Modulation of calcium oxalate dihydrate growth by phosphorylated osteopontin peptides

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ABSTRACT

Osteopontin (OPN) is a significant component of kidney stone matrix and a key modulator of stone formation. Here, we investigated the effects of different phosphorylated states of a synthesized peptide of OPN (the ASARM peptide; acidic, serine- and aspartate-rich motif) on calcium oxalate dihydrate (COD) crystals, a major mineral phase of kidney stones. *In vitro*, phosphorylated OPN-ASARM peptides strongly inhibited COD crystal growth in solution as compared to the nonphosphorylated state, with increasing inhibitory potency correlating with the degree of peptide phosphorylation. Scanning electron microscopy revealed that the inhibition from the phosphopeptides resulted in distinctive, rosette-like crystal aggregates called spherulites. The OPN-ASARM peptides preferentially bound and specifically inhibited the {1 1 0} crystallographic faces of COD, as identified by combining atomic force microscopy and computational simulation approaches. These {1 1 0} surfaces of COD have high lattice calcium occupancy (exposure), providing preferential binding sites for the highly acidic peptides; binding and inhibition by OPN-ASARM peptides at the {1 1 0} faces led to crystal aggregation and intergrowth. The crystal spherulite formations obtained *in vitro* when using the most phosphorylated form of OPN-ASARM peptide at a high concentration, resembled crystal morphologies observed *in vivo* in a rat model of urolithiasis, in which crystal deposits in the kidney contain abundant OPN as revealed by immunogold labeling. A mechanistic model for spherulite formation is proposed based on the symmetry and crystallographic structure of COD, where the phosphate groups of OPN-ASARM bind to calcium atoms at [1 1 1] step risers on the COD {1 1 0} surface, inducing the periodic emergence of new COD crystals to form spherulites.

1. Introduction

Kidney stones (renal calculi) are pathologic crystal aggregations formed through a complex biomineralization process that involves organic biomolecules found in the extracellular milieu of the kidney. The composition of renal calculi typically consists of a majority mineral phase by dry weight (up to ~95%), and smaller amounts of an organic phase (Boyce, 1968). Calcium oxalate is the most common mineral phase found in kidney stones (Wesson et al., 1998), with the monohydrate form (calcium oxalate monohydrate, COM) occurring about twice as frequently as the dihydrate form (COD).

Both COM and COD precipitates can be found in normal urine, and these two mineral phases have different affinities for binding and interacting with urinary proteins (Asplin et al., 1998; Fisher et al., 2001; Ryall et al., 2005; Wesson et al., 1998). It is widely recognized that osteopontin (OPN) – a highly acidic, glycosylated phosphoprotein abundant in stone organic matrix – plays an essential and regulatory role in inhibiting calcium oxalate growth and stone formation, where its prominent intra-stone localization at specific locations and zones can be readily identified (Chien et al., 2009; Kohri et al., 1993; McKee et al., 1995; Thurgood et al., 2012, 2010). The expression and production of OPN in the kidney is stimulated by the formation of calcium oxalate stones (Kohri et al., 1993; Yasui et al., 2001), and the secreted OPN incorporates into the renal calculus. At physiologically relevant concentrations, OPN is known to inhibit COM nucleation and reduce the growth and aggregation of COM crystals (Asplin et al., 1998; Qiu et al., 2004; Wang et al., 2008; Wesson et al., 2003). Accumulating evidence indicates that while inhibiting COM growth, OPN influences calcium

oxalate growth in favor of COD, and in turn this COD has a higher affinity to bind and incorporate OPN than does COM in normal urinary precipitates (Chan et al., 2012; Durrbaum et al., 2001; Grohe et al., 2011; Pedraza et al., 2008; Ryall et al., 2005; Wesson et al., 1998). More specifically in this regard, Grohe and colleagues demonstrated that the carboxylate groups of OPN are particularly effective in inhibiting COM crystal growth and aggregation, but that conversely, phosphate and carboxylate combine to promote COD growth in urine (Chan et al., 2012; Grohe et al., 2011). These investigators proposed that proteins and peptides having high hydrophilicity and high net-negative charge inhibited most effectively the formation of COM. Taken together, these findings suggest that phosphorylated OPN and/or its enzymatically released peptides rich in aspartic acid, glutamic acid and phosphoserine residues, may act to preserve the COD form at the expense of the COM form to reduce stone recurrence rates (Berg et al., 1976). Indeed, COD is more readily excreted via the urine before retention occurs and stone formation develops (Finlayson and Reid, 1978), possibly because COD crystals are less prone than COM crystals to adhere to cell surfaces (Cerini et al., 1999; Mandel, 1994; Verkoelen et al., 2000; Wesson and Worcester, 1996).

Like at most soft-tissue ectopic calcification sites seen in various pathologies (Giachelli, 2004, 2005; Jahnen-Dechent et al., 2008), OPN has long been identified as a prominent component of kidney stones (Kohri et al., 1993; Konya et al., 2003; McKee et al., 1995; Rittling and Denhardt, 1999; Wesson et al., 2003). A role for OPN in inhibiting stone formation *in vivo* was obtained using comparative experimental mouse models for kidney stone formation, as induced by glyoxylate injections; for example, OPN-knockout mice produced less stones compared to wildtype mice (Hamamoto et al., 2010; Okada et al., 2008). In contrast, other reports showed that OPN can immobilize onto the surface of seed crystals to enhance their adherence and aggregation (Konya et al., 2003), in this way acting as a promoter of kidney stone formation (Yamate et al., 1998; Yasui et al., 2001), proposed as likely being attributable to various functional domains of OPN (Hamamoto et al., 2010; Okada et al., 2008). From these and other reported discrepancies, additional work is clearly required to better understand the exact roles of OPN in regulating specifically calcium oxalate crystal growth, and also to know how it is involved in the multiple complex processes leading to kidney stone formation.

In OPN, carboxylate side-groups from aspartic and glutamic acid residues, and phosphate side-groups from phosphoserine, both can bind to surface lattice calcium exposed at the surface of mineral crystals, and in this way modulate crystal growth through inhibition (Addison et al., 2010; Chien et al., 2009; Kazanecki et al., 2007; O'Young et al., 2009; Qiu et al., 2004; Sodek et al., 2000; Wang et al., 2006). OPN contains a remarkably high (~25%) acidic amino acid content (Asp and Glu), and its overall net negative charge and hydrophilicity arising from these residues, as well as similar features residing in proteolytic fragments and peptide motifs of OPN, are widely considered to be responsible for its mineral-binding and inhibitory properties (Fisher et al., 2001; Hoyer et al., 2001; Rittling and Denhardt, 1999; Wesson and Worcester, 1996). Post-translational phosphorylation of OPN can make it even more negatively charged, and enhance even further its mineral-binding and mineralization-inhibiting capacity (Addison et al., 2010; Christensen et al., 2008, 2005; Gericke et al., 2005; Hoyer et al., 2001; Kazanecki et al., 2007; Lasa et al., 1997; Qin et al., 2004; Sorensen et al., 1995; Wang et al., 2006). Human urinary osteopontin contains about 8 phosphorylated residues distributed over 30 potential phosphorylation sites (Christensen et al., 2008), where the phosphorylation of OPN is rather variable both within and between tissues (Sodek et al., 2000; Sorensen and Petersen, 1993; Sorensen et al., 1995).

A conserved acidic, serine- and aspartate-rich motif (ASARM) has been identified in the mineral-regulating family of Small Integrin-Binding Ligand N-linked Glycosylated proteins (SIBLINGs) (Addison et al., 2010, 2008; Fisher et al., 2001; Rowe et al., 2000, 2004), based largely on the work by P.S. Rowe and colleagues investigating matrix extracellular glycoprotein (MEPE) (Liu et al., 2007; Rowe et al., 2000, 2006, 2004). In addition to its acidic amino acids, the ASARM motif contains at least three potential kinase phosphorylation sites (Argiro et al., 2001; Rowe et al., 2000). In osteoblast cultures, calcium-phosphate mineralization is blocked by addition of phosphorylated MEPE- and OPN-ASARM peptides (Addison et al., 2010, 2008; Boukpessi et al., 2010; Bresler et al., 2004). *In vivo*, elevated levels of inhibitory ASARM peptide and OPN have been shown in the soft, hypomineralized bones and teeth of patients and mice with X-linked hypophosphatemia (human XLH, and the *Hyp* mouse model) (Boukpessi et al., 2010, 2017). When phosphorylated, the OPN-ASARM peptide contains a cluster of phosphate and carboxylate groups that forms an extended 18-residue motif with a particularly high density of negative charges (Addison et al., 2010; Christensen et al., 2008) that can inhibit mineralization. The OPN-ASARM peptide inhibits hydroxyapatite mineralization by binding to crystals in a phosphorylation-dependent manner, and this inhibitory peptide, as well as full-length OPN (Barros et al., 2013), can be extensively cleaved (inactivated) by the enzyme PHEX (Addison et al., 2010), an enzyme whose inactivating mutations lead to the soft bones and teeth found in XLH/*Hyp* where ASARM and OPN accumulate (Boukpessi et al., 2010, 2017). For calcium-carbonate mineral, we have shown that the OPN-ASARM peptide interacts with calcite step edges, modulating its dissolution pattern to likely alter the dissolution kinetics of calcite biominerals (Nelea et al., 2014).

Given the abundance of OPN and OPN fragments in the organic matrix of renal calculi, we considered that it was important to evaluate the inhibitory/modulatory actions on calcium oxalate of mineral-binding motifs found in this protein. Thus, in the present study, an acidic OPN peptide motif (the ASARM peptide) – identified also as being susceptible to phosphorylation - was selected to evaluate its effects on calcium oxalate crystal growth (Chan et al., 2012; Grohe et al., 2011, 2007; Nene et al., 2013; Wang et al., 2008). Here, with these studies, we show inhibitory but variable effects of phosphorylation on calcium oxalate crystal growth and morphology.

2. Materials and methods

2.1. Peptides

COD crystals were grown from aqueous $CaCl_2$-$Na_2C_2O_4$ solutions (see below) in the presence of four synthesized peptide motifs of human OPN, the first three being variably phosphorylated acidic ASARM peptides (Addison et al., 2010; Rowe et al., 2000), and the fourth being a control, neutrally charged peptide sequence. Synthetic peptides were synthesized (NeoMPS Inc., San Diego, CA) by Fmoc solid-phase chemistry according to standard peptide synthesis procedures to obtain 0-phosphoserine peptide (DDSHQSDESHHSDESDEL), 3-phosphoserine peptide (DDSHQpSDESHHpSDEpSDEL) or 5-phosphoserine peptide (DDpSHQpSDEpSHHpSDEpSDEL); these peptides were designated as OpnAs0, OpnAs3 and OpnAs5, respectively. Phosphorylations at specific serine residues in the chosen ASARM peptide sequence were achieved using preformed, protected phosphoserine amino acids. Peptides were purified by high-performance liquid chromatography and the integrity of the peptides was verified by mass spectroscopy (data not shown). As a control, a similarly sized peptide sequence from a neutrally charged region of OPN was synthesized (residues 198–215, LNGAYKAIPVAQDLNAPS, designated as OpnN). A synthetic poly-aspartic acid-rich (poly-Asp) peptide was also synthesized based on the aspartate-rich domain sequence of bovine OPN (DDLDDDDD, residues 86–93). Commercial poly-Asp (average MW 15 kDa; Sigma, St. Louis, MO) was also used to examine the specificity of acidic peptide modulation of COD growth. Synthetic peptides were added to the sodium oxalate solution prior to crystal growth experiments, with various final concentrations ranging from 0.15 to 12.5 μM, which spans the physiologic range (Thurgood et al., 2008) and extends to pathologic, elevated levels.