

Post-Translational Modification Analysis of Native Rat Bone Osteopontin

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Introduction

Osteopontin (OPN) is a highly acidic protein found in many tissues including kidney, smooth muscle, blood, urine, milk, and bone (Sodek *et al.*, 2000). It is involved in many biological functions including developmental processes, immunological responses, wound healing, tumorigenesis, and bone resorption. It is found at sites of pathological calcification such as that found in atherosclerotic plaque, kidney stones, dental plaque, and tumour associated calcification.

OPN function is tissue dependent and thought to be modulated by post-translational modifications (PTMs), especially phosphorylation. Other PTMs (*i.e.* glycosylation, sulfation) have been reported for various forms of this protein, but have not been localized for rat bone OPN (Sorensen *et al.*, 1995; Nagata *et al.*, 1989). The relationship between structure and function of OPN is of great interest to many researchers. Thus it is desirable to comprehensively characterize this protein with respect to its post-translational modifications. This work was carried out on OPN isolated from rat bone.

Experimental

OPN was isolated from rat bone using EDTA and guanidine extraction, FPLC, and dialysis (Goldberg and Sodek, 1994). Intact protein was analysed using both MALDI and LC-ESI-MS prior to and after treatment with calf alkaline phosphatase (1 milliunit per μg OPN, 37°C, overnight). MALDI was performed on a Bruker Reflex III mass spectrometer operating in positive ion linear mode and externally calibrated with cytochrome c, trypsinogen, and BSA using sinapinic acid as the matrix. MALDI results were processed and peaks were assigned using XTOF software (Bruker). ESI-MS analysis was performed on a Q-TOF Micro mass spectrometer (Micromass) operating in positive ion mode. LC was achieved on a microbore C4 column (1 mm x 15 cm). ESI-MS data were acquired using MassLynx 3.5 and processed with the MaxEnt1 algorithm of MassLynx.

Several proteolytic digests (trypsin, Asp-N, trypsin + Asp N, before and after sialidase and/or phosphatase treatment, 1:50 enzyme:OPN) were analysed on a Micromass Q-TOF Global mass spectrometer operating in positive ion electrospray mode and calibrated with MS/MS spectra of Glu-fibrinopeptide B. Peptides were separated using a standard precolumn (0.5 x 5 mm, LC Packings) + analytical column (75mm x 15 cm, LC Packings) setup. MS experiments were performed with parent ion discovery (PID) and data dependent acquisition (DDA, with or without specific precursor ion inclusion) modes. For PID experiments, spectra were obtained at low collision energy (CE, 10V) and high CE (30 V). MS/MS of the parent ion was triggered by single or multiple neutral losses of 98 observed in the high CE spectra. For DDA experiments, MS/MS was obtained for multiply charged (+2 to +4) species in the spectra. Data were acquired with MassLynx 4.0 and analyzed using ProteinLynx Global Server 2.0 (Micromass). Peak list files were used also analysed using PEAKS (Ma *et al.*, 2003) and Mascot (www.matrixscience.com). All MS data were verified manually.

Results

MALDI-MS analysis of rat bone OPN shows a shift in average mass from 37 621 to 36 790 Da after enzymatic phosphorylation corresponding to the removal of approximately 10.4 moles of phosphate per mole of OPN (Figures 1A and B). The width in the MALDI peaks can be explained by ESI-MS data (Figure 1C) which clearly shows that, even after dephosphorylation, OPN is very heterogeneous. The different forms observed by ESI-MS show indicate various amounts of glycans are present.

ESI-MS and MS/MS spectra indicate that sites of phosphorylation are heterogeneous with several differentially modified forms being observed for most peptides. An example of this is shown in Figure 2 (A and B). For one peptide, a mixture of phosphorylation and sulfation was observed (Figure 2C). This site (Y150) corresponds to the only Tyr residue in OPN that is found in a sulfation consensus sequence.

Earlier studies have indicated that sulfation may be involved in bone formation (Nagata *et al.*, 1989). In total, 29 sites of phosphorylation and 1 sulfation site were identified. Peptides from OPN 67-128 could only be observed after sialidase treatment possibly due to the highly acidic nature of this region. Analysis of two peptides in this region after removal of sialic acid and phosphate revealed glycans of the type (NH)_n where N corresponds to N-acetylhexosamine and H corresponds hexose (Figures 3 A-C). Based on all of the evidence, the following four residues are O-glycosylated (T107, T110, T116, T121) with the largest glycan structure being that proposed in Figure 3D. These results are summarized within the context of the protein sequence in Figure 4.

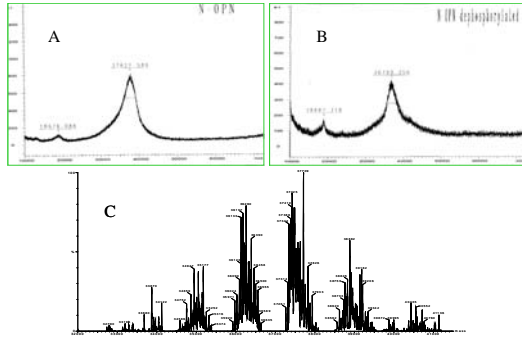


FIGURE 1: MALDI-MS spectra for native rat bone OPN A) as purified and B) after treatment with calf alkaline phosphatase. The shift in average mass from 37621 to 36790 Da corresponds to the liberation of approximately 10.4 moles of phosphate per mole of OPN. C) ESI-MS spectrum for dephosphorylated OPN. The different forms observed indicate various amounts of glycosylation. Attempts to analyze phosphorylated OPN by ESI-MS failed presumably due to the extreme heterogeneity of the sample. The expected theoretical mass of OPN is 33248 Da indicating that approximately 9% of OPN's mass comes from glycans and PTMs other than phosphate.

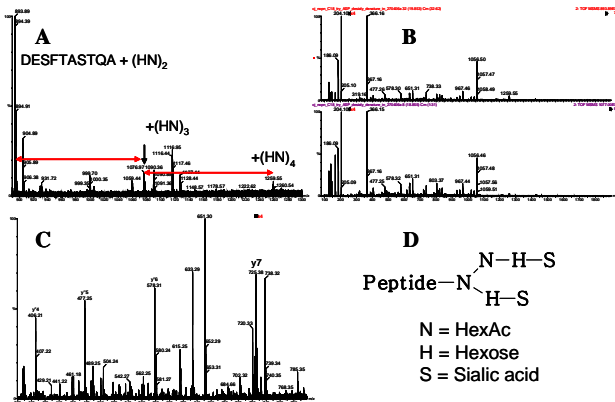


FIGURE 3: A) MS data showing +2 charge state of OPN 103-112 (DESFTASTQA) with various glycans after treatment with sialidase and phosphatase. B) MS/MS data for m/z 894 (2+) and m/z 1077 (2+) showing characteristic glycan ions at m/z 204 (H) and 366 (HN) and fragmentation ions that are identical for both precursor ions. The y-ion series in C (FTA) verifies the peptide identity. From the differences of approximately 1300 Da in the protein MS and other data, the maximum structure of each O-linked glycan is likely to be that shown in D.

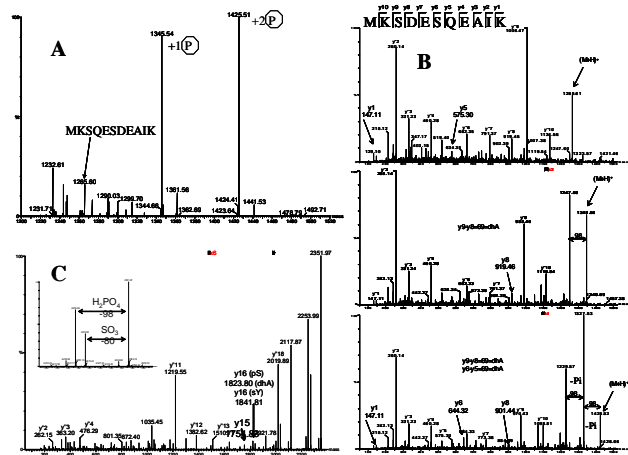


FIGURE 2: A) ESI-MS and B) MS/MS spectra showing heterogeneity for OPN residues 162-172 (MKSQESDEAIK). Non-phosphorylated, singly phosphorylated, and doubly phosphorylated species are observed. For the singly modified species, S167 is the preferred site of phosphorylation. C) MS/MS data for residues 142-161 (SFPVSEQYPDATDEDLTSR) + 80. The loss of 80 and 98 correspond to losses of sulfate and phosphate respectively indicating this is actually a mixture of peptides. The modifications are on S147 (phosphorylation) and Y150 (sulfation). The doubly charged precursor ion at m/z 1176.5 is still present after phosphatase treatment. According to the Sulfinator (Monigatti *et al.*, 2002, <http://ca.expasy.org/tools/sulfinator/>), this region contains the only Tyr residue in OPN that lies within a sulfation consensus sequence.

References

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1 LPVKVAEFGSSEEKAHYSKHSDAVATWLKPDPSQKQNLAPQNSVSSEETDDFKQETLPS

61 NSNESHDHMDDDDDDDGGDHAESDSVNSDESDESHHSDSDESFTASTQADVLTPIAP

121 TVDVPDGRGDSLAYGLRSKRSFPVSDEQYPDATDEDLTSRMKSQESSDEAIKVIPVAQRL

181 SVPSDQDSNGKTSHESSQLDEPSVETHSLEQSKEYKQRASHESTEQSDAIDSAEKPDAD

241 SAERSDAIDSQASSKASLEHQSHEFHSHEDKLVLPKSKEDDRYLKFRISHELESSSSEV N

FIGURE 4: OPN sequence showing location of phosphorylation (red), sulfation (yellow), and glycosylation (blue) sites. Underlined sequences have been fully mapped.