Identification of the Phosphorylated Sites of Metabolically 
\(^{32}\)P-Labeled Osteopontin from Cultured Chicken Osteoblasts*

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Osteopontin (OPN) is one of the major secretory phosphoproteins in both calcifying and non-calcifying tissues. Evidence has accumulated for the biological importance of the phosphoproteins and, in particular, the phosphate groups in bone formation, resorption, and calcification. The precise locations of the phosphate groups in the OPN molecule were determined by metabolically labeling OPN with \(^{32}\)P in cultured chicken osteoblasts, followed by purification to homogeneity. N-terminal sequencing showed a single sequence of WPVSKRQHAISA, consistent with that deduced from both cDNA, and previous amino acid sequencing of the protein isolated from chicken bone. Three \(^{32}\)P-labeled peptides were isolated by reverse-phase high performance liquid chromatography of thrombin-digested, \(^{32}\)P-labeled OPN. The N-terminal sequencing of each of these thrombin fragments gave single sequences as follows: WPVSKRQHAISA, SHHTHRHQLDHD, and ASKLRKAAKRL, with approximate molecular masses of 5, 30, and 20 kDa. These data demonstrate that \(^{32}\)P was incorporated throughout the N- to C-terminal sequence of the protein. Thrombin specifically cleaved chicken OPN at two sites: between Arg-22 and Ser-23, which generated the 5-kDa N-terminal end fragment, and another between Lys-138 and Ala-139, which generated the 30- and 20-kDa fragments. To further define the exact locations of the phosphorylated amino acids and the surrounding amino acid sequences, OPN was digested with trypsin, which generated seven major \(^{32}\)P-labeled peptides whose amino acid sequences were determined. The phosphorylated peptide regions of osteopontin were identified as amino acids 8–18 (QHAISA\(^{*}\)AS\(^{*}\)SEEK), 39–54 (LASQQTHTY\(^{*}\)S\(^{*}\)EENAD), 150–171 (LIEDDAT\(^{*}\)A-EVGDSQIALGLWLPK), 179–191 (ELAHIQSVENDSD), 194–205 (FDS\(^{*}\)PEVGDSK), 214–219 (ES\(^{*}\)LASR), and 239–248 (HSIENNEVTR). The phosphorylated amino acid sites are followed by an asterisk (*). Of the seven identified phosphorylated peptide regions, three were localized on the N-terminal end of the osteopontin molecule (with five phosphorylated serines) and contained the sequence motifs that were phosphorylated by casein kinase II type(s), whereas the remaining four peptides are concentrated toward the C-terminal half of the molecule (with five phosphorylated residues) and contained recognition motifs for other kinases as well as casein kinase II.

It has been well established that the processes of phosphorylation and dephosphorylation of proteins catalyzed by protein kinases and phosphatases, respectively, play a major role in the initiation, regulation, and termination of a wide range of intracellular biochemical processes with significant functional consequences. Such processes may also play an important role in a wide variety of intercellular mechanisms, including general cell-cell signal transduction of extracellular agonists via specific transmembrane receptors, often causing alterations of intracellular concentrations of cAMP, calcium, inositol polyphosphates, and/or diacylglycerol. These intracellular modulators in turn induce a cascade of biochemical processes by mechanisms that also involve phosphorylation of specific rate-determining enzymes or proteins by protein kinases (1–3). Phosphoproteins containing \(\alpha\)-phosphoserine and \(\alpha\)-phosphothreonine are not only found in the intracellular compartments of cells, but have also been identified in extracellular matrices, particularly those of normal vertebrate mineralized tissues and in experimental and human pathologically calcified tissues (4–16). There is uncertainty as to the biological functions of these proteins and the precise role of the covalently bound phosphate groups per se. The presence of these phosphorylated proteins in all of the normal and pathologically mineralized tissues of vertebrates, and their ultrastructural localization (17–23), have combined to suggest that one biological function of these phosphoproteins and specifically of the covalently bound phosphate groups is their critical role in the nucleation and growth of inorganic calcium-phosphate crystals (24–26). This view is also supported by in vitro nucleation experiments, which attempted to simulate the postulated in vivo nucleation substrate of bone tissue by cross-linking the resident phosphoproteins in situ in their native positions and comparing the efficacy (induction or lag time) of this nucleation substrate with samples containing the collagen-phosphoprotein complexes after the covalent phosphate groups were enzymatically cleaved. Not only did the decalcified bone samples containing the collagen-phosphoproteins complexes markedly decrease the nucleation induction time, but this property was lost when the phosphate groups alone were removed enzymatically, leaving the dephosphorylated collagen-phosphoprotein completely intact and, hence, pinpointing the role of the phosphate groups in this in vitro crystal nucleation event (27, 28). In addition to their postulated role in calcification, these phosphoproteins have been implicated in many other biological functions (13–16, 29).

Two major glycosylated phosphoproteins in bone and many other mineralized tissues, osteopontin (OPN)\(^1\) and bone sialo-

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\(^{1}\) The abbreviations used are: OPN, osteopontin; BSP, bone sialopro-
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protein (BSP), have been extensively studied in the past decade. The potential roles of these two phosphoproteins in the calcification process have not been clearly defined. Data have been reported demonstrating that these two components can induce or inhibit calcification in several different in vitro models of calcification (30–33). The results may be due to the experimental techniques and models utilized to assess crystal nucleation and the accumulation of increasing amounts of the solid crystalline phase of calcium phosphate by secondary nucleation and possibly by growth of the crystals. However, their presence in pathologically calcified soft tissue such as breast tumors, urinary stones, and atherosclerotic plaques (13–16) supports the general hypothesis that they are involved in the deposition of a solid phase of calcium phosphate in normal and pathologically calcified tissues.

OPN is one of the most characterized of the secreted phosphoproteins. It is synthesized in a highly phosphorylated form by osteoblasts (34, 35), and can be isolated from the mineralized extracellular matrix of bone tissue (12, 26). In contrast, except for milk, it is essentially unphosphorylated in non-mineralized tissues (37, 38). Clearly, such differences in the extent of phosphorylation of the same protein when expressed in widely different tissues suggests different functional properties of both the unphosphorylated and phosphorylated protein, and further suggests the presence of tissue-specific kinase(s) that modify this protein. The protein kinase activity found in the bone, which phosphorylates the endogenous proteins of bone, was first observed three decades ago (39). More recently, the presence of membrane-bound casein kinase type II activity was detected in 14-day-old embryonic chicken bone, which phosphorylated endogenous bone phosphoproteins and dephosphorylated casein (40). Further studies using cultured chicken osteoblasts as the source of protein kinase from bone cells led to the conclusion that the microsomal casein kinase II (CKII) type activity of chicken osteoblasts was the predominant enzyme that phosphorylated purified chicken OPN and recombinant mouse OPN (41, 42). In these studies assessment of this enzyme with other protein substrates, including casein and synthetic peptide substrates, indicated that, while its activity is very similar to that of casein kinase II, it also possessed unique enzymatic activities that distinguished it from that of classical casein kinase II (41, 42). This enzyme was found to be localized in the Golgi apparatus of chicken osteoblasts (43). Furthermore, in vitro studies also identified the major protein kinase responsible for the phosphorylation of both bovine OPN and BSP to be a CKII (44). Other investigators using ROS 17.28 osteosarcoma cells have also isolated a membrane-associated CKII, which phosphorylates the major dentin phosphoprotein, phosphophoryn (45). This enzyme was also found in the endoplasmic reticulum and Golgi apparatus (46, 47). The subcellular location of the CKII to the endoplasmic reticulum and Golgi apparatus is significant mechanistically, since both OPN and BSP are secretory proteins and the enzyme participating in their post-translational modification must encounter them.

Extensive studies have been carried out in this laboratory to isolate, purify, and characterize the protein kinase(s) responsible for the phosphorylation of the secreted extracellular matrix phosphoproteins of bone. Quantitative amounts (mg) of 32P-secreted osteopontin from cultured chicken osteoblasts were isolated and purified to define both the location of the phosphorylated amino acids in the protein and the amino acid sequences adjacent to the phosphorylated residue. This paper reports on the isolation and purification of 32P-labeled peptides of OPN and the solid-phase amino acid sequencing of seven peptides, which unequivocally identify the peptide motifs and the specific sites of the phosphorylated OPN.

EXPERIMENTAL PROCEDURES

Materials
Sources for materials were as follows: GF/F glass filters (5.0 mm diameter) (Whatman, Ltd.); p-aminoephosphorimethoxysilane (Petrach Co.), radioactive orthophosphate (32P, 3000 Ci/mmol, ICN Inc.); reverse-phase Vydac C-4 column (15 × 0.38 cm) (Nerst Group); reverse-phase Dynax C-4 column (25 × 0.4 cm) (Du Pont); and trypsin (toxophilylalenyl chloromethyl ketone-treated), human thrombin (5000 units/mg), endoprotease Asp-N, Kemptide (LR-RASSAV), and CAM-dependent kinase, water-soluble coupling agent N-ethyl-N’-(dimethylaminopropyl)carbodiimide (EDAC) (all obtained from Sigma).

Methods
Labeling and Isolation of Secreted 32P-Labeled Osteopontin from Mineralized Cultured Chicken Osteoblasts—Chicken calvarial osteoblasts were isolated by sequential collagenase/trypsin treatment of 17-day-old embryonic calvaria. Cells were initially maintained in minimal essential media supplemented with 10% fetal bovine serum until confluence. They were then put into BGE media supplemented with 10% fetal bovine serum, 12.5 μg/ml ascorbic acid, and 10 μM β-glycerol phosphate. Cultures were grown for 3 weeks in the latter medium to promote extracellular matrix mineralization. To generate 32P-labeled OPN, mineralized cultures (15 100-mm culture dishes, 15 × 10^6 cells/dish) were initially incubated with phosphate-free Dulbecco’s modified Eagle’s medium for 1 h, followed by incubation of Dulbecco’s modified Eagle’s phosphate-free medium (5 ml/dish) containing 12.5 μg/ml ascorbic acid (1 μCi of 32P/superoxide) and incubated for 18 h. All protein extraction buffers and wash buffers were ice-cooled to 4 °C. The medium was removed, and the mineralized cell layers were washed (three times) with 10 ml of 10 mM phosphate buffer, pH 7.2. The cells were lysed with ice-cold phosphate buffer (2 ml/dish), pH 7.2, containing 0.5% Tween 20, 0.05% deoxycholate, 50 mM NaCl, 1 mM MgCl2, 50 mM NaVO3, 10 mM NaF, and 1 mM phenylmephlysulfonil fluoride for 10 min on ice. The lysis buffer was removed, and the mineralized cell layer was washed (three times) with 10 ml of 10 mM phosphate buffer (pH 7.2/dish). The mineralized cell layer was then extracted with 0.1 M HCl (adjusted to pH 2–2.0 with citric acid) 10 ml/dish over ice for 3 h. The acid extracted proteins were lyophilized, and 32P-labeled OPN was purified by reverse-phase HPLC on a C-4 column as described below.

Purification of Secreted 32P-Labeled Osteopontin by Reverse-phase HPLC on a C-4 Column—The lyophilized acid extract was reconstituted in ~0.5 ml of H2O, 0.1% tririrucuicatric acid (v/v), and aliquots (0.25 ml) were subjected to reverse-phase HPLC on a C-4 column (15 × 0.38 cm). After injection, the column was washed for 10 min with H2O, 0.1% tririrucuicatric acid (v/v) followed by elution using a linear gradient from H2O, 0.1% tririrucuicatric acid (v/v) to 60% CH3CN, 0.55% tririrucuicatric acid (v/v) at 60 min at a flow rate of 0.72 ml/min. Fractions of 0.72 ml were collected, and absorbance at 230 nm was measured by a continuous on-line chart recorder/integrator. Aliquots of 0.025 ml from each fraction were counted for 32P. The radiolabeled peaks were pooled, freeze-dried, and rechromatographed under similar conditions as described above. An aliquot of the purified osteopontin was then N-terminally sequenced.

Thrombin Digestion of Purified 32P-Labeled Osteopontin and Isolation of Thrombin Fragments by HPLC on a C-4 Column—Approximately 200 μg of purified 32P-labeled OPN was incubated with bovine thrombin (1 unit/4 μg of protein) in 0.20 ml of 50 mM Tris buffer, pH 8, containing 2 mM CaCl2 for 2 h at 37 °C. The reaction products were treated with 50 μl of H2O, 0.1% tririrucuicatric acid (v/v) and HPLC-chromatographed on a C-4 column (15 × 0.38 cm) as described above. The 32P-radiolabeled fragments were identified by counting for 32P, separately pooled, and freeze-dried. Aliquots of each thrombin fragment were N-terminally sequenced.

Trypsin Digestion of Purified 32P-Labeled Osteopontin and Isolation of the Labeled Peptides by Reverse-phase HPLC on a C-18 Column—The purified 32P-labeled OPN (~0.5 mg) from the HPLC C-4 column was digested with trypsin (2% w/v) in 0.2 ml of 50 mM NH4HCO3, pH ~8.0, for 20 h at 37 °C. The reaction products were then treated with 50 μl of H2O, 0.1% tririrucuicatric acid (v/v) and subjected to HPLC on a C-18
column (25 × 0.46 cm). After injection the column was washed for 10 min, followed by linear gradient elution from H₂O, 0.1% trifluoroacetic acid (v/v) to 60% CH₃CN, 0.055% trifluoroacetic acid (v/v) over 120 min, with a second gradient from 60% CH₃CN to 80% CN₃CN over 30 min at a flow rate of 0.5 ml/min. The absorbance at 219 nm was recorded on-line and recorded on an automated model 477A sequenator (Applied Biosystems Inc., Foster City, CA) under the same conditions as described previously (49). The absorbance at 219 nm was recorded on-line and recorded on an automated model 477A sequenator (Applied Biosystems Inc., Foster City, CA) under the same conditions as described previously (49). Glass filters coated with Biobrene were used, and the proteins or peptides were adsorbed on these filters. This approach was used for N-terminal sequence analysis in which the filter was the only filter identified the phosphorylated residues. The filters were treated with trifluoroacetic acid overnight at 37 °C. These filters were then treated with trifluoroacetic acid, air-dried in a fume hood, and treated with p-aminophenyltrimethoxysilane overnight at 37 °C. Therefore, there is significant limitation in attaining sufficient 32P counts for identification of the site of phosphorylation. A solid-phase sequencing technique utilizing GF/F glass filters derivatized by p-aminophenyltrimethoxysilane, followed by covalent attachment of the phosphorylated peptide through its C terminus (50), was used, which allowed the use of a sufficiently hydrophilic extraction solvent (Sₓ) without loss of the peptide, which would be the case if the adsorbent Biobrene method was used.

The glass GF/F Whatman filters (5.0 cm diameter) were treated with trifluoroacetic acid overnight at 37 °C. These filters were then freed of trifluoroacetic acid, air-dried in a fume hood, and treated with p-aminophenyltrimethoxysilane overnight at 37 °C. The filters were removed from the solution and extensively washed with MeOH and dried. In the present study, trimethoxysilane was used instead of triethoxysilane since the latter reagent was no longer commercially available. The derivatized filters were stored in dry, dark glass dishes until needed. The covalent attachment of the 32P-labeled peptides was carried out on derivatized GF/F glass filters cut in circular sizes of ~1 cm in diameter and using water-soluble carbodiimide (EDAC) (50). The automated N-terminal sequencing of the 32P-labeled peptides were performed directly from these filters. Further modifications of the sequencing technique included the following. The extraction solvent Sₓ was CH₃CN instead of 1-chlorobutane and contained 0.1% thiourea, since the latter reagent was no longer commercially available. The derivatized samples were stored in dry, dark glass dishes until needed. The covalent attachment of the 32P-labeled peptides was carried out on derivatized GF/F glass filters cut in circular sizes of ~1 cm in diameter and using water-soluble carbodiimide (EDAC) (50). The automated N-terminal sequencing of the 32P-labeled peptides were performed directly from these filters. Further modifications of the sequencing technique included the following. The extraction solvent Sₓ was CH₃CN instead of 1-chlorobutane and contained 0.1% thiourea, since the latter reagent was no longer commercially available. The derivatized samples were stored in dry, dark glass dishes until needed. The covalent attachment of the 32P-labeled peptides was carried out on derivatized GF/F glass filters cut in circular sizes of ~1 cm in diameter and using water-soluble carbodiimide (EDAC) (50). The automated N-terminal sequencing of the 32P-labeled peptides were performed directly from these filters. Further modifications of the sequencing technique included the following. The extraction solvent Sₓ was CH₃CN instead of 1-chlorobutane and contained 0.1% thiourea, since the latter reagent was no longer commercially available. The derivatized samples were stored in dry, dark glass dishes until needed. The covalent attachment of the 32P-labeled peptides was carried out on derivatized GF/F glass filters cut in circular sizes of ~1 cm in diameter and using water-soluble carbodiimide (EDAC) (50). The automated N-terminal sequencing of the 32P-labeled peptides were performed directly from these filters. Further modifications of the sequencing technique included the following. The extraction solvent Sₓ was CH₃CN instead of 1-chlorobutane and contained 0.1% thiourea, since the latter reagent was no longer commercially available.
a particular phosphorylated residue was accomplished during sequencing by collecting two thirds of the Edman degradation products of each cycle as ATZ-amino acid derivative and radioactive counting for $^{32}$P radioactivity; one third was converted to the PTH-amino acid derivative and analyzed by on-line HPLC to identify the amino acids. $^{32}$P-Labeled Kempfite (1.95 nmol, 17, 200 dpm), covalently attached through its C terminus to derivatized GF/F glass filter, was sequenced as LR-RASAV, with an initial yield of $^{32}$P released during sequencing and counting of two thirds of the Edman degradation and $^{32}$P radioactivity (dashed line). The isolated $^{32}$P-labeled peptide was freeze-dried and rechromatographed under the same conditions (inset).

To identify the specific sequences adjacent to the phosphorylated serine residue, $^{32}$P-labeled OPN was digested with trypsin and the $^{32}$P-labeled peptides isolated by reverse-phase HPLC on a C-18 column (Fig. 4). Seven major radiolabeled peptides were isolated and N-terminally sequenced. Typically, 200–300 pmol of the $^{32}$P-labeled peptides from secreted OPN were recovered and covalently attached to derivatized GF/F glass filters, representing 65–85% of the total as determined from the $^{32}$P counts after washing the filters. The total $^{32}$P radioactivity on the filter ranged from 2000 dpm to 6000 dpm. $^{32}$P released during sequencing and counting of two thirds of each cycle gave a background of 30–40 dpm, with the cycle containing the phosphorylated residues releasing 100–300 dpm depending on the initial $^{32}$P level in a given peptide. Typical data are shown in Fig. 5, which demonstrates that the repetitive yields of sequencing were approximately 85%, with initial yields ranging between 50 and 300 pmol, depending on the amount of a given peptide. It is worth noting that during sequencing, some of the Ser(P) led to the formation of some dehydroalanine by $\beta$-elimination and thus the $^{32}$P counts of a specific cycle reflected a combination of $^{32}$P and $^{32}$P-Ser. This method permitted simultaneous identification of the amino acid sequence of the phosphorylated peptide and the phosphorylated residue. While unequivocal identification of each peptide sequence and the site of phosphorylation was possible, the size of some peptide (Fig. 4, fraction 6) $^{32}$P radioactivity sequence starting at Tyr-29 and extending to Lys-78 did not allow the unequivocal identification of the site of phosphorylation. The sequencing of this peptide up to 20 amino acid did not release any $^{32}$P radioactivity, indicating that the phosphate label was not on the Thr-26, Ser-36, Ser-38, or Thr-45. The $^{32}$P-labeled peptide containing residues Tyr-29 to Lys-78 was then further digested with endopeptidase Asp-N, from which a
Fig. 4. Isolation and purification of tryptic peptides of purified $^{32}\text{P}$-labeled OPN. $^{32}\text{P}$-Labeled OPN ($\sim 0.5$ mg) was digested with trypsin (2% w/w) in 0.2 ml of 50 mM NH$_4$HCO$_3$, pH 8.0, for 20 h at 37 °C. The peptides were separated by reverse-phase HPLC on a Dynamax C-18 column (25 × 0.46 cm) using a linear gradient from H$_2$O, 0.1% trifluoroacetic acid (v/v) to 60% CH$_3$CN, 0.055% trifluoroacetic acid (v/v) in 120 min, at a flow rate of 0.5 ml/min. Absorbance at 219 nm was recorded continuously (solid line). Fractions of 0.5 ml were collected and aliquots (0.05 ml) counted for $^{32}\text{P}$ radioactivity (dashed line). Inset, rechromatography of fraction F6 using a linear gradient from H$_2$O, 0.1% trifluoroacetic acid (v/v) to 60% CH$_3$CN, 0.055% trifluoroacetic acid (v/v) in 80 min. All of the other radiolabeled fractions were also rechromatographed prior to sequence analyses (data not shown).

Fig. 5. Solid phase N-terminal sequence analysis of purified $^{32}\text{P}$-labeled tryptic peptides of metabolically labeled OPN from cultured chicken osteoblasts. A, peptide F$_2$ from Fig. 4. Initially the total $^{32}\text{P}$ on the filter was 3200 dpm; at the end of 16 sequencing cycles the $^{32}\text{P}$ remaining on the filter was 1005 dpm (30% of the initial $^{32}\text{P}$). Initial yield, $I_o = 50$ pmol; repetitive yield, $R = 85\%$. The total radioactivity recovered from the sequencing and counting of the two thirds of Edman degradation products (862 dpm) + one third analyzed as PTH-amino acid (431 dpm) = 1293 dpm (46%). B, peptide F$_6$ from Fig. 4 had an initial total $^{32}\text{P}$ count on the filter of 4,915 dpm; after sequencing, 1820 dpm (37%) remained on the filter, recovered from sequencing and counting of two thirds of Edman degradation products (1685 dpm) and one third analyzed as PTH-amino acid (643 dpm) = 2,528 dpm (52%). Initial yield, $I = 216$ pmol; repetitive yield, $R = 92\%$. $\log_{10} (\text{pmol of amino acid})$ at each cycle: $\log_{10} (\text{pmol of dehydroalanine})$; generated from Ser(P); $\bullet$, $\log_{10} (\text{pmol of dehydroalanine + Ser})$.

DISCUSSION

Our initial survey of the culture medium and the cell-calcified matrix layer during the isolation and purification of quantitatively traceable amounts of $^{32}\text{P}$-labeled secreted OPN from cultured chicken osteoblasts indicated that >90% of the secreted OPN was in the calcified matrix of the cell layer. Our quantitative analysis of OPN in the medium and mineral layer during purification indicated that 93% of the secreted $^{32}\text{P}$-labeled OPN was in the mineral and 7% in the medium. These results were based on calculations from the HPLC-purified material in terms of the $^{32}\text{P}$ counts and protein content. Furthermore, from such data the calculated ratio of $^{32}\text{P}$ label/OPN protein for medium and mineralized layer were comparable, suggesting that the phosphorylated forms of OPN in these two compartments were of very similar nature.

The unique cleavage of OPN by thrombin (19, 53–57), which cleaved chicken OPN at two specific sites (one between Arg-22, and Ser-23 and the other between Lys-138 and Ala-139) (Fig. 6), was used to establish that phosphorylated sites were distributed throughout the length of the OPN molecule, which is synthesized and secreted by cultured chicken osteoblasts. In vitro studies of the extent of phosphorylation and of the protein kinases that predominantly phosphorylate bovine bone OPN
showed that bovine bone OPN was highly phosphorylated (9 mol of phosphate/mol of OPN) by CKII, and that these phosphates were distributed on both the N-terminal and C-terminal halves of the molecule (44), consistent with the results obtained from metabolically labeled chicken OPN of cultured osteoblasts.

The present results indicate that three peptides (8–18, 48–65, and 149–162) concentrated in the N-terminal half of the molecule are phosphorylated only by a CKII, while the remaining four peptides concentrated in the C-terminal half of the molecule appear to be phosphorylated by a combination of CKII and cGMP-dependent kinase (peptide 179–191). Peptide 194–205 can be phosphorylated on Ser-196, either by an CKII or a Ca$^{2+}$/calmodulin-dependent kinase, and Ser-204 of this peptide can be phosphorylated by cGMP-dependent protein kinase. The results indicate that the CKII is the predominant kinase that post-translationally modifies chicken bone OPN (6 serines and 1 threonine residue; 70% of the total phosphorylated residues), while the remaining 30% of the total phosphorylated residues (3 serines) are phosphorylated by other kinases. The determination of the total number of moles of phosphate/mol of metabolically phosphorylated OPN calculated from the values in Table I (including an unidentified peptide between F5 and F6; see Fig. 4) was 6.1. This occurred despite the fact that there were a total of seven different phosphorylated peptides identified, containing up to 10 phosphorylated residues, as well as an eighth unidentified phosphorylated peptide. These findings indicate that not all the poten-
tial phosphorylation sites are phosphorylated on every OPN molecule in a given population. On average, most of the peptides contain approximately 0.76 mol of phosphate/mol peptide, with an average overall extent of phosphorylation per peptide site per peptide site of ~53%. The biological significance of the variable extent phosphorylation of the potential phosphorylation sites and its variation with age and rate of synthesis and the possible rate of dephosphorylation are not known at this time, but present intriguing avenues for future research.

*In vitro* phosphorylation of purified OPN obtained from mineralized chicken bone and recombinant mouse OPN by four pure protein kinases, namely cAMP-dependent kinase, protein kinase C, cGMP-dependent kinase, and CKII, showed that there was essentially no phosphorylation of OPN by cAMP-dependent kinase, minor phosphorylation by protein kinase C and cGMP-dependent kinase (each introducing ≤1 mol of phosphate/mol of OPN), whereas CKII significantly phosphorylated OPN (~9 mol of phosphate/mol of OPN) (41, 42). These data are completely consistent with the conclusions obtained in the present study on the extent and sites of phosphorylation in *32P*-labeled OPN synthesized by chicken osteoblasts. Similar *in vitro* phosphorylation data were obtained for bovine bone OPN (44). It was of interest that *in vitro*, CKII phosphorylated all the potential sites (9 mol of phosphate/mol of chicken OPN in contrast to the phosphorylation of OPN in osteoblast cell culture, in which case only partial phosphorylation of the potential sites for phosphorylation was observed (6.1 mol of phosphate/mol of [32P]OPN (61%)). A possible explanation of the more complete phosphorylation that occurs *in vitro* is that the overall reaction conditions are optimized *in vitro*. For example, the relative concentrations of the reactants such as the enzyme, ATP, and OPN would be very different when compared with those in the secretory pathway of the protein in cell culture and/or the length of the reaction time.

Although there are some conflicting reports with respect to whether phosphorylation of matrix phosphoproteins occurs extracellularly or intracellularly (40, 46), recent work carried out in our laboratory with OPN and BSP and a series of purified protein kinases isolated from osteoblasts, as well as the present study, strongly support the conclusions that phosphorylation of OPN and BSP occurs “intracellularly.”

Recently, 27 phosphorylated serines and one phosphorylated threonine were identified in bovine milk OPN (58). The predominant sites for phosphorylation contained recognition sequence for casein kinase II. Fig. 6 compares the phosphorylated regions of chicken bone OPN obtained in the present study with those of bovine milk OPN. Although most of the phosphorylated regions in both proteins are highly conserved, bovine milk OPN was more phosphorylated than chicken bone OPN. This is probably related to the fact that chicken OPN is only ~60% homologous with the primary structure of bovine milk OPN. Of the 28 phosphorylated sites in bovine milk OPN, representing 60% of the potential sites for phosphorylation, 12 of the serines with recognition regions SXE/SXSSXE are either absent in chicken bone OPN or the serine residue is substituted by amino acids such as I, P, or A. However, the phosphorylated sites in the chicken bone OPN were similar to those found in bovine milk OPN. Therefore, it is apparent that the lower level of phosphorylation of chicken OPN is due to the absence of the specific amino acid sequence, SXE/SXSSXE, or to the substitution of serine residues by other amino acids. In view of the fact that bovine milk OPN has a very high homology with OPN from other mammalian species, it may be expected that the extent of phosphorylation in mammalian bone OPN may be similar to milk OPN. Nevertheless, although the number of potential sites for phosphorylation in mammalian OPN may be generally the same in each species (regardless of tissue origin), the extent of phosphorylation can vary due to differences in the types and numbers of protein kinases in the various tissues or organs. As a result of different levels of phosphorylation, interactions of the OPN from various tissues with other proteins and conformational changes collectively may reflect different biological functions and open new avenues of research.

As often is the case in complex biological systems, the local environment and type of matrix with which OPN is interacting may play an important role whereby a given state of phosphorylation is optimal for induction or inhibition of calcification. One may therefore intuitively postulate that OPN at a particular state of phosphorylation in different matrix environments can behave and function very differently. The variable extent of phosphorylation of OPN from different tissues or organs and the variable degree of phosphorylation in a given population of OPN molecules, all signify the possibility that an important and a wide range of biological functions of this molecule may be exerted frequently by the capacity of a given cell population to secrete OPN at a particular state of phosphorylation. Additionally, the biological functions of OPN may be further modulated in the extracellular matrix by the varying degrees of dephosphorylation that can take place by the actions of the phosphatases.

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**TABLE 1**

Phosphorylated peptides of secreted *32P*-labeled OPN from cultured chicken osteoblasts identified by solid-phase sequence analysis

| Residues     | Peptide                  | Mol of phosphates/mol of peptide | Phosphorylation on Each Residue |
|--------------|--------------------------|----------------------------------|--------------------------------|
| 8–18         | QHAIS*A5*S*E               | 0.88                | 30, 30, 30                      |
| 45–54        | THYS*S*DENAD             | 1.0                  | 67, 33                          |
| 150–161      | LIEADAT*EAVG             | 0.81                | 31                              |
| 180–191      | LAQHGS*EVENDSR           | 0.31                | 31                              |
| 194–205      | FDS*PEVGGSDE            | 1.0                  | 100                             |
| 214–219      | ES*LASR                  | 0.88                | 88                              |
| 239–248      | HSINEVNEVTR             | 0.43                  | 43                              |
|              | Average                   | 0.76                | 53                              |
Identification of the Phosphorylated Sites of Metabolically $^{32}$P-Labeled Osteopontin from Cultured Chicken Osteoblasts

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