Phosphorylation of Purified Bovine Bone Sialoprotein and Osteopontin by Protein Kinases*

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The large number of covalently bound phosphates on the extracellular phosphoproteins osteopontin (OPN) and bone sialoprotein (BSP) have been implicated in biological functions such as mineral deposition and osteoclast binding. In the present study the state of phosphorylation of BSP and OPN was evaluated by in vitro 32P labeling using a series of protein kinases and quantification. Both the purified bovine BSP and OPN were radiolabeled by [32P]ATP and factor-independent protein kinase. Quantification of 32P radioactivity incorporated on dephosphorylated BSP and OPN provided 6.6 and 8.9 mol of phosphate incorporated/mol, respectively. Native OPN incorporated 1.07 and BSP 2.46 mol of phosphate/mol by factor-independent protein kinase. These data led to calculations that OPN and BSP, respectively, contain 7.83 and 4.14 mol of phosphate/mol in their natural state. Thrombin digests of radiolabeled BSP showed radioactivity to be associated with fragment of molecular mass value of 30 kDa (N-terminal half), with no observable radioactivity associated with the 40-kDa fragment (C-terminal half). Similar experiments with 32P-labeled OPN provided two radiolabeled thrombin fragments, with molecular mass 30 kDa (N-terminal half) and 20 kDa (C-terminal half), both were radioactive. The major phosphorylation was associated with the N-terminal half containing 7.0 mol of phosphate, and 1.9 mol of phosphate were associated with the C-terminal half. Additional experiments of in vitro phosphorylation of OPN and BSP by several other known protein kinases were carried out. cAMP-dependent protein kinase showed no phosphorylation of OPN or BSP, while protein kinase C and cGMP-dependent protein kinase led to minor phosphorylation, each of the latter introduced about 1 mol of phosphate/mol of OPN and BSP molecule.

In addition to a major collagen matrix, bone contains several other non-collagenous proteins. Of the non-collagenous proteins, glycosylated phosphoproteins have been the subject of intense study in the past decade or so. Two such proteins, osteopontin (OPN)1 and bone sialoprotein (BSP) are probably the best known (1–7). The importance of these phosphoproteins in initiation, regulation, and stability of hydroxyapatite crystals has received increasing attention (8–11). BSP has been purified from several species including chicken, bovine, rat, human, and rabbit bones (12–16), and the cDNA primary amino acid sequence deduced for BSP from bovine, porcine, human, and rat (5, 14, 17). Both OPN and BSP possess an "RGD" (Arg-Gly-Asp) sequence region found to be involved in cell binding (6, 18, 19). The susceptibility of OPN to thrombin cleavage has been observed for OPN from human (20), porcine (14), mouse (21), rat (22–24), and chicken (25) leading to fragments of molecular size ranging between 23 and 45 kDa. However, the physiological importance of thrombin cleavage of OPN is not yet clearly understood. Suggestions have been made that since the thrombin cleavage sites are "close" to the RGD cell binding region, it may be involved in altering the cell attachment properties of OPN (14, 20, 22, 26, 27). BSP also possesses the RGD cell binding sequence and has been shown to have cell attachment properties (6, 18, 19). However, thrombin action on BSP from chicken bone (13), a rat osteosarcoma cell line (28), and porcine bone (14) led to no observable cleavages, and BSP from bovine bone is found to be specifically cleaved by thrombin 140 amino acids from the RGD cleavage sites, as reported in the present work.

The number of acidic amino acids and covalently bound phosphates in both OPN and BSP seems to enable them to bind simultaneously to hydroxyapatite and cells (2, 7). This process may become particularly significant when the cells in question are osteoclasts (involved in bone resorption) or metastatic tumors. At present, there is little known about the nature of the phosphorylated regions and how these may be coupled with the above function, or the protein kinases responsible for the phosphorylation of these proteins. The sites of phosphorylation of purified bovine milk OPN have been determined by an indirect method using S-ethanethiol derivatization followed by sequencing, which led to determinations of 27 phosphoserine and 1 phosphothreonine residues (29). In our laboratory, a more direct method was used to metabolically 32P label secreted OPN from cultured chicken osteoblasts, and sequence analysis led to identification of phosphorylated peptide regions with up to a total of 8 phosphorylated residues (7 P-Ser and 1 P-Thr) (30, 31). This work on metabolically 32P-labeled secreted OPN from cultured chicken osteoblasts revealed that the phosphorylation regions and sites on chicken OPN were predominantly with sequences SSEE and SXEE (i.e. recognition aa sequences for FIPK). However, there were also several other phosphorylated regions with recognition amino acid sequences for other protein kinases. In a separate study in this laboratory using cultured chicken osteoblast cytosolic and microsomal enzyme preparations, we have shown that the cytosolic preparations contained several protein kinases (FIPK, cGMP- and cAMP-dependent kinases, protein kinase C, and Ca2+/calmodulin-dependent kinase), whereas the microsomal preparation contained predominantly FIPK. Use of these osteoblast enzyme...
preparations in phosphorylation of purified chicken OPN and recombinant mouse OPN in combination with specific inhibitors (heparin in particular) and "qualitative analysis" led to the conclusion that these proteins were predominantly phosphorylated by FIPK (31, 32). Despite significant advances that have been made in the study of OPN and BSP, there is still uncertainty and controversy with respect to the state of phosphorylation and protein kinases that are involved. In the present work, both purified bovine bone OPN and BSP were subjected to in vitro phosphorylation by several known protein kinases. We report on the quantification of phosphates (moles of phosphates/mole of OPN and BSP) that different protein kinases introduce, and use native and dephosphorylated forms of OPN and BSP to determine the extent of naturally occurring phosphorylation. Additionally, thrombin cleavage of \(^{32}P\)-labeled OPN and BSP was used to evaluate the proportions of the phosphates on different domains.

**MATERIALS AND METHODS**

Isolation and Purification of Bovine BSP and OPN—Cortical bone of the midportion of the femora of 5- to 6-week-old calves was prepared and extracted in dilute HCl, and initially fractionated by DE52 ion exchange chromatography as described previously (3, 4), with the exception that 7 M urea was included in the buffers. The fractions containing the majority of the phosphoproteins were dialyzed free of salt, dried, redisolved in a solution containing 7 M urea, 0.4 M NaCl, 0.05 M KH2PO4/KOH buffer, pH 6.8, and aliquots of 0.25 ml molecularly filtered on a TSK-3000 HPLC column (60 × 0.75 cm) at a flow rate of 1 ml/min using the same buffer solution. The major peak was pooled, concentrated, and adjusted to 0.3% trifluoroacetic acid in H2O. Samples were then chromatographed by reverse-phase HPLC using a Brownlee C-4 column (22 × 0.46 cm) with a linear gradient: 10% acetonitrile and 0.3% trifluoroacetic acid in H2O to 60% acetonitrile and 0.3% trifluoroacetic acid, at a flow rate of 1 ml/min.

The sample was purified by a similar procedure, with the exception that the DE52 ion exchange chromatography was carried out at pH 4 as described previously (2). Further purification of OPN was achieved by reverse phase HPLC on a Vydac C-4 column (15 × 0.38 cm).

Thrombin Digestion— Aliquots of 200 μg of purified BSP were digested separately with highly purified human and bovine thrombin (5000 and 2000 units/mg, respectively, Sigma), 1 unit of thrombin was used per 8 μg of OPN or 50 μg of BSP protein in 0.1 M NaAc, pH 8.0, or in 0.05 M Tris-HCl, pH 8.0, buffer containing 10 mM CaCl2 for 2 h at 37°C. A control sample of BSP or OPN was also incubated in the same buffer without the addition of thrombin. An aliquot of the thrombin digest (5 μg of protein) and of the control sample was subjected to SDSPAGE on 10% acrylamide gels (5 × 7 cm) at 10 mA for 3 h, fixed, stained, and destained as described previously (3). The thrombin-digested sample of OPN and BSP (105 μg of protein) was chromatographed by reverse-phase HPLC on a Brownlee C-4 column (22 × 0.46 cm) using a linear gradient from 0.3% trifluoroacetic acid in H2O to 80% acetonitrile and 0.3% trifluoroacetic acid with a flow rate of 1 ml/min (Fig. 2). Fractions 1–4 were separately pooled, freeze-dried, and an aliquot subjected to SDS-PAGE (Fig. 1). N-terminal sequences were obtained from fractions 1, 2, and 3. Fraction 4 is predominantly thrombin which co-elutes with the small amounts of uncleaved BSP.

The bovine OPN was digested similarly and the OPN fragments were separated by SDS-PAGE (Fig. 3) followed by blotting onto Immobilon P and N-terminal sequencing directly from strips of Immobilon P. An amino acid analysis was carried out of digested thrombin-digested samples of purified BSP and OPN, and the peptides generated after digestion with thrombin were carried out by Edman degradation (33) using an Applied Biosystems model 477A automated sequenator essentially as described in Ref. 34.

**Picot HPLC Analysis for Phosphorylated Residues and Total Amino Acid Analysis**— Purified bovine OPN or BSP was mildly acid hydrolyzed in 0.5 ml of 4 M HCl + 1% phenol for 4 h at 110°C in a Pyrex-sealed tube. The hydrolysate was dried in vacuo, resuspended in 20 μl of a mixture of 200 proof ethanol:water:triethylamine (2:2:1) and dried in vacuo. The sample was then derivatized to phenylthiocarbamyl (PTC) derivatives for 30 min at room temperature with a solution of 200 proof ethanol:water:triethylamine:phenylisothiocyanate (7:1:1) as described in the Waters Associates Picot Tag manual. After redrying in vacuo, the PTC-derivatives were dissolved in 20 μl of Waters sample diluent buffer and analyzed using the Waters HPLC system and Pico Tag column (0.39 × 15 mm) maintained at 38°C. The PTC-derivatives were eluted by a set of gradients, from 94% buffer A to 51% buffer B in 10 min (flow rate 1.0 ml/min), from 51% buffer B to 100% buffer B in 0.2 min, maintained at 100% buffer B for 1.3 min at 1.0 ml/min flow rate, followed by 100% buffer B for 0.8 min at a flow rate of 1.5 ml/min.

The PTC-phosphoamino acids were identified by analyzing initially the phospho derivatives of phenyllithionine and phosphothreonine, and phosphoserine of 125 pmol of each sample, and in a mixture of standard PTC-derivatives (65 pmol each), followed by analysis of the PTC-derivatives of the bovine BSP or OPN acid hydrolysates. Comparison of the elution times of the standard PTC-phosphoamino acids with that of the PTC-derivatives of BSP amino acids enabled determination of the absence or presence of particular phosphoamino acids.

For total amino acid analysis 50 μg of BSP or OPN was hydrolyzed in 6 N HCl at 110°C for 24 h. The derivatization to PTC-derivatives was as described above. The Picot Tag analysis in quantifying the total amino acids was accomplished using standard PTC-derivatives.

**Dephosphorylation of Bone OPN and BSP**— Acid Phosphatase—OPN (90 μg) and BSP (100 μg) were each suspended in 0.2 ml of NaAc buffer, pH 5.0, I (ionic strength) = 0.1 mol/liter and 10 units of acid phosphatase (potato, Sigma) was included and incubated for 3 h at 37°C. Each sample (OPN and BSP) was then isolated rapidly by reverse-phase HPLC on a Vydac-C-4 column (15 cm × 0.38 cm). This step separates both the cleaved phosphates and the acid phosphatase from the original OPN or BSP. The extent of dephosphorylation was determined by analyzing for phosphoamino acids after partial HCl hydrolysis as described above.

In a separate experiment, 20 μg of bovine BSP and 15 μg of OPN were first dephosphorylated with 5 units of acid phosphatase at pH 5.0 for 3 h at 37°C twice, followed by isolation of each dephosphorylated protein using reverse-phase HPLC on a Vydac-C-4 column (Nest Co.). Both BSP and OPN were then \(^{32}P\) phosphorylated using FIPK and \(^{32}P\) JATP as described below and passed through a HPLC Vydac C-4 column. The radiolabeled OPN and BSP were then incubated with 5 units of acid phosphatase in NaAc buffer, pH 5.0, for 3 h at 37°C twice. The reaction mixture was then chromatographed using a HPLC Vydac C-4 column. Fractions were collected and aliquots counted for \(^{32}P\) radioactivity. The degree of dephosphorylation was calculated from the loss of \(^{32}P\) after acid phosphate treatment.

**Phosphorylation of Bovine OPN and BSP by cGMP-dependent Protein Kinase**— The dephosphorylated OPN (40 μg) and BSP (39 μg) were phosphorylated by \(^{32}P\) JATP (specific activity 100 mCi/mmol) and factor-independent protein kinase (FIPK) (50 ng, Upstate Biotechnology, Inc.) in 0.2 ml of 0.1 M NaAc, pH 7.4, 1 mM MgCl2, 1 mM EGTA, 45 μg of bovine OPN and 15 μg of BSP by FIPK is virtually complete. After incubation of 1 h, the phosphorylated OPN and BSP were rapidly isolated by reverse-phase HPLC on a Vydac-C-4 column with total \(^{32}P\) radioactivity 1.38 \times 10^5 dpm (9.82 \times 10^3 Ci) /on OPN and BSP, respectively. Using specific activity of \(^{32}P\) JATP = 11 mCi/mmol led to 5.69 \times 10^{-9} mol and 8.93 \times 10^{-9} mol of \(^{32}P\) incorporated in OPN and BSP, respectively.

**Phosphorylation of Bovine OPN and BSP by cGMP-dependent Protein Kinase**— The dephosphorylated OPN and BSP by FIPK were phosphorylated by cGMP-dependent protein kinase (Promega), or by cGMP-dependent protein kinase (Promega) and protein kinase C (Promega). The reaction mixture contained no EGTA, but 1 mM CaCl2 + 2.0 μg CPM + 50 ng of cGMP-dependent protein kinase (Promega), or (b) for protein kinase C + 1 mM CaCl2 + 0.1 mM l-phosphatidylserine + 50 ng of protein kinase C (Promega). In a separate experiment, dephosphorylated OPN and BSP were first thrombin digested followed by \(^{32}P\) labeling by the above protein kinases.
Reverse-phase HPLC of Thrombin-digested $^{32}$P-Labeled OPN and BSP and Quantification of Moles Phosphate/Mol of Protein on the Fragments—Alliquots of the original $^{32}$P-labeled OPN and BSP and thrombin digests were subjected to SDS-PAGE followed by autoradiography (Fig. 5). The $^{32}$P-labeled OPN and BSP and thrombin fragments were separated by reverse-phase HPLC on a Vydac C-4 column and aliquote counts were counted for $^{32}$P radioactivity (Fig. 6). Furthermore, two different thrombin digests of both OPN and BSP $^{32}$P-labeled by FIPK were subjected to reverse-phase HPLC separation on a Vydac C-4 column (Fig. 6). One thrombin digest used 1 unit of thrombin/8 μg of OPN or BSP of which aliquots were run on SDS-PAGE (Fig. 5), the other thrombin digest was using 5 units of thrombin/8 μg of OPN and BSP (i.e. 5 times more thrombin). For this latter case, SDS-PAGE was also run (Fig. 6). The reverse-phase-HPLC separation profiles of the two different thrombin digestions are compared (Fig. 6) and the $^{32}$P radio-labeled thrombin fragments of both OPN and BSP were N-terminal sequenced, as well as $^{32}$P radioactivity and total protein amount of each fragment were determined (from Pico Tag total amino acids) for calculations of moles of phosphate/mol of thrombin fragment.

Quantification of Number of Moles of Phosphate/Mol of OPN or BSP Introduced by FIPK—$^{32}$P-Labeled samples of OPN and BSP (free of excess $^{32}$PATP, and phosphorylation reaction buffers from “Phosphorylation of Bovine OPN and BSP by Catalytic Subunit, Sigma” and “Phosphorylation of Bovine OPN and BSP by Factor-independent Protein Kinase C, Sigma” by cAMP-dependent kinase, Sigma) were subjected to reverse-phase HPLC separation on a Vydac C-4 column (Fig. 6). One thrombin digest used 1 unit of thrombin/8 μg of OPN or BSP of which aliquots were run on SDS-PAGE (Fig. 5), the other thrombin digest was using 5 units of thrombin/8 μg of OPN and BSP (i.e. 5 times more thrombin). For this latter case, SDS-PAGE was also run (Fig. 6). The reverse-phase-HPLC separation profiles of the two different thrombin digestions are compared (Fig. 6) and the $^{32}$P radio-labeled thrombin fragments of both OPN and BSP were N-terminal sequenced, as well as $^{32}$P radioactivity and total protein amount of each fragment were determined (from Pico Tag total amino acids) for calculations of moles of phosphate/mol of thrombin fragment.

Evaluation of the State of Phosphorylation of OPN and BSP—Further studies with protein kinases (Table II). Further studies with protein kinases: (a) CAMP-dependent, (b) protein kinase C, and (c) cGMP-dependent led to no phosphorylation by cAMP-dependent kinase and minor phosphorylation (–1.0 mol of phosphate/mol of BSP) by each of protein kinase C and cGMP-dependent kinase. Similar study carried out using FIPK and the above enzymes using native (non-dephosphorylated) BSP led to significantly reduced amounts of $^{32}$P incorporation compared with the dephosphorylated form. FIPK introduced 2.46 mol of phosphate/mol of native BSP, indicating that BSP contained 62% naturally phosphorylated sites. Therefore, native purified BSP contained 4.14 mol of phosphate/mol that were naturally occurring and phosphorylated by FIPK type enzyme in vivo (Table II). The overall results indicate that the major protein kinase that phosphorylates BSP is FIPK.

Thrombin Cleavage of Native and $^{32}$P-Labeled BSP—Previous reports from several laboratories have utilized the cleavage of OPN by thrombin (13, 14, 20, 22, 25) to distinguish between OPN and BSP since it has been uniformly reported that BSP is not cleaved by thrombin. When purified bovine BSP was incubated with thrombin under conditions usually used for thrombin cleavage of OPN (14, 20, 22–24), we found that bovine BSP amounts of O-phosphoserine (27 residues/1000 total residues) were observed, with only very small amounts of O-phosphothreonine (0.5 residues/1000 total residues). Analysis in the present report for phosphorylation in purified bovine BSP led to similar results, with O-phosphoserine (19 residues/1000 total residues) and O-phosphothreonine (0.3 residues/1000 total residues). The above values reflect nanomoles of amino acids recovered and were not corrected for loss due to partial acid hydrolysis.

RESULTS AND DISCUSSION

Bone Sialoprotein

Purified bovine BSP was isolated from the major phospho-protein containing fractions separated by DE52 ion exchange chromatography followed sequentially by molecular filtration and reverse-phase HPLC. SDS-PAGE of the major BSP peak obtained by reverse-phase HPLC chromatography revealed either a single band or two of very closely adjacent bands of 66 kDa molecular mass (Fig. 1), with a single N-terminal amino acid sequence (LMSKKNLNRAK) identical to the sequence of bovine BSP derived from the cDNA sequence (6). This approach provided ~ 2.0 mg of BSP per 100 g of bovine bone powder. Both O-phosphoserine and O-phosphothreonine have been identified in the non-collagenous phosphoproteins (3, 35, 36). Significant amounts of both phosphoamino acids were also identified in purified OPN (12, 13, 37). However, the only previous analyses of phosphoamino acids in BSP have been discussed in the report by Heinegard (38), where significant

| std | 1 | 2 | 3 | 4 |
|-----|---|---|---|---|
|    | 97 | 66 | 45 | 31 |
|    | 21 | 21 | 21 | 21 |

Fig. 1. SDS-PAGE (10% acrylamide slab gel, 5 × 7 cm) of protein fractions containing BSP from DE52 anion exchange, TSK-3000 gel filtration, reverse-phase HPLC on C-4, and thrombin fragments isolated on C-4 reverse-phase column. Lane 1, 10 μg of purified BSP Coomassie Bluestained; Lane 2, 10 μg of purified BSP stained with Stains All; and Lanes 3 and 4, 10 μg of purified BSP digested with human and bovine thrombin, respectively, stained with Stains All. Panel A, thrombin fragments of BSP isolated by reverse-phase HPLC from Fig. 2; (a) 5 μg of fraction 1, (b) 5 μg of fraction 2, and (c) 5 μg of fraction 3, respectively, stained with Stains All.
also undergoes such specific fragmentation by thrombin. Both human and bovine thrombin fragmented bovine BSP and in the same way (Fig. 1, lanes 3 and 3', respectively). The thrombin-generated polypeptides were separated and isolated by reverse-phase HPLC on C-4 column (Fig. 2, fractions 1–4). Thrombin generated two fragments of very similar molecular mass (40 kDa) eluted from reverse-phase HPLC at 16 and 20% acetonitrile concentrations, respectively (Fig. 2, fractions 1 and 2), and a third smaller molecular mass (30 kDa) fragment eluted around 32% acetonitrile (Fig. 2, fraction 3). The original residual 66-kDa BSP and thrombin co-eluted at 40% acetonitrile (Fig. 2, fraction 4). The N-terminal amino acid sequencing of thrombin fractions 1 and 2 led to a single sequence KAGATAK-AGKKA in each case. This sequence corresponds to the sequence starting with Lys-123, indicating cleavage to be between Arg-122 and Lys-123. The third lower molecular mass fragment (Fig. 2, fraction 3) provided a sequence of LSMKLN-RRARAK, corresponding to the N-terminal of the original 66-kDa BSP. The presence of two C-terminal end thrombin fragment sequences with the same N-terminals and very similar molecular masses (40 kDa), but eluting at sufficiently enough different acetonitrile concentrations suggests that the naturally occurring BSP may exist in at least two different molecular forms similar to findings reported for OPN (22).

32P-labeled BSP by FIPK was subjected to thrombin cleavage followed by HPLC analysis and SDS-PAGE (Figs. 5 and 6, SDS-PAGE gel insets). In Fig. 5, lane 3, the small amount (<5%) of the total radioactivity present in that lane associated with high molecular mass region represent aggregates of BSP, which is not observed in lane 4 of thrombin cleaved BSP, and not observed for OPN or its thrombin cleaved samples. Although BSP is known to not stain easily by Coomasie Blue, with the amounts of BSP (10 μg) used which gave very intense staining by "Stains All," we observe some staining by Coomasie Blue at levels 10–15% that of Stains All (Fig. 1). This observation is probably partly due to the large amount of pure BSP used and possibly some desulfated/deglycosylated states being present in the purified BSP sample. Analysis of 32P-labeled bovine BSP thrombin fragments by HPLC and SDS-PAGE (Fig. 6B and Table II) showed that of the total 6.6 mol of phosphate incorporated by FIPK (or 4.14 mol of naturally occurring phosphates) on this protein, no significant phosphorylation was observed on the 40-kDa (C-terminal half) fragment. The predominant phosphorylation was associated with the 30-kDa (N-terminal half) thrombin fragment and minor phosphorylation (25%) found on a lower molecular mass fragment (22 kDa). This latter 22-kDa fragment was found to increase when higher amounts (5 ×) of thrombin were used. For instance, compare HPLC profiles of 32P counts, solid line and dashed lines, and autoradiographs (Fig. 5 and Fig. 6B) for two different thrombin concentrations. The proportions on the 30- and 22-kDa N-terminal fragments of BSP were ~75 and ~25%, respectively, when 1 unit of thrombin/8 μg of BSP was used, and this proportion changed to ~55 and ~45% when 5 units/8 μg of BSP (5 × more) thrombin was used. The N-terminal sequence analysis of this fragment showed that it has the N-terminal amino acid sequence of the original BSP. Therefore, it appears that the easily generated N-terminal half residues Leu-1 to Arg-122 (30 kDa) can further fragment at a much lower rate to generate the 22-kDa fragment which still has the N-terminal sequence of BSP. Similar treatment of OPN, that is, 5 × more thrombin, however, led to no further fragmentation of either the 30- or 20-kDa fragments (Fig. 6A).

Studies of thrombin action on BSP from chicken bone (13), a rat osteosarcoma cell line (28), and porcine bone (14) apparently lead to no observable cleavage. We have repeated the experiment using purified chicken BSP and thrombin and thrombin in the same experimental conditions used in this study which lead to specific cleavage of bovine BSP and OPN, and found that, consistent with the previous report, chicken BSP is not cleaved by thrombin. The present report is the first to date to demonstrate thrombin cleavage of BSP and identify the site of cleavage. Furthermore, while the cleavage site of the bovine BSP contains the recognition sequence (underlined), Arg-Lys-Ala-Gly, for thrombin specificity, the site of cleavage (i.e. between Arg and Lys) has not been observed previously for other proteins susceptible to thrombin action, e.g. OPN or fibrinogen, where cleavage takes place between Arg/Lys and residues Ala, Gly, and Ser. Thus, it appears that the thrombin cleavage of bovine BSP is unique and may be referred to as atypical.

The physiological importance of thrombin cleavage of OPN found in bone has been emphasized as a modulator of functionality of this protein through possibly changing its cell-binding property (12, 22, 26, 38). However, both OPN and BSP possess the RGD cell-binding sequence found to be involved in cell attachment through specific receptors on the cells. It appears that modulation by thrombin cleavage does not seem to have the same applicability for BSP as that reported for OPN in the literature. BSP from several species are found not to be susceptible to thrombin cleavage and bovine BSP is cleaved 140 amino acids removed from the RGD cell-binding sequence. In the case of rat BSP, it has been suggested that the non-RGD peptide region containing the sequence KKAGDA (residues 123–128) may be involved in cell attachment (28), in particular osteoclasts (39, 40). This sequence is also found in human BSP and is analogous to the non-RGD sequence KQAGDV in fibrinogen known to be involved in cell attachment (41). It is interesting to note that this sequence in bovine BSP is RKAGAT (residues 122–127) and the thrombin cleavage occurs between Arg and Lys (Table I). The substitution of Lys-122 found in BSP from other species by Arg-122 in bovine BSP is probably responsible for cleavage at this region, since Arg is a better recognition residue for thrombin cleavage. There are indications that in OPN, additional cell binding regions (other than RGD) are involved during cell attachment (42–44). Studies of OPN, BSP, and the non-RGD fragments of BSP (40, 44, 45),

Fig. 2. HPLC purification on a C-4 reverse-phase column of thrombin-digested bovine BSP. Thrombin-digested (1 unit/8 μg of BSP) bovine BSP (200 μg) at 37 °C for 2 h was chromatographed using a C-4 reverse-phase HPLC column. The HPLC conditions were as described under "Materials and Methods."
Table II. Similar studies were carried out using OPN and corporation of 8.9 mol of phosphate/mol of OPN, Fig. 4 and Kinases—FIPK phosphorylated provided LPVKPTSSGSKE, and on SDS-PAGE 64 kDa (Fig. 5). Purified similarly to BSP. The N-terminal sequence analysis of protein kinase C, and \( \text{cGMP-dependent protein kinase} \) and protein kinase \( \text{C}. \) Evaluation of the degree of phosphorylation by FIPK and by each of cGMP-dependent protein kinase and protein kinase C. The bovine bone OPN (0.5 mg/100 g of bone powder) was phosphorylated in vitro bovine OPN with incorporation of 8.9 mol of phosphate/mol of OPN, Fig. 4 and Table II. Similar studies were carried out using OPN and protein kinases: (a) cAMP-dependent protein kinase, (b) protein kinase C, and (c) cGMP-dependent protein kinase. \( \text{cAMP-dependent protein kinase} \) showed no phosphorylation, and minor phosphorylation (1.0 mol of phosphate/mol) was observed by each of cGMP-dependent protein kinase and protein kinase C. Evaluation of the degree of phosphorylation by FIPK and other enzymes of native (non-dephosphorylated) OPN led to incorporation of \( ^{32} \text{P} \) at a significantly reduced level compared to BSP postulated non-RGD cell-binding sequence found for bovine BSP and OPN (46).

### Osteopontin

The bovine bone OPN (0.5 mg/100 g of bone powder) was purified similarly to BSP. The N-terminal sequence analysis provided LPVKPTSSGSKE, and on SDS-PAGE 64 kDa (Fig. 3).

Evaluation of the State of Phosphorylation of OPN by Protein Kinases—FIPK phosphorylated in vitro bovine OPN with incorporation of 8.9 mol of phosphate/mol of OPN, Fig. 4 and Table II. Similar studies were carried out using OPN and protein kinases: (a) cAMP-dependent protein kinase, (b) protein kinase C, and (c) cGMP-dependent protein kinase. cAMP-dependent protein kinase showed no phosphorylation, and minor phosphorylation (1.0 mol of phosphate/mol) was observed by each of cGMP-dependent protein kinase and protein kinase C. Evaluation of the degree of phosphorylation by FIPK and other enzymes of native (non-dephosphorylated) OPN led to incorporation of \( ^{32} \text{P} \) at a significantly reduced level compared to BSP postulated non-RGD cell-binding sequence found for bovine BSP and OPN (46)."
half of OPN (30 kDa) starting with Leu-1 and extending to Arg-163, contained about 6.9 mol of the total phosphates incorporated by FIPK (or 6.19 mol naturally occurring phosphates). The C-terminal half (20 kDa) starting with Ser-104 and ending with the C-terminal contained 1.9 mol of the total phosphates incorporated (or 1.64 mol naturally occurring phosphates) on OPN. Thrombin cleavage showed that of the total phosphates introduced (−1.1) by each of cGMP-dependent kinase and protein kinase C on OPN, −75% (0.8 mol) of the total phosphate was associated with the N-terminal half and remaining −0.3 mol was on the C-terminal half.

Implications of the in Vitro Phosphorylation of Bovine OPN and BSP—The total number of phosphates introduced in vitro by FIPK on OPN and BSP is clearly different, about 25% less phosphate on BSP compared to OPN. The naturally occurring phosphates calculated using the difference in the extent of phosphorylation on native and dephosphorylated forms by FIPK also emphasized these differences, where OPN was found to contain −7.8 and BSP 4.1 mol of phosphate/mol. This indicates that BSP has −47% less naturally occurring phosphates compared with OPN. Whether these natural variations are indications of important physiological processes such as, the two proteins are phosphorylated on the potential phosphorylation sites to different extent prior to secretion or that BSP specifically undergoes partial dephosphorylation while it is resident in the extracellular matrix is not easy to discern. The present in vitro phosphorylation experiments were carried out on samples of OPN and BSP using the uncleaved molecules and prior thrombin cleaved samples followed by phosphorylation. It was found that as far as the phosphorylation was concerned regarding several kinases used, there was no significant difference in the extent of phosphorylation on the two halves of OPN and BSP. Thus a single cleavage of OPN or BSP did not alter significantly the ability of the kinases to recognize the specific phosphorylation regions. This may have been expected since small synthetic peptide substrates of 10–15 amino acid residues size with recognition sequences toward different kinases are phosphorylated with relative ease, e.g., Kemptide, syntide 2, etc. It is noteworthy that the possibility of some of the observed phosphates introduced in vitro reactions by FIPK were the result of contaminant kinases phosphorylating Tyr residues may be excluded, since our analysis of the 32P-labeled proteins for phosphoamino acids led to identification of no P-Tyr, but predominantly P-Ser. Furthermore, the P-Ser peak elution time of Pico Tag analysis was correlated with the 32P count released from HPLC during analysis. Similarly, the possibility of sugars on OPN and BSP being phosphorylated can be excluded since we observe no other additional 32P-peak in the Pico Tag HPLC analysis profile than P-Ser/P-Thr. It may be perceived that the presence of significant amounts of glycosylation on both OPN and BSP, and since these glycosylations occur on Ser/Thr sites, the possible deglycosylation naturally occurring in the matrix or by contamination of acid phosphatase (by glycosidases) may expose the Ser/Thr sites normally occupied by glycosyl moieties.

In vitro phosphorylation of bovine OPN and BSP by protein kinases, quantification of phosphate introduced and proportional distribution of the phosphates on the N-terminal and C-terminal halves of these proteins after specific thrombin cleavage

Quantification of moles of phosphate incorporated by protein kinases per mol of OPN and BSP before and after dephosphorylation. Proportion of the total phosphates incorporated on two halves of OPN and BSP was determined after thrombin cleavage and quantifying 32P label after isolation of each thrombin fragment from HPLC using Vydac C-4 column. The naturally occurring phosphates per mol of OPN and BSP were calculated by utilizing the difference between amount of phosphate introduced in vitro for native and dephosphorylated forms: (a) phosphate incorporated in vitro and (b) calculated naturally occurring.

**TABLE II**

| Enzyme         | OPN Original 64-kDa | OPN Thrombin fragments | 66-kDa | 66-kDa Thrombin Fragments | 30 kDa | 20 kDa |
|----------------|---------------------|------------------------|--------|---------------------------|--------|--------|
|                | Native | Dephosphorylated | Naturally occurring | N-terminal 30 kDa | C-terminal 20 kDa | Native | Dephosphorylated | Naturally occurring | N-terminal 30 kDa | C-terminal 40 kDa |
| FIPK           | 1.07   | 8.9          | 7.83                  | 7.07     | 1.9                    | 1.64   | 2.46                | 6.6                 | 4.1           | 6.6          |
| cGMP-dependent | 0.14   | 1.1          | 0.96                  | 0.87     | 0.3                    | 0.27   | 0.40                | 1.2                 | 0.8           | 1.2          |
| Protein kinase C | 0.06   | 0.5          | 0.44                  | 0.43     | 0.1                    | 0.09   | 0.01                | 1.0                 | 0.9           | 1.0          |
| cAMP-dependent | No phosphorylation | 5.84           | 8.8                  | 2.96                | 8.8          | 5.84     | 8.8                | 5.84                |

*NS, no significant phosphorylation.*
et al. (29). Also, the recognition amino acid sequences around OPN are distinctly different than those of phosphorylation of (that overall cell attachment/modulation involves participation in the thrombin digests. Lanes 1 and 3 are original 32p-labeled OPN and BSP before thrombin digestion, and lanes 2 and 4 are thrombin digested (1 unit of thrombin per 8 μg of OPN or BSP) OPN and BSP, respectively.

Fig. 5. Autoradiography following SDS-PAGE (10% acrylamide slab gel, 17 × 20 cm) of 32P-labeled OPN and BSP (by FIPK) and the thrombin digests. Lanes 1 and 3 are original 32p-labeled OPN and BSP before thrombin digestion, and lanes 2 and 4 are thrombin digested (1 unit of thrombin per 8 μg of OPN or BSP) OPN and BSP, respectively.

Fig. 6. Reverse-phase HPLC profiles of thrombin digested 32P-labeled OPN and BSP. A: OPN, 32P-labeled by FIPK and digested by human thrombin followed by reverse-phase HPLC on a Vydac C-4 column using linear gradient from 100% H2O + 0.1% trifluoroacetic acid to 60% CH3CN + 0.055% trifluoroacetic acid in 60 min at a flow rate of 1.0 ml/min. Column was washed for 10 min with 100% H2O + 0.1% trifluoroacetic acid after injection and prior to linear gradient. Fractions of 1.0 ml were collected and aliquots of 0.05 ml counted in 5 ml of biodegradable counting scintillant. Solid line, 32P count of thrombin fragments when 1 unit of thrombin was used per 8 μg of OPN (total OPN used 20 μg) and dotted line represents counts when 5 units of thrombin were used per 8 μg of OPN (total OPN used 8 μg). The inset represents the SDS-PAGE (10% acrylamide slab gel, 17 × 20 cm) of 32P-labeled OPN thrombin digests (5 units of thrombin per 8 μg of OPN) without HPLC. Lane a, phosphorylated by FIPK; Lane b, phosphorylated by cGMP-dependent kinase; and Lane c, phosphorylated by protein kinase C. B: BSP, 32P-labeled by FIPK and digested by human thrombin followed by reverse-phase HPLC on a Vydac C-4 as A. Solid line, 32P count of thrombin fragments when 1 unit of thrombin was used per 8 μg of BSP (total BSP used 6 μg) and dotted line represents counts when 5 units of thrombin were used per 8 μg of BSP (total BSP used 6 μg). Inset: autoradiography following SDS-PAGE (10% acrylamide slab gel, 17 × 20 cm) of 32P-labeled BSP thrombin digest (5 units of thrombin per 8 μg of BSP) without HPLC. Lane a, phosphorylated by FIPK; Lane b, phosphorylated by cGMP-dependent kinase; and Lane c, phosphorylated by protein kinase C.

OPN and BSP are important in mineral deposition (48, 49) and osteoclast attachment to OPN. This region appears to have a coupled fashion. The precise functional consequence of coupling or synergistic effect of these different moieties is as yet not known. However, involvement of the RGD sequence in cell attachment is well established, and thus far there is considerable evidence that the covalently bound phosphates on OPN and BSP are important in mineral deposition (48, 49) and osteoclast cell attachment (46). Since these proteins are intimately involved in biological events in the extracellular matrix, then the state of phosphorylation can affect the functional properties of OPN and BSP with perturbations of the matrix biology. Although the KQAQD region was found not to affect osteoclast attachment to OPN, this region appears to have a signaling effect toward change of osteoclast behavior, i.e. osteoclast cell “rounding” has been reported in response to short peptides KQAQD and AGDV (40).

It has been well established that the recognition sites of FIPK for phosphorylation are predominantly Ser/Thr-X-Glu-Glu-Glu or Ser/Thr-XX-Glu-Glu (50–52). Recent findings of the naturally occurring sites of phosphorylation of bovine milk OPN (29) revealed several regions of OPN phosphorylated with sequences Ser-Glu-Glu or Ser-Ser-Glu-Glu with up to 27 phosphoserine residues. On the N-terminal half of the molecule up to the thrombin cleavage site found in the present work of bovine bone OPN, bovine milk OPN contained phosphorylation
of peptide regions: residues 5–14 PTSSGSSEEK and 42–50 E. Salih.

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