We have developed and validated a method that uses liquid chromatography/electrospray ionization-mass spectrometry to quantify site-specific protein phosphorylation. The method uses selected ion monitoring to determine the chromatographic peak areas of specific tryptic peptides from the protein of interest. The extent of phosphorylation is determined from the ratio of the phosphopeptide peak area to the peak area of an unmodified reference peptide that acts as internal standard, correcting for variations in protein amounts and peptide recovery in the digest preparation procedure. As a result, we refer to this protocol as the native reference peptide method. Mole of phosphate at the selected site per mole of protein is obtained from this ratio, using calibration curves of synthetic peptides to determine relative responses. Our method begins with protein separation by SDS-PAGE and is carried out on amounts of peptide produced by an in-gel digestion of single Coomassie blue-stained bands. To illustrate the utility of the method and provide validation, we used cardiac troponin I as analyte and monitored the time course of a protein kinase C IIH1 reaction. Those analyses appropriately demonstrate the time-dependent increase of phosphorylation at a PKC-preferred site, Ser44 in the peptide EISASpR45 and the concomitant consumption of the nonphosphorylated peptide. We believe that this method provides a novel tool to directly measure specific phosphorylation sites in proteins in different physiological states and expect that the method will be adaptable not only to a variety of samples types (i.e., culture cells, tissues, etc.) but to a variety of posttranslational modifications as well.

Cell signaling by means of protein phosphorylation regulates a multitude of cellular functions at the level of receptors, ion channels, transcription factors, kinases, and contractile proteins. This regulation may occur via protein–protein interactions, autoinhibition of protein kinases by blocking either substrate or ATP binding, or modulation of cooperative binding of contractile proteins. A common theme of all these interactions is the altered charge of the newly formed phosphorylated amino acid that induces either local or global changes. Thus, phosphorylation of a single amino acid is capable of triggering complex cellular signals involved in almost any physiological process: growth, differentiation, metabolism, contractility, etc. For example, a recent report of a molecular dynamics assay showed that phosphorylation of a single Tyr regulates the coupling of SH2 and SH3 domains in Src kinases. These investigators showed that the conversion of a specific C-terminal tyrosine to a phosphotyrosine residue directly disrupts the interaction of the SH2 and SH3 domains, allowing them to bind substrate and converting inactive to active kinase. As the degree of phosphorylation increases, the amount of active Src kinase would also be increased. This and similar reports clearly illustrate the general need to understand protein phosphorylation qualitatively (i.e., where is the modification taking place?) and quantitatively (to what degree is that site modified?).

To date, various methods have been used to study protein phosphorylation. Methods such as NMR, radiolabeling with 32P, and immunochemical methods have all been used in different types of systems. The goal of these experiments has most often been to map phosphorylation sites so that subsequent site-directed mutagenesis studies can demonstrate the biochemical effects of the modification. None of these methods is routinely used to provide quantification of phosphorylation at specific sites, although limited examples of quantitation have been reported.

Mass spectrometric techniques have also been applied to the characterization of protein phosphorylation sites. Electro spray

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ionization mass spectrometry and matrix-assisted laser desorption/ionization-time-of-flight mass spectrometry offer high-sensitivity methods for the characterization of proteins and peptides. In the case of electrospray ionization mass spectrometry, the combination of capillary column liquid chromatography and tandem mass spectrometry allows the detection and characterization of atomole amounts of peptides, including phosphopeptides. In these experiments, peptide phosphorylation is readily identified based on the characteristic increase of the peptide molecular mass by 80 Da/ments, peptide phosphorylation is readily identified based on the amount of peptides, including phosphopeptides. In these experiments, peptide phosphorylation is readily identified based on the amount of peptides, including phosphopeptides.

In this paper, we describe a new method for the determination of phosphorylated peptides. The method uses liquid chromatography (LC)/mass spectrometry (MS) for the analysis of peptides produced by the in-gel digestion of proteins separated by SDS-PAGE. The internal standard that is used is a peptide present in the digest from the protein being studied but is unmodified by the modification reaction of interest. As a result, we refer to this method as the "native reference method". The method is distinct from typical internal standardization experiments where an exogenous species is added at some point in the sample processing and assumed to equilibrate with the analyte. Not only does the polycarylamide gel matrix condense such an equilibration but also standardization by addition of an exogenous peptide would be compromised by any variation in the amount of protein present in the band. The use of a reference peptide that is an intrinsic part of the protein being studied provides the reference point for the LC/MS signal that is needed in a manner that effectively compensates for any variation in the amount of protein taken for analysis. Quantitation is achieved by measuring the ratio of the phosphorylated peptide to the native reference peptide by LC/MS analysis based on the chromatographic peak area given by the mass spectrometer detector. This value can be utilized directly to determine the relative differences in phosphorylation states in different samples. Alternatively, a calibration curve produced with synthetic peptide standards can be used to quantify the degree of phosphorylation as mole of phosphorylation at that site per mole of protein.

As an illustration of the native peptide reference method, we have quantified the time-dependent phosphorylation of Ser44 in cardiac troponin I (cTnl) by protein kinase C/jii (PKC/jii). cTnl is a substrate for CAM P-dependent protein kinase A and PKC. cTnl is the inhibitory subunit of the regulatory troponin complex, which also contains a Ca<sup>2+</sup>-binding protein (troponin C) and tropomyosin-binding subunit (troponin T). cTnl provides an exquisite example of intramolecular communications among phosphorylation sites, whereby phosphorylation of a single site modulates the phosphorylation of other sites and thus regulates fundamental physiological processes. Our analyses demonstrate the time-dependent increase of phosphorylation at this PKC-preferred site in the tryptic peptide (1415SAIR45, with the concomitant consumption of the nonphosphorylated peptide, by independently measuring the amounts of each peptide. Each assay was carried out on the amount of protein present in a single Coomassie blue-stained gel band (20 pmol). The degree of phosphorylation was from 0% phosphorylated at the initiation of the PKC/jii reaction to 100%phosphorylation following a 2-h treatment, with relative standard deviations of ~15% at each time point tested.

**MATERIALS AND METHODS**

**PKC/jii Phosphorylation of cTnl.** The reaction time course as well as enzyme/substrate ratio were designed based on 2D phosphopeptide-mapping experiments of free bovine cTnl-phosphorylation by PKC isoforms. Briefly, 5 µg of purified cTnl (Sigma) was incubated in 20 mM Tris-HCl, pH 7.5, that contains 10 mM MgCl<sub>2</sub>, 1 mM CaCl<sub>2</sub>, 5 µg of phosphatidylinserine, 0.3 unit of PKC/jii, and 40 µM ATP in a total volume of 200 µL at 30 °C for 0, 5, 30, and 120 min. The reactions were stopped by precipitating aliquots of the reaction mixture with >80%acetone. After centrifugation, pelleted proteins (cTnl, PKC/jii) were dissolved in Laemmli buffer and loaded on 12%SDS–PAGE (BioRad). Typical loadings were 20 pmol of cTnl per lane. After electrophoresis, the gels were stained with Gel Code Blue Reagent (Pierce).

**In-Gel Digestion of cTnl.** The cTnl bands were digested with trypsin, in gel, for 18 h at 37 °C as previously described. The peptides produced in the digest were collected by successive extractions with 50 µL of 50 mM ammonium bicarbonate and 0.1% trifluoroacetic acid.
μL of 50% acetonitrile/5% formic acid. The total extract was concentrated in a vacuum centrifuge to less than 5 μL and redissolved in 50 μL of 1% acetic acid for analysis.

Liquid Chromatography/Electrospray Ionization-Mass Spectrometry. A capillary column LC/electrospray ionization-tandem mass spectrometry system was used for all analyses. The LC/MS system was a ThermoFinnigan LCQ-Deca ion trap mass spectrometer system with a Protana ion source interfaced to a 10 cm × 50 μm i.d. Phenomenex® Jupiter C-18 reversed-phase capillary chromatography column that was slurry-packed in our laboratory. In a typical experiment, a 0.2-μL aliquot of the digest was injected onto the capillary column and the column eluted at a flow rate of 0.2 μL/min with a gradient of acetonitrile in 50 mM acetic acid. The phosphopeptides produced by the tryptic digestion were no longer than 10 amino acids, with most in the range of 5–7 amino acids. The ion source was operated at ~3 kV, without sheath gas or liquid.

For the qualitative mapping of the phosphorylation sites, the digest was analyzed using a data-dependent acquisition routine in which full-scan mass spectra, used to measure peptide molecular weights, were acquired in one scan, and product ion spectra were obtained with collision-induced dissociation at 30%collision energy, used to determine amino acid sequence, were acquired in the three subsequent scans. The acquisition cycle was repeated with a total of ~1500 spectra acquired during the 55-min chromatographic run.

For the quantitative measurement of the degree of phosphorylation, full-scan mass spectra were acquired with scanning from m/z 200 to 1400. Automatic gain control was used with three microscans and a maximum injection time of 100 ms. As a result, spectra were typically acquired at a rate of approximately two scans/s. From these spectra, selected ion chromatograms for the following cTnl tryptic peptide ions were extracted: 41[ISSA][R][S][A]267.3 [M + 2H]2+ and m/z 533.3 [M + H]+, 41[ISAR][S][A]307.2 [M + 2H]2+ and m/z 613.3 [M + H]+, 121[NITELADLTK]131 m/z 623.5 [M + 2H]2+, and 132[FDLR]136 m/z 332.2 [M + 2H]2+. As indicated in the figures, each chromatogram was plotted with a 3 m/z window. This window included not only the entire isotope cluster of each ion but was also tolerant of minor scan-to-scan variations in the exact m/z assignments made by the mass spectrometer data system. The chromatographic peak area was determined by integration, using the instrument software.

Synthetic Peptides. The synthetic peptides that were used as standards, 41[ISAR][S][A]267.3 [M + 2H]2+ and m/z 533.3 [M + H]+, 41[ISAR][S][A]307.2 [M + 2H]2+ and m/z 613.3 [M + H]+, 121[NITELADLTK]131 m/z 623.5 [M + 2H]2+, and 132[FDLR]136 m/z 332.2 [M + 2H]2+, were purchased from Anaspec, Inc. (San Jose, CA) in 5–10-mg quantities as HPLC-purified material. The identity of each peptide was confirmed by mass spectrometric analysis in our laboratory.

Each synthetic peptide was dissolved in water to produce a series of concentrated stocks that were stored at −20 °C until use. The concentrations of these stocks were determined relative to primary standards made of the respective N-terminal amino acids. The standardization step was needed because of the small amounts of each peptide that could be synthesized, and their variable physical characteristics, and because the unknown salt content of each preparation made gravimetric standardization unreliable. For this standardization, each peptide stock was diluted 10-fold, and the concentration was measured spectrophotometrically, using the 2,4,6-trinitrobenzenesulfonic acid (TNBS) method for free amino groups.23 Briefly, the peptide stocks were diluted in 4%NaHCO3 to a total volume of 200 μL, 50 μL of 0.1%TNBS was added, and the mixture was incubated for 1 h at 37 °C. The reaction was stopped by addition of 100 μL of 1 N HCl and 10 μL of 10% SDS, and the absorbance was measured at 340 nm.

Calibration. The response ratio of each analyte peptide (41[ISAR][S][A] and 41[ISAR][S][A]) relative to the native reference peptide (121[NITELADLTK]131) was determined, using calibration curves produced from synthetic peptide standards. A series of standards with increasing molar ratios of the analyte peptide relative to the native reference peptide was produced. The standards were analyzed, as described above, and calibration curves were plotted and used to convert the measured chromatographic peak area ratios into mole fraction ratios.

RESULTS AND DISCUSSION

Sequence Analysis of cTnl. The sequencing of cTnl, using LC/MS analysis of an in-gel tryptic digest, covered more than 95% of the protein sequence. In repetitive analyses, using a variety of digestion conditions, two important observations were made: the digest included a number of peptides that contained what would be considered missed cleavage sites, and the pattern of peptides produced by the digestion was highly reproducible.

Cardiac Tnl is a difficult analyte for the quantitative analyses described below. Of the 210 amino acids in the protein, 23 are lysine residues and 25 are arginine residues. As a consequence of this high proportion of lysine and arginine residues, there are nine instances of adjacent lysine or arginine and nine instances of RXR, RXK/KXR, and KXX motifs (where X could be any amino acid), all of which provide opportunities for missed cleavages.24 As a reference, a more typical protein of ~20 kDa would have approximately four such sites.24 Indeed, many of the cTnl tryptic peptides detected in the digests contained additional arginine or lysine residues, and the digestion of the phosphorylated protein produced more of this type of peptide than digestion of the unphosphorylated protein.

Because the goal of these experiments was to quantify these phosphopeptides, an initial test was to assess the effect of these missed cleavages on the reproducibility of the digestion. No changes in the pattern of digestion, relative to the standard conditions, were observed by manipulations such as increasing the amount of protease, re-treating the digest with additional protease, increasing the time of digestion, or increasing the temperature of the digestion, those results suggest that an end point digestion was achieved, as previously proposed.24 Excised proteins bands were also digested with the endoproteases Asp-N and Glu-C. The digest produced by these proteases, however, was considered inferior to the tryptic digests in terms of the amount of cTnl sequence that was covered and the reproducibility of the digest. As a result, we found that the standard in-gel digestion strategy described in the methods was well-suited to this type of analysis, and all subsequent experiments used this tryptic digestion method.

Identification of Ser44 as a Preferred Site of PKCɔ II-Dependent Phosphorylation of cTnl In Vitro. A number of phosphorylation sites in cTnl were identified, using tandem mass

spectrometry after an in vitro reaction with PKC\textsubscript{II}. One such site was identified as Ser44, as shown in the product ion spectrum presented in Figure 1. In this product ion spectrum, the presence of a phosphoamino acid is clearly indicated by the ion at m/z 515 that is produced by the signature loss of 98 Da from (M + H\textsuperscript{+}) at m/z 613 (H\textsubscript{3}PO\textsubscript{4}). The sequence of the peptide and the site of phosphorylation at Ser44 (as opposed to Ser42) is given by the observation of the y-ion series, which includes the y\textsubscript{1}-ion at m/z 175, the y\textsubscript{2}-ion at m/z 342, the y\textsubscript{3}-ion at m/z 413, and the corresponding y\textsubscript{3}-ion minus H\textsubscript{3}PO\textsubscript{4} at m/z 315, to give the AS\textsubscript{P} portion of the peptide 41ISAS\textsubscript{P}R.\textsuperscript{45} In vitro PKC phosphorylation of cTnI at Ser42 could not be detected in any of our experiments. Previous studies have shown that PKC phosphorylation of Ser42 or Ser44 is responsible for the inhibition of Ca\textsuperscript{2+}-stimulated actomyosin MgATPase activity.\textsuperscript{18} However, this previous study could not identify whether one or both (Ser42 and Ser44) were phosphorylated by PKC. As seen in Figure 2, Ser44 is conserved in cardiac and slow skeletal muscle TnI's over a number of species, with the flanking sequences highly conserved as well.

**Native Reference Peptide Method**. **Experimental Design.** As the qualitative pattern of PKC\textsubscript{II} phosphorylation of cTnI was determined, subsequent studies focused on the use of those data for the design of a quantitative method for specific phosphorylation sites. The operating principle of the method that was developed, shown schematically in Figure 3, is relatively straightforward: use a peptide derived from an unmodified region of the protein as an internal standard in an LC/MS experiment, with selected ion monitoring, to compensate for factors such as variances in mass spectrometer response, amount of protein taken for digestion, recovery of peptides out the digest, and so on. Because the peptide used for normalization is an unmodified portion of the protein of interest, as opposed to an exogenously added protein or peptide, we refer to this approach as the native reference peptide method. Quantitation is achieved by measuring the chromatographic peak area for each peptide of interest and by calculating the peak area ratios of the analyte peptides relative the native reference peptide. These peak area ratios are ultimately converted to molar fractions, using calibration curves produced with synthetic peptide standards.

We propose that the ideal native reference peptide should have the following characteristics: (i) the reference peptide should be a proper tryptic peptide that does not include a missed cleavage, (ii) the reference peptide should not contain a modifiable amino acid such as methionine (which can be oxidized before or during electrophoresis procedure and, indeed, during sample storage) or cysteine (which is alkylated during the digestion procedure), (iii) the reference peptide should elute at approximately the same time as the analyte peptides, and (iv) the response characteristics

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**Figure 1.** Product ion spectrum of 41ISAS\textsubscript{P}R.\textsuperscript{45} The product ion spectrum, produced by collision-induced dissociation, of the (M + H\textsuperscript{+}) ion of the peptide 41ISAS\textsubscript{P}R.\textsuperscript{45} The m/z of the b- and y-ions are inset.

**Figure 2.** Sequence alignment of the Ser44 region of cardiac and slow skeletal muscle TnI. A map of the primary structure and the exon organization of human cTnI is shown together with the binding sites for troponin C, (TnC), troponin T, (TnT), and actin and the TnI inhibitory peptide. The segments encoded by each exon are outlined by the vertical bars. The exon 4-encoded amino acid sequences of cardiac (cTnI), slow skeletal (ssTnI), and fast skeletal (fsTnI) TnI isoforms from a wide range of vertebrate species are aligned. The data show that Ser44 is conserved in cardiac and slow skeletal muscle TnIs with the flanking sequences also highly conserved.

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for the reference peptide. Digest the protein band in-gel with trypsin. Analyze the digest by LC-MS using selected ion monitoring. Calculate chromatographic peak areas for the phosphorylated peptide(s) relative to the native reference peptide. Determine the relative degree of peptide phosphorylation. Convert the chromatographic peak area ratios into molar fraction values using the response ratios taken from the calibration curves. Absolute quantitation? Determine the relative response ratios for each phosphorylated peptide with calibration curves of synthetic peptides. Identify the modified peptides using tandem mass spectrometry.

In the analysis of the cTnI digests, the peptide \(121\text{NITEIADLTQK}^{131}\) was chosen as the reference peptide. Desirable characteristics of this peptide are that it is a classic tryptic peptide that does not contain either methionine or cysteine. Unfortunately, as shown in Figure 4, because of the early elution of the analyte peptides of interest in these experiments, the elution of the reference peptide is considerably removed (\(-40\) min) from the elution of the analyte peptides. The early elution of the analyte peptides is a unique property of the small, hydrophilic peptides of interest in these experiments. In addition, as seen in calibration curves presented below, the mass spectrometer response for reference peptide was significantly better than the response for the analyte peptides. As with the elution characteristics, this less than ideal characteristic is probably traceable to properties of the analyte peptides, as opposed to deficiencies in the reference peptide. On the whole, however, the choice of \(121\text{NITEIADLTQK}^{131}\) as the reference peptide gave an optimum combination of properties for these analyses.

Method Validation. The performance of the method was validated through the analysis of the time course of an in vitro phosphorylation of cTnI by PKC\(\beta\)II. Digests of phosphorylated cTnI were prepared in triplicate following 0, 5, 30, and 120 min of reaction time, and the quantitative data for selected cTnI peptides were evaluated. The specific phosphorylation of Ser44 by PKC\(\beta\)II was monitored by the quantitation of the appearance of the phosphopeptide \(41\text{ISASpR}^{45}\) and by the disappearance of the corresponding unphosphorylated peptide \(41\text{ISASR}^{45}\). Selected ion monitoring data acquired using capillary column LC/electrospray ionization—ion trap mass spectrometry were evaluated for the peptides \(41\text{ISASR}^{45}\) and \(41\text{ISASpR}^{45}\). These peptides were monitored as both singly and doubly charged ions and the chromatographic peak areas for each calculated relative to the chromatographic peak area of the native reference peptide. These data are given in Figure 5. As seen in the figure, the peak area ratio of the phosphopeptide increased from 0 at 0 min to 0.050 \(\pm 0.006\) (mean \(\pm\) standard deviation) after 5 min, 0.083 \(\pm 0.025\) after 30 min, and 0.110 \(\pm 0.031\) after 120 min of reaction. Conversely, as the phosphopeptide was formed, the substrate region of the cTnI, \(41\text{ISASR}^{45}\), was consumed, with the peak area ratio progressing from 0.110 \(\pm 0.021\) at 0 min to 0.033 \(\pm 0.006\) after 5 min, and was undetectable at 30 and 120 min. The relative standard deviations of these triplicate measurements were \(-15\%\).

Other peptides, unaffected by the phosphorylation reaction, were also monitored to further assess the precision of the measurements. For example, the chromatographic peak area ratio of the peptide \(132\text{FDLR}^{136}\) remained unchanged over the course of the reaction. The average relative standard deviation of the four triplicate measurements was 7%.
Calibration and Conversion of Peak Area Ratio to Mole Fraction Phosphorylation. The peak area ratios are a direct result of the LC/MS analyses and are directly proportional to the relative degree of phosphorylation of a specific site. As a result, these peak area ratios can be utilized, without further processing, to monitor quantitatively the progression of the phosphorylation reaction. The peak area ratios can also be converted to mole fraction values if the responses of the analyte peptides, relative to the native reference peptide, are determined from calibration curves. The calibration curve for the analyte peptide 41ISASR45 is given in Figure 6A. Standard linear regression of calibration data for the 41ISASR45 and 41ISASPR45 peptides allows conversion of the measured peak area ratios into molar equivalents to give the reaction time course shown in Figure 6B.

Utility of the Native Reference Peptide Method. The combination of chromatography and mass spectrometry is a powerful, traditional tool for quantitative analyses. In general, quantitative methods that utilize mass spectrometry include some type of internal standard to compensate for factors such as variations in recovery and variations in instrument response that prevent the direct translation of amount of signal to amount of analyte. With the inclusion of an internal standard, good results are obtained—provided that the internal standard is incorporated into the analytical scheme as early as possible in the sample processing and that the chemical properties of the standard very closely match the properties of the analyte. The method presented here begins with a protein sample that has been isolated by SDS-PAGE. The SDS-PAGE separation is important because it not only facilitates the most direct analysis possible of mixtures but it is also generally advantageous for making high-quality digests. Common methods of adding internal standards are not possible with protein preparations that are separated by SDS-PAGE unless the standard begins with an added protein that migrates to the same position in the gel as the analyte protein. Proteins or peptides added after the SDS-PAGE experiment would not be suitable because they would either not equilibrate into the gel matrix or not accurately model the protein digestion. The native reference peptide is an ideal internal standard for gel-separated proteins because it experiences all of the same processing steps as the peptide that bears the posttranslational modification, including the SDS-PAGE separation, any reduction/alkylation steps, and the

Figure 5. (A) Time course of the in vitro PKC/II phosphorylation of cTnI at Ser44. The chromatographic peak area ratios of 41ISASR45 and 41ISASPR45 relative to the native reference peptide 121NITEIADLTQK131 plotted over the reaction time course. For the analyte peptides, 41ISASR45 (filled circles) and 41ISASPR45 (open circles), the chromatogram was reconstructed by adding the ion current for both the singly and doubly charged ions. For the native reference peptide, the chromatogram was reconstructed from the ion current for the double charged peptide alone. The differences in these values reflect the progression of the kinase reaction, i.e., the conversion of the nonphosphorylated peptide (41ISASR45) into the corresponding phosphorylated peptide (41ISASPR45). Each value is plotted as the mean ± standard deviation for the result of three independent samples. (B) 132IFDLR136 as a control peptide. The chromatographic peak area ratio for the peptide 132IFDLR136 relative to the native reference peptide 121NITEIADLTQK131 plotted over the reaction time course. This peptide showed no change during the time course of the kinase reaction.

Figure 6. (A) Calibration curve for the conversion of the chromatographic peak area ratio to moles of a specific analyte peptide per mole of protein for the absolute quantitation of the degree of phosphorylation. The chromatographic peak area ratios for 41ISASR45 relative to the native reference peptide were determined in duplicate for each standard mixture. (B) The time course of Ser44 phosphorylation. The calibration data were subsequently used to calculate moles of each analyte per mole of protein at the different time points of the PKC/II kinase reaction. The data shown in Figure 5A were converted to mole fractions of 41ISASR45 (filled triangles) and 41ISASPR45 (open triangles).
digestion. As a result, the native reference peptide rigorously compensates for variations in the amount of protein in the gel band.

Other quantitative methods for proteins and peptides that use mass spectrometry have been described. Relative quantification of proteins has been achieved with isotope-coded affinity tags (ICAT). These biotin-based reagents label cysteine-containing tryptic peptides from different treatment groups with either of two stable isotope labels, not only facilitating isolation with avidin binding but also allowing quantitation of the source protein by determining the isotope ratio.25 Although the ICAT approach is well-suited for making comparisons of the amounts of specific phosphorylated peptides in different samples, the method does not give the mole fraction of phosphorylation of a given site and would appear dependent on the strict control of the amount of protein in each sample. Further, the multistep derivatization chemistry might not be feasible for low levels of phosphorylation.26 Other investigators have monitored changes in protein expression and the degree of phosphorylation of the PAK-related Ste20 protein kinase in yeast through differential labeling, using cells grown in 15N-enriched media, versus unenriched controls.27 Again, quantitation is achieved by measuring the isotope ratios with mass spectrometry, but this method is limited by the requirement for cell growth in the enriched media. Finally, a method that does not require isotope labeling has been reported by Tsay and coworkers.28 In these experiments, quantification of peptide phosphorylation was carried out with LC/MS analyses to determine the ratio of phosphopeptide to the corresponding nonphosphorylated peptide. Such an approach has the advantage that no labeling is required but is dependent on the detection of the phosphorylated and nonphosphorylated species. Unfortunately, one effect of protein phosphorylation is to alter the pattern of proteolytic digest;14 that alteration potentially limits the applicability of this approach.

CONCLUSIONS

We propose the use of the native reference peptide method for site-specific quantitative analysis of protein phosphorylation. Using LC/MS analysis with selected ion monitoring, we have shown relative and absolute quantification of the degree of phosphorylation of cTnI by PKC\(\beta\)II at the Ser44 site. The method is completely compatible with SDS–PAGE-separated protein mixtures, utilizes the amounts of protein that are present in a single, Coomassie blue-stained gel band, and should be applicable to any protein—provided that an appropriate native reference peptide can be chosen. We would also speculate that similar results could be obtained for other types of modification, once the modified peptide is detected and characterized. Finally, the precision of the method seen in these analyses was in the range of 10–15% relative standard deviation. This level of precision would appear useful for most biological problems.

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