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Review

Acidic Phosphoproteins from Bone Matrix: A Structural Rationalization of Their Role in Biomineralization

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Summary. Osteopontin, bone sialoprotein, and bone acidic glycoprotein-75 are three acidic phosphoproteins that are isolated from the mineralized phase of bone matrix, are synthesized by osteoblastic cells, and are generally restricted in their distribution to calcified tissues. Although each is a distinct gene product, these proteins share aspartic/glutamic acid contents of 30–36% and each contains multiple phosphoryl and sialyl groups. These properties, plus a strict relationship of acidic macromolecules with cell-controlled mineralization throughout nature, suggest functions in calcium binding and nucleation of calcium hydroxyapatite crystal formation. However, direct proof for such roles is still largely indirect in nature. The purpose of this review is to present two speculative hypotheses regarding acidic phosphoprotein function. The goal was to use new sequence information along with database comparisons to develop a structural rationalization of how these proteins may function in calcium handling by bone. For example, our analysis has identified a conserved polyacidic stretch in all three phosphoproteins which we propose mediates metal binding. Also, conserved motifs were identified that are analogous with those for casein kinase II phosphorylation sites and whose number correlates well with that of phosphoryl groups/protein. A two-state conformational model of calcium binding by bone matrix acidic phosphoproteins is described which incorporates these findings.

Key words: Bone — Matrix — Phosphoproteins — Biomineralization — Calcium — Nucleation.

The discovery, isolation, and characterization of acidic phosphoproteins osteopontin (2ar, Sppl) [1–3], bone sialoprotein [4, 5], and bone acidic glycoprotein-75 [6, 7] from the mineralized phase of bone has naturally led to questions about the functions of these interesting macromolecules in biologically controlled mineralization. Shared aspartic/glutamic acid and basic amino acid contents of 30–36% and 8–10%, respectively, and the presence of multiple phosphoryl and sialyl groups give rise to a common overall high negative charge density. However, the sequences of osteopontin and bone sialoprotein do not show evidence of an evolutionary relationship [8, 9], although a limited homology is shared at the N-termini of osteopontin and bone acidic glycoprotein-75 [6]. Given the restricted tissue distribution of these acidic phosphoproteins to calcified tissues, their co-localization to areas undergoing mineralization, and a strict correlation of acidic macromolecules with cell-controlled mineralization, it has been suggested that they singly or together participate in the nucleation and growth of hydroxyapatite crystals in bone and calcifying cartilage [10–12]. Despite such suggestions, proof for such roles is still largely indirect in nature. The purpose of this review is to present two speculative hypotheses regarding acidic phosphoprotein function. The goal was to use new sequence information along with database comparisons to develop a structural rationalization of how these acidic phosphoproteins may function in calcium handling. No attempt has been made to provide a complete review of the topic of biomineralization; the reader is instead referred to several recent comprehensive treatments [10–12].

Hypothesis 1

Acidic phosphoproteins directly mediate specific functions through particular structural motifs. This position is contrasted with that which states osteopontin, bone sialoprotein, and bone acidic glycoprotein-75 serve a generalized function, i.e., to provide a source of inorganic phosphate following enzymatic release by alkaline phosphatase or to act as regional localizers of calcium to an area undergoing mineralization. Hypothesis 1 is based on the predominant tissue localization (and synthesis) of bone sialoprotein, osteopontin, and bone acidic glycoprotein-75 to calcifying tissues, as well as on a structural analysis of phosphoprotein sequences. The highest rates of synthesis of bone acidic glycoprotein-75 in explant cultures correlate well with the preponderance of protein antigen in bone and growth plate [7]. Detectable, although much lower levels of synthesis and antigen quantity were also observed in spleen, liver, lung, kidney, heart, and brain. Studies by Franzen and Heinegard [4], Bianco et al. [13], Fisher et al. [14], Nomura et al. [15], Mark et al. [16], Ecarot-Charrier et al. [17], Yoon et al. [18], and Nagata et al. [19] have provided a similar picture for bone sialoprotein and osteopontin. In these cases, bone and car-

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tilage were found to contain the highest amounts of the respective antigens of all normal tissues examined, although antigen and message could also be detected in placenta, kidney, uterus, and ear. Histological evidence has previously demonstrated an enrichment of acidic and sulfated groups within mineralizing nodules [20]. Osteopontin, bone sialoprotein, and bone acidic glycoprotein-75 are all synthesized by osteoblastic cells in culture [3, 4, 7, 17, 19]. Cell transformation appears to increase expression of osteopontin protein since Senger et al. [21] identified pp69 (i.e., osteopontin) as a serum marker for sarcoma tumor burden in man. However, this finding does not negate the quantitative differences in synthesis and distribution observed among normal tissues.

The primary structures of acidic phosphoproteins from bone matrix [6, 8, 9, 14, 22–24] contain several conserved features that may mediate specific cell adhesion and metal binding functions. For example, both osteopontin and sialoprotein contain an -RGD- cell binding motif which has been shown to interact with a vitronectin receptor present on cultured osteosarcoma cells [25]; consensus integrin receptor binding sequences are absent from noncollagenous components osteocalcin, osteonectin, proteoglycans I/II, and matrix GLA protein. Horton and Davies [26] recently reviewed the subject of substrate adhesion receptors on osteoclastic and osteoblastic cells. The three acidic phosphoproteins from bone matrix represent prime candidates for matrix ligands for developing osteoclast precursor cells. Second, in addition to an overall elevated content of acidic amino acids, bone acidic glycoprotein-75, bone sialoprotein, and osteopontin, each contain unusual polyaspartate/polyglutamate sequences (Table 1). Comparison of polyacidic sequences from different species reveals almost complete conservation between both species of bone sialoprotein and good conservation among four species of osteopontin (Table 1). Polyacidic regions in osteopontin and bone sialoprotein show a predominance of aspartic acid and glutamic acid, respectively. Based on an overall sequence identity of about 70% between any two species for both proteins, respectively, the observed greater degree of conservation and residue preference for these polyacidic stretches lends support to our hypothesis that these regions may comprise functional binding sites. Though such regions are not unique in nature, a survey of protein and nucleic acid databases indicates that polyacidic stretches of eight residues or greater in length are found predominantly in metal/cation binding proteins. Specifically, a search of GenBank and EMBL libraries for sequences coding for either $(GLU)_8$ or $(ASP)_8$ identified the 12 proteins noted in Table 2, along with bone matrix phosphoproteins (Table 1). These search criteria excluded many acidic proteins that bind metals or cations; however, I have done so with a bias that this aspect of bone matrix phosphoprotein structure may be an important determinant of function. Citations for noncoding sequences and for sequences within variable regions of immunoglobulins were not considered. Seven of the proteins selected on this basis function as cation binding or transporter proteins. For parathymosin, zinc ion binding co-localizes with a fragment containing a polyacidic amino acid region [37] and appears to ligand with carboxyl side chain groups. Polyacidic stretches have been suggested to participate in spermine binding, calcium binding, and voltage sensing by the spermine binding protein [30], calsequestrin [28, 29], and sodium channel protein [38], respectively. Calsequestrin is also devoid of the EF-hand motif and acts as a weak affinity, large capacity calcium binder in the junctional sarcoplasmic reticulum [39]; calcium binding by a C-terminal polyacidic region in calsequestrin alters its conformation and reversibly blocks binding to junctional membrane proteins. Calspermin exhibits calcium-dependent binding of calmodulin [33]; interestingly, the 11 residue polyacidic stretch closely follows the calmodulin binding site in the primary sequence. Finally, the function of polyacidic sequences in prothymosin [27], aspartactin [34], ubiquinol cytochrome c reductase subunit VI [36], and two nucleolar proteins [35] is unknown, although a role in complex formation is possible as each of the last four proteins forms multimeric complexes. Thus, it is predicted that polyacidic stretches in acidic phosphoproteins from bone participate in calcium binding due to a structural analogy with other metal/cation binding proteins, an expected surface or accessible location, and an abundance of carboxyl side chain ligands.

Table 1. Comparison of polyacidic sequences in acidic phosphoproteins from different species

Bone sialoprotein		
Human [14]	EEEEEEEE (res. 77–84)	DEEEEEEEEGN (res. 151–160)
Rat [9]	EEEGEEEE (res. 78–85)	DEEEEEEEEEE (res. 155–164)
Osteopontin		
Human [22]	(res. 86–96)	DDMDDEDDDE
Rat [8]	(res. 70–79)	DDDDDDDDDG
Pig [23]	(res. 80–89)	DDVDDDDDED
Mouse [24]	(res. 71–80)	DDDDDDDDGD
Bone acidic glycoprotein-75		
Rat	(res. 10–17)	EEEEDDED/E

Finally, bone acidic glycoprotein-75, osteopontin, and bone sialoprotein contain 44–4 mol phosphoamino acids/mol protein [3–6], which are primarily seryl-phosphate in the latter two proteins [19 moles organic phosphate/mol BAG-75 if the molecular weight is taken as 33,000 instead of 75,000 (a value based on electrophoretic studies with globular protein standards)]. Surveys of the primary sequences of bone sialoprotein and osteopontin suggest an absence of consensus sequences for well-characterized kinases such as A and C; however, three types of casein kinase II consensus phosphorylation sites can be identified (Table 3). Casein kinase II is found in both the cytosol and nucleus of a wide variety of cell types. Enzyme substrates include proteins participating in translational control, in metabolic regulation, as cytoskeletal elements, oncoproteins, and transcription factors [40]. The preferred phosphorylation site for casein kinase II is a seryl or threonyl residue located on the N-terminal side of several glutamyl and/or aspartyl residues, although phosphoserine may substitute for glutamyl or apartyl residues [41, 42]. Type 1 sites (Table 3) are not only present in the first half of both osteopontin and bone sialoprotein, but are entirely conserved in all species of these proteins examined. A similar -S-S-E-E- sequence exists in SV40 large T antigen, where studies with the protein and model peptides indicate that the adjacent serines can be phosphorylated sequentially by casein kinase II [40]. Type 2 sites represent a series of variant sequences with a single serine/threonine followed by two to four acidic residues. Excellent conservation of type II sites is also observed. A third type of consensus phosphorylation site is present near the C-terminus of all four species of osteopontin (Table 3). Though our analysis is based entirely on an analysis of presumptive casein kinase II phosphorylation sites, the predicted numbers correlate well with the actual numbers of phosphoamino acids in bone sialoprotein and osteopontin. For example, the measured phosphoamino acid content of rat osteopontin is 13 mol/mol, whereas a maximum of 12 casein kinase II acceptor sites are predicted (Table 3).

Table 2. Other proteins containing polyacidic amino acid sequences

Protein and sequence
Parathymosin [27] (res. 53–74) EDGEDDDEG*DEEDEEEEEEEDE*
Calsequestrin
Cardiac [28] (res. 362–391) i) DDG*DDDEDDDDDD*GNNSDEESNDDSDDDDE
Skeletal [29] (res. 364–377) ii) *EDDDDEDDDDDDD*
Spermine-binding protein [30] (res. 228–268) DDNEENVDDERDDK*DDDEEDDD*NDKENDKDDGEGSGDDDDN
Potassium ion transporter protein [31] (res. 509–526) DM*DDDDDDDD*NDGDNNEE
Sodium channel protein [32] (res. 942–953) *EEEEEEEEE*PE
Calspermin [33] (res. 87–119) SEEVEEEEGVK*EEEEEEEEEEEE*T
Prothymosin [27] (res. 54–82) DEEEEEGG*EEEEEEEE*GDGEEEDGDEDEE
Aspartactin [34] (res. 374–406) *DDDDDDDDDDDDDDDDDDDDDDDDDDDDDDDDD*
Nucleolar nucleolin [35] (res. 120–149) KEES*EEDEDDEDDEEDEDEEEE*SDEEEEP
Nucleolar NO38 [35] (res. 160–186) S*EDDEDDDEDEDDDEDDEDD*LDDDEEEI
Ubiquinol-cytochrome c reductase [36] (res. 53–79) *GDEDEDEDEDEDDDDDDDEDEEEEEE*V

Table 3. Presumptive serine/threonine phosphorylation sites within bone sialoprotein, bone acidic glycoprotein-75, and osteopontin

Type	Description
Type 1.	Conserved sequence SSEE: two of such sites are present in the N-terminal half of bone sialoprotein and osteopontin from all species[a] characterized
Type 2.	TEE, SEE, SDE, and TED sequences: two found in human and rat sialoprotein in identical locations, whereas 7–8 of such sequences are found in all species[a] of osteopontin. TEEEE sequence: exists in the first 15 residues of rat bone acidic glycoprotein-75
Type 3.	SSE sequence: one site present near the C-terminus of all species[a] of osteopontin

These potential phosphorylation sites are similar to the consensus substrate sequence for casein kinase II, Thr/Ser-Glu/Ser-P-Glu/Asp, a cyclic nucleotide-independent enzyme with wide distribution. Based on model peptide studies, both serines of Type 1 and Type 2 sites could be phosphorylated by this enzyme

[a] Human, rat, pig, and mouse osteopontin sequences

This analysis of the primary sequences of osteopontin, bone sialoprotein, and bone acidic glycoprotein-75 yields a picture in which polyacidic regions and consensus phosphorylation sites have been conserved during evolution. Combined with structural analogies with other proteins, it is reasonable to propose that acidic phosphoproteins from bone may mediate specific functions through these conserved regions. As one approach to testing this general hypothesis, polyacidic and presumptive casein kinase II sites should represent primary targets of future site-directed mutagenesis studies on acidic phosphoproteins. However, appropriate *in vitro* assays of function will be needed to evaluate such mutants.

Although much information is available about structure/function relationships for intracellular calcium binders, far less is known about the mechanisms by which calcium binding regulates extracellular processes such as biomineralization and blood coagulation.

Hypothesis 2

The calcium binding function of acidic phosphoproteins in bone formation and mineralization is dependent on the formation of specific protein-protein complexes within bone matrix. Because it is not yet possible to assign separate functions to individual acidic phosphoproteins, I have chosen for purposes of this discussion to deal with them as a group. The rationale behind Hypothesis 2 can be summarized as follows. First, most earlier studies of the effect of aqueous solutions of proteoglycans and glycoproteins on nucleation and growth of hydroxyapatite crystals *in vitro* emphasized the inhibitory action of these anionic macromolecules [43–47]. Results were commonly ascribed to a general competition of polyanionic proteins for available free calcium ions, thereby blocking hydroxyapatite deposition, an interpretation that tended to reinforce a nonspecific role. It is now clear from recent work by Addadi et al. [48] and Linde et al. [49] that experimental conditions can dramatically affect the outcome of such experiments. For example, dentin phosphoprotein can act as a nucleator of calcium crystallization when bound to a surface, whereas, inhibition is observed in solution studies. Second, data on exchangeable potassium ion levels are incompatible with the existence of a separate membrane interposed between bone extracellular fluid and the general extravascular fluid compartment [50]. Thus, because the concentration of blood ionic calcium is regulated tightly around 1.2 mM and bone extracellular fluid calcium is predicted to be in equilibrium with the blood calcium concentration, it is expected that the concentration of calcium ion in bone extracellular fluid is maintained relatively constant. Acidic phosphoproteins in bone matrix would be maintained under these conditions as partly saturated calcium complexes [51], raising a question as to the nature of the extracellular signal by which such steady-state binding could initiate hydroxyapatite nucleation/formation. The nature of the intracellular calcium second messenger elicited by IgE-antigen complexes on mast cells, e.g., is thought to be a pulse in concentration which acts directly or by inducing a reversible conformational change in calcium sensor proteins, i.e., calmodulin [52]. Activation of the blood coagulation system requires calcium, but relies on protease-mediated zymogen activation to initiate conformational changes in individual proteins, thereby maturing cofactor and substrate binding sites [53]. In the case of gamma-carboxyglutamic acid-containing factors, calcium ion binding to activated coagulation factors is required to induce or stabilize conformations with membrane binding sites. Assuming a relatively static extracellular calcium ion concentration, we reason that a change in acidic phosphoprotein conformation, i.e., through specific protein-protein interactions, is necessary to yield a form with decreased flexibility/motion and capable of orientating ligand groups into a molecular scaffold for epitaxic growth of an inorganic hydroxyapatite phase (Fig. 1). The monomer could act as a regional localizer of calcium (Fig. 1). These features of the two-state model are attractive even if one assumes that the local concentrations of calcium and phosphate are controlled by osteoblasts and increased to saturation at the time of matrix mineralization. The concept of phosphoprotein complexation was introduced by Curley-Joseph and Veis [54] who documented the existence of covalent complexes between

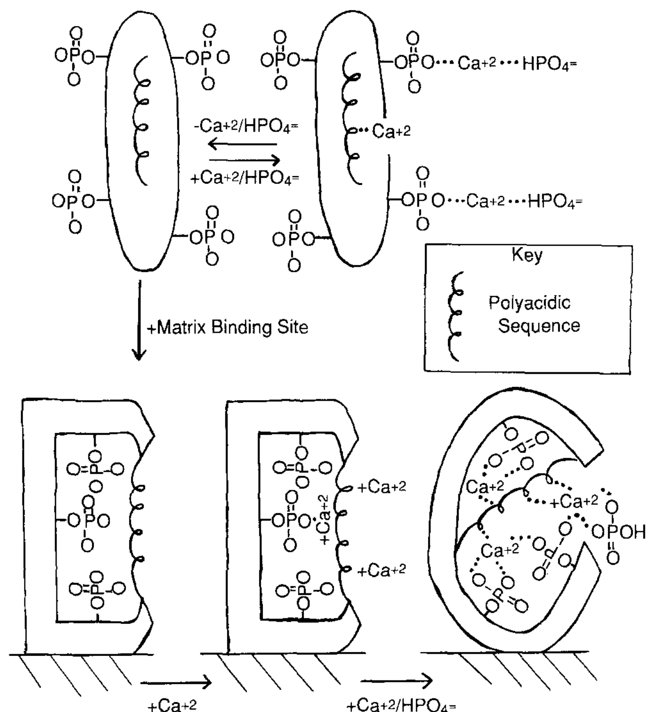

Fig. 1. Two State Model of Calcium Binding for Bone Matrix Phosphoproteins. Diagram depicts hypothetical model for different conformational states of acidic phosphoproteins in bone matrix: a monomeric form and a complexed form. It is suggested that monomeric forms act as calcium, and indirectly phosphate, localizers. In contrast, complexation with matrix binding sites, i.e., collagen, induces or selects a form with decreased flexibility/motion and capable of orienting ligand groups into a molecular scaffold for epitaxic growth of mineral phase. Though metal binding sites on the monomer are predicted to show little selectivity, it is hypothesized that the geometry and identity of coordination ligands in complexed phosphoproteins will favor preferential binding of calcium and phosphate ions. Although calcium ion can ligand with up to seven groups or water molecules, only a portion of these ligands are shown in the drawing. For simplicity, metal counter ions other than calcium are not shown associated with carboxyl and phosphate groups.

phosphophoryn and collagen. Although the molecular mechanism by which proteins nucleate crystal mineralization is unknown, work with other acidic phosphoproteins, i.e., phosphophoryn, suggests they are conformationally flexible in a monomeric state and adopt a β-conformation when precipitated with calcium [55]. As the three dimensional structures of the proteins in Tables 1 and 2 are not yet known at atomic resolution, estimates of the secondary structure of such polyacidic stretches must rely on reference to model compounds such as polyglutamic acid. Molecular modeling of $(ASP)_9$ as an α-helix based on energy minimization procedures shows that the minimum distance between carbonyl oxygens on adjacent carboxyl side chains is less than 5.6 Å for residues 1–4 and 5–8 (Doskey and Gorski, unpublished result). This analysis indicates these pairs of adjacent side chains are close enough to ligand to the same calcium atom as each oxygen atom in helix-loop-helix calcium binding sites is located about 2.4 Å from the central calcium ion (1.0 Å diameter) [56]. Coordinating ligands in the latter binding sites, however, arise from nonadjacent residues and water molecules. It is proposed that a polyacidic stretch in acidic phosphoproteins serves as an organizing part of the nucleation site (Fig. 1). In this way, complexation of phosphoprotein with collagen or other matrix components would select for or induce a conformation in which the polyacidic stretch would be located at the mouth of a pocket, with serine-phosphate groups distributed around the inside wall. Calcium ions are expected to be attracted first to the electronegative polyacidic side chain carboxyl groups to which they would bind. Ligand formation with other protein sites including phosphate groups would follow progressively and cooperatively, effectively increasing the overall calcium affinity constant. Arrangement and identity of coordination ligands within the pocket are viewed as determinants of calcium, phosphate, and hydroxyl ion addition, over that of other ions (Fig. 1).

This model shares some features with that for iron-core formation in ferritin [57]. Ferritin can be thought of as a protein shell surrounding a core of hydrous ferric oxide and variable amounts of phosphate containing up to 4,500 iron atoms. Rather than acting purely as an inert protective shell, ferritin influences nucleation of the iron core, as well as rates of iron uptake and release. Spectroscopic [58] and side chain modification [59] studies indicate that ferritin initially binds single Fe(II) atoms at a small number of acidic sites. Oxidation of Fe(II) coincides with formation of Fe(III) clusters bound to ferritin. The hydrolysis of hydrated Fe(III) and formation of core crystallites from oxo-bridged Fe(III) atoms lead to growth of the inorganic iron core in the hollow center of apoferritin. Sites with sufficient carboxylate side chains to facilitate this process are localized at subunit dimer interfaces [60]. In contrast to ferritin, growth of a hydroxyapatite crystalline phase is expected to occur out and away from an acidic phosphoprotein-protein complex. This model (Fig. 1) could provide a means to direct mineralization to specific matrix areas through restricted distribution of matrix binding sites for acidic phosphoproteins or a weak association constant for acidic phosphoprotein complexation. Although a simple explanation, a requirement for an elevated local concentration of acidic phosphoproteins to favor complexation would explain why mineralization *in vivo* occurs within condensed collagenous areas and not in adjacent loose mesenchyme of healing limbs [61], and within long-term multilayer [62] but not monolayer calvarial cell cultures.

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Note added in Proof: The sequences of bovine [J. M. Kerr, personal communication] and chicken osteopontin [Moore MA, Gotoh Y, Rafidi Y, Gerstenfeld LC (1991) Biochemistry 30:2501–2508] were determined recently. Both sequences contain polyaspartate sequences and Type 1 and Type 2 casein kinase substrate motifs. However, only bovine osteopontin contains a C-terminal kinase motif (Type 3).

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