

Localization of Phosphorylated Residues in Rat Bone Osteopontin

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Elucidation of the relationship between the amount and location of phosphorylation in osteopontin (OPN) to its mineral-modulating function is an ongoing project in our lab. Previous studies performed in our lab (Hunter *et al.*, 1994) and by others (Boskey *et al.*, 1993) demonstrated that native rat bone OPN is a potent inhibitor of *de novo* hydroxyapatite (HA) crystal formation. However, different forms of OPN (bone and milk isoforms in kidney) have shown different degrees of HA-inhibiting activity. These observations suggest that differences in both the levels and specific site(s) of phosphorylation between the various tissue-specific forms is an important modulator of its function. The only form of OPN in which the phosphorylation sites have been mapped is that from bovine milk (Sorensen, 1995). To gain further insight into the profile of phosphorylation in various forms of OPN, we set out to map the sites of phosphorylation on serine and threonine residues of native rat bone by mass spectrometry. OPN is generally acidic in character and by observation there are numerous potential serine and threonine phosphorylation sites (Figure 1). In addition, the sequences surrounding many of these potential phosphorylation sites conform to casein kinase (CK2) consensus sequences of D/E-S/T-X-X-D/E/pS/pY where X is any amino acid except basic amino acids or proline. Correlation of the mass spectrometric analysis described above with the ongoing functional studies will allow us to identify the HA-inhibiting "site" of the protein. This information will facilitate us in the development of specific inhibitors of crystal formation that can be used in the prevention or treatment of disorders such as atherosclerosis and kidney stone disease.

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1  LPVKVAEFGSSEEKAHYSKHSDAVATWLKPDPSQKQNLLAPQNSVSSEETDDFKQETLP
61  SNSNESHDMDDDDDDDDGDHAESEDSVNSDESDESHHSEDESDESFTASTQADVLTIAP
121 TVDVPDGRGDSLAYGLRSKRSRFPVSDEQYPDATDEDLTSRMKSQESDEAIKVIPVAQRL
181 SVPSDQDSNGKTSHESSLDEPSVETHSLEQSKEYKQRASHESTEQSDAIDS AEKPAID
241 SAERSDAIDSQASSKASLEHQSHEFHSHEDKLVLPKSKEDDRYLKFRISHELESSSSEV
301 N
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Figure 1. Primary structure of rat bone osteopontin (OPN). OPN is acidic in character due to numerous aspartic and glutamic acid residues. Color-coded conserved regions of OPN correspond to the RGD integrin - binding motif (green), a single thrombin-cleavage site (yellow) and a polyaspartic region responsible for calcium binding site of hydroxyapatite (pink). Many serine and threonine residues in this protein conform to potential CK2 phosphorylation sites. Additionally, OPN is thought to contain at least one N-linked glycan and numerous O-linked glycans.

Recombinant His-tagged rat OPN was expressed in *E. coli* and purified using Ni²⁺ affinity chromatography followed by anion exchange chromatography and gel filtration. *In vitro* phosphorylation was carried out using CK2. Native rat bone OPN (n-OPN) was isolated from rat bone using EDTA and guanidine extraction prior to purification by FPLC. Native OPN was treated with alkaline phosphatase to determine level of phosphorylation. Proteins were analysed by either ESI-MS or MALDI-MS. Recombinant OPN, *in vitro* phosphorylated OPN, and native OPN were digested with trypsin and Asp-N (separate digests) and analysed using nano-LC-MS and nano-LC-MS/MS.

There appear to be a minimum of seven phosphorylation sites in CK2-phosphorylated OPN. Of these, six sites have been localized to specific residues (Table 1). Phosphatase treated native OPN shows an average loss of ten phosphates when compared to untreated OPN (Figure 2). MS and MS/MS data show that native OPN is variably phosphorylated with more than twenty modified residues. Of these, thirteen have been tentatively assigned (Table 1). Additional sites are in the process of being characterized. Preliminary functional studies with in vitro phosphorylated recombinant OPN indicate some inhibitory activity when compared to the non-phosphorylated form.

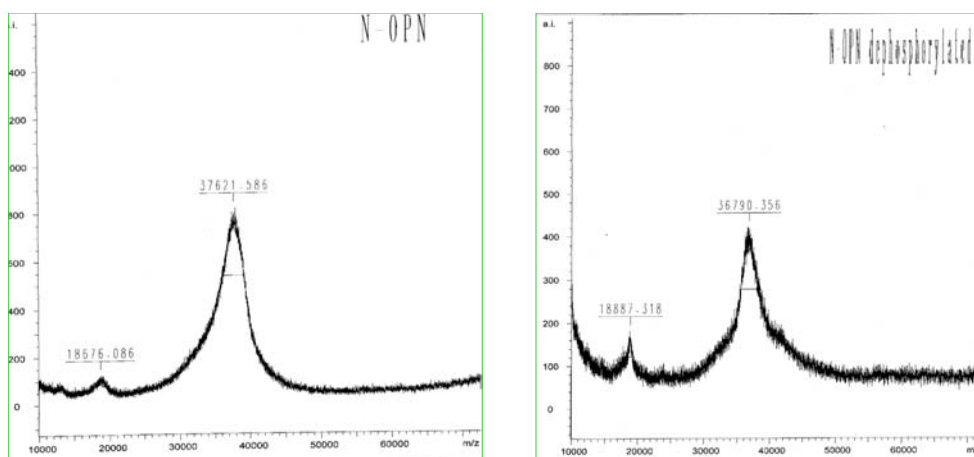


Figure 2. MALDI-MS of native OPN and phosphatase treated OPN (obtained by Dyanne Brewer, Biological Mass Spectrometry Facility, University of Guelph, Guelph, Ontario).

Table 1. Phosphorylation sites in CK-2 treated recombinant OPN (A) and native OPN (B).

(A) PHOSPHORYLATED rp-OPN

Residues	Observed MW	Expected MW	Sequence	Modified residues
5-14	1081.53	1081.49	VAEFG S SEEK	S10
20-35	1858.94	1858.86	HSDAVATWLKPDPSPQK	1 Phospho (ST)
142-161	2351.03	2350.95	SFPVSDEQYPDAT D EDLTSR	T154
219-244	2838.32	2838.18	ASHESTEQ S DAIDSAEKPDIDSAER	S 228
289-301	1496.68	1496.60	ISHELES S SSSEVN	S295
289-301	1576.68	1576.57	ISHELES S SSSEVN	S296,S297

(B) n-OPN

Residues	Observed MW	Expected MW	Sequence	Modified residues
22-50	3312.46	3312.50	DAVATWLKPDPSPQKQNLAPQN	S44,S46
130-151	2581.17	2581.18	DSLAYGLRSKRSFPV QYP	S146
142-179	2350.89	2350.95	SFPVSDEQYPDATDEDLT	S160,S164,S167
157-167	1360.58	1360.57	DLTRMKSQE	S167
162-179	2188.00	2187.97	MK EAIKVIPVAQR	S164,S167
228-236	1024.41	1024.45	DAID P	S232
237-245	955.38	955.40	DAID S	S241
249-269	2536.97	2536.96	DSQASSKASLEHQ FH	S262,S267
281-301	2670.14	2670.14	DDRYLKFRILE SSEVN	S290,S295
287-301	1959.65	1959.71	FRI E SSEVN	S290,S294,S295

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