MS experiments, the full m/z range is detected, and numerous species are present simultaneously.

Phosphorylated versus Sulfated Nucleotide. Figure 3 shows a broadband positive-ion mass spectrum of ATP and PAPS in a binary mixture. The spectrum is highly congested, exhibiting protonated, sodiated, and lithiated dimers of ATP and PAPS. An expansion of the $555 < m/z < 585$ region (Figure 3, inset) shows that ATP and PAPS both bind alkali metal ions.

A highly mass scale-expanded segment for the monoisotopic sodiated nucleotide ions (Figure 4) baseline-resolves the phosphorylated and sulfated nucleotides, even after truncation of the original time domain data from 8 Mword to 4 Mword. Again, the mass difference between the two peaks (0.009 34 Da) is in excellent agreement with the theoretical difference (0.0095 Da).

In conclusion, we are able to distinguish phosphorylated from sulfated peptides or nucleotides, whether present individually or (experimentally much more demanding) simultaneously. Although the present mass resolution (0.0095 Da) is exceeded by (our own) prior record (two other peptides differing by 0.000 45 Da at ~904 Da),[26] the latter result required narrowband heterodyne detection, whereas the present experiments were performed with broadband (1000 Da) direct-mode detection, which is much more relevant to analysis of proteolytic digests and other biological mixtures of peptides. Although the present data were acquired on a time scale too slow (3 min/scan) for direct on-line chromatographic interface, such interface could become feasible following increased speed and efficiency of recovery of externally stored ions. The present result can be obtained only by FTICR mass analysis—other mass analyzers lack the resolution to separate phosphorylated from sulfated peptides or nucleotides when present together.

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