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Open Tubular Immobilized Metal Ion Affinity Chromatography Combined with MALDI MS and MS/MS for Identification of Protein Phosphorylation Sites

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Protein phosphorylation is one of the most important known posttranslational modifications. Tandem mass spectrometry has become an important tool for mapping out the phosphorylation sites. However, when a peptide generated from the enzymatic or chemical digestion of a phosphoprotein is highly phosphorylated or contains many potential phosphorylation residues, phosphorylation site assignment becomes difficult. Separation and enrichment of phosphopeptides from a digest mixture is desirable and often a critical step for MS/MS-based site determination. In this work, we present a novel open tubular immobilized metal ion affinity chromatography (OT-IMAC) method, which is found to be more effective and reproducible for phosphopeptide enrichment, compared to a commonly used commercial product, Ziptip from Millipore. A strategy based on a combination of OT-IMAC, sequential dual-enzyme digestion, and matrix-assisted laser desorption/ionization (MALDI) quadrupole time-of-flight tandem mass spectrometry for phosphoprotein characterization is presented. It is shown that MALDI MS/MS with collision-induced dissociation can be very effective in generating fragment ion spectra containing rich structural information, which enables the identification of phosphorylation sites even from highly phosphorylated peptides. The applicability of this method for real world applications is demonstrated in the characterization and identification of phosphorylation sites of a Na^+/H^+ exchanger fusion protein, His182, which was phosphorylated in vitro using the kinase Erk2.

Characterization of posttranslationally modified proteins is an analytical challenge in proteomics. As one-third of the thousands of proteins expressed in a typical mammalian cell are thought to be phosphorylated, protein phosphorylation is one of the most important posttranslational modification.[1,2] Proteins are mainly phosphorylated at specific serine or threonine residues and, less commonly, at tyrosine as well as other amino acid residues.[3] Reversible protein phosphorylation is important for regulation of the activity of proteins in signal transduction, metabolism, or apoptosis development in the cell.[4−9]

Gel electrophoresis remains the most powerful technique for phosphoprotein separation. Detection of phosphorylation in gel-separated proteins can be done by either ^{32}P radiolabeling or Western blotting with antibodies. Autoradiography or phosphorimaging is very sensitive in detecting ^{32}P-labeled phosphoproteins. Traditionally, phosphoamino acid analysis[10,11] and manual Edman sequencing[12,13] were used to identify phosphorylated residues. However, these techniques require large quantities of sample, and the identification of phosphorylation sites is indirect. Confirmation of the sequence is usually not accomplished.[14]

Mass spectrometry (MS) has recently become an important tool for characterization of phosphorylation in native and recombinant proteins. Not only can phosphorylation be identified, but phosphorylation sites can also be assigned. However, direct analysis of phosphopeptides in crude enzyme-digested complex mixtures is problematic due to "ion suppression" effects and low abundance of phosphopeptides. Separation and enrichment of phosphopeptides from a digest mixture is desirable and often a critical step for subsequent MS analysis. Reversed-phase (RP)-HPLC is commonly used to separate the phosphopeptides as well as the other coexisted peptides obtained from an enzymatic digest of a protein.[15−19] Using electrospray ionization MS/MS, separated

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phosphopeptides can be selectively detected with precursor ion scan,[1,20,21] neutral loss scan,[22] or selected ion monitoring of m/z 79 in the negative ion mode.[1,23−25] However, a reversed-phase C18 column does not retain highly phosphorylated peptides due to their hydrophilic nature, and RP-HPLC/MS may fail to detect them.[26,27]

To improve selectivity, affinity-based techniques have been applied to purify or enrich phosphopeptides from crude samples. Affinity anti-phosphotyrosine antibodieshave been used to enrich phosphotyrosine proteins[28] or used in a "reactor" column to concentrate the phosphotyrosine prior to LC−MS/MS.[29] However, the enrichment of phosphoserine/threonine-containing proteins with antibodies has not been routinely possible. Biotin affinity chromatography was also applied to isolate phosphopeptides after the phosphate was chemically replaced by biotin.[30] The most frequently used affinity technique is immobilized metal ion affinity chromatography (IMAC) first introduced by Andersson and Porath.[31] The retention of phosphopeptides in IMAC is based on an electronic interaction of analyte molecules with metal ions immobilized (chelated) on a stationary phase.

Fe(III)-IMAC columns have been used in combination with RP-HPLC and detected by MALDI-MS or LC−MS for phosphopeptide purification and characterization at picomole and subpicomole levels.[32−34] Ga^{3+}-IMAC was reported as a selective method for phosphopeptide purification prior to MS analysis.[35,36] IMAC was also used to isolate phosphopeptides in complex mixtures, by itself or combining with strong anion exchange chromatography, in large-scale analysis of membrane proteins.[37−40] To analyze (sequence) femtomole levels of phosphopeptides, nanoscale chromatographic columns with packing resin in a centrifugal capillary[25,41] or in a constricted GELoader pipet tip[42,43] have been constructed. Nanoscale Fe(III)−nitrilotriacetic acid−silica was applied for phosphoprotein characterization.[44−47] In most work published, trypsin has been used to generate phosphopeptides for IMAC enrichment. To facilitate the assignment of phosphorylation sites, the less specific enzyme elastase or multiple enzyme digestions have been performed on proteins to obtain higher amino acid sequence coverage and cross-identification.[27,48]

For routine practical applications, IMAC experiments are often performed using chromatographic beads containing immobilized metal ions. Iminodiacetic acid and nitrilotriacetic acid (NTA) are the most common functional groups used to chelate metal ions. Commercially available IMAC beads or packed columns are available from several suppliers. The skeletons of beads or supports are commonly gels such as Sepharose,[31,49−53] agarose,[54,55] and agarose gel chelating Sepharose[56] and less commonly polystyrene resin,[57] cellulose,[58] or inorganic silica.[59]

Despite the availability of commercial IMAC products and some reported successes, the use of IMAC/MS for phosphopeptide analysis is far from routine. Specifically, the extent of success in using IMAC to capture phosphopeptides varies considerably, dependent on many factors including the protein properties, sample impurities, skill of the operator, and quality of the IMAC capture media. A recent report of ABRF on the failure of using IMAC to detect an unknown phosphopeptide, in a blind test by a number of sophisticated laboratories highlights the difficulty associated with the routine use of IMAC for phosphopeptide analysis.[60] In a recent review discussing the practical aspects of phosphoprotein characterization, Campbell and Morrice pointed

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out that phosphopeptides in "model proteins" can be enriched by metal chelate chromatography, but this is notoriously unreliable for "real life" proteins.[61] Others have also documented the difficulty in using IMAC for real world sample applications.[62] In our own experience in using Ziptips from Millipore at the Alberta Cancer Board Proteomics Resource Facility, we have also had limited success in analyzing unknown phosphoproteins. One major contributing factor is likely related to the quality and reproducibility of the IMAC capture media or device. For a miniature column packed at the tip of a pipet such as Ziptip, the surface area of the tip and supporting matrixes used to hold together the IMAC beads that are not chemically modified with IMAC functionality can be comparable to or even larger than that of the IMAC materials, thus causing nonspecific binding. The extent of nonspecific binding is varied depending on many factors such as sample and elution conditions. Since commercial products for microscale sample preparation are disposable items for one-time use only, the quality of each product for successful capturing of phosphopeptides cannot be individually checked. However, for a real world sample, the sample amount is often limited and only a few, if any, repeat experiments can be performed.

To improve the reliability of the IMAC method, we have developed an open tubular (OT) IMAC column where the inner surface of a glass tube is uniformly modified by chemically attaching a metal chelate group. Compared with the packed IMAC where the IMAC medium is placed inside a tube that is not chemically modified with IMAC functionality, the functional groups are attached directly to the glass surface through a chemical linker in OT-IMAC. No frits or embedding matrixes or non-IMAC tubes are present; thus, no contamination (e.g., those via nonspecific binding) will result from the supporting matrix such as a gel, polymer, or glue. The surface chemistry can be more readily controlled due to surface homogeneity associated with the glass tube, compared to less uniform surfaces in chromatographic porous beads. With less back pressure, the analyte solution can be freely moved inside the immobilized glass tube and extensive washing can be easily carried out after phosphopeptide binding.

In this work, we describe the construction of OT-IMAC, present a strategy of identifying modification sites of highly phosphorylated peptides based on sequential enzyme digestion, followed by MALDI MS and MS/MS analysis, and demonstrate its application for selective enrichment and identification of phosphopeptides in a Na^+/H^+ exchanger fusion protein, His182, which was phosphorylated in vitro with active kinase Erk2. The Na^+/H^+ exchanger is a pH regulatory protein that removes excess intracellular acid and is involved in cell growth, cell differentiation, and cell migration.[63] Phosphorylation of Na^+/H^+ exchanger cytoplasmic domain leads to a shift in the pH*i* sensitivity of the modifier site, accounting for activation of exchange activity and cytosolic alkalinization.[64,65] The His182 protein used in this study

1	11	21	31
MILRNNLQKT	RQRLRSYNRH	**TLVADPYEEA**	**WNQMLLRRQK**
41	51	61	71
ARQLEQKINN	**YLTVPAHKLD**	**sPTMSRARIG**	**SDPLAYEPKE**
81	91	101	111
DLPVITIDPA	SPQSPESVDL	**VNEELKGKVL**	**GLSRDPAKVA**
121	131	141	151
EEDEDDDGGI	**MMRsKETsSP**	**GTDDVFtPAP**	**SDsPSSQRIQ**
161	171	181	
RCLSDPGPHP	**EPGEGEPFFP**	**KGQHHHHHH**	

Figure 1. Amino acid sequence of His182 and phosphorylation sites determined from this work.

encompassed all of the amino acids known to be involved in regulation of the protein by phosphorylation.[66,67] Deducing the amino acids that are targeted by Erk-mediated phosphorylation is crucial to understanding the physiological mechanism of activation and regulation of the protein.

EXPERIMENTAL SECTION

Chemicals and Reagents. 2,5-Dihydroxybenzoic acid (DHB), (3-glycidoxypropyl)trimethoxysilane (Catalog No. 44,016-7), and iminodiacetic acid (Catalog No. 22,000-0) were from Aldrich Chemicals (Milwaukee, WI). Trypsin, (porcine; modified sequencing grade) was from Promega (Madison, WI). All water used was obtained from a Milli-Q system (Millipore, Bedford, MA). All other chemicals used were ACS or HPLC grade. Coommassie blue stain was obtained from Bio-Rad (Hercules, CA) and used according to the manufacturer's instructions. Endoproteinase Asp-N (from *Pseudomonas fragi* mutant strain sequencing grade), α-casein, EDTA, TFA, DTT, α-cyano-4-hydroxy-*trans*-cinnamic acid (HCCA) and iodoacetamide were obtained from Sigma (St. Louis, MO). All solvents were obtained from Fisher Scientific and were of HPLC grade. Glass tubings used were made of borosilicate glass with o.d. of 1.0 or 1.2 mm, i.d. of 0.58, 0.68, and 0.85 mm, and length of 10 cm (Sutter Instrument Co.). Capillary tubing used has an o.d. 360 μm and i.d. 50 μm. (Polymicro Technologies). The phosphopeptide sample ABRF-PRG03 was a gift from Dr. Kaye D. Speicher, the Wistar Institute Proteomics Facility.

Expression and Purification of Fusion Proteins. The carboxyl-terminal 182 amino acids of the human Na^+/H^+ exchanger (NHE1) was expressed as a fusion protein with a C-terminal hexahistidine tag (His182) using the plasmid pDest 14 and the Gateway Cloning System as described earlier.[68] In this case, the *Escherichia coli* strain BL21-SI strain was induced with 0.3 M NaCl for 3 h. His182 protein was harvested using standard conditions, and the protein was purified from the supernatant of *E. coli* via Ni-NTA affinity chromatography as described by the manufacturer (Qiagen). The sequence of His182 is illustrated in Figure 1. Residues 2−183 correspond precisely to amino acids 634−815 of the NHE1 isoform of the human Na^+/H^+ exchanger.[69] An initiator methionine residue was necessary at the N-terminus,

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and a hexahistidine tag at the C-terminus was necessary for protein purification.

In Vitro Phosphorylation of the Na^+/H^+ Exchanger Fusion Proteins. The standard reaction conditions for phosphorylation of His182 contained 10.0 μg of His182, 0.03 μg of Erk2 (Biomol, Plymouth Meeting, PA), 12.5 mM 3-(4-morpholino)propanesulfonic acid (MOPS) pH 7.2, 0.5 mM EGTA, 2 mM DTT, 8.5 mM magnesium chloride, 6 μM okadaic acid, 0.24 mM sodium fluoride, 500 μM ATP, and 1 μL of 10 μCi/μL of [γ-^{32}P]ATP (3000 Ci/mmol) in a final volume of 24 μL (modified after ref 66). Samples were incubated at 30 °C for 90 min, and the reaction was terminated by the addition of SDS loading buffer. Samples were run on a 12% SDS gel, dried, and exposed for autoradiography.

In-Gel Trypsin Digestion of Phosphoprotein Separated by SDS−PAGE. The protein bands were excised from gels into 0.6-mL siliconized vials and washed three times with distilled water. The gel bands were dehydrated with acetonitrile and dried under vacuum. The dried gels were incubated with 4.5 mM DTT in 0.1 M NH_4HCO_3 for 30 min at 56 °C, cooled to ambient temperature, and resuspended in 0.1 M NH_4HCO_3 containing 9 mM iodoacetamide for 30 min in the dark. The solution was removed, and gel slices were washed twice with water, dehydrated with acetonitrile, and dried under vacuum. Trypsin (10 ng/μL) was prepared in 0.1 M NH_4HCO_3 and 5 mM $CaCl_2$, and kept in an ice bucket. The ice-cold trypsin was added to the vials to cover the gels. The vials were kept in ice for 40 min. Excess liquid around the gel was removed and replaced with 50 mM NH_4HCO_3. The vials were then placed in an incubator at 30 °C overnight. The resulting peptides were extracted sequentially for 20 min with 45% acetonitrile in 20 mM NH_4HCO_3, 45% acetonitrile in 0.5% TFA, and 75% acetonitrile in 0.25% TFA with agitation. The pooled extracts containing the peptides were evaporated to a final volume of 1−2 μL under vacuum. The binding buffer for IMAC (50 mM MES in 10% acetonitrile, pH 5.5) was added to each vial, which was then vortexed.

Sequential Digestion of Phosphopeptides with Asp-N. When phosphorylation sites could not be assigned from the product ion spectra of tryptic phosphopeptides, 10 μL of the 100 mM ammonium phosphate eluate containing OT-IMAC enriched phosphopeptides from the tryptic digest of His182 was adjusted to pH 7.0 with 2% ammonia solution. One microliter of 20 ng/μL endoproteinase Asp-N in 100 mM phosphate buffer (pH 7.0) was then added. Peptides were digested for 48 h at 37 °C; 0.4 μL of the digest was taken out at intervals and checked by MALDI-MS.

Synthesis of OT-IMAC. The metal ion immobilization chemistry used in this work was based on that used in making Sepharose-bead IMAC.[70−72] The surface of the glass or capillary tubing was first cleaned with a solvent mixture of $NH_3 \cdot H_2O/H_2O_2/H_2O$ (1:1:5) overnight and then rinsed with Milli-Q water. The tubing inner surface was reacted with 6 M HCl overnight and rinsed with Milli-Q water until neutral. A 5:1 mole ratio of NaOH to iminodiacetic acid was mixed in 100 mL of ice water with stirring. While keeping the temperature at 273 K, 10 mL of (3-glycidoxypropyl)trimethoxysilane was slowly added to the mixture with stirring. The reaction mixture was allowed to warm to room temperature and kept stirring for 4 h. After this, the temperature was raised to 338 K and the solution was stirred overnight. The above solution was adjusted to pH 3.5 with 6 M HCl and used to immerse glass tubing or flush the capillary tubing at 368 K overnight. The glass tubing or capillary was rinsed with a large quantity of Millli-Q water and then cured overnight at 473 K under nitrogen gas. It was then cooled to room temperature and stored in a dry place until use. We have not examined the exact shelf life of OT-IMAC; but the performance of OT-IMAC does not degrade after at least one year of storage.

Phosphopeptide Enrichment and Determination of Binding Capacity. The inner surface of the OT-IMAC tube was first acidified with 50% ACN containing 0.1% HAc and then charged with 200 mM $FeCl_3$ solution. Excess Fe^{3+} was removed by washing with pure water. After equilibration with binding buffer (50 mM MES in 10% ACN, pH 5.5), samples were loaded into the glass tubing and washed twice with binding buffer and 1% acetic acid containing 10% ACN, before eluting phosphopeptides with 100 mM ammonium phosphate at pH 4.5. The OT-IMAC column was used like a pipet, and the solution was moved inside the glass tube forward and backward for usually 10 times. When a standard phosphopeptide of less than 100 pmol was used, we did not see phosphopeptide left in the residual solution.

Inductively coupled plasma (ICP) MS was used to determine the binding capacity of OT-IMAC to phosphopeptides. Because the determination of Fe^{3+} by ICPMS has serious interference from ArO^+, Cu^{2+} was used as the immobilized metal ion instead of Fe^{3+}. The bound Cu^{2+} was stripped from the OT-IMAC column with 60 μL of 200 mM EDTA and determined with ICPMS. The binding capacity was calculated with assumption of 1 phosphate/Cu atom bound.

Mass Spectrometry. Peptide mass mapping was done on a Bruker Reflex III time-of-flight mass spectrometer (Bremen, Leipzig, Germany) equipped with a 337-nm nitrogen laser in positive ion mode with delayed extraction in the reflectron mode. Spectra were obtained by averaging 100−300 individual laser shots and then processed with the Bruker supporting software and reprocessed using Igor Pro software (WaveMetrics, Lake Oswego, OR). The spectra were internally calibrated with trypsin autolysis peptide peaks and matrix cluster peaks. MALDI MS/MS spectra were collected on an Applied Biosystems MDS-Sciex QSTAR Pulsar QqTOF instrument equipped with an orthogonal MALDI source employing a 337-nm nitrogen laser (Missisauga, ON, Canada). The instrument was operated in positive ion mode, and CID of peptides was achieved with argon as collision gas. Spectra were acquired and processed using Sciex supporting software and reprocessed with Igor Pro software. Both peptide mapping and sequencing were done using the ProteinProspector Tools MS-Digest and MS-Product (http://prospector.ucsf.edu/) for protein identification.

The sequence information obtained for each peptide was used to determine the phosphorylation sites. If the location was ambiguous, further digestion with endoproteinase Asp-N was performed on the ammonium phosphate effluent. MS and MS/MS spectra were then acquired once more from the resulting peptides.

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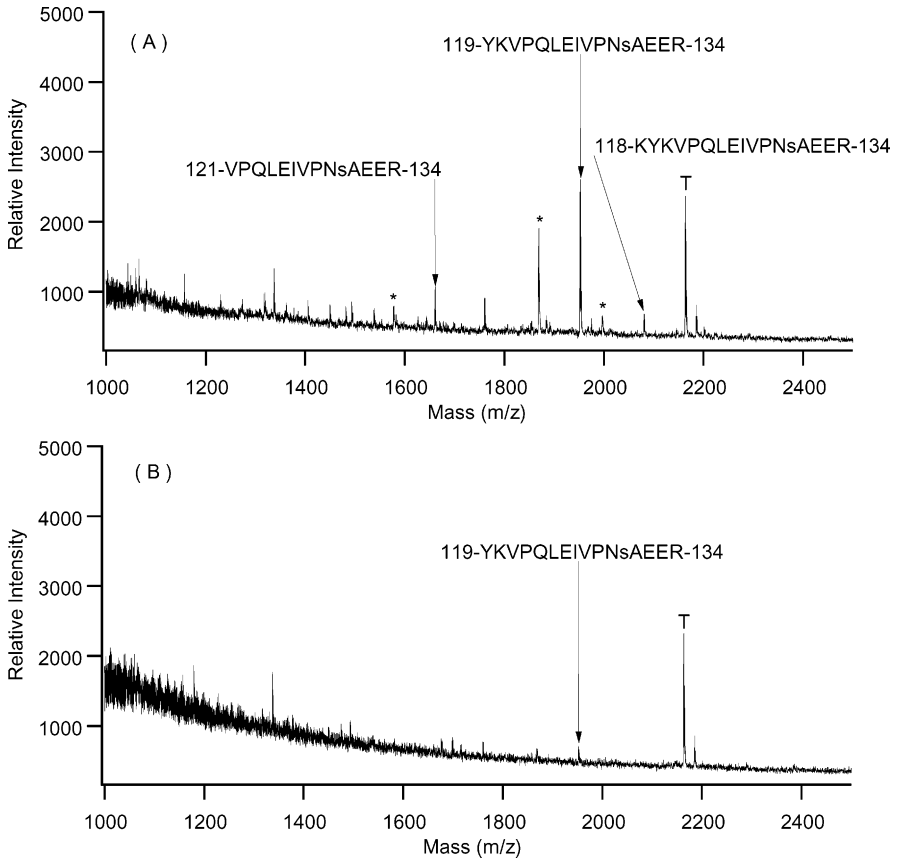

Figure 2. Sensitivity comparison between (A) OT-IMAC and (B) commercial Ziptip IMAC from Millipore on enrichment of 200 ng of α-casein in-gel tryptic digest.

Peptide extracts were deposited on the MALDI target using the two-layered method. The first layer was prepared with 12 mg/mL HCCA matrix in methanol/acetone (1:4 v/v) and the second layer with a saturated solution of matrix in methanol/water (2:3 v/v). The second layer was added to the samples in a 1:1 ratio of matrix to analyte, and the mixture was then vortexed. One microliter of the first-layer matrix solution was deposited on the sample plate to form a thin layer, and 0.5 μL of the sample matrix mixture was then deposited onto this layer and allowed to air-dry, before finally washing twice with distilled water.

For MS/MS experiments, the solution eluted from OT-IMAC with 100 mM ammonium phosphate (pH 4.5) was mixed with 20 mg/mL DHB in water/ethanol (4:1 v/v), and 0.5 μL of the sample matrix mixture was then deposited onto the plate directly.

RESULTS AND DISCUSSION

There are several commercial products of IMAC for phosphopeptide separation. For analyzing small amounts of sample, IMAC media packed at the tip of a pipet tip is commonly used. Such a mini-IMAC column can be prepared in a laboratory or purchased from a commercial source such as Ziptip IMAC from Millipore. α-Casein was used as model phosphoprotein to compare the performance of OT-IMAC with the commercially available Ziptip IMAC. Different masses of α-casein were loaded onto a 15% SDS−PAGE gel, separated, and in-gel digested with trypsin. The phosphopeptides in the digests were enriched by OT-IMAC or Ziptip IMAC. The results are shown in Figure 2. In this figure, the amino acid sequences of phosphopeptides enriched by IMAC are shown and the peaks marked with asterisks are the metastable peaks arising from the phosphopeptides. The peaks marked with "T" are trypsin autolysis peaks. For the in-gel tryptic digest from 200 ng (~8 pmol) *oϕ* α-casein, which was the total amount of protein loaded on the gel, phosphopeptide peaks are intense following enrichment with OT-IMAC (Figure 2A). However, only one weak phosphopeptide peak was observed with the commercial IMAC minicolumn (Figure 2B). At the 100-ng level of sample loading, several phosphopeptides can still be observed with OT-IMAC, but none with Ziptip IMAC (data not shown). The above results are representatives of many trials from different operators using many OT-IMAC and Ziptip IMAC tips of different batches. Thus, OT-IMAC appears to be generally more efficient and, hence, more sensitive than the Ziptip IMAC for enrichment of phosphopeptides. An additional feature of OT-IMAC is that the quality of these tubes is very consistent. Repeat experiments from the disposable OT-IMAC on the same sample give very similar results.

It should also be noted that, by reducing the column dimension and using a microspot sample deposition method (to be submitted for publication), we can increase the sensitivity of OT-IMAC to the level that we can routinely detect phosphopeptides from 10 ng of sample loading (400 fmol), which is approaching the limit of silver stain detection for gel electrophoresis. The problem of handling even a lower amount of sample lies in the digestion—efficient in-gel digestion from a gel spot containing <10 ng of protein is not routine.

In a related experiment in gauging the detection sensitivity and utility of OT-IMAC, we examined the phosphopeptide ABRF

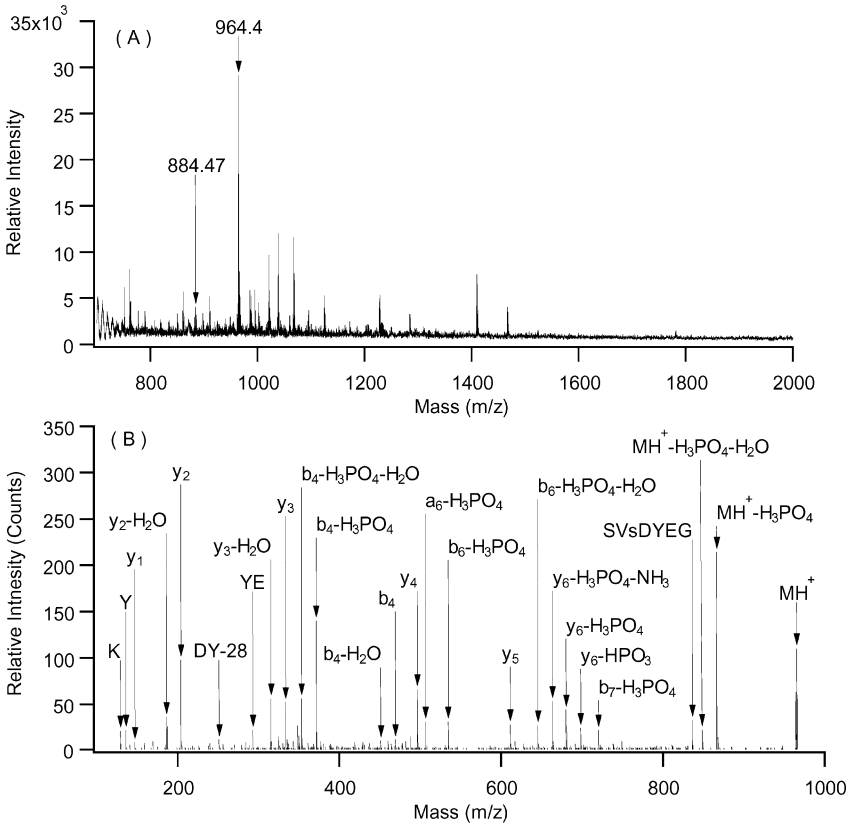

Figure 3. (A) MALDI mass spectrum of the ABRF-PRG03 phosphopeptide sample after enrichment with glass tube OT-IMAC. (B) MALDI QqTOF MS/MS spectrum of the phosphopeptide at *m*/*z* 964.4 (SVsDYEGK) shown in (A).

sample, which was the material previously used for the round-robin exercise.[60] In this case, 10 μL of binding buffer (50 mM MES in 10% ACN, pH 5.5) was used to dissolve the peptides in the vial received and the solution was then divided into two. One microliter of the solution was directly applied to OT-IMAC using 1-cm capillary tubing (o.d. 360 μm and i.d. 50 μm) to enrich the phosphopeptides. For OT-IMAC using 1-cm glass tubing (o.d. 1.0 mm and i.d. 0.58 mm), 5 μL of solution was further diluted to 10 μL with the binding buffer before it was used to enrich the phosphopeptides. One phosphopeptide was always detected in the ammonium phosphate eluents from both capillary and glass OT-IMAC experiments. The results are shown in Figure 3. No phosphopeptides were detected without enrichment by OT-IMAC (data not shown). The peptide at *m*/*z* 964.4 appears as the strongest peak in the spectrum shown in Figure 3A and is regarded as a phosphopeptide candidate. This peak is accompanied by another peak with mass difference of −80 Da, likely corresponding to the unphosporylated peptide at *m*/*z* 884.4. The fragment ion spectrum of the *m*/*z* 964.4 peptide is shown in Figure 3B. Based on this MS/MS spectrum, the phosphorylation site can be assigned to the serine residue at y_6. Although we did not identify the other phosphopeptide in the sample, we have proved that OT-IMAC is effective for direct enrichment of 1 pmol of phosphopeptide present in a 5-pmol mixture of unphosphorylated tryptic peptides.[60] In the study published by Arnott et al., it was stated that only one group using commercial IMAC products was able to achieve this after methyl esterification of acid residues.[60]

OT-IMAC was developed mainly for analyzing phosphopeptides from in-gel digestion of gel-separated proteins where the sample amount used is usually low. To determine the binding capacity of OT-IMAC, we did an experiment where ICPMS was used to analyze the metal ions after it was eluted out of the column (see Experimental Section). The binding capacity of an OT-IMAC column (i.d. 0.85 mm, o.d. 1.2 mm, length 10 cm) was found to be ∼300 ± 27 pmol (triple experiments) of phosphopeptide with assumption of 1 phosphate/metal ion bound.

Prior to the development of OT-IMAC, several real world samples including His182 had been subjected to many attempted analyses by the Alberta Cancer Board proteomics resource laboratory; but very few phosphopeptides were consistently detected using the standard commercial IMAC protocol. A new protocol has been developed, based on the use of OT-IMAC, MALDI MS, and MS/MS. In the following, we use the example of characterizing His182 to illustrate the experimental strategy and data interpretation involved in mapping phosphorylation sites by OT-IMAC MALDI. This strategy was developed with a goal of mapping out as many phosphorylation sites as possible. As shown below, multiple experiments had to be performed, which required a much larger amount of starting protein material than that used to merely determine whether a protein was phosphorylated. The starting material used was ∼10 μg. However, the stoichiometries of phosphorylation for all sites were unknown. It would not be surprising that some of the sites had low ratios of phosphorylation. Thus, the actual amount of each form of phosphorylated proteins loaded onto the gel was most likely less than 10 μg.

The gel-separated His182 was digested with trypsin, and the phosphopeptides in the digest were enriched by OT-IMAC. Considering that desalting, such as treatment by C_{18}, may result in the loss of some high phosphorylated peptides, the ammonium phosphate eluate from OT-IMAC was directly deposited on the

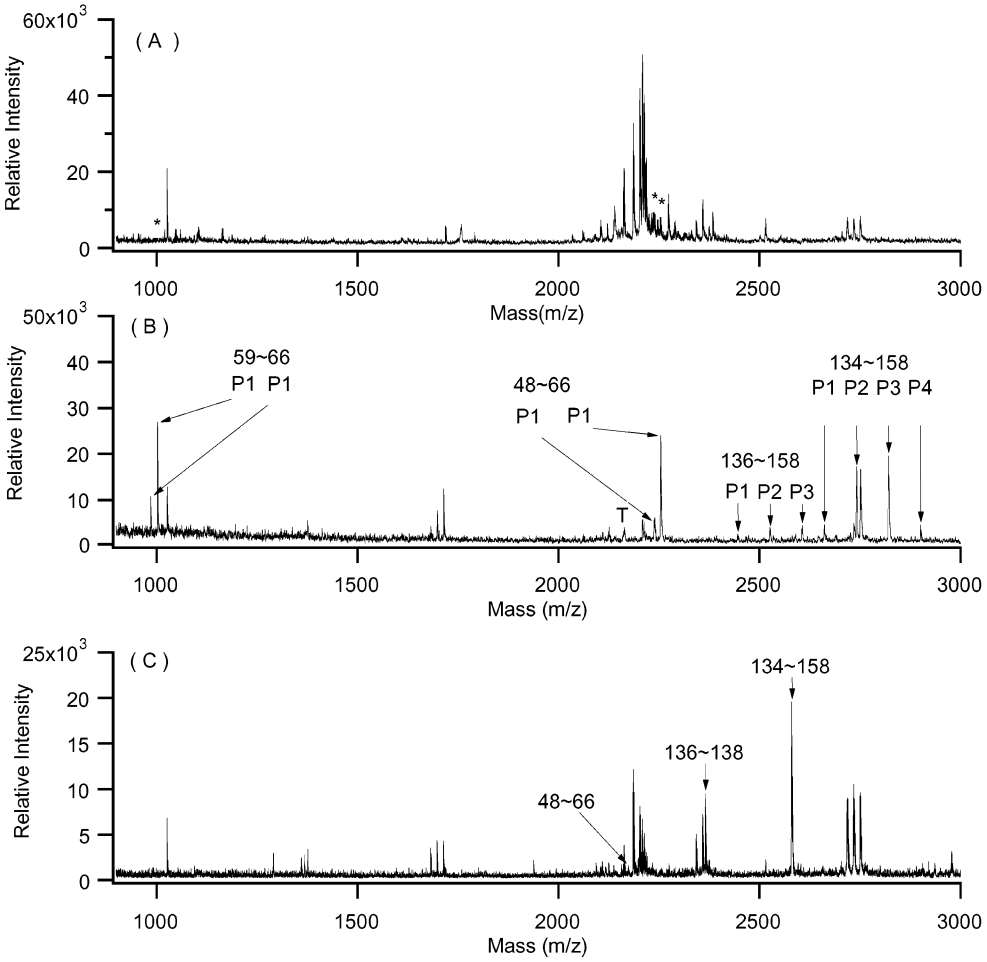

Figure 4. MALDI-TOF MS analysis of His182. The spectra were obtained by directly depositing the sample solution on the plate after mixing with DHB matrix. (A) MALDI mass spectrum of Erk2-treated His182 in-gel tryptic digest before OT-IMAC enrichment. (B) MALDI mass spectrum of Erk2-treated His182 after OT-IMAC enrichment. (C) MALDI mass spectrum of Erk2 untreated His182 after OT-IMAC enrichment.

target after mixing with matrix and analyzed with MALDI-TOF MS. After comparing the peptide masses with the theoretical trypsin digest peak list in which the phosphorylation of threonine, serine, and tyrosine was selected, possible phosphopeptide peaks were tentatively assigned. The phosphopeptide assignment was verified with the sequence information obtained from MALDI-QqTOF-MS/MS.

Figure 4 shows the MALDI spectra of His182 digest with and without OT-IMAC enrichment. As Figure 4A shows, without OT-IMAC enrichment, phosphopeptide peaks marked with an asterisk are difficult to distinguish. After OT-IMAC enrichment, the intensity of these peaks increases significantly, and other phosphopeptide peaks become detectable (see Figure 4B). None of the phosphopeptide candidate peaks exist in the control of nonphosphorylated His182 digest (Figure 4C), and the peak intensities of the corresponding nonphosphorylated peptides are very strong. It is well known that IMAC can selectively enrich phosphopeptides, but at the same time, it also concentrates other peptides, mostly those containing many carboxylic acid residues (such as aspartic acid and glutamic acid).

In Figure 4B, besides the phosphopeptide peaks at m/z 2253.07 and 2237.07, two other phosphopeptide peaks, m/z 1002.39 and 986.40, were observed. These two new peaks belong to the peptide from residue 59−66 (Figure 1), with one oxidized methionine. For the peptide whose sequence is from residue 134 to 158, there are four phosphopeptide peaks observed in the spectrum. Among them, four adjacent peaks were found 80 mass units apart, which is the characteristic loss of phosphate ($-HPO_3$) observed in reflectron mode MS. This result indicates that these four peaks are likely from a quadruply phosphorylated peptide.

From our observations, DHB is better than HCCA as a matrix for phosphopeptide analysis for both MALDI TOF and MALDI QqTOF MS, as the intensities of phosphopeptide peaks were observed to be significantly higher. The reason for this lies in the fact that the DHB is a "cool" matrix that imparts less energy to analyte molecules.[73]

The fragment ion spectra of the phosphopeptides were obtained by MALDI QqTOF-MS/MS. For the singly phosphorylated peptide m/z 2253.07, the sequence is 48-INNYLTV-PAKLDSPTMSR-66. There are five possible phosphorylated residues within this peptide, but the phosphorylation site was unambiguously assigned to be at Ser61, from the y_6 ion. This is shown in Figure 5. Peaks that resulted from loss of H_3PO_4 (m/z 98), HPO_3 (m/z 80) and loss of $SOCH_4$ from the parent ion were observed. No characteristic loss of phosphate [H_3PO_4 (m/z 98) or HPO_3 (m/z 80) or HPO_2 (m/z 64)] was observed for either

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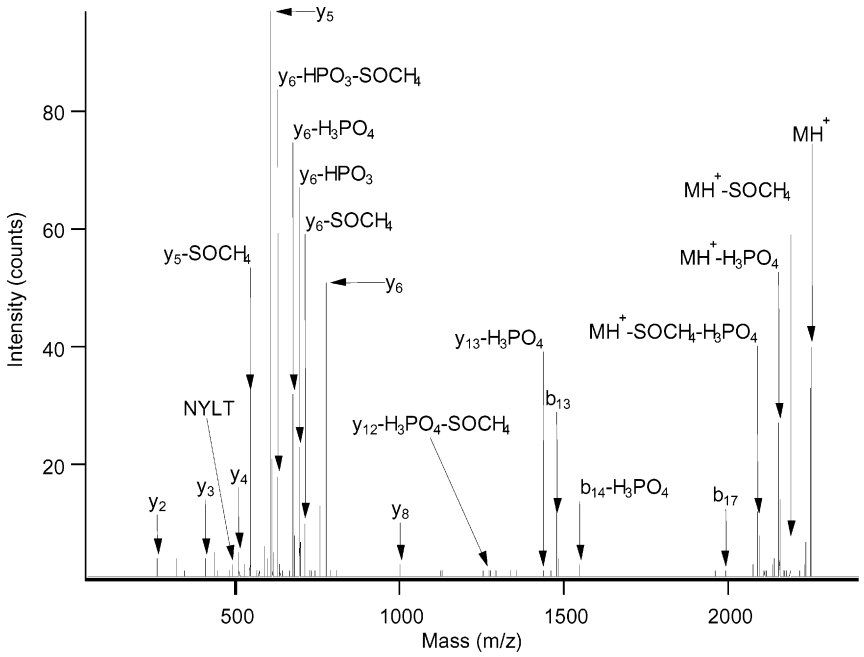

Figure 5. MALDI QqTOF MS/MS spectrum of a phosphopeptide at *m*/*z* 2253.07 (48-INNYLTVPAHKLDsPTmSR-66).

the y_4 or y_5 ions; therefore, Thr62 is not phosphorylated. For the y_5 ion, a loss of $SOCH_4$ was observed, which is from the oxidized methionine. For y_6, losses of both H_3PO_4 (*m*/*z* 98) and HPO_3 (*m*/*z* 80) were observed. Therefore, we conclude that Ser61 is phosphorylated. The very intense peak corresponding to the b_{13} ion also confirms that there is no phosphorylation from residues 48−60 (INNYLTVPAHKLD). Fragmentation of the peak at *m*/*z* 2237.08 (spectrum not shown), which corresponds to the same sequence without the methione oxidation, further confirmed our assignment.

Figure 6A shows the MS/MS spectrum of the phosphopeptide at mass 2820.06 (134-SKETsSPGTDDVFtPAPSDsPSSQR-158). Although the intensity of the peak resulted from triple loss of H_3PO_4 from the parent ion is low, its presence indicates that the peptide is triply phosphorylated. From y_1 to y_5, no −80(− HPO_3) or −98 (− H_3PO_4) peak was observed; hence, there is no phosphorylation in the sequence PSSQR. From the y_6 ion, peaks with the characteristic loss of phosphate [H_3PO_4 (*m*/*z* 98) or HPO_3(*m*/*z* 80)] are observed; therefore we conclude that Ser153 is phosphorylated. Observation of the y_{11} ion (*m*/*z* 1208.49) excludes the possibility of phosphorylation of Ser151. The assignment of the y_{15} ion (*m*/*z* 1750.67) indicates that Thr147 is phosphorylated. From the y_{19} ion (*m*/*z* 2120.82), it was inferred that there are only two phosphorylated sites in its sequence PGTDDVFtPAPSDsPSSQR and that residue 142 does not contain a phosphate. Because the spectrum is dominated by y-series ion peaks, and since no fragments for b_1 to b_7 and y_{20} to y_{25} were observed, the third phosphorylation site can only be narrowed down to three Ser and one Thr in the sequence, SKETSS.

To assign the third phosphorylation site, endoproteinase Asp-N was added to the solution containing OT-IMAC enriched phosphopeptides from the tryptic digest of His182. After digestion at 37 °C for 15 h, the peak at *m*/*z* 1088.41 was observed, which corresponds to the singly phosphorylated peptide, SKETSSPGTD. The peptide was fragmented with the QqTOF instrument. The spectrum obtained is shown in Figure 6B. For clearance and simplicity, peaks corresponding to the loss of water (such as b_3, y_3, y_4, y_5, b_5 − H_3PO4) were not labeled in the figure. The loss of *m*/*z* 98 from the parent ion confirms that the peptide is singly phosphorylated. From the appearance of the b_2 and b_3 ions, Ser134 is excluded as a possible phosphorylation site; y_4 excludes the possibility of phosphorylation Thr142. If the phosphorylation assignment of threonine 137 had been made, the peak labeled KET could not be assigned. The y_5 − H_2O ion excludes the serine 139 as a phosphorylation site. The appearance of the b_5 − H_3PO_4 ion confirms that the serine 138 is phosphorylated, which excludes the possibility of phosphorylation of Ser139. From these analyses, we conclude that the third phosphorylation site is at serine 138.

The MS/MS spectrum of peptide at mass 2900.03 (134-sKETsSPGTDDVFtPAPSDsPSSQR-158) was also obtained (data not shown). In this case, there are four sequential losses of phosphate (− H_3PO_4), confirming that the sequence from residues 134 to 158 contains four phosphate groups. Most of peaks in the spectrum were assigned. Because the intensity of peak *m*/*z* 2900.03 in the MS spectrum shown in Figure 4B is low, and since the Qq-TOF MS/MS sensitivity is poor in the high-mass range, fragment ion information obtained from the MS/MS spectrum of the peptide ion at mass 2900.03 is not sufficient to successfully assign all the phosphorylated sites. However, after Asp-N digestion, the peak at *m*/*z* 1221.46 (126-DDGGImmRsK-135) was observed and this peptide ion was selected for fragmentation. The MS/MS spectrum of this ion (data not shown) indicated that the serine at residue 134 was phosphorylated. From the combination of examining the tryptic peptides and the subsequent peptide fragments generated by Asp-N digestion, the four phosphorylation sites in the sequence 134−158, corresponding to the sKETsSPGTDDVFtPAPSDsPSSQR peptide, were assigned as serines at residues 134, 138, and 153, and threonine at residue 147.

Table 1 summarizes the mapping results obtained for His182. As Table 1 illustrates, the phosphorylation sites can be assigned by using OT-IMAC MALDI MS and MS/MS. With dual-enzyme trypsin and Asp-N digestion, every possible phosphorylated

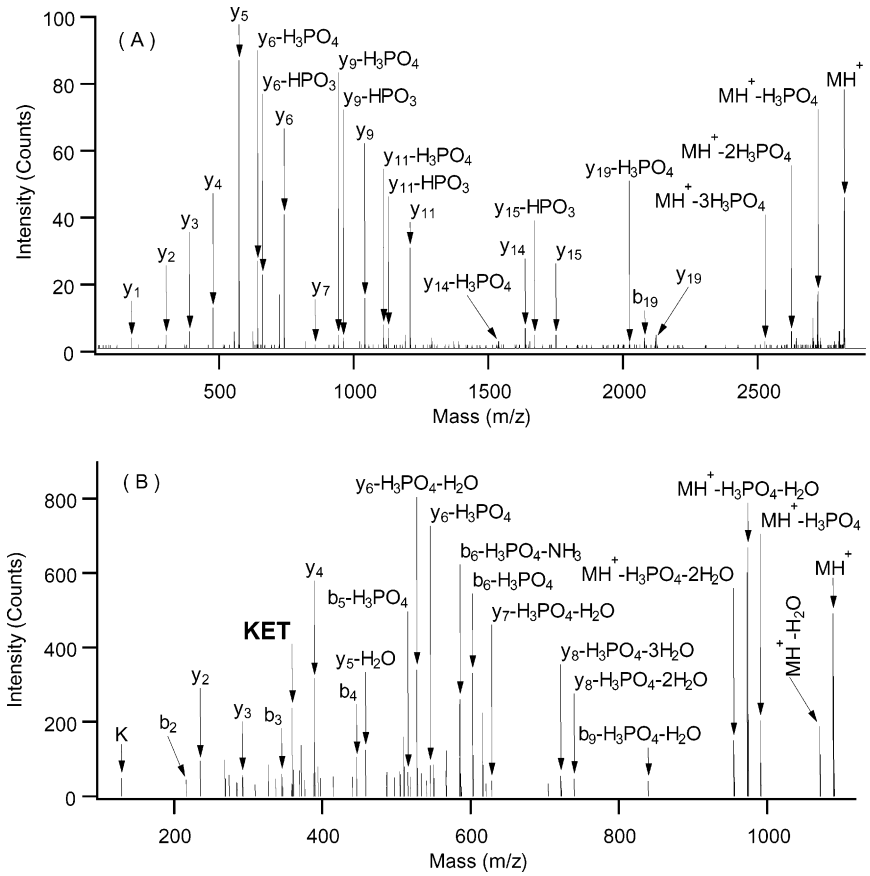

Figure 6. (A) MALDI QqTOF MS/MS spectrum of a phosphopeptide at *m*/*z* 2820.06 (134-SKETsSPGTDDVFtPAPSDsPSSQR-158) generated from the in-gel tryptic digestion of His182. (B) MS/MS spectrum of peak 1088.41 (134-SKETsSPGT-142). This peptide was generated from the endoproteinase Asp-N digestion of the tryptic digest.

Table 1. List of Phosphopeptides Identified from the In-Gel Digestion of His182

peptide peaks (m/z)	residue start	residue end	no. of phosphorylation sites	identified phosphopeptides[a]
986.40	59	66	1	LDsPTMSR
1002.40	59	66	1	LDsPTmSR
2237.08	48	66	1	INNYLTVPAHKLDsPTMSR
2253.07	48	66	1	INNYLTVPAHKLDsPTmSR
2660.14	134	158	1	SKETSSPGTDDVFTPAPSDsPSSQR
2740.10	134	158	2	SKETSSPGTDDVFtPAPSDsPSSQR
2820.07	134	158	3	SKETsSPGTDDVFtPAPSDsPSSQR
2900.04	134	158	4	sKETsSPGTDDVFtPAPSDsPSSQR
741.29	153	158	1	sPSSQR
1635.64	145	158	2	VFtPAPSDsPSSQR
1750.67	143	158	2	DVFtPAPSDsPSSQR
1088.41	134	143	1	SKETsSPGTD
1203.44	134	144	1	SKETsSPGTDD
1221.46	126	135	1	DDGGImmRsK

[a] The lowercase letters “s” and “t” stand for phosphorylated serine and threonine, respectively. Lowercase m stands for oxidized methionine.

peptide observed in the mass spectra had been fragmented and analyzed by MS/MS. In Figure 1, detected sequences are highlighted in bold letters. There is still ~20% of the sequence not detected. In these regions, there are potentially additional phosphorylation sites present. The use of other enzymes for digestion may improve the sequence coverage as well as enhance positive identification of phosphorylation sites. However, achieving 100% site assignment still remains a major challenge in using the bottom-up MS/MS approach for mapping phosphorylation sites. Nevertheless, the ability of the method to map out a large proportion of phosphorylation sites in a protein can greatly aid in subsequent investigation of the biological functions associated with phosphorylation and dephosphorylation. Future experiments will involve mutation of the identified phosphorylation sites in the full-length Na^+/H^+ exchanger protein. The residues identified as phosphorylated by Erk in this study Ser 61, Ser 134, Ser 138, Thr 147, and Ser 153, correspond to amino acids Ser 693, Ser 766, Ser 770, Thr 779, and Ser 785 in the intact full-length Na^+/H^+ exchanger protein. These residues can be mutated to nonphosphorylatable amino acids of equivalent size (Ser to Ala, and Thr

to Val). The intact mutated protein can then be expressed in Na^+/H^+ exchanger-deficient cells, and the effects on regulation of activity of the protein will be examined as described earlier.[74] These experiments will lead to a greatly improved understanding of the regulation of this clinically important protein.

CONCLUSIONS

OT-IMAC has been developed and successfully applied to enrich phosphopeptides produced by in-gel trypsin digestion of phosphoproteins. A protocol based on the use of OT-IMAC combined with MALDI MS and MS/MS as well as sequential dual-enzyme digestion is reported. In the case of His182, for the singly phosphorylated peptides, phosphorylation sites were unambiguously assigned from the MS/MS data. For the quadruply phosphorylated peptide with sequence SKETSSPGTDDVFTPAPSDSPSSQR, detailed structural information was obtained by MALDI MS/MS. However, only two out of four possible phosphorylation sites could be assigned based on the MS/MS data of this tryptic peptide. By using sequential dual-enzyme digestion, this phosphopeptide was cleaved into shorter sequences, allowing all four sites (134 y_{26}, 138 y_{21}, 147 y_{12}, 153 y_6) to be successfully assigned.

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