Nucleation and inhibition of hydroxyapatite formation by mineralized tissue proteins

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Many proteins found in mineralized tissues have been proposed to function as regulators of the mineralization process, either as nucleators or inhibitors of hydroxyapatite (HA) formation. We have studied the HA-nucleating and HA-inhibiting properties of proteins from bone [osteocalcin (OC), osteopontin (OPN), osteonectin (ON) and bone sialoprotein (BSP)], dentine [phosphoryn (DPP)] and calcified cartilage [chondrocalcin (CC)] over a wide range of concentrations. Nucleation of HA was studied with a steady-state agarose gel system at sub-threshold $[Ca] \times [PO_4]$ product. BSP and DPP exhibited nucleation activity at minimum concentrations of 0.3 $\mu\text{g/ml}$ (9 nM) and 10 $\mu\text{g/ml}$ (67 nM) respectively. OC, OPN, ON and CC all lacked nucleation activity at concentrations up to 100 $\mu\text{g/ml}$. Inhibition of HA

formation *de novo* was studied with calcium phosphate solutions buffered by autotitration. OPN was found to be a potent inhibitor of HA formation [$IC_{50} = 0.32 \mu\text{g/ml}$ (0.01 μM)] whereas OC was of lower potency [$IC_{50} = 6.1 \mu\text{g/ml}$ (1.1 μM)]; BSP, ON and CC all lacked inhibitory activity at concentrations up to 10 $\mu\text{g/ml}$. The effect of OPN on HA formation *de novo* is mainly to inhibit crystal growth, whereas OC delays nucleation. These findings are consistent with the view that BSP and DPP may play roles in the initiation of mineralization in bone and dentine respectively. OPN seems to be the mineralized tissue protein most likely to function in the inhibition of HA formation, possibly by preventing phase separation in tissue fluids of high supersaturation.

INTRODUCTION

The mineralized tissues of vertebrates include bone, dentine and calcified cartilage. All three tissues have hydroxyapatite (HA; $Ca_{10}[PO_4]_6[OH]_2$) as their mineral component, but each has a different organic matrix composition. Numerous proteins isolated from these tissues have been proposed to function in the mineralization process, either as nucleators or inhibitors of HA formation.

The extracellular matrix of bone is principally composed of Type I collagen. Bone also contains large amounts of some serum proteins, including albumin and α_2 -HS-glycoprotein, that accumulate in bone because of their affinity for HA. Among the proteins synthesized by bone cells are osteonectin (ON), osteopontin (OPN), osteocalcin (OC) and bone sialoprotein (BSP), all of which are acidic proteins that have Ca- and HA-binding properties [1]. ON (also known as SPARC: secreted protein, acidic, rich in cysteine) is a glycoprotein that is present at high concentration in bone [2]. OC (also known as bone Gla protein, or BGP) is a protein of 49 residues that contains two or three residues of γ -carboxyglutamic acid (Gla) [3]. OPN and BSP are both phosphorylated sialoproteins rich in acidic amino acids and containing an RGD cell-attachment sequence [4,5]. OPN has a higher proportion of aspartic acid residues, some of which occur contiguously to form a 'polyaspartate' site, whereas BSP has more glutamic acid residues, including two or three 'polyglutamate' sites [6,7]. Of these osteoblast-derived proteins, only OC and BSP are specific to mineralized tissues [8,9].

Like bone, the extracellular matrix of dentine also has Type I collagen as its major constituent. However, certain non-collagenous proteins found at high concentrations in bone, such as BSP and OPN, are much less abundant in dentine [10]. Conversely, dentine contains some proteins that have been found in

no other tissues. One of these is dentine phosphophoryn (DPP), a 150 kDa protein containing approx. 40% aspartic acid and 50% serine [11]. Most of the serine residues are phosphorylated, resulting in a very high negative charge density.

The extracellular matrix of non-calcified cartilage consists principally of Type II collagen and the large aggregating proteoglycan aggrecan. Calcifying cartilage, however, undergoes a matrix remodelling process characterized by the accumulation of the C-terminal propeptide of Type II collagen [also known as chondrocalcin (CC)] [12,13] and the expression of certain extracellular matrix proteins including OC, OPN, ON and BSP [9,14,15].

In previous studies, we have shown that porcine BSP is a potent nucleator of HA in a steady-state agarose gel system [16]. This activity is associated with the poly(glutamic acid) sequences [17,18]. Porcine OPN, in contrast, is a potent inhibitor of HA formation *de novo* in solution [19]. The inhibitory activity seems to involve both the poly(aspartic acid) sequence and phosphate groups. In the present study we have analysed a variety of mineralized tissue proteins (OC, ON, OPN, BSP, DPP and CC) over a wide range of protein concentrations for HA-nucleating activity in the steady-state agarose gel system and for HA-inhibiting activity in calcium phosphate solutions.

EXPERIMENTAL

Purification and characterization of mineralized tissue proteins

Mineral-associated proteins were extracted from guanidine-treated adult pig calvaria with 0.5 M EDTA [20]. BSP, OPN and ON were purified from the EDTA extract by FPLC as previously described [20–22].

DPP was extracted from dissected pig coronal dentine with 0.5 M EDTA [20] and purified by a combination of the

Abbreviations used: BSP, bone sialoprotein; CC, chondrocalcin; DPP, dentine phosphophoryn; Gla, γ -carboxyglutamic acid; HA, hydroxyapatite; OC, osteocalcin; ON, osteonectin; OPN, osteopontin.

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Table 1 Amino acid compositions of mineralized tissue proteins

Amino acid compositions (residues/1000) measured in the present study (M) are compared with previously reported (R) compositions. For BSP, OPN and OC the reported compositions are based on cDNA sequences. For ON, DPP and CC the reported compositions are based on amino acid analysis. Note that the reported DPP data are from the bovine protein, whereas the DPP used in this study was porcine. Abbreviation: n.d., not determined.

	BSP		OPN		OC		ON		DPP		CC	
	(M)	(R) [45]	(M)	(R) [22]	(M)	(R) [47]	(M)	(R) [21]	(M)	(R) [46]	(M)	(R) [26]
Asx	154	150	183	170	108	102	108	124	386	381	163	127
Thr	75	77	81	80	0	0	28	49	0	11	74	51
Ser	71	93	111	139	42	41	34	26	421	421	74	136
Glx	241	213	209	174	193	184	176	179	61	33	128	100
Pro	59	53	41	52	106	102	83	66	9	11	65	42
Gly	122	117	40	24	88	82	63	52	40	45	97	118
Ala	49	47	50	49	125	122	61	53	18	14	74	72
Cys	n.d.	0	n.d.	0	n.d.	41	n.d.	35	n.d.	2	n.d.	26
Val	24	23	57	59	64	61	67	65	0	9	44	39
Met	n.d.	3	n.d.	0	n.d.	0	n.d.	11	n.d.	2	n.d.	9
Ile	16	17	15	17	20	20	37	38	1	3	55	43
Leu	38	33	62	63	63	61	96	93	3	7	89	66
Tyr	52	70	10	14	52	61	22	26	0	0	23	23
Phe	12	17	13	17	46	41	46	44	0	3	37	27
His	11	17	35	38	24	20	37	54	1	5	11	25
Lys	51	43	58	66	5	0	62	64	60	52	18	67
Arg	24	27	34	35	63	61	35	31	0	1	43	32
Trp	n.d.	0	n.d.	0	n.d.	0	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.

methods of Dimuzio and Veis [23] and Butler et al. [24]. Briefly, EDTA-extracted proteins were fractionated by ion-exchange chromatography on Q-Sepharose. DPP-containing fractions were precipitated with 1 M CaCl_2 . The precipitated protein was fractionated sequentially by gel filtration (Superdex 200PG) and ion-exchange chromatography (Mono-Q).

OC was isolated from chicken bone as previously described [25]. CC was isolated from bovine cartilage as previously described [26].

Aliquots of all proteins were hydrolysed in 6 M HCl at 118 °C for 18 h and analysed with a Beckman 119CL single-column amino acid analyser. The compositions obtained are shown in Table 1. These amino acid analyses were used to calculate the polypeptide concentrations given below.

Assay for nucleation of HA

Nucleation activity was assayed with a modification of the steady-state agarose gel system previously described [16,17]. In brief, mineralized tissue proteins were added at various concentrations (0.3–100 $\mu\text{g/ml}$) to solutions containing 1% (w/v) agarose, 150 mM NaCl, 20 mM Hepes, pH 7.4, and 0.01% NaN_3 . Approx. 1 ml aliquots of these solutions were then poured into the central cavities of modified equilibrium dialysis cells and allowed to gel. On both sides of the gels were placed squares of dialysis membrane (SpectraPor-3, 3500 Da nominal molecular mass cut-off). A group of cells (normally 12–16) was then connected in series with polyethylene tubing. Each series normally included gels containing three or four different concentrations of protein in triplicate and triplicate negative controls (no added protein). Each series also included, as a positive control, one gel containing 10 $\mu\text{g/ml}$ poly(L-glutamic acid) (83 kDa, P4886; Sigma Chemical Co.). Poly(L-glutamic acid) is a known nucleator of HA in this system [17].

Reservoir solutions were prepared containing 150 mM NaCl, 20 mM Hepes, pH 7.4, and 0.01% NaN_3 , and either 6.5 mM CaCl_2 or 3.9 mM sodium phosphate, pH 7.4. The pH of the

reservoir solutions was adjusted to pH 7.4 immediately before final volume correction. The calcium- and phosphate-containing solutions were perfused through the cells on opposite sides of the agarose gels, and from opposite ends of the series of cells, at a flow rate of 1 ml/h per cell without recirculation. The entire apparatus was incubated at 37 °C for 5 days.

At the end of the 5-day incubation period, gels were harvested, ashed and analysed for Ca and phosphate as previously described [16]. Results are expressed as Ca + PO_4 content per gel. Statistical analysis by the Dunnett multiple correlations test was performed on the entire data set, using the pooled negative control data.

Assay for inhibition of HA formation *de novo*

The inhibition of HA formation *de novo* was quantified by a modification of the method previously described [19]. Aliquots (8 ml) of a filtered solution containing 2.0 mM CaCl_2 , 1.20 mM sodium phosphate and 150 mM NaCl, plus mineralized tissue proteins in the concentration range 0.01–10 $\mu\text{g/ml}$, were placed in double-walled glass titration vessels attached to Mettler DL-21 autotitrators programmed to maintain pH 7.4 by the addition of 50 mM NaOH. Water was circulated through the titration vessels from a water bath at 37 °C. After temperature equilibration, the pH of the calcium phosphate solution was adjusted to 7.4–7.5, using a gel-filled epoxy electrode. Titration was then performed for a period of 1000 min. The volume of NaOH added after 1000 min (V_{1000}) was plotted against concentration of test substance to give a curve from which the concentration causing half-maximal inhibition of HA formation (IC_{50}) was calculated [19].

RESULTS

Purification and characterization of mineralized tissue proteins

The proteins used in this study were BSP, OPN and ON from pig bone, OC from chicken bone, DPP from pig dentine and CC from bovine cartilage. In Table 1 are listed the measured (M)

Table 2 Effects of mineralized tissue proteins on nucleation of hydroxyapatite in steady-state agarose gels

Results shown are Ca + PO₄ contents in mg per gel, means ± standard deviation ($n = 3$ except for pooled negative controls, where $n = 16$). Significance levels compared with pooled negative control data, by Dunnett's multiple correlations test: * $P < 0.05$; ** $P < 0.01$. The pooled positive control (poly-L-Glu, 10 $\mu\text{g/ml}$) value was 0.942 ± 0.056 ($n = 5$). Abbreviation: n.d., not determined.

Concn. ($\mu\text{g/ml}$)	BSP	DPP	CC	OC	OPN	ON
0	0.260 ± 0.034					
0.3	$0.585 \pm 0.32^{**}$	n.d.	n.d.	n.d.	n.d.	n.d.
1	$0.890 \pm 0.25^{**}$	0.263 ± 0.04	0.273 ± 0.02	0.237 ± 0.01	0.285 ± 0.02	0.246 ± 0.03
3	$1.31 \pm 0.261^{**}$	0.300 ± 0.02	n.d.	n.d.	n.d.	n.d.
10	$1.33 \pm 0.043^{**}$	$0.458 \pm 0.24^*$	0.275 ± 0.01	0.280 ± 0.01	0.276 ± 0.02	0.267 ± 0.06
30	n.d.	$0.871 \pm 0.01^{**}$	n.d.	n.d.	n.d.	n.d.
100	n.d.	n.d.	0.313 ± 0.02	0.292 ± 0.01	0.296 ± 0.03	0.272 ± 0.01

amino acid compositions of the protein preparations used in the present study and, for comparison, the amino acid compositions previously reported (R) for these proteins. The amino acid analyses given in Table 1 were also used to calculate the protein concentrations given below. Therefore these concentrations relate to the polypeptide rather than to the fully modified, post-translationally processed protein.

Effects of mineralized tissue proteins on the nucleation of HA in steady-state agarose gels

The nucleation of HA was assayed with a modification of the method previously described [16,17]. A higher buffer concentration and an additional pH adjustment step were used to ensure better control of pH. Also, a Ca/P ratio of 1.67 (similar to that in HA) and perfusion of the calcium- and phosphate-containing reservoir solutions from opposite ends of the cell series were used to minimize differences due to position in the series.

To determine whether the mineralized tissue proteins of interest had the ability to induce the formation of HA *in vitro*, various concentrations of each protein were incorporated into steady-state agarose gels containing calcium and phosphate concentrations below the threshold for spontaneous precipitation of HA (see Table 2). BSP caused a significant increase in Ca + PO₄ content even at the lowest concentration studied, 0.3 $\mu\text{g/ml}$. This nucleation effect of BSP appears to be maximal at 3 $\mu\text{g/ml}$, in agreement with previous studies [16]. DPP also caused a dose-

dependent increase in Ca + PO₄ content, reaching statistically significant levels at 10 $\mu\text{g/ml}$. Note that considerable variation in Ca + PO₄ content occurs at intermediate BSP and DPP concentrations (Table 2); this is due to precipitation occurring in only one or two of the three replicate gels, suggesting that nucleation in this system is generally an 'all-or-none' phenomenon. The relative nucleation potencies of BSP and DPP in weight and molar terms are expressed in Table 3 as the minimum protein concentration necessary to give a significant increase in Ca + PO₄. BSP is approx. 30-fold more potent in weight terms, and approx. 7-fold more potent in molar terms, than DPP.

At concentrations from 1 to 100 $\mu\text{g/ml}$, OPN, OC, ON and CC had no significant effect on Ca + PO₄ content (Table 2). In all cases, however, the Ca + PO₄ content at 100 $\mu\text{g/ml}$ was higher than at lower protein concentrations. This seems to be largely due to sequestration of calcium in gels containing high concentrations of these proteins, each of which has been shown to bind Ca²⁺ ions ([27–29]; P. V. Hauschka and L. C. Rosenberg, unpublished work). The lack of observed nucleation by OC, OPN, ON and CC indicates that these proteins are 1/300 as effective (or less) as BSP in weight terms, and 1/280–1/2000 as effective (or less) in molar terms, in inducing HA formation (Table 3).

The CC used in this study was prepared in 4 M guanidine/HCl, a potent denaturing agent. CC-containing gels were perfused with Ca- and phosphate-free reservoir buffer to remove the guanidine/HCl and renature the protein. As a positive control in this experiment, BSP was treated with 4 M guanidine/HCl before being incorporated into gels. Guanidine/HCl treatment did not

Table 3 Constants for nucleation and inhibition of HA formation *de novo* by mineralized tissue proteins

Values were calculated from the data shown in Table 2 and Figure 2. IC₅₀ is the concentration of effector required for 50% inhibition of HA formation. C_{min} is the minimum concentration of effector to give significant elevation of Ca + PO₄ content. Abbreviation: n.d., not determined.

Protein	Polypeptide molecular mass (Da)	Reference	Inhibition			Nucleation	
			Hill Coeff.	IC ₅₀ ($\mu\text{g/ml}$)	IC ₅₀ (μM)	C _{min} ($\mu\text{g/ml}$)	C _{min} (μM)
BSP	33 000	[45]	–	> 10	> 0.3	0.3	0.009
OPN	32 000	[22]	–1.3	0.32	0.01	> 100	> 3.1
OC	5500	[48]	–9.9	6.1	1.1	> 100	> 18
ON	29 000	[29]	–	> 8	> 0.28	> 100	> 3.4
CC	39 000	[49]	–	> 10	> 0.26	> 100	> 2.6
DPP	150 000	[11]	n.d.	n.d.	n.d.	10	0.067

Figure 1 Inhibition of HA formation *de novo* by mineralized tissue proteins

The volume of 0.05 M NaOH added to calcium phosphate solutions at 1000 min (V_{1000}) is plotted against the logarithm of protein concentration. Curves shown are sigmoid dose–response (variable-slope), and straight lines are linear regression; both were generated by the Prism graphics package (GraphPad). (A) OPN (○) and OC (△); (B) ON; (C) CC; (D) BSP.

Figure 2 Representative titration curves for HA formation in the presence of (A) OPN and (B) OC

OPN or OC was added to calcium phosphate solutions buffered by autotitration and incubated for 1000 min. The numbers shown beside the titration curves are the protein concentrations added, in $\mu\text{g/ml}$.

affect the nucleation activity of BSP (result not shown). However, the possibility cannot be excluded that a nucleation activity associated with CC is lost through irreversible denaturation.

Effects of mineralized tissue proteins on HA formation *de novo* in solution

Inhibition of HA formation *de novo* was measured by a modification of the autotitration method previously described [19]. In previous studies it was observed that conventional pH electrodes leak significant amounts of KCl into the calcium phosphate solutions during a 1000 min incubation period. This suppresses HA formation by dilution of the calcium phosphate and by increasing the ionic strength. To prevent this effect, gel-filled pH electrodes were used in the present study. This change allowed the use of a lower $[\text{Ca}] \times [\text{PO}_4]$ product than that used previously [19].

HA formation was followed by autotitration for a period of 1000 min. Control solutions exhibited a sigmoidal titration curve that reached a plateau value by 1000 min. To determine their relative HA-inhibiting potencies, OPN, OC, BSP, CC and ON, in the range 0.01–10 $\mu\text{g/ml}$, were added. The volume of 0.05 M NaOH added at 1000 min (V_{1000}) is plotted against protein concentration in Figure 1. OPN and OC caused a dose-dependent decrease in V_{1000} , whereas BSP, CC and ON had no discernible effect. From the results shown in Figure 1, IC_{50} values were calculated; these are shown in Table 3.

OPN caused inhibition of HA formation at much lower concentrations than OC, which is reflected in its lower IC_{50} (0.32 $\mu\text{g/ml}$ compared with 6.1 $\mu\text{g/ml}$). Expressed in molar units, this difference in inhibitory potency is even more pronounced (0.01 μM compared with 1.1 μM) (Table 3). However, the

inhibition curve for OC was much steeper than that for OPN, as shown by the Hill coefficient (-9.9 for OC compared with -1.3 for OPN) (Table 3). The reason for this difference can be seen from the titration curves plotted in Figure 2. With increasing concentrations of OPN the titration curve becomes flatter, so that the final amount of NaOH added is decreased (Figure 2A). However, increasing concentrations of OC cause the titration curve to shift to the right, with little effect on the shape of the curve (Figure 2B). Therefore the main effect of OPN is on the final amount of precipitate formed, with little effect upon the nucleation lag time, whereas OC mainly affects the lag time with little effect upon the final amount of HA formed. Under the experimental conditions used, therefore, OC has an 'all-or-none' effect on HA formation, depending whether or not precipitation occurs before or after the arbitrary 1000 min end-point, resulting in the very steep inhibition curve shown in Figure 1(A).

DISCUSSION

Numerous studies with a variety of different experimental systems have attempted to determine the effects of mineralized tissue proteins on HA formation *in vitro*. The most exhaustively studied of these proteins is DPP. DPP has been shown to inhibit the formation of HA *de novo* in solution [30] and agarose gels [31], and the growth of HA seed crystals [32]. In a gelatin gel system, however, HA formation *de novo* was decreased by high concentrations (100 $\mu\text{g/ml}$) of DPP but increased by low concentrations (0.01–1 $\mu\text{g/ml}$) [33]. HA formation was also promoted by DPP covalently attached to agarose beads [34–36] and collagen sheets [37].

In general it appears that the formation of HA is inhibited by soluble DPP but promoted by immobilized DPP. Similar considerations seem to apply to other mineralized tissue proteins. OC inhibits the seeded growth [38–40] and formation *de novo* [30,41,42] of HA. When attached to agarose beads, however, OC either promoted [35] or had no effect on [36] HA formation *de novo*. ON also inhibited the seeded growth [29,38,40] and formation *de novo* [30] of HA. Attachment of ON to agarose beads resulted in no effect on HA formation *de novo* [36]. Attachment to collagen, however, has been reported both to promote [2] and inhibit [43] HA formation.

Less information is available on the other mineralized tissue proteins studied here. OPN has been shown to inhibit HA formation *de novo* in gelatin gel [44], agarose gel [19] and solution [19] systems. BSP has been shown to nucleate HA in the steady-state agarose gel system [16]. No studies on the effect of CC on HA formation have been reported.

It should be clear from the foregoing discussion that the diversity of experimental systems used makes any overall conclusions very difficult to find. Clearly, an understanding of the role of mineralized tissue proteins in the initiation and/or inhibition of HA formation would be facilitated by the use of well-characterized protein preparations and experimental systems. The present study has used a well-established steady-state agarose gel system to study HA nucleation. In this system, the $[\text{Ca}] \times [\text{PO}_4]$ product is below the threshold for spontaneous precipitation. This is an important distinction, because a nucleating agent might not increase HA formation when the $[\text{Ca}] \times [\text{PO}_4]$ product is above the spontaneous precipitation threshold. We have recently shown that high concentrations of poly(L-glutamic acid) nucleate HA under sub-threshold conditions but inhibit HA formation under super-threshold conditions (G. K. Hunter, unpublished work).

To study the inhibition of HA formation, we have used formation *de novo* rather than seeded growth of HA. The

rationale here is that a physiological inhibitor of calcification should act primarily to prevent the generation of crystal nuclei. Again, it is not obvious that an inhibitor will have even qualitatively similar activities against both crystal nucleation and crystal growth.

The results obtained from the steady-state agarose gel system indicate that two of the proteins studied, BSP and DPP, have significant HA-nucleating activity in the concentration range studied. The minimum concentration of BSP required for significant increase in $\text{Ca} + \text{PO}_4$ content was 0.3 $\mu\text{g/ml}$ (approx. 9 nM, based on a polypeptide molecular mass of 33 kDa [45]). With DPP, 10 $\mu\text{g/ml}$ (approx. 67 nM, based on a molecular mass of 150 kDa [11]) was required for significant elevation of $\text{Ca} + \text{PO}_4$ content. Therefore DPP is approx. 1/30 as potent as BSP in weight terms, and approx. one-seventh as potent on a molar basis. The other four proteins studied (OPN, OC, ON and CC) had no nucleation activity at concentrations as high as 100 $\mu\text{g/ml}$. The lack of nucleation by OPN is particularly noteworthy, as the structure of this protein is in many ways very similar to that of BSP. However, the HA-nucleating activity of BSP apparently involves poly(glutamic acid) sequences [17], a structural motif that is absent from OPN. The observation that BSP is at least 300-fold more potent than OPN in the nucleation of HA therefore supports the view that poly(glutamic acid) sequences are required for nucleation.

Only very limited sequence information is available for DPP, a protein that has so far defied genetic analysis. Based on its amino acid composition, however, it seems very unlikely that DPP contains a poly(glutamic acid) sequence. It does contain poly(aspartic acid) sequences and phosphoserine residues [46], structural features that have been correlated with the inhibition of HA formation by OPN [19]. Therefore DPP seems to contain a HA-nucleating 'site' that is distinct from and weaker than that in BSP.

With calcium phosphate solutions buffered by autotitration, the abilities of five mineralized tissue proteins to inhibit HA formation *de novo* were also studied. At concentrations up to 10 $\mu\text{g/ml}$, BSP, ON and CC had no effect on HA formation. Over the same concentration range, OPN and OC both exhibited inhibitory activity. Plotting the amount of HA formed at 1000 min against protein concentration allowed IC_{50} values to be calculated. By this criterion, OPN was approx. 20-fold more potent than OC in weight terms, and approx. 100-fold more potent on a molar basis (Table 3).

However, the mechanisms by which OPN and OC inhibit HA formation seem to be quite different. From the shapes of the titration curves in the presence of various concentrations of OPN and OC, it seems that OPN mainly inhibits growth of HA crystals, whereas OC mainly delays nucleation. This finding suggests that these proteins might interact with HA crystals and crystal nuclei in different ways. The inhibition of HA formation *de novo* by OPN has been shown to involve carboxylate and phosphate groups [19], whereas the dicarboxylate groups of OC Gla residues are important for the inhibition of HA growth [40].

Previous observations that OC inhibits both formation *de novo* [30] and seeded growth [38–40] of HA suggest that this protein inhibits both the nucleation and growth processes. In contrast, our findings indicate an effect only on nucleation. The previous studies cited also demonstrated inhibition of HA nucleation and growth by ON, which had no effect in the present study. It should be noted, however, that ON was more effective than OC in inhibiting seeded growth [38,40], whereas OC was more effective than ON in inhibiting formation *de novo* [30]. Taken together, these studies are consistent with the present findings in suggesting that the effect of OC on HA formation is mainly to

delay nucleation. The lack of effect of ON observed in the present study is presumably because the protein concentrations used were too low to produce an effect on formation *de novo*.

In conclusion, proteins of mineralized tissues differ greatly in the ability to modulate HA formation *in vitro*. The HA-nucleating activities of BSP and DPP suggest that these are the proteins most likely to be involved in the initiation of mineralization in bone and dentine respectively. However, the differences in structure and potency between BSP and DPP indicate that these proteins might have different nucleating sites. Inhibition of HA formation might also occur by more than one mechanism. OPN and OC were the only proteins to exhibit inhibition of formation *de novo* under the conditions of the present study. OPN seems to act principally on crystal growth, whereas OC seems to act principally on nucleation. The observed effects of mineralized tissue proteins on nucleation and inhibition of HA formation suggest that proteins can modulate biological crystal formation in a variety of ways.

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