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Rapid Communication

Osteopontin binds multiple calcium ions with high affinity and independently of phosphorylation status

Eva Kläning [a,c], Brian Christensen [a], Esben S. Sørensen [a], Thomas Vorup-Jensen [c], Jan K. Jensen [a,b,*]

[a] Department of Molecular Biology and Genetics, Aarhus University, Denmark
[b] Danish-Chinese Centre for Proteases and Cancer, Aarhus University, Denmark
[c] Department of Biomedicine, Aarhus University, Denmark

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ABSTRACT

Osteopontin (OPN) is an acidic, intrinsically disordered extracellular matrix protein with a capacity to modulate biomineralization in vitro and in vivo. The role of posttranslational modification of osteopontin has been intensively studied. Phosphorylation of OPN has been demonstrated to play a role in inhibition of biomineral formation and growth in vitro. Here, we used isothermal titration calorimetry (ITC) to investigate the ability of OPN to bind the divalent cations Ca^{2+} and Mg^{2+}, both essential components of inorganic minerals in vivo. We found, that bovine OPN binds ~10 Ca^{2+} ions with an apparent affinity ~50-fold tighter than Mg^{2+}, both regardless of OPN phosphorylation, and with affinities significantly stronger than previously reported. These results were confirmed using human derived OPN. This implies that a majority of the acidic residues within OPN must be engaged in calcium interaction under physiological conditions.

Introduction

The formation and deposition of biominerals is of great importance in normal tissue as well as under pathological conditions. Osteopontin (OPN) has been demonstrated to be involved in both scenarios. OPN is an acidic intrinsically disordered extracellular glycoprotein found in many human tissues and body fluids including bone, skin, urine, milk and blood [1,2]. In the normal kidney, OPN is secreted by renal epithelial cells after it has been produced by renal tubular cells [3]. OPN is present at a concentration of approximately 0.1 μM in normal human urine [4]. In kidney stone formers, the expression of OPN is upregulated and OPN has been identified as an integral part of kidney stones [5]. OPN levels have also been implicated in the risk of calcification of renal allografts [6]. Calcium oxalate is a major constituent of kidney stones [7] and phosphorylated OPN is a more potent inhibitor of calcium oxalate monohydrate crystal growth in vitro than the non-phosphorylated counterpart [8,9].

During the in vitro formation of hydroxyapatite, the major component of human bone [10], phosphorylated OPN can play a modulatory role [11]. Evidence suggests that OPN interacts with both calcium oxalate monohydrate and hydroxyapatite crystals in ways involving negatively charged residues coordinating divalent cations on the surface of the crystals [12]. The peptide chain of OPN is highly negatively charged with one-fourth of residues being acidic. In addition, OPN is varyingly phosphorylated, with the highest level identified in milk-derived OPN of both human and bovine origin [13,14]. In humans, OPN is phosphorylated by the secreted kinase Fam20C [15]. It remains unclear how the addition of phosphates to the already highly acidic OPN can influence biomineral formation [8,16]. OPN could fulfill the Flexible Polyelectrolyte Hypothesis, proposed in [12], stating that the overall density of negative charges and protein flexibility may be determining factors in protein biomineral interactions.

Ca^{2+} and Mg^{2+} ions have important physiological functions including cellular signaling, as enzyme cofactors and as modulators of biomineral properties by incorporation into hydroxyapatite of bone. In body fluids, both ions are present as free ions or as protein-bound forms and are found in concentrations of 2.2–2.6 mM and 0.65–1.05 mM for Ca^{2+} and Mg^{2+}, respectively [17]. In two pioneering studies, membrane-immobilized OPN was shown to bind Ca^{2+} in competition with Mg^{2+}, via an aspartate-rich N-terminal domain of OPN [18,19]. Moreover, both cations bound a synthetic OPN fragment, indicating that phosphorylation is irrelevant for binding [18]. An average affinity (K_D) of 1 mM was reported for binding of Ca^{2+} to rat OPN [19]. A

Abbreviations: OPN, osteopontin; dOPN, dephosphorylated osteopontin; ITC, isothermal titration calorimetry.

* Corresponding author at: Gustav Wieds Vej 10C, 8000 Aarhus C, Denmark.
E-mail address: jkj@mb.au.dk (J.K. Jensen).

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more recent isothermal titration calorimetric (ITC) study suggested that bovine OPN binds Ca^{2+} with an even weaker affinity (K_D ~2.8 mM) [20].

In the present study we designed a method to obtain the apo-form (cation free) of native bovine and human OPN. Using ITC, we show that more than 10 Ca^{2+}-binding sites, with an average affinity orders of magnitude higher than previously reported, reside in OPN. We provide strong evidence that phosphorylation is irrelevant for the divalent cation binding. We conclude that OPN in vivo is always saturated with Ca^{2+}. Our observations are not only important for understanding of physiological functions of OPN, but also represents a fundamental knowledge when studying OPN in vitro.

Methods and materials

Purification and dephosphorylation of bovine OPN and preparation of human OPN

OPN from bovine milk was purified as described in [21] (for further information see the Supplementary data (SD)). After purification, OPN still contained various cleavage fragments, amounting to approximately 40% of the total protein, as evaluated by SDS–PAGE (data shown in SD). Dephosphorylated OPN (dOPN) was prepared using bovine alkaline phosphatase (Sigma) (30 milliunits/μg OPN) in 10 mM NH_4HCO_3 pH 8.5 overnight at 37 °C. Complete dephosphorylation was confirmed by mass spectrometry (data not shown). Unfragmented human OPN (see SDS–PAGE in SD) was used for supporting experiments and was purified as described in [13]. Apo-forms of bovine OPN and dOPN as well as human OPN were generated under calcium free environment by extensive EDTA dialysis as described in SD. Buffer from the final dialysis step was used to prepare the metal solutions. Calcium and magnesium concentrations were below the detectable concentration of 10 nM in our ITC apo-OPN stock and milliQ water, as determined by Inductively Coupled Plasma Optical Emission Spectrometry (ICP-OES, Spectro Analytical Instrument GmbH) analysis on an instrument located at Department of Chemistry, Aarhus University (See SD for further information).

ITC of OPN and dOPN

ITC experiments were performed on a VP-ITC instrument (Microcal, Northampton, MA, USA) in 20 mM HEPES pH 7.5, 150 mM NaCl at 25 °C. Titrations were performed by stepwise 5 μl injections of buffer containing 1.5 mM $MgCl_2$ or $CaCl_2$ into the ~1.4 ml sample cell. The molar binding enthalpies (ΔH) were obtained by integration of the injection peak areas using the manufacturer's software. Average association constants ($K_A = K_D^{-1}$) for binding were estimated by fit to a 1:1 binding isotherm of the integrated values as a function of metal:OPN ratio. OPN and dOPN concentrations of 13.4 μM and 14.8 μM were used, respectively. Due to limitation in human OPN preparation, a concentration of 1.6 μM was used in the ITC. Protein concentration was determined by dry-weight, OD_{280} ($\varepsilon = 22{,}910, 84{,}080\ M^{-1}\ cm^{-1}$, human and bovine respectively) theoretical extinction coefficients were predicted using the ProtParam tool (http://web.expasy.org/protparam/) and BCA quantification (Pierce), (33,500 g/mol for bovine OPN and 45,000 g/mol for human OPN).

Results and discussion

OPN phosphorylation does not influence binding of divalent cations

To generate OPN starting material (apo-forms) completely free of divalent cations, we designed an extensive EDTA-dialysis routine (see SD).

Apo-forms of bovine OPN and dOPN were titrated with $CaCl_2$ and $MgCl_2$, the prevalent physiological relevant cations, in separate ITC experiments and for both metal ions, saturable and indistinguishable titration curves were observed (Figs. 1A and B) and (Figs. 2A and B). These results clearly confirm previous reports using membrane-coated OPN, stating that phosphorylation has no effect on Ca^{2+} and Mg^{2+} binding [18,19]. OPN prepared from native source without EDTA-treatment yielded no signal (Fig. 1C). This illustrates that OPN generated by a standard purification procedure remains saturated with divalent cations. As a control, $CaCl_2$ titration into the buffer from the final dialysis step did not yield a signal (Fig. 1D), confirming that the EDTA used in the initial dialysis steps was effectively removed by the final dialysis step.

OPN binds multiple Ca^{2+} ions with high affinity

To estimate the average binding parameters, a One Set of Sites model (Origin software) was used to fit the integrated ITC data (Figs. 1E and F) and (Figs. 2C and D). The resulting molar enthalpies (ΔH), affinity constants (K_D) and the stoichiometries (N) are reported in Table 1. Assuming that all sites are equivalent, OPN and dOPN interacts with Ca^{2+} with an average K_D of ~30–50 nM and a stoichiometry (Ca^{2+}:OPN) of 9–12. In the case of Mg^{2+}, an average K_D of ~2 μM was estimated and a stoichiometry of 12–13 (Table 2). Both K_D values must be considered as estimates due to the high number of binding sites, however, it can be directly observed that OPN is completely saturated at ten-fold surplus of Ca^{2+} in the ITC experiment (~130 μM, injection number ~30), demonstrating that an average K_D must be >100-fold below this concentration (Figs. 1E and F). Importantly, our ITC data show that Mg^{2+} and Ca^{2+} must bind with markedly different affinities (Figs. 1 and 2). At a near-constant OPN concentration, the steepness of the curves approaching saturation illustrate the difference in the overall affinity for Mg^{2+} and Ca^{2+} according to the Wiseman value [22] also termed the c-value ($c = [OPN] * K_A$) as described in the ITC protocol (Microcal). In fact, given the apparent large c-value (~500) in the case of Ca^{2+}, the N value can be extrapolated from the molar ratio at which saturation is complete. Hence, based on estimated c-values, Ca^{2+} affinity is likely to be ~50-fold stronger than for Mg^{2+} (c ~ 10). Unlike the previously reported mM affinities for Ca^{2+} [19,20], we here report much stronger binding with K_D values in the mid-nanomolar range. Using standard purification procedures, the previously reported low affinities must stem from cation binding to additional low-affinity sites.

Distinctive differences in the thermodynamic parameters for Ca^{2+} and Mg^{2+}-binding suggest a similarity between the metal binding sites of OPN and EDTA

OPN titration with Mg^{2+} results in strong endothermic (positive injection peaks) signals as opposed to the strong exothermic (negative injection peaks) signals observed for the Ca^{2+} titration (Figs. 1 and 2). Interestingly, this observation and the overall binding parameters and estimated affinities reported here correlate well with what reported for EDTA. Here, a K_D of 22.3 nM and 1.7 μM was reported for EDTA interaction with Ca^{2+} and Mg^{2+}, respectively [23]. These values are very compatible with the approximate average K_D-values, 30–50 nM and 2 μM, for OPN interaction with Ca^{2+} and Mg^{2+} we report. Additionally, the ΔH-values reported for EDTA, −6.0 kcal/mol and 4.4 kcal/mol for the interaction with Ca^{2+} and Mg^{2+} [23], respectively, are also similar to those reported here. In EDTA, four carboxyl groups and two amines chelate the divalent cation (Fig. 3A) [24]. These observations and the fact the fact that OPN is flexible and contain ~24% acidic residues, suggests that OPN may interact with divalent cations in a manner similar to EDTA. Another calcium-binding protein, calsequestrin, has been demonstrated to bind multiple Ca^{2+} ions via an intrinsically disordered, acidic stretch located in the C-terminal part of the protein. However, the affinities reported for the Ca^{2+} interaction with the C-terminal part of calsequestrin are markedly lower than those reported here for OPN [25]. Sites with higher affinity were also reported for calsequestrin,