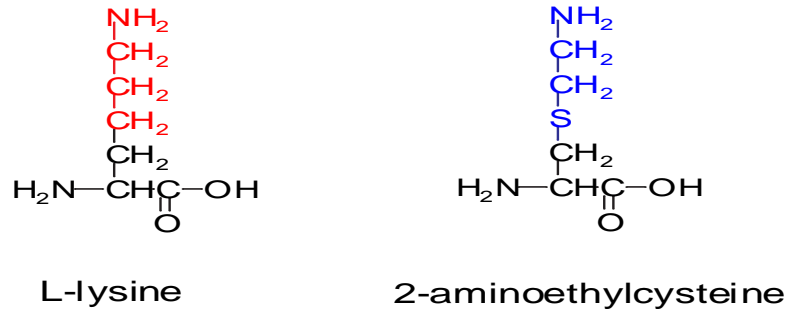


# **Identification of Phosphorylated and Glycosylated Sites in Peptides by Chemically-Targeted Proteolysis (CTID)**

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## 2-AMINOETHYLCYSTEINE IS AN ANALOG OF LYSINE

As shown by R.D.S. Cole in 1967, 2-aminoethylcysteine, produced by the reaction of cysteine containing peptides with ethyleneimine, <sup>1</sup> could serve as a substrate for trypsin.



Later it was shown by Masaki et al. that this residue was cleaved with even greater efficiency by Lysine endopeptidase <sup>2</sup>.

**This poster demonstrates that 2-aminoethylcysteine, formed upon  $\beta$ -eliminating phospho- or *O*-glycosylserines and threonines in the presence of 2-aminoethanethiol, are cleaved by lysine endopeptidase. Advantage can be taken of these reactions as an analytical tool for investigating these PTM's in proteins and peptides.**

- 1.) Cole, R.D.S. "Aminoethylation". Hirs.CHW. (ed): Methods Enzymol. Vol. 11. New York:Academic Press, 1967:315-317.
- 2.) Masaki, T, Takiya, T, Tsunasawa, S, Kuwahara, S, Sakiyama, F, Soehma, M. "Hydrolysis of S-2-Aminoethylcysteinyl Peptide Bond by Achromobacter Protease I". Biosci. Biotech. Biochem. 1994;58(9):215-216.

## Abstract

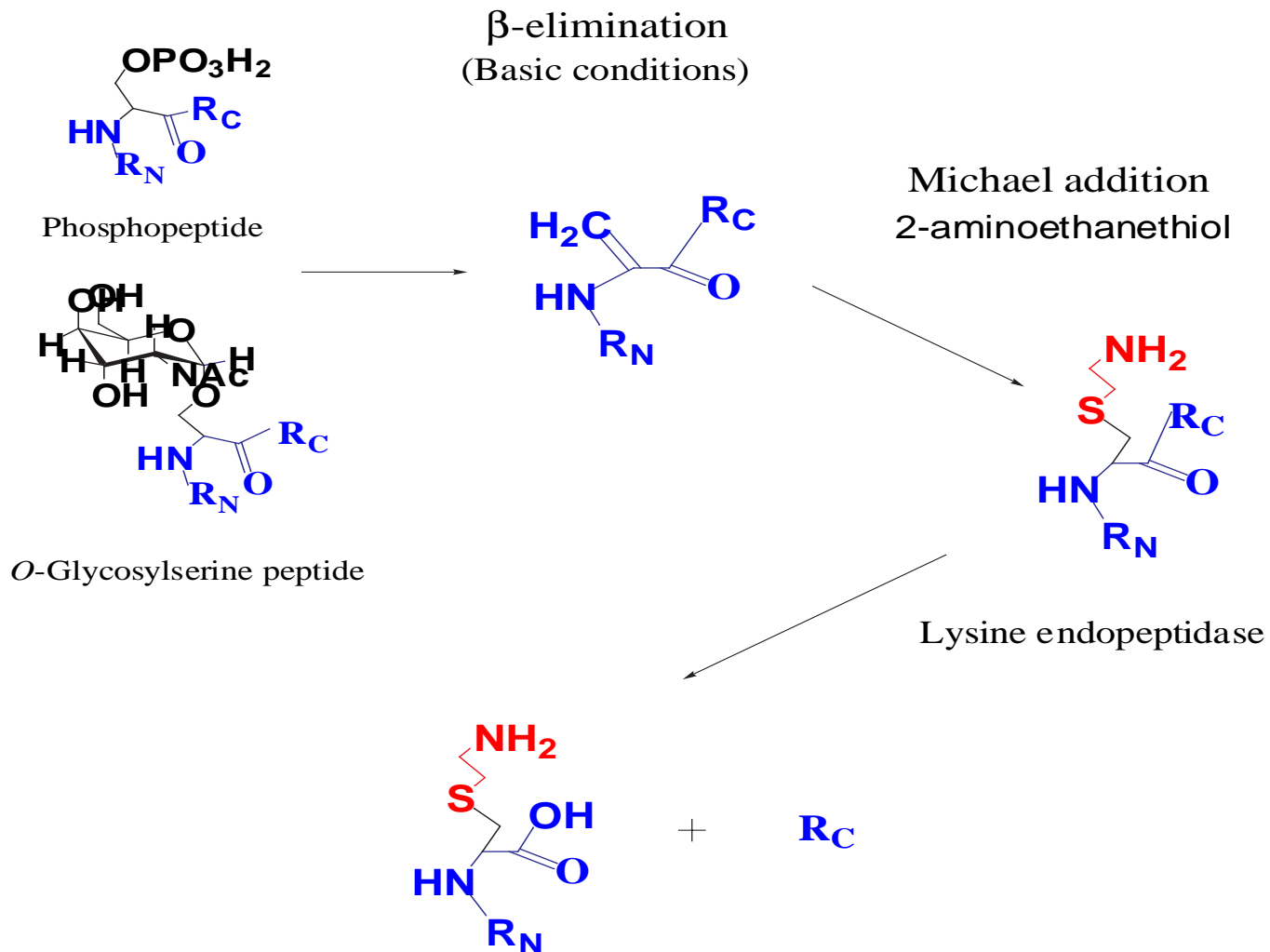
The increasing number of known sequences makes it possible to use a new approach to determine the site of certain post-translational modifications (PTM) by chemically targeting them for specific proteolytic cleavage. Analysis of the digest allows one to obtain correct structures by inference. We call this approach chemically-targeted-identification or **CTID**.

Peptides, which were phosphorylated, or glycosylated on serine or threonine residues underwent  $\beta$ -elimination in the presence of the nucleophile 2-aminoethanethiol to produce a 2-aminoethylcysteine analog of lysine. Both serine and threonine derivatives were specifically cleaved by lysine endopeptidase. The products of digestion were analyzed by mass spectrometry and/or Edman sequencing. Prior knowledge of the sequence allowed site identification either from peptide mass, or from a few residues sequenced from the newly liberated amino terminus. Oxidation of the modified peptide allowed its detection in digest mixtures by precursor ion, or neutral loss linked scans.

Mass analysis of specific (enzymatic) fragments combined with computerized searching of genomic databases to gain specific structural or sequence information has become the standard approach in Proteomics. Unfortunately, with few exceptions, the genomic sequence provides little help in predicting the site or extent of post-translational modifications. However, if site-specific cleavage of the modified site could be achieved, then genomic sequence information could be used to identify the amino acids involved.

In this study, phosphorylated and *O*-glycosylated peptides were  $\beta$ -eliminated in the presence of the nucleophile, 2-aminoethanethiol. The product was the 2-aminoethylcysteine analog of lysine, which was cleaved by lysine endopeptidase. The products of digestion were analyzed either by mass spectrometry, Edman sequencing or both. Site identification could be inferred from the peptide masses, or from a few residues sequenced from the newly liberated amino terminus and confirmed by tandem mass spectrometry.

**CONVERSION OF PHOSPHO- AND O-GLYCOSYLPEPTIDES TO  
SMALLER FRAGMENTS FOR TANDEM MASS AND/OR  
N-TERMINAL SEQUENCING**



1. Smaller, stable fragments for MS/MS
2. New aminotermminus (chemical sequencing)

## **Materials**

Phosphatase substrate (PS) and protein kinase C substrate (PKC) phosphopeptides were obtained from Anaspec Labs (San Jose, CA). Calcineurin substrate (CNS) was obtained from Calbiochem. (San Diego, CA). Lysine Endopeptidase from *Achromobacter lyticus* (E.C. 3.4.21.50) was purchased from WAKO Chemicals (Richmond, VA).

N-hydroxysuccinimide and 2-aminoethanethiol hydrochloride (2-AET) were from Sigma Aldrich Chemical Co. N-acetoxysuccinimide was synthesized according to the procedure of Geng et al. (2000).

## **Methods**

### Elimination and Addition Reactions

Beta-elimination and addition reactions for phosphoserine and phosphothreonine peptides were carried out with a modification of the procedure of Molloy and Andrews (2001).<sup>3</sup>

#### *Phosphopeptides:*

To the dry peptide, 10 µl 1M NaOH, 3 µl saturated Ba(OH)<sub>2</sub> and 12 µl of 0.5M 2-AET were added. Incubation was for 1-5 hr at 45-47 C.

#### *O-glycosylpeptides:*

Peptides were incubated at 45 C for 5-12 hr in a 25% solution of ammonium hydroxide, 0.14 M 2-AET.<sup>4</sup>

<sup>1</sup> Geng, M., Ji, J. Regnier, F.E. (2000) J. Chromatogr. (A) 870, 295-313.

<sup>2</sup> Ji, J., Chakraborty, A., Geng, M., Zhang, X., Amini, A., Bina, M., and Regnier, F. (2000) J. Chromatogr. (B) 745, 197-210.

<sup>3</sup> Molloy, M.P. and Andrews, P.C. (2001) Anal. Chem. 73, 5367-5394.

<sup>4</sup> Rademaker, G.J., Pergantis, S.A., Blok-Tip, L. Langridge, J.L., Kleen, A., and Thomas-Oates, J.E. (1998) Anal. Biochem. 257, 149-160.

**REACTION SCHEME FOR PROTEIN  
KINASE C SUBSTRATE  
PHOSHOPEPTIDE**

**KRPpSQRHGSKY**

↓  
Acetylation  
β- elimination  
Michael addition

**Ac-KRPJQRHGSKY**

↓  
Endopeptidase  
Mass spectrometry

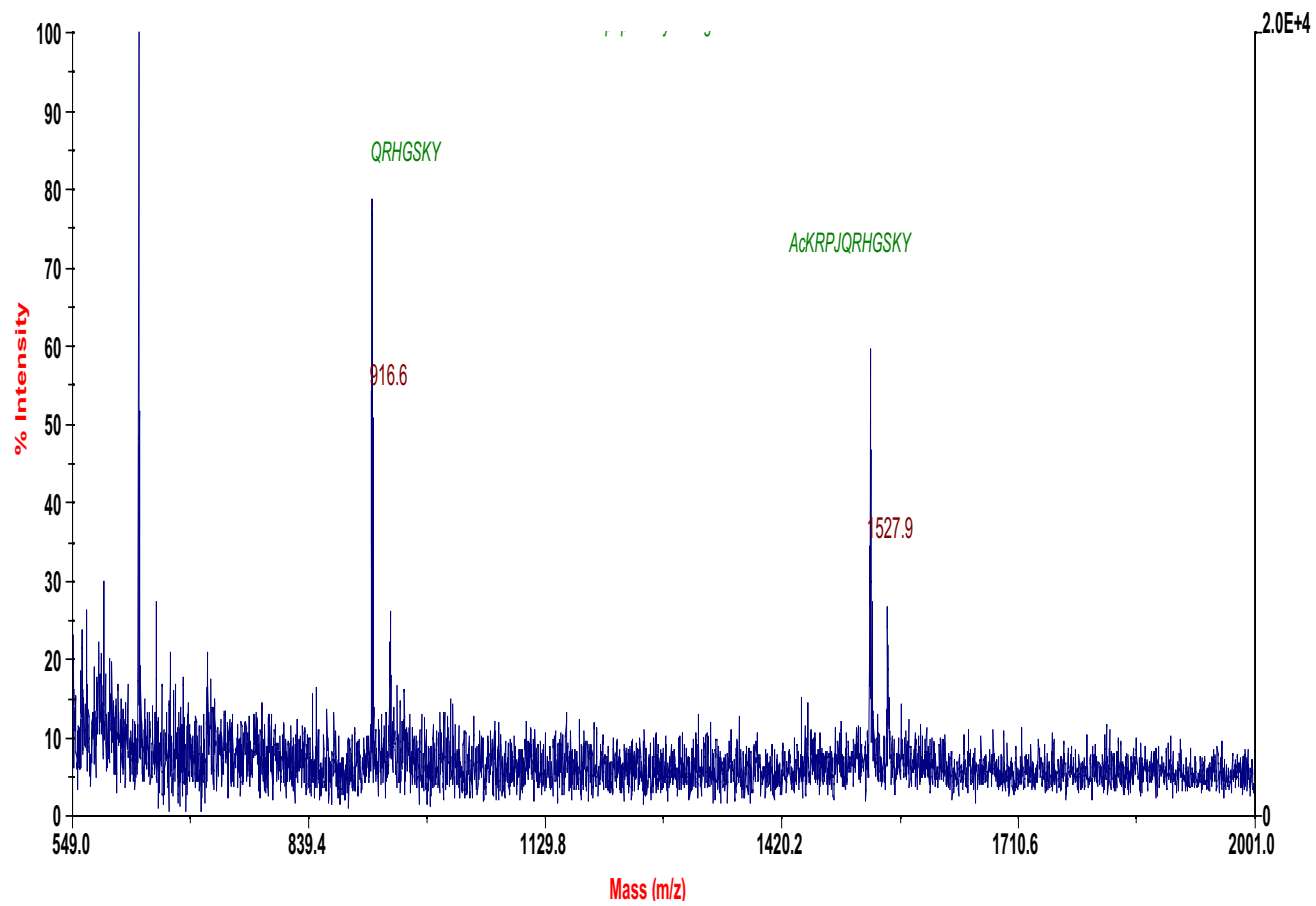
**Ac-KRPJ**  
629.3

**QRHGSKY**  
915.5

↓  
Edman degradation of mixture

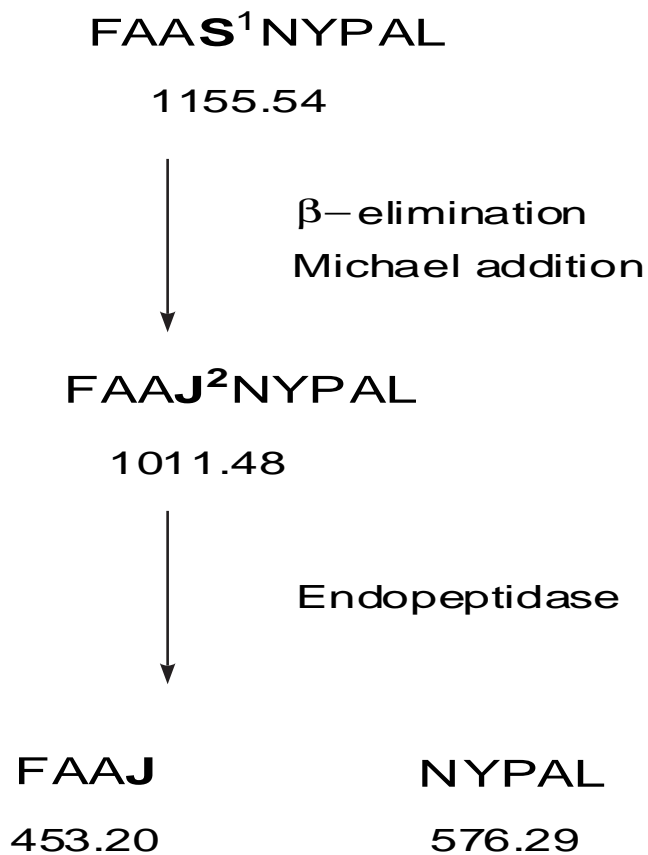
Identify Phosphoamino acid as Residue 4  
From Mass And Sequence Data

# PKC Phosphopeptide Modified and Lysine Endopeptidase Digested



# Reaction Scheme For a 9 AA *O*-Glycosylpeptide

Phe-Ala-Ala-Ser-Asn-Tyr-Pro-Ala-Leu



1. *O*-Glycosylserine
2. 2-aminoethylcysteine

**TABLE 1**

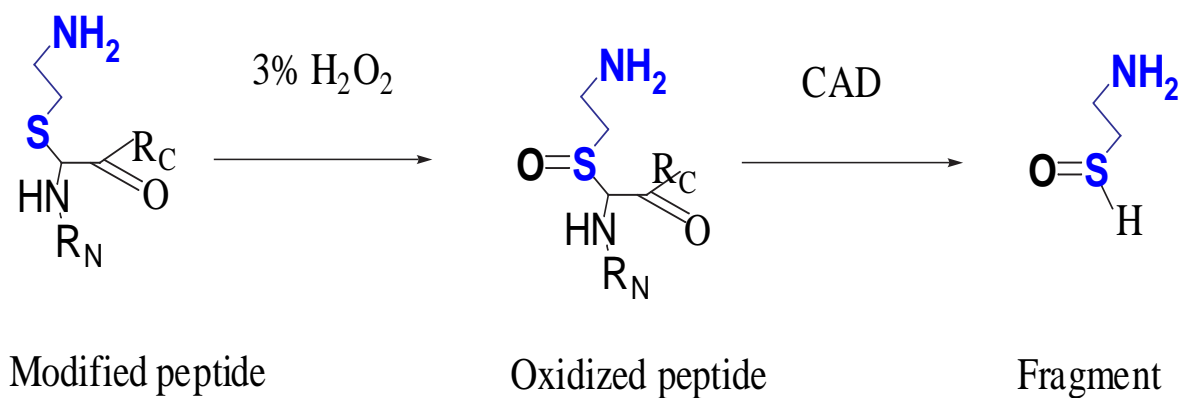
Peptides were modified as described in the methods section, digested with lysine endopeptidase, and analyzed by matrix-assisted, laser desorption ionization, time-of-flight mass spectrometry

<b>Peptide</b>	<b>N-Terminus</b>			<b>C-Terminus</b>		
	(sequence)	(calc.)	(obs.)	(sequence)	(calc.)	(obs.)
<b>PS</b> peptide	LKRAJ(1)	730.4	730.5	LG	187.1	
<b>PKC</b> peptide	KRPJ	629.3	629.4	QRHGSKY(2)	915.5	915.6
<b>CNS</b> peptide	DLDVPIPGRRFDRR	1841.9	1842.1	VAAE(2)	388.2	Not Obs.
<i>O</i> -hexSer	FAAJ	453.2	453.2	NYPAL	576.3	576.3

(1) J is the 2-aminoethanethiol modified residue.

(2) Sequence VAAE and QRHGSKY by Edman degradation

## CONVERSION FOR NEUTRAL LOSS OR PRECURSOR ION SCAN \*



\* <5 min. at room temp. followed by speedvac drying; conditions were similar to those given in: Steen, H. and Mann, M., J. Am. Soc. Mass Spectrom. (2002)13:996-1003.

## Conclusions

The methods proposed here provide new approaches for identifying sites of phosphorylation and *O*-glycosylation particularly for large, difficult to analyze polypeptides. Either the mass of the products of digestion or the amino acid sequence immediately C-terminal to the modified site can be used to infer the modified residue from the known sequence. Multiple sites can be distinguished by adjusting the conditions used for  $\beta$ -elimination.. Although the reactions employed here showed a high level of completion, quantitative conversion is not necessary to get a valuable readout.

In this work, no non-specific cuts and no sequences from peptidase autolysis were observed. Sites with proline, or acidic residues C-terminal to the phosphoamino acid can be expected to hydrolyze with slower kinetics (Sakiyama et al.) Thus it may be necessary to extend the time for digestion in these cases.

Sakiyama, F, Masaki, T, Lysine Endopeptidase, in Barrett, AJ, Rawlings, ND, Woessner, JF (eds.): *Handbook of Proteolytic Enzymes*, Academic Press, 1998:261-263.

## **SUMMARY**

**1.) CHEMICAL MODIFICATION COUPLED WITH THE SPECIFICITY OF LYSINE ENDOPEPTIDASE WAS USED TO IDENTIFY PHOSPHO- AND O-GLYCOSYL-SITES ON BOTH SERINE AND THREONINE RESIDUES.**

**2.) ALTHOUGH MASS ALONE WAS SUFFICIENT, EDMAN SEQUENCING ALSO IDENTIFIED THE SITES BY READING SEQUENCE IMMEDIATELY C-TERMINAL TO THE MODIFICATION.**

**3.) FOLLOWING OXIDATION, HIGH SENSITIVITY LINKED SCAN, DIFFERENTIAL MASS ANALYSIS (e.g. NEUTRAL LOSS) COULD BE USED TO ASSIGN THE SITES IN MIXTURES.**