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Self-Association of Calcium and Magnesium Complexes of Dentin Phosphophoryn†

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ABSTRACT: Self-association of rat dentin phosphophoryn in the presence of calcium and magnesium ions was examined by chemical cross-linking and electron microscopy. Highly phosphorylated phosphophoryn (HP) binds a maximum of 1.33 calcium ions or 1.07 magnesium ions per organic phosphate residue at pH 7.4-8.0. The Ca-HP complexes are predominantly linear when the calcium content of the complex is less than about 65% of the saturation level. At higher calcium levels, the protein has a folded conformation, and transient protein-protein interactions occur. The equilibrium mixture of monomers and oligomers is predominantly monomeric unless the protein is saturated with calcium. The saturated Ca-HP complex forms discrete high molecular weight particles about 25 nm in diameter. The particles are electrically neutral and generally occur in clusters. Mg-HP complexes appear predominantly linear by electron microscopy at all concentrations of bound magnesium up to about 99% of the saturation level; however, protein-protein interaction is measurable when the magnesium content is as little as 65% of the saturation level. At saturation, Mg-HP complexes form high molecular weight particles which are negatively charged. Because of the negative charge, these particles form a stable colloidal suspension and have a rather stellate configuration.

High-capacity calcium-binding proteins are associated with calcium or mineral ion metabolism in extracellular or intravesicular environments where the free calcium concentration is in the millimolar range. In some tissues the function of the protein is obvious or interpretable from theoretical considerations. For example, the colloidal aggregates of casein and calcium phosphate in milk provide the fluid with a high content of suspended mineral ions (Schmidt, 1982). In muscle cells, calsequestrin is localized in the terminal cisternae of the sarcoplasmic reticulum and is thought to act as a calcium buffer by maintaining a low free calcium ion concentration (1-2 mM) and providing a large pool of readily dissociable bound calcium (20-30 mM) (MacLennan et al., 1983). In other tissues the function of the high-capacity calcium-binding proteins is unclear. The egg yolk protein phosvitin binds many calcium ions in vitro due to its high content of phosphoserine residues (Grizzuti & Perlmann, 1973), and presumably, a majority of the yolk calcium is associated with phosvitin in vivo. Dentin and bivalve phosphoproteins localized at sites of calcium phosphate and calcium carbonate deposition, respectively, have an undefined role in skeletal mineralization (Rahima et al., 1988; Marsh, 1986a).

High-capacity calcium-binding proteins self-associate in the presence of calcium ions. Native casein micelles are nearly spherical particles of variable size composed of submicelles ranging from 8 to 20 nm. Schmidt (1982) postulates that the subunits are linked together by $Ca_9(PO_4)_6$ clusters which interact with calcium phosphoserine complexes on adjacent submicelles. Consistent with this model, dephosphorylated casein does not aggregate in the presence of colloidal calcium phosphate (Aoki et al., 1987). In vitro calsequestrin crystallizes in the presence of 0.5-2.0 mM $CaCl_2$ (Maurer et al., 1985), and five different crystal forms have been identified (Williams & Beeler, 1986). In vivo calsequestrin forms an aggregated network in the terminal cisternae of the sarcoplasmic reticulum (Franzini-Armstrong et al., 1987) which appears paracrystalline in some preparations (Saito et al., 1984). In vivo the bivalve phosphoprotein occurs as discrete 40-nm particles which have a rather angular contour (Marsh & Sass, 1984). The integrity of the bivalve phosphoprotein particles is maintained by both calcium ions and covalent interchain cross-links through histidinoalanine residues (Sass

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& Marsh, 1983; Marsh, 1986b).

In vitro the dentin phosphoprotein termed phosphophoryn is precipitated at calcium ion concentrations as low as 2.0 mM (Fujisawa et al., 1986), but the structure of the calcium–phosphophoryn aggregate has not been examined. No structures identifiable as aggregated phosphophoryn have been observed by electron microscopy of intact tissue in thin section. However, isolated phosphophoryn contains small amounts of the covalent cross-linking residues lysinoalanine and histidinoalanine (Kuboki et al., 1983; Fujisawa et al., 1985) which indicates that the protein is aggregated in situ if these cross-links are intermolecular. Since the mineralized aggregate is the functional form of casein, calsequestrin, and the bivalve phosphoprotein in vivo, phosphophoryn may also be functionally active in an aggregated state.

In this study the self-association of phosphophoryn is examined by chemical cross-linking and ultrastructural analysis as a function of the fractional saturation of the protein with calcium and magnesium ions. The onset of protein–protein interaction occurs when the mineral ion content of the protein is about 65% of the saturation level. At saturation, the uncharged calcium–phosphophoryn aggregates are similar to casein micelles, and the negatively charged magnesium–phosphophoryn particles have a rather stellate configuration.

Materials and Methods

Isolation of Rat Dentin Phosphophoryn. Phosphophoryn was isolated from rat incisors essentially as described by Linde et al. (1980), Tsay and Veis (1985), and Kuboki et al. (1979). Incisors were removed from 100 frozen rat heads obtained from Pel Freeze, Inc. The pulp was discarded. The teeth were crushed with pliers and extracted for 24 h at 4 °C with 200 mL of 4.0 M guanidine hydrochloride–50 mM Tris,[1] pH 8.0, containing the following protease inhibitors: 2.5 mM benzamidine hydrochloride, 50 mM ϵ-amino-*n*-caproic acid, 0.6 mM *N*-ethylmaleimide, and 0.3 mM phenylmethanesulfonyl fluoride. The extract was discarded, and the residue was demineralized by dialysis for 3 weeks at 4 °C against 6 × 500 mL of 0.5 M EDTA, pH 8.0, containing the protease inhibitors. The nondialyzable fraction was centrifuged for 20 min at 10000*g*. The supernatant fluid was filtered through a 0.45-μm membrane and then concentrated and equilibrated with 20 mM Tris, pH 8.0 by diafiltration using an Amicon YM10 membrane. Phosphophoryn was precipitated by addition of an equal volume of 2.0 M $CaCl_2$. The precipitate was collected by centrifugation for 30 min at 100000*g* and redissolved in 6 mL of 0.2 M EDTA, pH 8.0. EDTA was removed by dialysis against 50 mM NaCl and then against 50 mM Tris, pH 8.0. Phosphophoryn was further purified by chromatography on a 2.5 × 20 cm column of DEAE-cellulose. The column was eluted with 150 mL of start buffer (50 mM Tris, pH 8.0) and then with 600 mL of a linear gradient buffer ranging from 0 to 0.7 M NaCl in start buffer. Phosphophoryn was eluted in a single biphasic band at about 0.25 M NaCl. Phosphophoryn concentration is conveniently expressed as the concentration of organic phosphate residues (P_o). In this preparation 550 μmol of P_o was obtained from 400 incisors.

Fractionation of Phosphophoryn. Phosphophoryn was resolved into a highly phosphorylated (HP) and moderately phosphorylated (MP) fraction by two methods. In the first method phosphophoryn (117 μmol of P_o) dissolved in 5 mL of water was dialyzed against 10 mM $MgCl_2$, 30 mM NaCl, and 10 mM Tris, pH 7.4, and then charged onto a 1.6 × 200 cm column of Sephacryl S-500 and eluted with the same buffer. Five-milliliter fractions were collected at a flow rate of 25 mL/h, and the effluent was monitored at 230 nm. HP was eluted in a single band and MP in a biphasic band at eluent volumes of 185 and 280 mL, respectively (Figure 1). Eighty percent of the organic phosphate was recovered in the HP fraction. Phosphophoryn, chromatographed under the same conditions in the absence of $MgCl_2$, eluted in a single broad asymmetric band in an eluent volume of about 250 mL (Figure 1).

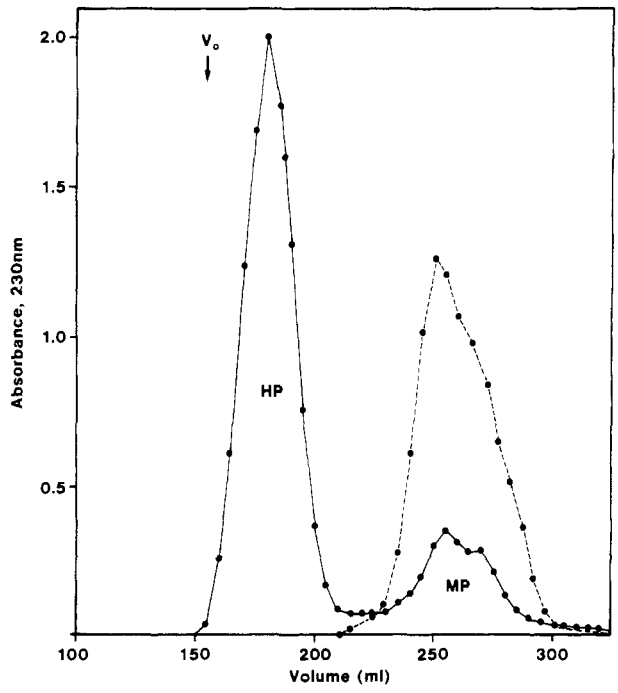

FIGURE 1: Chromatography of rat dentin phosphophoryn on a 1.6 × 200 cm column of Sephacryl S-500. Eluted with 50 mM NaCl–10 mM Tris, pH 7.4 (– –), or with 10 mM $MgCl_2$, 30 mM NaCl, and 10 mM Tris, pH 7.4 (—). Highly phosphorylated fraction (HP). Moderately phosphorylated fraction (MP). V_0 indicates the void volume.

In the second method, 3.0 mL of 0.25 M $MgCl_2$, 0.2 M NaCl, and 50 mM Tris, pH 7.4, was added to phosphophoryn (398 μmol of P_o) dissolved in 17 mL of water. The final solution had a free magnesium concentration of about 18 mM, since the protein binds about one magnesium ion per organic phosphate residue (Figure 7). The solution was centrifuged for 1 h at 100000*g* to sediment HP. The pellet (HP) and the supernatant fluid (MP) contained 82% and 18% of the ogranic phosphate, respectively. HP and MP fractions recovered from both molecular sieve chromatography and ultracentrifugation were treated with EDTA to remove magnesium ions and dialyzed extensively against 50 mM NaCl and then water to remove EDTA. Both methods were equally efficient in separating HP and MP as judged by SDS–polyacrylamide gel electrophoresis; however, differential ultracentrifugation was much faster and more suitable for large-scale separations. HP fractions obtained from the chromatographic and centrifugation procedures were combined and utilized in all subsequent studies. Amino acid and organic phosphate analyses were performed as previously described (Marsh, 1986b). The lyophilized sodium salts of HP and MP contained 2.93 and 1.47 μmol of P_o/mg, respectively.

Electrophoresis. SDS–polyacrylamide slab gel electrophoresis was performed on 7.5% gels, 0.75 mm thick, in the

[1] Abbreviations: EDTA, ethylenediaminetetraacetic acid; Tris, tris(hydroxymethyl)aminomethane; P_o, organic phosphate; HP, highly phosphorylated protein; MP, moderately phosphorylated protein; SDS, sodium dodecyl sulfate.

Laemmli (1970) system. Gels were double stained with Stains-All and silver as described by Tsay and Veis (1985). Agarose gel electrophoresis was performed in 1% slab gels, 4 mm thick, with 0.25 M Tris–0.192 M glycine, pH 8.3, and stained with Stains-All as described by Green et al. (1973).

Calcium- and Magnesium-Binding Studies. All mineral ion binding experiments were performed in a total volume of 1.0 mL and at an HP concentration of 0.2 or 1.0 mM P_o. The pH was 7.4 or 8.0. The solutions were buffered with 22.5 mM Tris and 0, 63, or 126 mM NaCl to give an ionic strength of 0.019, 0.082, or 0.145 M, respectively. The total calcium or magnesium concentration ranged from 0.10 to 8.0 mM. ^{45}Ca at a specific activity of about 1.5×10^5 dpm/μmol was used for calcium-binding experiments. Mineral ion binding was initiated by addition of $^{45}CaCl_2$ or $MgCl_2$ to the protein solution, and 0.5 mL of ultrafiltrate was collected by centrifuging the solutions for 5 min at 1000*g* in Amicon micropartition cells (MPS-1) equipped with YMT membranes. Free ^{45}Ca or Mg ions in the ultrafiltrates were measured radiometrically or by atomic absorption spectroscopy, respectively. Protein-bound calcium or magnesium ions were determined from the difference in the total and free ions. The data are expressed as the number of calcium ions ν_{Ca} or magnesium ions ν_{Mg} bound to the protein per organic phosphate residue. In these experiments, no protein was detected in the ultrafiltrate, and the YMT membranes did not bind detectable amounts of mineral ions or protein.

Chemical Cross-Linking. Chemical cross-linking was performed under the same conditions as the mineral ion binding experiments, except that the solutions also contained 0.5% glutaraldehyde and 25 mM triethanolamine instead of Tris. After equilibration for 1 h at room temperature, the solutions were diluted with an equal volume of electrophoresis buffer containing 20% glycerol and 25 mM EDTA. Covalent polymers were detected by electrophoresis in 1% agarose gels as described above.

Electron Microscopy. Three microliters of the HP–mineral ion solutions (without glutaraldehyde) was applied to carbon- or Formvar-coated grids. The solution was left in contact with the grid for 0.5–3.0 min depending on the protein concentration to allow the HP–mineral ion complex to bind (or settle) to the substrate. Then the solution was withdrawn by touching the tip of a wedged-shaped piece of filter paper to the edge of the grid and maintaining contact until the substrate appeared perfectly dry (about 20 s). The grids were rotary coated with Pt–Pd (80:20) at an angle of 6° and a pressure of 5×10^{-6} Torr and then examined with an Hitachi 11E electron microscope operated at 75 kV. The appearance and distribution of the protein were similar on carbon and Formvar substrates. However, the carbon substrates were much more stable in the electron beam.

Results

SDS–polyacrylamide gels of total rat dentin phosphophoryn show three major bands with apparent molecular weights of 90 000–100 000 (Figure 2). The highly phosphorylated fraction (HP) has two bands, and this fraction polymerizes in the presence of magnesium ions. The moderately phosphorylated fraction (MP) has a major band with an apparent molecular weight of about 90 000 and several minor bands of lower molecular weight (Figure 2). The MP fraction is not polymerized by magnesium ions. After equilibration of total phosphophoryn with magnesium, HP and MP are separable by either molecular seive chromatography (Figure 1) or differential ultracentrifugation. About 50% and 25% of the amino acid residues are phosphorylated in HP and MP, respectively (Table I). All mineral ion binding experiments were performed with HP only.

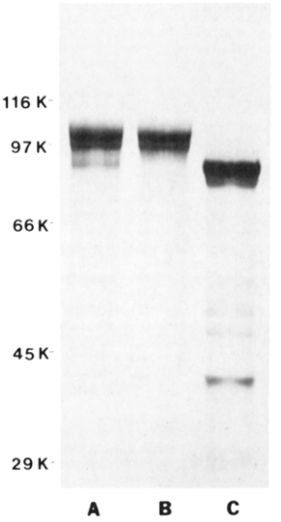

FIGURE 2: SDS–polyacrylamide gel stained with Stains-All and silver. (A) Total rat dentin phosphophoryn. (B) Highly phosphorylated fraction (HP). (C) Moderately phosphorylated fraction (MP). Ten nanomoles of organic phosphate was applied to each lane. Position of molecular weight markers is indicated at the left of gel. In this gel the standards and phosphoproteins were equilibrated with 2-mercaptoethanol before electrophoresis in the presence of SDS. However, neither SDS nor 2-mercaptoethanol effects the mobility of the phosphoproteins.

Table I: Amino Acid Composition of HP and MP[a]

residue	HP	MP
aspartic acid	349	293
threonine	7	15
serine[b]	557	458
glutamic acid	33	100
proline	1	14
glycine	24	39
alanine	7	18
valine		7
isoleucine	1	3
leucine		10
tyrosine		2
phenylalanine		3
lysine	12	13
histidine	9	10
arginine		15
P_o[c]	478	234

[a] Units are residues per 1000 amino acid residues. [b] Includes serine and phosphoserine. [c] P_o is organic phosphate residues.

HP binds a maximum of 1.33 calcium ions per organic phosphate residue, and this value is independent of ionic strength (0.019–0.145 M), protein concentration (0.2–1.0 mM P_o), and pH (7.4–8.0) in the indicated ranges (Figure 3). Under the same range of conditions, the affinity constant is independent of pH and protein concentration, but not ionic strength. Note that curves describing ν_{Ca} as a function of the free calcium ion concentration [Ca] are coincident within experimental error at pH 7.4 and 8.0 and at protein concentrations of 0.2 and 1.0 mM P_o, but at ionic strength 0.019 and 0.145 M the curves are distinct (Figure 3). Scatchard plots describing $\nu_{Ca}/[Ca]$ as a function of ν_{Ca} are nonlinear (Figure 4), demonstrating that the "binding sites" are not identical and independent. Note that the curve has an abrupt slope change at $\nu_{Ca} = 0.90$ (67% saturation) and at $\nu_{Ca} = 1.33$ (100% saturation). The apparent dissociation constant K is defined here as the free calcium ion concentration in equilibrium with the Ca–HP complex at 50% saturation. Values of K_{Ca} and K_{Mg} are listed in Table II.

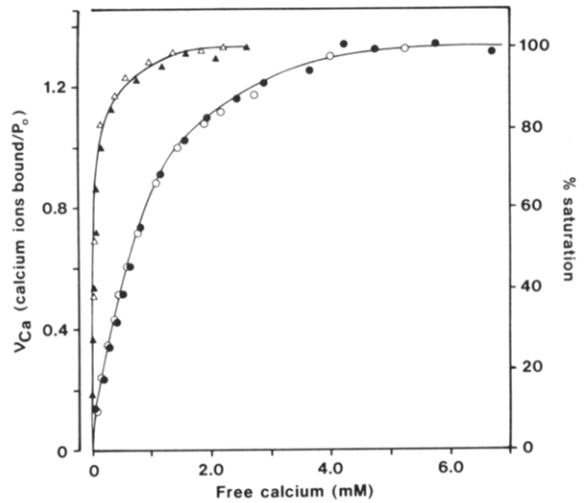

FIGURE 3: Titration of HP with calcium ions. Graph shows ν_{Ca} as a function of the free calcium concentration. Conditions are (●) 1.0 mM P_o, ionic strength 0.145 M, pH 7.4; (○) 1.0 mM P_o, ionic strength 0.145, pH 8.0; (▲) 1.0 mM P_o, ionic strength 0.019, pH 7.4; and (△) 0.20 mM P_o, ionic strength 0.019, pH 7.4. P_o is organic phosphate residues. Units of ν_{Ca} are calcium ions bound per P_o.

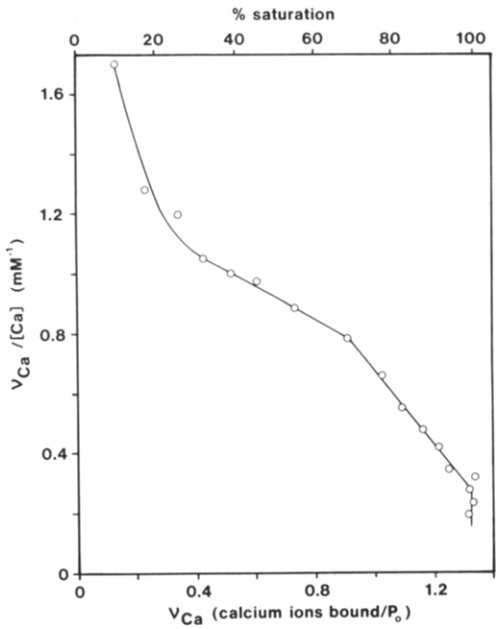

FIGURE 4: Scatchard plot showing $\nu_{Ca}/[Ca]$ as a function of ν_{Ca}, where [Ca] is the free calcium ion concentration. Conditions are 1.0 mM P_o, ionic strength 0.145 M, and pH 7.4.

When HP was treated with glutaraldehyde in the presence of increasing calcium ion concentrations, covalent interchain cross-linking was first observed when the protein was 60–75% saturated. The degree of cross-linking increased as ν_{Ca} increased. Covalent dimers, trimers, etc., as well as very high molecular weight multimers, were resolved by electrophoresis in agarose gels after removal of calcium ions with EDTA (Figure 5). A faint band with the mobility of a dimer was also observed with the untreated protein (lane A, Figure 5). This band may represent a natural dimer covalently cross-linked by lysinoalanine or histidinoalanine residues (Fujisawa et al., 1985; Kuboki et al., 1983). The onset of polymerization always occurred between 60 and 75% saturation, irrespective of the pH, ionic strength, and protein concentration in the ranges given above. The abrupt slope change in the Scatchard plot at 67% saturation (Figure 4) occurs at the onset of protein–protein interaction. Since the slope decreases (becomes more negative), the apparent affinity for calcium ion increases at the polymerization point.

Table II: Apparent Dissociation Constants for HP–Mineral Ion Complexes

ionic strength (M)	pH	P_o (mM)[a]	K_{Ca} (mM)[b]	K_{Mg} (mM)[b]
0.019	7.4	1.0	0.055	0.13
	7.4	0.20	0.049	
0.082	7.4	1.0		0.63
0.145	7.4	1.0	0.71	1.4
	8.0	1.0	0.69	

[a] P_o is protein concentration expressed as the concentration of organic phosphate residues. [b] Calculated from the equation $K = (n - \nu)[M]/\nu$, where [M] is the free Ca^{2+} or Mg^{2+} concentration at 50% saturation, so that $(n - \nu)/\nu = 1$. n is the maximum value of ν.

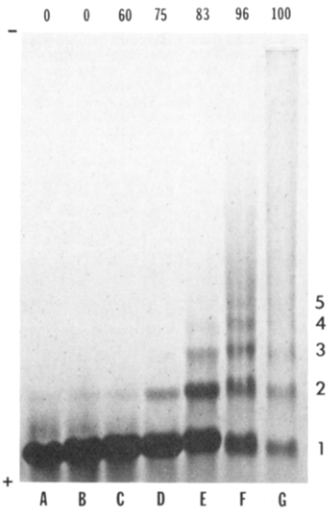

FIGURE 5: Agarose gel electrophoresis of HP treated with 0.5% glutaraldehyde at pH 7.4, ionic strength 0.145 M, for 1 h. The reaction mixtures had a free calcium ion concentration of 0 (A and B), 1.0 (C), 1.5 (D), 2.0 (E), 3.5 (F), and 5.5 mM (G). The percent saturation of the protein with calcium ions is indicated for each lane above the gel. Numbers to the right of gel indicate monomers (1), dimers (2), etc. Reaction mixtures were treated with EDTA to remove calcium ions immediately before electrophoresis. Lane A is the untreated protein, i.e., minus glutaraldehyde.

In electron micrographs of rotary-coated Ca–HP complexes, structures with both linear and globular regions are observed when the protein is about 0–65% saturated with calcium. Above about 65% saturation, the protein is predominantly globular (Figure 6). In the 65–99% saturation range, the majority of the globular structures may represent Ca–HP monomers, since treatment of the complexes with glutaraldehyde gives a mixture of monomers and small covalent oligomers with the monomeric form predominating (Figure 5, lanes D–F). Between 65 and 99% saturation, protein–protein interactions are probably transient with monomers dominating the equilibrium mixture. When HP is saturated with calcium, the protein assumes a dense compact particulate structure (Figure 6C). The abrupt slope change at 100% saturation in the Scatchard plot (Figure 4) corresponds to the aggregation of the Ca–HP complex. The Ca–HP particles are about 25 nm in diameter and occur singly or clustered. Assuming the particles are approximately spherical and have a density of about 1.88 g/cm^3 similar to the bivalve phosphoprotein particles (Marsh & Sass, 1984), the Ca–HP particles have a molecular weight of about 9.2×10^6. The saturated Ca–HP monomers have a molecular weight of 1.19×10^5 on the basis of an HP molecular weight of 10^5. Therefore, the Ca–HP particles may contain about 75 monomers.

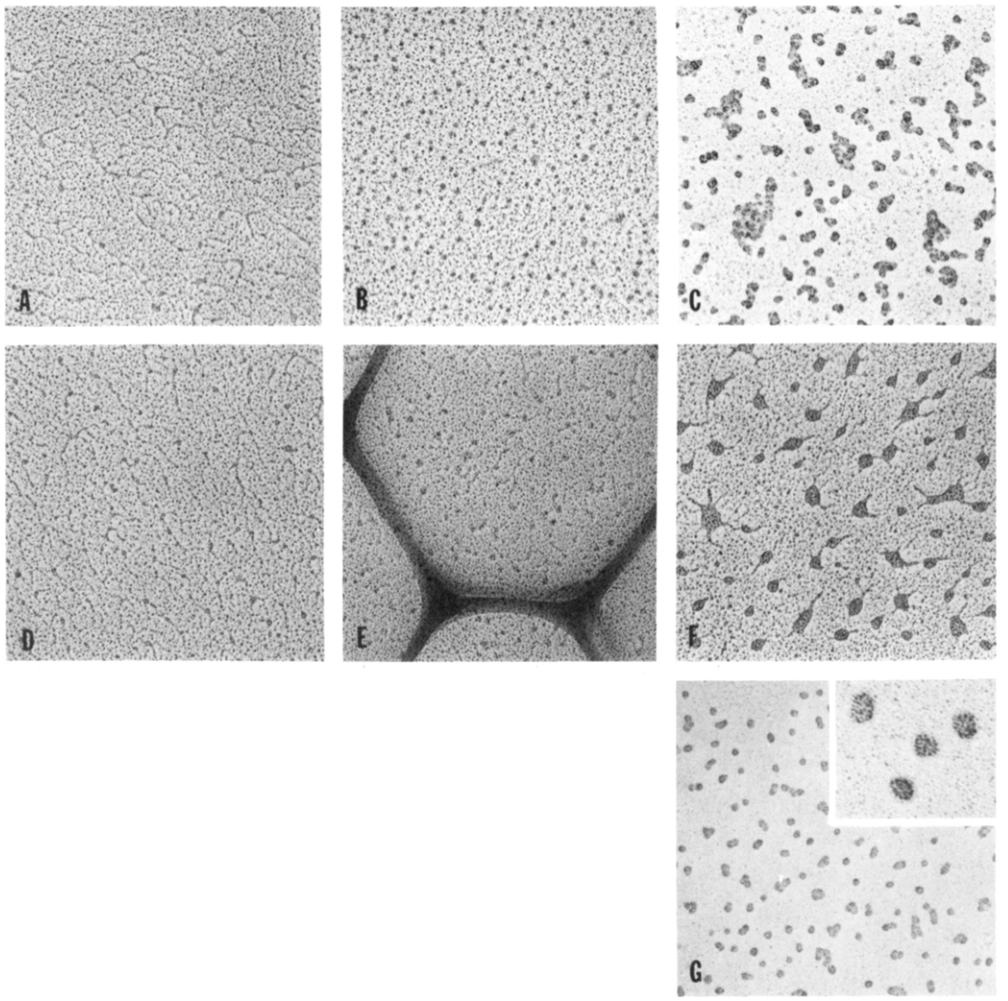

FIGURE 6: Electron micrographs of HP–mineral ion complexes rotary shadowed with Pt–Pd. The protein is 30%, 85%, and 100% saturated with calcium ions in (A), (B), and (C), respectively, and 40%, 87%, and 100% saturated with magnesium ions in (D), (E), and (F), respectively. In (G) the protein was equilibrated with an equal molar mixture of calcium and magnesium at a combined concentration sufficient to saturate the protein. In (E) the carbon film is supported by a lacy Formvar network. The size and shape of the HP complexes were independent of the time that the protein was equilibrated with mineral ions. Equilibration periods ranged from less than 1 min to 24 h. All magnifications are 42000×; inset in (G) is 135000×.

The interaction of magnesium ions with HP is somewhat different. The protein binds a maximum of only 1.07 magnesium ions per organic phosphate residue at pH 7.4 (Figure 7). The maximum value of ν_{Mg} is independent of the ionic strength, but the dissociation constant is not (Table II). Direct plots of ν_{Mg} as a function of the free magnesium concentration are biphasic (Figure 7). There is a plateau at 60–70% saturation (ν_{Mg} = 0.65–0.75) and another one at saturation (ν_{Mg} = 1.07). The plateau at 60–70% saturation can be correlated with the onset of protein–protein interaction by chemical cross-linking and agarose gel electrophoresis (Figure 8), and at saturation the protein aggregates into discrete particles (Figure 6F).

Ultrastructurally, Mg–HP and Ca–HP are indistinguishable when the mineral ion content of the complexes is less than about 65% of the saturation level; i.e., both complexes have linear and globular domains (Figure 7A,D). However, at higher levels of bound ions, Mg–HP complexes still have prominent linear domains (Figure 6E), but Ca–HP complexes do not. The Mg–HP particles, which appear abruptly at saturation, average about 50 nm in diameter. They are considerably larger and have a less compact appearance than Ca–HP aggregates. Even as large particles, Mg–HP has linear segments protruding from the main body of the aggregate, giving the particles a rather stellate configuration. Ca–Mg–HP particles, formed by equilibrating the protein with an equal molar mixture of calcium and magnesium ions, are about 25 nm in diameter (Figure 6G); they have little tendency to clump like the Ca–HP particles, and they have no linear protrusions characteristic of Mg–HP particles.

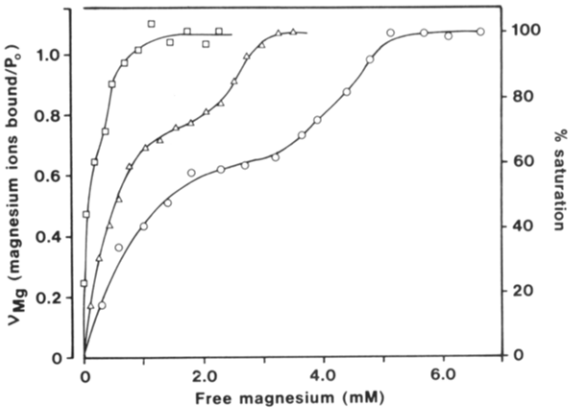

FIGURE 7: Titration of HP with magnesium ions. Graph shows ν_{Mg} as a function of the free magnesium ion concentration. Conditions are 1.0 mM P_o, pH 7.4, and ionic strength 0.019 (□), 0.082 (△), and 0.145 (○).

Discussion

Rat dentin phosphophoryn is a heterogeneous mixture of aspartic acid rich phosphoproteins (Dimuzio & Veis, 1978).

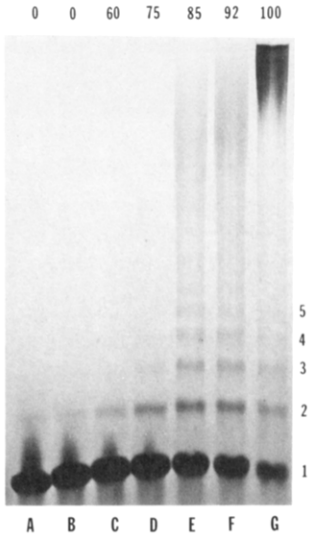

FIGURE 8: Agarose gel electrophoresis of HP treated with 0.5% glutaraldehyde at pH 7.4, ionic strength 0.145 M, for 1 h. The reaction mixture had a free magnesium concentration of 0 (A and B), 3.0 (C), 4.0 (D), 4.5 (E), 4.75 (F), and 5.5 mM (G). The percent saturation of the protein with magnesium ions is indicated for each lane above the gel. Reaction mixtures were treated with 25 mM EDTA to remove magnesium ions immediately prior to electrophoresis. Numbers to the right of gel indicate monomers (1), dimers (2), etc. Lane A is the untreated protein, i.e., minus glutaraldehyde.

By ion-exchange chromatography on a sulfonated polystyrene resin, Butler et al. (1983) resolved rat phosphophoryn into a highly phosphorylated fraction and a moderately phosphorylated fraction which they called HP and MP, respectively. Their HP fraction gave two closely spaced bands on polyacrylamide gel electrophoresis with apparent weights of 95 000. About 46% of the HP amino acid residues were phosphoserine, and the fraction contained no valine, leucine, phenylalanine, arginine, or sulfated amino acids. After chromatography on Sepharose CL-6B, their MP fraction contained a single band with a slightly greater mobility on polyacrylamide gels than the HP bands. About 25% of the amino acid residues in MP were phosphorylated, and MP contained three times as much glutamic acid as HP in addition to small amounts of valine, leucine, phenylalamine, and arginine which were absent in HP. In the present study, rat phosphophoryn was resolved into a highly phosphorylated fraction and a moderately phosphorylated fraction by equilibration of phosphophoryn with magnesium ions and fractionation by either molecular seive chromatography or differential ultracentrifugation. The highly phosphorylated fraction was strikingly similar to the HP fraction of Butler et al. (1983), in both electrophoretic bands and amino acid composition (see Figure 2 and Table I); therefore, this preparation was labeled HP, using Butler's designation. The moderately phosphorylated fraction isolated in this study was designated MP, because it was similar to Butler's MP fraction, except for the presence of additional minor electrophoretic bands (Figure 2) which were removed from the MP fraction by Sepharose CL-6B chromatography in the earlier study. The magnesium fractionation and ion-exchange chromatography methods are equally efficient for separating HP and MP; however, the former method is faster and easier. Butler et al. (1983) further resolved dephosphorylated HP (dP-HP) into two subfractions designated dP-HP1 and dP-HP2. In both subfractions aspartic acid and serine represented about 90% of the total amino acid residues, but there were clear differences in the aspartic acid/serine ratios, in the minor amino acid contents, and the NH_2-terminal amino acid sequences. In the present study HP1 and HP2 were not resolved by magnesium ion fractionation. HP1 and HP2 either coaggregate or form distinct colloidal particles of similar size.

HP binds a maximum of 1.33 calcium ions per organic phosphate residue with similar affinity at pH 7.4 and 8.0. Insensitivity to pH in this range demonstrates that the phosphoserine residues are completely ionized at pH 7.4 in the presence of calcium. HP bears a negative charge of 2.7 electrons per organic phosphate residue, assuming that nearly all of the residues analyzed as aspartic acid were derived from aspartate as opposed to asparagine residues. Therefore at saturation, the net charge on the Ca–HP complex is essentially zero. The Ca–HP complex aggregates into discrete particles only at saturation, when charge neutralization occurs. The Mg–HP complex also forms particles at saturation, but here the protein saturates with only 1.07 magnesium ions per organic phosphate residue, so Mg–HP particles are negatively charged. Kuboki et al. (1979) observed that phosphophoryn was precipitated by calcium but not magnesium ions. The negative charge on Mg–HP particles probably prevents their flocculation, because they form a stable colloid even in the presence of a large excess of free magnesium. The uncharged Ca–HP particles flocculate at relatively low calcium ion concentrations, particularly at low ionic strength. For instance, in 22.5 mM Tris, pH 7.4, the protein saturates at a free calcium ion concentration of about 1.5 mM, and at about 3.0 mM calcium, the particles precipitate.

The shape and distribution of Ca–HP and Mg–HP particles as observed in electron micrographs are consistent with their electrostatic charges. The Ca–HP particles are small (about 25 nm in diameter) compact structures with a rather angular contour, and the majority occur in clumps. The Mg–HP particles are about twice as large and have a rather stellate configuration due to linear segments protruding from the central aggregate. Little if any clumping is observed, and the linear segments probably correspond to negatively charged domains. Ca–Mg–HP particles are similar in size and shape to Ca–HP particles, except that clumping is greatly reduced in the former. The Ca–HP particles and clumps resemble the small and large casein micelles of milk (Schmidt, 1982). The Ca–Mg–HP particles resemble the native phosphoprotein particles of heterodont bivalves (Marsh, 1986b).

Formation of Ca–HP particles requires a free calcium concentration of about 5.0 mM under physiological conditions, which is considerably higher than the expected calcium ion concentration in the dentin matrix (Neuman, 1980). However, under the same conditions, protein–protein interaction occurs at calcium ion concentrations as low as 1.2 mM, corresponding to about 65% saturation of the protein with calcium ions. At this point there is also a shift in the protein conformation as judged by electron images from a predominantly linear structure characteristic of the free protein (Cocking-Johnson et al., 1983) to a folded conformation. Protein–protein interaction is probably both conformation and charge dependent. As shown in the following paper, HP aggregates are stable at much lower calcium ion concentrations in the presence of inorganic phosphate.

The Mg–HP complex also begins to polymerize at about 65% saturation. Here, however, there is no obvious change in protein conformation by electron microscopy. From 0 to 99% saturation, Mg–HP appears predominantly linear. Even the Mg–HP particles which form at saturation contain linear segments. Some conformational change is probable, but it is too subtle to be detected by electron microscopy. Ca–HP and Mg–HP complexes probably have different conformations, since Ca–phosvitin and Mg–phosvitin complexes are confor-

mationally different, on the basis of circular dichroism studies (Grizzuti & Perlmann, 1973). In addition phosvitin, like HP, has a higher capacity for calcium ions than magnesium ions.

The calcium-binding capacity of calsequestrin (MacLennan & Wong, 1971; Aaron et al., 1984), phosvitin (Grizzuti & Perlmann, 1973), and HP (as measured in this study) are in good agreement; i.e., at saturation each protein binds enough calcium to approximately neutralize the negatively charged amino acid residues. In contrast, the calcium-binding capacity of phosphophoryn observed in previous studies is far greater than that required to neutralize the aspartic acid and phosphoserine residues. Zanetti et al. (1981), using a rat dentin HP similar to the one studied here, estimated a total calcium capacity about 4-fold greater than required to neutralize the negative residues. Lee et al. (1977) and Stetler-Stevenson and Veis (1987) also found that bovine phosphophoryn binds a large excess of calcium, and they postulated that chloride or hydroxide ions were included in the complex to maintain electrical neutrality. The previous calcium-binding studies on phosphophoryn used equilibrium dialysis and/or calcium-specific electrode methods, while in the present study an ultrafiltration technique was used. Ultrafiltration is a much faster and simpler procedure. It is also theoretically equivalent to equilibrium dialysis, if binding is independent of protein concentration (Sophianopoulos et al., 1978), since the concentration of the protein increases as ultrafiltration proceeds. In this study, calcium binding was independent of protein concentration, so there is no theoretical basis for reconciling the present results with previous studies. Interestingly, Cookson et al. (1980) found that rat dentin HP bound an approximately electrostatic equivalent amount of manganese. Here the free manganese concentration was determined by electron paramagnetic resonance spectroscopy (EPR); Mn^{2+} bound to HP did not contribute significantly to the amplitude of the EPR signal due to relaxation effects. Presumably, if there were additional Mn^{2+} ions associated with the Mn–HP complex, their relaxation behavior was characteristic of free ions and not bound ions. As seen in the following paper, HP binds excessive calcium in the presence of PO_4^{3-} ions, but this phenomenon is related to the low solubility of calcium phosphate salts.

Registry No. Ca, 7440-70-2; Mg, 7439-95-4.

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