Cross-linking of Osteopontin by Tissue Transglutaminase Increases Its Collagen Binding Properties*

(Mari T. Kaartinen‡, Arja Pirhonen, Annikka Linnala-Kankkunen, and Pekka H. Miinppää
From the Department of Biochemistry and Biotechnology, University of Kuopio, FIN-70210 Kuopio, Finland)

Osteopontin, a major noncollagenous bone protein, is an in vitro and in vivo substrate of tissue transglutaminase, which catalyzes formation of cross-linked protein aggregates. The roles of the enzyme and the polymeric osteopontin are presently not fully understood. In this study we provide evidence that transglutaminase treatment significantly increases the binding of osteopontin to collagen. This was tested with an enzyme-linked immunosorbent assay. The results also show that this increased interaction is clearly calcium-dependent and specific to osteopontin. In dot blot overlay assay 1 pg of collagen type I was able to bind 420 ng of in vitro prepared and purified polymeric osteopontin and only 83 ng of monomeric osteopontin, indicating that the transglutaminase treatment introduces a 5-fold amount of osteopontin onto collagen. Assays using a reversed situation showed that the collagen binding of the polymeric form of osteopontin appears to be dependent on its conformation in solution. Circular dichroism analysis of monomeric and polymeric osteopontin indicated that transglutaminase treatment induces a conformational change in osteopontin, probably exposing motives relevant to its interactions with other extracellular molecules. This altered collagen binding property of osteopontin may have relevance to its biological functions in tissue repair, bone remodeling, and collagen fibrillogenesis.

Tissue transglutaminase (TG)1 (EC 2.3.2.13) is a widely distributed intra- and extracellular calcium-dependent enzyme, which catalyzes the formation of high molecular mass complexes of its substrate proteins by creating isopeptide cross-links from glutamine and lysine residues and releasing ammonium (1, 2). TG is suggested to be involved in matrix maturation and stabilize the tissue with cross-links that are resistant to normal proteolysis (1, 2). TG is closely related to wound healing which suggests a role for it in tissue remodeling and repair (3, 4). Immunohistochemical data have also demonstrated the presence of TG in mineralizing cartilage and bone (5, 6) and the enzyme is thought to participate in matrix cross-linking before the tissue undergoes calcification (5, 6). The number of proteins serving as glutaminyl substrates for TG is restricted indicating the physiological importance of its functions (1). The roles of TG and the actions of its enzymatic products, meaning high molecular weight proteins, are still unclear.

Osteopontin (OPN), a prominent and potentially multifunctional acidic phosphoglycoprotein (7, 8), is a substrate of TG (9–11). OPN is a major product of bone forming cells, osteoblasts, but is not specific to bone. It is also synthesized in other types of tissues and found in, e.g. inner ear, brain, kidney (7), and atherosclerotic plaques (13, 14), and it is also secreted into milk (12) and urine (15). Its production is also related to immunity, infection, and cancer (8). Osteoblasts express OPN at an early developmental stage of bone formation (16, 17). In bone, OPN is deposited into unmineralized matrix prior to calcification and thereon localized at various tissue interfaces, e.g. cement lines, lamina limitans, and between collagen fibrils of fully matured hard tissues (18). Recent knock-out mice experiments by Liaw et al. (19) indicate that OPN, more specifically, functions in tissue repair, matrix organization, and collagen fibrillogenesis.

The role of polymeric OPN, resulting from cross-linking by TG, is unknown. We have previously demonstrated that osteocalcin inhibits TG activity in vitro as measured by cross-linking of osteopontin (20). Since recent gene knock-out experiments have demonstrated that osteocalcin is an inhibitor of bone formation (21), our results suggest that TG activity and the OPN aggregates may be involved in enhancement of biomineralization or matrix maturation that precedes it. In this study the collagen binding properties of polymeric and monomeric OPN were investigated since this feature could be pivotal for the maturation and organization of the bone matrix as well as for the mineralization event. The collagen types examined were the fiber forming collagens, types I and II, III and V, which are synthesized in, e.g. bone, cartilage, and vascular smooth muscle cells (22), and type IV, which is a basement membrane collagen (23). We provide evidence that OPN, as a high molecular weight complex form, exhibits significantly increased binding ability to all tested collagens. This may result from an altered conformation of the OPN after polymerization as observed by circular dichroism measurements. A more stable structure and amplified collagen binding property, after treating OPN with an enzyme that is intimately involved in tissue repair, brings further support to OPN’s role as a tissue remodeling protein and gives an insight into the functions of the polymeric OPN.

EXPERIMENTAL PROCEDURES

Materials—OPN antibody was a gift from Dr. E. Sørensen (University of Aarhus, Denmark). TG antibody was generously donated by Dr. D. Aeschlimann (University of Wisconsin). Anti-collagen type I, anti-rabbit IgG alkaline phosphatase conjugate, guinea pig tissue transglutaminase.
taminase (specific activity; 2 units/mg), collagen types I (from calf skin), II (from bovine tracheal cartilage), III (from human placenta), IV (from human placenta), V (from human placenta), bovine serum albumin, 5-bromo-4-chloro-3-indolyphosphate/nitro blue tetrazolium, and FAST p-nitrophenyl phosphate alkaline phosphatase substrate were purchased from Roche Molecular Biochemicals (Indianapolis, IN). Casein and anti-casein IgG were from ANAWA Trading SA (Wangen, Switzerland). 125I-Radionuclide was obtained from NEN Life Science Products, Inc. (Boston, MA). IODO-BEADS™ were from Pierce, Inc.. The membrane blocking reagent from digoxigenin luminescent detection kit for nucleic acids was from Roche-Mannheim GmbH (Mannheim, Germany). PD-10 desalting columns were from Bio-Rad and polyvinylidene difluoride Immobilon P membranes from Millipore (Bedford, MA). Centrisul™ concentrates were purchased from Amicon Inc. (Beverly, MA). Nunc-Immuno™ microtiter plates with MaxiSorb surface were from Nalge Nunc International (Naperville, IL). Instaview Universal protein stain was from BDH Laboratory Supplies (Poole, United Kingdom).

**Instrumentation—**Chromatography was performed with Pharmacia LKB GradiFrac chromatography system and Pharmacia LKB FPLC instrument. HPLC purifications were done with Hewlett-Packard 1050 HPLC system. Protein detection was performed with Applied Biosystems 477 A sequencer, amino acid analysis with LKB 4151 Alpha Plus amino acid analyzer, and mass spectrometry with Bruker Biflex MALDI-TOF mass spectrometer. Circular dichroism was measured with JASCO J-720 spectropolarimeter (Institute of Biotechnology, University of Helsinki, Finland).

**Preparation and Purification of High Molecular Weight Osteopontin—**OPN (1 mg) was treated with TG (enzyme:substrate ratio, 1:2.5 (w/w)) for 24 h at 37 °C. The 2-mL reaction mixture contained 50 mM Tris-HCl, pH 8.0, 2.5 mM CaCl2, and 1 mM dithiothreitol. After incubation the reaction mixture was subjected to desalting using PD-10 column and 20 mM Tris-HCl, pH 8.0, as an eluent. The elution was monitored by measuring the absorbance at 280 nm. The selected fractions containing OPN were pooled and concentrated with a microconcentrator having a molecular mass cut-off of 10 kDa. The homogeneity of OPN was confirmed by amino acid analysis, N-terminal sequencing, SDS-PAGE, and MALDI-TOF mass spectrometry.

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**Radiiodination of Proteins—**Osteopontin, purified polymeric osteopontin and collagen type I were radiolabeled with 125I using IODO-BEADS™ following the protocol provided by the manufacturer. Shortly, 20 μg of each protein was labeled with 2 mCi of 125I (100 μCi/μg of protein). The labeling was performed in 0.1 Tris-HCl, pH 6.5, for 30–40 min. Free 125I was removed from the reaction mixture by Sephadex G-25 chromatography using a PD-10 column. Monomeric and polymeric osteopontin were eluted with 5 mM NaHCO3, pH 8.0, and collagen type I with 50 mM Tris-HCl, pH 6.5. Radiolabeling was confirmed by running the labeled proteins in 8.5% SDS-PAGE followed by overnight autoradiography of the gel.

**Cross-linking of Osteopontin and Casein with Increasing Amounts of Tissue Transglutaminase—**Cross-linking reactions were conducted in a total volume of 100 μl of 50 mM Tris-HCl, pH 8.0, containing 2.5 mM CaCl2 and 1 mM dithiothreitol. Reaction mixtures contained 2.5 μg of OPN or casein and 5, 10, 20, 50, 100, 500, and 1000 ng of the enzyme, yielding increasing amounts of cross-linked substrates of the substrates. The reaction mixture was incubated at 37 °C for 2 h. The reactions were terminated with 0.25% EDTA before applying to ELISA plates. OPN cross-linking reactions that were analyzed by Western blotting were terminated by lyophilization. Western blotting was performed as described previously (20). Casein cross-linking was analyzed by 12% SDS-PAGE, each sample containing 20 μg of casein.

Collagen Binding ELISA—Collagen types I, II, III, IV, and V, originally in 10 mM acetic acid, were neutralized by diluting them in 50 mM Na2CO3/NaHCO3, pH 9.7, buffer and then applied to microtiter wells in a 10 μg/ml concentration (1 μg/well). The plates were incubated with proteins overnight at 4 °C. Wells were washed with 0.1% Tween 20 in 50 mM Tris-buffed saline, pH 8.0, and blocked with 1% BSA in the washing buffer. After washing, the wells were subjected to a treatment with anti-rabbit IgG alkaline phosphatase conjugate (for OPN) or anti-ovine IgG alkaline phosphatase conjugate (for casein). The bound antibody was detected with FAST p-nitrophenyl phosphate alkaline phosphatase substrate, following instructions of the manufacturer. The absorbance values were measured at 405 nm. Negative control experiments included binding of OPN (or casein) to BSA, binding of TG-treated OPN (or casein) to BSA (background) and to plastic and experiments without OPN (or casein), i.e. studying recognition of TG by OPN (or casein) antibody.

To test the binding of soluble collagen type V to immobilized OPNs, the wells were coated with 1 μg of monomeric and purified polymeric OPN (or casein) to BSA (background) and to plastic and experiments without OPN (or casein), i.e. studying recognition of TG by OPN (or casein) antibody.

**RESULTS**

**Cross-linking of Osteopontin and Casein with Increasing Amounts of Transglutaminase—**To study the collagen binding properties of high molecular weight OPN, we gradually increased the state of OPN cross-linking by treating it with increasing amounts of TG. In the reaction series the amounts of TG corresponded to ratios of 1:500, 1:250, 1:125, 1:50, 1:25, 1:5, and 1:2.5 (w/w of TG protein to OPN protein). Fig. 1A of the Western blotted reaction series shows that the OPN band above 120 kDa increases as the amount of cross-linking enzyme increases in the mixture. The 2-h treatment did not cross-link...
all available monomeric OPN since this form was still present in the reaction mixture. According to the molecular weight markers, the high molecular weight OPN is a heterogenous mixture of OPN complexes with molecular masses ranging from 120 to over 250 kDa. However, the migratory properties of the monomeric OPN in SDS-PAGE analysis are altered and the electrophoretic markers are indicated on the left margin. Lane 1, OPN; lanes 2-8, OPN cross-linked with 5, 10, 20, 50, 100, 500, and 1000 ng of tissue TG, respectively. Molecular weight markers are shown on the left.

Fig. 1. Osteopontin and casein cross-linked with increasing amounts of tissue transglutaminase. A bovine OPN (2.5 μg) was incubated with tissue TG for 2 h at 37 °C. Reaction products were resolved on 8.5% SDS-PAGE, electroblotted onto a polyvinylidene difluoride membrane, and stained with a polyclonal antibody against bovine OPN. Lane 1, OPN; lanes 2–8, OPN cross-linked with 5, 10, 20, 50, 100, 500, and 1000 ng of tissue TG, respectively. Molecular weight markers are shown on the left margin. B, casein was cross-linked with TG for 2 h at 37 °C and reaction products were analyzed by 12% SDS-PAGE. Lane 1, casein; lanes 2–8, OPN cross-linked with 5, 10, 20, 50, 100, 500, and 1000 ng of tissue TG, respectively. Molecular weight markers are shown on the left.

In Vitro Produced Polymeric Osteopontin—For further studies we cross-linked OPN with TG and purified the reaction products by Mono-Q anion exchange chromatography. The chromatogram is illustrated in Fig. 2A and the electrophoretic analysis of the collected peaks, including their Western analysis with OPN and TG antibodies in Fig. 2B. Polymers were found in two fractions, peaks B and C. SDS-PAGE and Western analyses with OPN antibody showed that peak C contains more higher molecular mass OPN polymers (Fig. 2B, lane 8), is more homogenous, and contains less impurities (Fig. 2B, lane 7) than the polymer in peak B (Fig. 2B, lane 4). Further Western analysis showed slight staining of the polymer in peak C with the TG antibody indicating the presence of TG-TG or TG-OPN cross-linked complexes in the preparation (Fig. 2B, lane 9). The polymer preparation from peak C was used in further studies.

**FIG. 2.** Purification of polymeric osteopontin with Mono-Q anion exchange chromatography. OPN was cross-linked with TG (1:2.5 w/w) TG to OPN) at 37 °C overnight. After concentrating and desalting, the protein mixture was adsorbed into a Mono-Q anion exchange column in 20 mM Tris-HCL, pH 8. Proteins were eluted using a FPLC system with a NaCl gradient and the elution was monitored at 280 nm. Fractions were collected manually, desalted on a PD-10 column, and lyophilized. A, Mono-Q chromatogram. Three peaks obtained were designated as A, B, and C. B, electrophoretic analysis of the protein peaks collected from the Mono-Q chromatography. Lanes 1, 4, and 7, represent peaks A, B, and C, respectively, resolved by 8.5% SDS-PAGE. Lanes 2, 5, and 8, represent the same peaks subjected to Western analysis after 8.5% SDS-PAGE separation and detected with the OPN antibody. Lanes 3, 6, and 9, represent Western blots of the same peaks detected with the TG antibody.

**FIG. 3.** Assay to compare the collagen binding properties of different collagens. (A) An enzyme-linked immunosorbent assay was used to compare the collagen binding properties of the monomeric and polymeric forms of OPN. Different collagens (types I-V) were immobilized onto microtiter wells and incubated with OPN samples cross-linked with increasing amounts of TG. To confirm that all samples contained equal amounts of OPN, no purification steps were performed for the cross-linked samples, but they were directly applied onto collagen after incubation. Fig. 3A shows that the binding of OPN to different collagens clearly increased as a function of the amount of TG in the reaction mixture that contains 2.5 mM CaCl2. Collagens also appeared to be saturated with OPN. Since the OPN antibody did not detect TG (data not shown), the increase in the binding of OPN to collagens is due solely to the presence of the polymeric forms of OPN in the sample. When Ca2+ was chelated from the reaction mixtures with EDTA prior
to incubation with the ligand proteins, a drastic decrease in binding was observed. In this case, the TG-treated OPN showed binding only to collagen type V (Fig. 3B). Casein reaction series, used as a comparison and control, behaved in an opposite way (Fig. 4, A and B). Casein appeared to bind to collagens both in the presence and absence of calcium ions, but the binding decreased as the amount of TG increased in the reaction mixture, suggesting that TG was competing with casein in collagen binding. This was confirmed by detection of increasing amounts of collagen-bound TG in the wells (data not shown). TG was bound to collagens also in experiments with OPN (data not shown), however, as seen in Fig. 3A, TG does not block the binding of OPN.

These results indicate that casein does not possess specific affinity for collagens either as monomeric or polymeric forms and that binding of OPN to collagens before and after TG treatment seems to be a specific action of this particular protein and this post-translational modification.

Since the elevated OPN polymer binding to collagens can also partly depend on the tendency of the OPN aggregates to bind more antibody, we quantified the collagen binding in a dot blot overlay assay using purified radioiodinated proteins. We were also interested in finding out how collagen types I and V would behave when they were in a soluble form. This interest was directed to collagen types I and V as they seemed to bind polymeric OPN most efficiently, collagen type V even in the absence of calcium ions (Fig. 3B). We radioiodinated monomeric and polymeric OPN as well as collagen type I as illustrated in Fig. 5. Radioiodination of collagen type V was not, however, successful (data not shown) and, therefore, we used an ELISA binding assay to study the binding of soluble collagen V to pure immobilized polymeric and monomeric OPN.
We have previously reported that the TG-catalyzed cross-linking of OPN is inhibited in vitro by osteocalcin (20). In light of the finding that osteocalcin apparently functions as a mineralization inhibitor in the mouse model (21), the TG-mediated protein aggregation event might have an advantageous effect on mineralization or matrix maturation that precedes it. The results of this study demonstrated that OPN aggregates exhibited a property of increased binding to collagen as compared with the monomeric form. Another acidic phosphorylated TG-substrate, casein, did not possess this property, indicating the specificity of collagen binding of TG-treated OPN. TG treatment appeared to introduce a 5-fold amount of OPN onto collagens, but predominantly when OPN and its polymer were in solution. This might indicate that a specific conformation, achieved in solution, might be required for binding. This elevated collagen binding property of polymeric OPN can result from: 1) its increased affinity for and association with collagen fibrils, therefore resulting in more rapid coating of collagen during the incubation; 2) or the polymer may have more binding sites on collagen resulting in a more efficient coating. Both could be explained by a conformational change observed in the CD experiments. Alteration in the OPN conformation of the monomer unit and/or several OPN molecules packed together could expose or create motives relevant to its interactive properties with collagen. Most interestingly, in comparison with other types of collagens, collagen type V appeared to have a very distinctive and different behavior. It seemed to bind the polymeric OPN even in the absence of calcium, but only when the polymer was in solution. Soluble collagen type V also seemed to have the greatest affinity for immobilized monomeric OPN. This might not only reflect a special function of collagen type V in extracellular matrix maturation (or bone formation) sequence, but also demonstrates the difference between the two forms of OPN.

OPN’s in vitro behavior with TG clearly shows that OPN is a substrate of this enzyme. OPN functioning also as an in vivo substrate is supported by several studies. The transglutaminase reactive acceptor glutamines are well conserved in all known OPN sequences, indicating the significance of the motif to its functions (27). OPN polymers have been found in different physiological sources such as bone (10), secreted by smooth muscle cells (28), and in milk as shown by our study. The observation that TG has been found to be active in bone in areas undergoing mineralization (5, 6) where also OPN has been localized in high concentrations (29), gives us a reason to believe that TG and OPN are able to interact in these areas. Indeed, Sørensen et al. (10) have shown with Western blotting that EDTA extracts of bovine bone contain high-molecular mass OPN complexes. Functional or basic biochemical studies of OPN aggregates have not been performed earlier and the functions of these complexes have only been speculative.

FIG. 6. Electrophoretic analysis and autoradiography of radioiodinated monomeric and polymeric osteopontins and collagens type I. 20 μg of purified osteopontin, its polymeric form, and collagen type I were labeled with 2 mCi of 125I. Proteins were resolved on 5.5% gel and autoradiographed. Lane 1, 10 μg of OPN; lane 2, 10 μg of polymeric OPN; lane 3, 10 μg of collagen type I. Lanes 4, 5, and 6, each lane contains 200,000 counts/min of the respective 125I-labeled protein.

Fig. 6A represents results from a dot blot overlay assay, showing that the polymeric OPN bound more efficiently to immobilized collagen type I than the monomeric OPN. In the reversed situation only a slight difference in binding of collagen type I to OPNs was observed. Quantification results are presented in Table I indicating a 5-fold increase in OPN binding to collagen type I as a polymer than as a monomer. Similarly, the polymeric OPN was more efficiently bound to immobilized collagen type V in the ELISA assay (Fig. 6B). In the reversed situation, however, soluble collagen type V was more readily bound to the monomeric form of the immobilized OPNs.

Transglutaminase Treatment of Osteopontin Produces Polymers with Altered Conformation—The increased collagen binding property of OPN after TG treatment led us to test the hypothesis that OPN may undergo a conformational change during polymerization. Conformational analyses of both monomeric and polymeric forms of OPN were performed with CD spectroscopy in water to obtain information also at lower wavelengths, where salts usually interfere with the signal. Circular dichroism is expressed as millidegrees, since calculation of OPN’s molar concentration was not possible due to the uncertainty of the exact molecular weight of the polymeric OPN. The spectrum of the polymeric OPN (Fig. 7) exhibits a clear shift in ellipticity between 210 and 240 nm as compared with the spectrum of the monomeric OPN, indicating a more ordered structure for the polymer. Secondary structure estimation based on the spectra (Table II) suggested that the random coil structure had decreased in the polymeric OPN from 43.4 to 30.6%. OPN has been observed to undergo structural alterations at higher concentrations (17.9 mg/ml) by Gorski et al. (26). In our experiments the concentrations corresponded to about one-third of that and, yet, a change in the CD spectrum was clearly observed and the spectrum was different from the CD spectrum of Gorski et al. (26). In conclusion, the CD data and the secondary structure estimations suggest that cross-linking of OPN by TG produces covalent polymers, which have a more organized structure than OPN has as a monomer.

DISCUSSION

Based on the results of this study, we suggest that the TG-mediated cross-linking of OPN may be directed to enhance the “glue-like” adhesion properties required in the processes that need collagen binding, e.g. in the adherence of different tissue interfacial structures (new and old bone), collagen fibrillogenesis, and wound healing. The enhanced adhesive property would be highly important for a protein postulated to function as a “mortar between bricks” (18). Although OPN is predominantly localized between collagen fibrils in fully matured hard tissue (29), the reports on its affinity for collagens have shown evidence of no or only moderate attachment (7, 30). The results of our study suggest that OPN has a significant affinity for collagens, but predominantly as a polymeric form. TG has been recently characterized as a biological glue for cartilage-carti-
lage interfaces (31). Therefore, OPN as a TG substrate could be an essential component of the "glue" and the polymerization indeed a prerequisite for its functioning as an adhesive protein. Since isopeptide bonds produced by TG are resistant to normal proteolysis (1, 2), the polymeric OPN may substantially contribute to the overall integrity and strength of the extracellular matrix where it is present. This kind of strengthening might be required, for example, in wound healing. In bone, it is also possible that the binding of OPN to collagen could be further stabilized by additional cross-linking by TG since covalent collagen-phosphoprotein complexes have been found in vivo in bone (32) and several collagens have been identified as substrates of TG (2, 33). Osteonectin (6) and fibronectin (11) are also TG substrates indicating the presence of non-collagenous protein-protein cross-links in the extracellular matrix.

In addition to the plausible adhesive and matrix-stabilizing (or matrix organizing) properties of the polymeric OPN/TG activity, a collagen-bound acidic cross-link network could also provide suitable bedding for mineral growth in bone (34–36). Calcification of the matrix follows matrix deposition and maturation in the bone formation sequence, distinguishing it from other types of extracellular matrices. It has been shown that collagen per se is not able to calcify (37). To accumulate and crystallize calcium and phosphate into hydroxyapatite, collagens seem to require other charged molecules on their surface.

**TABLE I**

Binding of monomeric and polymeric osteopontin to collagen type I in soluble and immobilized forms

| Immobilized protein | Soluble protein bound (ng/μg of immobilized protein) |
|---------------------|-----------------------------------------------------|
|                     | OPN monomer  | OPN polymer  | Collagen type I |
| Collagen type I     | 83 ± 7       | 420 ± 22     | 59 ± 18         |
| OPN monomer         | 35 ± 8       | 440 ± 22     |                |
| OPN polymer         | 37 ± 8       | 450 ± 22     |                |

**FIG. 6.** Binding of osteopontin and its polymeric form to collagen types I and V analyzed by dot blot overlay and ELISA assays. Collagen types I and V, OPN, and the OPN polymer were immobilized onto polyvinylidene difluoride membranes for 30 min. Each collagen dot contained 1 μg of protein and OPN and the polymeric OPN dots 5 μg of protein. The membranes were blocked with 1% BSA in 10 mM imidazole buffer, pH 6.8, containing 60 mM KCl and 2.5 mM CaCl₂. After washing, the immobilized proteins were overlaid with an excess of soluble labeled proteins; collagen type I with soluble monomeric and polymeric OPN and immobilized OPNs with soluble collagen type I. Each solution contained 10⁶ cpm/ml of radiiodinated protein as a tracer. Incubation was carried out for 1 h. The membranes were subsequently washed, dried, and autoradiographed. ELISA assay was performed as described under "Experimental Procedures" and Fig. 3. A, binding of soluble monomer OPN and the polymeric OPN to immobilized collagen type I and vice versa analyzed by dot blot overlay assays. B, the binding of soluble monomeric and polymeric OPN to immobilized collagen type V and vice versa analyzed by ELISA.

**FIG. 7.** Circular dichroism analysis of monomeric and polymeric osteopontin. Far UV CD spectra (250–190 nm) of OPN and the purified OPN polymer from the TG treatment were recorded at room temperature in water using the following concentrations: OPN, 0.5 mg/ml; OPN polymer, 6.15 mg/ml. The measured rotations are expressed as millidegrees.
Bone phosphoproteins have been postulated to function as such molecules (34). Indeed, phosphoproteins have been observed to initiate in vitro mineral formation when bound to collagen (37) and to inhibit crystal growth in vitro when in solution (38–40). OPN, as a phosphoprotein, could function as a protein scaffold together with collagen for other bone matrix macromolecules, such as bone sialoprotein, to bind and initiate calcification. The calcium binding properties of OPN were not specifically altered after polymerization (data not shown). However, OPN is known as a high capacity calcium binder (30).

A protein aggregation event may have an important role in bone formation, maturation, and calcification in general. Gorski et al. (41, 42) have reported that bone acidic glycoprotein-75 (BAG-75) undergoes a spontaneous Ca²⁺-induced polymerization, which increases its collagen binding activity. The aggregated forms of BAG-75 are resistant to reducing and denaturing conditions indicating their common nature, which suggests that intramolecular cross-links could be present. High molecular weight complexes of BAG-75 were detected in extracts of mineralizing calvarial explant cultures (41). More interestingly, newly synthesized BAG-75 from these cultures was present entirely in large macromolecular complexes, whereas nonmineralizing ROS 17/2.8 cultures produced only monomeric BAG-75 (41). Other kinds of enzymatic modulation have also recently been reported to have an effect on bone matrix protein interaction property. Sasaki et al. (43) demonstrated, e.g., that cleavage of osteonectin by metalloproteinases results in a 7–20-fold increase in its binding to collagens.

Those results together with ours suggest that biological functions of bone matrix proteins may largely depend on their post-translational modifications, conformational alterations, and surrounding ion concentrations, and that especially a covalent aggregation event may have a pivotal role for tissue maturation and development. In light of our findings, we suggest that TG activity could result in an altered function of OPN resulting from altered conformation accompanied with amplified collagen binding property. Instead of monomeric OPN, the complex form of OPN might be the OPN that is involved in collagen fibrillogenesis, matrix maturation, and possibly mineralization. Importantly, the function of OPN could therefore be regulated by TG expression and tissue distribution during different stages of tissue remodeling or bone formation.

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