Phosphopeptide Enrichment by Covalent Chromatography after Derivatization of Protein Digests Immobilized on Reversed-Phase Supports

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A rugged sample-preparation method for comprehensive affinity enrichment of phosphopeptides from protein digests has been developed. The method uses a series of chemical reactions to incorporate efficiently and specifically a thiol-functionalized affinity tag into the analyte by barium hydroxide catalyzed β -elimination with Michael addition using 2-aminoethanethiol as nucleophile and subsequent thiolation of the resulting amino group with sulfosuccinimidyl-2-(biotinamido) ethyl-1,3-dithiopropionate. Gentle oxidation of cysteine residues, followed by acetylation of α - and ϵ -amino groups before these reactions, ensured selectivity of reversible capture of the modified phosphopeptides by covalent chromatography on activated thiol sepharose. The use of C18 reversed-phase supports as a miniaturized reaction bed facilitated optimization of the individual modification steps for throughput and completeness of derivatization. Reagents were exchanged directly on the supports, eliminating sample transfer between the reaction steps and thus, allowing the immobilized analyte to be carried through the multistep reaction scheme with minimal sample loss. The use of this sample-preparation method for phosphopeptide enrichment was demonstrated with low-level amounts of in-gel-digested protein. As applied to tryptic digests of α -S1- and β -casein, the method enabled the enrichment and detection of the phosphorylated peptides contained in the mixture, including the tetraphosphorylated species of β -casein, which has escaped chemical procedures reported previously. The isolates proved highly suitable for mapping the sites of phosphorylation by collisionally induced dissociation. β -Elimination, with consecutive Michael addition, expanded the use of the solid-phase-based enrichment strategy to phosphothreonyl peptides and to phosphoserine/phosphothreonyl peptides derived from proline-directed kinase substrates and to their *O*-sulfonyl- and *O*-linked β -N-acetylglucosamine (*O*-GlcNAc)-modified counterparts. Solid-phase enzymatic dephosphorylation proved to be a viable tool to condition *O*-GlcNAcylated peptide in mixtures with phosphopeptides for selective affinity purification. Acetylation, as an integral step of the sample-preparation method, precluded reduction in recovery of the thiolation substrate caused by intrapeptide lysine-dehydroalanine cross-link formation. The solid-phase analytical platform provides robustness and simplicity of operation using equipment readily available in most biological laboratories and is expected to accommodate additional chemistries to expand the scope of solid-phase serial derivatization for protein structural characterization.

KEY WORDS: mass spectrometry, β -elimination/Michael addition, multistep solid phase, affinity purification, phosphorylation-site determination, alkaline phosphatase, *O*-glycopeptides, sulfonopeptides

INTRODUCTION

Among the many protein post-translational modifications (PTMs), phosphorylation has been studied most extensively because of its critical role in the modulation of cellular processes.¹ For example, aberrant phosphorylation

of components of signaling complexes has been implicated in the genesis, progression, and metastasis of the most commonly occurring cancers. Therefore, the determination of phosphorylation sites has become increasingly important. Various strategies using electrospray ionization (ESI) tandem mass spectrometry (MS/MS) coupled to liquid chromatography (LC) have been used, including precursor ion and neutral-loss scanning for diagnostic ions generated under the conditions of collisionally induced dissociation (CID).² However, the ionization inefficiency of phosphopeptides, especially in complex mixtures, and

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their low stoichiometry limit the information content of the MS/MS spectra as a result of the intrinsic lability of the phosphate group upon CID. To address these limitations, several methods to enrich phosphopeptides have been developed. One approach involves the use of ion metal affinity chromatography (IMAC). This technique is easy to implement and relies on ionic binding of phosphopeptides to metal ion-chelating resins.³ However, in many instances, this approach had been inefficient as a result of coadsorption of nonphosphorylated (acidic) peptides that contaminate the IMAC fractions and is complicated further by variability of phosphopeptide elution. Phosphopeptide enrichment by titanium dioxide (TiO₂) chromatography is widely used currently, but this technique also suffers from nonspecific peptide adsorption and has been shown to poorly enrich phosphopeptides derived from basophilic kinase substrates.⁴ Although the above strategies afford improved phosphopeptide detection, issues still persist with regard to neutral loss of phosphate, because as the favored fragmentation event, it dominates over peptide backbone cleavage. Subjecting the resultant neutral-loss product ion from the MS/MS spectrum to a further cycle of fragmentation (MS/MS/MS) may improve the peptide sequence information content and thus, aid in phosphorylation-site determination.⁵ Alternative fragmentation schemes have been introduced, such as electron transfer dissociation (ETD),⁶ electron transfer dissociation with supplemental activation (ETcaD),⁷ and higher-energy collisionally activated dissociation cells (HCD),⁸ to alleviate the neutral loss problem. These methods have often been used in combination with CID in a complementary manner. However, these sophisticated instruments are not available to all experimental biology laboratories. Thus, a phosphopeptide-enrichment method rendering the isolates amenable to CID on MALDI-TOF or ESI instruments would be highly desirable.

Chemically induced β -elimination of phosphate coupled with Michael addition has re-emerged as a strategy to address the limitations of the existing enrichment methodology by exploiting the nucleophilic substitution to preclude neutral loss of the phosphate as the preferred pathway of peptide backbone fragmentation. In an early method, 1,2-ethanedithiol was used as nucleophile, followed by thiol-directed biotinylation, tryptic digestion of the modified proteins, and avidin-based purification of the tagged, formerly phosphorylated, peptides.⁹ There are several drawbacks associated with this method. Reagent excess is removed from the modified proteins by centrifugal filtration or precipitation, manipulations well-recognized to cause adsorptive sample loss. Furthermore, limitations inherent to the use of protein-based retrieval systems include nonspecific peptide adsorption, as well as reduced sample

recovery as a result of irreversible analyte binding. To overcome these unfavorable effects, sample loads at the 1- to 2-nmol level were processed, limiting the general use of these biotin/avidin retrieval methods. To address these issues, disulfide groups or base-labile moieties have been incorporated into the biotin tag to facilitate the release of the enriched species.^{10,11} However, model protein digests processed with the former protocol were still at the nanomole level, and data required to illustrate the selectivity of the method were not provided. The base-labile moiety used in an alternative approach was a 4-carboxy Fmoc group linked via a spacer to a cysteine residue to generate the dehydroalanyl reactive species.¹¹ As compared with the TiO₂ enrichment, the modified phosphopeptides could be isolated from 10 pmol of a tryptic digest of model protein at higher selectivity. However, issues persist with the reactivity of the sulfhydryl moiety. Furthermore, sample purification before Michael addition was required to preserve the stability of a base-labile linker. An additional biotin probe features an acid-cleavable Wang-type (4-hydroxymethylphenoxy) linker and maleimide functionality incorporated for a chemoselective thiol reaction.¹² The enrichment efficiency of this method was evaluated with a peptide mixture containing 1 nmol of each species spiked with 1 nmol phosphopeptide. As assessed by MALDI-MS, an ~25% of the nonphosphorylated peptides were found in the enriched fraction. In addition, the maleimide moiety proved partially stable to CID, complicating MS/MS data interpretation. Alternative efforts to improve the selectivity of analyte enrichment use nonprotein-based retrieval systems. One such approach has features similar to the cleavable isotope-coded affinity tag (cICAT) developed for the isolation and quantitation of cysteinyl peptides.¹³ This solid-phase tag incorporates an iodoalkyl group to capture thiolated peptides that are then released from the solid-phase support by ultraviolet photocleavage.¹⁴ Compared with the earlier biotin/avidin-based methods, sample recovery was improved significantly, but 1–3 nmol starting material was needed for routine operation, and 90 pmol β -casein was still required to identify T6, the monophosphorylated species. The α -S1 casein phosphopeptide spanning residues 121–134 and its miscleavage product spanning residues 19–134 were not identified unequivocally at this sample level. Notably, the isolation of the tetraphosphorylated tryptic peptide of β -casein was not demonstrated in the report.

Improvements to the classical β -elimination reaction have also been pursued. Barium hydroxide was reported to provide for more efficient β -elimination, allowing the reaction to proceed under mildly alkaline conditions in pure aqueous solution.¹⁵ This advantage was exploited to combine IMAC with concurrent β -elimination/Michael addi-

tion using 2-aminoethanethiol as the nucleophile. The resultant 2-aminoethylcysteine derivatives, upon CID, fragmented considerably more informatively than their phosphorylated analogs, enabling facile phosphorylation-site determination.¹⁶ The milder reaction conditions were used in the protocol reported by McLachlin and Chait¹⁷ to conjugate the Michael acceptors with DTT in a concurrent reaction scheme. The derivatization was preceded by performic acid oxidation to avoid co-isolation of cysteine-containing peptides. The thiol-functionalized peptides were reversibly captured by covalent chromatography on activated thiol sepharose.¹⁸ This method allowed routine sample purification at the 10- to 50-pmol level of digest. At lower protein levels, autoproteolytic tryptic peptides that were also isolated by the method became dominant in the spectra. In addition, minor signals were observed in the spectra of the enriched fractions, attributable to elimination of water from serine and subsequent addition of the nucleophile. Others have also adopted barium hydroxide as a β -elimination reagent, combined with solid-phase capture supports that use acid labile linker incorporated into a polyethylene glycol dimethylacrylamide copolymer or contain the 2-pyridyl disulfide-functionalized agarose gel for reversible analyte binding.^{19,20} Model phosphopeptides analyzed by these protocols were at the 10-pmol level, whereas typically 1 nmol protein digest was required for routine operation. The selectivity of analyte retrieval, as demonstrated in these reports, was relatively modest at the lower sample level, as the MALDI spectra of the enriched fractions were contaminated frequently by nonphosphorylated species and polymer byproducts that caused signal suppression. In the method developed by Tseng,²¹ a 6-(mercaptoacetylamine)-hexanoic-functionalized RINK resin was used as a solid-phase Michael addition donor that allows capture of the peptide dehydroderivatives in a one-pot reaction. The analyte was then retrieved from the support by acidolysis, which confers an amide tag onto the site of phosphorylation. Advantages of this approach include ease of operation and the potential of automation. The capability of the method to tag phosphopeptides was evaluated with a synthetic phosphoserine peptide. Although the labeled peptide formed the main ion in the MALDI-MS spectrum, several additional components were released from the resin. To demonstrate the capture/release selectivity of the approach, tryptic peptides from 100 pmol in vitro-phosphorylated tau protein were subjected to enrichment. MALDI-MS revealed the phosphorylated species, but the spectrum was dominated by several additional signals that were considerably more prominent, compromising the selectivity of the method. In parallel experiments, the digest was subjected to IMAC purification. In comparison, the selectivity of the solid-phase retrieval system

proved to be only slightly better than afforded by the IMAC approach.

More recently, the well-known phosphoramidate chemistry (PAC) has been adopted to conjugate phosphopeptides to an amino group-functionalized dendrimer in a one-pot reaction step, which is preceded by carboxylate methyl esterification. In the final step, nonphosphopeptides are removed from the dendrimer by centrifugal filtration, followed by acidolytic release of the bound fraction.²² The capture/release selectivity and the recovery efficiency of the procedure were evaluated with 100 pmol of a β -casein tryptic digest. MALDI-MS of the enriched fraction revealed a prominent ion corresponding to the protein's methyl-esterified, monophosphorylated fragment, with very little background contamination. However, the isolation of the protein's tetraphosphorylated tryptic peptide was not demonstrated in this report. The overall recovery efficiency of the method, as assessed with a synthetic peptide at 10 pmol sample loads, was $\sim 35\%$, and 0.5 pmol of a synthetic peptide within a mixture of 500 pmol of a BSA tryptic digest could be selected. In a variation of this strategy, PAC was used to generate cystamine-modified phosphoramidate preparations, which were immobilized on C18 cartridges, reduced in situ, eluted, and then covalently linked to maleimide-functionalized glass beads.²³ Nonphosphorylated peptides were depleted by a solvent wash, followed by on-bead acidolytic release of the enriched fraction. As a result of the lengthy phosphate-conjugation step, >10 h were needed to complete these procedures. In a subsequent study, the PAC-based approach and the two noncovalent methods were applied in parallel to identical aliquots of a tryptic digest from *Drosophila melanogaster* Kc167 cells.²⁴ Each method detected nearly the same number of phosphopeptides; however, the overlap between the data sets was relatively low (35%), indicating that none of the methods alone was capable of analyzing comprehensively a complex mixture. Moreover, all three methods preferentially isolated singly phosphorylated species. In the case of IMAC and TiO_2 , the poor overlap of the isolates is expected because of the difference in their ionic isolation concepts, whereas phosphopeptide selection by PAC, based on covalent analyte capture, appears to reflect a structure-reactivity modulation effect that impacts the efficiency of the covalent reaction. In addition, the method regenerates the original phosphopeptide, rendering site mapping subject to ambiguity as a result of phosphate neutral loss under the conditions of CID.

As mentioned above, DTT has been used as a nucleophile to introduce a thiol tag into phosphopeptides in a single-reaction step for subsequent reversible affinity purification by covalent chromatography.¹⁷ This technique has been used in several studies to map phosphorylation/*O*-

glycosylation events in isolated proteins and on the proteome-wide scale.^{20,25–29} In an application to a tryptic digest of the rat organic anion-transporting protein 1a1 (Oatp1a1), however, the method failed to enrich a doubly-phosphorylated species.²⁸ As demonstrated by our experiments discussed below, we encountered the same complication in attempts to isolate the DTT conjugate of the tetraphosphorylated peptide of β -casein by covalent chromatography. This shortcoming prompted us to devise an alternative strategy, which is discussed in detail below. In brief, the phosphoprotein digest was first subjected to α - and ϵ -amino group acetylation, followed by barium hydroxide-catalyzed β -elimination with Michael addition (BEMAD), using 2-aminoethanethiol as nucleophile. The amine-blocking step renders the substituted serine and threonine residues as the sole targets for the subsequent acylation with sulfosuccinimidyl-2-(biotinamido)-ethyl-1,3-dithiopropionate. In the subsequent steps, the digest was exposed to hydroxylamine for reversal of unwanted hydroxyl group acylation, followed by reductive release of the disulfide-linked biotinamido moiety from the derivative. The selectively thiolated phosphopeptide analogs were then reversibly enriched from the digest by covalent chromatography. Co-isolation of cysteinyl peptides was precluded by performic acid oxidation of the digests used in the protocol as initial reaction step. Alternatively, the reactivity of this residue was arrested by in-gel performic oxidation.

Multistep chemical reaction schemes, such outlined above, are in general, perceived as problematic because of the high potential of adsorptive sample loss associated with intermittent, postreaction sample purification. As demonstrated in our study, we have addressed this issue successfully by using reversed-phase C18 supports as reaction beds on which reagents are replaced directly after each reaction step. We have used this minimal sample handling format earlier for phosphopeptide detection in MALDI mass maps of an unfractionated protein digest and to prepare digest for phosphorylation-site determination by chemical-targeted proteolysis.^{30,31} Benefits gained from of this approach include ease of operation, completeness of derivatization, improved throughput, and efficient use of dilute samples. It is noteworthy that these advantages had been recognized already one decade ago in conjunction with off-line and on-line derivatization on various sorbents and have since then been exploited by use of chromatographic trapping columns to improve significantly the accuracy, sensitivity and throughput of the quantitative analysis of bioorganic compounds.^{32,33}

A significant portion (>50%) of cellular proteins is targeted by proline-directed kinases known to be associated intimately with cell-cycle control.³⁴ Reversible phosphory-

lation at threonine, although relatively infrequent in occurrence, is involved in diverse cellular reactions, such as the regulation of cell adhesion, cell attachment, and cell migration.³⁵ The structural characterization of these kinase targets is therefore of considerable interest. The notable resistance of phosphothreonine, particularly when positioned adjacent to proline to the barium hydroxide-catalyzed β -elimination/Michael addition using 2-aminoethanethiol or other thiols, is well-recognized.^{20,36} In the accompanying report, we have successfully expanded our solid-phase derivatization approach to this subset of phosphopeptides using the consecutive BEMAD reaction format, which provided the requisite chemical basis for their affinity enrichment from phosphoprotein digests. Application of this protocol to phosphopeptides containing lysine positioned adjacent to the site of phosphorylation revealed the formation of intramolecular cross-links between dehydroalanine and the proximal lysine. As shown in our study, amine acetylation, as integral step of the sample-preparation method, prevented this effect that would reduce the level of analyte captured on the affinity support.

MATERIALS AND METHODS

TFA, formic acid, *N*-hydroxysulfosuccinimide ester of acetic acid (sulfo-NHS acetate), sulfosuccinimidyl-2-(biotinamido) ethyl-1,3-dithiopropionate (EZ-Link Sulfo-NHS-SS-Biotin), hydroxylamine hydrochloride, Bond Breaker Tris(2-carboxyethyl)phosphine hydrochloride (TCEP) solution (0.5 M), Spin Columns-Screw Caps (0.8 ml internal volume), and GelCode Blue Stain Reagent were obtained from Pierce (Rockford, IL, USA). 2-Aminoethanethiol hydrochloride, barium hydroxide octahydrate, and ammonium hydrogen carbonate were from Sigma-Aldrich (St. Louis, MO, USA). Alkaline phosphatase (AP), from calf intestine (20 U/ μ l), and *N*-octyl glucoside (OGS) were obtained from Roche Diagnostics (Indianapolis, IN, USA). Hydrogen peroxide (H₂O₂) 30% (vol/vol), ammonium hydrogen carbonate, and sodium phosphate dibasic dodecahydrate were purchased from Fluka (Ronkonkoma, NJ, USA). Methanol and acetonitrile were from Burdick & Jackson (Muskegon, MI, USA). DTT, iodoacetamide, sodium phosphate monobasic monohydrate, 1 M Tris-HCl/0.1 M EDTA buffer, pH 8.0, sodium chloride, sodium carbonate, bovine α -S1-casein, bovine β -casein, HSA, horse heart myoglobin, β -lactoglobulin from bovine milk, carbonic anhydrase from bovine erythrocytes, OVA, human angiotensin I acetate salt hydrate [DRVYIHPFHL, mass:charge ratio (*m/z*) 1296.5], human fibrinopeptide B (pyrGVND-NEEGFFSAR, *m/z* 1552.5), the somatostatin fragment 3–10 (CKNFFWKT, *m/z* 1073.2), the monophosphorylated peptide T6 of β -casein, and the tetraphosphorylated

peptide of β -casein were purchased from Sigma-Aldrich. Activated thiol sepharose 4B was from GE Healthcare (Piscataway, NJ, USA). α -Cyano-4-hydroxycinnamic acid was from Agilent Technologies (Palo Alto, CA, USA). Trypsin (modified sequencing grade) was purchased from Promega (Madison, WI, USA). Polyacrylamide gels (Criterion precast gel, 1 mm, 10%) were from Bio-Rad (Hercules, CA, USA). ZipTip_{C18} pipette tips (0.6 μ l bed volume) and ZipTip _{μ -C18} pipette tips (0.2 μ l bed volume) were purchased from Millipore (Billerica, MA, USA). OMIX pipette tips (1 μ l bed volume) were obtained from Varian (Walnut Creek, CA, USA). The phosphorylated cholecystokinin fragment, residue 10–20 (IKNLQpSLDPSH, m/z 1331.4), was obtained from Protea Biosciences (Morgantown, WV, USA). The O-linked β -N-acetylglucosamine (O-GlcNAc)-modified peptide SVES(O-GlcNAc)GSADAK, m/z 1152.2, was custom-synthesized by Sussex Research Laboratories (Ottawa, Ontario, Canada). The model peptides P1, RADpSHEGEVA, m/z 1150.4; P2, SHNSALYpSQVQK, m/z 1441.2; and P3, GIKSHNpSALYSQVQK, m/z 1739.5, were synthesized in-house. The UOM9 PKC substrate-3 phosphopeptide (KRpSQRHGSKY amide, m/z 1422.5); the DAM1, outer kinetochore protein DAM1 phosphopeptide (SFVLNPTNIGMpSKSSQGH-VTK, m/z 2312.6); and the myristoylated alanine-rich PKC substrate (MARCKS) phosphopeptide (KKKKKRFpSFKKpSFKLSGFpSFKKNKK, m/z 3321.7), were obtained from AnaSpec (San Jose, CA, USA).

In-Gel Performic Acid Oxidation

GelCode Blue-stained bands, from 5 to 50 pmol protein, were destained, reduced, dehydrated in acetonitrile, and dried briefly, as described below. Performic acid reagent was prepared by mixing 30% aqueous H₂O₂ with 96% formic acid (5:95, vol/vol). After 30 min at room temperature, the reagent was diluted 1:9 with water and the dried bands rehydrated for 10 min in a 20- μ l reagent at 4°C. The reaction was continued for 50 min at 4°C, with an additional 40 μ l reagent. After incubation, the supernatant was removed and the bands washed three times for 15 min with 500 μ l water with agitation. The supernatant and the combined washes were taken to dryness and subjected to tryptic digestion to assess potential protein loss. The performic acid-treated gel bands were equilibrated twice for 10 min at room temperature with 200 μ l 25 mM ammonium bicarbonate, dehydrated for 10 min in 100 μ l acetonitrile, dried in a SpeedVac, reswollen in 20 μ l 25 mM ammonium bicarbonate/0.01% OGS containing 50–400 ng Promega-modified trypsin, and processed for tryptic digestion and peptide extraction as described below.

Protein In-Gel Proteolytic Digestion

The procedure, as described previously, was used, except that the alkylation step was omitted from the protocol.³⁰ GelCode Blue-stained bands, from 5 to 50 pmol protein loaded onto the gels, were destained twice with 200 μ l 25 mM ammonium bicarbonate in 50% aqueous acetonitrile for 30 min at 37°C. Bands were dried briefly in a SpeedVac and incubated for 15 min at 37°C in 100 μ l 2 mM TCEP/25 mM ammonium bicarbonate; the supernatant was then removed, leaving the bands diffused with the reductant. Bands were then dehydrated for 10 min in 100 μ l acetonitrile and dried briefly in a SpeedVac. Dried gel bands were reswollen in 20 μ l 25mM ammonium bicarbonate/0.01% OGS containing 50–400 ng Promega-modified trypsin. After 20 min reswelling at room temperature, 40 μ l 25mM ammonium bicarbonate was added, and the digestion continued for 18 h at 37°C. After incubation, 50 μ l 0.1% TFA was added. The supernatant was removed, and another 50 μ l 0.1% TFA was added. The gel bands were incubated for 30 min 37°C. The combined extracts were reduced in volume to 35 μ l and acidified by addition of 5 μ l 10% TFA before solid-phase immobilization, as described below.

Protein In-Solution Proteolytic Digestion

In-solution tryptic digestion was performed in 40 μ l 25 mM ammonium bicarbonate/0.01% OGS, typically at an enzyme-substrate ratio of 1/100. After 18 h incubation at 37°C, the digests were acidified with 5 μ l 10% TFA before solid-phase immobilization, as described below.

Sample Immobilization

ZipTip_{C18} pipette tips and ZipTip _{μ -C18} pipette tips were wetted six times with 10 μ l methanol, followed by six washes with 0.1% TFA, following the manufacturer's instructions. Model peptides (10–100 pmol), prepared in 100 μ l 1% TFA/0.01% OGS solutions were transferred in 10 μ l aliquots into 0.5 ml microfuge tubes and subjected to 10 sample aspiration-dispense cycles (for each cycle, the 10- μ l aliquot was withdrawn and dispensed once). The pipette tips loaded with the immobilized peptides were then washed three times to waste with 10 μ l 0.1% TFA and once with 10 μ l water. Dilute samples (i.e., some peptides and all in-gel digests and fractions recovered from the affinity resin) were aspirated sequentially in 10 μ l aliquots onto ZipTip_{C18} pipette tips and dispensed into a 0.5-ml microfuge tube. The partially stripped peptide solution was then transferred back in this manner into the original sample tube. This enrichment cycle was repeated five times to maximize peptide recovery. The ZipTip pipette tips were then washed five times with 10 μ l 0.1% TFA before derivatization. For more dilute samples, ZipTips were

press-fitted onto 200 μ l pipette tips and operated with a 200- μ l pipette, as suggested by the manufacturer, using 10–20 aspiration dispense cycles. Up to 200 μ l sample can be processed for binding in this manner.³⁷

General Procedure for Solid-Phase Peptide Derivatization

The various reagents (10 μ l) were aspirated three times onto the ZipTip_{C18} pipette tips or ZipTip _{μ -C18} pipette tips and dispensed to waste. The reagents (10 μ l) were then loaded onto the tips from 60 μ l that had been placed into 0.5-ml microfuge tubes. During incubation, the tips were left immersed in the reagents. The tips were desalted subsequently by a solvent wash, passed over the resin, as specified below. The products were eluted in 5–10 μ l 50% acetonitrile/0.1% TFA/0.01% OGS, of which 1 μ l was typically used for MALDI-MS analysis. ZipTip _{μ -C18} pipette tips were eluted in matrix, supplemented with 0/1% TFA onto the target.

On-Column Performic Acid Oxidation

The reagent was prepared by mixing 30% aqueous H₂O₂ with 96% formic acid (5:95, vol/vol). The reagent was left standing at room temperature for 30 min and then diluted 1:10 with water (pH 3.1). ZipTip_{C18} pipette tips were loaded with 10 μ l reagent and incubated for 1 h at 4°C. To halt the reaction, the tips were washed 10 times with 10 μ l water and then five times with 0.1% TFA. Sample processing for MALDI-TOF was as described above.

On-Column Acetylation

A 20-mM sodium phosphate buffer, pH 8.0, was supplemented with 20 mM sulfo-NHS acetate. ZipTip_{C18} pipette tips loaded with 10 μ l reagent were incubated for 20 min at 55°C and then washed 10 times with 10 μ l 0.1% TFA. Sample processing for MALDI-TOF was as described above.

On-Column β -Elimination with Concurrent Michael Addition

The reagent mixture was prepared by mixing 100 μ l aqueous 100 mM barium hydroxide with 50 μ l aqueous 100 mM 2-aminoethanethiol hydrochloride to give a final concentration of 66 mM barium hydroxide and 33 mM 2-aminoethanethiol (pH 12.3). In some experiments, 2-aminoethanethiol was substituted for DTT in the reagent mixture. Barium hydroxide was finely ground to facilitate dissolution by vigorously vortexing for 1.5 min. The stock solution (31.54 mg/ml) was then centrifuged for 1 min at 13 000 rpm to remove carbonate precipitates. The barium hydroxide and the 2-aminoethanethiol hydrochloride stock solution (11.36 mg/ml) were prepared fresh for daily use.

The ZipTip_{C18} pipette tips were loaded with 10 μ l of the reagent mixture, incubated for predetermined times at 37°C or 55°C. After incubation, the tips were washed 10 times with 10 μ l 0.1% TFA and prepared for MALDI-TOF analysis, as described above.

On-Column β -Elimination with Consecutive Michael Addition

The β -elimination reaction was carried in 10 μ l 50 mM barium hydroxide (pH 12.6) for 30 min at 55°C. ZipTip_{C18} pipette tips were subsequently flushed three times to waste with 10 μ l of the Michael addition reagent, prepared by mixing 100 mM aqueous barium hydroxide with 400 mM aqueous 2-aminoethanethiol hydrochloride (45.44 mg/ml) in a 3:1 ratio (100 mM 2-aminoethanethiol/75 mM barium hydroxide, pH 10.6). The addition reaction was allowed to proceed in 10 μ l of the reagent mixture, loaded onto the support for 2–3 h at 55°C. The reaction was halted by passing 100 μ l 0.1% TFA in 10 μ l aliquots over the resin. The tips were prepared for MALDI-TOF analysis, as described above.

On-Column Amino Group Thiolation

A 20-mM sodium phosphate buffer solution, pH 8.0, was supplemented with 20 mM Sulfo-NHS-SS-Biotin. ZipTip_{C18} pipette tips were incubated in 10 μ l reagent for 30 min at room temperature and then washed 10 times with 10 μ l 0.1% TFA. Sample processing for MALDI-TOF was as described above.

On-Column O-Deacylation

Hydroxylamine hydrochloride was dissolved in 1 M sodium carbonate, pH 9.4, to a final concentration of 2%. ZipTip_{C18} pipette tips were incubated in 10 μ l reagent for 15 min at 37°C, washed 10 times with 10 μ l 0.1% TFA, and then five times with 10 μ l water. Sample processing for MALDI-TOF was as described above.

On-Column Disulfide Reduction

A 20-mM sodium phosphate solution, pH 8.0, was supplemented with 5 mM TCEP. Reagent (10 μ l) was loaded onto the ZipTip_{C18} pipette tips. After incubation for 15 min at 37°C, the tips were washed 10 times with 10 μ l of a 2-mM aqueous EDTA solution and then 10 times with 10 μ l 0.1% TFA. Samples were processed for MALDI-TOF, as described above.

On-Column Carboxyamidomethylation

ZipTip_{C18} pipette tips were loaded with 10 μ l of a 20-mM iodoacetamide solution in 25 mM sodium phosphate buffer, pH 8.0, incubated for 30 min at 37°C, and then desalted by passing 100 μ l 0.1% TFA in 10 μ l aliquots over

the resin. Samples were prepared for MALDI-TOF, as described above.

Consecutive On-Column Derivatization: Sample Preparation for Thiol-Directed Affinity Isolation

Performic acid oxidation, acetylation, BEMAD, *N*-thiolation, *O*-deacylation, and disulfide-bond reduction were combined into the sequential reaction scheme, as outlined in Fig. 1. The intermittent, desalting steps before reagent loading were as specified for the individual chemistries described above, except that sample clean-up after acetylation was omitted. Instead, the acetylation step was terminated by flushing the ZipTips three times with 10 μ l 66 mM barium hydroxide/33 mM 2-aminoethanethiol for concurrent BEMAD or with 50 M barium hydroxide when the consecutive reaction mode was used. Immobilized digests from in-gel-oxidized proteins were flushed five times with 10 μ l 0.1% TFA. After thiolation, the desalted ZipTips can be stored at least overnight at -20°C in 10 μ l 0.1% TFA, while leaving the ZipTips immersed in solvent. At the conclusion

of the reaction scheme, care must be taken to remove the reductant thoroughly from the support by sequentially passing 100 μ l aqueous 2 mM EDTA and 100 μ l 0.1% TFA in 10 μ l aliquots over the resin. The final reaction products were eluted in 10 μ l 50% acetonitrile/0.1% TFA/0.01% OGS, directly into 40 μ l 50 mM sodium phosphate/2 mM EDTA, pH 8.0, used as coupling buffer for covalent chromatography (see Covalent Chromatography).

Covalent Chromatography

Activated thiol sepharose (1 g) was allowed to swell for 10 min in 10 ml water. The gel was washed by vacuum filtration with a total of 150 ml water added in 15 ml aliquots, suspended in 10 ml 10% aqueous ethanol, and stored, refrigerated, until use. A 75% gel slurry (100 μ l) was placed into a spin column that had been washed with 200 μ l acetonitrile by centrifugation at 1500 rpm for 1 min before use, the centrifugation speed and time setting that was used throughout the protocol. The affinity medium was centrifuged, resuspended in 200 μ l 50 mM sodium

FIGURE 1

(A) Flow chart of phosphopeptide sample preparation. The individual solid-phase reaction steps are numbered and highlighted with boxes. After solid-phase extraction (SPE) on ZipTip_{C18} or ZipTip_{μ-C18} pipette tips, the phosphoprotein digest is subjected to performic acid oxidation to preclude co-isolation of cysteinyl peptides (1). Alternatively, the reactivity of this residue is arrested by in-gel performic acid oxidation, followed by in-gel digestion and peptide adsorption to the reversed-phase support. The oxidized phosphoprotein digest is subjected to α - and ϵ -amino group acetylation (2) and subsequently, to barium hydroxide-catalyzed BEMAD using 2-aminoethanethiol as nucleophile (3). The amine-blocking step renders the substituted serine and threonine residues as the sole target for the subsequent acylation with sulfo succinimide-2-(biotinamido)-ethyl-1,3-dithiopropionate (4). In the subsequent steps, the digest is exposed to hydroxylamine for reversal of unwanted hydroxyl group acylation (5), followed by reductive release of the disulfide-linked biotinamido moiety from the derivatives (6). After sample transfer to activated thiol Sepharose, the phosphopeptides' thiolated analogs are reversibly enriched from the digest by covalent chromatography (7). AP denotes the AP reaction step. (B) Representation of the structure of the analyte and its derivatives. The reaction steps are indicated in boxes. The affinity tag is highlighted in red.

phosphate/2 mM EDTA, pH 8.0, or in 50 mM Tris-HCl/5 mM EDTA, pH 8.0, followed by centrifugation; the flow-through was discarded. The spin column was then sealed with the plastic plugs supplied by the manufacturer. The thiolated sample was eluted with 10 μ l 50% acetonitrile/0.1% TFA/0.01% OGS, directly into 40 μ l 50 mM sodium phosphate/2 mM EDTA, pH 8.0, or in 50 mM Tris-HCl/5 mM EDTA, pH 8.0, used as thiol-coupling buffer. The mixture was transferred to the spin column, which was then gently agitated to resuspend the pellet. The spin column was capped, inserted into 1.5-ml microfuge tubes, secured with Parafilm, and end-over-end-incubated for 1 h at room temperature using a rotary mixer and subsequently centrifuged to recover unbound material. The affinity medium was then washed consecutively with 50 μ l of the thiol-coupling buffer, 50 μ l 60% aqueous acetonitrile/0.1% TFA, and 50 μ l 80% aqueous acetonitrile/0.1% TFA. The combined flow-through fractions were reduced in volume by SpeedVac evaporation to 35 μ l, acidified with 5 μ l TFA to a final concentration of 1%, and stored, refrigerated. The affinity medium was then washed with 50 μ l thiol-coupling buffer to neutralize residual TFA. The flow-through was discarded. For analyte release, 50 μ l of the coupling buffer containing 5 mM TCEP was added to the sealed spin column, which was then incubated for 30 min at room temperature with end-over-end mixing. In some experiments, 10 μ l 300 mM iodoacetamide was added to the spin column to a final concentration of 50 mM. The incubation was continued for 30 min at room temperature with end-over-end mixing. The enriched thiol peptide derivatives, with and without alkylation, were recovered from the affinity support by centrifugation, which was then washed with 50 μ l of the reductant. Carboxyamidomethylated samples were washed with coupling buffer. The affinity resin was subsequently washed with the organic solvents, as described above. The combined fractions were reduced in volume to 35 μ l and acidified with 5 μ l 10% TFA to a final concentration of 1%. The enriched as well as the nonbound material was subsequently immobilized on the reversed-phase supports. ZipTip_{C18} pipette tips were washed with 100 μ l 0.1% TFA, passed over the resin in 10- μ l aliquots. Peptides were eluted from the support with 10 μ l 50% acetonitrile/0.1% TFA/0.01% OGS before MALDI-TOF analysis. Low-level amounts of analyte were enriched with micro ZipTips, washed 10 times with 10 μ l aliquots of 0.1% TFA, and deposited in matrix containing 0.1% TFA directly onto the MALDI target.

MS

A Voyager-DE STR (Applied Biosystems, Foster City, CA, USA) was used and operated in the reflector mode at an accelerating voltage of 20 kV. Laser intensity was typically

set at 1690–2400, and spectra were acquired using 100–200 laser shots/spectrum. The data shown are based on three accumulated acquisitions. Some spectra were obtained in the linear mode using 80 laser shots/spectrum, and five to 10 spectra acquired from spots at different positions were averaged. For peptide fragmentation, a 4800 Proteomics Analyzer was used and operated in the MS and MS/MS modes; typical laser power for MS was $\sim$ 3700; for MS/MS, $\sim$ 4500. Usually 1000–2000 shots were acquired for MS; 2000–20,000 for MS/MS.

RESULTS AND DISCUSSION

Reaction Scheme

Sample handling during sequential derivatization is exceedingly simple involving analyte adsorption on the reversed-phase C18 pipette tips, followed by solvent washes to remove unwanted matrix components. The tips are then loaded with reagents and left immersed in reagent during incubation. The subsequent reaction steps are initiated by on-phase reagent exchange, eliminating sample transfer between the reaction steps. The reaction cycle is concluded by a clean-up step, followed by product elution.

As schematically illustrated in Fig. 1, the consecutive on-column sample-preparation scheme is initiated by subjecting the phosphopeptides or phosphoprotein digest to on-column performic acid oxidation (Step 1). Alternatively, the reactivity of the sulfhydryl group is arrested by protein in-gel performic acid oxidation, followed by in-gel digestion. The oxidized digest is then subjected to α - and ϵ -amino group acetylation (Step 2) and then to BEMAD using barium hydroxide as an elimination base and 2-aminoethanethiol as a nucleophile (Step 3). The amine-blocking step renders the amine-functionalized serine and threonine residues as sole targets for reaction with sulfosuccinimidyl-2-(biotinamido)-ethyl-1,3-dithiopropionate (Step 4). The digest is subsequently exposed to hydroxylamine for reversal of unwanted hydroxyl group acylation (Step 5). The biotin-amido moiety, along with its linker arm, is then reductively released from the derivatives (Step 6), which are subsequently isolated from the digest by covalent chromatography (Step 7). The use of dephosphorylation by AP for selective affinity purification of *O*-glycosylated- or *O*-sulfonated peptides is discussed below.

Sample Handling

As discussed in an earlier report and in the companion article,^{30,31} the solid-phase derivatization format offers some notable advantages over the classical in-solution-based methods, the predominant sample-preparation technique in current proteomic studies. In brief, the solid-phase strategy obviates the need for sample dry-down, commonly practiced to concentrate peptide mixtures before de-

rivatization in solution-based proteomics studies solution. As noted for low-level samples (<2 pmol), this sample-handling step can cause substantial adsorptive peptide loss, ranging up to 50% or more of the starting solution after a single evaporation step.³⁸ The process of sample adsorption concentrates the analyte on the support, allowing the reaction to proceed *in situ*, in general, at higher efficiency and faster kinetics than in solution.^{30,31,39,40} Protein digests can be enriched effectively on the solid phase from dilute solutions, in which chemical reactions inherently proceed at slow reaction rates. As demonstrated in this report, in the companion article, and in an earlier communication,⁴¹ the solid-phase format proved particularly advantageous for combining distinct chemistries into serial reaction schemes. Classical desalting methods, such as microdialysis, reversed-phase HPLC, and centrifugal sample concentration, which were used initially to remove excess reagent from the intermediate- and final-reaction products of in-solution serial derivatization, resulted in substantial adsorptive sample loss.^{38,42,43} Also, the use of intermittent, reversed-phase-coated pipette-tip extraction was met with limited success. As assessed by a stable isotope-dilution technique, an average of 20% of the bound sample could not be recovered in the eluted fractions (see Fig. 5 and companion article³¹), whereas once the starting material was bound to the reversed-phase support, this cumulative sample loss was avoided by on-phase reagent exchange, eliminating intermittent sample-transfer steps. As a result, the analyte could be carried through the multistep reaction scheme with minimal sample loss, providing a significant advantage in sample limited situations. The use of solid-phase derivatization has evolved as the predominant sample-preparation technique for trace analysis of bioorganic compounds and has found wide-spread application in pharmaceutical and toxicological studies.^{32,33}

Test peptide solutions used in our study were supplemented with trace amounts of OGS, a MALDI-TOF-compatible nonionic detergent, to minimize peptide adsorption to plastic surfaces during sample immobilization.⁴⁴ OGS, in low concentration (0.005–0.01%), has also been shown to be fully compatible with LC-MS, owing to its strong retention on reversed-phase columns.^{44,45}

Evaluation of β -Elimination-Based Affinity Purification using DTT as Nucleophile

In this procedure, phosphoprotein digests are subjected to BEMAD for 1 h at 37°C, using DTT as a Michael donor in the Ba^{2+} -catalyzed concurrent reaction scheme.¹⁷ The resultant mercaptobutanediolcysteine derivatives are then purified by covalent chromatography. Because of the inherent advantages of the immobilization approach, we were interested in adapting this in-solution procedure to the

on-column method. To test this, the monophosphorylated tryptic fragment (FQpSSEQQQTEDELQDK, m/z 2061.5) and the tetraphosphorylated tryptic fragment β -casein (RELEELNVPGEIVEpSLpSpSpSEESITR, m/z 3122.2) were selected as model peptides. The latter peptide was barely discernible in MALDI-TOF spectra, even at high sample loads but became clearly detectable in its β -eliminated form, consistent with previous results.³¹ Unexpectedly, the acetylated dehydroalanyl derivative ionized in MALDI-MS more efficiently than its unmodified counterpart. The on-resin acetylation conditions (20 mM sulfo-NHS acetate in a buffered sodium phosphate solution, pH 8.0, for 20 min at 55°C) were shown previously to be highly effective for acetylation of protein digests bound to reversed-phase supports (see accompanying report³¹). The acetylation reagent could be then replaced on the ZipTip_{C18} pipette tips by the β -elimination base, 66 mM barium hydroxide, and allowed to proceed at 37°C for 30 min and for 1 h. As assessed by MALDI-TOF MS, the elimination product was fully formed after 30 min incubation and then used as a control to monitor the reactions described in the experiments below (Fig. 2A).

After the 1-h incubation at 37°C in 66 mM barium hydroxide/33 mM DTT, the ZipTip_{C18} pipette tips, loaded with the monophosphorylated and the modified tetraphosphorylated peptides, were washed briefly. The reaction products were eluted and analyzed by MALDI-TOF. The MALDI-TOF spectra showed that the monophosphorylated species at m/z 2061.2 reacted to completion (Fig. 2B, inset). The derivatization of the multiply phosphorylated peptide gave rise to the dominant ion at m/z 3080.4 (Fig. 2B). The resultant mass addition of 308 Da, imparted on the β -elimination product at m/z 2772.3, indicates that two of its phosphates had been substituted by the nucleophile. Only a minor fraction of the peptide was derivatized further, resulting in the formation of the triply substituted species at m/z 3234.6 (Fig. 2B, arrow). Prolonged incubation (2 h) at the same or at elevated temperature (55°C) did not improve the reaction yield (data not shown). To gain insight in kinetics of the reaction, the ZipTip tip-bound derivatives were incubated for 30 min at 37°C in 20 mM iodoacetamide/25 mM sodium phosphate buffer, pH 8.0, a procedure that we found highly effective to alkylate thiol-containing peptides (e.g., somatostatin). The MALDI-TOF spectra illustrated in Fig. 3C revealed that the triply substituted species at m/z 3234.6 shifted in mass by 114 Da, indicating the presence of two reactive thiols, whereas the predominant derivative proved impervious to alkylation. When the derivatized peptides were submitted to covalent chromatography (see Fig. 7), the DTT adduct of the monophosphorylated peptide was retained effectively by the affinity support (data not shown),

FIGURE 2

Evaluation of β -elimination-based affinity purification using DTT as a nucleophile. The monophosphorylated tryptic fragment $^{48}\text{FQpSEEQQTEDELQDK}^{63}$ and the acetylated tetraphosphorylated tryptic fragment of β -casein $^{16}\text{RELEELNVPGEIVEpSLpSpSpSEESITR}^{40}$, bound to Ziptip_{C18} pipette tips, were loaded with 66 mM barium hydroxide/33 mM DTT or with 66 mM barium hydroxide/33 mM 2-aminoethanethiol. After incubation for 1 h at 37°C, the reaction products were eluted and analyzed by MALDI-TOF. The acetylated tetraphosphorylated tryptic fragment was incubated in parallel in 66 mM barium hydroxide for 30 min or 60 min at 37°C. MALDI-TOF spectra of (A) acetylated tetraphosphorylated fragment after 30 min β -elimination; (B) acetylated tetraphosphorylated fragment after β -elimination with concurrent Michael addition using DTT as nucleophile; and (C) after iodoacetamide treatment of the Michael addition reaction products illustrated in B. Note that the monophosphorylated peptide was fully converted to its mercapto derivative at m/z 2118.2 (B, inset). Note that the tetraphosphorylated fragment's reaction product at m/z 3080.4 (B) proved impervious to alkylation (C). Asterisk and arrow in B designates single-substituted derivative at m/z 2926.3 and the triple-substituted derivative at m/z 3234.6, respectively. Asterisk and arrow in C denote the carboxyamidomethylated products of these species, respectively. Reaction products recovered in B were submitted to covalent chromatography (see Fig. 7). (C, inset at left margin) Spectrum of the flow-through fraction. Note that the derivatives, owing to intrapeptide cross-link formation, were not retained by the thiol affinity support. (C, inset at right margin) The location of the cross-links within the peptide's sequence, as determined by MS/MS. Dha, Dehydroalanine; C, mercaptobutanediolcysteine. One-tenth of the eluates corresponding to ~ 5 pmol peptide was applied to the target.

whereas its tetraphosphorylated counterpart was recovered in the flow-through fraction (Fig. 2C, inset at left margin). This result was expected, as this product proved resistant to alkylation. Collectively, the data indicate that intramolecular Michael addition had occurred within the peptide's multiply phosphorylated region, resulting in covalent-bond formation. This interpretation is consistent with the kinetics of the concurrent BEMAD, which renders the nucleophilic substitution the time-limiting step of the overall reaction.¹⁵ During this time lag, the intrapeptide Michael addition of mercaptobutanediol cysteine to dehydroalanine was favored heavily over attack on dehydroalanine by the exogenous nucleophile. MALDI TOF-TOF analysis of

the fragment unambiguously assigned the location of mercaptobutanediol cysteine, the Michael donor, by the unique residue mass of 223 Da to Positions 30 and 32 and the targeted dehydroalanine bearing the characteristic mass signature of 69 Da to Positions 33 and 34 (Fig. 2C, inset at right margin). A nearly uninterrupted y and b ion series was observed in the spectrum, albeit produced from the cross-linked region at relatively low abundance, indicating that the covalent bonds were resistant to fragmentation (data not shown). By analogy, disulfide fragmentation has been shown to occur with much lower efficiency than cleavage of the backbone peptide bonds under the conditions of low-energy CID.⁴⁶ No difference in chemical reactivity among

FIGURE 3

Sample preparation for covalent chromatography: peptide acetylation coupled to β -elimination with concurrent Michael addition. The model phosphopeptide (P2, SHNSALYpSQVQK, m/z 1441.5) bound to ZipTip_{C18} pipette tips was incubated in 20 mM sodium phosphate solution, supplemented with 20 mM sulfo-NHS acetate (pH 8.0) for 20 min at 55°C. The acetylation reagent was exchanged on the solid phase for 66 mM barium hydroxide/33 mM 2-aminoethanethiol, and the incubation continued at 37°C for 1 h. Samples were eluted after a solvent wash, and eluates were analyzed by MALDI-TOF. MALDI-TOF spectra of (A) unmodified peptide; (B) after acetylation; (C) after acetylation, followed by β -elimination with concurrent Michael addition. Note the predominant ion at m/z 1567.6 representing the triple-acetylated species. Arrow and open arrow in B denote the double-acetylated species at m/z 1525.6 and the quadruple-acetylated species at m/z 1609.6, respectively. Asterisks in B and C denote reaction products of a peptide synthesis byproduct recognized in A at m/z 1274.5. Note effective reversal of *O*-acylation of the product at m/z 1567.6 and of the product at m/z 1609.6 in the alkaline β -elimination/Michael addition medium and resultant formation of the predominant ion at m/z 1504.9, corresponding to the *N*-acetylated 2-aminoethylcysteine derivative. PSD, Postsorce decay products. One-tenth of the eluates corresponding to ~ 2 pmol peptide was applied to the target. (C, inset) Sequential experiment with 200 fmol peptide loaded onto ZipTip _{μ -C18} pipette tips. The reaction products were eluted in matrix directly onto the target.

the multiple sites and thus, no cross-link formation were observed when 2-aminoethanethiol was used as a nucleophile. The S-2-aminoethylcysteine derivative was fully formed, consistent with previous data,^{31,47} and proved highly susceptible to subsequent acetylation (data not illustrated).

Under the described conditions, the intramolecular linkage occurred in high yield, suggesting that the nucleophile was present predominantly in its most reactive, anionic form. However, the ionization potential of mercaptobutanediol cysteine and hence, the efficiency of cross-link formation may be subject to modulation by the surrounding amino acid mi-

croenvironment. Clearly, further studies with model phosphopeptides, currently pursued in our laboratories, are needed to identify sequence characteristics that may impact the intramolecular Michael addition's reaction rate. Furthermore, the propensity of a peptide to form cross-links is evidently dictated by the distance between phosphorylation sites to be bridged by the cross-linker's spacer arm (8.2 Å). The α -carbons of adjacent amino acid residues are 3.8 Å apart; hence, the distance of more than two amino acids between the reactants would exert constraint on cross-link formation. A statistical assessment of phosphosite distribution of the $\sim 51,000$ nonredundant sites from unique

eukaryotic proteins revealed that a significant fraction (32–37%) of phosphoserine/phosphothreonine sites were, such as observed in the tetraphosphorylated fragment of β -casein, separated from each other by one or two amino acids residues, a distance well within the reach of the intermolecular nucleophilic attack.⁴⁸ The strong tendency for phosphorylation sites to cluster in such close proximity suggests that a subpopulation of these phosphopeptides is likely excluded from isolation by the dithiol-based affinity-enrichment approach. This method has been used more recently to profile the phosphoproteome of human whole saliva.²⁹ The isolates contained mostly single phosphorylated peptides. It is noteworthy that the PAC also exhibited a strong bias against multiple phosphorylated species selection. This method has been used extensively for large-scale phosphoproteome profiling of *D. melanogaster* Kc167 cells.^{22–24} As proof of method, a tryptic digest of β -casein was examined.²² Notably, the isolation of the protein's tetraphosphorylated tryptic species was not demonstrated in this report.

Solid-Phase Sample Preparation for Phosphopeptide Enrichment by Covalent Chromatography

Phosphopeptide acetylation coupled with concurrent β -elimination/Michael addition

The reaction scheme devised to address the shortcoming of the dithiol-based phosphopeptide-enrichment strategy is centered on exploiting the BEMAD to introduce into phosphopeptides a thiol-functionalized affinity tag. As noted previously, all amino groups in the protein digests need to be initially blocked to render the substituted serine and threonine residues as the sole targets for the subsequent amine-directed chemical modification. Representative data from this two-step reaction scheme are shown in Fig. 3, using P2 (SHNSALY**p**SQVQK, m/z 1441.2) as a model peptide. The peptide was adsorbed on ZipTip_{C18} pipette tips and reacted with sulfo-NHS acetate in a buffered sodium phosphate solution (pH 8.0) under the conditions described in Fig. 2A. The acetylation reaction was terminated by a brief solvent wash, followed by product elution and MALDI-TOF analysis of the recovered fractions. In a parallel experiment, the acetylation reaction was halted by replacing the reagent *in situ* with 66 mM barium hydroxide/33 mM 2-aminoethanethiol. The β -elimination with concurrent Michael addition was allowed to proceed for 1 h at 37°C. The tips were then washed briefly and eluted and the eluates submitted to MALDI-TOF. The MALDI-TOF spectra of the acetylated samples revealed a set of ions that was distinguishable by the mass increments of 42 Da, as doubly, triply, and quadruply acetylated species observed at

m/z 1525.6, at m/z 1567.6, and at m/z 1609.6, respectively, indicating that in addition to the desired *N*-acetylation of the peptide's N- and C-terminal amines giving rise to the product at m/z 1525.6, *O*-acetylation had occurred to a considerable degree (Fig. 3B). This side-reaction has been shown to be promoted by sequence features, such contained in the peptide examined, in which histidine is located in close proximity to serine, threonine, or tyrosine.⁴⁹ As shown in Fig. 3C, this unwanted side-reaction was fully reversed by alkali-induced ester-bond hydrolysis upon subsequent BEMAD, resulting in the formation of the dominant ion at m/z 1504.9, corresponding to the *N*-acetylated S-2-aminoethylcysteine derivative. As illustrated further in Fig. 3C, a small fraction of the triply acetylated peptide (~10%) proved resistant to the sequential reactions, and extended incubation is expected to drive the conversion to completion. The low-intensity ion at m/z 1385.9 represents the thiol adduct of the peptide's synthesis byproduct, observed in the starting material at m/z 1274.5. The data showed that the acetylation reaction and subsequent thiol adduct formation proceeded to near completion. To explore the overall mass sensitivity of the method, 200 fmol peptide was immobilized on ZipTip_{μ-C18} pipette tips and carried through the sequential reaction scheme, which could be completed in <3 h. The eluates were deposited directly in matrix onto the MALDI plate. The high quality of the spectrum suggests that lower quantities of peptide should be amenable to derivatization (Fig. 3C, inset).

Additional model peptides examined with the method were the DAM1 phosphopeptide SFVLNPTNIGM**p**-SKSSQGHVTK, m/z 2312.6; the phosphorylated cholecystokinin fragment IKNLQ**p**SLDPSH, m/z 1331.4; and the MARCKS phosphopeptide KKKKKRF**p**SFKK**p**SFKLSGF**p**SFKKNKK, m/z 3321.7, of which the former two peptides reacted to completion. The MARKS phosphopeptide, containing 13 acylation targets, was recovered at a ~70% efficiency, as its fully acetylated 2-aminoethylcysteine analog (results not illustrated).

Peptide thiolation

Our sample-preparation strategy uses a thiolation step to introduce a disulfide moiety into the analyte (see Fig. 1). Sulfosuccinimidyl-2-(biotinamido)-ethyl-1,3-dithiopropionate, commonly used for protein and peptide biotinylation,⁴⁹ was evaluated for this purpose using angiotensin I (DRVYIHPFHL, m/z 1296.5) as a model peptide. In the experiments, the peptide was immobilized on ZipTip_{C18} pipette tips and exposed to 20 mM Sulfo-NHS-SS-Biotin in a sodium PBS (pH 8.0). After 30 min incubation at room temperature, the reaction was halted by a solvent wash. The reaction product was eluted and submitted to

FIGURE 4

Sample preparation for covalent chromatography: sequential peptide biotinylation, *O*-acylation reversal, and reductive release of thiol derivative. Angiotensin I (DRVYIHPFHL, m/z 1296.9) was immobilized on the ZipTip_{C18} pipette and incubated with 20 mM Sulfo-NHS-SS-Biotin in a sodium phosphate-buffered solution (pH 8.0) for 30 min at room temperature. The biotinylated peptide was then exposed for 15 min at 37°C to an alkaline 2% hydroxylamine solution (pH 9.4), followed by incubation in 20 mM sodium phosphate solution, supplemented with 5 mM TCEP (pH 8.0). MALDI-TOF spectra of (A) unmodified peptide; (B) after biotinylation; (C) after *O*-deacylation; and (D) after disulfide reduction. Mass shifts accompanying the reactions are highlighted with boxes. Open arrow in B denotes *O*-acylated peptide. Note that this side-reaction was reversed effectively by hydroxylamine treatment. Note that a small fraction of the thiol derivative at m/z 1385.5 is prereleased during the initial reaction (arrow in B). Asterisks in B and D designate the reaction product of a peptide synthesis byproduct and partially regenerated peptide, respectively. Note that the sequential reaction scheme may be used to introduce thiol-reactive stable isotope-coded labels into peptides (see text and Fig. 7 for details). One-tenth of the eluates corresponding to ~2 pmol peptide was allied to the target.

MALDI-TOF, along with untreated controls. The MALDI-TOF spectra, illustrated in Fig. 4A and B, show that the peptide reacted to near completion. Incubation at 37°C over the same time period or extended incubation at room temperature markedly promoted hydroxyl group acylation (see below). The mass difference of 398 Da between the starting material at m/z 1296.9 and its biotinylated counterpart at m/z 1686.4 was in accord with the expected mass addition. Furthermore, the native peptide and its derivative ionized in MALDI-MS at comparable efficiency. As illustrated in Fig. 4B, angiotensin I was susceptible to transesterification of its tyrosine hydroxyl group (*O*-acylation), giving rise to the product at m/z 2075.6. To address this unwanted side-reaction, the sample was exposed for 15 min at 37°C to an alkaline 2% hydroxylamine solution (pH 9.4), followed by incubation in a 20-mM sodium phosphate solution, supple-

mented with 5 mM TCEP (pH 8.0). This treatment resulted in hydrolysis of the *O*-linked ester bond (Fig. 4C) and subsequent formation of the thiol-functionalized derivative observed in the spectrum at m/z 1385.5 (Fig. 4D). As illustrated further in Fig. 4D, a small percentage (<10%) of the peptide was regenerated after disulfide reduction. The data highlight the use of the solid-phase platform to ensure for efficiency of derivatization and to carry the analyte through the sequential reaction scheme at minimal sample loss. The procedure was completed in <70 min.

On-Resin and In-Gel Performic Acid Oxidation

Cysteine-containing peptides have been shown to be highly susceptible to β -elimination/Michael addition reaction, resulting in unwanted co-isolation of these peptides during β -elimination-based phosphopeptide affinity purification.

FIGURE 5

Solid-phase performic acid oxidation. The phosphopeptide DAM1 (SFVLNPTNIGMpSKSSQGHVTK) was bound to ZipTip_{C18} pipette tips and incubated in a 0.15% performic acid solution at 4°C for 1 h. After incubation, the ZipTips were processed for MALDI-TOF. The MALDI spectra of (A) untreated peptide and (B) after performic acid oxidation. Note that the peptide's methionine residue was fully oxidized to its stable sulfone form. Low-intensity ions in A represent peptide synthesis byproducts observed in B as oxidized derivatives. Arrow in A denotes the peptide's sulfoxide derivative formed upon air oxidation. Angiotensin I was N-thiolated, as described in Fig 4 and subjected to performic acid oxidation. MALDI-TOF spectra of (A, inset) unmodified thiol derivative and (B, inset) after performic acid oxidation. Note completeness of cysteine acid formation. One-tenth of the eluates corresponding to ~2 pmol digest was applied to the target.

Carboxyamidomethylation, often used in proteomics studies to facilitate protein digestion, proved ineffective to protect thiols from β -elimination,³⁰ in contrast with previous results.^{15,29} Performic acid oxidation has emerged as the method of choice in proteomics studies to arrest the reactivity of this residue.⁵⁰ With the use of model peptides, we explored the feasibility to perform the reaction on the solid phase. This derivatization format abrogates the need for extended sample dry-down, as used after in-solution phase oxidation to remove residual reagents, a sample handling step may cause adsorptive sample loss. In the experiments, the DAM1 phosphopeptide (SFVLNPTNIGMpSKSSQGHVTK, m/z 2312.6) was selected, as its methionine proved prone to partial oxidation, resulting in unwanted signal dilution (Fig. 5A). The formation of the sulfoxide derivative at m/z 2328.4 was especially promoted upon peptide storage. The peptide was bound to ZipTip_{C18} pipette tips and incubated in a 0.15% performic acid solution at 4°C for 1 h. The MALDI spectrum showed that the peptide was fully oxidized to its sulfone derivative at m/z 2344.2. Low-intensity ions observed in the spectra represent peptide synthesis byproducts and their reaction products.

We subsequently evaluated the protocol with thiolated angiotensin I, prepared as described in Fig. 4. As illustrated in Fig. 5A and B, insets, the thiol derivative at m/z 1385.5 shifted upon oxidation in mass by 48 Da, corresponding to the incorporation of three oxygen atoms. Residual peptide

was not observed in the spectrum, indicating that the thiol group had been quantitatively oxidized essentially. The results show that methionine residues as well as thiols can be oxidized effectively on the solid phase. Partial methionine oxidation is well-recognized to occur during protein gel electrophoretic separation and upon storage. As a general application to protein digests, the solid-phase protocol provides for a simple and effective tool to eliminate the resultant sample heterogeneity, thereby facilitating spectral data interpretation.⁵⁰ As an integral step of our sample-preparation method, the protocol prevents false-positive phosphorylation-site determination by precluding co-isolation of cysteinyl-containing peptides during activated thiol-affinity purification.

With the use of gel-separated OVA as a test protein, we next developed an in-gel protocol of this method. We reasoned that this alternative format would be advantageous, as proteins are presented frequently in gel-separated form for proteomic analysis. When performing the oxidation initially at the performic acid reagent concentration of 1.5%, as used in reported protocols,⁵⁰ MALDI backgrounds were excessively high, obscuring the signals of interest (data not shown). Backgrounds were found to be much lower, down to the same low level as observed for the control digest (Fig. 6A), when a 1:10 reagent dilution was used (Fig. 6B). The MALDI spectra show that the minor species at m/z 1190.5 and m/z 1465.7 shifted by 48 Da, a mass change characteristic for the oxidation of cysteine-to-cysteic

FIGURE 6

In-gel performic acid oxidation of gel-separated OVA. Gel-separated protein was incubated in 0.15% performic acid for 1 h at 4°C. The samples were subjected to in-gel tryptic digestion, along with untreated protein used as a control. Digests were bound to ZipTip_{C18} pipette tips. Oxidized samples were subjected to concurrent BEMAD for 1 h at 37°C. MALDI-TOF spectra of (A) digest of untreated protein; (B) digest of oxidized protein; and (C) digest of oxidized protein after β -elimination with concurrent Michael addition. Peptides assigned as oxidized at methionine and/or tryptophan are labeled in A, according to their native counterparts, and identified by database search as follows: P2, AFKDEDTQAMPFR (m/z 1555.7); P3, LTEWTSSNVMEER (m/z 1581.6); P4, ELINSWVESQTNGIIR (m/z 1858.9); and P5, VTEQESKPVQMMYQIGLFR (m/z 2284.1). Corresponding labels in B denote the oxidized species separated by 16 Da from the lower oxidation states indicated in italics. Peptide P1 (m/z 1546.8), which had no detectable signal in A, was identified as a nonspecific cleavage product containing two methionine residues. The number of oxygen atoms incorporated into the various peptides is indicated in parenthesis. Note that oxidation renders the peptide P5 distinguishable from its near-isomass counterpart at m/z 2281.1. Open arrow and arrow in A denote cysteinyl peptides at m/z 1190.5 (ADHPFLFCIK) and at m/z 1465.7 (YPILPEYLQCVK), respectively. Arrowhead and asterisk in B and C denote the corresponding cysteine acid derivatives at m/z 1238.6 and at 1513.7, respectively, impervious to β -elimination with concurrent Michael addition. Arrows in C indicate alkali-degraded species assigned as oxidized in B. Cross-arrow indicates the phosphopeptide at m/z 2088.9 and its thiol adduct at m/z 2067.9. Note prominent signal enhancement after derivatization. One-tenth of the eluates corresponding to ~ 2 pmol digest was applied to the target.

acid (Fig. 6B). Upon β -elimination with concurrent Michael addition, the oxidized cysteinyl peptides at m/z 1238.6 [ADHPFLF(CO₃)IK] and at m/z 1513.7 [YPILPEYLQ(CO₃)VK] were, as expected, unaffected by the chemical treatment (Fig. 6C). The results prove in-gel oxidation as an effective strategy to protect cysteinyl peptides from unwanted β -elimination, thereby preventing their co-isolation during β -elimination-based phosphopeptide-affinity purification.

As denoted in Fig. 6B, representing the mass map of the oxidized digest, several other peptides were shifted by mass additions relative to their nonoxidized counterparts (Fig. 6A), indicative of modification of methionine (MetO₂, +32 Da) and tryptophan (TrpO₃, +48 Da). It

should be noted that oxidation of these residues was complete with the exception of the tryptophan residue contained in peptides P3 at m/z 1581.6 (LTEWTSSNVMEER) and P4 at m/z 1858.9 (ELINSWVESQTNGIIR), which remained partially in the lower dioxidation state (TrpO₂), consistent with an earlier report.⁵⁰ In addition, the oxidation product at m/z 1546.7 had no discernible native counterpart and was assigned preliminarily by the FindPept analysis as a nonspecific cleavage product containing two methionine residues with an apparent mass of 1482.7 Da. Furthermore, the performic acid oxidation rendered P5 at m/z 2284.1 (VTEQESKPVQMMYQIGLFR), bearing two methionines, distinguishable from its near-isomass counterpart at m/z 2281.1 (Fig. 6B, arrow). As evident

from the significant signal reduction, the oxidation products of peptides P3 and P4 proved unstable under the conditions of β -elimination with concurrent Michael addition, consistent with earlier results (Fig 6C, arrows).¹⁷ The unknown chemistry side-reaction, targeting doubly and triply oxidized tryptophan, warrants further investigation as a location for potential cleavage sites. To assess potential sample loss during in-gel oxidation, reagent and wash supernatants from the gel bands were combined, dried, and subjected to tryptic digestion. MALDI analysis of this material revealed only low-intensity signals corresponding to autoproteolytic products (data not shown). The data show that performic acid oxidation could be performed effectively in the gel matrix under mild-reaction conditions, leaving the protein intact for further manipulation.

ZipTip_{C18} Pipette-Tip Alkali-Compatibility Evaluation

Long-term C18 reversed-phase column testing (>1 week) with mobile phases at pH 9–12.3 has been shown diminish, to a minor extent (~2%), the retention of the silane-bonded stationary phase.⁵¹ This effect was attributed to mechanical attrition around the covalently attached silanes, eventually causing splitting off of the bonded organic phase, and could therefore potentially reduce in our applications the analyte recovery from the silica-based reaction bed. In earlier work, we assessed the relative recovery of the model peptide angiotensin I (DRVYIHPFHL, *m/z* 1296.6) from ZipTips before and after concurrent BEMAD, using a stable isotope-dilution technique.³⁰ In the experiment, the peptide was derivatized for 1 h at 55°C or for 2 h at 37°C in 66 mM barium hydroxide/33 mM 2-aminoethane thiol (pH 12.3). The data showed that only a small fraction of the sample (average 10%) could not be retrieved from the support after the alkali exposure. As noted in the accompanying study, a similar, minor alkali-induced sample loss (average 13.9%) occurred under the more stringent conditions of the consecutive BEMAD, in which the peptide was first exposed for 30 min at 55°C to 50 mM barium hydroxide (pH 12.6) and subsequently, at the same temperature for 2 h to 100 mM 2-aminoethanethiol/75 mM barium hydroxide (pH 10.6). Accordingly, one would expect the analyte, under the considerably less alkaline-reaction conditions described in Figs. 3A and B and 4, to remain firmly bound to the reversed-phase support. To test the validity of this assumption, fibrinopeptide B, N-terminally blocked by pyroglutamate and devoid of lysine, was loaded onto ZipTip_{C18} pipette tips in three replicates and carried through the sequence of the chemical reactions. Untreated samples were left aside as controls. For MALDI-TOF MS of the eluates, a total of 640 laser shots, acquired from eight different spot positions, was averaged for each

spectrum. Comparison of the spectra showed that the peptide was recovered at comparable signal strength from the alkali-exposed and the untreated samples. Variations in signal intensity between the replicates were <15% (results not shown). Comparable results were obtained upon peptide exposure to the mildly acidic conditions of performic acid oxidation (pH 3.1; data not shown). Taken together, the data strongly suggest that the peptide retention on the reversed-phase support was little-affected by the chemical treatment, allowing the analyte to be carried through the serial reactions with minimal sample loss.

Covalent Chromatography

The method relies on capture of the analyte from a mixture on the activated thiol affinity support by thiol-disulfide interchange, removal of the nonbound material by a solvent wash, followed by reductive release of the species of interest.^{17,18} Nonspecific peptide adsorption during this process is of concern, as it could compromise the selectivity of the method and reduce the recovery of the species of interest. We reasoned that micro adaptation of the method would minimize this effect expected to become especially noticeable with low-level analytes. To this purpose, a miniaturized spin-column format was adopted to accommodate a relatively small reaction bed (<100 μ l). To evaluate its use to isolate low-level amounts of peptide from a complex peptide mixture, 5 pmol immobilized angiotensin I was thiolated as described in Fig. 4 and eluted in 10 μ l 50% acetonitrile/0.1% TFA/0.01% OGS into 40 μ l 50 mM sodium phosphate/2 mM EDTA containing 50 pmol of a HSA tryptic digest. A slurry of 100 μ l-activated thiol sepharose was placed into the micro spin column, briefly centrifuged and resuspended with the peptide mixture. End-over-end incubation was then carried out for 1 h at room temperature. The reaction vessel was then submitted to centrifugation to collect the depleted, nonphosphorylated peptides. After an organic solvent wash of the resin, the combined fractions were reduced in volume and subjected to ZipTip_{C18} pipette-tip purification. The bound peptide was released reductively during 30 min incubation in sodium phosphate buffer supplemented with 5 mM TCEP. Iodoacetamide was then added to a final concentration of 50 mM to alkylate the derivative. After 30 min end-over-end incubation, the material was collected by centrifugation. The flow-through fraction was combined with the subsequent organic solvent wash, concentrated, and submitted to ZipTip_{C18} pipette-tip purification. The eluates from the starting material and from the depleted and enriched fraction were analyzed by MALDI-TOF. The MALDI spectra, illustrated in Fig. 7, show that the enriched fraction was essentially devoid of nonbound material found exclusively in the flow-through fraction, indicat-

FIGURE 7

Covalent chromatography: evaluation of miniaturized spin-column format. *N*-Thiolated angiotensin I (5 pmol) was prepared as described Fig. 4 and spiked into 50 pmol of a HSA tryptic digest. The sample was submitted to covalent chromatography (see text for details). The reductively released analyte was carboxyamidomethylated on the affinity reaction bed before collection by centrifugation. The eluates from starting material, from the depleted, and from the enriched fraction were analyzed by MALDI-TOF. MALDI-TOF spectra of (A) starting material; (B) flow-through fraction; and (C) enriched fraction. The analyte at m/z 1385.5 is indicated in A by an arrow. Note that the enriched fraction containing the isolate at m/z 1442.0 is devoid of any background contamination. One-tenth of the eluates was applied to the target. In a different but related application, the digest was carried through the serial reactions, as described in Fig. 4, and subjected to covalent chromatography and *in situ* carboxyamidomethylation. (C, inset) Spectrum of the enriched fraction. Peptides are annotated corresponding to their native counterparts in A. One-tenth of the eluates corresponding to ~5 pmol digest was applied to the target.

ing that nonspecific peptide binding was negligible in the miniaturized reaction vessel.

To assess the relative coupling/release efficiency of the method, 5 pmol of the cysteinyl peptide-somatostatin (CKNFFWKT, m/z 1073.2) was processed with this protocol. Fractions were concentrated on ZipTip_{C18} pipette tips along with controls, and ~1 pmol peptide was deposited onto the MALDI target. A total of 640 laser shots sampled from eight different spot positions was summed for each spectrum. The MALDI-TOF spectra revealed that the signals of the starting material and the retrieved peptide were of nearly equal abundance. An estimated 10% of the starting material was typically found in the flow-through

fraction, indicating that an ~90% coupling/release efficiency could be achieved (data not illustrated). Taken together, the data validate the use of the miniaturized, covalent chromatographic system to enrich the analyte effectively from a complex mixture with minimal sample loss.

To probe the capability of the retrieval system to process a wide range of peptides, the HSA tryptic digest was carried through the sequence of chemical reactions, as described in Fig. 4, and submitted to covalent chromatography. The thiol derivatives were carboxyamidomethylated on the affinity support as described above. MALDI-TOF analysis of the reductively released fraction revealed the

same set of several prominent ions as observed in the spectrum of the starting material, of which the ions at m/z 2202.7 and m/z 2223.8 were recognized as doubly acylated species (Fig. 7C, inset). The peptide at m/z 1721.6 had no discernible native counterpart and was assigned preliminary by FindPept analysis as a nonspecific cleavage product with an apparent mass of 1431.6 Da.

Covalent Affinity Chromatography of Protein Digests

To test the applicability of the enrichment protocol to protein digests, in-gel tryptic digests, derived from equimolar amounts of α -S1 and β -casein (25 pmol), were combined, immobilized on ZipTip_{C18} pipette tips, and carried through the multistep reaction scheme, as outlined in Fig. 1, using on-resin performic acid oxidation as an initial reaction step. The preparations were then submitted to covalent chromatography. One-tenth of the eluates from the starting material and from depleted and enriched fraction was analyzed by MALDI-TOF. As illustrated in Fig. 8C, the phosphopeptides were recognized in the MALDI spectrum of the enriched fraction as their thiolated counterparts at m/z 1770.2, m/z 2103.1, and m/z 3432.8, corresponding to the α -S1 fragment spanning residues 121–134 (VPQLEIVPNpSAEER), its miscleavage product spanning residues 119–134 (YKVPQLEIVPNpSAEER), and the β -casein tetraphosphorylated species (RELEEL-NVPGEIVEpSLpSpSpSEESITR) spanning residues 16–40. The data show that the species of interest could be isolated selectively from the protein digest, including the tetraphosphorylated tryptic peptide of β -casein that proved impervious to enrichment by the dithiol-based affinity approach (Fig. 8C, arrowhead). We note that the thiol derivative of the monophosphorylated peptide of β -casein (FQpSEEQQQTEDELQDK, residues 48–63) was not detected in the isolates. However, the synthetic peptide was readily converted to its thiolated counterpart at m/z 2212.8, indicating that β -casein in this preparation was a protein variant exclusively phosphorylated at its multiple sites (Fig. 8A, inset). The peptide at m/z 2260.5 represents an acetylated fragment of β -casein, corresponding to DMPIQAFLLYQEPVLGPVR (residues 199–217) that had been fully oxidized to its methionine sulfone analog during the initial on-resin performic acid oxidation step. Comparable results were obtained after in-gel oxidation of the protein, followed by in-gel tryptic digestion (data not shown).

To assess the overall sensitivity of the phosphopeptide strategy, tryptic digests, generated by in-solution proteolysis of 2 pmol α -S1 casein, were carried through the multistep sample-preparation scheme and submitted to covalent chromatography. The samples were subjected to carboxyamidomethylation, as described in Fig. 7, collected by

centrifugation, and concentrated on ZipTip_{C18} pipette tips. One-tenth of the eluates was applied to the target. The data show that the protein's miscleavage product, spanning residues 119–134, exclusively formed during digestion, was selected effectively from the peptide mixture (Fig 8C, inset). The high quality of spectrum, produced from $\sim$ 200 fmol peptide, suggests that lower quantities of digest should be amenable to the multistep phosphopeptide purification strategy.

After reversible capture on the affinity support, the thiol derivatives become highly suitable targets for labeling with a stable isotope-coded alkylation reagent, such as $^{13}\text{CD}_3$ -methyl iodide and $^{13}\text{C}_2\text{D}_2$ iodoacetamide. As noted above, the alkylation reaction can be performed conveniently, directly on the thiol-affinity resin before collection of the enriched fraction or optionally, on the ZipTip-bound isolates. Carbodiimide-mediated postdigestion carboxylate labeling with stable isotope-coded amine nucleophiles, such as aniline- $^{13}\text{C}_6$ hydrochloride, may be used as an alternative method to expand the scope of the method to relative phosphopeptide quantitation.⁵² The reaction conditions for solid-phase carboxyl group labeling of digests with aniline hydrochloride were as earlier reported for the conjugation of glycineamide hydrochloride, except that an eightfold-lower nucleophile concentration was used, and the reaction was allowed to proceed for 3.5 h to reach completion (data not illustrated).

Phosphorylation-Site Determination

We next examined the use of our phosphopeptide-enrichment strategy for MS/MS-based phosphorylation-site determination. Representative MALDI-TOF/TOF data obtained from the enriched tryptic peptide at m/z 1770.2 are illustrated in Fig. 9. As exemplified by the resultant product ion spectrum of the derivative, a nearly uninterrupted y ion series was produced in high abundance. These fragment ions included y5, representing the first ion that contains the chemical tag, as well as y4, which is contiguous to the modification. The location of the modification could be readily identified by its unique residue mass of 234 Da, and hence, serine in position 130 within the peptide sequence $^{121}\text{VPQLEIVPNpSAEER}^{134}$ is the site of phosphorylation. Substituted phosphothreonine would be recognized by its characteristic signature mass of 248 Da. The affinity tag proved stable under the conditions of CID, aiding MS/MS data interpretation. The data show that our phosphopeptide-enrichment method provides isolates highly suitable for unambiguous phosphorylation-site determination.

FIGURE 8

Covalent affinity chromatography of protein digests. A tryptic in-gel digest (25 pmol; 1.15 μ g) of a prepared-from-equimolar mixture of bovine α -S1 and β -casein was bound to ZipTip_{C18} pipette tips and carried through the six step preconditioning cycle and enrichment protocol (Fig. 1). Samples were analyzed by MALDI-TOF MS. MALDI-TOF spectra of (A) digest after preconditioning reactions; (B) affinity resin flow-through fraction; and (C) affinity resin-enriched fraction. The phosphopeptides are recognized in the spectrum as their thiolated adducts at m/z 1770.2, m/z 2103.1, and m/z 3432.8 (arrowhead) and mass-matched within the known protein sequences to the α -S1 casein fragments VPQLEIVPpSAEER (residues 212–134) and YKVPQLEIVNPpSAEER (residues 119–134) and to the β -casein fragment RELEELNVPGEIVEpSLpSpSpSEESITR (residues 16–40). Apart from three minor ions, the enriched fraction was devoid of nonphosphorylated fragments, as they were depleted effectively from the affinity support. One-tenth of the eluates corresponding to ~ 2.5 pmol digest was applied to the target. (A, inset) Spectrum the thiol derivative of the synthetic monophosphorylated β -casein fragment (FQpSEEQQQT-EDELQDK; residues 48–63). Note that this derivative was not observed in MALDI spectrum of the isolates (see text). Arrowhead in A denotes the acetylated β -casein fragment at m/z 2260.5, corresponding to 199 DMPIQAFLLYQEPVLGPVR 217 , fully oxidized at methionine. (C, inset) Expanded section of spectrum containing the α -S1 miscleavage product, selected by covalent chromatography from 2 pmol preconditioned digest. Note that the thiolated peptide was carboxyamidomethylated on the affinity support. Approximately 200 fmol isolate was applied to the target.

Comprehensive Phosphopeptide Sample Preparation

The structural characterization of proline-containing phosphopeptides is of considerable interest, as a significant portion (>50%) of cellular proteins are targeted by proline-directed kinases.³⁵ In the accompanying report,³¹ we have adopted the consecutive BEMAD format to systematically optimize the *in situ* reaction conditions and showed that these peptides, as well as phosphothreonyl peptides,

were notably more efficiently and faster derivatized with this protocol than with protocols reported in the literature.^{20,36,42,43} This two-step, solid-phase reaction can be readily incorporated in the sample-preparation method to condition this class of peptides for affinity enrichment by covalent chromatography (see Fig. 1). The entire sample-preparation procedure can be readily completed within <8 h. After reaction with Sulfo-NHS-SS-Biotin, the desalted

FIGURE 9

Phosphorylation-site determination by MALDI-MS/MS. MALDI-TOF/TOF spectrum of thiol derivative at m/z 1770.2 enriched from α -S1/ β -casein tryptic digest, as described in Fig. 8. The y ion series annotated in the spectrum is nearly uninterrupted and yielded the peptide sequence shown. The derivatization discriminates the site of phosphorylation as the unique residue mass of 234 Da contained in the product ion y_5 indicated in red in the spectrum, as is y_4 , which is contiguous to the modification. The presence of this ion pair affords unambiguous assignment of serine in position 130 as the site of phosphorylation. Ions indicating fragmentation of the label were not observed in the spectrum. One-tenth of the eluate corresponding to ~ 2 pmol isolated peptide was applied to the target.

ZipTips can be stored overnight at -20°C for further manipulation (see Materials and Methods). If site determination of phosphoserine is of main interest,¹⁵ then the faster sample-preparation protocol using the concurrent BEMAD allows the procedure, including affinity purification, to be completed within one working day.

Intraphosphopeptide Cross-Link Formation

We have demonstrated previously that phosphoserine peptides reacted to completion under the relatively mild conditions of concurrent BEMAD.³⁰ One would expect the phosphoserine peptides to react equally efficiently under the consecutive, more stringent reaction conditions required to convert phosphothreonyl peptides with and without adjoining prolines. To test this perception, the phosphoserine peptides listed in Materials and Methods were bound to ZipTip_{C18} pipette tips and loaded with 50 mM barium hydroxide. After 30 min incubation at 55°C , the elimina-

tion base was exchanged on the solid-phase by 100 mM 2-aminoethanethiol/75 mM barium hydroxide, and the incubation continued for 2 h at 55°C . The MALDI-TOF spectra showed that the oxidized DAM1 phosphopeptide (SFVLNPTNIGM**p**SKSSQGHVTK, m/z 2344.9) and the MARCKS phosphopeptide (KKKKKRF**p**SFKK**p**SFKLSGF**p**SFKKNKK, m/z 3321.6) were under these conditions, partially converted. In contrast, all other phosphopeptides reacted to completion (data not shown). Similar results were obtained when the β -elimination was allowed to proceed for 2 h at 37°C . As illustrated in Fig. 10B, $\sim 50\%$ of the DAM1 phosphopeptide, in which the phosphoserine is followed by lysine, remained in its β -eliminated form. Such missed cleavage products, in which the phosphorylated residue is positioned adjacent to lysine (or arginine) or separated by a single amino acid residue from the proteolysis-resistant cleavage site, are derived predom-

FIGURE 10

Intramolecular phosphopeptide cross-link formation. The oxidized DAM1 phosphopeptide (SFVLNPTNIGMp-SKSSQGHVTK, m/z 2344.9) and the MARCKS phosphopeptide (KKKKKRFpSFKKpSFKLSGFpSFKKNNKK, m/z 3321.6) were subjected with and without prior acetylation to β -elimination with consecutive Michael addition. The peptides were β -eliminated for 30 min at 55°C in 50 mM barium hydroxide (pH 12.6) and subsequently reacted with 100 mM 2-aminoethanethiol/75 mM barium hydroxide (pH 10.6) for 2h at 55°C. (A and D) MALDI-TOF spectra; oxidized DAM1 and MARCKS phosphopeptide, respectively. (B and E) Oxidized DAM1 and MARCKS phosphopeptides after consecutive BEMAD, respectively. Note incompleteness of Michael addition product formation. (C and F) Oxidized DAM1 and MARCKS phosphopeptide peptides after consecutive BEMAD, preceded by on-resin acetylation, respectively. Note completeness of reaction after lysine acetylation, preventing intrapeptide cross-link formation. Arrows in B and E denote β -elimination products rendered unreactive by intrapeptide cross-link formation. Open arrow and asterisk in E denote single- and double-substituted peptide, separated in mass by 77 Da, respectively. Inset in B depicts the peptides' sequence region affected by cross-link formation with cross-linked residues highlighted in bold. Arrow in F designates partially acetylated thiol adduct.

inantly from basophilic kinase substrates and accounted for 42% of the 1435 tryptic phosphopeptides identified in digests of human embryonic kidney 393T cells.^{7,53} In the case of the MARCKS phosphopeptide, selected as an example of a nontryptic lysine-rich phosphopeptide, the β -elimination product at m/z 3027.2 and its singly substituted derivative at m/z 3104.3 were observed as dominant ions in the spectrum (Fig 10E, arrow and open arrow). Only a small fraction of the peptide was converted to its doubly substituted derivative (Fig. 10E, asterisk). These data suggest that during the β -elimination phase of the sequential reaction, a significant fraction of the dehydroalanine intermediate became cross-linked to the proximal lysine serving as an intramolecular Michael donor. This interpretation is in agreement with earlier results.⁵⁴ Consequently, blockage of lysine by acetylation should prevent this effect that would exclude the cross-linked portion of these phosphopeptides from affinity purification by cova-

lent chromatography. To test this assumption, the peptides were acetylated, as described in Fig. 3, before consecutive BEMAD. The MALDI spectra revealed that the nucleophilic addition proceeded to near completion when the peptides were reacted in their acetylated form (Fig. 10C and F). Likewise, performic acid oxidation should provide a viable means to arrest the reactivity of cysteine that had been shown in human-lens proteins to affect a nucleophilic attack on proximal dehydroalanine.⁵⁵ The data highlight the benefit of the amine-blocking step, as an integral component of the sample-preparation method, to render this subpopulation of phosphopeptides fully amenable to affinity enrichment by covalent chromatography.

Peptides modified by *O*-GlcNAc and *O*-sulfonated peptides have been shown to β -eliminate as readily as their phosphorylated counterparts, making these peptides highly susceptible to reaction with the nucleophile.^{42,56} In consequence, our solid-phase analytical platform should provide

for a viable means to prepare digests derived from *O*-GlcNAcylated and *O*-sulfonated proteins for isolation of the *O*-glycosylated and *O*-sulfonated components by thiol-directed affinity purification.

Current and Future Developments

AP treatment of protein digests has been an early method to identify phosphopeptides in MALDI-MS by the resultant characteristic mass shift of 80 Da/liberated residue. In the accompanying report, we have adapted this technique to the solid phase and demonstrated its use for comprehensive dephosphorylation of a variety of substrates, including highly basic phosphopeptides, previously found resistant to dephosphorylation. The enzymatic digestion inherently renders phosphopeptides insensitive to the β -elimination/Michael addition, an effect that had been exploited for selective affinity purification of *O*-glycosylated fragments from phosphoglycoprotein digests using DTT as nucleophile.²⁵ We began, in pilot experiments, to test this strategy on our solid-phase analytical platform using a model system mimicking a glycoprophosphoprotein digest. To this purpose, we spiked an in-gel OVA digest (10 pmol) with 2 pmol of the glycosylated peptide SVES(*O*-GlcNAc)GSADAHar that had been guanidinated, as described in the companion report. The peptide mixture was adsorbed on the ZipTip_{C18} pipette tip, exposed to a solution containing 2 U/ μ L AP, and allowed to react for 15 min at 37°C. The digestion was halted by a brief, solvent wash. The samples were then carried through the series of chemical reactions, as described in Figs. 3 and 4, followed by covalent chromatography of the recovered material. MALDI-TOF of the enriched fraction revealed that the glycopeptide was reversibly captured by the affinity support, whereas the dephosphorylated species was found in the initial flow-through fraction. Conversely, β -*N*-acetylglucosaminidase treatment of this sample would leave the phosphopeptide as sole target for incorporation of the thiol affinity tag. We anticipate the enzymatic deglycosylation to be readily adaptable to the solid phase. Furthermore, the specificity of these enzymes may be exploited to afford the selective retrieval of sulfonopeptides from mixtures with phospho- or *O*-glycosylated peptides. The peptides depleted from the affinity support in this manner may then be recovered by covalent chromatography from preparations that received no enzymes. The isolates rendered by this strategy, distinguishable from each other in MALDI-MS, may then be selected from the mass maps for MS/MS modification-site determination. Differentiation and site determination of these PTMs have gained considerable interest to gain insight in the potential, functional role of the dynamic interplay perceived to occur between these *O*-linked modification events in cellular responses.^{25,26} We note here that the nearly complete

neutral loss of the sulfono moiety (80 Da) from the parent upon CID precluded site determination of sulfonated peptides under the conditions of low-energy, collisionally induced fragmentation, highlighting the advantage of the chemical proteomics approach to characterize this PTM.⁵⁶

Large-Scale Phosphoproteome Profiling

We are currently exploring the use of commercially available, reversed-phase spin columns for chemistry scale-up needed to use the sample-preparation method for large-scale phosphoproteome profiling. These spin columns accommodate up to 300 μ g digest, corresponding to amounts typically available for further analysis after prerequisite sample prefractionation of proteomics mixtures.⁵⁷ Our preliminary experiments with model systems, spiked with phosphopeptide, showed that the sample-preparation method can be readily adapted to the spin-column derivatization format. In recent work, we began to evaluate the scaled-up sample-preparation method with prefractionated digests prepared from cell lysates, with a future view to use differential, stable-isotope labeling to measure protein expression levels in complex biological systems.

Conclusions

A method for affinity enrichment of phosphopeptides from protein digests has been developed based on the Ba^{+2} ion-catalyzed β -elimination/Michael addition reaction. In this strategy, 2-aminoethanethiol was used as nucleophile, and all amino groups in the protein digest were blocked initially by acetylation to render the amino group of the Michael addition products the sole target for reaction with sulfo succinimidyl-2-(biotinamido) ethyl-1,3-dithiopropionate. The disulfide-linked biotin moiety, along with its linker arm, was released reductively from the derivatives, which were then enriched by covalent chromatography on activated thiol sepharose. Multistep chemical reaction schemes are, in general, perceived as problematic because of the high potential of adsorptive sample loss incurred during the requisite intermittent sample purification. We have addressed this issue by using reversed-phase supports as a venue for derivatization. Sample handling during the sequential derivatization was minimal, as confined to reagent exchanges *in situ* and elution of the final derivatives, allowing the immobilized analyte to be carried through a sequential reaction cycle with minimal sample loss. The use of this sample-preparation method for phosphopeptide enrichment was demonstrated with low-level amounts of in-gel digests of α -S1 and β -casein. The thiol derivatives were selectively enriched from the digest and proved highly suitable for phosphorylation-site determination by MALDI-TOF/TOF-MS/MS. Alternatively, ZipTip_{C18} pipette tips or ZipTip _{μ -C18} pipette tips can be readily interfaced with

nanospray MS techniques, or the eluates can be optionally analyzed by LC-MS/MS. On-resin and in-gel performic acid oxidation methods were developed to preclude co-isolation of cysteinyl proteolytic fragments. β -Elimination with consecutive Michael addition incorporated in the sample-preparation scheme expanded the use of the solid-phase-based enrichment strategy to phosphothreonyl peptides, to phosphoseryl/phosphothreonyl peptides derived from proline-directed kinase substrates, and to their *O*-sulfonyl- and *O*-GlcNAc-modified counterparts. The acetylation step, as an integral step of the sample-preparation method, prevented unwanted phosphopeptide signal dilution induced by lysine-dehydroalanine cross-link formation that would reduce the recovery of the isolates. Solid-phase enzymatic dephosphorylation coupled with the sample-preparation method is anticipated to provide for a viable tool to render *O*-glycosylated or *O*-sulfonopeptides in mixtures with phosphopeptides amenable to selective affinity purification. The solid-phase analytical platform combines robustness with simplicity of operation using equipment readily available in most biological laboratories and is anticipated to be used in a scaled-up format for quantitative phosphoproteome profiling of complex biological mixtures.

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DISCLOSURE

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