



# Association of bovine dentine phosphophoryn with collagen fragments

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## KEYWORDS

Dentine;  
Phosphophoryn;  
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**Summary** Bovine dentine phosphophoryn (BDP), a protein rich in aspartyl (Asp) and *O*-phosphoseryl (Ser(*P*)) residues, is synthesized by odontoblasts and believed to be involved in matrix-mediated biomineralization of dentine. Phosphophoryn was purified from bovine dentine using EDTA extraction, Ca<sup>2+</sup> precipitation, anion exchange and size exclusion chromatography. The purified protein migrated on SDS–PAGE as a single band. The protein was dephosphorylated using a chelex alkaline dialysis procedure, repurified using anion exchange and size exclusion chromatography and then subjected to cleavage with trypsin. The digest was subjected to reversed-phase HPLC and analysed by Q-TOF mass spectrometry. The only non-trypsin peptides that could be identified were two collagen Type I α2 peptides whose sequence was determined by fragmentation analysis. The association of collagen fragments with highly purified phosphophoryn suggests that the EDTA extraction method yields BDP that is strongly bound to collagen fragments. This association now helps explain discrepancies in molecular weight and amino acid composition data for various phosphophoryn preparations compared with the same data calculated from the C-terminal extension of mouse, rat and human dentine sialophosphoprotein (DSPP) gene products. Analysis of the mutation pattern of the clinical disorder Osteogenesis Imperfecta within the region enclosed by the identified collagen fragments reveals that phosphophoryn associates with a segment of collagen that is crucial for structure and/or function.

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*Abbreviations:* BDP, bovine dentine phosphophoryn; PAGE, polyacrylamide gradient gel electrophoresis; SDS, sodium dodecyl sulphate; DSPP, dentine sialophosphoprotein; HA, hydroxyapatite; DGI, dentinogenesis imperfecta; OI, osteogenesis imperfecta

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## Introduction

Bovine dentine phosphophoryn (BDP), a protein rich in aspartyl (Asp) and *O*-phosphoseryl (Ser(*P*)) residues is synthesized by odontoblasts and believed to be involved in matrix-mediated biomineralization of

dentine.<sup>1</sup> Autoradiographic<sup>2</sup> and immunohistochemical<sup>3</sup> studies have shown that dentine phosphophoryn is secreted directly into the mineralizing dentine extracellular matrix through the odontoblast cellular processes. Characterization of this protein has proved difficult due to the high degree of phosphorylation, extreme negative charge ( $pI$  1.1<sup>4</sup>) and redundant amino acid composition (80–90% Asp and Ser residues).<sup>1</sup> Due to its high negative charge<sup>4</sup>, it binds calcium with high affinity. When immobilised on a stable support and incubated in solutions containing physiologically relevant concentrations of calcium and phosphate, phosphophoryn can induce the formation of hydroxyapatite (HA).<sup>5</sup> However, at high phosphophoryn concentrations, when phosphophoryn is free in solution, phosphophoryn tends to inhibit the crystallisation of HA. It has been demonstrated that phosphophoryn binds to the (1 0 0) and (0 1 0) faces of hydroxyapatite, preventing crystal growth perpendicular to these faces thus promoting crystal growth along the *c*-axis.<sup>6</sup>

Phosphophoryn is also believed to have a specific affinity for collagen<sup>7,8</sup>, in particular it is believed to be associated in situ with the 'e' band of collagen.<sup>7</sup> This association along with phosphophoryn's dual ability to initiate or inhibit HAP formation has led to the currently widely held view that phosphophoryn plays an important role in the mineralization process. Phosphophoryn has been proposed to induce the formation of initial apatite crystals when attached to the gap regions of collagen fibrils, while the free form has been suggested to control the size, shape and orientation of crystals during growth by binding to specific crystal faces. It also has been reported that bovine phosphophoryn activates the differentiation of pulp cells to odontoblast-like cells possibly via enhancement of attachment of the cells to a matrix.<sup>9</sup>

Recently a gene (*dspp*) has been characterized in mouse, rat and human that encodes dentine sialophosphoprotein (DSPP) with a C-terminal extension that contains a repeat motif: Asp-[Ser]<sub>*n*</sub>- where  $n = 1-3$ .<sup>10,11</sup> The presence of the C-terminal extension rich in aspartyl and seryl residues, like the purified phosphophoryn protein has led to the hypothesis that the *dspp* gene encodes for both DSP and phosphophoryn.<sup>10</sup> This hypothesis was strengthened recently by the determination of the N-terminal sequence of bovine phosphophoryn which was almost identical to the deduced amino acid sequence of the start of the C-terminal extension of mouse, rat and human DSPP.<sup>12</sup> However, the reported amino acid composition of purified phosphophoryn<sup>1</sup> and its relative size do not match that deduced from the *dspp* gene (Tables 1 and 2). In rat

another DSPP homologue, termed dentine matrix protein 3 (DMP3) has also been reported.<sup>13</sup>

The only way to definitively assign the purified phosphophoryn protein to its encoding gene is to obtain further sequence information for the purified protein. Obtaining protein sequence information for phosphophoryn remains problematic due to the technical difficulties of sequencing long stretches of clustered phosphoserine residues. In addition, phosphophoryn appears to be considerably resistant to cleavage by enzymes. In this study, we have dephosphorylated phosphophoryn using a chelex alkaline dialysis procedure and then shown that enzymatic hydrolysis released peptides that were identified as derived from bovine Type I  $\alpha 2$  collagen. The presence of these peptides now helps explain the discrepancies between the reported bovine phosphophoryn amino acid composition and relative molecular weight and the corresponding information calculated from the C-terminal extensions of the human, rat and mouse *dspp* gene products.

## Materials and methods

### Extraction of dentine phosphophoryn

Bovine mandibles were obtained from local abattoirs. Unerupted teeth were extracted, cleaned free of soft tissue, freeze-dried, then powdered in a ball mill. The following procedures were carried out at 4 °C. The powder was equilibrated in 500 ml of 4.0 M guanidine-HCl, 50 mM Tris-HCl with a cocktail of protease inhibitors (2.5 mM benzamide hydrochloride, 50 mM  $\epsilon$ -amino-*n*-caproic acid, 0.5 mM *N*-ethylmaleimide and 0.3 mM phenylmethylsulfonyl fluoride) for 24 h. The residue was washed six times with Milli-Q water and then freeze-dried.

Demineralization of the dried residue was performed by diafiltration with an EDTA solution using an Amicon Stirred Cell containing a 150 mm Amicon YM10 membrane (MWCO 10,000) combined with an Amicon RC800 Mini-Reservoir. Pressure was applied using N<sub>2</sub> gas. Dry tooth powder (40 g) was demineralized using a total of 5 l of a solution containing 0.5 M EDTA-disodium salt, pH 7.0 and the cocktail of protease inhibitors. To remove EDTA, the retentate was diafiltered with the same membrane but using 2.5 l of a solution containing 50 mM Tris-HCl and the protease inhibitor cocktail. The retentate was removed and centrifuged for 30 min at 18,000  $\times g$  to separate the soluble and insoluble EDTA fractions. The supernatant (soluble EDTA extract) was decanted and filtered through a Millipore 0.22  $\mu m$  membrane.

**Table 1** Comparison of molecular weights of purified proteins or deduced molecular masses from DNA sequences for dentine phosphoprotein/phosphophoryn from bovine, rat mouse and human.

Values (kDa)		Methods	Reference
Protein <sup>a</sup>	cDNA <sup>b</sup>		
Bovine			
95		Gel filtration	58
155		SDS-PAGE	1,27
167		Sedimentation velocity	1
151		Viscosity	1
95		Gel filtration	58
Human			
	121.7		28
96		SDS-PAGE	59
140		SDS-PAGE	60
Mouse			
	69		10
72		SDS-PAGE	61
Rat			
	22.5 (PP171)		62
	74.7 (PP523)		63
	32 (PP240, rDSPP)		11
	29 (rDMP3)		13
65–71		Gel filtration	64
90–27		SDS-PAGE	26
30		Sedimentation equilibrium	65
Dephosph72		SDS-PAGE	23
Dephosph90		SDS-PAGE	24
Dephosph28		Analytical centrifuge	26
Dephosph28		Gel filtration	26

<sup>a</sup> Purified phosphophoryn.

<sup>b</sup> Deduced molecular mass from cDNA of the fully phosphorylated phosphophoryn-like domain of the DSPP gene.

The soluble EDTA extract was combined with an equal volume of 2.0 M CaCl<sub>2</sub> to precipitate the crude BDP. The precipitate was collected by centrifugation in a Beckman L8-70 Ultracentrifuge (125,000 × *g* for 30 min) and resuspended in 80 ml of 0.5 M EDTA-disodium salt, pH 8.0 containing the cocktail of protease inhibitors. The solution was diafiltered with 400 ml of 0.5 M EDTA containing the cocktail of protease inhibitors in an Amicon 8050 magnetically stirred cell using a 43 mm YM10 membrane. EDTA was removed by diafiltration with ten volumes (800 ml) of 50 mM Tris-HCl, pH 8.0. The diafiltrate was filtered through a 0.22 μm membrane prior to anion exchange chromatography.

### Chromatography

Anion exchange chromatography was carried out using a Pharmacia-LKB FPLC<sup>TM</sup> system with a Mono Q HR 5/5 column. Buffer A was 20 mM NH<sub>4</sub>HCO<sub>3</sub>, pH 8.0, 20 mM NaCl, and buffer B was 20 mM NH<sub>4</sub>HCO<sub>3</sub>, pH 8.0, 1.0 M NaCl. The diafiltrate containing the

crude phosphophoryn was applied to the Mono Q column and eluted with a linear gradient of 20–60% B over 50 min at a flow rate of 1 ml/min. The eluant was monitored at 214 nm wavelength. The major peak from the anion-exchange FPLC was then applied to a Superose 12 gel filtration column and eluted at a flow rate of 0.4 ml/min using 25 mM NH<sub>4</sub>HCO<sub>3</sub> pH 7.8 buffer.

### SDS-PAGE

Chromatographic fractions were subjected to SDS polyacrylamide gradient gel system (SDS-PAGE) using 4–15% linear acrylamide gradient gels (Bio-Rad Mini-Protean<sup>TM</sup> II Ready Gels) and the discontinuous buffer system of Laemmli.<sup>14</sup> The fully phosphorylated phosphophoryn was stained with Stains-All<sup>15</sup> or ammonium molybdate and rhodamine B according to the method of Debruyne.<sup>16</sup> The gel was subsequently stained in Coomassie Blue<sup>17</sup> overnight for the detection of the molecular weight markers.

**Table 2** Amino acid compositions reported for purified BDP<sup>1,27</sup>, and calculated from the h(DSPP)<sup>28</sup> mDSPP<sup>10</sup> rDSPP<sup>11,29</sup>, rDMP3<sup>13</sup> rPP523<sup>63</sup>, rPP171<sup>62</sup> genes.

RESIDUE	BDP	hDSPP	mDSPP	rDSPP	rDMP3	rPP523	rPP171
ALA	7	3	4	4	4	4	4
ARG	3	2	1	0	0	0	0
ASN	0	75	17	16	16	19	14
ASP	452	215	138	74	66	167	52
CYS	1	0	2	0	0	0	0
GLN	0	1	0	0	0	0	0
GLU	14	13	12	10	8	11	10
GLY	27	15	14	12	13	12	10
HIS	6	1	3	4	4	4	4
ILE	3	0	0	1	1	1	1
LEU	4	0	0	0	0	0	0
LYS	45	9	5	5	5	5	5
MET	1	0	0	0	0	0	0
PHE	2	0	0	0	0	0	0
PRO	6	1	1	1	1	1	1
PSER	518	—	—	—	—	—	—
THR	8	4	4	9	5	5	5
TRP	0	0	0	0	0	0	0
TYR	2	1	1	1	1	1	1
VAL	3	0	0	0	0	0	0
SER	27	498	281	103	92	293	64
Total aa	1129	838	483	240	216	523	171
Mass	—	81828	47021	24062	21578	51533	17387
Fully phosphorylated mass	154825	121668	69501	32302	28938	74773	22507

## Dephosphorylation

Phosphophoryn was dephosphorylated using a procedure already described.<sup>12</sup> Briefly, the procedure involved 4 day dialysis with a 500 MW cut-off Spectra/Por Float-a-lyzer dialysis bag in 0.5 M EDTA, pH 8.0 followed by a 10 day dialysis against 50 mM Tris, pH 8.0 in the presence of 10 g/l Chelex 100. The dialysis buffers were changed daily. The dephosphorylated phosphophoryn was repurified using anion exchange and size exclusion chromatography. Dephosphorylation was confirmed by several techniques: NMR spectroscopy, staining with ammonium molybdate/Rhodamine B<sup>16</sup> or Stains-All, organic phosphorus analysis<sup>18</sup>, and matrix assisted laser desorption ionisation-time of flight (MALDI-TOF) mass spectrometry (MS).<sup>12</sup>

## Digestion with trypsin

The freeze-dried, dephosphorylated phosphophoryn (10 ug) was denatured with 7 ul of 6 M Guanidine HCl for 1 h. After dilution with 93 ul of 20 mM NH<sub>4</sub>HCO<sub>3</sub> pH 7.8, the protein was digested with trypsin with an E:S ratio of 1:10 for 4 h. Digestion was stopped using conc. HCl. The digest was separated on a 4.6 mm × 150 mm Eclipse Zorbax XDB-C8 column (Agilent Technologies, Vic., Australia) using a Hew-

lett-Packard 1100 series HPLC instrument. Buffer A consisted of 5% CH<sub>3</sub>CN, 0.1% TFA and buffer B consisted of 85% CH<sub>3</sub>CN and 0.7% TFA. Bound material was eluted with a gradient applied at a flow rate of 0.2 ml/min as follows: 5% (v/v) solvent B for 8 min, a linear gradient of 5–85% (v/v) solvent B for 40 min, 85–100% (v/v) solvent B for 3 min, 100% (v/v) solvent B for 2 min, 100%-5% (v/v) solvent B for 3 min. The eluant was monitored at 214 nm.

## Q-TOF mass spectrometry

The collected tryptic peptides were injected onto a LC pre-column (300 um i.d., 5 mm, C18). The peptides were desalted and concentrated using an isocratic flow of (93%A:7%B) at a rate of 30 ul/min for 3 min. Buffer A consisted of 5% CH<sub>3</sub>CN, 0.1% HCOOH and buffer B consisted of 85% CH<sub>3</sub>CN and 0.7% HCOOH. The desalted peptides were eluted from the pre-column at a flow rate of 200 nl/min and then injected onto an analytical C18 column (75 nm i.d., 15 cm, C18 pepmap packing material) The peptides were eluted at a flow rate of 200 nl/min using linear gradient of 7–40% B in 30 min.

MS/MS spectra were acquired using a Q-TOF mass spectrometer (Micromass, Manchester UK) equipped with a Z-spray type ESI source. A capillary voltage of 3.5 kV was applied. Data were collected using the

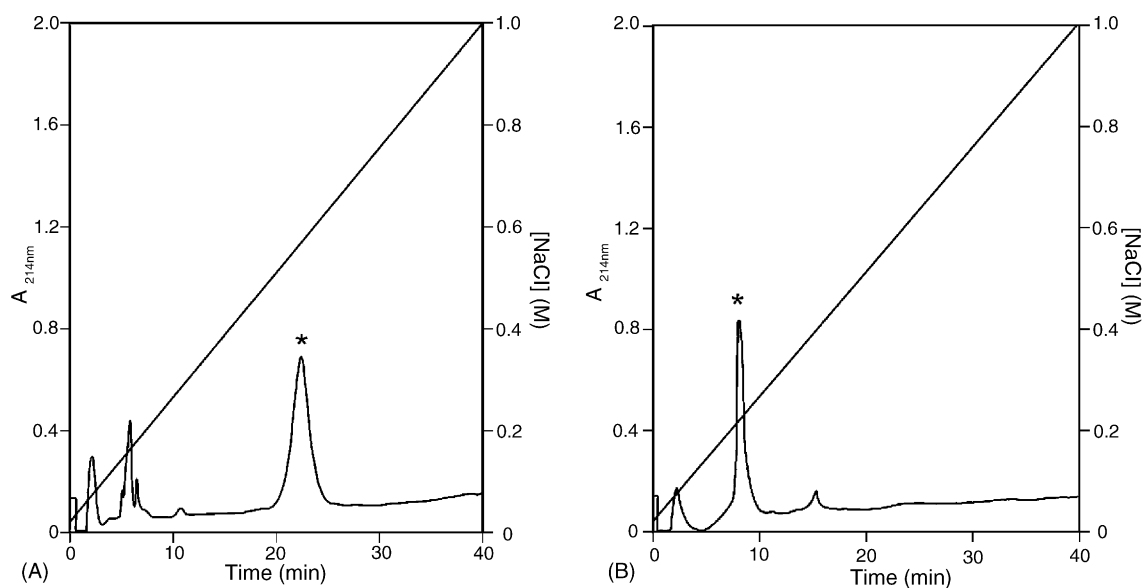
directed analysis mode of acquisition. Only doubly and triply charged peptides were selected for MS/MS analysis. Charge state recognition software was used to determine the optimal collision energy for each peptide selected for MS/MS analysis. The mass range for MS was 400–1500  $m/z$  and MS/MS was 50–2000  $m/z$ . Data were processed automatically by means of ProteinLynx software (Micromass, Manchester, UK) and identification was achieved by analysis with ProteinLynx Global Server version 1.1 (Micromass, Manchester, UK). Tolerances of 0.25 Da were set on the parent and fragment ions.

## Results

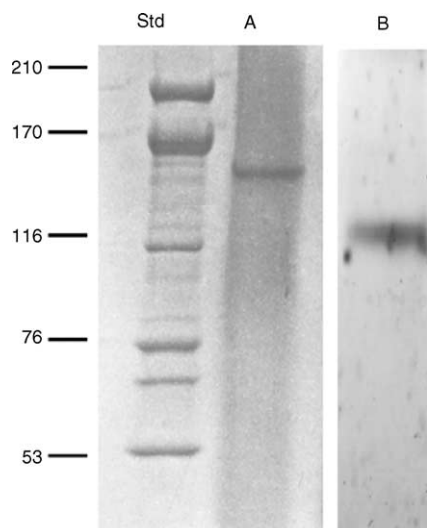
### Purification of BDP

The EDTA extract of bovine dentine was selectively precipitated with  $\text{Ca}^{2+}$  to separate the high affinity calcium binding proteins including phosphophoryn from the positively charged collagen and then diafiltered extensively to remove the  $\text{Ca}^{2+}$ . The demineralised protein extract was subjected first to anion-exchange FPLC to separate the highly acidic phosphophoryn from other less acidic proteins and any remaining positively charged proteins including collagen. The elution profile of phosphophoryn from the Mono Q anion-exchange column is shown in Fig. 1A. The major protein fraction was a highly negatively charged protein

at pH 8.0 as the peak eluted at approximately 0.57 M NaCl. This fraction was then subjected to size exclusion FPLC on a Superose 12 column to remove any smaller proteins and contaminating peptides and again the protein eluted as a single peak in the void volume (data not shown). The migration of this purified protein on SDS gradient PAGE is shown in Fig. 2A. The protein is highly phosphorylated as indicated by intense staining with Stains-all (Fig. 2A) and ammonium molybdate with rhodamine B (data not shown). The protein would not stain with Coomassie Blue. The phosphorylated protein migrated as a single band with an approximate molecular weight of 150 kDa, calculated from a linear regression of molecular weight standards. Dephosphorylation by extensive dialysis with chelex 100 at pH 8.0 resulted in a 104–107 kDa protein that eluted earlier on anion exchange (0.22 M NaCl) as shown in Fig. 1B and was able to be stained with Coomassie Blue after SDS-PAGE as shown in Fig. 2B as described previously.<sup>12</sup> The level of organic phosphate of the dephosphorylated protein was below the limit of detection.<sup>18</sup> MALDI-TOF MS analysis of the dephosphorylated phosphophoryn gave a molecular mass of 105 kDa. Using the Stetler-Stevenson and Veis<sup>1</sup> data the number of each amino acid type and their total number (Table 2) corresponding to a molecular mass of 155 kDa can be calculated as reported previously.<sup>12</sup> Assuming all the phosphoserine residues are present as dehydroalanine



**Figure 1** Elution profile of bovine dentine phosphophoryn using a Mono Q column with a flow rate at  $1 \text{ ml min}^{-1}$  and a linear gradient from 0 to 100% B. Buffer A was 0.02 M  $\text{NH}_4\text{HCO}_3$  pH 8.0, 0.02 M NaCl and Buffer B was 0.02 M  $\text{NH}_4\text{HCO}_3$  pH 8.0, 1 M NaCl, pH 8.0. (A) Fully phosphorylated phosphophoryn eluted at  $\sim 0.57$  M NaCl. (B) Dephosphorylated phosphophoryn eluted at  $\sim 0.22$  M NaCl.



**Figure 2** SDS polyacrylamide (4–15%) gradient gel. The gel shows: (A) fully phosphorylated phosphophoryn stained with Stains-All and (B) dephosphorylated phosphophoryn stained with Coomassie Blue. Track 1 shows high molecular weight markers ~212,000 Myosin (rabbit muscle); ~170,000 alpha 2-macroglobulin; ~116,000  $\beta$ -galactose (*E. coli*); 76,000 transferrin (human); ~53,000 Glutamic dehydrogenase (bovine liver).

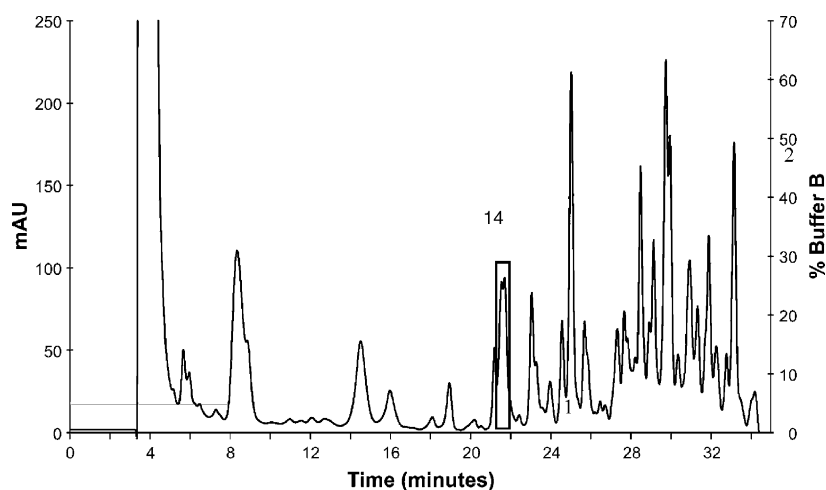
residues in our dephosphorylated form of the protein, through beta-elimination, then the Stetler-Stevenson and Veis<sup>1</sup> data predicts a molecular mass of 105,111 kDa for the dephosphorylated protein.<sup>12</sup> This value is extremely consistent with our MALDI-TOF MS measurement of 105 kDa. NMR spectroscopy was also consistent with the lack of Ser(P)

in the dephosphorylated protein due to the presence of a strong signal assigned to the dehydroalanyl methine protons.<sup>12</sup>

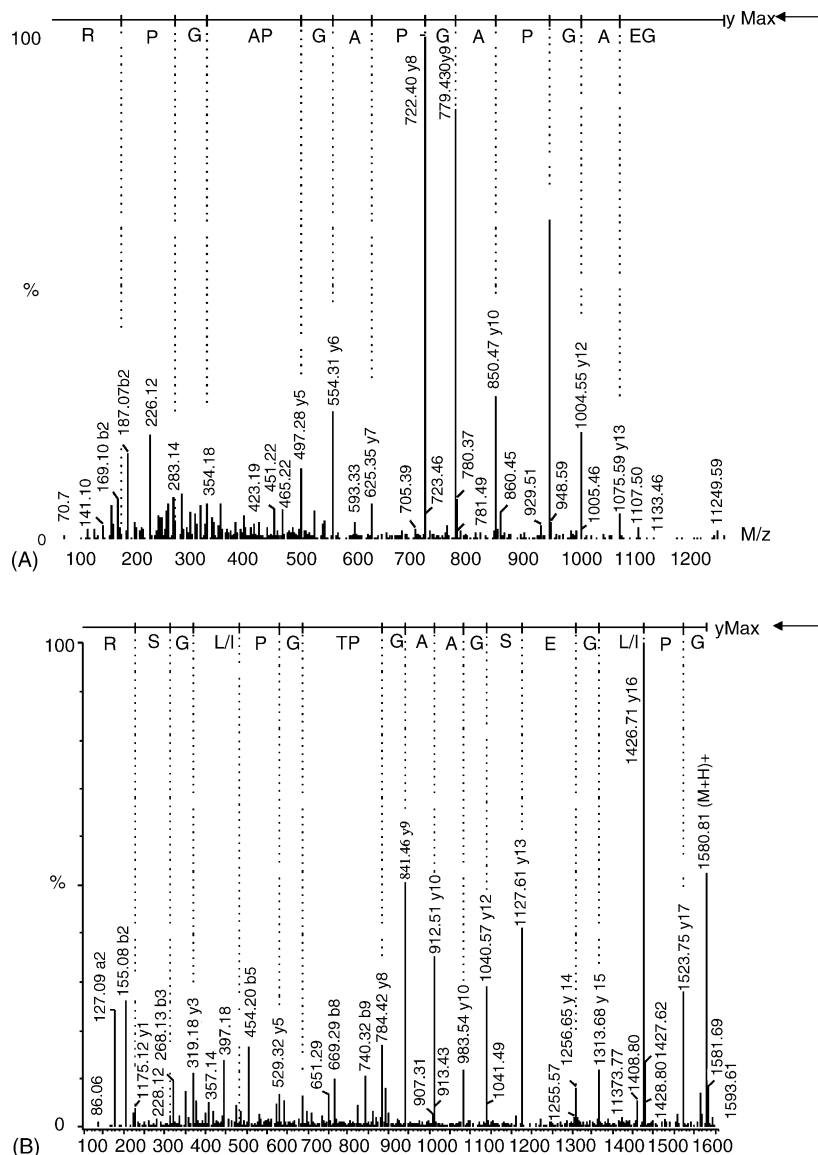
### Enzymatic digestion and mass spectrometry

The purified dephosphorylated protein was denatured in guanidine hydrochloride prior to digestion with trypsin. Fig. 3 shows the elution profile of the digest from Reversed Phase HPLC. MS analysis of the peaks collected indicated that the majority of the peaks were autolytic trypsin peptides. However, peak 14 in Fig. 3 contained peptides that could not be assigned to trypsin. This fraction therefore was subjected to Q-TOF MS/MS analysis. Fig. 4A and B shows the MS/MS spectrum of the fragmentation of two peptides 1260.621 and 1579.80 Da, respectively. BLAST searches against the sequence databases revealed that these two peptides assigned as GEAGPAGPAGPAGPR and GPLGESGAAGPTGPI/LGSR were derived from collagen. These collagen tryptic peptides are shown highlighted within the sequence of bovine collagen Type I  $\alpha$ 2 chain in Fig. 5.

These two peptides are devoid of the common G-X-P motif for proline hydroxylation in collagen. Of the expected collagen tryptic fragments, most contain this motif and hence are likely to be cross-linked in vivo. This cross-linking would produce branched peptides that would not be easily identified by the search algorithms used for the MS analysis. This may account for the lack of identification of other collagen tryptic peptides.



**Figure 3** Elution profile of the dephosphorylated phosphophoryn digest separated on a 4.6 mm  $\times$  150 mm Eclipse Zorbax XDB-C8 column (Agilent Technologies, Vic., Australia) using a Hewlett-Packard 110 series HPLC instrument. Buffer A consisted of 5% CH<sub>3</sub>CN, 0.1% TFA and buffer B consisted of 85% CH<sub>3</sub>CN and 0.7% TFA. A linear gradient of 5–85% buffer B was applied at a flow rate of 0.2 ml/min. The eluant was monitored at 214 nm. Fraction 14 was subjected to mass spectrometry.



**Figure 4** MS/MS spectrum of collagen peptides identified in fraction 14. (A) Fragmentation of peptide 1260.621 Da. The y-ions identified and the amino acids they represent are indicated. (B) Fragmentation of peptide 1579.8021 Da and the de novo sequence it produced. The y-ions identified and the amino acids they represent are indicated.

## Discussion

### Significance of collagen attachment

In this paper we have demonstrated that the EDTA extracted BDP that had undergone several steps of purification, is strongly associated with collagen fragments. The in situ co-location of collagen and phosphophoryn has been suggested.<sup>19–21</sup> However, previous studies have indicated that phosphophoryn associated with collagen, is found only in the EDTA-insoluble fraction of dentine with a ratio of free versus collagen bound phosphophoryn estimated at 4:1.<sup>21</sup> In contrast our results indicate that soluble phosphophoryn derived from EDTA extracts of den-

tine may also be bound to collagen fragments. The purification processes employed in this study would have adequately purified the highly negatively charged phosphophoryn from any collagen fragments that may have been contaminating the sample. Therefore, the presence of the collagen fragments in the phosphophoryn preparation could not have arisen by the reactive dehydroalanine formed during the dephosphorylation procedure covalently attaching to contaminating collagen fragments.

Obtaining accurate mass data for phosphophoryn has been difficult using mass spectrometry. Attempts using conventional techniques other than MS, report considerable variation and anomalous

MLSFVDTRTLTLLAVTSLATCQSLQEATARKGSPGDRGFRGERGPPGPP	50
GRDGDGIPGPPGPPGPPGPPGLGGNFAAQFDAKGGGPGMGLMGPRGPP	100
GASGAPGPPQGFQGGPPGEPGEPGQTGPAGARGPPGPPGKAGEDGHPGKPR	150
PPERGVVPPQGARGFPGTFFGLPGFKGIRGHNGLDGLKGGPPGAPGVKGEFG	200
APGENTPQQTGARGLPGERGRVGPAGARGSDGSPVGPVGPAGFIGSA	250
GPPGFFGAPGPKGELGVPVGNPAGPAGPRGEVGLPGLSGPVGPPGNPGA	300
NGLPGAKGAAGLPVAGAPGLPGRGIPGPVGAAGATGARGLVGEPGPAG	350
SKGESGNKGEPAVGQPPGPPGSGEEGKRSTGEIGPAGPPGPPGLRGNP	400
GSRGLPGADGRAGVMGPAGSRGATGPAGVRGPNDSGRPGEPGLMGPRGF	450
PGSPGNIGPAGKEGVPVGLPGIDGRPGPIGPAGARGEFGNIGFPPGPKGSPG	500
DPGKAGEKGHAGLAGARGAPPDGNNGAQGPPGLQGVQGGKGEQGPAGPP	550
GFQGLPFPAGTAGEAGKPPGERGIPGEFGLPFPAGARGER <u>PPGESGAAGP</u>	600
<u>TGPIGSRGSPGPPGPDGNKGEPPVVGAPGTAGPSGSPGLPGERGAAGIPG</u>	650
<u>GKGEKGETGLRGDIGSPGRDARGAPGAI GAPFPAGANGDRGEAGPAGPA</u>	700
<u>GPAGPRGSPGERGEVGPAGPNGFAGPAGAAGQPGAKGERGTPKPKGENP</u>	750
VGPTGPVGAAGPSGPNP <b>PPGPAGSRGDGGPPGATGFPGAAGRTGPPGSPG</b>	800
<b>ISGPPGPPGPAKKEGLRGRGDQGPVGRSGETGASGPPGFVGEKGPSPGEP</b>	850
GTAGPPGTPGPPQGLLGAAPGFLGLPGRGERGLPGVAGSVGEPGLGIAGP	900
PGARGPPGNGVNPVNGAPGEAGRDGNPNNDGPPGRDQGFHKGERGYPG	950
NAGPVGAAGAPGPPQGVGVPVGHGNGRGEPPAGAVGPAGAVGPRGSPGPPQ	1000
GIRGDKGEPGDKGPRGLPGLKGHNGLQGLPGLAGHHGDQGAPGAVGPAGP	1050
RGPAGSPGPAK <b>DGRIGQ</b> PGAVGPAGIRGSQGSQGPAGPPGPPGPPGPPG	1100
PSGGGYEFGFDGDFYRADQPRSPSLRPKDYEV DATLKLNNQIETLLTP	1150
EGSRKNPARTCRDLRLSHPEWSSGYWIDPNQCTMDAIKVYCDFSTGET	1200
CIRAQPEDI PVKNWYRNSKAKKHVVVGETINGTQFEYNVEGVTTKEMAT	1250
QLAFMRLLANHASQNI TYHCKNS IAYMDEETGNLKKAVILQGSNDVELVA	1300
EGNSRFTYTVLVDGCSKKTNEWQKTI IEYKTNKPSRLPILDIAPLDIGGA	1350
DQEIRLNIGPVCFK	1364

**Figure 5** Sequence of bovine collagen Type I  $\alpha 2$  chain<sup>57</sup>, double underlined highlighting the position of the two collagen peptides identified in this study. The corresponding region of rat collagen in contact with rat incisor phosphophoryn reported by Dahl et al.<sup>8</sup> is highlighted in single underline, and that reported by Di Lullo et al.<sup>34</sup> is highlighted in bold. The peptide equivalent to the Type I  $\alpha 1$  chain reported to be associated with phosphophoryn is highlighted in bold and italic.<sup>7</sup>

behaviour. The post-translational event of binding to collagen may explain the discrepancies that have been reported in the literature for: (a) the molecular weights for phosphophoryns measured using a variety of techniques; (b) the amino acid compositions of phosphophoryn from different species; and (c) the NMR spectral data obtained for bovine phosphophoryn.

### Discrepancies in reported molecular weights of phosphophoryns

Table 1 shows the comparison of molecular weights of purified dentine phosphophoryn preparations obtained from bovine, rat, mouse and human dentine using a variety of techniques. The variation in molecular weight observed for the different preparations from the same species may be explained by the unique physicochemical properties of the protein. For example, selected methods including sedimentation velocity and ultracentrifugation actually measure hydrodynamic volume rather than molecular weight. Globular proteins are used as standards for most techniques, however due to the high negative charge, it is unlikely that phosphophoryn exhibits characteristics of globular proteins in solution. Recent studies of the protein dynamics of phosphophoryn have demonstrated that phosphophoryn is a molecule of uniform mobility,

thus belonging to a recently identified class of intrinsically disordered proteins that are characterised by sequences of low complexity and rich in polar and charged residues.<sup>22</sup>

Nevertheless, these experimentally determined values for the purified protein are greater than the deduced molecular mass calculated from the cDNA of the phosphophoryn-like domains of the known DSPP genes (Table 2). For example, the human DSPP-like sequence yields a theoretical mass of 121 kDa assuming 100% phosphorylation; however the most recent experimentally determined molecular weight for human phosphophoryn using SDS-PAGE is 140 kDa. Similarly, the reported molecular weights for dephosphorylated rat phosphophoryn 72<sup>23</sup> and 90 kDa<sup>24</sup> (Table 1) are considerable greater than the deduced masses of the unphosphorylated phosphophoryn domains of the rDSPP (24 kDa) and rDMP3 (21.6 kDa) genes (Table 2). It has been reported that intrinsically unstructured proteins bind less SDS than usual because of their unusual amino acid composition, consequently their apparent Mr is often 1.2–1.8 times higher than the real molecular mass deduced from sequence data.<sup>25</sup> However, the presence of collagen fragments may also help explain these discrepancies and the reported anomalous behaviour in SDS-PAGE, analytical gel filtration in 4 M Gdn-HCl buffer<sup>1</sup> and in the analytical ultracentrifuge.<sup>26</sup>

## Discrepancies in reported amino acid compositions of phosphophoryns

The first reported amino acid composition of bovine phosphophoryn indicated that 95% of the Ser residues were phosphorylated.<sup>1</sup> Table 2 shows the amino acid composition obtained for BDP<sup>1,27</sup> and the theoretical amino acid composition of human, mouse and rat phosphophoryn-like domains based on the h(DSPP1)<sup>28</sup>, mDSPP<sup>10</sup>, rDSPP<sup>11,29</sup> and rDMP3<sup>13</sup> genes, respectively. For the phosphophoryn-like domains deduced from the human, mouse and rat phosphophoryn genes, the number of glycines and prolines are fewer than that experimentally measured for BDP.<sup>1</sup> The human DSPP-like sequence contains only 16 glycines and 1 proline in the phosphophoryn-like domain. Similarly, the mouse and rat sequences have only 12–14 glycyl and 1 prolyl residues in the phosphophoryn-like domains. In contrast, the reported bovine phosphophoryn amino acid compositions contain 22–27 glycines and 5–6 prolines.<sup>1,27</sup> Since collagen is rich in glycines and prolines, the attachment of collagen fragments may therefore account for this discrepancy between the observed amino acid compositions of the purified phosphophoryns and the expected amino acid compositions from the reported DSPP genes.

## Discrepancies in reported nmr spectra of bovine phosphophoryn

Based on the reported amino acid composition of BDP and the results of an NMR study of bovine phosphophoryn, Evans et al.<sup>27</sup> postulated that BDP behaved in solution as a protein having regions of differing mobility. It was suggested that the mobile regions in the protein represented 'hinge regions' that interrupt the long stretches of rigid polyanionic sequences made of aspartyl and phosphoseryl residues.<sup>27</sup> They further proposed that the hinge regions were comprised of seryl, prolyl and glycyl residues. However, this interpretation is difficult to reconcile with the relatively featureless phosphophoryn-like domains of the published DSPP sequences consisting of long uninterrupted stretches of aspartyl and phosphoseryl residues. The results of the current study are therefore consistent with the spin systems for prolines, glycines and serines that were observed by Evans et al.<sup>27</sup> being derived from the bound collagen fragments and not from phosphophoryn.

## Location of binding to collagen

The Type I collagen molecules are supercoiled assemblies of three polypeptide chains of over 1000 residues. These molecules aggregate with their

long axes in parallel to form fibrils of various thickness in different tissues. Within the fibril, there is a linear shift of some 67 nm (D-period) between neighbouring molecules. This arrangement of staggered fibril leaves gaps of 0.6 D between the ends of the co-linear collagen molecules and a complementary 0.4 D overlap region for every 67-nm fibril repeat as reviewed recently.<sup>30</sup>

The binding of phosphophoryn to collagen has been investigated using a variety of methods including rotary shadowing and electron microscopy<sup>8,31</sup> and immunolabelling.<sup>7,32</sup> In vitro binding studies of biotin-labelled bovine phosphophoryn to bovine collagen CNBr peptides, suggested that the  $\alpha$ 1CB6 collagen peptide, had the highest affinity for the labelled phosphophoryns.<sup>7</sup> The matrix-bound phosphophoryn prepared from CNBr digests of bovine dentine insoluble matrix was also analyzed by chromatography, SDS-PAGE, immunoblotting and finally subjected to enzymic digestion, purification and sequence analysis. One peptide sequence was reported to be Asp–Gly–Leu–Asn–Gly–Leu–Hyp corresponding to the sequence starting from Asp975 of the  $\alpha$ 1(I) chain within the  $\alpha$ 1CB6 region.<sup>7</sup> This was the only identification of a collagen sequence reported to bind phosphophoryn to date.

Traub et al.<sup>33</sup> observed phosphophoryn binding to the 'e' band of complexes formed in vitro of turkey tendon and rat dentine phosphophoryn. Another study reported binding of monomeric rat incisor phosphophoryn to monomeric rat Type I collagen at a single interaction site 210 nm from the N-terminus where the sequence contains both positively and negatively charged residues further extrapolated as residues  $72 \pm 26$  or 698–750 as shown in Fig. 5.<sup>8</sup> This corresponds to the 'c' band of the collagen fibril at the N-terminal edge of the overlap zone. CLUSTAL alignment of the rat (accession no.: CAB01633) and bovine Type I  $\alpha$ 1 chains suggests that this corresponds to residues 619–671 within the bovine sequence as shown in Fig. 5. Furthermore, a recent review of collagen binding sites highlighted residues 768–838 as the binding sequence for phosphophoryn based on the reports of Traub et al.<sup>33</sup> and Dahl et al.<sup>8</sup> as shown in Fig. 5.<sup>34</sup>

In contrast, a later study suggested two collagen binding sites of rat phosphophoryn close to the gap and overlap boundaries; thus extrapolating to residues >750.<sup>32</sup> It is difficult to accurately correlate distances measured from electron micrographs with residue positions. This is due to inconsistencies caused by dehydration and demineralization of dentine during sample preparation, and also due to the variation of the structure, organization and composition of the collagen matrix from the proximal to the distal zones of predentine prior to

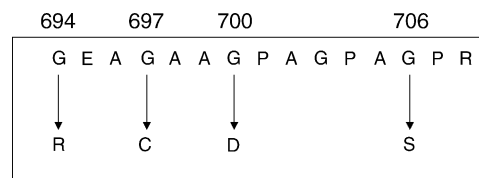
mineralization as detailed by Beniash et al.<sup>32</sup> The collagen peptides we have identified in this report appear to be consistent with the extrapolations made by Dahl et al.<sup>8</sup>

The two collagen peptides identified in this study were constructed in silico using Sybyl 6.8 with  $\phi$  and  $\varphi$  angles observed previously in reported X-ray structures of synthetic peptides (1DZI and 1QSU)<sup>35,36</sup> in triple helix conformation. The peptides measured ~40 and 50 Å, respectively. The minimum stretch of 117 residues comprising the two peptides span 325 Å within a triple helix. This would suggest an additional minimum molecular weight of 10,218 Da attached to the phosphophoryn molecule. Since phosphophoryn is a large molecule with long uninterrupted stretches of aspartyl and phosphoserine residues, the binding of phosphophoryn with multiple strands and multiple fibrils of collagen cannot be overlooked. It has previously been estimated that the ratio of collagen to phosphophoryn is 2:3 based on the high molecular weight of matrix bound phosphophoryn and amino acid composition data.<sup>7</sup> However, to date, the extent of intermolecular contact between collagen and phosphophoryn has not been determined.

Recently the binding of the protein DMP1 with collagen fibrils was investigated.<sup>37</sup> Using a solid phase binding assay, the C-terminal domain of DMP1 demonstrated high affinity towards Type 1 collagen. Subsequently, the C-terminal DMP1 was digested with trypsin and passed through a collagen-immobilized column. MALDI-TOF MS analysis revealed three peptides corresponding to two highly acidic regions<sup>349</sup>DSEDDDEDR<sup>357</sup> and <sup>424</sup>SEENRSDSQDSSR<sup>437</sup> that were designated “collagen interactive clusters”.<sup>37</sup> Site-directed mutagenesis of these two clusters with neutral sequences resulted in reduced affinity for collagen suggesting that the binding is driven by electrostatic interactions. In the case of phosphophoryn, the majority of the sequence is highly and evenly negatively charged, therefore, similar “collagen interactive clusters” may not be specific or localized.

### Significance of bound collagen peptides

Recently, the binding sites for nearly 30 ligands and over 300 disease-associated mutations have been mapped to the human Type 1 collagen.<sup>34</sup> The human equivalent of the bovine collagen peptide associated with BDP <sup>590</sup>GPLGESGAAGPTGPI/LGSR<sup>607</sup> is reported to be part of the binding site for  $\alpha 1\beta 1$  and  $\alpha 1\beta 2$  integrins.<sup>34</sup> Similarly, the human Type I  $\alpha 1$  equivalent of the other BDP associated collagen sequence <sup>691</sup>GEAGPAGPAGPAGPR<sup>705</sup> is reported to



**Figure 6** Reported mutations within the fragment resulting in Type II osteogenesis imperfecta.

be part of the binding site of cartilage oligomeric protein (COMP).<sup>34</sup>

The vast majority of mutations of Type I collagen result in osteogenesis imperfecta (OI).<sup>38,39</sup> This is a heritable disorder characterized by increased bone fragility, thus giving rise to its more common name “brittle bone disease”. It is generally classified according to severity ranging from a mild (Type I) to a lethal form (Type II) that does not usually allow survival beyond the neonatal period. Type III is progressive and deforming, with severe osteopenia and growth deficiency while Type IV is more moderate.<sup>40–42</sup>

The most common type of mutation on *COL1A1* and *COL1A2* genes are single base changes causing substitutions of glycines that are essential for correct folding of the collagen triple helix.<sup>43,44</sup> It has been observed that substitutions by D, V and R are more deleterious than S, C and A.<sup>45,46</sup> The collagen mutation database<sup>43,44</sup> (<http://www.le.ac.uk/genetics/collagen>) was examined for mutations within the observed peptides. Four mutations within the peptide sequence GEAGAAGPAGPAGPR have been reported to be associated with OI Type II as shown in Fig. 6.<sup>47–50</sup> The distribution of the reported mutations for Type II OI supports a regional model of alternating non-lethal/lethal regions along the chain.<sup>50</sup>

### Relationship between dentinogenesis imperfecta and osteogenesis imperfecta

The autosomal dominant disorder, dentinogenesis imperfecta (DGI) is classified into three types based on clinical features. DGI-I is the least severe and always is associated with OI whereas the more severe forms II and III are restricted to dentine.<sup>10</sup> The restricted expression of *Dspp* and its physical localization on the human chromosome 4 within DGI-III locus, implicated *Dspp* as a potential candidate gene.<sup>51,52</sup>

In patients with DGI-II and dentine dysplasia II disorders, mutations have been found in the dentine sialoprotein part of the *Dspp* gene.<sup>53,54</sup> So far no mutations have been found in the C-terminal phosphophoryn-like coding region. This is not

unexpected, as single residue mutations within the highly charged and featureless sequence of phosphophoryn, are unlikely to have a dramatic effect on its function or on the binding to collagen. Recently, a DSPP knock-out mouse produced teeth with widened pre-dentin zone and defective dentine mineralization similar to that observed in human DGI-III.<sup>55</sup> It was suggested that the *Dspp* gene has a crucial role in orchestrating the events during dentinogenesis including regulation of proteoglycan levels.<sup>55</sup>

Current evidence suggests that DGI is present in all children with OI, with a continuum from minimal to severe dentine pathology.<sup>56</sup> The teeth from patients with OI and DGI were recently investigated using light and polarized light microscopy, scanning and electron microscopy (SEM, TEM) selected area diffraction (SAD) and X-ray spectroscopy (EDX). These studies showed structurally normal enamel with severe pathologic changes in the dentine.<sup>56</sup>

Given the localisation of lethal mutations of Type II OI within the sequence of Type I  $\alpha 2$  collagen that was found associated with phosphophoryn in this study (Fig. 6), it is likely that this region is extremely important for collagen structure and function. It is tempting to speculate that mutations within this region of collagen may affect the proper formation of dentine by preventing template formation through phosphophoryn cross-linking to collagen; this would account for the prevalence of the mild form of DGI with OI. Furthermore, there may be other proteins that also bind to this region eg. the nucleating equivalent of phosphophoryn in bone, whose interactions are affected.

## Conclusion

We have demonstrated the strong attachment of collagen fragments with phosphophoryn purified from bovine unerupted teeth in the soluble fraction of the EDTA extract. These fragments of collagen have been retained throughout a purification process involving anion exchange and size exclusion chromatography and have been released only after extensive dialysis to remove calcium, dephosphorylation, further purification and enzymatic digestion with trypsin. The presence of collagen is likely to account for discrepancies in the reported literature on the amino acid composition and molecular weight of phosphophoryn when compared with that information calculated from the C-terminal extension of the *dspp* gene product. Furthermore we have established that phosphophoryn associates with a segment of collagen that is crucial for structure

and/or function, based on the mutation pattern of the clinical disorder OI.

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