Isolation of a novel bone glycosylated phosphoprotein with disulphide cross-links to osteonectin

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An 80 kDa protein was purified from calf bone by HCl-de-mineralization followed by 0.5 M EDTA/1.0 M NaCl extraction and sequential chromatography on DE-52, hydroxyapatite, and TSK-gel G3000SW HPLC columns. From the DE-52 column the protein was eluted at three different fractions, of which one further separated into two fractions on the hydroxyapatite column, indicating that the protein is present in four different molecular forms designated as 80 k-I-1, k-I-2, k-II, k-III. The N-terminal sequence analysis of all four forms gave the same sequence, SEQYNQEPNNV. Several tryptic internal peptides were also generated, purified and sequenced, leading to the identification of several repeat sequences, IFLGXXEI. Homology searching of the N-terminal and internal sequences indicates that this is a novel protein. Both 80 k-I-2 and k-III had similar amino acid composition with high contents of Asx, Glx and Leu and contained 7 and 16 phosphoserines per 1000 total amino acids, respectively. The 80 k-I-1 and 80 k-II forms were stained

with Rhodamine B specific for phosphoproteins. The four forms contained different contents of neutral sugars ranging from 5.5 to 26% (w/w protein) and ~1.7% sialic acid. These data indicated that the 80 kDa protein exists in four isomeric forms, at least based on the different post-translational modifications. The evaluation of the 80 kDa glycosylated phosphoprotein under alkylating, reducing and non-reducing conditions indicated that this protein undergoes polymerization through intermolecular disulphide bonds. Furthermore, the 80 kDa protein and osteonectin (ON), both of which are cysteine-rich proteins, can cross-link with each other via disulphide bonds, and this process can be induced to take place *in vitro* under experimental conditions. The occurrence of such a phenomenon *in vivo* was confirmed from the presence of similar high M_r components containing both 80 kDa and ON in the same SDS/PAGE bands, detected by the respective antibody reactions in crude bone extracts which were extracted in the presence of alkylating agent.

INTRODUCTION

In addition to a major collagen type-I matrix, approximately 90%, bone contains several other non-collagenous extracellular matrix (ECM) proteins (10%). Of the non-collagenous proteins, the glycosylated phosphoproteins of ECM, of which probably the best known and characterized are osteopontin (OPN) and bone sialoprotein (BSP) [1–5], have been the subject of intense study in the recent years. The conceptual considerations and *in vitro* studies postulated importance of these phosphoproteins in initiation, regulation and stability of hydroxyapatite crystals [6–8], and their involvement in cell attachment and bone resorption [9–15] are probably responsible for the extensive studies being carried out in an attempt to understand some of the biological functions of these proteins. The importance of covalently-bound phosphates on the ECM phosphoproteins in mineral deposition has been postulated and supported by studies initially in controlled *in vitro* nucleation experiments, whereby complexes of chemically and structurally native bone collagen and bone phosphoproteins markedly influenced the rate of nucleation of Ca-P apatite crystals as a function of the total phosphoserine and phosphothreonine contents of the complex [8]. Studies *in vitro* using BSP and OPN indicated that while BSP appears to induce Ca-P apatite formation [16] OPN lacks this property or is inhibitory [16,17]. These studies continue to be of interest and present different results from study to study;

these differences may be attributed to the nature of the *in vitro* system used and the interpretation of the physico-chemical data [16–18]. Further studies related to the importance of the phosphate groups were recently reported where phosphorylated and dephosphorylated forms of OPN and BSP were found to have different cell-binding capacity to osteoclasts [15]. The presence of an RGD (Arg-Gly-Asp) sequence region in both OPN and BSP found to be involved in cell binding led to the conclusion that these two proteins can regulate osteoblastic and osteoclastic cell activity during bone formation and resorption respectively [4,5,9,10,15,19,20]. Indeed, studies have confirmed that the RGD containing proteins promote cell attachment through interactions with $\alpha_v\beta_3$ integrin receptors [11–13]. In addition, it has also been shown that the RGD sequence can initiate intracellular biochemical processes such as Ca^{2+} signalling [14] and modulate cellular behaviour towards osteoclastic bone resorption [12].

In studies from this laboratory, embryonic chick tibia has been shown to contain membrane-associated casein kinase II (CKII) activity that can phosphorylate 32 kDa bone phosphoprotein and dephosphorylated casein [21]. More recent series of studies defined categorically that the major protein kinase that phosphorylates OPN is a microsomal CKII (also referred to as factor-independent protein kinase) [22,23]. The extensive quantitative analysis of the state of phosphorylation in *in vitro* studies of both purified bovine bone OPN and BSP in their native and dephosphorylated states and using a series of purified protein

Abbreviations used: ECM, extracellular matrix; CKII, casein kinase II; BSA, bovine serum albumin; OPN, osteopontin; BSP, bone sialoprotein; ON, osteonectin; OC, osteocalcin; PG, proteoglycan; BAG-75, bone acidic glycoprotein with M_r 75 kDa; HAP, hydroxyapatite; DTT, dithiothreitol; IA, iodoacetic acid; CBB, Coomassie Brilliant blue R-250; PAGE, polyacrylamide gel electrophoresis; RP-HPLC, reversed-phase high performance liquid chromatography.

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kinases clearly defined that both of these secretory proteins are highly phosphorylated and contained 7.8 and 4.1 mol of phosphate/mol of naturally occurring OPN and BSP, respectively [24]. In this same study it was shown that dephosphorylated OPN and BSP can be phosphorylated by CKII, which adds 8.9 and 6.6 mol of phosphate/mol of OPN and BSP, respectively, with additional phosphorylation by protein kinase C and cGMP-dependent protein kinase, each of the latter introducing about 1 mol of phosphate/mol of OPN or BSP. Further evaluation led to the conclusion that the majority of the phosphates on OPN resided on the N-terminal half of the molecule, which is the non-RGD domain, while for BSP all of the phosphates were concentrated on the N-terminal half [24]. Consistent with these, the precise sites of identification of ^{32}P -labelled OPN secreted from cultured chicken osteoblasts indicated that the majority of the phosphorylation sites were those with recognition sequences towards CKII [25]. Overall, when these findings were assessed in conjunction with a recent report in which the phosphates on OPN and BSP were found to be important in osteoclast binding [15], led to our postulate that osteoclast binding is strongly assisted by the highly phosphorylated N-terminal halves of both OPN and BSP, which are the non-RGD domains [24]. Hence, this in turn has led us to propose that the overall cell attachment/modulation involved participation of (a) the RGD sequence region, (b) phosphorylated regions and (c) the non-RGD amino acid sequence KKAGDA, in a coupled fashion [24]. The regulation of the above biological functions therefore becomes dependent on the state and extent of phosphorylation of OPN and BSP, since this is a variable component.

Despite the extensive general studies carried out on the best characterized ECM phosphoproteins of bone, OPN and BSP, their precise biological functions are still unknown, and even less understood are the roles of the post-translational modification, in particular covalently bound phosphates. The number of bone ECM proteins being reported and characterized continues to increase, e.g. BSP, OPN, osteonectin (ON), osteocalcin (OC), matrix Gla protein, 75 kDa bone acidic glycoprotein (BAG-75), proteoglycan I (PG I), proteoglycan II (PG II), bone morphogenetic proteins, etc. However, these are still only a small number compared with the presence of over 200 unknown and uncharacterized proteins in the bone matrix [26], of which perhaps there exist ECM non-collagenous proteins with some unique characteristics and very significant biological functions specific to bone still to be discovered.

The present study reports on isolation, purification and characterization of bone glycosylated phosphoprotein with unique amino acid sequences and physico-chemical characteristics hitherto undiscovered and/or undescribed.

EXPERIMENTAL PROCEDURES

Materials

DE-52 was obtained from Whatman Ltd. (Maidstone, U.K.). YM10 (M_r 10000 cut-off) membrane for ultrafiltration was from Amicon (Beverly, MA, U.S.A.) and nitrocellulose membranes for immunoblotting from Schleider and Schuell Co. (Keene, NH, USA). A TSK-gel G3000SW (TSK-3000) HPLC column (0.75 × 60 cm) and a C18 reversed-phase (RP) HPLC Dynamax-300A column from TosoHaas Co. (Montgomeryville, PA, U.S.A.) and Rainin Co. (Woburn, MA, U.S.A.), respectively were used. Econo-Pac HTP cartridge (5 ml), a HAP column and electrophoresis chemicals were products of Bio-Rad (Richmond, CA, U.S.A.). Iodoacetic acid (IA), dithiothreitol (DTT), β -mercaptoethanol, Coomassie Brilliant Blue R-250 (CBB), Rhodamine B, bovine serum albumin (BSA), chicken ovalbumin, D-(+)-glucose,

N-acetylneuraminic acid, trypsin (TPCK treated), bovine skin gelatin (75 bloom), goat anti-rabbit IgG antibodies conjugated with alkaline phosphatase, 5-bromo-4-chloro-3-indolyl phosphate and Nitro Blue Tetrazolium were purchased from Sigma Co. (St. Louis, MO, U.S.A.). Rabbit polyclonal anti-bovine ON antiserum was provided by Drs. Takita and Kuboki (Hokkaido University, Japan).

Tissue preparation and extraction

The cortical parts of calf tibiae (1–3 weeks postnatal) were crushed into powder and demineralized with HCl at a constant pH $\sim$ 2.0 as described previously [27]. Normally 100 g of bone powder was processed with several repeats up to 1 kg in order to purify 0.2–1.0 mg quantities of each of the 80 kDa forms. The extraction including the following procedures was performed at 4 °C in 3–5 volumes of solvent relative to bone powder. After centrifugation, the residual demineralized bone powder was extracted with 0.5 M EDTA, 1.0 M NaCl in 50 mM Tris/HCl buffer, pH $\sim$ 7.5, containing protease inhibitors (1 mM phenylmethylsulphonyl fluoride, 1 mM ϵ -amino-n-caproic acid, 1 mM *p*-hydroxymercuribenzoic acid, 1 mM benzamidinium hydrochloride, 1 mM levamisole, 5 mM sodium pyrophosphate) and the pH was adjusted to $\sim$ 7.5 by addition of solid Trizma base followed by extraction for 1 day. The residual bone powder was recovered by centrifugation and extracted for 24 h with 1.0 M NaCl, 50 mM Tris/HCl, pH $\sim$ 7.5, containing the same protease inhibitors. The EDTA/NaCl and NaCl extracts were dialysed against distilled water for 1 day (extract:H₂O = 1:30, v/v), lyophilized, and stored at -20 °C until use. Before any chromatographic step, the lyophilized extract was redissolved in 50 mM

Figure 1 DE-52 chromatography of EDTA/NaCl and NaCl extracts

The extracts were redissolved in 50 mM Tris/HCl, pH 7.4, concentrated by ultrafiltration over a YM10 membrane on ice, diluted with the same buffer and chromatographed on a DE-52 column as described in the text (A). An equal volume of the central portion of each fraction was electrophoresed and visualized with CBB (B).

Figure 2 HAP chromatography of the partially purified 80 kDa protein

(A) The fraction 80 k-I (Figure 1A) was concentrated, brought to 5 mM KH_2PO_4 , pH 6.8, and applied on a HAP column as described in the text. An aliquot of each fraction was electrophoresed and visualized with CBB (inset). (B) Fraction 80 k-III (Figure 1A) was chromatographed and analysed by SDS/PAGE (inset). (C) Fractions 80 k-II (Figure 1A) and 1 (Figures 2A and 2B) were pooled together and chromatographed.

Tris/HCl buffer, pH 7.4, concentrated, diluted and reconcentrated using an Amicon ultrafiltration system with a YM10 membrane.

In a separate experiment, 0.1 g of bone powder was extracted for 10 days in a microcentrifuge tube containing 1 ml of 0.5 M EDTA, 1.0 M NaCl, 10 mM IA, protease inhibitors, 50 mM Tris/HCl, pH 7.7, by changing the solvent every 2 days. The extract was obtained by centrifugation. The residue was washed twice with 1.0 M NaCl, protease inhibitors, 50 mM Tris/HCl, pH 7.4, then 3 times with 0.15 M NaCl, protease inhibitors, 50 mM Tris/HCl, pH 7.4, and placed for 24 h in 150 μl of the latter buffer containing 50 mM DTT. The supernatant was obtained by centrifugation and frozen until use. In the same way, the control NaCl extract was prepared in the absence of DTT.

Column chromatography

A DE-52 column (0.9×6.0 cm) was eluted at 4 °C either in 50 mM Tris/HCl, pH ~ 7.5 , using a linear gradient from 0.1 to 0.5 M of NaCl followed by a stepwise elution at 0.7 M NaCl or in 7 M urea, 50 mM Tris/HCl, pH ~ 7.5 , using a linear gradient (0.05–0.2 M) at a flow rate of 1 ml/min and monitored continuously at 280 nm. Fractions of 2.0 ml were collected. A HAP column was run at 4 °C by a linear gradient (0.02–0.2 M) and a stepwise (0.6 M) gradient of KH_2PO_4 (pH 6.8). Further purification was done on a TSK-3000 HPLC in 7 M urea, 0.4 M NaCl, 50 mM KH_2PO_4 , pH 6.8, at room temperature. This latter chromatography was repeated for the final purification. ON was purified on DE-52, HAP and TSK-3000 HPLC columns in the absence of urea and DTT. Commercial BSA was passed through the TSK-3000 HPLC column in 0.4 M NaCl, 50 mM KH_2PO_4 , pH 6.8, before use. Each of the purified proteins was concentrated and the buffer was changed to deionized distilled water containing 2 mM NaCl (~ 10 ml final volume) and freeze-dried. BSP was purified by chromatography as described previously [24].

SDS/polyacrylamide gel electrophoresis (SDS/PAGE) and Western blotting

SDS/PAGE was carried out by the method of Laemmli [28], using 7.5 % and 10 % mini-gels ($1.5 \times 55 \times 80$ mm) at 9 mA for ~ 4 h. All the samples were treated with β -mercaptoethanol unless indicated otherwise. The gels were stained with CBB or with Rhodamine B following the method of Debruyne [29]. In another set of experiments, the proteins separated in 7.5 % gels were transferred to nitrocellulose membranes and immunodetected as described previously except gelatin was used for the blocking solution [30].

Antibody preparation

Antibody to the protein of interest was raised in a rabbit by cutting the corresponding band from SDS/PAGE gels as described elsewhere with some modifications [30]. About 200 μg of the purified protein were electrophoresed in 10 lanes in a 7.5 % mini-gel. The protein bands were visualized with CBB, collected and lyophilized. The dried gels were cut into small pieces (< 0.5 mm) with scissors and used for two injections (two-thirds for the first time). The second injection was given 2 weeks later and the antiserum was obtained 4 weeks after the first injection.

Internal peptide generation and isolation

The purified 80 kDa protein (~ 20 μg) was partially digested with trypsin (2 % w/w) in 0.5 ml of 0.1 M NH_4HCO_3 at 37 °C for 3 h and lyophilized. The peptides were isolated by RP-HPLC on a C18 column using a linear gradient from $\text{H}_2\text{O}/0.1$ % (v/v) trifluoroacetic acid (TFA) to 60 % $\text{CH}_3\text{CN}/0.055$ % TFA (v/v) in 60 min at a flow rate of 0.5 ml/min. The column was washed for 10 min with $\text{H}_2\text{O}/0.1$ % TFA after injection followed by the above gradient. Fractions of 0.5 ml were collected and absorbance at 219 nm was continuously recorded. The separated peptides were concentrated using a SpeedVac freeze-drier and stored at -20 °C until analysis.

Amino acid composition and N-terminal sequence analysis

The purified proteins were each hydrolysed in 6 M HCl in sealed Pyrex tubes for 2 and 24 h for phosphoamino acid and total amino acid analyses, respectively. The hydrolysates were analysed using a Waters HPLC system for phosphoamino acid as described

Figure 3 DE-52 chromatography of the partially purified 80 kDa protein in the presence of urea of the four fractions obtained from Figure 2

The fractions were concentrated and left overnight at 4 °C after the buffer was changed to 7 M urea, 20 mM DTT, 50 mM Tris/HCl, pH 7.4. The samples were rechromatographed on the DE-52 column in the presence of 7 M urea. (A) Fraction 80 k-I-1 (Figure 2A); (B) fraction 80 k-I-2 (Figure 2A); (C) fraction 80 k-II (Figure 2C); (D) fraction 80 k-III (Figure 2B). Fraction 1 in chromatograms A–D all contained 55 and 65 kDa proteins as judged by SDS/PAGE (inset).

previously [24] and a Beckman Model 121-M amino acid analyser for total amino acid detection following instructions accompanying the analyser. The N-terminal and internal sequences were determined using an Applied Biosystem Model 477A automated sequenator as described previously [24].

Protein, neutral sugar and sialic acid determination

The purified protein ($\sim 100 \mu\text{g}$) was each dissolved in 10 mM DTT, 0.1 M NH_4HCO_3 . An aliquot was used for protein assay by the method of Read and Northcote [31], using chicken ovalbumin as standard. Neutral sugars and sialic acid were determined as reported previously [32,33]. D-(+)-glucose and N-acetylneuraminic acid were used as standards.

Evaluation of disulphide bond formation

(i) About 50 μg of protein from the crude bone extracts (isolated in the absence of IA and DTT or in the presence of IA or DTT) was treated for 2 h at room temperature ($\sim 22^\circ\text{C}$) with 40 μl of 8 M urea, 0.2 M Tris/HCl, pH 8.0, in the absence and presence of 30 mM DTT. (a) The sample without DTT was alkylated by 30 mM IA for 30 min at the same temperature, and (b) the sample with DTT was treated with 90 mM IA, since this sample contained 30 mM DTT equivalent to 60 mM free thiol groups, which would consume 60 mM of IA, the remaining 30 mM IA is as in (a). (ii) The purified 80 kDa protein (5 μg) and ON (10 μg) were incubated in 40 μl of 0.155 M KCl, 10 mM Hepes, 0.01 % streptomycin sulphate, pH 7.4 at 37 °C for 3 days. After centrifugation, the supernatant was alkylated with 20 μl of 8 M urea, 0.2 M Tris/HCl, pH 8.0, containing 30 mM IA. The residue was washed three times with 0.155 M KCl, 10 mM Hepes, 0.01 % streptomycin sulphate, pH 7.4, and extracted with 15 μl of the same buffer containing DTT (final 30 mM) at 37 °C for 60 min. The supernatant was obtained by centrifugation and mixed with 25 μl of 8 M urea, 0.2 M Tris/HCl, pH 8.0, containing 90 mM

IA for alkylation. As controls, ON and BSA were treated in the same way. All the above samples were incubated with 10–20 μl of 8 M urea, 15 % sugar, 2 % SDS, 50 mM Tris/HCl, pH 6.8, at 100 °C for 3 min, subjected to SDS/PAGE, and visualized by CBB staining or Western blotting.

RESULTS

Extraction and purification of the 80 kDa protein

Most of the mineral-binding proteins such as OPN, BSP, OC and blood-derived albumin, major non-collagenous ECM components of bone are extracted by acid demineralization [24,34]. In contrast, the 80 kDa protein and most of ON were still in the HCl residue composed mainly of type-I collagen and extracted with 0.5 M EDTA/1.0 M NaCl, pH ~ 7.5 . The 80 kDa protein was not readily observed in EDTA/NaCl extract by SDS/PAGE due to the presence of predominant ON, 55 and 65 kDa proteins unless the extract was chromatographed on a DE-52 ion-exchange column (Figure 1).

The 80 kDa protein was coeluted with a variety of proteins having molecular weights of about 30, 43 (ON), 47, 55, 65, 95 and 120 kDa on the DE-52 column in 50 mM Tris/HCl, pH 7.4 (Figure 1). Interestingly, the 80 kDa protein was eluted in two peaks which were divided into three fractions designated as 80 k-I, 80 k-II and 80 k-III on the DE-52 column under non-denaturing conditions when a shallow linear gradient was used (Figure 1). The prevailing ON was also eluted in the same manner; had not been reported for ON, suggesting the heterogeneity of ON, as in the case of the 80 kDa protein as discussed later. A similar elution pattern was observed for the 55 and 65 kDa proteins, however, they appeared earlier.

The 80 k-I fraction was further separated on a HAP column into two different HAP-affinity forms, 80 k-I-1 and 80 k-I-2 (Figure 2A). The 47, 95 and 120 kDa proteins were removed by HAP chromatography (Figure 2). The eluting concentrations for

Figure 4 TSK-3000 HPLC of the partially purified 80 kDa protein

Fraction 80 k-I-1 (Figure 3A) was concentrated and brought into 7 M urea, 0.4 M NaCl, 20 mM DTT, 50 mM KH_2PO_4 , pH 6.8 and chromatographed on a TSK-3000 HPLC in the same buffer without DTT at room temperature as described in the text (A). The fraction containing 80 kDa protein was rechromatographed (B). Similarly, chromatograms C, E and G were obtained from fractions 80 k-I-2 (Figure 3B), 80 k-II (Figure 3C), 80 k-III (Figure 3D) and the final purified chromatograms were D, F and H, respectively. Proteins were electrophoresed and stained with CBB (insets). Five μg of the purified protein as applied. —/+ Indicates without or with β -mercaptoethanol when samples were heated at 100 °C.

80 k-I-1, 80 k-I-2, 80 k-II and 80 k-III were 0.08–0.11, 0.12–0.15, 0.04–0.07 and 0.09–0.14 M KH_2PO_4 , respectively. The predominant components, 55 and 66 kDa proteins were almost completely removed on the DE-52 column in the presence of urea and DTT (Figure 3). After TSK-3000 HPLC, a single broad band for the four forms of the 80 kDa protein was observed around 80 kDa on SDS/PAGE gels under the reducing conditions together with small amounts (< 2%) of a ~ 43 kDa contaminant protein in the 80 k-I-1 and -II, which was identified as ON as described later (Figure 4). However, under the non-reducing con-

Table 1 Amino acid compositions and N-terminal and tryptic internal sequences of the purified 80 kDa protein

AA ^a	80 k-I-2	80 k-III	PG I ^b	PG II ^b			
Asx ^c	155	152	116	133	80 k-I-1	N-terminal	SEQYNQEXN
Thr	39	42	46	52	80 k-I-2	N-terminal	SEQYNQEPNNV
Ser	58	60	77	75	80 k-II	N-terminal	S(D)EQYNQEPN
Glx ^d	150	157	114	122	81 k-III	N-terminal	SEQYNQEPNNV
Pro	66	64	67	70		Peptide 1	L(E)(X)V(A)L(F)HSNS
Gly	46	37	75	94		Peptide 6	<u>IFLGISEIR</u>
Ala	47	41	47	50		Peptide 7	<u>IFLGYYXI</u>
Cys ^e	12	14	N.D. ^f	N.D.		Peptide 8	<u>IFLGSGXI</u>
Val	33	40	56	51			
Met	2	1	N.D.	N.D.			
Ile	66	65	46	42			
Leu	120	115	120	109			
Tyr ^e	33	36	27	10			
Phe	43	47	31	29			
His	52	51	51	20			
Lys	55	51	70	54			
Arg	24	25	50	42			

^a Amino acid residues/1000; ^b Data from ref. [44]; ^c Asp + Asn; ^d Glu + Gln; ^e Uncorrected; ^f Not determined.

ditions three more bands appeared, probably dimer, trimer and tetramer of the 80 kDa protein while the 43 kDa protein disappeared. The approach of extraction and purification used here provided ~ 0.3, 0.2, 0.9 and 0.8 mg of 80 k-I-1, 80 k-I-2, 80 k-II and 80 k-III per 1 kg of wet bone powder, respectively. In comparison, 20 mg of BSP, 5 mg of OPN [24] and 3.5 mg of 18 kDa lysine-rich protein (a growth and differentiation factor) [19] were obtained from 1 kg of wet bovine bone.

Amino acid compositions and sequences of the 80 kDa protein

Both 80 k-I-2 and -III had similar amino acid composition (Table 1), sharing high contents of Asx and Glx similar to other Ca^{2+} /HAP-binding bone proteins such as OPN, BSP, ON, and OC. However, unlike these proteins, the 80 k-I-2 and -III contained strikingly high content of leucine residue, 120, 115 per 1000 total amino acid residues. These features are reminiscent of PG I (biglycan) and PG II (decorin) (Table 1). Also, the two forms of the 80 kDa protein were shown to be rich in cysteine (Table 1). All four forms of the 80 kDa protein had the same N-terminal sequence (Table 1). Partial tryptic digestion of the 80 k-III generated eight internal peptides as separated by C18 HPLC (Figure 5). Four of the peptides were sequenced. Peptide 1 contained two N-terminal sequences at a ratio of ~ 2:3 (Table 1). Interestingly, peptides 6, 7 and 8 were shown to be different but contain the same region, IFLGXXEI. No sequences similar to these N-terminal and internal sequences were found in the non-redundant GenBank CDS translations + PDB + SwissProt + SPupdate + PIR database, when the computation was performed at the National Center for Biotechnology Information using the Blast network service. When Swiss Prot database using the FASTA program in the Wisconsin package was used low homology sequences were defined towards a single internal peptide, however, combination of the internal peptides and the N-terminal sequences could not be matched to a known protein.

Post-translational modifications of the 80 kDa protein

Post-translational modifications in proteins are a common phenomenon. So far collagen cross-linking, phosphorylation,

Figure 5 RP-HPLC of the trypsin-treated 80 k-III
The purified 80 k-III was partially digested with trypsin and chromatographed by RP-HPLC on a C18 column as described in the text.

glycosylation, sulphation, γ -carboxylation, transglutaminase-catalysed cross-linking, etc. have been reported for bone ECM proteins. When we examined whether the 80 kDa protein undergoes such modifications, we found that 80 k-I-2 and -III contained 7 and 16 phosphoserines per 1000 total amino acid residues, respectively (Table 2); the latter is close to that of BSP. The 80 k-I-1 and -II were stained to an extent similar to BSP with Rhodamine B, an indicator of phosphoproteins (Figure 6). The four forms of the 80 kDa protein were also shown to contain different contents of neutral sugars and sialic acid (Table 2).

Evaluation of intermolecular disulphide formation

Surprisingly, SDS/PAGE showed that three bands of higher molecular weights, apparently consistent with dimers, trimers and tetramers of the 80 kDa protein, were observed in all different forms of the purified 80 kDa protein under the non-reducing conditions and disappeared when reduced (Figure 4, insets). Similarly ON, rich in cysteine, also formed polymers, when the purified ON was incubated for 3 days at 37 °C under physiological

Figure 6 Rhodamine B staining of the purified 80 kDa protein
The proteins (10 μ g for each lane) were electrophoresed and stained with Rhodamine B as described in the text.

conditions of pH and ionic strength (Figure 9, lane 8). The treatment of the sample with DTT led to disappearance of such high M_r complexes (Figure 9, lane 12). In contrast, BSA containing 35 cysteine residues per molecule under the same experimental conditions showed no oligomer formation *in vitro* (Figure 7, lane 4). ON has been shown to migrate faster on SDS/PAGE gels under non-reducing ($\sim$ 40 kDa) than under reducing conditions ($\sim$ 43 kDa) [35]. Our data also revealed the same result when ON was incubated for less than 60 min at 37 °C (Figure 1B and Figure 7, lane 2), except the molecular weight was a little larger ($\sim$ 46 kDa) in the 7.5% gels in the presence of reducing agent (Figure 7, lane 1). The 43 kDa contaminant protein ($<$ 2%) in the purified 80 k-II showed positive reaction with anti-ON antibody and an additional band at $\sim$ 95 kDa was also found, which was not visible in the CBB-stained gels (Figure 9, lane 1). When 80 k-II was added during the incubation of purified ON for 3 days under physiological conditions, two new high M_r bands were observed in the stacking gels and just below the border of stacking and running gels (Figure 9, lane 9). Also, the density of ON dimer and trimer bands decreased and the tetramer and pentamer disappeared. Furthermore, ON was

Table 2 Contents of phosphate and carbohydrates in the 80 kDa protein compared with other bone proteins

	80 k-I-1	80 k-I-2	80 k-II	80 k-III	BSP	OPN	BAG-75	ON
Phosphate ^a	N.D. ^b	N.D.	N.D.	N.D.	1.8 ^c	7.7 ^c	7.9 ^d	0.5 ^e
P-Ser ^f	N.D.	7	N.D.	16	19 ^g	27 ⁱ	N.D.	N.D.
P-Thr ^f	N.D.	0	N.D.	0	0.3 ^g	0.5 ^j	N.D.	N.D.
Neutral sugars ^a	9.7	26.3	5.5	16.2	26.2	N.D.	N.D.	5 ^h
Sialic acid ^a	1.6	N.D.	1.7	N.D.	36.9, 33.6 ^c	10.6 ^c	7 ^d	3 ^e

^a Values as percentages per unit protein; ^b Not determined; ^c Data recalculated from ref. [2] to be able to compare on the same basis; ^d Data from ref. [40]; ^e Data from ref. [45]; ^f Values as phosphoserine (P-Ser) or phosphothreonine (P-Thr) per 1000 residues; ^g Data from ref. [24]; ^h Data from ref. [35]; ⁱ Data from ref. [46].

Figure 7 SDS/PAGE and CBB staining

Lanes 1, 2, reduced and non-reduced ON (1 μ g for each lane), respectively; lanes 3, 4, BSA (10 μ g/lane) was incubated for 3 days under physiological conditions, reduced (lane 3) or non-reduced (lane 4), and both alkylated; lanes 5, 9, EDTA/NaCl extract (50 μ g/lane), from HCl-residue without IA treatment during extraction, non-reduced (lane 5) or reduced (lane 9) and alkylated; lanes 6, 10, EDTA/NaCl extract (50 μ g/lane), directly from bone powder in the presence of IA, non-reduced (lane 6) or reduced (lane 10) and alkylated; lanes 7, 8 (30 μ l/lane), an extract from IA-treated EDTA/NaCl residue in the absence (lane 7) and presence (lane 8) of DTT, both reduced and alkylated.

Figure 8 Western-blot using anti-80 k-II

Lane 1, purified non-reduced 80 k-I-1 (5 μ g); lanes 2, 4, EDTA/NaCl extract (50 μ g/lane), from HCl residue without IA treatment during extraction, non-reduced (lane 2) or reduced (lane 4) and alkylated; lanes 3, 5, EDTA/NaCl extract (50 μ g/lane), from bone powder in the presence of IA, non-reduced (lane 3) or reduced (lane 5) and alkylated; lanes 6, 7 (both 30 μ l/lane), an extract from IA-treated EDTA/NaCl residue in the absence (lane 6) and presence (lane 7) of DTT, both reduced and alkylated. Arrows indicate that the reacting bands in lanes 2 and 3 are in the same positions as observed in Figure 9, lanes 2, 3 and 9.

detected in the insoluble fraction when incubated in the presence of 80 k-II (Figure 9, lane 11) but not alone (Figure 9, lane 10); this fraction was solubilized in the presence of DTT. The presence of 80 kDa-ON, 80 kDa-80 kDa and ON-ON disulphide bonded complexes in bone matrix was established by initial extraction carried out in the presence and absence of IA (Figure 8, lanes 2, 3; Figure 9, lanes 2, 3). Use of 80 kDa and ON antibodies in Western blots showed that the high M_r bands were reactive towards both 80 kDa and ON antibodies within each of these bonds, indicating that these high M_r bands contained complexes

Figure 9 Western blot using anti-ON

Lane 1, purified reduced 80 k-II (10 μ g); lanes 2, 4, EDTA/NaCl extract (50 μ g/lane), from HCl-residue without IA treatment during extraction, non-reduced (lane 2) or reduced (lane 4) and alkylated; lanes 3, 5, EDTA/NaCl extract (50 μ g/lane), from bone powder in the presence of IA, non-reduced (lane 3) or reduced (lane 5) and alkylated; lanes 6, 7 (30 μ l/lane), an extract from IA-treated EDTA/NaCl residue in the absence (lane 6) and presence (lane 7) of DTT, both reduced and alkylated; lanes 8, 10, ON (10 μ g) was incubated for 3 days, centrifuged and separated into two fractions, one (supernatant) alkylated (lane 8) and the other (residue) extracted with DTT and alkylated (lane 10); lanes 9, 11, ON (10 μ g) + 80 k-II (5 mg) were incubated for 3 days, centrifuged and separated into two fractions, one (supernatant) alkylated (lane 9) and the other (residue) extracted with DTT and alkylated (lane 11); lane 12, reduced and alkylated ON (10 μ g) after 3 days incubation. Arrows indicate that the reacting bands in lanes 2, 3 and 9 are in the same positions as those observed in Figure 8, lanes 2 and 3.

between 80 kDa and ON (Figure 8, lanes 2, 3; Figure 9, lanes 2, 3, arrows). When the above samples were analysed under reducing conditions, the high M_r bands containing both 80 kDa and ON proteins disappeared (Figure 8, lanes 4, 5; Figure 9, lanes 4, 5). Thus, it appears that 80 kDa-80 kDa, ON-ON and 80 kDa-ON disulphide bonded complexes can occur both *in vitro* and *in vivo*, and such phenomena appear specific. As seen in the *in vitro* insolubilized complex consisting of ON and the 80 kDa protein (Figure 9, lane 11), both the 80 kDa protein and ON were detected in the DTT-treated NaCl extract, which was obtained by DTT addition under physiological ionic strength from the EDTA/NaCl residue (Figure 8, lane 7; Figure 9, lane 7), whereas they were weaker in the control (Figure 8, lane 6; Figure 9, lane 6).

DISCUSSION

More than 200 non-collagenous ECM proteins have been shown to exist in bone by two-dimensional gel electrophoresis [26]. However, to date only a few have been purified and characterized. In the present study we report on isolation and characterization of an 80 kDa glycosylated phosphoprotein with unique physico-chemical characteristics hitherto undescribed for any of the known bone proteins. Both the unusual characteristics and the N-terminal and internal amino acid sequences of the 80 kDa protein were unmatched to any known protein, indicating that this protein is another novel component of the bone. The separation characteristics of the 80 kDa protein on anion-exchange and HAP columns led to purification of four different forms, 80 k-I-1, -2, -II, and -III with the same N-terminal sequence and almost the same molecular weight, suggesting evidence of four isoforms resultant from different degrees of post-translational modification. This was supported by the quantitative analyses of covalently-bound phosphates and carbohydrates (Table 2). Although the amino acid composition indicated similarities to PG I and PG II (Table 1), ABC

chondroitinase has no effect on the 80 kDa protein (data not shown). Sato et al. [36] have reported an 80 kDa acidic glycoprotein from bovine bone and this protein can be separated from ON on a DEAE-Sephacel column. However, the 80 k-I-1, -I-2, -II, and -III could not be separated from ON on a DE-52 column, and the amino acid contents of Ile, Leu and His in both 80 k-I-2 and -III are almost double that in the 80 kDa acidic glycoprotein. Also, neither polymer formation nor HAP-binding has been shown for the 80 kDa acidic glycoprotein. These data indicate that the 80 kDa protein in the present work is different from the previously reported 80 kDa acidic glycoprotein.

The importance of the covalently-bound phosphate groups on the ECM proteins as discussed in the Introduction combined with our extensive studies on the state and sites of phosphorylation of OPN and BSP suggest significant roles of phosphates in the biology of bone. The domains that contain the majority of the phosphates of OPN and BSP and the determination of the precise sites of phosphorylation with variations of the extent of phosphorylation on each site between 30 and 100% [25], led to the postulate that an important and a wide range of biological functions of these ECM molecules may be exerted frequently by their state of phosphorylation [25]. Whether the isomeric forms of 80 kDa proteins with different extents of phosphorylation present such behaviour is yet to be determined. However, it is clear that the 80 kDa glycosylated phosphoprotein we are reporting on in the present work follows very closely the state and varying degrees of phosphorylation exemplified by OPN and BSP, including the heterogeneity of the protein from the chromatographic elutions, with the overall amount of phosphates on 80 kDa protein more closely correlated with that of BSP than OPN. The phosphate groups may contribute to the affinity of 80 kDa protein for HAP as well as a possible regulatory effect on mineral deposition analogous to those postulated for OPN and BSP. However, the ability of the 80 kDa protein to form polymers through intermolecular disulphide formation as discussed below sets this unique protein apart from OPN and BSP, with possible yet unknown additional functionalities.

Under the non-reducing conditions, three high M_r bands (> 100 kDa) were observed in all different forms of the purified 80 kDa protein (Figure 4, insets), indicating that these proteins contain free thiol groups which can participate in 'intermolecular' disulphide bond formation in an appropriate environment to form polymers. The 80 k-I-2 and -III forms appear to form polymers more easily than 80 k-I-1 and -II since the partially purified former fraction contained larger amounts of high M_r fraction (> 80 kDa), observed as an 80 kDa band in reducing SDS/PAGE, which was obtained in the second gel chromatography (Figure 4). ON can also undergo polymerization in a similar manner (Figure 9, lane 8). This inconsistency with the previous report [35], is due to the reaction time over which observations were made. In contrast, BSA, which also contains a large number of cysteine residues, cannot form disulphide-bonded polymers under the same conditions (Figure 7, lane 4). The intermolecular disulphide cross-links between the same protein molecules to form complexes have been previously reported, e.g. fibronectin [37]; however, such occurrences are of low frequency in nature. Since both the 80 kDa protein and ON are coeluted chromatographically (Figures 1–3) and the purified proteins have the ability to form homo-oligomers *in vitro* through disulphide bonds, it is possible that these two proteins may undergo disulphide interchange to form hetero-oligomer complexes. To address this question, purified ON was incubated with the purified 80 kDa protein under physiological conditions, which led to the appearance of two additional bands with a higher M_r than those of ON and that of the 80 kDa protein (Figure 9,

lanes 8, 9; Figure 8, lane 1), indicating the disulphide bond formation between these two proteins. Such interactions between the 80 kDa protein and ON appear to take place *in vivo* as determined in experiments not treated by DTT and when alkylating agent was used at the time of extraction. To prevent the thiol–disulphide and disulphide–disulphide interchange, of which the former can occur at neutral pH and the latter at acidic, IA and alkaline pH were used at the time of extraction. Western-blot analyses of the extracts using anti-80 kDa protein and anti-ON showed that multiple high M_r bands (> 90 kDa) were reactive in each of these bands toward both antibodies under non-reducing conditions and most of these bands disappeared in the presence of reducing agent (Figure 8, lanes 3, 5; Figure 9, lanes 3, 5, arrows). The same results were obtained in the extract without IA treatment during extraction (Figure 8, lanes 2, 4; Figure 9, lanes 2, 4, arrows), indicating that the intermolecular disulphide bond complexes between 80 kDa and ON existed naturally in the bone matrix.

The existence of insoluble, intermolecular disulphide-bonded complexes of ON was supported from the results in the present study where (a) ON was coextracted with the 80, 55 and 65 kDa proteins from the insoluble EDTA/NaCl residue in the presence of DTT (Figure 7, lane 8; Figure 8, lane 7; Figure 9, lane 7) and (b) ON and the 80 kDa protein formed insoluble complexes under physiological conditions which were solubilized by DTT treatment (Figure 9, lane 11). These data suggest that the intermolecular disulphide-bond formation is required for these proteins to provide insoluble complexes. Such insoluble forms are probably important for different functions of these proteins, since there is sufficient evidence to suggest that certain bone ECM proteins in an insoluble state can induce mineral deposition, while the soluble forms lack such properties [35,38,39]. Self-association to form large macromolecular complexes has been reported for BAG-75 [40]; however, these complexes were apparently of the non-covalent type and can be prevented by denaturants such as SDS alone. The multiple functions of ON that have been postulated [19,41–43] suggest that the disulphide-bonded complex formation of ON and the 80 kDa protein are highly likely to be important in modulating such activities of ON and other biological functions in bone as yet unknown. It is noteworthy that the intermolecular disulphide bonds between monomers of different ECM proteins in bone forming 'hetero-oligomers' has not been reported to date.

In summary, the findings in the present work indicate that the 80 kDa protein is another novel component of non-collagenous ECM proteins of bone. The protein undergoes the same post-translational modifications, such as glycosylation and phosphorylation as those well-known and characterized ECM proteins of bone, e.g. OPN, BSP, BAG-75 and ON. However, the 80 kDa protein has unique characteristics not shared by these proteins, i.e. it possesses ability to form polymers with itself through disulphide bonds, and intermolecularly disulphide bonds to ON resulting in possible new structures and functions that set this 80 kDa protein apart from any of the thus far characterized bone non-collagenous ECM proteins, with possible unique and important biological functions specific to bone as yet undetermined.

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