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Dentin Phosphoprotein: An Extracellular Calcium-Binding Protein[†]

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ABSTRACT: The (ethylenedinitrilo)tetraacetic acid extracted phosphoprotein of bovine dentin contains 37.6 residue % of aspartic acid and 41.8 residue % serine, most of which is phosphoserine. The phosphoprotein binds calcium ions to both high- and low-affinity sites, with association constants 3.6×10^4 and 5.1×10^2 mol^{-1}, respectively. Alkaline-hydrolysis studies showed that γ-carboxyglutamic acid was absent from this calcium-binding protein. The binding interactions are nonideal, showing a dependence of the number of high-affinity sites per milligram of protein on the protein concentration. This appears to be the result of a calcium-ion mediated concentration-dependent aggregation of the phosphoprotein. Conformational studies, using circular dichroism, show the protein to have a random chain conformation in aqueous solution. The addition of either methanol (to 50%, v/v) or Ca ion caused a change in the circular dichroism spectra, suggesting a transition to a more ordered extended chain conformation. ^{31}P nuclear magnetic resonance spectra of the phosphoprotein showed resonances corresponding only to the presence of orthophosphates. Spectra determined during the titration of an alkaline solution of the sodium ion salt of the phosphoprotein showed the presence of a single set of acidic groups with a pK_a of 6.8, also characteristic of orthophosphates. The addition of calcium ions caused a significant signal broadening of the orthophosphate resonance, indicating a specific calcium ion–orthophosphate interaction. A calcium–phosphoprotein complex precipitates upon standing at neutral pH at room temperature. Infrared spectra of this precipitate showed the presence of bands at 500–600 cm^{-1}, characteristic of calcium–orthophosphate interaction. Examination of the infrared bands in the carboxylate region also established the presence of calcium–carboxylate interactions in the calcium–phosphoprotein complex. Both carboxylate and orthophosphate groups thus appear to be involved in providing high-affinity binding sites for calcium ions. In the trans-extended chain conformations suggested by the circular dichroism data, sheet-like arrays would present high-affinity calcium-binding faces that could initiate crystal phase growth.

A variety of noncollagenous proteins is present in the organic extracellular matrix of dentin (Veis et al., 1972; Leaver and Shuttleworth, 1966; Jones and Leaver, 1974; Butler et al., 1972). A major component of these noncollagenous proteins of human and animal dentin is a group of acidic, phosphorus-containing proteins, referred to as dentin phosphoproteins. The phosphoprotein isolated from unerupted bovine molars contains large amounts of serine, phosphoserine, and aspartic acid. These residues comprise at least 70% of the total amino acid composition of the bovine dentin phosphoprotein.

The interaction between the dentin phosphoprotein and calcium ion is of interest in connection with the mechanism of mineralization of the collagenous matrices of bone and dentin. Mineralization involves the deposition of calcium phosphate as amorphous calcium phosphate or crystalline calcium hydroxylapatite in and around the collagen fibers (Posner, 1973; Posner and Betts, 1975; Termine and Posner, 1965). The newly synthesized dentin phosphoprotein in the developing rat incisor is secreted and appears rapidly at the mineralizing front (Weinstock and Leblond, 1973). Moreover, some of the phosphoprotein binds directly to the collagen matrix (Dickson et al., 1975; Carmichael et al., 1971). These considerations all point to a calcium ion-organic matrix interaction as a key element in the nucleation of calcium phosphate deposition in dentin. Nawrot et al. (1976) have recently shown that the addition of dentin phosphoprotein to a metastable solution of calcium phosphate catalyzes the formation of hydroxylapatite crystals. The matrix–mineral phase interaction in bone may be of similar nature, since bone contains strongly polyanionic noncollagenous proteins which also interact strongly with collagen (Shuttleworth and Veis, 1972) and with divalent metal ions (Peacocke and Williams, 1966).

Since phosphate and carboxylate groups are known to chelate divalent cations (Chaberek and Martell, 1959), it was suggested that a possible biological function of the phosphoprotein was in the initiation of calcification of dentin. It was proposed that phosphoproteins could initiate calcification by acting as a nucleation site for the initial epitactic localization of calcium within the collagen-fibril network (Veis et al., 1969). Alternatively, it was suggested that the phosphoproteins could inhibit calcification by complexing calcium ions, thereby preventing nucleation within the collagen-fibril network (Veis et al., 1969). In either case, the same questions arise: What is the affinity of the dentin phosphoprotein for calcium ions, and how does the phosphoprotein relate to the precipitation of calcium phosphates? The experiments summarized in this

report were designed to determine the calcium-ion binding, solubility, and conformational properties of the phosphoprotein isolated from unerupted bovine molars and to determine the extents of carboxylate and phosphate group participation in calcium-ion coordination.

Materials and Methods

EDTA[1] *Demineralization of Unerupted Bovine Molars.* The EDTA-soluble components of dentin were extracted by a modification of the procedure of Veis and Schlueter (1964). All operations were conducted in a cold room (4 °C). Unerupted molars were removed from jaws of 2-year-old heifers. The molars were rinsed in water and soaked overnight in 15% (w/v) NaCl. They then were rinsed thoroughly in water and lyophilized. The dried teeth were splintered with a hammer, and adhering pulp, sac, and gingiva were removed mechanically. The cleaned fragments were transferred to prepared dialysis bags (Spectrapor-3, Spectrum Medical Industries) and demineralization with cold 0.5 M EDTA, pH 7.4, was begun. The external EDTA solution was decanted and replenished daily. After 9 days of EDTA dimineralization in this manner, the dialysis bags were removed and the suspension was centrifuged in a Sorvall RC-2-B centrifuge, using the SS-34 rotor, at 10 000 rpm for 30 min at room temperature. The EDTA extract was desalted by preparative gel filtration, using Bio-Gel P-2 (Bio-Rad, 5 × 60 cm) in 0.05 M triethylammonium bicarbonate buffer, pH 9.5.

DEAE-Cellulose Chromatography. The desalted EDTA extract was dissolved in 0.05 M Tris-HCl, pH 8.2, at 40 °C and the solution was pumped into a DEAE-cellulose column (Whatman DE-52, 2.0 × 30 cm), which was maintained at 40 °C. Fractions of 2 mL were collected and the absorbance at 230 nm was determined for each fraction. One hundred milliliters of starting buffer was collected and a linear NaCl gradient (to 0.05 M) in starting buffer was begun. The NaCl concentration was determined by conductivity, measured by the resistance of every tenth fraction. Pooled fractions were desalted by gel filtration on Bio-Gel P-2 (Bio-Rad, 0.9 × 60 cm) in triethylammonium bicarbonate buffer, pH 9.5.

Characterization of the Bovine Phosphoprotein. Amino Acid Analysis. The amino acid composition of the phosphoprotein was determined by a modification of the original method of Spackman et al. (1958). One-milligram samples were hydrolyzed for 22 h in triple-distilled 6 N HCl in vacuo at 108 °C, and the acid hydrolysate was analyzed using a single-column procedure on the JEOL 6 AH amino acid analyzer. To determine the presence of γ-carboxyglutamic acid, a 1 mg sample of the phosphoprotein was hydrolyzed in 2 N KOH (Hauschka et al., 1975) and the alkaline hydrolysate was neutralized and analyzed using our standard single-column procedure. In this system, γ-carboxyglutamic acid, the gift of Dr. John Stenflo, is well resolved from aspartic acid.

Phosphorus Analysis. Total phosphorus was measured after wet ashing, which converts organic phosphorus to inorganic phosphate (Kirkpatrick and Bishop, 1971). The inorganic phosphate content of the intact protein was measured by eliminating the wet-ash step. Inorganic phosphate was quantitated as the phosphomolybdate complex, using KH_2PO_4 as the standard (Kirkpatrick and Bishop, 1971).

Agarose–Guanidine Hydrochloride Chromatography of

Reduced and Alkylated Phosphoprotein. The procedure for reduction and alkylation was taken from Monson and Bornstein (1973). The derivatized phosphoprotein was desalted by chromatatography on Bio-Gel P-2 (Bio-Rad Laboratories, 0.9 × 60 cm) in 0.05 M triethylammonium bicarbonate buffer, pH 9.5, and was lyophilized. Amino acid analysis of an acid hydrolysate of the reduced and alkylated phosphoprotein revealed no residual half-cystine. The reduced and alkylated phosphoprotein was then subjected to chromatography on Bio-Gel A-5m (Bio-Rad Laboratories, 1.5 × 90 cm) in 6 M guanidine hydrochloride (Heico, Inc.) at pH 7.0, using absorbance at 230 nm to monitor elution. For molecular weight estimation, two phosphoprotein markers were used: phosvitin (Nutritional Biochemicals Co.) and the major CNBr peptide of phosvitin, prepared by the method of Volpin and Veis (1973).

Calcium-Binding Experiments. A calcium-ion selective electrode (Orion, Model 92-20), single-junction reference electrode (Orion, Model 90-01), and pH meter (Radiometer, pH M-62) were used to measure the calcium-ion binding property of the phosphoprotein at room temperature (Carr, 1973). All samples and standards were dissolved in 8×10^{-4} M KCl, pH 6.5-7.0, as recommended by the manufacturer. The calcium-ion selective electrode exhibited the expected Nernstian behavior in the $CaCl_2$ concentration range 1×10^{-4} to 1×10^{-1} M. The response time of the electrode, i.e., the time required to achieve 95% of the final potential, was less than 10 s and the potential readings were accurate to ±0.15 mV when the entire apparatus was placed in a Faraday cage. To determine the effect of protein concentration on calcium-ion binding, binding experiments were conducted at two protein concentrations, 0.1 and 0.5 mg/mL. Values for the amount of calcium ion bound were obtained by subtraction of the calcium-ion activity curve obtained in the presence of phosphoprotein from the calcium-ion activity curve obtained in the absence of phosphoprotein. Measurement of the pH of the phosphoprotein solution before and after calcium titration revealed only a small pH decrease (<0.2 pH unit).

Circular Dichroism. The circular dichroism (CD) spectrum of the phosphoprotein was recorded using a Cary Model 60 recording spectropolarimeter with a Cary Model 6002 circular dichroism accessory. Measurements were made at room temperature (21 °C) in a 1-mL path-length quartz cell (Luminon, Inc.). Residue concentrations were determined by amino acid analysis of acid hydrolysates of the samples. An aqueous solution of d-10-camphorsulfonic acid (0.01%, w/v) was used to check the calibration of the circular dichrometer (Adler et al., 1973).

Phosphorus-31 Nuclear Magnetic Resonance Spectroscopy. ^{31}P NMR spectra were recorded using a Bruker HFX-5 spectrometer with ^2D stabilization operating at 36.43 MHz for ^{31}P (21-kG magnetic field, ^1H frequency 90.00 MHz) and containing facilities for Fourier transform, signal-averaging, and broad-band and continuous-wave heteronuclear-^1H decoupling (Henderson et al., 1972; Glonek et al., 1970). Deuterium oxide, added to all final samples, was used for field-frequency stabilization. Phosphoprotein samples, containing 16 to 21 mmol of phosphorus, were filtered through glass wool immediately prior to introduction into 13-mm diameter spinning sample tubes. Samples were maintained at 25 ± 1 °C. The chemical-shift data are reported relative to 85% H_3PO_4 as has been customary (Crutchfield et al., 1967; Van Wazer and Glonek, 1972) in ^{31}P NMR. In all of the NMR samples, the countercation for the phosphoprotein was the sodium ion. To minimize the effects on the ^{31}P spectrum of sample contami-

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[1] Abbreviations used are: EDTA, (ethylenedinitrilo)tetraacetic acid; Tris-HCl, 2-amino-2-hydroxymethyl-1,3-propanediol hydrochloride; DEAE, diethylaminoethyl; CD, circular dichroism; NMR, nuclear magnetic resonance.

TABLE I: Composition of Bovine Dentin Phosphoprotein.

Residue[a]	Residues/1000 residues
Acid hydrolysis	
Asp	376
Ser(P) + Ser	418
Glu	37
Lys	49
Gly	44
Pro	T
Ala	13.2
Thr	13.7
Leu	7.9
Phe	4.9
His	9.8
Arg	5.6
½-Cys	3.6
Val	5.5
Ile	3.5
Tyr	4.1
Met	1.8
Hyl	
Hyp	
Met(O)	
Alkaline hydrolysis	
Glu(CO₂)	
Trp	
Phosphorus analysis	
Total P	4.8%
P$_i$	<0.01%

[a] Abbreviations used: Ser(P), phosphoserine; ½-Cys, half-cystine; Hyl and Hpl, hydroxylysine and hydroxyproline, respectively; P$_i$, inorganic phosphate.

nation by trace amounts of metal cations (Van Wazer and Glonek, 1972), especially transition metals, 10 mg of the sodium salt of the phosphoprotein was dissolved in a solution containing 1 part of 0.1 M NaEDTA, pH 9.5, and 4 parts D$_2$O. For the hydrogen-ion titration experiments, 20 mg of the sodium salt of the phosphoprotein was dissolved in water–D$_2$O (1.3:0.2, v/v) and aliquots of NaOH and HCl were added as needed. In the calcium-ion titration experiment, aliquots of aqueous CaCl$_2$ were added to the pH 7.0 phosphoprotein solution. The addition of CaCl$_2$ to the phosphoprotein solution caused a small decrease in pH (<0.3 unit), but the final pH of the calcium-containing solution was adjusted to 7.0 prior to scanning. To preclude the possibility that the CaCl$_2$ solution was contaminated by paramagnetic transitional-metal ions, the solution was tested for its effect on inorganic orthophosphate and several other simple alkyl phosphate mono-, di-, and triesters. Thus tested, the solution exhibited no evidence for paramagnetic contamination (Henderson et al., 1975).

Infrared Spectroscopy. Infrared spectra (400–2000 cm^{-1}) were recorded using a Beckman IR-12, double-beam, double-prism, grating spectrophotometer. Mixtures of solid samples and crystalline potassium bromide were pressed at 100 000 lb/in^2 for 1 min, to form transparent KBr wafers containing 1% protein. Crystalline hydroxylapatite was obtained from Bio-Rad (Bio-Gel HT). For the D$_2$O-solution spectra, Irtran-2 windows (International Crystal Laboratories, Irvington, N.J.) and Teflon spacers (0.05-mm path length) were used with water-jacketed cell holders. The temperature of the jacketed cell holders was maintained at 26 °C by circulating water while the actual temperature was monitored by a thermistor (YSI). The sodium salt of the phosphoprotein
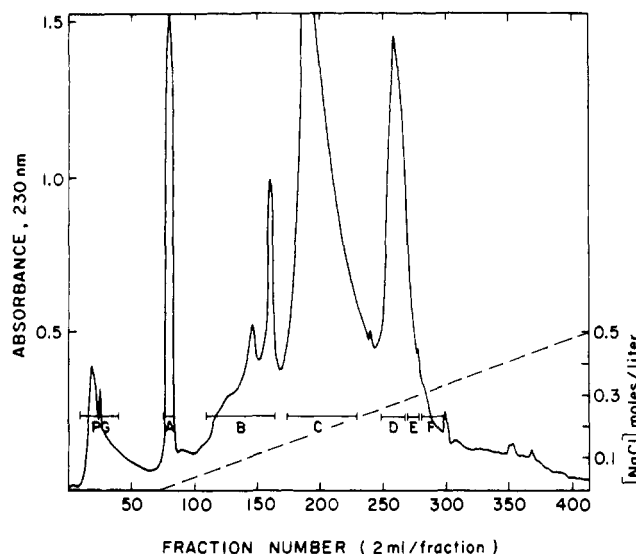

FIGURE 1: DEAE-cellulose chromatography of desalted EDTA extract, in 0.05 M Tris-HCl, pH 8.2, at 40 °C, using a linear NaCl gradient in 0.05 M Tris-HCl, pH 8.2. All peaks were collected separately, desalted, and hydrolyzed, and their amino acid compositions were determined. Only peak D contains the dentin phosphoprotein.

(0.5 mg/mL) was dissolved in D$_2$O (Bio-Rad, 99.8 mol % D$_2$O). One-half of the D$_2$O solution of the sodium phosphoprotein was immediately scanned in the infrared frequency range 1500–1640 cm^{-1}. One microliter of 0.1 M CaCl$_2$ was added to the remaining half of the D$_2$O–phosphoprotein solution (Ca/P ratio 1:2) and the calcium-containing solution was scanned in the same frequency range.

Results

Characterization of the Bovine Dentin Phosphoprotein. The EDTA extract of bovine molars contains a number of proteins originating from the dentin and the enamel. These proteins are resolved on DEAE-cellulose chromatography, as shown in Figure 1. Previous work in this laboratory (Veis et al., 1972; Dickson et al., 1975) showed that the dentin phosphoprotein eluted at approximately 0.3 M NACl, corresponding to peak D of Figure 1. Amino acid analysis of peak D, shown in Table I, confirmed that this was the aspartic acid–serine enriched protein previously identified as originating in the dentin. Enamel proteins rich in proline, glycine, and glutamic acid (Glimcher et al., 1964) appear, with other dentin proteins in peaks PG, A, B, and C which elute at lower ionic strength.

The amino acid analysis data in Table I are presented as the directly determined values for the numbers of residues of aspartic acid, serine, and phosphoserine, uncorrected for losses during acid hydrolysis. It is particularly difficult to obtain a precise value for the seryl residue content in a protein containing both seryl and *O*-phosphoseryl residues (Shainkin and Perlmann, 1971). Serine is lost by destruction during acid hydrolysis, while *O*-phosphoserine may be directly degraded or converted first to serine before destruction. Thus, even though destruction of serine may occur, timed hydrolysis studies of mixtures of serine and phosphoserine first show an increase in serine content, making a zero-time extrapolation difficult. Nevertheless, the sum of serine and phosphoserine determined under standard conditions is quite reproducible, indicating that net destruction rates of both serine and phosphoserine are similar. The reproducible content of total seryl residues in the phosphoprotein is 1.47 μmol of seryl residues/ mg of protein. Assuming a standard acid hydrolytic loss of
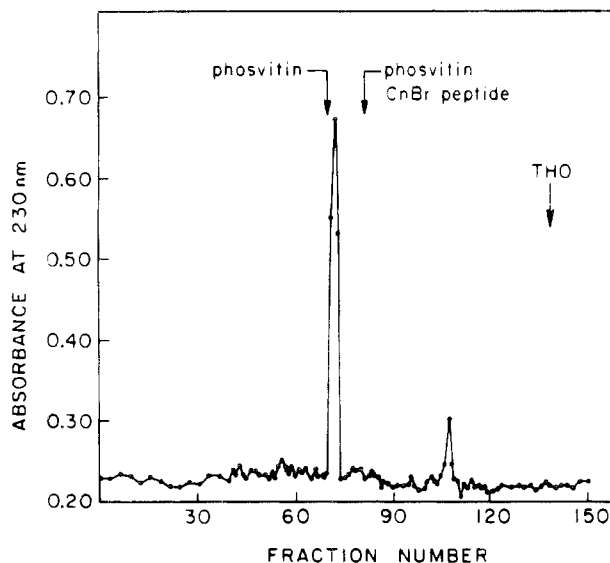
FIGURE 2: Gel filtration chromatography of the reduced and alkylated bovine dentin phosphoprotein on agarose A5m. Elution with 6.0 M guanidine hydrochloride arrows indicate elution peaks for the phosvitin markers.

10-12% (Blackburn, 1968), the phosphoprotein contains 1.62 μmol of seryl residues/mg. The phosphoprotein contains 4.8 + 0.1% phosphorus, corresponding to 1.6 μmol/mg of protein, indicating nearly equimolar concentrations of organic phosphorus and serine residues. Since no inorganic phosphate was detected, these data suggest that practically all of the serine is present in the phosphoprotein as phosphoserine. Thus, aspartic acid and phosphoserine account for 78 residue % of the protein.

To determine the presence of other acid-labile residues, such as γ-carboxyglutamic acid and tryptophan, the phosphoprotein was subjected to alkaline hydrolysis. Neither γ-carboxyglutamic acid nor tryptophan was found in the hydrolysate. Both amino acids would have been detected if present at the level of 1 residue per 1000, given the amount of protein taken for analysis.

Ultraviolet absorption spectroscopy confirmed the earlier observation of the presence of a 260-270-nm absorbing moiety in the isolated protein (Veis et al., 1972). The origin of this absorbance is still undetermined.

After isolation, the phosphoprotein appears to be less soluble and aggregates at low ionic strength. In order to determine an approximate minimum molecular weight, and since the phosphoprotein contains some cysteine, the phosphoprotein was reduced and alkylated and then subjected to gel filtration chromatography under minimally aggregating conditions in 6 M guanidine hydrochloride. The reduced and alkylated phosphoprotein elutes as a single component on Agarose A-5m chromatography in 6 M guanidine hydrochloride, Figure 2. Using the egg-yolk phosphoprotein, phosvitin (molecular weight 36 000), and the major CNBr peptide of phosvitin (molecular weight 31 000) to calibrate the agarose column (Taborsky, 1974), a provisional minimum molecular weight range for phosphoprotein is 34 000-35 000.

Calcium-Ion Binding to the Phosphoprotein. The potentiometric binding data are presented in the form of double-reciprocal plots in Figure 3 in terms of ν, the moles of calcium ion bound per milligram protein. This form of computation avoids uncertainties due to the state of aggregation of the phosphoprotein. In the ideal case, binding of a ligand to a

protein with a single set of independent calcium-ion binding sites is characterized by a linear plot of $1/\nu$ vs. $1/[Ca]$. In the present case, each binding plot is biphasic and this can be interpreted as indicating at least two classes of calcium-ion binding sites on the phosphoprotein. A second and distinctly different type of nonideal behavior is also shown in Figure 3. In the ideal case, ν should be independent of the protein concentration at any given ligand concentration. This is clearly not the case and different binding plots are obtained at the two different protein concentrations examined.

The binding parameters n_i (maximum number of ions bound at sites of class i) and k_i (the intrinsic association constants for sites of class i) were determined using the formulation of Klotz

$$1/\nu_i = \frac{1}{n_i k_i [Ca]} + \frac{1}{n_i}$$

by linear least-squares fit of the data. At the lower protein concentration (0.1 mg/mL), n_i = 2.7 μmol of calcium-ion bound to the high-affinity binding sites, with a binding constant, k_1 = 3.6 × 10^4 mol^{-1}. At the higher protein concentration (0.5 mg/mL), the binding constant for these sites is essentially unchanged, but n_i, the number of calcium ions bound, with k_1 = 1.3 μmol/mg of protein. The second class of lower-affinity binding sites, with k_2 = 5 × 10^2 mol^{-1}, apparently binds a larger maximum number, n_2, of calcium ions. The value of n_2 could not be reliably calculated for this class of sites because the data did not fit a linear plot.

It is of interest to compare the number of anionic groups on the phosphoprotein with the actual numbers of bound calcium ions. Two types of anionic groups are present in the phosphoprotein: divalent phosphomonoesters and monovalent aspartyl or glutamyl side-chain carboxylates. There is a total of 2.9 μmol of such groups per mg of protein. The number of high-affinity binding sites at low protein concentration, 2.7 μmol/mg, is thus approximately equal to the number of available anionic groups. Binding to the low-affinity sites clearly exceeds the maximum number of anionic groups in the protein. It is possible that backbone polar groups may be involved in the low-affinity binding sites, but it is more likely that these additional calcium ions are absorbed to the protein-calcium surface, along with an electrostatic equivalent of microcounteranions. It appears that the class 2, low-affinity sites are indefinite in number, representing condensation of excess calcium salt on the protein.

During the potentiometric measurements the calcium phosphoproteins appeared stable. Upon standing, however, turbidity developed and precipitates ultimately were seen.

Conformation of the Phosphoprotein. The calcium binding was accompanied by a red shift in the peptide-bond absorption, suggesting that a conformational transition paralleled binding. Upon addition of 1 × 10^{-2} M CaCl$_2$ to an aqueous solution of the phosphoprotein (0.5 mg/mL), the λ_{max} shifted from 222 to 228 nm and the extinction at λ_{max} was doubled. Another indication that a conformational change in the phosphoprotein accompanied calcium binding was revealed by circular dichroism. In aqueous solution at pH 7, the CD spectrum of the phosphoprotein resembles that of a random-chain molecule, with a weak negative dichroic band at 235 nm and a weak positive dichroic band at 218 nm (Figure 4). The addition of calcium chloride, in the range from 5 × 10^{-4} to 1 × 10^{-2} M CaCl$_2$, leads to a shift of the negative dichroic band to 227 nm and the disappearance of the positive dichroic band. The addition of ethanol to an aqueous solution of phosphoprotein engenders a comparable change in the CD spectrum. Direct
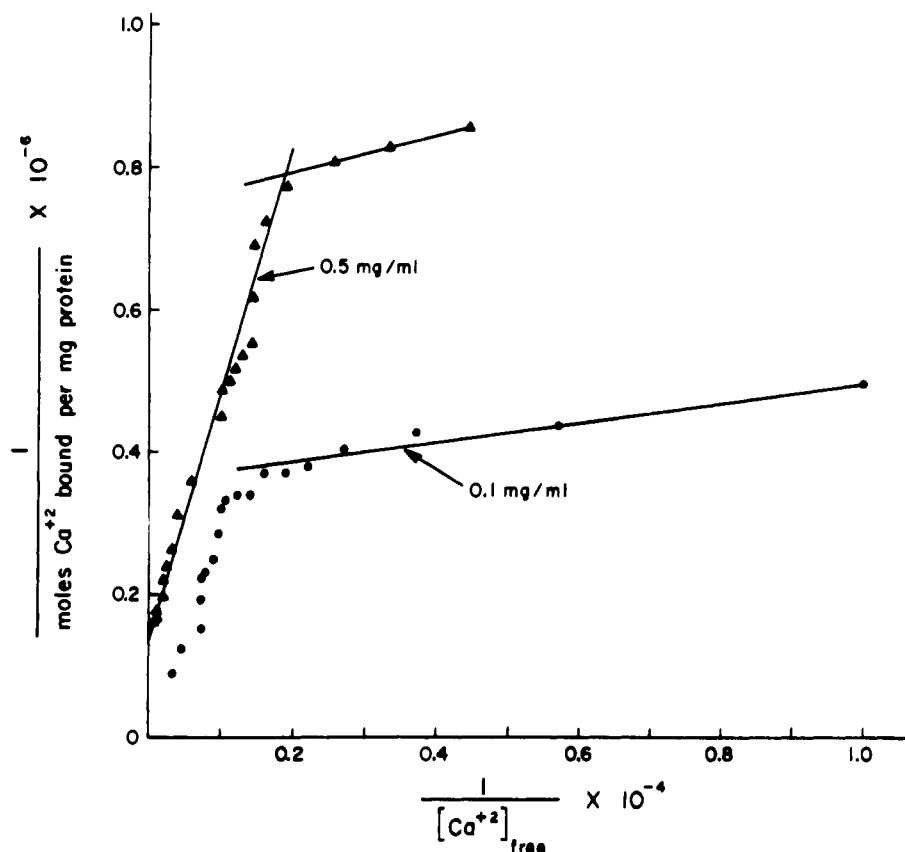
FIGURE 3: Double-reciprocal plots for calcium-ion binding to the bovine dentin phosphoprotein in 8×10^{-4} M KCl, at 21 °C. (●) 0.1 mg of protein/mL; (▲) 0.5 mg of protein/mL. Data are plotted as the reciprocal of moles of calcium-ion bound per milligram of protein vs. the reciprocal of moles of free calcium ion.
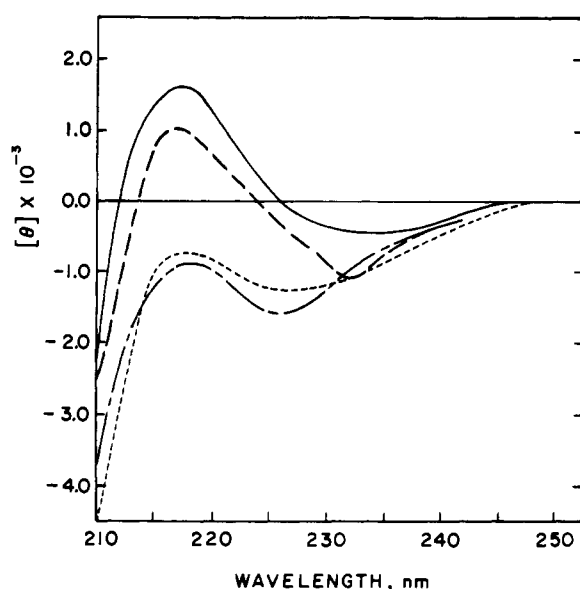
FIGURE 4: Circular dichroism spectrum of the bovine dentin phosphoprotein, 1 mg/mL, 21 °C. (-) In water; (- -) final concentration of CaCl₂ = 5.0 × 10⁻⁴ M; (- - -) final concentration of CaCl₂ = 1.0 × 10⁻² M; (- - -) in 50% methanol-water. Ellipticity, θ, is plotted as a function of wavelength, λ.

FIGURE 5: ^{31}P NMR spectrum of sodium phosphoprotein, 10 mg/mL, in D₂O-0.05 M NaEDTA, pH 9.5 (4:1, v/v), sweep width was 2500 Hz.

correlation of the conformational data in the presence of calcium ions to the binding data is not possible at present because, in order to obtain a sufficiently high signal to noise ratio in the CD studies with the apparatus available, the concentration of
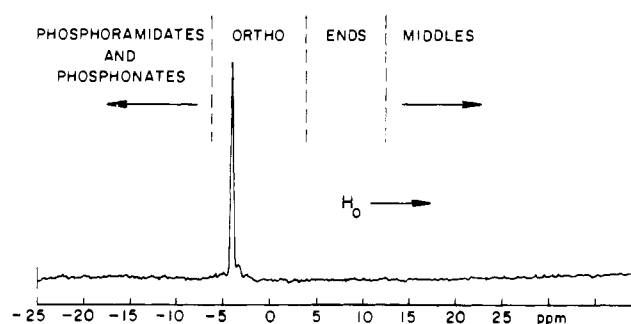
phosphoprotein for the conformational studies was in the higher range, 1-2 mg/mL.

Characterization of the Phosphorus in the Phosphoprotein. The ^{31}P NMR spectrum of the purified sodium phosphoprotein in EDTA-D₂O (1:4, v/v), pH 9.5, is shown in Figure 5. There is a single major phosphorus resonance at −4.2 ppm (−153 Hz, downfield from the 85% H₃PO₄ standard). This chemical shift is consistent only with the presence of orthophosphate monoesters. Resonances for free inorganic orthophosphate (−2.3 ppm), phosphodiesters (−0.5-2.0 ppm), and substituted pyrophosphates (10-12 ppm) are all absent (Crutchfield et al., 1967; Burt et al., 1976).

Further evidence supporting the interpretation that the phosphorus is in the phosphomonoester form in the phosphoprotein is obtained from an examination of the change in ^{31}P
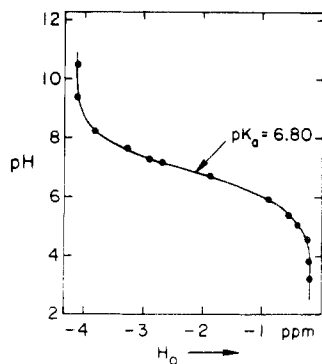
FIGURE 6: Hydrogen-ion titration of sodium phosphoprotein (20 mg/mL) in H$_2$O-D$_2$O (1.3:0.2, v/v) by ^{31}P NMR. Chemical shifts in ppm are those of major orthophosphate resonance.
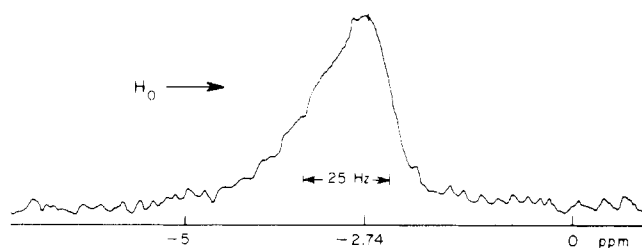

FIGURE 7: ^{31}P NMR spectrum of sodium phosphoprotein (10 mg/mL) in D$_2$O-0.05 M NaEDTA, pH 7.18 (4:1, v/v), sweep width was 200 Hz. Same sample as Figure 1.

chemical shift upon lowering the pH. The ^{31}P resonance band moves upfield as the solution becomes more acidic, so that at pH 3.46 the peak resonance is at −0.2 ppm.

The ^{31}P titration curve of Figure 6 was generated by the stepwise addition of acid to an alkaline sodium phosphoprotein solution, in the absence of EDTA. This curve shows a single class of ionizable phosphomonoester groups with an average pK_a of 6.80, uncorrected for the 10% D$_2$O. The results are surprisingly comparable to the titration of any single phosphate monoester possessing a single weak acid proton (Ho and Kurland, 1966; Ho et al., 1969). Phosphodiesters and unsymetrically substituted pyrophosphates, which are strong acids, can be excluded, since these compounds exhibit no pH dependence in their ^{31}P chemical shifts in the pH range examined (Ho et al., 1969). Spectra at selected pH values obtained in the presence of added EDTA were, with the exception of small chemical-shift changes, identical to those obtained in the absence of the chelating agent, demonstrating that the preparation was essentially uncontaminated by alkaline-earth and transition-metal cations.

The phosphomonoester resonance exhibits some fine structure about the main resonance peak, as can be seen at the base of the main resonance in Figure 5. In addition, the shape of the main resonance indicates that it is composed of multiple peaks. Figure 7 shows the sodium phosphoprotein at pH 7.18 using an expanded sweep width of 200 Hz, in contrast to the spectrum of Figure 5 recorded at a sweep width of 2500 Hz. The ^{31}P resonance peak is unsymmetric and roughly 25-Hz wide at half-height; a computerized curve analysis (Glonek et al., 1974) suggested at least three component resonance bands of differing intensity with the major resonance component being the farthest upfield.

Calcium Ion–Phosphomonoester Interactions. The ^{31}P NMR spectrum of a sample of the phosphoprotein, containing
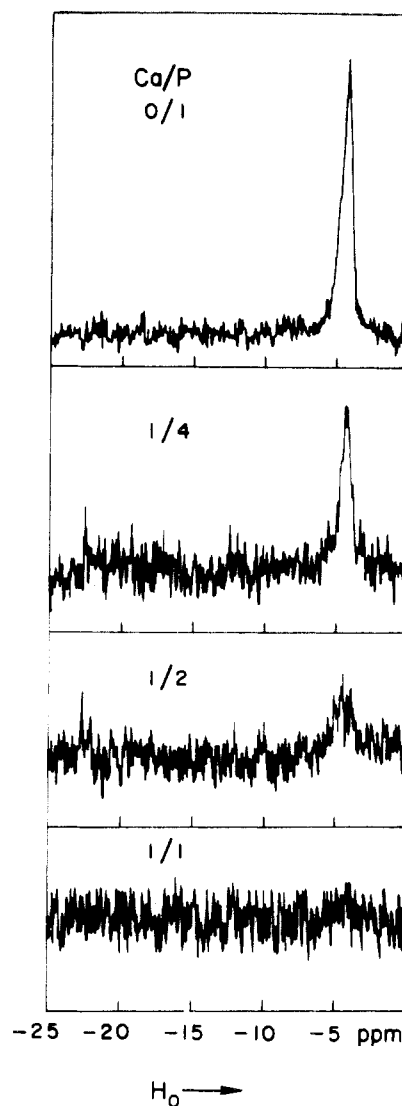

FIGURE 8: ^{31}P NMR spectrum of sodium phosphoprotein (20 mg/mL) in H$_2$O-D$_2$O (1.3:0.2, v/v) in the presence of added calcium chloride. Molar ratio of added calcium to organic phosphorus is noted in each panel.

15 mg/mL of protein, was determined in the presence of increasing concentrations of added calcium ion at pH 7.0 in the absence of EDTA. The four spectra of Figure 8 were recorded in identical manner and show that the peak height decreases and the peak width increases as CaCl$_2$ is added to the sodium phosphoprotein. At an equimolar ratio of calcium and phosphorus, the ^{31}P signal completely disappears. Throughout the 3-h titration and at the end point, the sample gave no evidence for precipitate formation; however, a precipitate did form upon overnight storage at room temperature, as in the potentiometric binding studies. In contrast, the sodium phosphoprotein is stable in solution at pH 7.0. These data leave no doubt that the phosphate groups participate in interactions with calcium ions and that such interactions lead to precipitation of a calcium–phosphoprotein complex.

Calcium Ion–Carboxylate Interactions. Infrared spectroscopy was selected for the examination of specific involvement of carboxylate groups in calcium-ion binding. The spectra of sodium and calcium phosphoproteins were compared with the expectation that calcium-ion association with carboxylate ions would shift the frequency of the carboxyl-group absorp-
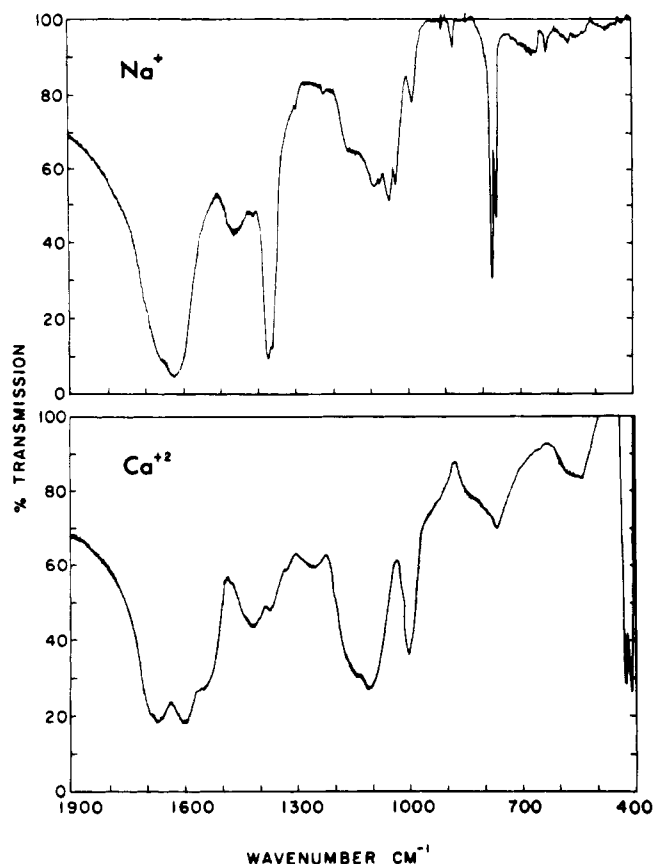
FIGURE 9: Infrared spectrum of solid phosphoprotein salts in KBr wafers. Protein concentration was 1% for each case. Na^+, the sodium salt of the phosphoprotein; Ca^{+2}, the calcium-ion precipitate.

FIGURE 10: Infrared spectrum of phosphoprotein salts in D_2O (0.5 mg/mL). Infrared scans for both salts in the carboxylate range.

tions. The first comparison was that of the solid precipitated calcium phosphoprotein formed after the ^{31}P NMR studies, with that of the lyophilized, calcium-free sodium phosphoprotein, both in the form of KBr wafers, Figure 9. In the sodium-ion form, Figure 9, the amide I band at 1650 cm^{-1} appears as a shoulder on the broader, more intense, ionized carboxylate carbonyl stretch at 1625 cm^{-1}. In the calcium salt, Figure 9, the amide I and carboxylate absorbances are more fully resolved and the relative intensity of the carboxylate absorbance at $\sim$1600 cm^{-1} is reduced, as expected if coordination of the carboxylate anion to the calcium cation had occurred.

Solution studies in the infrared confirm this interaction. The carboxylate absorbance is prominent in the sodium salt–D_2O solution at 1600 cm^{-1}, Figure 10, whereas this absorption is lost in the calcium salt in D_2O solution, Figure 10.

The infrared data of Figure 9 also are in agreement with the NMR data on phosphate–calcium interactions. Studies of the infrared spectra of calcium phosphates has led to the assignment of absorbances in the 600–500 cm^{-1} region to the antisymmetric P–O bend (Stutman et al., 1965; Termine and Posner, 1965). No absorbance in this range can be seen in the sodium phosphoprotein spectrum, Figure 9. In the calcium phosphoprotein, Figure 9, an absorbance at 550 cm^{-1} is prominent. In the spectra of Figure 9, the only phosphorus present in the samples is in the organic phosphomonoesters of the precipitated phosphoprotein; therefore, it can be concluded that the calcium ion–phosphomonoester interaction must be of the same nature and strength as that seen in inorganic calcium phosphates.
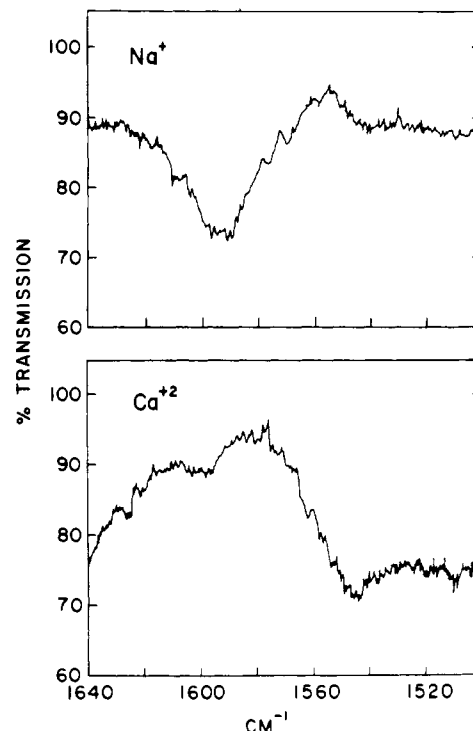
Discussion

The reduced and alkylated phosphoprotein has a molecular weight of about 35 000, based upon its elution on agarose A5 in 6 M guanidine hydrochloride. The amino acid analytical data are in reasonable agreement with these data; assuming 1 residue of methionine/mol, a molecule would contain 326 residues for a chain weight of $\sim$32 000. Even if the error is large, we can imagine the molecule in aqueous, calcium-free solution to be in a random-chain configuration, bristling with about 111 aspartic acid carboxylate side chains and somewhat more, perhaps 124, ionized phosphate groups present as phosphomonoesters of serine. At neutral pH and low ionic strength, such a molecule would behave as a fairly extended random-chain molecule due to charge repulsions. Moreover, with such a high density of negatively charged side chains, three charged residues per every four amino acid residues, it is not surprising that cations are avidly bound and that divalent calcium, in particular, binds strongly to the protein. There are, however, several features of the binding data which are both unexpected and of special interest.

First, as the double-reciprocal plots, Figure 3, show, at low protein concentration the phosphoprotein binds an equimolar rather than electrostatic equivalent amount of calcium ions with $k_1 = 3.6 \times 10^4$ mol^{-1}, that is, 1 mol of calcium ion for each mol of carboxylate or phosphate groups at pH 7.0. At higher calcium ion concentrations, an additional set of calcium ions condense on the calcium phosphoprotein, probably in an indefinite number and in a process related to the reversal of charge phenomena observed in other strongly interacting polyelectrolyte-counterions systems (Kruyt, 1948). Obviously, to maintain electrical neutrality, excess chloride or hydroxide ions must also be associated with the calcium–phosphoprotein complex. The apparent association constant for this undefined set of more weakly bound calcium ions is two orders of magnitude less than k_1, i.e., $k_2 = 5 \times 10^2$ mol^{-1}.
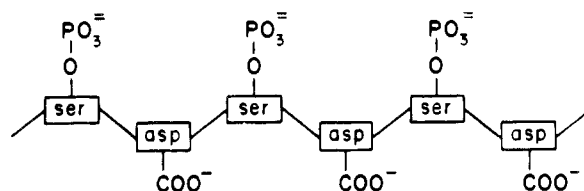
FIGURE 11: Proposed model for calcium-binding regions of bovine dentin phosphoprotein. The backbone is presumed to be in an extended trans-conformation, placing phosphate and carboxylates at regular intervals on either side of the plane of the extended peptide backbone. Hydrogen bonding to form sheet-like structures would run perpendicular to the plane of the paper.

Second, the nonideal dependence of the binding on protein concentration is probably an indication that, at the higher protein concentrations, phosphate or carboxylate ions on neighboring protein molecules replace chloride or hydroxide counterions and one obtains intermolecular calcium-mediated chelate complexes. Formation of such complexes reduces the number of calcium ions bound per protein molecule to the electrostatic equivalence value. The strong binding of many calcium ions reduced the solubility of the phosphoprotein and aggregation cannot be avoided when the phosphoprotein is exposed to a stoichiometric amount of calcium ions. The precipitation provides strong support for the concept of formation of intermolecular calcium chelate complexes.

Of equal interest, the circular dichroism data show that a conformational change accompanies binding of calcium ions. Although there is little data on other phosphoproteins in general, Grizzuti and Perlmann (1970, 1973) and Perlmann and Grizzuti (1971) have demonstrated that the egg-yolk phosphoprotein, phosvitin, which contains 50% phosphoserine, exhibits a random-chain structure in aqueous solution but forms a β sheet in aqueous methanol. In water, the sodium salt of the bovine phosphoprotein exhibits a CD spectrum characteristic of random chains. The addition of CaCl$_2$ or methanol (Figure 4) to the bovine phosphoprotein converts it to a more ordered structure, but, in contrast to phosvitin, the transition does not go entirely to a β sheet. More probably, the structure is that of trans-extended chains in sheet-like arrays over short intervals (Jirgensons, 1973; Blow, 1971).
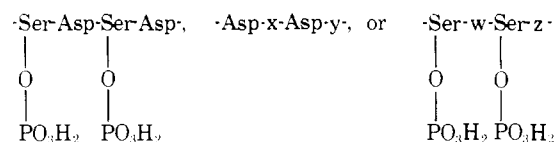
Two questions concerning the phosphate groups in the bovine dentin phosphoprotein have been answered in this study. First, the very sharp single ^{31}P NMR resonance peak −4.2-ppm downfield from the inorganic orthophosphate signal (Figure 5) characterizes the phosphate groups as being phosphomonoesters. Resonances for inorganic orthophosphate, phosphodiesters, and pyrophosphates were not seen. The minimum detectable concentration of these species was 1 mM, thus limiting their presence to less than 5% of the organic phosphorus. The 4.0-ppm chemical shift seen on adjusting the pH from 4 to 9 and the apparent pK_a of 6.8 (Ho et al., 1969) are also indicative of the presence of phosphomonoesters. The equimolar concentration of seryl residues and organic phosphorus coupled with the very small content of threonine (1.4 residue %, Table I) lead to the conclusion that most, if not all, the phosphorus occurs as phosphoseryl residues in this phosphoprotein. It is surprising in a system where the ionic side chains are so densely packed along the protein backbone that the phosphate esters have essentially the same pK as small molecule monoester phosphates. Since the sodium salts were used in the titration experiments, it is likely that sodium counterions must also be closely associated with the protein before displacement by specifically bound calcium ion. Second,

both the decrease in the resonance signal and the line-broadening effect (peak width at half-height) of calcium ion on the ^{31}P NMR resonance at pH 7.0 show unequivocally that the phosphomonoester groups are involved in strong calcium-ion interactions. These effects are similar to those seen in phosvitin at alkaline pH and α_s-casein at pH 7.0 (Ho et al., 1969). Line broadening may be the result of several factors which alter the spin–lattice and spin–spin relaxation times (Glonek et al., 1975a,b). Additional potential sources of line broadening are a distribution of chemical shifts resulting from the nonequivalence of phosphorus atoms in the sample or the formation of large molecular aggregates which give rise to dipolar broadening. Both of these latter factors may very well be involved. The expanded spectrum of Figure 7 shows that at least three component peaks must be present, indicating a multiplicity of chemical shifts at pH 7.18. The far infrared data showing the appearance of a 550-cm^{-1} absorbance in the calcium phosphoprotein spectrum (Figure 9) give further evidence of calcium–phosphate group interaction similar to those seen in calcium hydroxylapatite.

The infrared data also give an affirmative answer to the question of involvement of the carboxyl groups of aspartic acid in calcium-ion binding both in the solid phase and in D$_2$O solution.

An important qualitative observation was that the addition of calcium ion to a stable sodium phosphoprotein solution caused the protein to precipitate as a calcium–phosphoprotein complex.

Three out of every four residues in the dentin phosphoprotein are either aspartyl or phosphoseryl residues, in approximately equimolar amounts; hence, sequences such as

$$-Ser\text{-}Asp\text{-}Ser\text{-}Asp\text{-}, \quad -Asp\text{-}x\text{-}Asp\text{-}y\text{-}, \quad or \quad -Ser\text{-}w\text{-}Ser\text{-}z\text{-}$$

must be of frequent occurrence. The residues x, y, w, and z might also be Asp or Ser giving rise to small block polymer sections. Weiner and Hood (1975) have recently isolated an acidic protein from a mollusk shell matrix. This acidic protein is not a phosphoprotein but it appeared to be involved with calcium binding. Sequence studies showed it to contain regions in which aspartyl residues were regularly placed as every second residue. In a trans-extended chain conformation, these aspartyl side-chain carboxyl groups are on the same side of the chain at distances of ∼7 Å from each other. Weiner and Hood (1975) speculated that calcium ions, with a radius of ∼4.5 Å, could bind to each carboxylate acting as a bidentate ligand. In the dentin phosphoprotein, the seryl phosphate side chains are of nearly the same size and placement as the aspartyl side chains and, hence, in a trans-extended structure, as indicated by the CD data, the phosphate groups could bind calcium ions in the same fashion. Moreover, one of the specific repetitive sequences likely to occur in the dentin phosphoprotein, depicted in Figure 11, or any other trans-extended chain (phosphoserine, asp)$_n$ block would place properly spaced calcium-ion binding sites on either side of the plane of peptide bonds of a single chain. This type of structure, in which acidic groups are adjacent or alternating along peptide backbones, may be of general importance in all calcifying systems.

The observation that the phosphoprotein becomes insoluble upon binding calcium ions and at the same time is capable of sequestering more than an electrostatic equivalence of calcium ions is of profound physiological significance. Weinstock and

Leblond (1973) have found the phosphoprotein in mineralizing rat incisors to be located specifically at the mineralization front, the dentin-predentin junction, the site of appearance of hydroxylapatite crystals. Nawrot et al. (1976) have recently shown the bovine dentin phosphoprotein to catalyze the conversion of supersaturated solutions of calcium phosphate to the hydroxylapatite crystalline form. With the knowledge that the dentin phosphoprotein acts to sequester large amounts of calcium ion, we speculate with some confidence that this calcium-binding protein of the dentin matrix plays a very specific role in mineralization of dentin.

Acknowledgments

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