Modulation of crystal formation by bone phosphoproteins: structural specificity of the osteopontin-mediated inhibition of hydroxyapatite formation

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Osteopontin is a phosphorylated sialoprotein containing a conserved sequence of contiguous aspartic acid residues. This protein is expressed at high levels in mineralized tissues and has previously been shown to inhibit the *in vitro* formation of hydroxyapatite (HA). In the present study, protein modification and model compound studies have been used to identify the structural features of osteopontin that are responsible for its crystal-modulating properties. Using metastable calcium phosphate solutions buffered by autotitration, osteopontin caused half-maximal inhibition of HA formation at a concentration (IC_{50}) of 0.06 μg/ml. The hen egg yolk phosphoprotein phosvitin was a much weaker inhibitor, while dextran sulphate had no effect. The synthetic polypeptide poly(aspartic acid) was almost as effective an inhibitor of HA formation as osteopontin (IC_{50} 0.11 μg/ml), whereas poly(glutamic acid) was more than a thousand times less potent (IC_{50} 155 μg/ml). In a steady-state agarose gel system, much higher polypeptide concentrations were required for inhibition of HA formation, but a similar relative order of inhibitory effectiveness was observed. Treatment of osteopontin with alkaline phosphatase removed 84% of the covalently bound phosphate and reduced its HA-inhibiting activity by more than 40-fold. Treatment with glycine ethyl ester in the presence of carbodi-imide modified 86% of the carboxylate groups in osteopontin and reduced its inhibitory activity by 6-fold. These findings indicate that osteopontin is a potent inhibitor of HA formation. This activity requires phosphate and carboxylate groups, possibly including the conserved sequence of contiguous aspartic acid residues. Osteopontin may act as an inhibitor of phase separation in physiological fluids of high supersaturation.

INTRODUCTION

The mineralization of bone occurs by deposition of hydroxyapatite (HA) crystals in an organic matrix consisting of type I collagen and a variety of other proteins. The non-collagenous proteins include glycoproteins, phosphoproteins, Gla-containing proteins and proteoglycans (for a review, see Young et al., 1992).

Bone sialoprotein (BSP) and osteopontin are the major phosphoproteins of bone (Fisher et al., 1983; Prince et al., 1987). Both are phosphorylated sialoproteins with sulphated tyrosine residues and regions of contiguous acidic amino acids. BSP, however, contains more sialic acid and less phosphate than osteopontin (Franzen and Heinegard, 1985). In osteopontin the polycarboxylate sequences consist of aspartic acid (Oldberg et al., 1986), whereas in BSP they consist of glutamic acid (Oldberg et al., 1988). BSP is quite specific to bone, although it has also been found in growth plate cartilage and the embryonic trophoblast layer (Fisher et al., 1983; Bianco et al., 1991). Osteopontin is expressed in a variety of tissues, but at higher levels in bone than elsewhere (Nomura et al., 1988; Brown et al., 1992; Chen et al., 1993). The osteopontin of bone may differ from those of soft tissues in phosphorylation or by differential splicing (Singh et al., 1992).

Although the HA crystals of bone are intimately and specifically associated with type I collagen fibrils (Stuehler, 1937; Nylen et al., 1960; White et al., 1977), it is now generally accepted that collagen itself neither promotes nor inhibits the formation of HA (Glimcher, 1989). Therefore the non-collagenous proteins of bone have been studied for possible crystal-modulating activities. The HA formation systems that have been used can be generalized into three categories: *de novo* formation in calcium phosphate solutions or double-diffusion gel systems; growth of HA 'seed' crystals in calcium phosphate solutions; and transformations of amorphous calcium phosphate (ACP) into HA.

The bone proteins osteonectin and osteocalcin have been reported to inhibit the seeded growth (Menanteau et al., 1982; Romberg et al., 1986) and *de novo* formation (Doi et al., 1992) of HA. Another bone protein found to inhibit seeded growth of HA is albumin, but only at much higher concentrations than those reported for osteocalcin and osteonectin (Garnett and Dieppe, 1990). In the presence of fibrillar collagen, osteonectin has been reported to promote the formation of HA (Termine et al., 1981); however, others have found inhibition under these conditions (Doi et al., 1989). Immobilized anionic proteins can have different effects on crystal formation than those seen in solution. For example, covalent attachment of osteocalcin to agarose beads resulted in formation of HA from solutions of very low calcium phosphate supersaturation (Linde et al., 1989).

The major phosphoprotein of dentin, phosphophoryn, is characterized by extremely high contents of aspartic acid and phosphoserine (Veis et al., 1991). Different effects of phosphophoryn on HA formation have been reported. The ACP-to-HA transformation has been reported to be promoted (Nawrot et al., 1976) and inhibited (Termine and Conn, 1976) by phosphophoryn. This protein has also been found to inhibit the *de novo* formation (Doi et al., 1992) and seeded growth (Termine et al., 1980; Boskey et al., 1990) of HA and the formation of octacalcium phosphate in double-diffusion agar gels (Fujisawa et al., 1987). *De novo* formation of HA in gelatin gels has been reported to be increased by low concentrations (< 1 μg/ml) and inhibited by higher concentrations (> 1 μg/ml) of phosphophoryn (Boskey et al., 1990).

The effects of bone phosphoproteins on HA formation in a

Abbreviations used: HA, hydroxyapatite; ACP, amorphous calcium phosphate; BSP, bone sialoprotein; GEE, glycine ethyl ester.

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steady-state agarose gel system have recently been studied in our laboratory. It was shown that BSP induces the formation of HA at $Ca \times PO_4$ concentration products below the threshold for spontaneous precipitation in this system, whereas osteopontin has no effect (Hunter and Goldberg, 1993). At $Ca \times PO_4$ products above the threshold for spontaneous precipitation, osteopontin has a slight but statistically non-significant inhibitory effect on HA formation. Other workers have recently shown that higher concentrations of osteopontin inhibit the *de novo* formation and seeded growth of HA in a steady-state gelatin gel system (Boskey et al., 1993). In the present study, a quantitative analysis of the osteopontin-mediated inhibitors of HA formation has been performed using an autotitration system. Studies with model compounds and chemical and enzymic modification of osteopontin have been used to identify structural features of the protein that are involved in its crystal-modulating properties.

EXPERIMENTAL

Materials

Poly(L-aspartic acid) (P-6762; 28800 Da), poly(L-glutamic acid) (P-4761; 34000 Da), phosvitin, agarose-conjugated alkaline phosphatase (P-0762) and 1-ethyl-3-(3-dimethylaminopropyl)-carbodi-imide were obtained from the Sigma Chemical Company. SeaPlaque low-temperature agarose was obtained from Mandel Scientific. BSA (Cohn, crystallized) and dextran sulphate [48000 Da; 19% (w/w) S] were obtained from ICN. *N*-Hydroxysulphosuccinimide was obtained from Serva Biochemicals. [^{14}C]Glycine ethyl ester ([^{14}C]GEE; 40–60 mCi/mmol) was obtained from New England Nuclear.

Purification of osteopontin from porcine calvaria

Osteopontin was purified from adult porcine calvarial bone as previously described (Zhang et al., 1990). In brief, EDTA-extracted proteins were fractionated by fast liquid protein chromatography successively on Fast-Q, Superdex-200 and Mono-Q columns. The purity of the preparations was determined by Western blotting using affinity-purified antibodies and amino acid analysis. Typically, 2–3 mg of purified osteopontin was obtained from 100 g of bone. Protein concentrations determined by amino acid analysis were used to calculate molar absorption coefficients at 230 nm, which were in turn used for the estimation of the protein concentrations given below.

Dephosphorylation and chemical modification of osteopontin

To dephosphorylate osteopontin, aliquots of protein (approx. 100 μg) were incubated in 1 ml of 0.1 M Tris/HCl, pH 8.0, in the presence or absence of 0.5 unit of agarose-conjugated alkaline phosphatase at 37 °C for 4 h on a tube rocker. The samples were then centrifuged briefly to pellet the agarose beads, and the supernatants were dialysed against deionized water. Following lyophilization, samples were redissolved in 1 ml of deionized water. Aliquots of alkaline phosphatase-treated and control osteopontin were used for organic phosphate determination by the method of Van Veldhoven and Mannaerts (1987).

Blocking of carboxylate groups was performed by a modification (Staros et al., 1986) of the method of Hoare and Koshland (1967). Two 0.2 ml aliquots of osteopontin containing approx. 100 μg of protein were added to 0.8 ml of 12.5 mM GEE, pH 4.75, containing 6.25 μCi/ml [^{14}C]GEE. To one (experimental) sample was added solid 1-ethyl-3-(3-dimethylaminopropyl)carbodi-imide and *N*-hydroxysulphosuccinimide to final concentrations of 50 mM and 5 mM respectively. Both samples were then incubated for 4 h at 37 °C without mixing, and then dialysed extensively against deionized water at 4 °C. Following lyophilization, samples were redissolved in 1 ml of deionized water and the protein concentrations were determined by measuring absorbance at 230 nm. In order to determine the efficiency of carboxylate group modification, aliquots of the experimental sample and of the GEE/[^{14}C]GEE reaction solution were taken for liquid scintillation counting.

De novo hydroxyapatite formation in solution

The conditions used for *de novo* formation of HA from metastable calcium phosphate solution were similar to those previously described (Hunter and Szigety, 1992). Appropriate volumes of test substances dissolved in water were added to 2.15 ml of 10 mM $CaCl_2$/300 mM NaCl and deionized water was added to a volume of 4.3 ml. The solution was mixed, and 4.3 ml of 3 mM sodium phosphate, pH 7.4/150 mM NaCl was added. Final ionic concentrations were therefore 2.5 mM $CaCl_2$, 1.5 mM phosphate and 150 mM NaCl. The solution was then passed through a 0.22 μm nylon syringe filter, and 8 ml of filtrate was added to a custom-made double-walled glass titration vessel through which water was circulated from a 37 °C water bath. The vessel was attached to a Mettler DL-21 autotitrator programmed to maintain pH 7.4 by addition of 50 mM NaOH. The solution was pre-titrated to pH 7.40–7.45 before the experimental titration was begun. After 1000 min, the solution was removed, filtered as above, and added to 50 μl of concentrated HCl to prevent further precipitation during storage. In most cases, triplicate samples were run on titrators simultaneously. Following each run, titration vessels and all other components in contact with calcifying solutions were washed with 0.1 M HCl for at least 1 h, then rinsed extensively with deionized water. The autotitrators were recalibrated prior to each run.

The final amount of HA formation was quantified as the volume of 50 mM NaOH added by 1000 min (V_{1000}) (Hunter and Szigety, 1992). From a series of titrations in the absence of effector, a control V_{1000} value of 0.270 ± 0.013 ml ($n = 9$) was calculated. In the presence of high concentrations of inhibitor, a 'baseline' titrant addition of approx. 0.04 ml was observed (see Figure 1). From plots of V_{1000} against effector concentration, therefore, the effector concentration resulting in half-maximal inhibition (IC_{50}) corresponded to a V_{1000} value of 0.155 ml.

Growth of HA in steady-state agarose gels

HA formation in agarose gels was performed as previously described (Hunter and Goldberg, 1993), except that the agarose concentration was 1% and the $CaCl_2$ and sodium phosphate concentrations were 7.5 mM. The lower agarose concentration, which does not significantly affect the formation of HA, was used to facilitate pouring of the gels. In brief, gels containing 1% agarose, 150 mM NaCl, 0.01% NaN_3, 10 mM Tris/HCl, pH 7.4, and appropriate concentrations of test substances were cast in cylindrical chambers (approximate volume 1 ml) drilled in a block of acrylic plastic. A sheet of dialysis membrane covered each end of the gels. Solutions containing 150 mM NaCl, 0.01% NaN_3, 10 mM Tris/HCl, pH 7.4, and either 7.5 mM $CaCl_2$ or 7.5 mM sodium phosphate, pH 7.4, were pumped along opposite ends of the cells at a flow rate of 1 ml/h per cell without recirculation. The entire apparatus was incubated at 37 °C for 5 days. Agarose gels were then removed, ashed in 5 ml of concen-

trated nitric acid, and analysed for phosphate by the method of Chen et al. (1956) and for calcium by atomic absorption spectrometry using a Varian SpectrAA 30/40.

RESULTS

Effects of osteopontin and model compounds on HA formation from metastable calcium phosphate solution

De novo formation of HA from metastable calcium phosphate solution was achieved by incubation of solutions containing 2.5 mM $CaCl_2$, 1.5 mM sodium phosphate and 150 mM NaCl at 37 °C on autotitrators operating in the pH-stat mode. A pH of 7.4 was maintained by addition of 50 mM NaOH. As hydroxyl ions form part of the HA unit cell, the volume of NaOH added is a direct function of HA formation. Under the conditions used, precipitation of HA occurs during a standard incubation of 1000 min.

A series of titration curves produced in the presence of various concentrations of osteopontin is shown in Figure 1. Addition of increasing concentrations of osteopontin resulted in decreased formation of HA, with essentially complete inhibition achieved at 1.0 μg/ml. Previous studies on *de novo* HA formation have shown that the precipitation lag time is typically quite variable, but that the amount of titrant added at 1000 minutes of incubation (V_{1000}) is a convenient and reproducible measure of the amount of HA formation (Hunter and Szigety, 1992). In Figure 2, V_{1000} is plotted against osteopontin concentration. It can be seen that the concentration of osteopontin required for half-maximal inhibition of HA formation (IC_{50}) was approx. 0.06 μg/ml. Plots of phosphate incorporation against effector concentration were essentially identical to the plots of V_{1000} against effector concentration (result not shown).

To determine the specificity of the osteopontin-mediated inhibition of HA formation, several other anionic macromolecules were studied in this system. The egg yolk phosphoprotein phosvitin inhibited HA formation by 50% at approx. 1.9 μg/ml, whereas BSA had only a slight inhibitory effect and a highly sulphated dextran sulphate preparation had no effect at 250 μg/ml (Figure 2). For poly(L-aspartic acid), an IC_{50} value of 0.11 μg/ml was derived. The corresponding value for poly(L-glutamic acid) was 155 μg/ml, and complete inhibition was not achieved at concentrations as high as 250 μg/ml. Bone sialoprotein caused some inhibition of HA formation under these conditions, but with a much lower potency than osteopontin (results not shown).

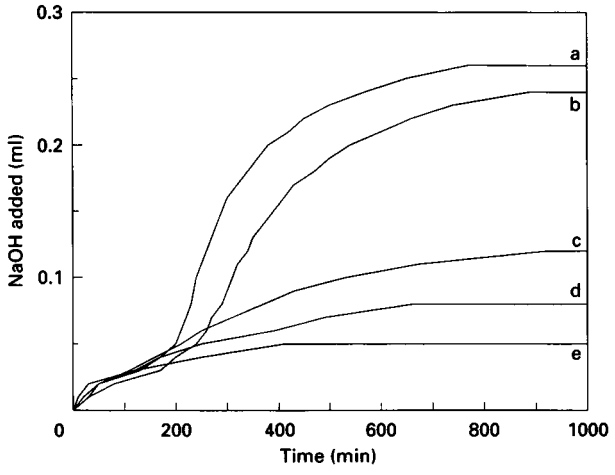

Figure 1 Titration curves for HA formation in the presence of osteopontin

Various concentrations of osteopontin were added to metastable calcium phosphate solutions, and HA formation was determined by autotitration. Concentrations: a, 0; b, 0.03 μg/ml; c, 0.1 μg/ml; d, 0.3 μg/ml; e, 1.0 μg/ml.

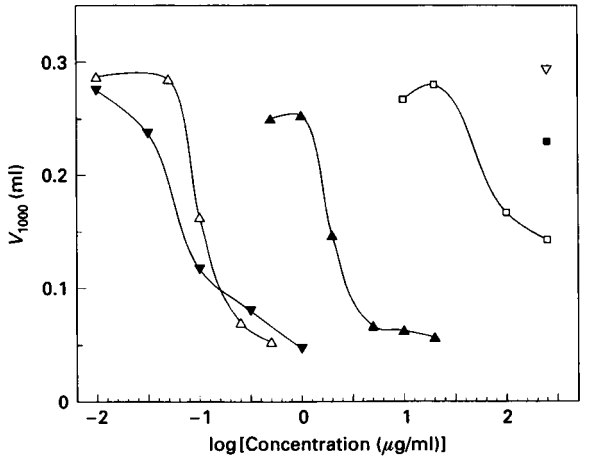

Figure 2 Effects of osteopontin and model compounds on HA formation in metastable calcium phosphate solution

Various concentrations of effector molecules were added to metastable calcium phosphate solutions, and HA formation was determined by autotitration. Results are expressed as volume of 50 mM NaOH added at 1000 min (V_{1000}), and are the means of three determinations. The coefficient of variation for these data was approximately 10%. △, Poly(Asp); ▲, phosvitin; □, poly(Glu); ■, BSA; ▽, dextran sulphate; ▼, osteopontin.

Effects of osteopontin and model compounds on HA formation in steady-state agarose gels

The effect of osteopontin on *de novo* formation of HA under steady-state conditions was studied as previously described (Hunter and Goldberg, 1993). Incorporation of osteopontin at 100 μg/ml into steady-state agarose gels at super-threshold $Ca \times PO_4$ product resulted in a 23% decrease in $Ca + PO_4$ content compared with control gels (Table 1). At the same concentration, poly(Asp) caused 31% inhibition and poly(Glu) had no significant effect. At 250 μg/ml, poly(Glu) and phosvitin both caused a slight but statistically significant inhibition of HA formation in steady-state gels, while albumin and dextran sulphate had no effect.

Effect of dephosphorylated and chemically modified osteopontin on HA formation from metastable calcium phosphate solution

Osteopontin was dephosphorylated by treatment with alkaline phosphatase. The phosphate contents of control and alkaline phosphatase-treated osteopontin were 72 and 11 ng/μg of polypeptide respectively. Therefore alkaline phosphatase digestion removed approx. 84% of the bound phosphate in osteopontin. On SDS/PAGE, dephosphorylated osteopontin migrated slightly faster than the native protein, and exhibited reduced staining with Stains-All, but not with Coomassie Blue (results not shown). Only trace amounts of lower-molecular-mass species were observed in the dephosphorylated preparation, indicating that the amount of proteolysis during alkaline phosphatase digestion was minimal.

Table 1 Effects of osteopontin and other model compounds on HA formation in steady-state agarose gels

Gels were incubated for 5 days before measurement of calcium and phosphate contents. Results shown are means ± S.D. Statistical significance (compared with the control value) was determined using one-way analysis of variance and the Tukey multiple correlations test. n.s., not significant.

	Concentration (μg/ml)	Ca + PO_4 (mg/gel)	n	P
Control	–	2.64 ± 0.08	9	–
Osteopontin	100	2.04 ± 0.15	3	< 0.001
Poly(Asp)	100	1.82 ± 0.04	3	< 0.001
	250	1.61 ± 0.09	6	< 0.001
Poly(Glu)	100	2.44 ± 0.11	3	n.s.
	250	2.40 ± 0.20	3	< 0.025
Phosvitin	250	2.33 ± 0.08	3	< 0.001
BSA	250	2.58 ± 0.06	3	n.s.
Dextran sulphate	250	2.68 ± 0.04	3	n.s.

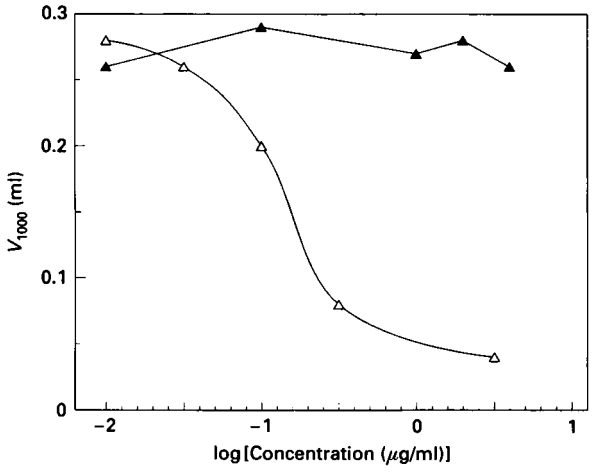

Figure 3 Effect of dephosphorylated osteopontin on HA formation in metastable calcium phosphate solution

Native and enzymically dephosphorylated osteopontin were added to metastable calcium phosphate solutions, and HA formation was determined by autotitration. Results are expressed as volume of 50 mM NaOH added at 1000 min (V_{1000}), and are single determinations. △, Control osteopontin; ▲, dephosphorylated osteopontin.

The effect on HA formation of dephosphorylated osteopontin is shown in Figure 3. Control osteopontin caused half-maximal inhibition at 0.15 μg/ml, while dephosphorylated osteopontin had no effect at concentrations between 0.01 and 4 μg/ml. Note that the inhibitory potency of the control osteopontin is less than that shown in Figure 2, indicating that some activity is lost, even in the absence of enzyme, during the incubation, dialysis and lyophilization procedures. As control osteopontin has a clear inhibitory effect at 0.1 μg/ml, and the alkaline phosphatase-treated protein had none at 4 μg/ml, it can be concluded that dephosphorylation reduces the inhibitory activity of osteopontin by at least 40-fold.

Carboxylate groups in osteopontin were modified by reaction with GEE. Assuming that the 74 aspartic acid and glutamic acid residues constituted all the carboxylate groups available for reaction, it can be calculated that this procedure was 86% efficient. The effect on HA formation of chemically modified osteopontin is shown in Figure 4. Control osteopontin and its GEE derivative caused half-maximal inhibition at concentrations of 0.19 and 1.2 μg/ml respectively. Therefore modification of carboxylate groups reduced the inhibitory activity of osteopontin by approx. 6-fold.

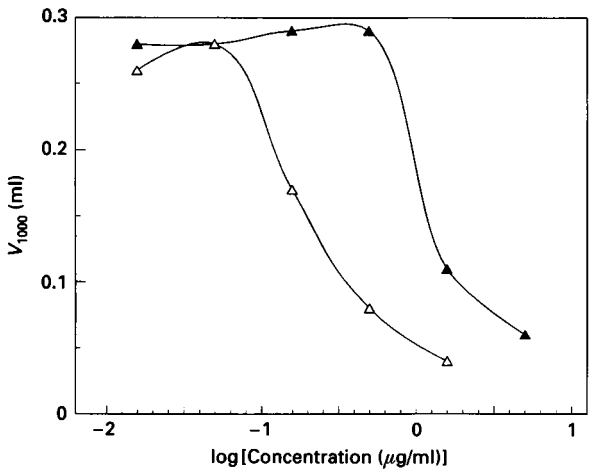

Figure 4 Effect of carboxylate-modified osteopontin on HA formation in metastable calcium phosphate solution

Native and GEE-derivatized osteopontin were added to metastable calcium phosphate solutions, and HA formation was determined by autotitration. Results are expressed as volume of 50 mM NaOH added at 1000 min (V_{1000}), and are single determinations. △, Control osteopontin; ▲, carboxylate-modified osteopontin.

Table 2 Inhibition constants for osteopontin and model compounds

Effector concentrations giving half-maximal inhibition of HA formation (IC_{50}) using the autotitration system were calculated from the data shown in Figures 2, 3 and 4.

	IC_{50} (μg/ml)	Molecular mass (kDa)	IC_{50} (μM)
Osteopontin	0.06	32[e]	0.0019
Phosvitin	1.9	35[f]	0.054
Poly(Asp)	0.11	28.8[g]	0.0038
Poly(Glu)	155	34[g]	4.56
Dextran sulphate	> 250	48[g]	> 5.21
BSA	> 250	67	> 3.73
AP-osteopontin[a]	> 4	32[e]	> 0.125
AP-osteopontin (C)[b]	0.15	32[e]	0.0047
GEE-osteopontin[c]	1.2	38.5[h]	0.031
GEE-osteopontin (C)[d]	0.19	32[e]	0.006

[a] Alkaline phosphatase-treated osteopontin.
[b] Control osteopontin for alkaline phosphatase experiment.
[c] GEE derivative of osteopontin.
[d] Control osteopontin for GEE experiment.
[e] Polypeptide molecular mass (Zhang et al., 1990).
[f] Taborsky (1983).
[g] According to manufacturer's specifications.
[h] Based on extent of carboxylate group modification measured.

The inhibitory potencies, in mass and molar concentrations, of the model compounds and native and modified osteopontin are listed in Table 2. Because the osteopontin concentrations used throughout this study related to the polypeptide backbone, not the fully modified protein, the polypeptide molecular mass of

osteopontin has been used to convert mass concentrations to molar units.

DISCUSSION

The effects of proteins and synthetic polypeptides on HA crystal formation have been extensively studied. It has consistently been found that acidic polypeptides inhibit HA formation to a greater extent than basic or neutral ones (Romberg et al., 1986; Mueller and Sikes, 1993). Phosphoproteins appear to be particularly inhibitory, suggesting that phosphate groups are of importance in crystal-modulating interactions. In the caseins, for example, inhibition of *de novo* HA formation is positively correlated with phosphoserine content (Van Kemenade and De Bruyn, 1989). Similarly, the N-terminal region of a proline-rich phosphoprotein from saliva, containing two phosphoserine residues, is a potent inhibitor of HA formation from dicalcium phosphate dihydrate, but dephosphorylation completely abolishes this activity (Hay et al., 1987). The synthetic polypeptide Asp_{20} was found to have no effect on the ACP-to-HA transformation, but addition of even one phosphoserine (pSer) to this molecule caused complete inhibition (Sikes et al., 1991). However, Asp_{20} and $Asp_{20}pSer_2$ did not differ in their inhibitory effects on the seeded growth of HA (Mueller and Sikes, 1993). Some other findings also minimize the role of phosphate groups and suggest that acidic amino acids are important in the inhibition of HA formation. For example, a study of the effects on HA formation of synthetic peptides corresponding to the N-terminal region of the salivary protein statherin showed that substitution of aspartic acid for the two phosphoserine residues did not affect its inhibitory activity, but substitution of serine completely abolished it (Raj et al., 1992).

The present study shows that porcine osteopontin is a potent and specific inhibitor of HA formation from metastable calcium phosphate solution. Two strategies were used to identify the structural features of osteopontin responsible for this activity. The first approach was to determine whether enzymic dephosphorylation or chemical modification of carboxylate groups affected the inhibitory activity. As shown above, removal of 84% of the phosphate groups reduced the inhibitory activity of osteopontin by at least 40-fold. Modification of 86% of the carboxylate groups with GEE reduced the inhibitory activity by 6-fold. These findings indicate that both phosphate and carboxylate groups are involved in the HA growth-modulating effect of osteopontin, but that phosphate groups are quantitatively more important. Boskey et al. (1993) found that alkaline phosphatase treatment abolished the HA-inhibiting activity of bovine osteopontin, but the extent of phosphate group removal was apparently not determined. Similarly, dephosphorylation of phosphophoryn reduces or completely abolishes its effects (both positive and negative) on HA formation (Nawrot et al, 1976; Fujisawa et al., 1987; Linde et al., 1989). It has also been shown that modification of carboxylate groups in mollusc shell aspartate-rich proteins with glycine methyl ester caused a reduction in the formation of oriented calcite crystals on polystyrene films (Addadi et al., 1987).

The second strategy for determining the structural motifs important in the inhibition of crystal formation involved the use of model compounds. These were phosvitin, a highly phosphorylated phosphoprotein from egg yolk; BSA, an anionic protein with known calcium- and HA-binding properties; dextran sulphate, a highly sulphated polysaccharide; and the synthetic polypeptides poly(Glu) and poly(Asp). In the titration system, the order of inhibitory potency of these compounds was poly(Asp) > phosvitin > poly(Glu) > BSA > dextran sulphate. As dextran sulphate is a highly anionic polymer, its lack of effect in this system indicates that inhibition of HA formation, at least in the concentration range used here (0.01–250 μg/ml), is not a non-specific polyelectrolyte effect. Phosvitin, which exhibited an intermediate inhibitory potency, has previously been shown to inhibit HA formation in a variety of systems (Termine and Conn, 1976; Hay et al., 1979; Termine et al., 1980; Doi et al. 1992). The complete primary structure of phosvitin has not been determined, but the amino acid composition and sequence data available suggest that it is much more similar to phosphophoryn than to the bone phosphoproteins (Taborsky, 1983).

Inhibition of *de novo* HA formation by osteopontin and model compounds was also demonstrated in a steady-state agarose gel system. The relative order of inhibitory potency was similar to that seen in the titration system: osteopontin $\approx$ poly(Asp) > poly(Glu) $\approx$ phosvitin > BSA = dextran sulphate. Under steady-state conditions, however, far higher concentrations of effector were required for inhibition: at 100 μg/ml, osteopontin and poly(Asp) caused decreases of 23% and 31% respectively, in $Ca+PO_4$ content. This is in agreement with the findings of Boskey et al. (1993), who showed that rat and bovine osteopontin at 100 μg/ml inhibited *de novo* HA formation and seeded HA growth in a steady-state gelatin gel system, whereas lower concentrations had no effect.

Two differences between the findings from the two experimental systems are of note. First, phosvitin, which is approx. 80-fold more potent than poly(Glu) in the titration system, is of similar potency in the steady-state agarose gel system. The reason for this difference is not clear. Secondly, poly(Asp), which is more than 1000-fold more potent than poly(Glu) in the titration system, is only 4-fold more potent in the steady-state system. This difference appears to reflect a diminished inhibitory activity of poly(Asp) under steady-state conditions. Such a conclusion would suggest that poly(Asp) is a better inhibitor of crystal nucleation than of crystal growth. Under steady-state conditions, the supply of lattice ions, and therefore the potential sites of homogeneous nucleation, is essentially infinite, and a finite amount of nucleation inhibitor will only be able to delay, not completely prevent, crystal formation.

The reason for studying poly(Asp) was to evaluate the importance of aspartic acid-rich sequences in the inhibition of HA by osteopontin. Such sequences have been found in the osteopontin of all species studied: in pig, DDVDDDDDED (residues 70–79; Wrana et al., 1989); in rat, DDDDDDDDDGD (residues 70–80; Oldberg et al., 1986); in human, DDMDDEDDDD (residues 72–81; Kiefer et al., 1989); in mouse, DDDDDDDDDDGD (residues 69–80; Miyazaki et al., 1989); and in chicken, DDDDDGDND (residues 83–91; Moore et al., 1991). In the autotitration system, the inhibitory potency of poly(Asp) was similar to that of osteopontin, and more than three orders of magnitude greater than that of poly(Glu). Similar differences between poly(Asp) and poly(Glu) in the inhibition of calcium phosphate precipitation (Hay and Schlesinger, 1977; Hay et al. 1979) and in binding to the HA crystals of polished enamel surfaces (Juriaanse et al., 1980a,b) have previously been reported. We have recently shown that poly(Asp) and poly(Glu) also differ in their abilities to nucleate HA formation at subthreshold calcium phosphate concentrations; in this case, nucleation activity is exhibited by poly(Glu) but not by poly(Asp) (G. K. Hunter and H. A. Goldberg, unpublished work). As aspartic acid and glutamic acid are chemically very similar, such profound differences in crystal binding and modulation of crystal formation seem difficult to explain. However, acidic amino acid homopolymers have been shown to adopt different secondary structures in solution. Poly(Glu) exists as a right-handed α-helix

at acidic pH and as a left-handed 'extended helix' at neutral pH (McDiarmid and Doty, 1966; Tiffany and Krimm, 1969), whereas poly(Asp) appears to lack ordered secondary structure (McDiarmid and Doty, 1966). Therefore the functional differences observed here and by others are probably due to conformational differences between poly(Asp) and poly(Glu).

In conclusion, it can be stated that osteopontin is a very potent inhibitor of *de novo* HA formation, and that this activity involves both phosphate and carboxylate groups. The dephosphorylation and chemical modification studies indicate that phosphate groups are quantitatively more important than carboxylate groups. However, phosvitin contains much more phosphate than osteopontin (27% versus 3%), but is approx. 20-fold less effective an inhibitor. The potent inhibitory effect of poly(Asp) suggests that the conserved aspartic acid-rich region of osteopontin may be involved in the inhibition of *de novo* HA formation. The exact protein sequence and conformation required for optimal inhibition of HA nucleation remain to be determined.

The physiological significance of the osteopontin-mediated inhibition of HA formation is not clear. It is interesting to note that the renal form of osteopontin (uropontin) inhibits the formation of calcium oxalate crystals (Shiraga et al., 1992) and is found as a component of kidney stones (Kohri et al., 1992). The presence of osteopontin in urine, breast milk (Senger et al., 1989) and bile (Brown et al., 1992) suggests that this protein may function to inhibit phase separation in physiological fluids of high supersaturation. In bone, osteopontin is found at high concentrations in the lamina limitans that underlies bone lining cells and in the reversal (cement) lines (McKee et al., 1993). This distribution, and the HA-inhibiting activity shown here and by Boskey et al. (1993), indicate that osteopontin may seal off growing HA surfaces once active bone formation has ceased.

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