Characterization of porcine osteonectin extracted from foetal calvariae

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Osteonectin, extracted from foetal porcine calvariae with 0.5 M-EDTA, was purified to homogeneity by using gel filtration and polyanion anion-exchange fast protein liquid chromatography under dissociative conditions without the need of reducing agents. The purified protein migrated with an M_r of 40 300 on SDS/polyacrylamide gels and was similar to bovine osteonectin in both amino acid composition and in its ability to bind to hydroxyapatite in the presence of 4 M-guanidinium hydrochloride (GdmCl). However, unlike the bovine protein, porcine osteonectin did not bind selectively to hydroxyapatite when EDTA tissue extracts were used. In addition, purified porcine osteonectin did not show any apparent affinity for either native or denatured type I collagen, but did bind to serum albumin. Primary sequence analysis revealed an *N*-terminal alanine residue, with approximately one-half of the subsequent 35 residues identified as small hydrophobic amino acids and one-quarter as acidic amino acids. The only significant difference between the *N*-terminal sequences of the bovine and porcine proteins was the deletion of the tripeptide Val-Ala-Glu in porcine osteonectin. In contrast with bovine osteonectin, far-u.v.c.d. of porcine osteonectin revealed considerable secondary structure, of which 27 % was α -helix and 39 % was β -sheet. Cleavage of the molecule with CNBr under non-reducing conditions generated five fragments, of which two major fragments (M_r 27 900 and 12 400) stained blue with Stains All, a reagent that stains sialic-acid-rich proteins/phosphate-containing proteins and/or Ca^{2+} -binding proteins blue while staining other proteins pink. The 12 400- M_r fragment bound $^{45}\text{Ca}^{2+}$ selectively, indicating a Ca^{2+} -binding site in this part of the molecule. The 27 900- M_r fragment did not bind Ca^{2+} , and since biosynthetic studies with $^{32}\text{PO}_4^{3-}$ did not show phosphorylation of porcine osteonectin, this fragment is likely to be highly acidic. The incomplete cleavage of the molecule with CNBr and the ability of the molecule to regain its secondary structure after exposure to 7 M-urea are features consistent with the molecule having a compact structure that is stabilized by numerous disulphide bridges. The chemical and binding properties of porcine osteonectin are closely similar to the recently described 'culture shock', SPARC and BM-40 proteins, indicating that these are homologous proteins.

INTRODUCTION

The isolation of bone proteins has been facilitated by the recent introduction of dissociative solvents to extract the proteins and the inclusion of proteolytic-enzyme inhibitors to prevent degradation during their extraction and subsequent purification (Linde *et al.*, 1980; Termine *et al.*, 1980). Several of the major bone proteins have been prepared in this way, including a small proteoglycan (Fisher *et al.*, 1983a), sialoprotein (Fisher *et al.*, 1983b), osteocalcin and osteonectin (Termine *et al.*, 1981a,b). More refined purification procedures have subsequently identified several forms of the proteoglycan (Franzén & Heinegård, 1984a,b; Sato *et al.*, 1985a) and sialoprotein (Franzén & Heinegård, 1985; Sato *et al.*, 1985b).

Osteonectin, which was originally characterized as a phosphorylated glycoprotein that binds Ca^{2+} , represents approx. 25 % of the non-collagenous proteins of foetal bovine bone (Termine *et al.*, 1981a). Particular interest in

this protein stems from early investigations indicating that osteonectin, as implied by its name, binds to the inorganic (hydroxyapatite) and organic (collagen) phases of bone and may also initiate the precipitation of $\text{Ca}^{2+}/\text{PO}_4^{3-}$ on to collagen fibrils (Termine *et al.*, 1981b). Osteonectin purified from adult bovine bone was subsequently shown to inhibit hydroxyapatite crystal formation more strongly than osteocalcin (Romberg *et al.*, 1986), and also to bind to a partially purified type I collagen preparation (Romberg *et al.*, 1985). Some structural characteristics of this protein, isolated under non-dissociative conditions, were also reported (Romberg *et al.*, 1985).

In previous work we studied the biosynthesis of osteonectin by calvarial cells (Otsuka *et al.*, 1984) and from mRNA (Kuwata *et al.*, 1985) prepared from foetal porcine calvariae. By using a cell-free translation system, the osteonectin was shown to be synthesized in a pre-osteonectin form (Kuwata *et al.*, 1985) that was processed to osteonectin by signal-peptidase activity. After removal

Abbreviations used: f.p.l.c., fast protein liquid chromatography; GdmCl, guanidinium chloride; PBS, phosphate-buffered saline solution deficient in Ca^{2+} and Mg^{2+} [KH_2PO_4 (0.20 g/l)/ $\text{Na}_2\text{HPO}_4 \cdot 7\text{H}_2\text{O}$ (2.16 g/l)/NaCl (8.00 g/l)/KCl (0.2 g/l), pH 7.2]; e.l.i.s.a., enzyme-linked immunosorbent assay; PBS/Tween 20, 0.05 % Tween 20 in PBS; TBS, Tris-buffered saline [Tris (1.21 g/l)/NaCl (9.0 g/l), pH 7.4]; EHS, Engelbreth-Holm-Swarm; SPARC, secreted protein acidic and rich in cysteine; SCAB protein, small collagenous apatite-binding protein.

of the signal peptide, the cell-synthesized osteonectin did not appear to be processed further. Like bovine osteonectin, the cell-synthesized osteonectin showed binding to hydroxyapatite in the presence of 4 M-GdmCl (Otsuka *et al.*, 1984). However, no apparent binding to various forms of collagen could be observed (Otsuka *et al.*, 1984). By using antibodies against osteonectin we have also shown that porcine osteonectin is synthesized by fibroblasts (Wasi *et al.*, 1983, 1984; Zung *et al.*, 1986; Otsuka *et al.*, 1988), and is present in various tissues other than bone (Tung *et al.*, 1985). Of relevance to these observations is the recent identification of three glycoproteins named 'culture-shock', 'SPARC' and 'BM-40' protein respectively, synthesized by bovine endothelial cells (Sage *et al.*, 1984), mouse parietal endodermal cells (Mason *et al.*, 1986a), and EHS tumour cells (Mann *et al.*, 1987). These soft-tissue-derived proteins appear to be closely related to, and show structural homology with, bovine osteonectin (Mason *et al.*, 1986a; Mann *et al.*, 1987), a protein thought to be bone-specific (Termine *et al.*, 1981b).

Here we describe chemical, physical and binding properties of porcine osteonectin. Some of these properties are distinctly different from those reported for bovine osteonectin and are not consistent with the proposed function of this protein. In addition, we show that osteonectin can be separated from proteins that appear to be associated with the mineralized collagenous matrix of bone.

MATERIALS AND METHODS

Extraction procedure

Foetal pigs were obtained from a local abattoir and within 2 h of slaughter the calvariae were excised and placed in ice-cold PBS, pH 7.4, deficient in Ca^{2+} and Mg^{2+} and containing proteinase inhibitors: 6-amino-n-hexanoic acid (100 mM), benzamidine hydrochloride (5 mM), *N*-ethylmaleimide (5 mM) and phenylmethanesulphonyl fluoride (1 mM). Soft tissue adhering to the bone was removed with tissue paper and the remaining hard tissue was freeze-thawed three times in PBS + proteinase inhibitors. The bone was then frozen in liquid N_2 and crushed with a mortar and pestle in order to ensure the exposure of the inner blood-containing cavities in bone to the extracting solutions. All subsequent steps were carried out at 4 °C with constant stirring. The bone chips (100 g) were washed further in PBS + proteinase inhibitors (three times for a total of 24 h) to remove soluble components, including cell debris and blood proteins that are weakly associated with bone. The tissue was then extracted four times with 4 M-GdmCl (Sigma)/50 mM-Tris/HCl, pH 7.4 (G-buffer) with proteinase inhibitors (50 ml/g of tissue) for a total of 96 h to remove non-covalently associated components in the remaining soft tissue, including osteoid (G_1 extract). The residue was then washed three times in PBS + proteinase inhibitors for 24 h before extraction with 0.5 M-EDTA (Sigma) in 50 mM-Tris/HCl, pH 7.4 (E-buffer) with proteinase inhibitors (four times for a total of 96 h at 50 ml/g of tissue) to release mineral-associated proteins (E extract). The bone residue was then washed three times with PBS + proteinase inhibitors and was then extracted again with G-buffer (four times over 96 h) to release proteins which remained associated with the exposed collagenous matrix (G_2 extract). The G_1 , E, and

G_2 extracts were then concentrated by ultrafiltration using a YM-10 membrane (Amicon Corp., Lexington, MA, U.S.A.). The G_1 , E, and G_2 concentrates were exhaustively dialysed against 10 mM- NH_4HCO_3 at 4 °C with constant stirring, using Spectrapor I acetylated dialysis tubing. The dialysed extracts were freeze-dried in preparation for gel filtration.

Sephacrose CL-6B chromatography

Chromatography on Sepharose CL-6B was performed either in 4 M-GdmCl as described by Termine *et al.* (1980) or in 7 M-urea. For each fractionation, 0.12 g of freeze-dried E-extract was dissolved in either G-buffer or 7 M-urea in 50 mM-Tris/HCl, pH 8.0, and chromatographed with the same buffer at 20 °C using two columns (each 2.6 cm × 95 cm) in tandem, packed with Sepharose CL-6B. A flow rate of 20 ml/h was used, and 6 ml fractions were collected. For the 4 M-GdmCl-fractionated sample, the column fractions containing osteonectin were pooled, concentrated by ultrafiltration on YM-10 Amicon filters and were further fractionated under the same conditions on tandem Sephacryl-200 columns (each 1.6 cm × 95 cm). A flow rate of 12 ml/h was used, with 2.0 ml fractions being collected. Fractions enriched in osteonectin were then pooled, dialysed, freeze-dried and were subjected to anion-exchange chromatography. For proteins separated in 7 M-urea, fractions containing osteonectin were directly applied to f.p.l.c. polyanion anion-exchange resin as described below. All column chromatography runs were monitored at A_{230} and by analysing portions of the collected fractions on SDS/polyacrylamide gels. Preparations chromatographed in 4 M-GdmCl were used for primary sequence analysis and experiments involving the binding of osteonectin to collagen- and gelatin-Sepharose resins. In all other experiments, osteonectin prepared in 7 M-urea was used.

Ion-exchange chromatography

Ion-exchange chromatography was performed at 20 °C with 7 M-urea in 50 mM-Tris/HCl buffer, pH 8.0, using a linear 0–1.0 M-NaCl gradient on an automated f.p.l.c. system (Pharmacia, Uppsala, Sweden) and either a preparatory grade polyanion SI column (1 cm × 10 cm) or an analytical-grade polyanion SI column (0.5 cm × 5.0 cm). Fractions enriched in osteonectin from the preparatory runs were pooled, concentrated and freed from NaCl on Sephadex G-25, in start buffer, and rechromatographed on the analytical column. Column runs were monitored by A_{230} , and the fractions were analysed on SDS/polyacrylamide gels. Protein fractions containing osteonectin were pooled, dialysed against 10 mM- NH_4HCO_3 and freeze-dried. An HR 5/5 Mono Q (Pharmacia) anion-exchange column could also be used instead of the polyanion column for the purification steps, with best results being obtained using a 20 mM-piperazine buffer, pH 6.0, a linear 0–1.0 M-NaCl gradient and a flow rate of 1.5 ml/min.

Polyacrylamide-gel electrophoresis

Electrophoresis in the presence of 0.1% SDS was carried out with 12.5% and 20% (w/v) linear, or 5–20% (w/v) gradient cross-linked polyacrylamide gels, using the discontinuous Tris/glycine buffer system of Laemmli (1970) on the Bio-Rad 16 cm Protean apparatus. Samples were dissolved in 40 μl of sample buffer containing 2%

(w/v) SDS, 2 M-urea, and 0.0032 % (w/v) Bromophenol Blue dye. For reduction, 10 μ l of a 75 mg/ml solution of dithiothreitol was added before heating the samples at 56 °C for 30 min. Gels were stained with 0.25 % (w/v) Coomassie Brilliant Blue R-250 (Sigma) by the method of Fairbanks (1971), or 0.01 % (w/v) Stains All (Eastman Organic Chemicals) as described by Campbell *et al.* (1983). In SDS/polyacrylamide-gel-electrophoretic analysis of the CNBr fragments, the gels were stained first with AgNO₃ (Merrill *et al.*, 1982), completely destained with Farmer's Reducer (Kodak Canada, catalogue no. 169 1492) and then re-stained with 0.010 % (w/v) Stains All using 200 ml of solution/gel. To remove completely the silver before Stains All staining, the gel was subjected to three 15 min washes with Farmer's Reducer. This was then followed by three 30 min washes in hypo-clearing agent (Kodak) to remove any residual Farmer's reagent in the gel. The gel was then washed with three 40 min changes of 25 % (v/v) isopropyl alcohol and restained with 0.01 % (w/v) Stains All. Low-molecular-mass protein standards (phosphorylase b, M_r 94000; albumin, 67000; ovalbumin, 45000; carbonic anhydrase, 30000; trypsin inhibitor, 21000; and lactalbumin, 14400 purchased from Pharmacia were used to calculate M_r values.

Amino acid analysis

Purified osteonectin was hydrolysed for 24 h at 110 °C with 5.7 M-HCl containing 0.4 % (v/v) phenol in Pyrex hydrolysis tubes, which were flushed with N₂ and sealed before hydrolysis. Solutions were dried *in vacuo* in the presence of NaOH pellets, the residue redissolved in sodium citrate (0.32 M, pH 2.2) and analysed on a Beckman 121 M automated amino acid analyser. To quantify recoveries, 5 nM-norleucine was added to the protein sample before hydrolysis. Cysteine was measured as cysteic acid in hydrolyses carried out in the presence of 2.5 % (v/v) dimethyl sulphoxide. Tryptophan was determined by hydrolysis of 40 μ g of osteonectin with mercaptoethanesulphonic acid (Pierce) as described by Penke *et al.* (1974).

Amino acid sequence analysis

The N-terminal sequence of porcine and bovine osteonectins were determined by applying 200 μ g of pure osteonectin to a gas-phase 470 A Protein Sequencer (Applied Biosystems). Porcine osteonectin for this analysis was purified in the presence of 4 M-GdmCl and was exposed to 7 M-urea only during ion-exchange chromatography. Residues were identified and quantified by h.p.l.c. to separate the amino acid phenylthiohydantoin derivatives. The bovine osteonectin used in the present study was generously supplied by Dr. John Termine of the National Institute of Dental Research, Bethesda, MD, U.S.A.

Determination of absorption coefficient

The absorption coefficient of porcine osteonectin at 280 nm $A_{280,1\text{ cm}}^{0.1\%}$ was determined from u.v.-absorption measurements on a Cary 219 spectrophotometer and from two independent methods of determining the protein concentration. One method employed differential refractometry on a Hilger and Watts Static Raleigh interference instrument, with data treatment as described by Babul & Stellwagen (1969). Alternatively, protein concentration was determined by amino acid analysis using

norleucine as an internal standard for protein recovery. The absorbance value at 280 nm was corrected for contribution of Rayleigh scatter by subtracting 1.7 times the value at 320 nm. In addition, using the molar absorption values at 280 nm for tryptophan and tyrosine of 5600 and 1250 M $\cdot$ cm⁻¹ respectively, an absorption coefficient for osteonectin was calculated from the amounts of these amino acids determined from the amino acid analysis. An estimated M_r value of 29000 [as determined by ultracentrifugation for bovine osteonectin (Romberg *et al.*, 1985)] was used in this calculation.

Secondary-structure analysis

Far- and near-u.v.c.d. spectra were obtained using a Jasco model J-41A spectropolarimeter at 20 °C for a 0.148 mg/ml solution of porcine osteonectin containing 10 mM-phosphate, pH 7.4, or a 0.094 mg/ml solution containing 5 mM-Tris/HCl, pH 7.4, in the presence or absence of 2 mM-Ca²⁺. The samples were scanned between 190 and 260 nm (far u.v.) in a 0.10 cm cuvette and were signal-averaged four times. Similarly, a far-u.v.c.d. spectrum for a 0.148 mg/ml solution of protein in 7 M-urea/5 mM-Tris/HCl, pH 8.0, with or without Ca²⁺ (2 mM-Ca²⁺) was also determined. The contribution of apparent α -helix and β -sheet to the secondary structure of osteonectin was calculated from the far-u.v.c.d. spectra using the curve-fitting procedure of Provencher & Glockner (1981). With a 0.148 mg/ml solution of protein, a near-u.v.c.d. spectrum was obtained by scanning the sample between 250 and 320 nm in a 1.0 cm cuvette and signal-averaged 16 times. For all samples the mean residue ellipticity, $[\theta]_r$, was calculated by using a mean residue weight of 110, which was determined from the amino acid composition. The concentration of protein was calculated by using an $A_{280,1\text{ cm}}^{0.1\%}$ value of 1.25 (determined by refractometry).

Hydroxyapatite-binding studies

Micro test tubes (Bio-Rad) containing 0.5 mg of synthetic hydroxyapatite (Bio-Gel HTP, Bio-Rad) were pre-washed with three 1 ml aliquots of G-buffer and then incubated, with gentle agitation, on a rotatory shaker at 20 °C for 1 h with 1.0 ml of a 5 μ g/ml solution of osteonectin in G-buffer or a sample of total E-extract proteins containing the equivalent of 10 μ g of osteonectin. The samples were centrifuged at 10000 *g* on a Microfuge (Brinkmann) for 30 s and were then washed three times with G-buffer to remove non-bound proteins. The hydroxyapatite was then re-suspended in 200 μ l of E-buffer and gently agitated on a rotatory shaker for a further 20 min at 20 °C, after which the hydroxyapatite was pelleted by centrifugation on a Microfuge at 10000 *g* for 30 s; this was repeated until the hydroxyapatite was completely dissolved. The E extracts and G-supernatants were then dialysed against 10 mM-NH₄HCO₃, freeze-dried, and analysed by SDS/polyacrylamide-gel electrophoresis on 12.5 % (w/v) gels.

Collagen binding

Type I collagen- and gelatin-Sepharose affinity resins were prepared as follows: 5 g of CNBr-activated Sepharose 4B resin (Pharmacia) was prepared according to the manufacturer's instructions. A solution of porcine gingival type I collagen (50 ml of a 1 mg/ml solution in 0.5 M-acetic acid) (Rao *et al.*, 1979), which was dialysed at 4 °C against a 0.5 M-NaCl/0.1 M-Na₂CO₃ buffer,

pH 8.5, was immediately added to 50 ml of similarly buffered resin. For the gelatin-affinity-resin preparation, the collagen was heated at 56 °C for 30 min and cooled to 20 °C before mixing with the activated Sepharose beads. The suspension was shaken gently for 2 days at 4 °C on a rotatory shaker. The resin was isolated by centrifugation at 1000 *g* for 30 s, re-suspended, and shaken for 2 h in 100 ml of 1 M-ethanolamine/0.5 M-NaCl/0.1 M-Na₂CO₃ buffer, pH 8.5. This was followed by washing with PBS/0.02 % (w/v) NaN₃ buffer in which the resin was stored at 4 °C. Before their use in the batch adsorption experiments, the resin beads were repeatedly washed in PBS. Osteonectin (40 µl of a 125 µg/ml solution in PBS) and human fibronectin (5 µl of a 323 µg/ml solution in PBS; used as a positive control) was incubated with 500 µl of either collagen/gelatin-Sepharose 4B affinity resins or Sepharose CL-4B (used as a control for non-specific adsorption) in the presence or absence of 5 mM-CaCl₂ for 1 h at 20 °C and shaken gently on a rotatory shaker. The samples were then centrifuged on a Microfuge at 10000 *g* for 30 s, and the supernatants were dialysed, freeze-dried and processed for SDS/polyacrylamide-gel electrophoresis, whereas the pellet was washed with five 1 ml aliquots of PBS to remove any non-bound material. Electrophoresis sample buffer (100 µl) was then added to the resin, which was then vortex-mixed and incubated at 56 °C for 30 min. The resin was then allowed to cool to 20 °C, centrifuged at 10000 *g* for 30 s, and the supernatants were analysed by SDS/polyacrylamide-gel electrophoresis for any affinity-bound material.

CNBr cleavage

Purified osteonectin (10 µg) was dissolved in 50 µl of 70 % (v/v) formic acid (flushed with N₂) to which was added 25 µl of a 400 mg/ml solution of CNBr in 70 % formic acid which was briefly flushed with N₂. The reaction was carried out at 30 °C for 4 h, and samples were agitated occasionally during the incubation. The contents were diluted 5-fold with water and freeze-dried. The residue was diluted and freeze-dried once again and then analysed by SDS/polyacrylamide-gel electrophoresis on 15 or 20 % gels. In addition, CNBr cleavage of osteonectin that was reduced and alkylated as described by Stevens *et al.* (1976) was also performed. The only modification of this method involved the micro-dialysis (Overall, 1987) of the reduced and alkylated samples (100 µl) against 10 mM-NH₄HCO₃ instead of using a desalting column.

Preparation of antibodies to purified osteonectin

Polyclonal antibodies to foetal porcine osteonectin were obtained by injecting female New Zealand White rabbits subcutaneously in the lower dorsal region of the back with osteonectin in complete Freund's adjuvant (80 µg/200 µl) once every 2 weeks for a total of 4 weeks, followed by one 80 µg/200 µl injection of 80 µg of osteonectin in 200 µl of incomplete Freund's adjuvant. Injections of osteonectin in PBS (100 µg/ml) and subsequent blood collections followed every 10 days. Similarly, mouse anti-(porcine osteonectin) polyclonal antibodies were prepared by injecting Balb/C (Charles River) mice every 2 weeks, first with 25 µg of osteonectin in Freund's complete adjuvant, followed by 25 µg of osteonectin in Freund's incomplete adjuvant, and then boosted with 30–50 µg of osteonectin in PBS.

Fig. 1. Analysis of proteins released from foetal-porcine calvariae using a modified extraction procedure

SDS/polyacrylamide-gel electrophoresis (12.5 % cross-linked gel) was performed under reduced conditions to separate proteins which were then stained with Coomassie Brilliant Blue. Lane 1, proteins extracted with 4 M-GdmCl (G₁ extract). This extract represents approx. 5 % of total extractable proteins; lane 2, proteins extracted subsequently with 0.5 M-EDTA (E extract). This extract represents approx. 90 % of total extractable proteins; lane 3, proteins extracted with 4 M-GdmCl a second time (G₂ extract); this extract represents approx. 5 % of total extractable proteins; lane 4, proteins extracted with 4 M-GdmCl/0.5 M-EDTA (G/E extract) with prior 4 M-GuHCl treatment using the dissociative extraction procedure of Termine *et al.* (1981a). Approx. 100 µg of protein was applied to each well.

Albumin and collagen binding

A modification of the enzyme-linked immunosorbent assay (e.l.i.s.a.) system described by Rennard *et al.* (1980) was used to test for osteonectin binding to various proteins. Bovine serum albumin (Pentex grade; Miles Laboratories) and type I collagen (porcine and rat) were dissolved at concentrations of 1.00, 0.25, 0.10 and 0.025 µg/150 µl in 20 mM-Na₂CO₃ buffer, pH 9.5, containing 0.02 % (w/v) NaN₃ and were allowed to adsorb to the microtitre wells [Linbro/Titertek poly(vinyl chloride) plates] for 48 h at 4 °C. Plates were then washed three times at 3 min intervals with PBS/Tween 20 (0.05 % Tween 20 in PBS) and 50 ng of osteonectin in 150 µl of PBS/Tween 20 was allowed to incubate for 1 h with the proteins adsorbed on to the microtitre wells. The plates were washed as described above and the procedure described by Kuwata *et al.* (1987) was followed, using 150 µl volumes of mouse anti-osteonectin antiserum (diluted 1:500 in PBS/Tween 20) as first antibody and rabbit horseradish-peroxidase-linked anti-

Fig. 2. Chromatographic profiles

(a) Chromatographic profile of E-extract proteins (0.12 g) on Sepharose CL-6B. Bars throughout the profile represent fractions enriched in various proteins: bar 1, enriched in proteoglycans; bar 2, enriched in sialoproteins; bar 3, enriched in osteonectin; bar 4, enriched in SCAB proteins; bar 5, enriched in osteocalcin. (b) F.p.l.c. chromatography of osteonectin pooled from Sepharose CL-6B gel filtration (a) using a Pharmacia HR10/10 column packed with Pharmacia preparatory-grade polyanion exchange resin run under dissociative conditions (7 M-urea) in 50 mM-Tris/HCl, pH 8.0, employing a linear gradient of NaCl. The bar represents fractions pooled for re-chromatography. (c) F.p.l.c. elution profile of osteonectin purification on analytical polyanion resin. Material [one-seventh of the pooled fractions from the preparatory run (Fig. 3b)] was run on an HR5/5 column run under dissociative conditions (7 M-urea) in 50 mM-Tris/HCl, pH 8.0, employing a linear NaCl gradient. The peak represents approx. 100 μ g of protein.

mouse IgG as second antibody (Cappel; diluted 1:1000 in PBS/Tween 20) for 1 h at 20 °C. As a positive control, binding of fibronectin (1.6 μ g) to the collagen/gelatin-coated wells was also tested.

Cell cultures and radiolabelling experiments

Bone cells from foetal porcine calvariae were prepared by using methods previously described (Brunette *et al.*, 1976; Rao *et al.*, 1977). Briefly, cells were grown to confluence and were then subjected to either pulse-chase experiments with [35 S]methionine (Otsuka *et al.*, 1984) or continuous $^{32}\text{PO}_4^{3-}$ labelling as described by Prince *et al.* (1987). The medium from these experiments was prepared for specific immunoprecipitation as described previously (Wasi *et al.*, 1984). The specific immunoprecipitates were then analysed by SDS/polyacrylamide-gel electrophoresis and fluorography or were electrophoretically transferred to nitrocellulose for $^{45}\text{Ca}^{2+}$ binding analysis or immunoblotting analysis.

Electrophoretic transfer, immunoblotting and $^{45}\text{Ca}^{2+}$ binding

For the electrophoretic transfer of proteins on to nitrocellulose membranes (0.45 μ m pore size), proteins were first run on linear 15% gels (0.75 mm thick, 5 cm long) using a Hoefer Scientific Instrument (San Francisco, CA, U.S.A.) Minilab gel system and then transferred on to the nitrocellulose sheets using an LKB 2117-250 Nova Blot electrophoretic transfer system. Immunoblotting (Western blotting) was performed using a modification of the original method by Towbin *et al.* (1979) as described by Kuwata *et al.* (1987), using rabbit anti-onectin antisera (1:50 dilution) as first antibody and peroxidase-conjugated sheep anti-rabbit IgG (Cappel; 1:1000 dilution) as second antibody. The ability of reduced and non-reduced forms of purified osteonectin and reduced CNBr cleavage fragments of osteonectin to sequester Ca^{2+} ions was determined by incubating nitrocellulose membranes containing the electrophoretically transferred proteins with $^{45}\text{Ca}^{2+}$ (1 mCi/litre) as described by Maruyama *et al.* (1984). The sequestered $^{45}\text{Ca}^{2+}$ was revealed by autoradiography.

RESULTS

Proteins sequentially extracted from calvariae with, first, 4 M-GdmCl (G_1 extract), then with 0.5 M-EDTA (E extract), followed by 4 M-GdmCl (G_2 extract) extraction were analysed by SDS/polyacrylamide-gel electrophoresis (Fig. 1). The E extract comprised a limited number of clearly resolved proteins, the major proteins migrating with M_r values of 67000–55000, 40000 and 14400. When compared with a 4 M-GdmCl/0.5 M-EDTA (G/E) extract, the E-extract protein profile was very similar, with the most notable difference being a lower content of collagen α -chains (M_r 110000) in the E extract (Fig. 1, lane 2). G_2 extracts, on the other hand, revealed strong bands for collagen α_1 - and α_2 -chains. Some 60000–55000- M_r material was also apparent, as were some minor bands having M_r values of 45000, 38000, 34000, 28000, 25000, 23000, and 14000. When tissues were extracted with EDTA without prior extraction with 4 M-GdmCl (i.e. under non-dissociative conditions), a similar profile to that of the G/E extract was generated; however, no collagen bands were evident (results not shown).

Proteins in the E extract were separated by gel filtration on Sepharose CL-6B in the presence of 7 M-urea. An elution profile characteristic of the chromatographic runs is shown in Fig. 2(a). The fractions containing osteonectin were identified from the mobility of the

Table 1. Amino acid compositions of calvarial osteonectin and proteins from other sources

Abbreviations used: Cal, calvariae; Lb, long bone; –, cannot be determined from sequence data; ND, amino acids not detected under the conditions described.

Amino acid	Composition (residues/1000 residues)				
	Foetal-porcine Cal-osteonectin	Foetal-bovine Lb-osteonectin†	Adult-bovine Lb-osteonectin‡	Bovine endothelial 'culture-shock' protein§	Murine endodermal SPARC protein
Hydroxyproline	0	0	0	0	–
Aspartate	124	130	140	135	116¶
Threonine	49	59	58	54	60
Serine	26	42	66	40	25¶
Glutamate	179	148	165	168	158
Proline	66	72	79	74	25¶
Glycine	52	65	83	69	56
Alanine	53	58	54	70	49
Half-cystine*	35	6	25	35	49
Valine	65	69	50	59	70
Methionine	11	11	4	8	18¶
Isoleucine	38	37	29	42	42
Leucine	93	90	62	93	84
Tyrosine	26	24	17	5	24
Phenylalanine	44	44	29	35	42
Histidine	54	44	37	33	42
Lysine	64	71	54	55	67
Arginine	31	28	41	ND	28
Tryptophan	10	ND	8	ND	10

* Determined as cysteic acid.

† From Termine *et al.* (1981a).

‡ From Romberg *et al.* (1985).

§ From Sage *et al.* (1984).

|| Based on amino acid sequence from Mason *et al.* (1986a).

¶ Major differences between SPARC protein and those from other sources.

Fig. 3. Analysis of foetal-porcine calvarial osteonectin purified as shown in Fig. 2(c)

SDS/polyacrylamide-gel electrophoresis (12.5% cross-linked gel) was performed under reducing conditions for

protein on SDS/polyacrylamide-gel electrophoresis (an apparent M_r of 40300 under reduced conditions and 37600 in the absence of reductant), and were pooled as shown in Fig. 2(a) (bar 3). Pooled material from the CL-6B runs was then applied on to a Pharmacia preparatory polyanion column run under dissociative conditions (Fig. 2b). Fractions highly enriched in osteonectin from these runs (bar in Fig. 2b) were re-chromatographed on a Pharmacia analytical polyanion column. As illustrated, a sharp symmetrical peak was obtained for the major component which eluted between 0.23 and 0.28 M-NaCl (Fig. 2c). The homogeneity of the protein in this peak was confirmed by SDS/polyacrylamide-gel electrophoresis using Coomassie Brilliant Blue (Fig. 3) and Stains All, both of which stained the single protein band blue; silver staining also showed a single protein band. The behaviour of the protein on SDS/polyacrylamide-gel electrophoresis under reducing and non-reducing conditions, the amino acid composition, and the Stains All staining properties were used to identify the purified protein as osteonectin. From 100 g wet weight of calvariae, approx. 6 mg of osteonectin was obtained in pure form. In addition, from other pools made from the Sepharose CL-6B separation, three small proteoglycans

proteins, which were stained with Coomassie Brilliant Blue. Lane 1, low- M_r markers; lanes 2–8, 50 μ l aliquots from 1 ml fractions collected through the f.p.l.c. profile represented by the bar in Fig. 2(c). The bar represents the fractions pooled for subsequent analyses.

Fig. 4. N-terminal amino acid sequences and cDNA-derived amino acid sequences for osteonectin from various sources

Alignment was made to maximize homology through nucleotide triplets. The horizontal line in the SPARC sequence represents a gap. Square brackets indicate amino acids also found in significant, but lower, amounts at that cycle number. 'X' represents an unidentified amino acid at that cycle number. Sources of sequences: * Young *et al.* (1986); †, Mason *et al.* (1986a); ‡, Mann *et al.* (1987). Note that there is a valine residue present at position 32 in bovine and porcine osteonectin, whereas methionine is found in the SPARC protein.

Fig. 5. Far-u.v.c.d. spectrum of osteonectin in the presence or absence of 2 mM- Ca^{2+} for samples dissolved in 5 mM-Tris/HCl, pH 7.4

Superimposed is the measurable part of the obtainable spectrum of osteonectin in the presence of 7 M-urea/5 mM-Tris/HCl at pH 8.0.

(H. A. Goldberg, C. Domenicucci, G. A. Pringle & J. Sodek, unpublished work), two or possibly three sialoproteins, osteocalcin (Domenicucci *et al.*, 1985; Domenicucci, 1986), SCAB proteins (Kuwata *et al.*, 1987) and $\alpha 1(\text{I})\text{pN}$ peptide (Goldberg *et al.*, 1988), have been isolated. Fractions enriched in these proteins are illustrated in Fig. 2(a).

In Table 1, the amino acid composition of the calvarial osteonectin is compared with the compositions of foetal (Terminé *et al.*, 1981a) and adult (Romberg *et al.*, 1985) bovine long-bone osteonectins and those of bovine endothelial culture-shock protein (Sage *et al.*, 1984) and murine SPARC protein (Mason *et al.*, 1986a). In each case the compositions were similar, the proteins being characteristically rich in acidic amino acids and/or their amides, hydrophobic residues and half-cystine. The half-cystine value for foetal-bovine osteonectin, however, was much lower than for the other proteins, whereas proline, serine and aspartic acid were low in the SPARC protein. An absorption coefficient ($A_{280.1}^{0.1\%}$) of 0.82 was found for a solution of porcine osteonectin whose concentration was determined by amino acid analysis, whereas a value of 1.01 was calculated from the tryptophan and tyrosine content of the molecule. Both of these values are lower than the value of 1.25 obtained by refractometry, but are much higher than the values reported for bovine long-bone osteonectin by Romberg *et al.* (1985) (0.36 and 0.46

respectively as determined by refractometry and amino acid analysis).

The amino acid sequence of the first 35 residues of porcine osteonectin and the first 38 residues of bovine osteonectin was determined by automated sequencing, with a repetitive yield of 96 and 89 % respectively for each of the two proteins. As shown in Fig. 4, the sequence is identical with that of bovine osteonectin, the only difference being the absence of a Val-Ala-Glu tripeptide after residue 20 of the porcine protein. Although there are notable differences with the short sequence reported for adult-bovine osteonectin (Romberg *et al.*, 1985), both the foetal-porcine and foetal-bovine osteonectin sequences (residues 1–19) are consistent with the bovine cDNA sequence (Young *et al.*, 1986). The porcine sequence, representing approximately one-tenth of the molecule, is enriched in small hydrophobic (approx. 50 %) and acidic (approx. 25 %) amino acids and is notably deficient in aromatic amino acids. Also shown in Fig. 4 are the *N*-terminal sequences of the murine SPARC protein and BM-40 and the corresponding DNA sequences of SPARC (Mason *et al.*, 1986a) and bovine osteonectin (Young *et al.*, 1986), the sequencing being aligned to maximize homology with the published DNA nucleotide sequences of SPARC and bovine osteonectin.

To determine the presence of secondary structure in the dissociatively extracted osteonectin, the c.d. spectra of the protein were studied in the presence or absence of Ca^{2+} . The far-u.v.c.d. spectrum of osteonectin in the presence of 10 mM-phosphate, pH 7.4, was similar to that in 5 mM-Tris/HCl, pH 7.4, shown in Fig. 5. When analysed by the c.d. curve-fitting procedure of Provencher & Glockner (1981) the following best-fit values were obtained: 27 % α -helical content, 39 % β -sheet structure; the remaining 34 % consisting of both unordered structure and β -turns. Although these values represent the chosen solution of the algorithm, visually acceptable fits of the data were also obtained in the following ranges: α -helix, 24–29 %, β -sheet, 35–45 %; unordered structure and β -turns, 26–41 %. Qualitatively, the far-u.v.c.d. spectrum was changed slightly in the presence of 2 mM- Ca^{2+} (Fig. 5), whereas no appreciable change could be detected in the near-u.v.c.d. spectrum (results not shown). In addition, when osteonectin was re-exposed to 7 M-urea, the far-u.v.c.d. spectrum revealed that all α -helical structure in the molecule was abolished (Fig. 5) and was not restored upon the addition of Ca^{2+} ions. In all cases the concentration of Ca^{2+} used in these experiments (i.e. 2 mM- CaCl_2) was well above the binding constant for Ca^{2+} determined for bovine osteonectin (Romberg *et al.*, 1985).

Osteonectin cleaved with CNBr generated a characteristic pattern of five silver-staining fragments with M_r values of 33100, 27900, 22800, 17600, and 12400 (Fig. 6, lane 2) as determined by SDS/polyacrylamide-gel electrophoresis under reducing conditions. When the gel was stained subsequently with Stains All, all bands except the 22800- M_r and 17600- M_r fragments, both of which were stained pink, were found to be stained blue (Fig. 6, lane 4). A 10300- M_r fragment was also observed in cleavage products transferred to nitrocellulose and stained with Amido Black (Fig. 7, lane 3). Analysis of CNBr fragments by SDS/polyacrylamide-gel electrophoresis under non-reducing conditions showed that most of the Stains All-staining bands, all of which were

Fig. 6. SDS/polyacrylamide-gel-electrophoretic analyses for CNBr cleavage fragments of osteonectin run under reducing and non-reducing conditions

Fragments were separated on a 20% cross-linked gel. Lane 1, 2 μ g of reduced osteonectin stained with silver; lane 2, reduced CNBr cleavage fragments (from 10 μ g of non-reduced osteonectin; see the text) stained with silver; lane 3, lane 1 destained of silver and re-stained with Stains All; lane 4, lane 2 destained of silver and re-stained with Stains All; lane 5, non-reduced CNBr cleavage fragments from 10 μ g of osteonectin which was stained with silver, destained and then re-stained with Stains All; lane 6, non-reduced osteonectin which was stained with silver, destained and then re-stained with Stains All. The relative molecular masses of osteonectin and the CNBr cleavage products are indicated. The colours generated by Stains All staining of the CNBr fragments in lane 4 are also labelled. Note that all bands in lane 5 are stained blue with Stains All and contain no detectable low- M_r fragments, indicating that the 22800-, 17000- and 12400- M_r fragments evident in lane 4 are linked to larger fragments before reduction and analysis. It is also evident that the Stains All staining of the 12400- M_r fragment in lane 4 is more sensitive than the staining with silver in lane 2.

blue, had M_r values between 40000 and 29000 (Fig. 6, lane 5). Notably, CNBr cleavage of reduced and alkylated osteonectin generated the same fragments as those described above, in addition to a 7200- M_r fragment, all of which were revealed by silver staining (results not shown).

Ca^{2+} -binding studies, using protein electrophoretically transferred on to nitrocellulose sheets, showed that both the reduced and the non-reduced forms of purified osteonectin were able to bind $^{45}\text{Ca}^{2+}$ (Fig. 7, lanes 8 and 9 respectively). Of the CNBr cleavage fragments, the 12400- M_r band was the only fragment to selectively sequester Ca^{2+} ions (Fig. 7).

Purified osteonectin incubated in the presence of 4 M-GdmCl bound quantitatively to hydroxyapatite crystals (results not shown). However, under similar conditions the osteonectin present in the whole E extract did not bind significantly to the hydroxyapatite. To study the binding of osteonectin to collagen, the purified protein was incubated with both native and denatured (gelatin) collagen-Sepharose 4B resin in the presence or absence of added Ca^{2+} ions. From SDS/polyacrylamide-gel-electrophoretic analyses (Fig. 8), only a small amount of osteonectin was found to be bound to the collagen and gelatin-affinity resins in the absence of Ca^{2+} (6 and 7% respectively) and in the presence of Ca^{2+} (14 and 11%

respectively) as measured by densitometric scanning. However, in each case the proportion bound was substantially less than was bound to the CL-4B resin alone (12% in the absence and 33% in the presence of Ca^{2+}), which was used as a control. Consequently there is no apparent affinity of osteonectin for collagen or gelatin, the latter in fact appearing to prevent osteonectin binding to the Sepharose 4B support resin. Control experiments with fibronectin, however, showed that 1.5 μ g (>90%) of fibronectin could bind to both the collagen- and gelatin-affinity resins. In agreement with these observations are the results of the e.l.i.s.a. binding assays, where the amount of osteonectin bound to native or denatured collagen over that in control wells could not be detected. By using this method, however, it could be shown that fibronectin binding to gelatin was 4-fold greater than to native collagen, as was previously described (Karp *et al.*, 1986). In addition, 50 ng of osteonectin was quantitatively bound by 1.0 μ g of highly purified albumin (Pentex) coated on to the e.l.i.s.a. plate.

To determine whether porcine osteonectin is phosphorylated, porcine and rat calvarial osteoblast-like cells were cultured in the presence of $^{32}\text{PO}_4^{3-}$. Although a number of phosphorylated proteins could be resolved by SDS/polyacrylamide-gel electrophoresis, no radio-

Fig. 7. Electrophoretic transfer, immunoblotting and $^{45}\text{Ca}^{2+}$ binding studies with reduced, non-reduced and CNBr-cleavage fragments of osteonectin

Purified porcine osteonectin ($2\text{ }\mu\text{g}$) and CNBr cleavage fragments ($10\text{ }\mu\text{g}$) generated under non-reducing conditions were electrophoresed on 15% Minislab SDS/polyacrylamide gels and transferred on to nitrocellulose membranes. Transferred proteins were tested for reactivity against polyclonal antibodies to foetal porcine osteonectin and the ability for osteonectin and CNBr fragments to sequester $^{45}\text{Ca}^{2+}$. Amido Black staining of proteins transferred on to nitrocellulose: lane 1, low- M_r markers; lane 2, reduced osteonectin; lane 3, fragments generated by CNBr cleavage obtained under non-reducing conditions, but run under reducing conditions; Lane 4, non-reduced osteonectin; immunoblot of proteins transferred on to nitrocellulose: lane 5, non-reduced osteonectin; lane 6, reduced osteonectin; lane 7, fragments generated by CNBr cleavage obtained under non-reducing conditions, but run under reducing conditions; $^{45}\text{Ca}^{2+}$ binding to proteins transferred on to nitrocellulose: lane 8, reduced osteonectin; lane 9, fragments generated by CNBr cleavage obtained under non-reducing conditions, but run under reducing conditions; lane 10, non-reduced osteonectin.

labelled proteins in the position of osteonectin could be detected in autoradiographs exposed for 2 weeks, nor in specifically immunoprecipitated protein. To confirm that the osteonectin had been immunoprecipitated, duplicate samples were first electrophoretically transferred to nitrocellulose and then the transferred protein was detected by immunoreactivity with antibodies. As expected, the immunoprecipitated proteins were found to behave identically with the EDTA-extracted protein run on SDS/polyacrylamide-gel electrophoresis under reducing and non-reducing conditions (results not shown).

DISCUSSION

In the present study, osteonectin, together with the other major non-collagenous proteins of mineralized bone matrix, including proteoglycans, sialoproteins and osteocalcin, could be extracted quantitatively with 0.5 M-EDTA under non-dissociating conditions (Fig. 2). These proteins, therefore, appear to be bound to the surface of hydroxyapatite crystals, where they may influence crystal growth or dissolution. More than 95% of the osteonectin in bone could be extracted with 0.5 M-EDTA, whereas only small quantities (< 5%) were recovered in the subsequent 4 M-GdmCl extract (G_2 extract). More exhaustive extractions with EDTA were found to completely remove the osteonectin. Consequently, osteonectin would not appear to have a marked affinity for any components in the demineralized collagenous matrix of bone. By using the same procedure we have also been able to quantitatively extract and purify osteonectin from porcine incisor dentine. The behaviour of the dentine protein on SDS/polyacrylamide-gel

electrophoresis under reducing and non-reducing conditions, and its immunoreactivity to anti-onectonectin antibody was used to identify the purified protein as osteonectin (C. Domenicucci & J. Sodek, unpublished work).

The G_2 extraction revealed the presence of a number of discrete proteins in the demineralized bone matrix (Fig. 1, lane 3), some of which are thought to be novel and require purification before they can be further characterized. This extract is likely to be enriched in collagen and collagen-associated proteins. Notably, components with an M_r of 34000 and 25000 are also found consistently in G_2 extracts of cementum and dentine, as well as in bone (C. Domenicucci & J. Sodek, unpublished work). It is probable that the G_2 -extract includes a protein with bone-inductive activity (Muthukumar *et al.*, 1985) similar to bone morphogenic protein (M_r 18000) (Urist *et al.*, 1984), cartilage-inducing factors (CIFs) of which CIF-A and CIF-B appear to be identical with two forms of TGF (transforming growth factor)- β (M_r 25000) (Seyedin *et al.*, 1987) and the 15000- M_r matrix Gla (carboxyglutamic-acid containing) protein (Price *et al.*, 1976). In addition, it might be anticipated that proteins that can simultaneously interact with collagen (because of the order of extraction) and Ca^{2+} /hydroxyapatite (blue Stains All staining) would also be present in the G_2 extract.

Initial interest in osteonectin was based on the apparent bone specificity of this protein as well as its putative role in hydroxyapatite crystal formation (Termin *et al.*, 1981a,b). Because of this proposed function in bone, the properties of osteonectin isolated from foetal-porcine calvariae were studied in more detail. The purified protein was found to behave similarly on

Fig. 8. Electrophoretic analyses for osteonectin bound to collagen, gelatin and Sepharose CL-4B in the presence or absence of Ca^{2+}

The SDS/polyacrylamide-gel-electrophoretic (12.5% crosslinked gels) analysis was under reducing conditions, and proteins were stained with Coomassie Brilliant Blue. Lane 1, low- M_r markers; lane 2, osteonectin not bound to collagen in the presence of Ca^{2+} ; lane 3, osteonectin not bound to collagen in the absence of Ca^{2+} ; lane 4, osteonectin not bound to gelatin in the presence of Ca^{2+} ; lane 5, osteonectin not bound to gelatin in the absence of Ca^{2+} ; lane 6, control (osteonectin not bound to Sepharose CL-4B in the presence of Ca^{2+}); lane 7, control (osteonectin not bound to Sepharose CL-4B in the absence of Ca^{2+}); lane 8, 'collagen-bound' osteonectin in the presence of Ca^{2+} ; lane 9, 'collagen-bound' osteonectin in the absence of Ca^{2+} ; lane 10, 'gelatin-bound' osteonectin in the presence of Ca^{2+} ; lane 11, 'gelatin-bound' osteonectin in absence of Ca^{2+} ; lane 12, control ('Sepharose CL-4B-bound' osteonectin in the presence of Ca^{2+}); lane 13, control ('Sepharose CL-4B-bound' osteonectin in the absence of Ca^{2+}). Collagen bands observed represent protein non-covalently associated with the collagen-Sepharose 4B-bound.

SDS/polyacrylamide-gel electrophoresis and to have an amino acid composition and an *N*-terminal sequence comparable with that of foetal-bovine osteonectin. The chemical similarities between bovine and porcine osteonectin are also apparent in the CNBr cleavage patterns generated under non-reducing conditions. Although not shown for foetal-bovine long-bone osteonectin, CNBr cleavage of porcine calvarial osteonectin (as illustrated in Fig. 6) releases relatively high-molecular-mass fragments indicative of incomplete cleavage of the molecule under these conditions. In addition, there appears to be preferential cleavage of the molecule into 27900 and 12400- M_r fragments, as shown by the relative staining intensity of fragments with Stains All. Since CNBr cleavage of methionine residues in proteins is usually highly efficient, the resistance of osteonectin cleavage to CNBr could reflect a compact tertiary structure stabilized by extensive disulphide bridging.

Interestingly, the major CNBr cleavage fragments (the 12400- and 27900- M_r fragments) retain the original staining properties of uncleaved osteonectin in that they are stained blue with Stains All. It has been shown that Stains All will stain sialic-acid-rich proteins (King & Morrison, 1976), phosphate-containing proteins (Cutting, 1984) and/or Ca^{2+} -binding proteins (Campbell

et al., 1983) blue, whereas other proteins stain pink. The blue-staining 12400- M_r fragment selectively sequestered $^{45}\text{Ca}^{2+}$ ions, indicating that it contains the Ca^{2+} -binding site in porcine osteonectin. Of note, an 'EF hand' Ca^{2+} -binding site has been predicted, from Chou-Fasman analysis of the cDNA-derived amino acid sequence, to be near the C-terminus of the SPARC protein (Mason *et al.*, 1986a), indicating that the 12400- M_r CNBr fragment is likely to be part of the C-terminal portion of the porcine osteonectin molecule. For the 27900- M_r fragment the blue staining with Stains All likely reflects the acidic character of this part of the osteonectin sequence, since the *N*-terminus is known to be acidic, and our findings indicate that osteonectin synthesized by porcine and rat osteoblast-like cells is not phosphorylated, as has also been observed for the 'culture-shock' protein (Sage *et al.*, 1984).

Analysis of the far-u.v.c.d. spectra of porcine osteonectin revealed appreciable amounts of α -helix (27%) and β -sheet (39%) structure. The β -sheet structure, which is considerable in porcine osteonectin, may provide the means whereby this protein binds to hydroxyapatite in bone, especially since the β -sheet structure has been shown to be a stereochemical requirement for the adsorption of a number of acidic proteins to hydroxy-

apatite (Addadi & Weiner, 1985). Furthermore, the refolding of osteonectin structure, after chromatography under dissociative conditions, is also indicated by the high content of secondary structure, which is abolished on re-exposure to 7 M-urea (Fig. 5). The recovery of secondary structure is likely to be facilitated by the extensive disulphide bridging which is apparent from the high half-cystine content (Table 1) and the behaviour of CNBr (Fig. 6, lane 5) and proteolytic (Kuwata *et al.*, 1985) fragments on SDS/polyacrylamide-gel electrophoresis in the presence or absence of dithiothreitol.

In contrast with our findings, adult-bovine osteonectin, isolated without dissociative agents, has been reported to lack α -helical structure and to have only 20% β -sheet structure (Romberg *et al.*, 1985). Although the spectra were analysed by different procedures, perhaps of more significance is the lower amount of negative ellipticity generated by a solution of bovine osteonectin (i.e. approx. 3.5 times less than porcine osteonectin at 202 nm). This difference is reflected in the absorption coefficients of these molecules: 1.25 for porcine osteonectin and 0.36 for bovine osteonectin, both measured in conjunction with refractometry. Since the tryptophan content appears to be the same for these proteins, the reason for this difference is not apparent. The lack of α -helical structure in bovine osteonectin (Romberg *et al.*, 1985) is surprising in view of its homology with the SPARC protein (Mason *et al.*, 1986a) in which the Ca^{2+} -binding domain appears to have an 'EF hand' structure, one with appreciable amounts of α -helix. In addition, Chou-Fasman analyses of amino acid sequences based on the cDNA of the SPARC protein predicts α -helical structure in the N-terminal segment of this protein (Mason *et al.*, 1986a), and 30% α -helix has been indicated in BM-40 protein (Mann *et al.*, 1987).

Although not readily apparent in the far-u.v.c.d. spectrum of adult-bovine osteonectin (Romberg *et al.*, 1985), foetal-porcine osteonectin shows a slight change in the secondary structure in the 217 nm c.d. region upon exposure to saturating amounts of Ca^{2+} . The lack of any spectral changes in the 'aromatic' region (250–320 nm) of the near-u.v.c.d. spectra (not shown) upon the addition of saturating amounts of Ca^{2+} suggests that a Ca^{2+} -binding site is not close to, nor does its binding affect, aromatic groups. Thus the Ca^{2+} -mediated effect on the far-u.v.c.d. spectrum is more likely to represent a small change in secondary structure rather than a perturbation of short-wavelength aromatic c.d. bands. Notably, a more prominent spectral shift in the presence of Ca^{2+} has been observed in BM-40 (Mann *et al.*, 1987).

In contrast with bovine osteonectin (Termine *et al.*, 1981a), porcine osteonectin was not selectively bound to hydroxyapatite (in the presence of 4 M-GdmCl) when compared with other proteins in the E extract, nor did osteonectin show any particular affinity for collagen or gelatin by either the e.l.i.s.a. binding assay or by affinity to collagen-Sepharose resins. Since the lack of affinity for collagen has also been shown for cell-synthesized porcine osteonectin (Otsuka *et al.*, 1984), the results obtained with the tissue-extracted osteonectin do not appear to be a consequence of exposure of the protein to denaturants and are consistent with the quantitative extraction of osteonectin from bone with EDTA alone.

Despite apparent differences in binding properties, spectral properties and some chemical properties between

porcine and bovine osteonectins, these proteins generally show closely similar chemical characteristics, many of which are shared by bovine endothelial-cell 'culture-shock' protein (Sage *et al.*, 1984), mouse endodermal SPARC protein (Mason *et al.*, 1986a,b) and BM-40 protein (Mann *et al.*, 1987). Some structural and immunological similarities between 'culture-shock' and SPARC protein have also been documented (Mason *et al.*, 1986a). All these proteins are acidic glycoproteins, rich in cysteine, with similar biosynthetic forms. They all show characteristic migration on SDS/polyacrylamide-gel electrophoresis under reducing and non-reducing conditions. Comparison of nucleotide sequences of SPARC and bovine osteonectin have revealed high homology (Holland *et al.*, 1987). Despite the homology between the murine SPARC and porcine/bovine osteonectins, there are major differences in the highly acidic N-terminal region which may also be involved in apatite binding. It is conceivable, therefore, that the low amounts of osteonectin in mineralized tissues of rodents (Zung *et al.*, 1986) may reflect a lower binding affinity to hydroxyapatite of the SPARC-like osteonectin.

Although osteonectin was thought to be specific to the osteoblastic phenotype (Whitson *et al.*, 1984), the same protein has been shown to be synthesized by various mesenchymal cells (Wasi *et al.*, 1984; Zung *et al.*, 1986) and may be extracted from platelets (Stenner *et al.*, 1986), dentine and periodontal ligament (Wasi *et al.*, 1984). Moreover, bovine 'culture-shock' protein does not bind to collagen or gelatin, but does bind to bovine serum albumin and a 67000- M_r serum component. Consistent with these observations are the results of porcine osteonectin tissue localization, which had shown the protein to be prominent in cells of soft as well as hard tissues (Tung *et al.*, 1985), and the present results showing the affinity of porcine osteonectin for albumin but not for collagen. Furthermore, like 'culture-shock' protein, cell-synthesized porcine osteonectin does not appear to be phosphorylated, whereas foetal-bovine osteonectin has been reported to be a phosphoprotein (Termine *et al.*, 1981a). In view of the homology of osteonectin with SPARC, BM-40 and 'culture-shock' protein, the apparent lack of specificity to bone, the absence of selective binding of porcine osteonectin to collagen and the unknown function of these proteins, it is recommended that, in accordance with the recently proposed nomenclature for bone proteins (Hauschka *et al.*, 1986), the functional term 'osteonectin' should be replaced by a more descriptive term: 'bone SPARC protein'.

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