Anal. Chem. **2001,** *73,* 170−176

Analysis of Protein Phosphorylation by a Combination of Elastase Digestion and Neutral Loss Tandem Mass Spectrometry

Andreas Schlosser,[†] Ruediger Pipkorn,[‡] Dirk Bossemeyer,[§] and Wolf D. Lehmann*,[†]

Central Spectroscopy, Central Peptide Synthesis, and Department of Pathochemistry, German Cancer Research Center (DKFZ), Im Neuenheimer Feld 280, 69120 Heidelberg, Germany

Loss of phosphoric acid is the most effective fragmentation reaction of pSer- and pThr-containing phosphopeptides of small size (up to 10−15 residues) in low-energy collision-induced dissociation. Therefore, tandem mass spectrometry with neutral loss scanning was evaluated for its utility to analyze protein phosphorylation using protein kinase A (PKA) catalytic subunit, which is phosphorylated at Thr197 and Ser338, as an example. Analysis of tryptic digests of phosphoproteins by tandem mass spectrometry with scanning for neutral loss of phosphoric acid resulted in spectra with poor signal-to-noise ratio, mainly because of the large size of the phosphopeptides formed (>2 kDa). This unfavorable size was caused by the distribution of tryptic cleavage sites in PKA and by interference of phosphorylation with tryptic cleavage. To generate a set of smaller peptide fragments, digestion was performed using the low-specificity protease elastase. Analysis of the total elastase digest with neutral loss scanning resulted in observation of a set of partially overlapping phosphopeptides with high abundance, providing a complete coverage of PKA phosphorylation sites. The peptide size generated by elastase (0.5−1.5 kDa) is ideally suited for this scan mode, which was found to provide the highest specificity for detection of singly charged phosphopeptides (neutral loss of 98). Identification of the PKA phosphorylation sites was performed by mass spectrometric sequencing of the elastase-derived phosphopeptides, which provided highly informative product ion spectra.

Reversible phosphorylation of proteins on serine, threonine, and tyrosine residues is a key principle for regulation of enzymatic activities and for signal transduction.[1] The vast majority of reversible protein phosphorylations occur at serine and threonine residues, the formation of phosphoserine (pSer) happening roughly 10 times more than formation of phosphothreonine (pThr). Mass spectrometry combined with soft ionization techniques has found numerous applications in the field of protein phosphorylation. Using reflector matrix-assisted laser desorption/ionization (MALDI) MS, pSer- and pThr-phosphopeptides are easily recognized by their characteristic metastable peaks indicating loss of phosphoric acid.[2,3] Phosphorylation sites can be assigned by utilizing MALDI in-source[4] or postsource[5] decay spectra. Using electrospray ionization (ESI), more complex mixtures of phosphopeptides can be characterized by on-line coupling to liquid chromatography[6] or capillary electrophoresis.[7,8] The specificity of this approach has been further improved by additional use of metal-affinity chromatography.[8]

ESI tandem MS is a powerful technique for detection of phosphopeptides in an unseparated phosphoprotein digest[9] due to the occurrence of phosphopeptide-specific fragmentation reactions. In the negative ion mode, the m/z 79 fragment can be used for detection of pSer-, pThr-, or pTyr-phosphopeptides either in the skimmer collison-induced dissociation (CID) mode[6,10] or with precursor ion scanning.[9,11,12] In the positive ion mode, scanning for neutral loss of phosphoric acid can be employed for pSer- and pThr-peptides.[13−15] Theoretically, this MS/MS technique should be the method of choice for detection of pSer/pThr-phosphopeptides, since H_3PO_4 loss is phosphopeptide-specific and represents the most abundant fragmentation reaction of these peptides, often much more abundant than competing backbone fragmentations. Moreover, the molecular ion intensities for most peptides are higher in the positive ion mode compared to negative ion

* Corresponding author: (tel) +49-6221-424563; (fax) +49-6221-424554; (e-mail) wolf.lehmann@dkfz-heidelberg.de.

[†] Central Spectroscopy.

[‡] Central Peptide Synthesis.

[§] Department of Pathochemistry.

(1) Hunter, T. *Cell* **2000**, *100*, 113−127.

(2) Schnoelzer, M.; Lehmann, W. D. *Int. J. Mass Spectrom. Ion Processes* **1997**, *169/170*, 263−271.

(3) Mueller, D. R.; Schindler, P.; Coulot, M.; Voshol, H.; van Oostrum, J. *J. Mass Spectrom.* **1999**, *34*, 336−345.

(4) Kinumi, T.; Niwa, H.; Matsumoto, H. *Anal. Biochem.* **2000**, *277*, 177−186.

(5) Annan, R. S.; Carr, S. A. *Anal. Chem.* **1996**, *68*, 3413−3421.

(6) Huddleston, M. J.; Annan, R. S.; Bean, M. F.; Carr, S. A. *J. Am. Soc. Mass Spectrom.* **1993**, *4*, 710−717.

(7) Figeys, D.; Corthals, G. L.; Galli, B.; Goodlett, D. R.; Ducret, A.; Corson, M. A.; Aebersold, R. *Anal. Chem.* **1999**, *71*, 2279−2287.

(8) Cao, P.; Stults, J. T. *Rapid Commun. Mass Spectrom.* **2000**, *14*, 1600−1606.

(9) Carr, S. A.; Huddleston, M. J.; Annan, R. S. *Anal. Biochem.* **1996**, *239*, 180−192.

(10) Jedrzejewski, P. T.; Lehmann, W. D. *Anal. Chem.* **1997**, *69*, 294−301.

(11) Wilm, M.; Neubauer, G.; Mann, M. *Anal. Chem.* **1996**, *68*, 527−533.

(12) Neubauer, G.; Mann, M. *Anal. Chem.* **1999**, *71*, 235−242.

(13) Covey, T.; Shushan, B.; Bonner, R. In *Methods in Protein Sequence Analysis*; Jörnvall, H., Höög, J.-O., Gustavson, A.-M., Eds.: Birkhauser Verlag: Basel, 1991; pp 249−256.

(14) Hunter, A. P.; Games, D. E. *Rapid. Commun. Mass Spectrom.* **1994**, *8*, 559−570.

(15) Tholey, A.; Reed, J.; Lehmann, W. D. *J. Mass Spectrom.* **1999**, *34*, 117−123.

10.1021/ac000826j CCC: $20.00
Published on Web 12/08/2000

detection. Despite all these favorable items, few applications of neutral loss scanning in the field of protein phosphorylation have been reported to date.[13−15] In view of this situation, neutral loss scanning for pSer- and pThr-phosphopeptide detection was studied using trypsin and elastase digests of the phosphoprotein protein kinase A catalytic subunit as an example.

MATERIALS AND METHODS

Protein Kinase A (PKA) Isolation and Digestion. The catalytic subunit of protein kinase A was affinity-purified as described.[16] About 10-pmol aliquots of the protein in 20 μL of buffer were subjected to digestion by elastase or sequencing grade modified trypsin (Roche Diagnostics, Mannheim, Germany). Digestion was performed overnight at 37 °C and pH 8.5, in 20 mM Tris using elastase and, at pH 7.5, in 1 mM $CaCl_2$ and 20 mM Tris using trypsin.

Mass Spectrometry. A 10-μL aliquot of the digests was purified using C_{18} ZipTips (Millipore, Bedford, MA). Following sample loading, two 10-μL aliquots of 1% aqueous formic acid were used for the cleaning step and samples were eluted with 10 μL of 1:1 water/acetonitrile containing 1% formic acid. Samples were immediately investigated by nanoESI-MS on a triple quadrupole TSQ 7000 (Thermoquest, San Jose, CA). Spray capillaries for nanoESI were prepared in-house using a type P-87 micropipet puller (Sutter Instruments, Novato, CA) and coated with a semitransparent film of gold in a sputter unit. The spray voltage applied was between +600 and +800 V, and for the MS/MS experiments, argon was used as collision gas at a nominal pressure of 2 mTorr. Mass calibration was performed using phosphoric acid clusters generated by spraying an aqueous solution of 20 mM phosphoric acid. Intact protein kinase A was analyzed by nanoESI using a hybrid quadrupole/time-of-flight instrument Q-TOF II (Micromass, Manchester, U.K.) operated at a resolution of ~10 000.

RESULTS AND DISCUSSION

Selected Strategy. For detection of protein phosphorylation sites we selected the general strategy outlined in Figure 1. After phosphoprotein digestion, the total digest is analyzed by nanoESI tandem mass spectrometry. Neutral H_3PO_4-loss scanning for different charge states of phosphopeptides is applied to obtain phosphopeptide candidate m/z values, which are then used to initiate product ion scans. Product ion spectra and the known sequence of the phosphoprotein finally result in identification of the phosphopeptide sequence and of the modified site. We decided to investigate total digests with nanoESI and MS/MS, since this combination allows extended spectra accumulation (of ~20−200 scans) compared to the use of LC−MS/MS (only ~1−5 scans). Thus, MS/MS spectra with improved signal-to-noise ratio can be recorded. Finally, the phospho-coverage was checked by analyzing the molecular weight of the intact phosphoprotein by nanoESI MS.

Trypsin Digestion. Bovine heart PKA catalytic subunit was purified[16] and subjected to trypsin digestion. The complete digest was analyzed by nanoESI MS and MS/MS with neutral loss scanning to detect doubly and triply charged phosphopeptides. The results are given in Figure 2. Among the peptides detected

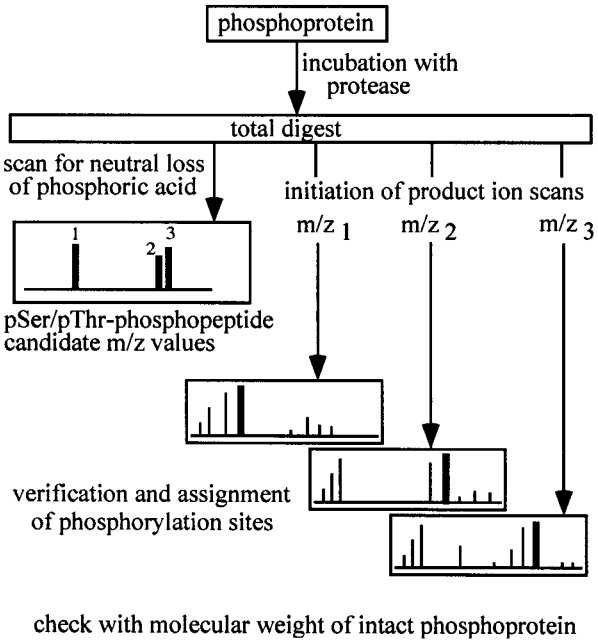

Figure 1. Strategy for localization of pSer/pThr phosphorylation sites. The phosphoprotein is cleaved with a protease, and the total digest is analyzed with nanoESI MS and scanning for neutral loss of phosphoric acid. The signals observed in the neutral loss spectrum are used to initiate product ion scans for verification of the presence of a phosphate group and assignment of the phosphorylated site. Finally, phospho-coverage is checked by comparison with the molecular weight of the intact phosphoprotein. This approach requires that either additional protein modifications can be excluded or are known.

in the normal ESI mass spectrum, two peptides are observed, the molecular weight of which corresponds to tryptic peptides with one phosphate group (mol wt average 2215.5 = T30; mol wt average 2999.0 = T45−47). These are listed in Table 1, which summarizes peptide fragments of PKA catalytic subunit with up to two uncleaved tryptic sites, which contain the known phosphorylation sites Thr197 and Ser338. The scans for neutral loss of m/z 49 and 32.7 (Figure 2b and c), selected to detect doubly and triply charged phosphopeptides, display the corresponding signals of the T30 and of the T45−47 fragment, however, with only moderate signal-to-noise ratio although acquisition was performed at optimal collision offset (see also Figure 6).

The question that arises in view of the calculated tryptic fragments in Table 1 is why none of the fragments T47, T47−48, and T47−49 are observed, which show a size well suited for their detection by neutral loss of phosphoric acid. We suspected that phosphorylation at Ser338 might interfer with tryptic cleavage at Arg336.[17] To check this hypothesis, we synthesized the peptides IRVSINEK and IRVpSINEK and analyzed their roughly equimolar mixture before and after tryptic digestion (see Figure 3). Comparison of the spectra given in Figure 3 clearly shows that phosphorylation at Ser338 blocks digestion of this model peptide. Thus, we conclude that the fragments T47, T47−48, and T47−49 are not observed in the tryptic digest of PKA because phosphorylation at Ser338 blocks this cleavage. We also observed a similar

(16) Olsen, S. R.; Uhler, M. D. *J. Biol. Chem.* **1989**, *264*, 8662−8666.

(17) Boyle, W. J.; van der Geer, P.; Hunter, T. In *Methods in Enzymology*; Hunter, T., Sefton, B. M., Eds.: Academic Press: London, 1991; Vol. 201, pp 110−149.

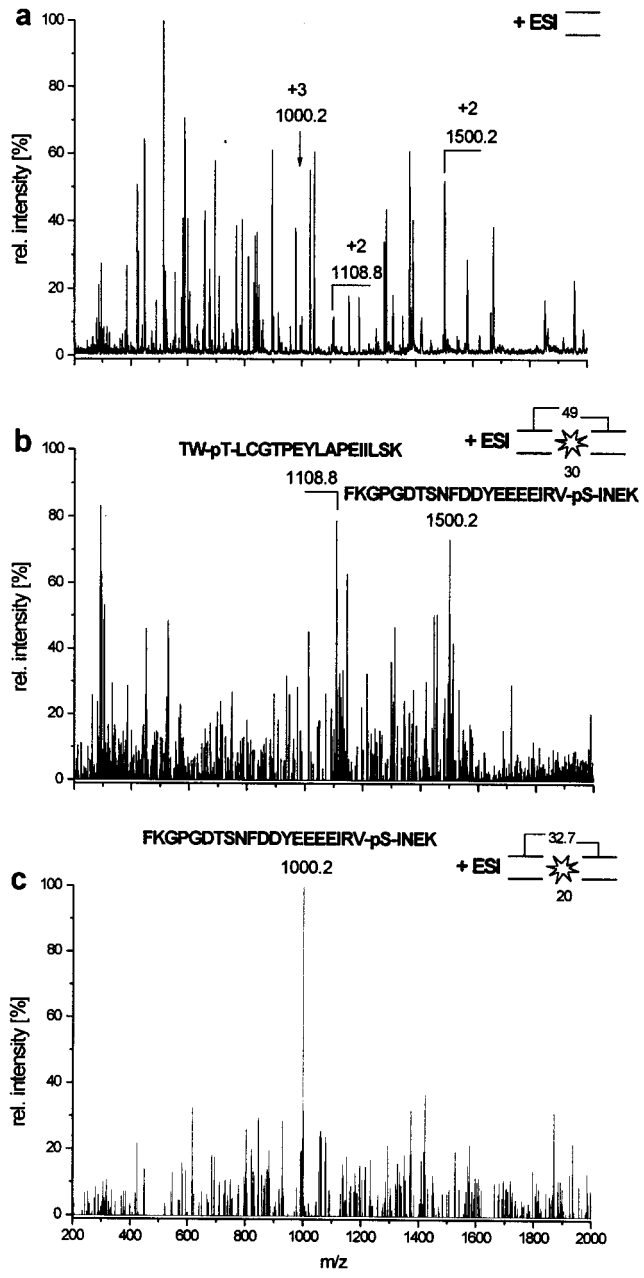

Figure 2. Positive ion nanoESI MS analysis of the unseparated tryptic digest of bovine PKA catalytic subunit: (a) mass spectrum; (b) scan for neutral loss of 49 Da for detection of doubly charged phosphopeptides, collision offset −30 V; (d) scan for neutral loss of 32.7 Da for detection of triply charged phosphopeptides, collision offset −20 V.

block of trypsin digestion for the sequence K−X−pS with the same strategy (data not shown) and thus conclude that trypsin cleavage is severely hampered at K and R residues which are part of a phosphorylated PKA consensus sequence K/R−X−pS/pT. This characteristic adds to the other limitations of trypsin cleavage which are no cleavage at K/R−P sites and less efficient cleavage at K/R−K/R and K/R−D/E sites. The conclusion from these observations is that trypsin proteolysis is not the method of choice for analyzing protein phosphorylation by neutral loss scanning, simply because large fragments are formed.

Elastase Digestion. To generate smaller phosphoprotein fragments better suited for neutral loss scanning, the broad-specificity protease elastase was selected for digestion.[18] Compared to trypsin, elastase generates a larger number of peptides with a lower average molecular weight. In addition, due to its broader cleavage specificity, it can be expected that the influence of phosphorylation on the product pattern might not be as pronounced as observed for trypsin. These characteristics should improve the sensitivity and phospho-coverage of the neutral loss scan approach selected. In addition, assignment of phosphorylation sites is facilitated in small compared to large fragments. Figure 4 shows the neutral loss scans for detection of singly and doubly protonated phosphopeptides applied to an elastase digest of protein kinase A catalytic subunit. These spectra indicate the presence of eight phosphopeptides with the molecular weights 654.3, 858.4, 915.4, 940.5, 1014.5, 1027.5, 1043.5, and 1426.8 (see also Table 3). Among these, three sequences carry one basic amino acid (K or R), four contain two, and one carries four basic amino acids. Those with one basic residue are only observed as singly charged ions (Figure 4b); of those with two basic residues, three are observed both as singly and doubly charged ions (Figure 4b and c), as well as the sequence with four basic residues. Phosphopeptides with a single basic amino acid are not detected in the neutral loss scan for 49 Da (Figure 4c), since they form doubly charged ions with minor abundance only. Using the SHERPA program and the PKA sequence, search for peptides/phosphopeptides results in a set of peptides as shown in Table 2 for mass 1014.5 as an example.

Identification of Peptides in an Elastase Digest. The list of isobaric peptides in Table 2 exemplifies the situation that using elastase, peptide assignment by mass alone is not possible due

Table 1. Calculated Tryptic Fragments (Two Undigested Sites Allowed) of PKA Catalytic α-Subunit, Containing the Phosphorylation Sites Thr197 and Ser338[a]

Tryptic Peptides (max. 2 undigested sites) containing Threonine 197

frag. no.	residues	mol.wt. (avg.)	sequence
28..30	191 - 213	2656.036	R <VKGRTW-pT-LCGTPEYLAPEIILSK>G
29..30	193 - 213	2428.729	K <GRTW-pT-LCGTPEYLAPEIILSK>G
29..31	193 - 217	2891.235	K <GRTW-pT-LCGTPEYLAPEIILSKGYNK>A
30	**195 - 213**	**2215.489**	**R <TW-pT-LCGTPEYLAPEIILSK>G**
30..31	195 - 217	2677.995	R <TW-pT-LCGTPEYLAPEIILSKGYNK>A
30..32	195 - 249	6363.257	R <TW-pT-LCGTPEYLAPEIILSKGYNKAVDWWA LGVLIYEMAAGYPPFFADQPIQIYEK> I

Tryptic Peptides (max. 2 undigested sites) containing Serine 338

frag. no.	residues	mol.wt. (avg.)	sequence
45..47	**318 - 342**	**2999.045**	**K <FKGPGDTSNFDDYEEEEIRV-pS-INEK> C**
45..48	318 - 345	3287.416	K <FKGPGDTSNFDDYEEEEIRV-pS-INEKCGK> E
46..47	320 - 342	2723.694	K <GPGDTSNFDDYEEEEIRV-pS-INEK> C
46..48	320 - 345	3012.065	K <GPGDTSNFDDYEEEEIRV-pS-INEKCGK> E
47	337 - 342	768.759	R <V-pS-INEK> C
47..48	337 - 345	1057.130	R <V-pS-INEKCGK> E
47..49	337 - 350	1696.792	R <V-pS-INEKCGKEFSEF>

[a] The observed fragments are given in boldface type.

(18) Barrett, A. J.; Rawlings, N. D.; Woessner, F. D. *Handbook of Proteolytic Enzymes*; Academic Press: San Diego, 1998.

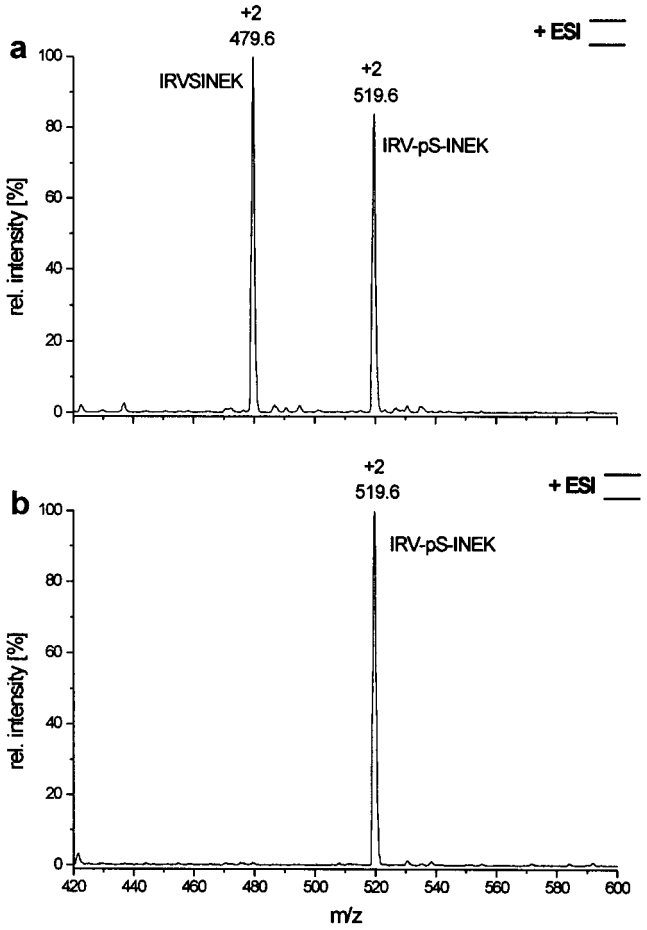

Figure 3. Positive ion nanoESI mass spectra of a roughly equimolar mixture of the peptides IRVSINEK and IRV−pS−INEK containing Ser338: (a) before incubation with trypsin; (b) after incubation with trypsin. It is observed that the nonphosphorylated peptide is cleaved, whereas the phosphorylated peptide remains intact.

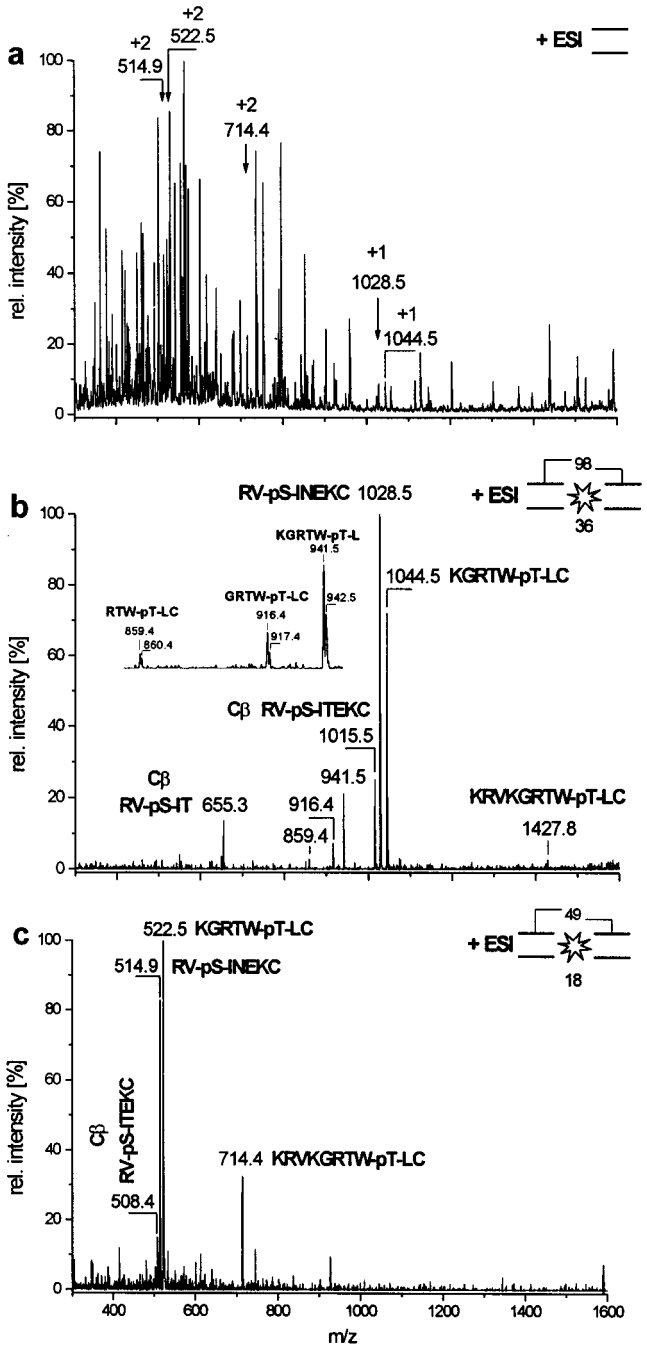

Figure 4. Positive ion nanoESI analyis of the elastase digest of bovine PKA catalytic subunit: (a) mass spectrum; (b) scan for neutral loss of 98 Da for detection of singly charged phosphopeptides, collision offset −36 V; (c) scan for neutral loss of 49 Da for detection of doubly charged phosphopeptides, collision offset −18 V.

to the broad cleavage specificity. For the mass values determined by neutral loss scanning in this study, up to 10 isobaric sequences (including nonphosphorylated sequences) were obtained within a mass error of ±0.5 Da. Among the strategies suited for assignment of the true sequence (see Discussion below), we selected product ion scanning. As an example, Figure 5 shows the results obtained for product analysis of m/z 508. Besides the expected neutral loss of H_3PO_4, a b-ion series extending from b_2 to b_6 is observed that identifies the sequence RV−pS−ITEKC with serine at position 3 as the site of phosphorylation, since the b_3-H_3PO_4 and b_4-H_3PO_4 ions are observed. RV−pS−ITEKC indicates the presence of the β-gene product of PKA. One of the differences between the PKA α- and β-gene product occurs at position 340, where the β-gene product exhibits a N → T exchange. Thus, the major signal in Figure 4b at m/z 1028.5 (or m/z 514.9 in Figure 4c) corresponds to the α-gene product RV−pS−INEKC. In the neutral loss spectra obtained by analysis of the trypsin digest, a signal for the β-gene product is not identified due to the poorer signal-to-noise ratio. For the pThr197-containing phosphopeptides observed after trypsin or elastase digestion, both gene products have the identical sequence.

Product ion scans were performed for four phosphopeptides, which allowed reliable identification of a single sequence from the list of isobaric peptides obtained using SHERPA. The occurrence of b ions appears to be characteristic for the product ion spectra of elastase-derived peptides, since these carry an uncharged residue at the C-terminus and often contain a basic site in the central part or near the N-terminus. The list of identified phosphopeptides is given in Table 3. Analyzing the ESI spectrum of the tryptic digest, unphosphorylated analogues of the identified phosphopeptides were not observed. This points toward a complete phosphorylation at Thr197 and Ser338. The presence of two completely phosphorylated sites was also supported by analysis of intact bovine PKA catalytic subunit by nanoESI MS which provided a molecular weight of 40 859.5. The value calculated for

Table 2. Isobaric Peptides Selected by SHERPA Using the Molecular Weight Data (mol wt mono 1014.50 as an Example) Derived from the Neutral Loss Spectra (See Figure 4) with an Open-Ended Sequence Search Performed on the Sequences of PKA Catalytic α- and *β*-Subunits (Mass Tolerance ±0.5 Da)

ΔM	mol.wt. (mono)	residues	origin	sequence
-0.01	1014.49	38 - 45	PKA alpha	T <AHLDQFER> I
0.12	1014.62	248 - 256	PKA alpha	Y <EKIVSGKVR> F
-0.01	1014.49	275 - 282	PKA alpha	G <pS-EQESVKE> F
-0.11	1014.39	10 - 17	PKA alpha	G <SEQE-pS-VKE> F
-0.05	1014.45	281 - 289	PKA beta	R <FGNLKNGV-pS> D
0.12	1014.62	248 - 256	PKA beta	Y <EKIVSGKVR> F
-0.01	1014.49	275 - 282	PKA beta	Q <VDL-pT-KRFG> N
-0.04	**1014.46**	**336 - 343**	**PKA beta**	**I <RV-pS-ITEKC> G**
-0.04	1014.46	336 - 343	PKA beta	I <RVSI-pT-EKC> G

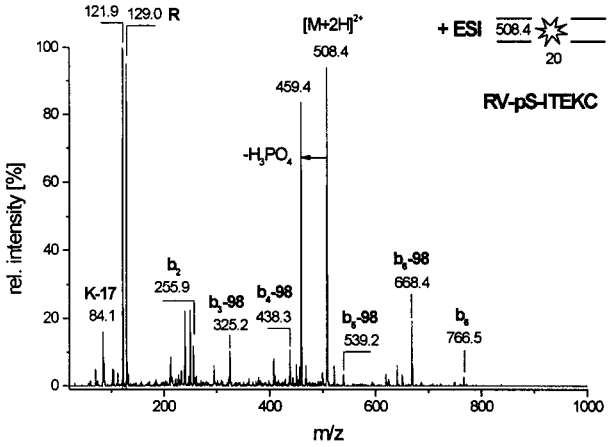

Figure 5. Product ion spectrum of *m*/*z* 508.4, collision offset −20 V. A b-ion series from b_2 to b_6 confirms the sequence of RV−pS−ITEKC. The presence of the $b_3 - 98$ and $b_4 - 98$ ions identifies the serine residue as phosphorylation site.

myristoylated, doubly phosphorylated PKA is 40 858.8. The minor *β*-gene product has a molecular mass 26 Da lower than the α-gene product. This signal was observed with an abundance of ~25% of the α-gene product.

In this study, informative product ion spectra could be obtained for elastase-derived peptides that were identified as phosphopeptide candidates in the neutral loss spectra. Using the product ion spectra, the correct peptide could be unambiguously selected from the software-selected list of isobaric PKA peptides. There are other methods to guide this selection. (i) Accurate mass determination, e.g., using ESI-TOF or ESI-Q-TOF instrumentation, narrows the list of candidate sequences considerably. (ii) The presence or absence of immonium ions is a useful parameter. In our experience, the immonium ions of L/I and of the amino acids containing a ring system (P, F, Y, H, W) are useful indicators for the presence or absence of the corresponding residue. However, if the precursor ion selected is a mixture of compounds, this hampers a reliable correlation of immonium ions with a certain sequence. (iii) Information about the C-terminal amino acid would also greatly

Table 3. Phosphopeptides Identified from an Elastase Digest of Bovine Heart PKA Catalytic Subunit[a]

mol. wt. (mono)	residues	sequence	origin
654.3	336-340	RV-pS-IT	PKA-beta
858.4	194-199	RTW-pT-LC	PKA_alpha
915.4	193-199	GRTW-pT-LC	PKA_alpha
940.5	192-198	KGRTW-pT-L	PKA_alpha
1014.5	336-343	RV-pS-ITEKC	PKA_beta
1027.5	336-343	RV-pS-INEKC	PKA_alpha
1043.5	192-199	KGRTW-pT-LC	PKA_alpha
1426.8	189-199	KRVKGRTW-pT-LC	PKA_alpha

[a] Phosphorylation at Thr197 and Ser338 is detected. In addition to the main α-gene product, the *β*-gene product is also detected. All identified phosphopeptides with pSer338 contain position 340, which carries N in the α- and T in the *β*-gene product.

facilitate the selection of a peptide sequence from a list such as given in Table 2. C-terminal sequencing by MS/MS starts from singly charged alkali-adducted[19−21] or argentinated peptide ions,[22] a method that in principle would also allow one to recognize or exclude an isobaric overlap. However, a method for generating peptide ions of this type with sufficient intensity is not available. (iv) After derivatization with the Edman reagent phenylisothiocyanate, the N-terminal amino acid can be recognized on the basis of an abundant b_1 ion.[23] Further development of these methods will definitely enhance the analytical use of broad-specificity proteases. Finally, software is available to use peptide fragments with low or no C-terminal specificity and their sequence tags for identification of the precursor protein.[24,25]

Phospho-Coverage. Complete coverage of all phosphorylation sites is an important goal in phosphoprotein analysis. Loss of a phosphopeptide during sample cleanup due to its hydrophilic nature is one reason for incomplete phospho-coverage.[26] One possibility for improving an incomplete phospho-coverage is to use a different protease, since there is a chance that the phosphorylation site originally lost is then present in a more hydrophobic peptide. Another strategy is to use a different packing material for the desalting column.[12] A straightforward control for completeness of the phospho-coverage achieved is the determination of the molecular weight of the intact phosphoprotein; however, other covalent modifications have to be known for this approach.

(19) Renner, D.; Spiteller, G. *Biomed. Environ. Mass Spectrom.* **1988**, *15*, 75−77.
(20) Tang, X.; Ens, W.; Standing, K. G.; Westmore, J. B. *Anal. Chem.* **1988**, *60*, 1791−1799.
(21) Lin, T.; Glish, G. L. *Anal. Chem.* **1998**, *70*, 5612−5615.
(22) Chu, K. I.; Guo, X.; Lau, T. C.; Siu, K. W. M. *Anal. Chem.* **1999**, *71*, 2364−2372.
(23) Summerfield, S. G.; Bolgar, M. S.; Gaskell, S. J. *J. Mass Spectrom.* **1997**, *32*, 225−231.
(24) Korostensky, C.; Staudenmann, W.; Dainese, P.; Hoving, S.; Gonnet, G.; James, P. *Electrophoresis* **1998**, *19*, 1933−1940.
(25) Perkins, D. N.; Pappin, D. J.; Creasy, D. M.; Cottrell, J. S. *Electrophoresis* **1999**, *20*, 3551−3567.
(26) Posewitz, M. C.; Tempst, P. *Anal. Chem.* **1999**, *71*, 2883−2892.

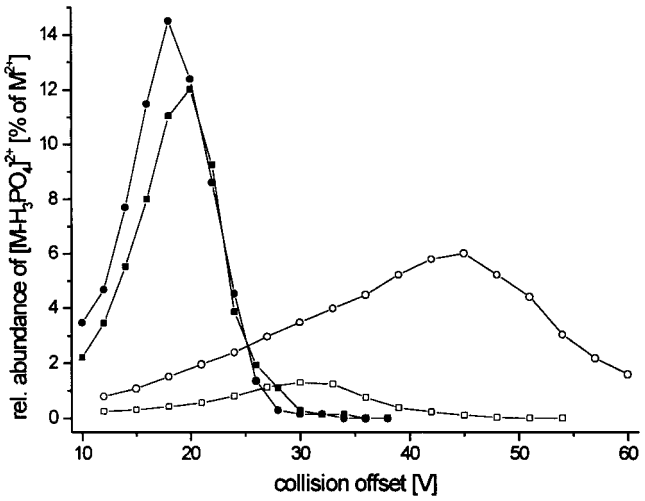

Figure 6. Collision offset plots for loss of H_3PO_4 from doubly charged phosphopeptides derived from protein kinase A catalytic α-subunit by elastase or trypsin digestion: (●) RVpSINEKC, (■) KGRTWpTLC, (□) TWpTLCGTPEYLAPEIILSK, and (○) FKGPGDTSN-FDDYEEEEIRVpSINEK. The trypsin-derived peptides show less abundant H_3PO_4 loss and require higher offset values compared to the elastase-derived peptides. Both effects are mainly caused by the larger size of the trypsin-derived peptides.

Collision Offset and Phosphopeptide Size. To investigate the efficiency of H_3PO_4 loss as a function of the phosphopeptide size in a quantitative manner, we recorded the intensity of the corresponding $[M + 2H - H_3PO_4]^{2+}$ signal of the trypsin- and elastase-derived phosphopeptides of PKA as a function of the collision offset. These intensities were then normalized to the intensity of the $[M + 2H]^{2+}$ ion. The resulting functions are given in Figure 6. It is evident that the elastase-derived phosphopeptides show a higher abundance for loss of H_3PO_4 than the trypsin-derived peptides and, thus, are detected with a higher sensitivity from total digest mixtures by neutral loss scanning. To investigate further the dependence of the optimal collision offset value from phosphopeptide size, we recorded collision offset plots for loss of phosphoric acid for a number of synthetic phosphopeptides in the molecular weight range from 750 to 2000. The results presented in Figure 7 imply that the optimal collision offset values show a dependency on molecular weight. For practical purposes, we fitted this dependency with a simple linear function for the singly and doubly charged ions. These functions are also given in Figure 7. Establishing a link between the neutral loss scan function and the collision offset using these functions, optimal fragmentation conditions can be established for detection of phosphopeptides over the complete mass range. The data presented in this study have been recorded with a fixed collision offset as documented in the spectra insets.

Sensitivity and Specificity of Scanning for Neutral Loss of Phosphoric Acid. The signal-to-noise ratio of phosphopeptide signals in a spectrum generated by scanning for neutral loss of phosphoric acid depends on the abundance of the fragmentation reaction and on the abundance of interfering ion signals. As shown above, the abundance of phosphoric acid loss can be optimized by a small phosphopeptide size and by selection of the optimal collision offset. With regard to interfering fragment signals, the ideal situation for application of neutral loss scanning would be that loss of phosphoric acid is the only or at least the most

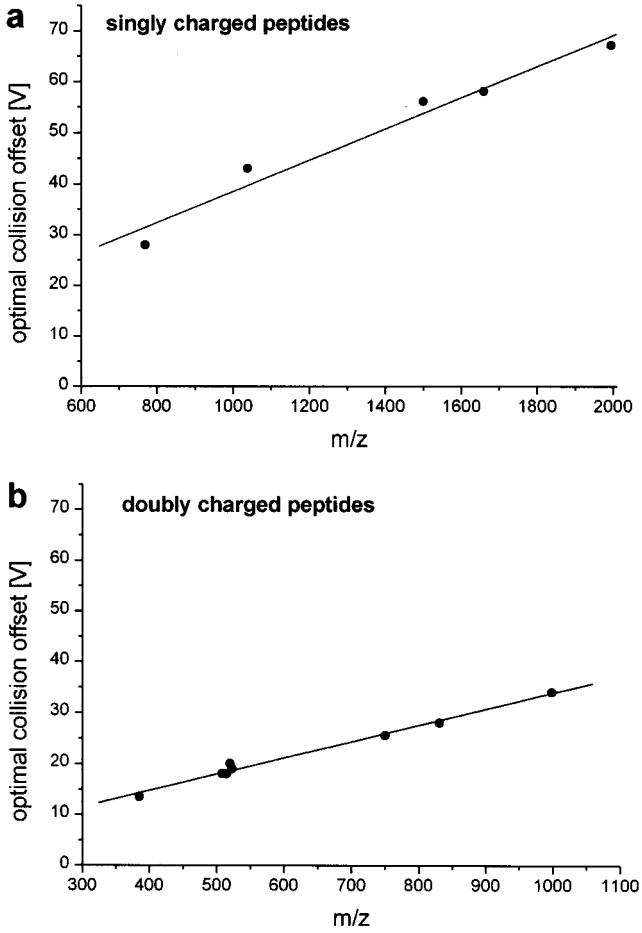

Figure 7. Optimal collision offsets for loss of H_3PO_4 for various phosphopeptides as a function of the *m*/*z* value. (a) Singly charged molecules of the following peptides: VpSINEK, IRVpSINEK, VRF-PpSHFSSDLK, KGpSEQESVKEFLAK, and myrGDAAAAKKGpSE-QEES. (b) Doubly charged molecules of the following peptides: VpSINEK, IRVpSINEK, RVpSINEKC, RVpSITEKC, KGRTWpTLC, VRFPpSHFSSDLK, KGpSEQESVKEFLAK, and myrGDAAAAKK-GpSEQEES. Linear regression analysis of the data points resulted in the functions $y = 8.0 + 0.0304x$ for singly charged molecules and $y = 2.0 + 0.0159x$ for doubly charged molecules, respectively.

prominent fragmentation reaction. However, since peptides show an increasing tendency to backbone fragmentation with increasing charge state, there is a risk that multiply charged nonphosphorylated peptides may show fragments located at $-98/z$ Da relative to the molecular ion (z, number of charges) and thus give rise to a signal in the neutral loss scan.

This effect is evident in Figure 4, showing that neutral loss scanning for 98 Da results in a spectrum with higher signal-to-noise ratio compared to neutral loss scanning for 49 Da. The neutral loss spectrum for detection of triply charged phosphopeptides shows a further increase in nonspecific background (data not shown). The main reason for this effect is that the low collision offsets required for optimal detection of doubly and triply charged phosphopeptides are also optimal for peptide backbone fragmentation of multiply charged peptides. Nevertheless, neutral loss scans for doubly and triply charged phosphopeptides are of value to support the findings of the neutral loss scan for 98 Da, which generally shows the best signal-to-noise ratio.

A possible source of error in scanning for neutral loss of H_3PO_4 is the presence of phosphoric acid. In the ESI spray process,

phosphoric acid tends to form relatively stable noncovalent complexes with proteins and peptides.[27,28] Upon collision-induced dissociation, these noncovalent complexes show an abundant loss of H_3PO_4 just as pSer- and pThr-phosphopeptides. These noncovalent complexes can be recognized as artifacts since they normally occur as a +98 Da satellite ions of intense peptide signals. In contrast, covalent phosphorylation causes a mass shift of +80 Da. Special attendance to these noncovalent H_3PO_4 complexes is recommended when in vitro phosphorylation assays are being analyzed. In these assays, generally high concentrations of ATP are present which upon hydrolysis generate phosphate ions.

An exception for the usual link between pSer- and pThr-phosphopeptide detection and neutral loss of 98/*z* occurs when a phosphopeptide shows simultaneous loss of water and phosphoric acid, mimicking a neutral loss of 116 Da. We observed this phenomenon upon analyzing the artificial sequence EAQAA−pS−AAQAK. This phosphopeptide could only be detected by scanning for neutral loss of 116/*z*, since loss of water from the N-terminal glutamate residue occurred almost quantitatively under the collision offset conditions optimal for loss of H_3PO_4. A neutral loss smaller than 98/*z*, namely, of 80/*z*, was observed studying a pSer-peptide, where the phosphorylation site was part of a SSS sequence. In this case, loss of HPO_3 and not of H_3PO_4 was the predominant process (unpublished results).

Little experience exists with identification by neutral loss scanning of peptides containing multiple pSer or pThr residues. So far, we only observed a simply additive behavior, a doubly phosphorylated peptide showing fragments for loss of one and two H_3PO_4 units. Highly phosphorylated peptides may show reduced sensitivity when detected in the positive ion ESI mode, and sequencing of multiply phosphorylated peptides is difficult since the multiple H_3PO_4 satellite ions of fragment ions hamper the recognition of ion series. To overcome this problem, dephosphorylation and subsequent product ion analysis of the dephosphorylated peptides has successfully been demonstrated.[29]

CONCLUSIONS

Elastase digestion, neutral loss, and product ion scanning have been successfully combined for analysis of phosphorylation sites in proteins. Use of a nonspecific protease generates small phosphopeptides (0.5−1.5 kDa), which are ideal for detection by scanning for neutral loss of H_3PO_4. For specific detection by neutral loss scanning, the singly charged ions are particularly useful. Sequencing byproduct ion spectra and localization of the phosphorylated site are best performed using doubly charged molecular ions. Generation of small proteolytic fragments by nonspecific digestion provides a higher specificity in localization of the phosphorylation site compared to the larger fragments obtained by trypsin digestion. The loss in proteolytic specificity using a protease such as elastase is balanced by the fact that almost complete sequence information can be extracted from the product ion spectra of small peptides. When applied in this context, neutral loss scanning represents a powerful supplement to other more established techniques in the field of phospho-proteomics.

ACKNOWLEDGMENT

We thank M. Karas for valuable discussions.

Received for review July 26, 2000. Accepted October 20, 2000.

AC000826J

(27) Henry, K. D.; Williams, E. R.; Wang, B. H.; McLafferty, F. W.; Shabanowitz, J.; Hunt, D. F. *Proc. Natl. Acad. Sci. U.S.A.* **1989**, *86*, 9075−9078.

(28) Chowdhury, S. K.; Katta, V.; Beavis, R. C.; Chait, B. T. *J. Am. Soc. Mass Spectrom.* **1990**, *1*, 382−388.

(29) Resing, K. A.; Johnson, R. S.; Walsh, K. A. *Biochemistry* **1995**, *34*, 9477−87.