Anal. Chem. **2003,** *75,* 6826−6836

Improved β-Elimination-Based Affinity Purification Strategy for Enrichment of Phosphopeptides

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Alkaline-induced β-elimination of phosphate from phosphoserine and phosphothreonine residues followed by addition of an affinity tag has recently been pursued as a strategy for enriching phosphorylated species from complex mixtures. Here we report the use of an introduced thiol tag as the ligand for affinity purification via disulfide exchange with an activated thiol resin and the development of a protocol to improve the sensitivity considerably over previous reports (i.e., to subpicomole levels.) During our experiments, we observed a side reaction in which water was eliminated from unmodified serine residues. This side reaction resulted in the introduction of the affinity tag into unphosphorylated proteins, confounding attempts to specifically purify phosphoproteins from mixtures. Unchecked, this side reaction will also prevent application of the β-elimination strategy to phosphopeptide samples where the phosphorylated species are minor components (i.e., most current phosphoproteomics applications). Quantitation of the side reaction products using three synthetic unphosphorylated peptides showed varying conversion efficiencies; at maximum, 1.7% of unphosphorylated peptide was converted to the affinity-tagged form. Inclusion of EDTA into the reaction reduced the side reaction but also greatly reduced the conversion efficiency of one of the phosphoserine residues of ovalbumin, suggesting a role for trace metal ions in the β-elimination chemistry. Despite the presence of the side reaction, the affinity strategy was shown to be effective at enriching phosphopeptides from fairly complex peptide mixtures. The strategy was applied to the analysis of in vitro phosphorylation of bovine synapsin I by Ca^{2+}/calmodulin-dependent kinase II, resulting in the identification of four phosphorylation sites, two of which have not been previously reported.

Phosphorylation of serine, threonine, and tyrosine residues is a key mechanism by which organisms regulate protein function.[1,2] Widespread interest in protein phosphorylation has led to the development of methods to detect and analyze phosphorylation sites. Traditional methods use radiolabeling to detect and track phosphorylation and Edman degradation of phosphopeptides to localize phosphorylation sites.[3,4] More recently, strategies involving mass spectrometry (MS) have emerged as a preferred method to analyze protein phosphorylation.[5,6]

Despite the variety of methods available, analysis of protein phosphorylation remains far from routine. Low phosphorylation stoichiometry, incomplete peptide coverage of an enzymatically digested protein, reduced mass spectrometric signal of phosphopeptides, and poor mass spectrometric fragmentation behavior contribute to the difficulty in analysis of phosphorylation sites. Usually it is difficult to be certain that comprehensive detection of all phosphorylation sites within a given protein sample has been achieved.

To improve the detection of phosphorylated proteins and peptides in mixtures, separation of the species of interest from unphosphorylated species is desirable. One method that has been used to enrich phosphopeptides from a mixture is immobilized metal affinity chromatography (IMAC).[7−10] In this method. the affinity of the phosphate group for metal ions (usually Fe^{3+} or Ga^{3+}) immobilized on a solid support is exploited. Acidic peptides are also retained by IMAC columns, frequently resulting in inefficient enrichment of phosphopeptides. To improve the specificity of IMAC, methyl esterification of acidic residues has been employed.[11] However, specific and complete esterification of acidic residues is not straightforward. IMAC is further complicated by preferential binding of multiply phosphorylated peptides and by variability in phosphopeptide recovery based on binding and elution conditions. Alternatively, phosphospecific antibodies may be used to enrich phosphorylated species.[12−14] Although general antibodies directed against phosphotyrosine have been used with

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10.1021/ac034989u CCC: $25.00
Published on Web 11/18/2003

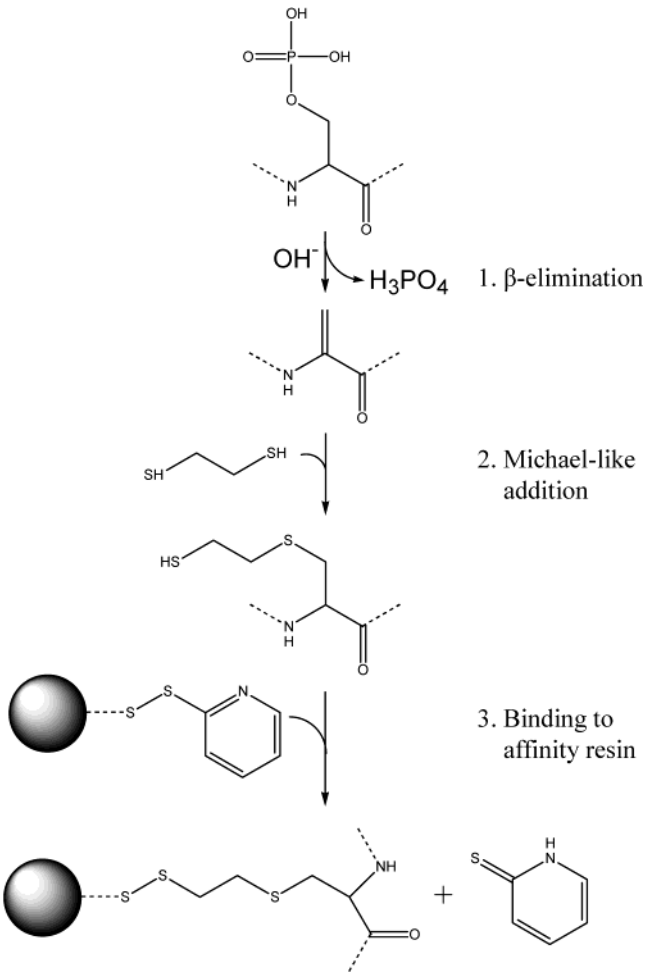

Figure 1. Chemical modification and affinity purification of phosphoserine-containing species. Phosphothreonine residues undergo an analogous reaction at a lower rate. Addition of EDT to the double bond is shown; DTT was also used.

some success,[13,14] antibodies suitable for immunoprecipitation that recognize phosphoserine or phosphothreonine residues independent of sequence context are only beginning to be characterized[15] and have not seen widespread use.

Two methods of chemically introducing affinity tags at sites of phosphorylation have also been demonstrated.[16−18] In the simpler of these methods,[16,17] H_3PO_4 was removed from phosphoserine or phosphothreonine residues at high pH via a β-elimination reaction (Figure 1, step 1). The resulting double bond was modified by a reagent containing two thiol groups to introduce a thiol tag at the site that was formerly phosphorylated (Figure 1, step 2). This thiol was alkylated by either a maleimido[16] or an iodoalkyl[17] group linked to a biotin moiety. The biotinylated peptides were enriched using avidin column chromatography. This enrichment strategy can provide relative quantitation of two samples through the use of an isotopically tagged dithiol reagent.[17,19]

When this strategy was applied, two difficulties were noted. First, the biotin−avidin interaction is very strong, resulting in inefficient recovery of the tagged species from the affinity column. Second, the maleimide moiety is unstable to fragmentation in the mass spectrometer, resulting in fragmentation patterns that are difficult to interpret. To overcome these difficulties, we describe here the use of the incorporated thiol group itself as the affinity reagent (Figure 1, step 3). We also describe a side reaction of the β-elimination chemistry in which unphosphorylated serine residues were modified by the affinity tag at levels up to 1−2%. Although this side reaction was problematic for the enrichment of phosphoproteins, we demonstrate that enrichment of phosphopeptides is still feasible. Finally, we have increased the sensitivity of the method such that phosphopeptides can be analyzed from subpicomole amounts of protein.

EXPERIMENTAL SECTION

General Information. Ovalbumin, bovine apotransferrin, bovine serum albumin, and yeast alcohol dehydrogenase were purchased from Sigma (St. Louis, MO). Dithiothreitol (DTT), ethanedithiol (EDT), NaOH, and $Ba(NO_3)_2$ used in β-elimination reactions were purchased from Sigma-Aldrich and were the highest grade available. Acetonitrile was HPLC grade from Pierce (Rockford, IL). Water was deionized with a Milli-Q water purification system (Millipore, Bedford, MA). Protein concentrations were assayed using the bicinchoninic acid absorbance assay[20] with reagents purchased from Pierce.

Mass Spectrometry. Matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) analyses were conducted on a Voyager DE-STR system (Applied Biosystems, Foster City, CA) using α-cyano-4-hydroxycinnamic acid as the matrix. MALDI-ion trap (IT) analyses were performed on a modified[21] LCQ ion trap mass spectrometer (Thermo Finnigan, San Jose, CA) using 2,5-dihydroxybenzoic acid as the matrix.

Affinity Purification of Phosphorylated Species. Three different protocols were used. In protocol I, the oxidation and β-elimination reactions were performed essentially as described previously,[16] but the affinity purification was accomplished using an activated thiol resin instead of the biotin/avidin system (Figure 1, step 3). Protocol II was similar to protocol I except that the β-elimination reaction conditions were altered to include Ba^{2+}, a lower concentration of NaOH, and DTT in place of EDT as the dithiol reagent. In protocol III, we adapted protocol II for use with smaller amounts of protein.

*Protocol I: β-Elimination Using Previously Published Reaction Conditions[16] Followed by Thiol Modification and Activated Thiol Affinity Chromatography***.** This protocol was used only on amounts of protein greater than 50 μg; typically 100 μg was used. Protein oxidation was accomplished essentially as described,[16] except that the performic acid solution was allowed to stand at room temperature for 2 h instead of 30 min prior to cooling on ice and subsequent overnight incubation. The oxidized protein was dialyzed as described[16] to remove performic acid and dried in a

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Speed-Vac. The β-elimination reaction mixture was added to the protein as described except that NaOH was used instead of LiOH. The final protein concentration during the β-elimination/alkylation reaction was typically 1 μg/μL.

Rather than continue with protein precipitation, biotinylation, and avidin affinity chromatography as described,[16] we removed the β-elimination reagents by size-exclusion chromatography and proceeded immediately to activated thiol affinity chromatography as follows. The β-elimination reaction was quenched by passing the protein through two consecutive Sephadex G-25 (Amersham, Piscataway, NJ) spin columns[22] equilibrated with 2.4% CHAPS, 50 mM Tris-HCl, 1 mM EDTA, pH 8.0 (CTE buffer). For a 100-μL reaction, a wet bed volume of 1.2 mL was used for each column. Then the sample was either applied to the activated thiol affinity resin as described in the next section or was alkylated to allow examination of the efficiency of the reaction. To alkylate the sample, acrylamide was added to a final concentration of 100 mM and the sample was incubated at room temperature in the dark for 1 h. The proteins were dialyzed against 1 L of 50 mM NH_4CO_3 at 4 °C for at least 16 h with several buffer changes. One-tenth of the sample was digested with sequencing grade modified bovine trypsin (Roche, Indianapolis, IN). using an enzyme/substrate ratio of 1:200 by weight based on the amount of protein initially oxidized. The digest was acidified with 0.1 volume of 5% trifluoroacetic acid (TFA; all concentrations of TFA are v/v), and the peptides were bound to Poros R2 beads (Applied Biosystems, Foster City, CA) in a pulled gel-loading tip for desalting. After washing with 20 μL of 0.1% TFA and elution with 5 μL of a 2:1 mixture of acetonitrile/water containing 0.1% TFA (TWA solution), the peptides were analyzed by MS.

Affinity Purification Using Activated Thiol Affinity Resin. The EDT-modified protein was added to activated thiol-Sepharose 4B affinity resin (Sigma, Catalog No. T8512) that had been washed with CTE buffer. The amount of resin used was ~1 μL of bed volume for every microgram of protein initially oxidized. The protein was incubated at room temperature with the resin for 1 h with gentle mixing. Unbound proteins were removed by successive washes of 20 bed volumes of CTE buffer, 20 bed volumes of 8 M urea, 50 mM Tris-HCl, 1 mM EDTA, pH 8.0 (UTE buffer), and 20 bed volumes of 50 mM NH_4HCO_3. To digest the bound proteins, trypsin was added to the affinity resin in a volume of 50 mM NH_4HCO_3 equal to the bed volume and at a 1:100 ratio by weight to the amount of protein initially oxidized. The column was sealed, and the digestion was allowed to proceed at 37 °C for at least 4 h, or overnight. The unbound peptides were removed with a few column volumes of 50 mM NH_4HCO_3 and collected, and then the resin was again washed with 20 bed volumes of CTE buffer, 20 bed volumes of UTE buffer, and 20 bed volumes of 50 mM NH_4HCO_3. To release the bound peptides, one bed volume of 10 mM DTT in 50 mM NH_4HCO_3 (ABC reducing buffer) was added and the resin was mixed at room temperature for 30 min. The resin was washed with a few column volumes of ABC reducing buffer, and the eluate was pooled. The unbound and DTT-eluted solutions were separately acidified with 0.1 volume of 5% TFA, and a portion of each (no more than 1/10) was applied separately to several hundred nanoliters of Poros R2 beads. After washing with 20 μL of 0.1% TFA, the peptides were eluted with 5 μL of TWA solution. The peptides were mixed with MALDI matrix solution and spotted by the dried droplet method. Spots were analyzed by MALDI-TOF or MALDI-IT MS.

To examine binding and release of proteins from the affinity resin, the tryptic digestion and subsequent removal of unbound peptides were omitted from the above procedure. Protein fractions were not acidified but were analyzed by SDS−PAGE.

Protocol II: β-Elimination Using New Reaction Conditions Followed by Activated Thiol Affinity Chromatography. This protocol was used only on amounts of protein greater than 10 μg; typically 100 μg was used. Protein oxidation was accomplished as described in protocol I. The β-elimination reaction mixture was prepared by mixing 20 volumes of CH_3CN, 2 volumes of 1 M DTT, 3 volumes of 2.17 M NaOH, and 75 volumes of 133 mM $Ba(NO_3)_2$. Assuming no change in volume upon mixing, the final concentrations of DTT, NaOH, and $Ba(NO_3)_2$ were 20, 65, and 100 mM, respectively. The stock solutions were prepared fresh daily. The mixture was added to the oxidized protein to give a final protein concentration of no more than 1 μg/μL. The reaction was allowed to proceed at 37 °C for 1 h. The reaction was quenched, and the proteins were bound to the affinity resin, digested, and eluted as described in protocol I.

Protocol III: More Sensitive β-Elimination Using New Reaction Conditions Followed by Activated Thiol Affinity Chromatography. This protocol was used on amounts of protein less than 10 μg. Performic acid solution was made by mixing 100 μL of 30% H_2O_2 with 900 μL of 95% formic acid and allowing the solution to stand at room temperature for 2 h. Dried protein was placed in an open tube in a vacuum desiccator along with a separate open tube containing the performic acid solution. After evacuation of the desiccator, it was sealed and allowed to stand at room temperature for 2 h. The tube containing oxidized protein was removed from the chamber and spun at low pressure in a Speed-Vac for 30 min to remove residual performic acid vapor. Trypsin (25−100 ng) was added to the protein in 5−20 μL of 50 mM NH_4HCO_3 buffer, and the digestion was allowed to proceed at 37 °C for 4 h. The sample was dried in a Speed-Vac overnight to remove solvent and buffer since failure to completely remove residual buffer impeded subsequent steps. The Ba^{2+}/NaOH/DTT reaction mixture was prepared as described in protocol II and added to the protein. A minimum volume of 10 μL was used. To include EDTA into the reaction mixture, the desired amount of a 250 mM EDTA solution at pH 12 was added to the 133 mM $Ba(NO_3)_2$ solution. After incubation at 37 °C for 1 h, the reaction was quenched with 1 μL of 5% TFA and diluted with 40 μL of 0.1% TFA. The peptides were applied to several hundred nanoliters of Poros R2 resin and were washed with three 20-μL washes of 0.1% TFA. The peptides were eluted from the R2 resin with 5 μL of TWA solution directly into 35 μL of 50 mM Tris-HCl, 1 mM EDTA, pH 8.0 buffer (TE buffer) containing 2−5-μL bed volumes of activated thiol affinity resin. The peptides were incubated with the resin at room temperature for 1 h with gentle mixing. The resin was placed in a pulled gel-loading tip for subsequent washing. After elution of the unbound peptides, the resin was washed with 100 μL of TE buffer, 100 μL of TWA solution, and 100 μL of TE buffer. Ten microliters of 10 mM DTT in TE buffer (TE reducing buffer) was added to the resin, and the slurry was mixed at room temperature for 30 min. The DTT-released peptides were collected, and the resin was

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washed with another 10 μL of TE reducing buffer. Finally, the resin was washed with 10 μL of TWA to ensure removal of peptides having a tendency to stick noncovalently to the thiol resin even after release with DTT. The eluted fractions were pooled, acidified with 1 μL of 5% TFA, and diluted with 20 μL of 0.1% TFA. The peptides were passed over ~100 nL of Poros-R2 resin, which was washed with 0.1% TFA. The peptides were eluted with 1 μL of half-saturated 2,5-dihydroxybenzoic acid in 50% methanol, 20% acetonitrile, 2% acetic acid, directly onto the MALDI−IT target.

Analysis of Side Reaction Using HPLC. Three synthetic peptides were used: DAEFRHDSGYE, EQKLISEEDL, and YEQLRNSRA (Anaspec, San Jose, CA). In a typical reaction, 100 pmol of one of the peptides was incubated in the presence of 10 μL of the Ba^{2+}/NaOH/DTT reaction mixture at 37 °C for 1 h. The reaction was quenched with 1 μL of 5% TFA and diluted 2-fold with 0.1% TFA. One-third of the resulting solution was injected onto a C_{18} PepMap column (15-cm length, 300-μm inner diameter, 3-μm particle size, 100-Å pore size, LC Packings, San Francisco, CA) connected to an UltiMate HPLC system (LC Packings). A linear gradient from 5 to 35% B at 1% B/min was used at a flow rate of 5 μL/min. Solvent A was 5% CH_3CN, 0.05% TFA in water whereas solvent B was 80% CH_3CN, 0.045% TFA in water. Absorbance was followed at 214 and 280 nm, and fractions were collected manually based on the absorbance readings and analyzed by MS.

Analysis of Synapsin Phosphorylation. Bovine synapsin I, rat Ca^{2+}/calmodulin-dependent kinase II (CaM kinase II) and rabbit calmodulin were kind gifts of Dr. Angus Nairn. About 11 μg of synapsin I was phosphorylated in vitro by CaM kinase II under conditions similar to those reported previously.[23] As an unphosphorylated control, the incubation was carried out in the absence of CaM kinase II. The final reaction volume was 15 μL. The reaction was quenched by addition of EDTA to a concentration of 20 mM and was stored at −20 °C until needed. For analysis, 1.5-μL aliquots were dried in a Speed-Vac, oxidized in vacuo, digested with trypsin as described above, and analyzed by MS. Alternatively 1.5-μL aliquots were dried and analyzed by β-elimination and affinity purification according to protocol III described above.

RESULTS AND DISCUSSION

Use of Disulfide Exchange for Affinity Purification. To avoid problems associated with tagging the phosphorylation sites with biotin, we decided to use the added thiol group itself as the affinity tag (Figure 1). The affinity resin used to capture thiol-containing polypeptides is available commercially and consists of a Sepharose matrix to which glutathione has been attached via its N-terminal amino group. The thiol of glutathione is activated through disulfide bond formation with 2-thiopyridine. Thiol-containing species added to the resin displace the thiopyridyl group by disulfide exchange (Figure 1, step 3); the leaving group then rearranges to form a thione that is unreactive to disulfide bonds. Upon binding of the thiol-containing species via the covalent disulfide bond, the resin may be washed extensively and under stringent conditions to remove all species that are not covalently bound. The disulfide linkage between the polypeptide and the affinity resin can be efficiently disrupted with a reducing agent such as DTT. The simple thioether tag that results is generally stable to tandem MS,[24,25] and its unique mass allows identification of the modified residue. Others have reported the addition of a biotin tag to the β-elimination-incorporated thiol via a disulfide bond, such that elution from the avidin column is effected by addition of DTT.[26] The present method eliminates the need to modify the thiol with biotin, simplifying the reaction protocol. A similar thiol-mediated affinity approach has been developed by others to study O-glycosylated proteins.[25]

To test the use of the thiol group as an affinity tag, the phosphoprotein ovalbumin, which contains two major sites of phosphorylation (Ser-68, Ser-344), was subjected to protocol I as outlined in the Experimental Section. Briefly, the protein was oxidized with performic acid and modified with EDT essentially according to the protocol described earlier.[16] After removal of excess EDT, the protein was bound to the activated thiol resin and washed extensively to remove unbound protein. The protein was digested with trypsin, and the unbound peptides were washed from the resin and collected. After further washing, the modified peptides were released with DTT. The flow-through and DTT-eluted peptides were desalted and analyzed by MS. Under the conditions used, there was no nonspecific binding to the resin, and all the thiol-containing peptides were retained by the resin. For example, the unphosphorylated tryptic peptide spanning residues 20−46 was found in the column flow-through but not in the DTT-eluted fraction (Figure 2). In contrast, the EDT-modified peptide 59−84, which was originally phosphorylated on Ser-68, did not appear in the flow-through but was found only in the DTT-eluted fraction. Residual phosphorylated 59−84 was observed in the column flow-through because the conversion to the EDT-modified form was not complete under these conditions (see Figure 3B). The oxidation of cysteine and cystine residues to cysteic acid was complete, as no cysteine-containing peptides from ovalbumin were observed to elute upon DTT treatment.

Detection of Side Reaction at Serine. We anticipated that the only peptides obtained in the DTT elution fraction would be those that were originally phosphorylated, i.e., those that had undergone β-elimination of H_3PO_4 and the addition of the dithiol reagent. Indeed, two EDT-modified peptides, arising due to incomplete tryptic digestion, were observed for each known phosphorylation site (Figure 2B). These peptides were shifted by −4 Da (loss of H_3PO_4 followed by addition of $C_2H_6S_2$) relative to their phosphorylated forms and were therefore 76 Da heavier than their unphosphorylated forms. A peak was also observed in which DTT had formed a disulfide bond with the abundant EDT-modified peptide 59−84. Tandem MS in the MALDI−IT instrument was used to confirm the identity of each peptide (not shown).

In addition to the modified phosphopeptides, several smaller peaks in the spectrum appeared to be shifted by 76 Da relative to expected tryptic peptides from ovalbumin (italicized labels in Figure 2B). We expected none of these peptides to be phosphorylated in the original sample, and all of them contained at least

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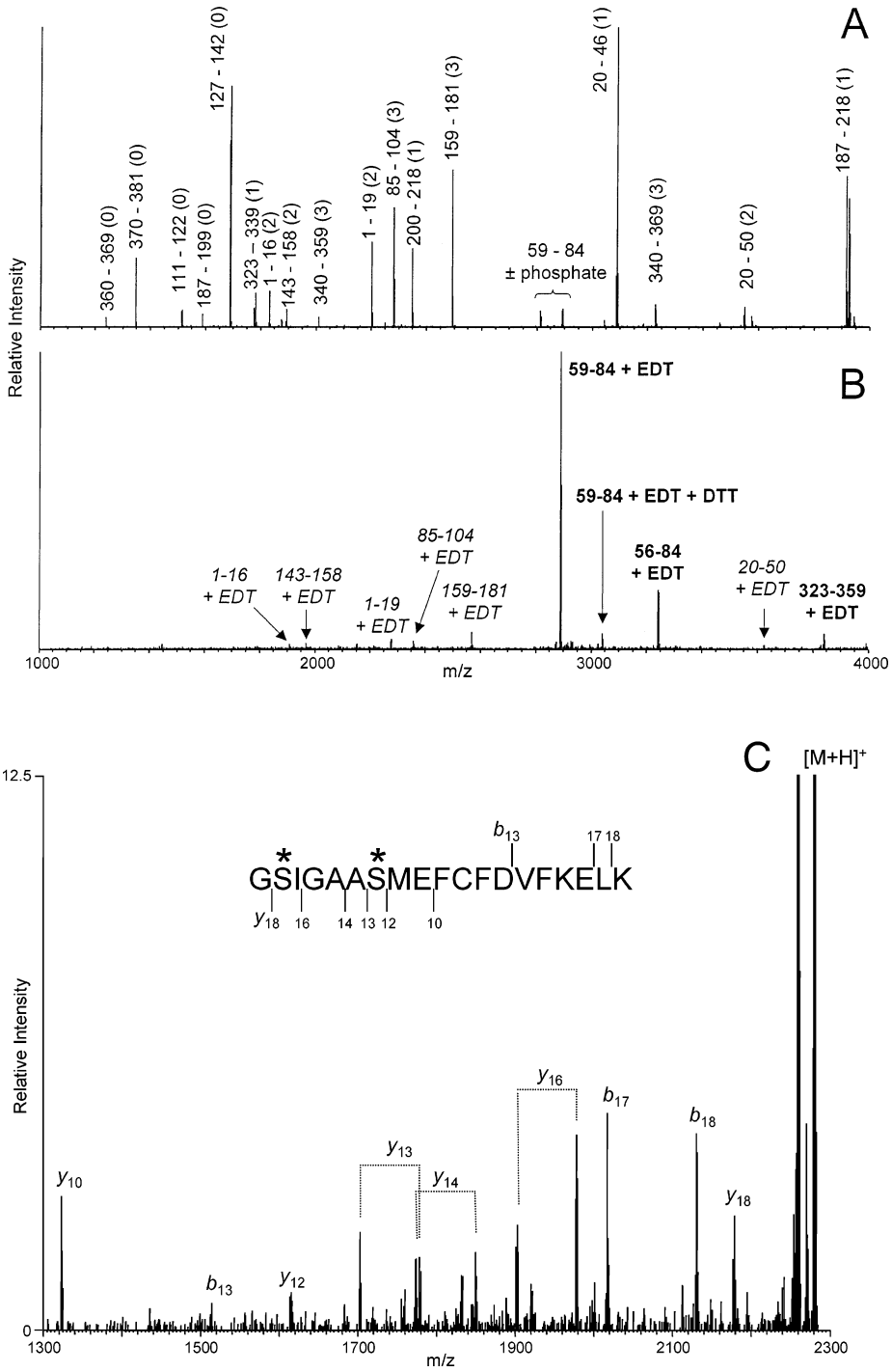

Figure 2. Enrichment of tagged peptides from ovalbumin. Ovalbumin was treated as described in protocol I. (A) Peptides that were washed from the affinity resin after tryptic digestion were analyzed by MALDI-IT-MS. (B) Peptides that eluted from the affinity resin upon treatment with DTT were analyzed by MALDI-IT-MS. In both panels, the amino acid residues spanned by selected tryptic peptides are indicated. The numbers in parentheses indicate the number of serine residues contained in the tryptic fragment. Formerly phosphorylated peptides are labeled in boldface type, while peptides arising due to side reaction at serine (see text) are italicized. Modification of a serine residue by β-elimination and addition of EDT is indicated by "+ EDT". The addition of DTT via a disulfide bond to the derivatized 59−84 peptide is indicated by "+ DTT". (C) MS fragmentation of a peptide modified by the side reaction. The ion at *m*/*z* 2277.5 in Figure 3B was subjected to fragmentation in the MALDI-IT mass spectrometer. The fragmentation pattern identified it as the acetylated N-terminal peptide 1−19 of ovalbumin. For each of the fragments y_{13}, y_{14}, and y_{16}, two ions separated by 76 Da were observed. These pairs of peaks are indicated by dotted lines. The ion of lower mass corresponds to the unmodified C-terminal fragment, and the ion of higher mass corresponds to the EDT-modified C-terminal fragment. Therefore, the EDT-modified 1−19 peptide was present as a mixture of two species, each one modified at a different site. The asterisks above the sequence indicate the deduced sites of EDT modification. For clarity, the *y*-axis of the spectrum has been magnified 8-fold.

one serine residue. For example, the peptide at m/z 2277.5 was identified from its fragmentation pattern (Figure 2C) as the tryptic peptide spanning residues 1−19 that had been modified by loss of H_2O and addition of EDT. The EDT modification appeared to be distributed approximately equally between the two serine residues in the peptide. The other unexpectedly modified peptides

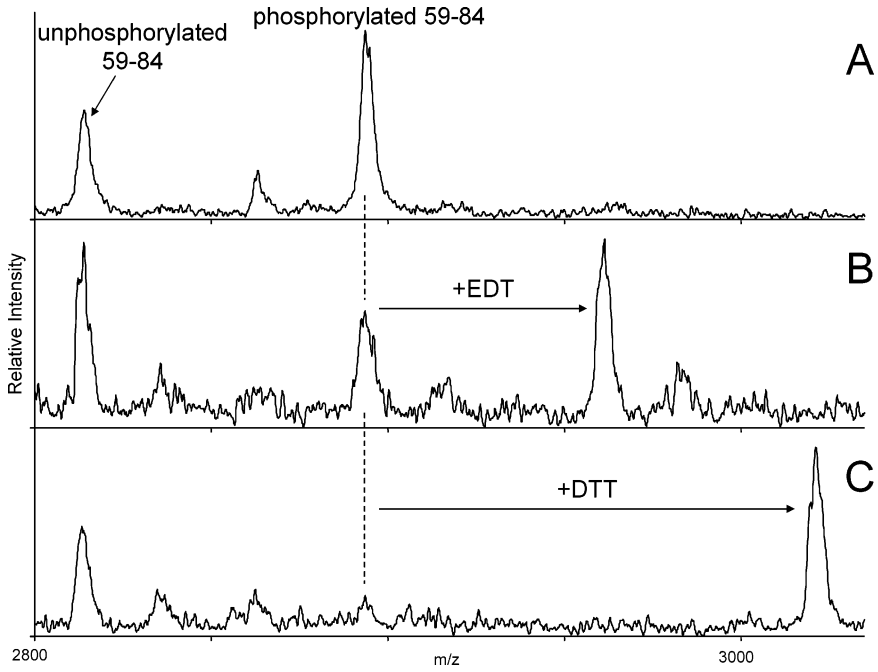

Figure 3. Efficiency of modification of phosphorylation sites. (A) Ovalbumin was oxidized with performic acid and digested with trypsin. The tryptic fragments were analyzed by MALDI-linear TOF MS. Only the region of the spectrum around the tryptic peptide spanning residues 59−84 is shown. (B) Ovalbumin was oxidized with performic acid, subjected to β-elimination as described in protocol I (see Experimental Section), alkylated with acrylamide, and digested with trypsin. The arrow indicates the shift in mass of the 59−84 phosphopeptide upon modification with EDT and acrylamide. (C) Ovalbumin was oxidized with performic acid, subjected to β-elimination as described in protocol II, alkylated with acrylamide, and digested with trypsin. The arrow indicates the shift in mass of the 59−84 phosphopeptide upon modification with DTT and acrylamide.

showed a similar distribution of EDT modification sites among their serine residues.

The observation of many EDT-modified peptides from ovalbumin implied either that there are a number of additional, low-stoichiometry phosphorylation sites within the protein or that unphosphorylated serine residues had undergone β-elimination of water to form dehydroalanine. We note that β-elimination of water from serine and threonine residues has, in fact, been observed previously during alkali treatment of proteins although the rates were observed to be relatively slow compared to that for glycosylated or phosphorylated residues (reviewed in ref 27). We searched for phosphopeptides of the expected m/z values in a tryptic digest of ovalbumin by MALDI-IT-MS/MS; phosphopeptides not visible in the single stage MS spectrum may be sensitively detected by observing their strong, characteristic loss of 98 Da in MS/MS mode.[28] We were unable to detect phosphopeptides of the expected masses corresponding to the peaks with italicized labels in Figure 2B. We concluded that unphosphorylated serine residues were undergoing β-elimination to a minor degree, because (i) most of the unexpected DTT-modified peptides contained at least two serine residues, (ii) the fragmentation data indicated a random distribution of serine modification within these peptides, and (iii) no phosphopeptides corresponding to the unexpected DTT-modified peptides could be detected by MS/MS.

A side reaction of the β-elimination chemistry that tags unphosphorylated serine residues, even at low efficiency, would render the strategy incapable of specifically separating phosphoproteins from unphosphorylated proteins within a mixture. Reaction at random serine residues throughout the unphosphorylated proteins would result in the tagging of a significant population of these proteins. The tagged proteins would then be co-purified along with the formerly phosphorylated proteins on the activated thiol resin. In fact, we were unable to use the β-elimination/affinity chromatography strategy to isolate ovalbumin from a simple mixture containing a 2-fold molar excess over ovalbumin of each of the unphosphorylated proteins yeast alcohol dehydrogenase, bovine serum albumin, and bovine apotransferrin (not shown). This finding contrasted with previous data from our laboratory showing enrichment of the phosphoprotein β-casein from a mixture of unphosphorylated proteins using the β-elimination chemistry.[16] We suspect that this difference is due to the different affinity tags used in the two experiments. β-Casein contains five phosphorylation sites, and therefore, in the previously published experiment, the protein would have been tagged with multiple biotin groups. In contrast, most unphosphorylated proteins modified by the β-elimination side reaction would have incorporated only one biotin moiety. The binding and washing steps employed in the previous experiment may have been stringent enough to disrupt the interaction between singly modified proteins and the monovalent avidin column, while leaving the β-casein protein (bound cooperatively by multiple sites) on the resin. Monovalent avidin binds biotin much more weakly than tetravalent avidin, with a dissociation constant of $\sim 10^{-7}$ M,[29] allowing cooperative binding effects. In the current experiment, using thiol affinity chromatography, all modified proteins were bound covalently to the resin,

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meaning that the strength of attachment was effectively independent of the number of modified sites. Therefore singly modified proteins could be captured and released with essentially the same efficiency as multiply modified proteins.

New β-Elimination Reaction Conditions. To improve the enrichment of phosphorylated species, we sought conditions under which the side reaction would be reduced or eliminated. Previous reports have indicated that β-elimination of phosphate from serine is facilitated by inclusion of certain metal cations, notably Ba^{2+}, in the reaction mixture.[30,31] We found that adding $Ba(NO_3)_2$ into the reaction mixture reduced the amount of NaOH required for modification of phosphorylation sites. However, the addition of Ba^{2+} also caused the formation of precipitates in the reaction vessel. The insolubility problems were solved by replacement of EDT by DTT as the dithiol nucleophile. After optimizing the new reaction conditions (which are defined in protocol II in the Experimental Section), the conversion of phosphopeptides to the thiol-tagged form was more efficient than under the previously published conditions (Figure 3). The efficiency of the reaction conditions can be estimated by comparing the intensity of the unphosphorylated 59−84 peptide, which should not undergo substantial change, with the intensity of the phosphorylated 59−84 peptide, which should decrease as the β-elimination reaction proceeds. As the relative intensity of the phosphorylated peptide decreased, a new signal was observed at higher mass representing the thiol-modified and alkylated peptide (Figure 3B and C). SDS−PAGE analysis of proteins treated under the new conditions (not shown) verified that these conditions maintained the integrity of the peptide backbone, which is susceptible to cleavage under very basic conditions.[27,32]

However, we found that the undesired side reaction persisted at levels comparable to those seen with the previously published conditions. By further decreasing the basicity of the reaction mixture, we could reduce the level of the side reaction, but under these conditions, the yield of the desired β-elimination products was unacceptably low. Despite the persistence of the side reaction at serine, in subsequent experiments we continued to use the new conditions (i.e., those of protocol II) rather than the previously published conditions, for several reasons. The new conditions use much less base and dithiol reagent, making it feasible to remove these reagents after an acid quench by binding the protein or peptide substrates to reversed-phase chromatography supports. DTT is more water-soluble than EDT, allowing more flexibility when designing reaction conditions. If isotopic tags are to be incorporated into the molecule for relative quantitation of two samples,[17,19] DTT allows inclusion of six deuterium atoms per molecule as opposed to four for EDT, resulting in a larger separation between tagged peptides. Finally, EDT has a very offensive smell, making it unpleasant to use.

Analysis of Unphosphorylated Synthetic Peptides. To test our conclusion that unmodified serine residues underwent β-elimination of water, and to quantify the extent of this reaction, we subjected unphosphorylated synthetic peptides individually to the newly defined reaction conditions and analyzed the products by HPLC. We selected three peptides for analysis, each of which contained one serine residue: DAEFRHDSGYE, EQKLISEEDL, and YEQLRNSRA. The treated peptides were injected onto a reversed-phase HPLC column, and absorbance at 214 nm was monitored. Fractions were collected and analyzed by MS. In each case, a major peak was observed containing a peptide of mass identical to that of the starting peptide as well as a minor peak corresponding to the side reaction (Supporting Information). The level of conversion of the unmodified, monomeric peptide to the DTT-modified form varied among the three peptides, but was at most 1.7%. The source of the variation in reaction efficiency among the three peptides is unknown but may relate to differences in the sequence surrounding the serine residue.[33,34]

Given that certain metals are known to enhance the β-elimination of phosphate from serine and threonine,[30,31] we hypothesized that trace metal ions present in the reaction mixture could enhance the undesired side reaction at serine. Also we noted that others using β-elimination to modify phosphorylated proteins have included the metal chelator EDTA in their reaction mixture.[17] Therefore, we tested the effect of adding EDTA into our Ba^{2+}/NaOH/DTT reaction mixture. Using the synthetic peptides, we found that the amount of side reaction product could be reduced by at least 10-fold by inclusion of 1 mM EDTA during the β-elimination reaction. Thus, it seems likely that trace metal ions contaminating our reagents (which were the highest grade available) were at least partially responsible for enhancing the side reaction efficiency. Chelation of certain metal ions by 1 mM EDTA in the presence of 100 mM Ba^{2+} is possible because EDTA binds Ba^{2+} much more weakly than it binds many other metal ions. For example, the pK values for complex formation of EDTA with Cu^{2+}, Fe^{3+}, and Ni^{2+} are 18.7, 24.2, and 18.6, respectively, as opposed to 7.8 with Ba^{2+}.[35] In reactions conducted with oxidized ovalbumin, 5 mM EDTA was able to reduce the level of side reaction by ∼10-fold. Whereas the presence of 5 mM EDTA in the reaction mixture produced a marginal reduction in the efficiency of the conversion of phosphoserine-68 of ovalbumin to DTT-modified serine, phosphoserine-344 appeared not to be converted at all in the presence of 5 mM EDTA. The reason for this difference in the behavior of the two phosphoserine residues is unknown. Therefore, although inclusion of EDTA can reduce the level of side reaction, there is the danger that certain phosphorylated residues may not undergo the β-elimination as desired in the presence of EDTA.

Enrichment of Phosphopeptides from a Mixture. Despite occurring at a relatively low level, as discussed previously, the side reaction made it unfeasible to use the β-elimination chemistry to separate phosphoproteins from unphosphorylated proteins because serine is a relatively common residue. However, digestion of proteins into peptides prior to the analysis would distribute the side reaction over numerous individual species. Since modification of the phosphoserine residues is heavily favored, enrichment of phosphorylated species from a mixture of peptides should be possible. To test this idea, we mixed three unphosphorylated proteins (bovine apotransferrin, bovine serum albumin, yeast alcohol dehydrogenase) with ovalbumin in a ratio of 2 mol of each unphosphorylated protein to 1 mol of ovalbumin. Only one

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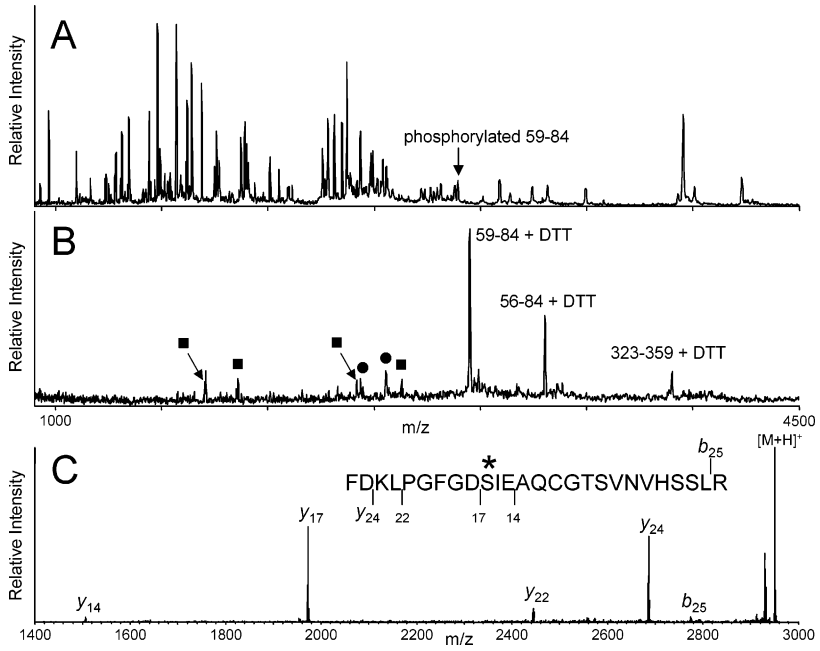

Figure 4. Enrichment of phosphopeptides from a moderately complex mixture. (A) Ovalbumin, bovine apotransferrin, bovine serum albumin, and yeast alcohol dehydrogenase were mixed in a molar ratio of 1:2:2:2 and dried in a Speed-Vac. The mixture was oxidized with performic acid and digested with trypsin. The tryptic peptides were desalted and analyzed by MALDI-TOF MS. The only observed phosphopeptide is labeled. (B) A mixture identical to the one described above was subjected to oxidation, β-elimination and affinity purification using protocol II. The DTT-eluted peptides were desalted and analyzed by MALDI-TOF MS. Three formerly phosphorylated peptides from ovalbumin are labeled according to the amino acid residues they contain. The symbols are as follows: (■) unphosphorylated peptide modified on serine; (●) peptide arising due to incomplete oxidation of cysteine. (C) The DTT-modified 59−84 peptide shown in panel B was fragmented in a MALDI-IT mass spectrometer. The asterisk indicates the site of DTT modification at the formerly phosphorylated serine residue.

phosphopeptide from ovalbumin was observed in the peptide map of the underivatized mixture after oxidation and tryptic digestion (Figure 4A). In contrast, after β-elimination (without EDTA) and enrichment by protocol II, three formerly phosphorylated peptides from ovalbumin were evident (Figure 4B), allowing identification of both phosphorylation sites. Fragmentation of the enriched peptides in the MALDI-IT mass spectrometer confirmed their identities and revealed the sites of modification; for example, fragmentation of peptide 59−84 identified Ser-68 as the site of DTT modification (Figure 4C). Some peptides observed in the DTT elution fraction were found to arise due to side reaction at serine-rich peptides (filled squares in Figure 4B) or incomplete oxidation of a cysteine residue (filled circles in Figure 4B). Unmarked peaks in Figure 4B were not identified. The peaks arising from unphosphorylated peptides suggest that background signals of unacceptable intensity would be observed if the procedure was applied to mixtures in which unphosphorylated peptides are present in amounts orders of magnitude greater than phosphopeptides.

Increasing the Sensitivity. As shown above, the enrichment protocol is maximally effective when used on mixtures of proteins in which the phosphoproteins are present at amounts comparable to unphosphorylated proteins, such as an in vitro kinase assay or an in vivo-derived complex that has undergone an initial purification procedure. For use with material obtained from in vivo sources, the sensitivity of the method is crucial. Previous reports using β-elimination-based protocols for affinity enrichment of phosphopeptides used at least 1 nmol of starting material.[16,17,26] Greater sensitivity is needed if this strategy is to be routinely useful. We found that the major losses occurred during sample handling steps; in particular, performic acid must be removed from the sample prior to adding the β-elimination reaction mixture, and DTT from the reaction mixture must be removed before application of the sample to the affinity resin.

To improve the sensitivity, we developed the following method (protocol III in the Experimental Section). First, the protein sample was taken to dryness in a Speed-Vac. The sample was oxidized by a novel procedure in which it was exposed to performic acid vapor in an evacuated chamber. The advantage of this in vacuo oxidation reaction was that the performic acid could be removed easily, resulting in minimal loss of sample and allowing substantial improvement in sensitivity. This oxidation method resulted in efficient addition of two oxygen atoms to methionine and tryptophan and essentially complete conversion of cysteine and cystine to cysteic acid. Tyrosine residues were mostly converted to an unknown form that bore an additional 34 Da; this conversion was not affected by inclusion of phenol in the sample or in the performic acid solution. In the presence of moderate amounts of organic buffers such as Tris, MOPS, or HEPES, tyrosine and tryptophan residues underwent further modifications, and after β-elimination, it was usually difficult to observe the tyrosine- and tryptophan-containing peptides. No formylation of any residue was observed. The oxidation reaction could be enhanced by incubation for longer times or at higher temperatures, but at the cost of fragmentation of the peptide backbone.

After oxidation, an ammonium bicarbonate buffer solution containing trypsin was added to the sample. The performic acid oxidation should denature almost any protein, allowing efficient proteolytic cleavage. After digestion, the solvent and buffer were removed by evaporation in a Speed-Vac. The β-elimination reaction

was then carried out for 1 h at 37 °C. The reaction was quenched by addition of trifluoroacetic acid, leaving the sample ready for desalting on a reversed-phase support. Washing the resin-bound peptides with a dilute TFA solution removed DTT from the sample. The peptides were eluted from the beads with an aqueous solution containing 67% acetonitrile and 0.1% TFA directly into a buffered suspension of the activated thiol affinity resin. After binding, the affinity resin was washed extensively to remove unbound peptides, and the specifically bound peptides were eluted efficiently with DTT. The DTT-eluted peptides were desalted and concentrated on a reversed-phase support. The peptides were then eluted directly onto a MALDI target with matrix solution.

Unlike protocols I and II, in protocol III the proteolytic digestion was performed before the β-elimination reaction. We found that removal of the β-elimination reagents from peptides using a hydrophobic resin was more convenient and allowed greater sample recovery than techniques used to remove the reagents from proteins. Attempts to perform the β-elimination reaction on proteins that were bound to an alkali-resistant HPLC resin (Source 15RPC from Pharmacia) resulted in very poor protein recovery.

Protocol III allowed detection of the major phosphopeptide of ovalbumin when starting with as little as 10 ng (~230 fmol) of protein (Figure 5), a considerable improvement in sensitivity over previously published β-elimination-based affinity purification protocols.[16,17,26] Naturally the stoichiometry of phosphorylation of a given site will influence the amount of material required to detect the phosphopeptide; successful observation of the minor site of phosphorylation of ovalbumin in MS mode required ~10-fold more material. A number of autoproteolytic tryptic peptides, either containing cysteine or modified by DTT at serine during the β-elimination reaction, were co-purified along with the phosphopeptide from ovalbumin (Figure 5). These peptides were easily recognized and possibly could be avoided by a second oxidation step after the tryptic digestion. Care must be taken to ensure that the pH of the proteolytic digest is not too low due to residual amounts of acid that may be present after the oxidation step, particularly when buffers or salts are present. Buffers that are not removed by evaporation can cause a reduction in the efficiency of the β-elimination, but increasing the concentration of NaOH in the reaction mixture compensated for this effect. It must also be noted that, due to rearrangements, the desired β-elimination reaction does not occur when the phosphorylated residue is at the N- or C-terminus of a peptide,[36] and therefore, digesting the protein prior to the reaction may result in failure of some phosphorylated residues to react. This problem may be ameliorated by analysis of samples digested with different proteases.

A Strategy for Phosphoprotein Analysis. Given the above findings, we propose the following strategy for identifying phosphorylation sites within proteins. A portion of the protein sample is treated using protocol III to preferentially tag sites of phosphorylation with a thiol moiety and enrich the sample for the formerly phosphorylated peptides. Analysis by MS gives a list of candidate phosphopeptides. By removing the phosphate moiety from the peptides of interest and separating these peptides from unphosphorylated species, one may obtain signals from candidate

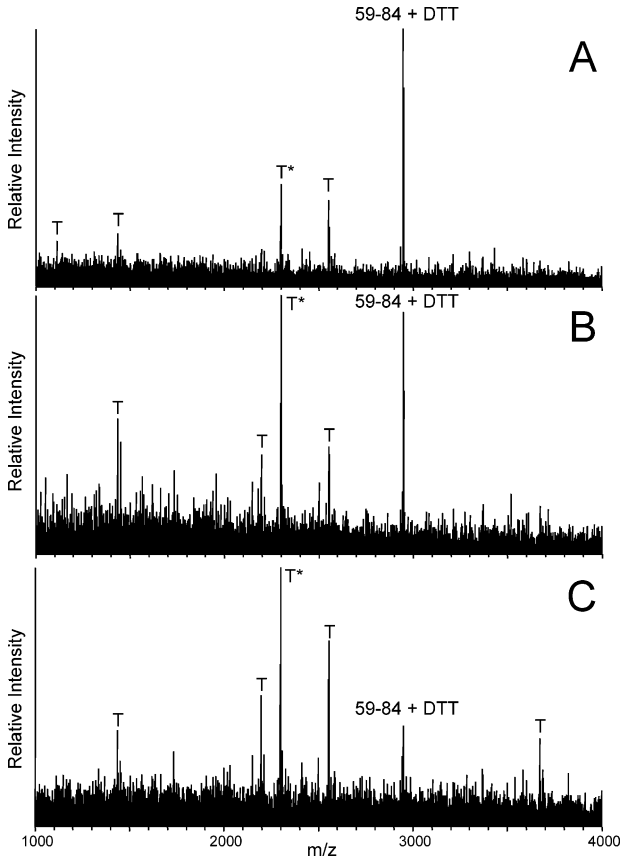

Figure 5. Sensitivity of the affinity purification protocol. Various amounts of ovalbumin (panel A, 50 ng or ~1.2 pmol; panel B, 25 ng or ~580 fmol; panel C, 10 ng or ~230 fmol) were treated as described in protocol III. The DTT-eluted peptides were analyzed by MALDI-IT-MS. The DTT-modified peptide spanning residues 59−84 of ovalbumin is labeled. The symbols are as follows: (T), autoproteolytic tryptic peptide containing cysteine; (T*), autoproteolytic tryptic peptide modified at serine by DTT during the β-elimination reaction.

phosphopeptides that are not seen in the spectrum of the entire mixture. Comparison of the sample with an unphosphorylated control or a second sample that has been treated with a phosphatase can help identify which candidate peptides are background signals due to side reaction or incomplete oxidation. Confirmation of phosphorylation on the candidate peptides may be obtained by digesting a portion of the original protein sample and using a hypothesis-driven approach to examine selected m/z values.[37] The intense, characteristic loss of 98 Da exhibited by phosphopeptides in MS/MS mode can allow detection of phosphopeptides not visible in the single-stage MS spectrum.[28] Using this method, it is not necessary to detect the phosphopeptide in MS mode in the underivatized mixture. In addition, the stable thiol tag allows more efficient sequencing of the enriched peptides since the labile phosphate group has been removed, as has been previously observed.[24]

To illustrate the proposed strategy for finding phosphorylation sites, bovine synapsin I was incubated with or without CaM kinase II and portions of the reactions were subjected to protocol III. A number of signals were observed in the presence of CaM kinase II that were not observed in its absence (Figure 6). A number of

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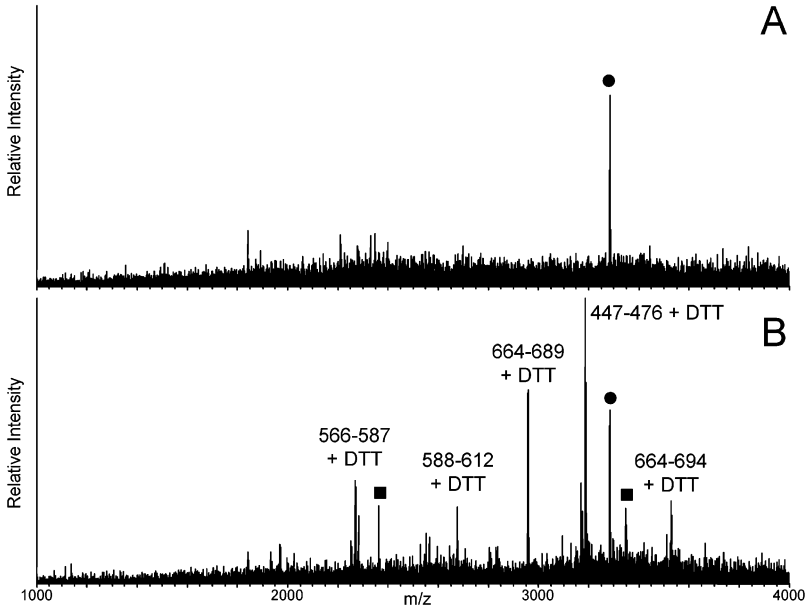

Figure 6. Analysis of phosphorylation of synapsin I by CaM kinase II. Bovine synapsin I was incubated in the absence (panel A) or presence (panel B) of CaM kinase II. A portion of each reaction was treated as described in protocol III (see Experimental Section). Peaks labeled with numbers are DTT-modified tryptic peptides of synapsin that were later confirmed by MALDI-IT-MS/MS to be phosphorylated in the underivatized +CaM kinase II sample. The symbols are as follows: (■) peaks for which phosphorylated counterparts were sought, but not found, in the tryptic digest of underivatized, CaM kinase II-treated synapsin; (●) an unidentified contaminant that bound to the affinity resin in each sample.

Table 1. Synapsin I Peptides Phosphorylated by CaM Kinase II

residues	peptide sequence[a]	m/z (MH^+)[b] measured	m/z (MH^+)[b] theoretical	phosphorylated sequence[a]
566−587	QT**pS**VSGQAPPKASGVPPGGQQR	2270.2	2270.1	ATRQT**pS**V
588−612	QGPPQKPPGPAGPTRQA**pS**QAGPMPR	2676.2	2676.3	PTRQA**pS**Q
447−476	QI**pS**QQPAGPPAQQRPPPQGGPPQPGPGPQR	3186.1	3186.6	LGRQI**pS**Q
664−689	SQ**pS**LTNAFNLPEPAPPRPSLSQDEVK	2957.6	2958.4	LNKSQ**pS**L
664−694	SQ**pS**LTNAFNLPEPAPPRPSLSQDEVKAETIR	3528.2	3528.7	LNKSQ**pS**L

[a] The suspected site of phosphorylation is indicated by "pS" in boldface type. [b] The masses were those measured from the MALDI-IT spectrum in Figure 6, i.e., after modification of the peptides by performic acid oxidation and addition of DTT via β-elimination. Therefore, the theoretical masses include these modifications.

these peptides (labeled with numbers in Figure 6B) could be identified based on their observed molecular masses (Table 1) and by their mass spectrometric fragmentation patterns. Other peptides (unlabeled or labeled with a filled circle or square in Figure 6B) could not be confidently identified. To look for phosphorylation of the major peptides of Figure 6B, the kinase reaction mixtures were oxidized and digested with trypsin, and the peptide maps were analyzed. No phosphopeptides corresponding to peaks marked with a filled circle or square in Figure 6B were observed by either MALDI-MS or MALDI-IT-MS/MS, and the origin of these peaks is uncertain. Peaks were observed in the single-stage mass spectrum corresponding to phosphorylated 447−476 and 664−694 in the sample that had received CaM kinase II but not in the negative control. Fragmentation of these peptides resulted in the loss of 98 units that is characteristic of phosphopeptides, and MS^3 spectra of the $(M + H - 98)^+$ species provided confirmation of the identity of the phosphopeptides (Supporting Information). Signals for the candidate phosphopeptides 566−587, 588−612, and 664−689 were not observed in single-stage MS mode, and therefore, these phosphopeptides were sought in MS/MS mode. In each case, the characteristic loss of 98 Da was observed in the MS/MS spectrum of the sample treated with CaM kinase II, confirming that these peptides were phosphorylated (while the MS^3 spectra of the $(M + H - 98)^+$ species provided confirmation of the identities of the putative phosphopeptides) and validating the results obtained with the enrichment protocol.

All of the identified phosphopeptides contain serine residues that are part of the consensus sequence known to be phosphorylated by CaM kinase II (Hyd − X − R/K− NB − X − S/T − Hyd, where Hyd = hydrophobic, NB = nonbasic, and X may be any residue)[38−40] (Table 1). Phosphorylation of Ser-568 and Ser-605 by CaM kinase II was described previously,[23] but phosphorylation of the other sites observed in this experiment has not been previously reported. With respect to these newly discovered phosphorylation sites, the peptide 447−476 contains only one serine and no threonine residues, making the assignment unambiguous. The fragmentation pattern of the DTT-modified peptide

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664−689 localized the site of modification to one of Ser-666 or Thr-668 (Supporting Information). Given the consensus sequence for CaM kinase II, we concluded that the residue phosphorylated was likely Ser-666. Assessing the physiological relevance of these newly discovered phosphorylation sites will require further experimentation in appropriate cellular systems.

CONCLUSIONS

Improved methods for analyzing phosphorylated proteins are continuously being sought. Most recently, a method has been demonstrated for β-elimination from phosphoserines followed by modification with aminoethylcysteine, the product of which can be cleaved by trypsin. The aminoethylcysteine reaction has been adapted to a solid-phase catch-and-release strategy to provide one-step modification and enrichment of phosphopeptides, although thus far only with large amounts of synthetic peptides.[41] The appeal of alkaline β-elimination methods for enriching species phosphorylated on serine and threonine lies in the relatively simple chemistry, improved mass spectrometric sequencing resulting from replacement of the phosphate group, and the possibility for incorporation of isotopic tags for relative quantitation. In the present work, we have brought the strategy closer to routine use by improving its sensitivity and identifying a side reaction that must be considered when planning experiments. Although the level of the side reaction could be reduced by using the metal chelator EDTA, the inclusion of EDTA into the reaction mixture also eliminated the modification of a bona fide phosphopeptide from ovalbumin. Until it is understood why this phosphopeptide was not modified, we cannot recommend the use of EDTA in β-elimination reactions where modification of phosphorylation sites is intended. Since we were unable to find satisfactory conditions that resulted in complete suppression of the side reaction at serine, this side reaction must be carefully accounted for by anyone using β-elimination as a method for protein modification analysis, particularly in samples where protein levels may vary by orders of magnitude. However, if experiments are designed appropriately, the β-elimination and affinity purification strategy described here may prove very useful for identifying phosphorylation sites.

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ACKNOWLEDGMENT

We thank Dr. Julio Padovan for suggesting the in vacuo oxidation method, for assistance with the HPLC system, and for helpful discussions. We also thank Dr. Michelle Trester-Zedlitz for helpful discussions and Dr. Angus Nairn for critical reading of the manuscript. D.T.M. was supported by a postdoctoral fellowship from the Canadian Institutes of Health Research. This work was funded by NIH Grants RR00862 and CA89810 to B.T.C. This work was presented in part in the Proceedings of the 49th ASMS Conferences on Mass Spectrometry and Allied Topics (May 27−31, 2001, Chicago, IL) and the 50th ASMS Conferences on Mass Spectrometry and Allied Topics (June 2−6, 2002, Orlando, FL.

SUPPORTING INFORMATION AVAILABLE

Additional information as noted in text. This material is available free of charge via the Internet at http://pubs.acs.org.

Note Added in Press. While this article was in press, Qian et al. (Quan, W.-J.; Goshe, M. B.; Camp, D. G., II; Yu, L.-R.; Tang, K.; Smith, R. D. *Anal. Chem.* **2003**, *75*, 5441−5450) described a procedure where they reported the ability to process and analyze phosphoproteins with amounts as low as 90 pmol of phosphoprotein and Li et al. (Li, W.; Backlund, P. S.; Boykins, R. A.; Wang, G.; Chen, H.-C. *Anal. Biochem*. In press) have also demonstrated that serine and threonine hydroxyl groups are susceptable to β-elimination/Michael addition under commonly used moderately high temperature conditions.

Received for review August 25, 2003. Accepted October 7, 2003.

AC034989U