Preparation, Detection, and Characterization of an Antibody to Rat α-Phosphophoryn†

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Received March 14, 1985

ABSTRACT: The phosphophoryn components of rat incisor dentin were extracted under stringent conditions to prevent proteolytic degradation during processing. Successive steps of $CaCl_2$ precipitation, ion-exchange chromatography, and gel filtration over Biosil TSK G4000SW high-performance liquid chromatography columns in 4.0 M guanidine hydrochloride yielded a mixture of phosphoryns that contained only trace amounts of other proteins, as shown by a very sensitive double stain procedure on acrylamide gels after electrophoresis. The highest weight phosphophoryn had a molecular weight of 90 000, on gradient polyacrylamide gels calibrated with globular protein standards. Polyclonal antibodies were produced in rabbits following a complex scheme. The specific antibodies were collected by passage over a Sepharose column conjugated to the purified phosphophoryn. The isolated antibody was used to prepare a second affinity column. Passage of the initial phosphophoryn fraction over the column led to the retention of a single component, identified as the M_r 90 000 α-phosphophoryn. Thus, a monospecific polyclonal antibody has been prepared. These data show that the other phosphoryns of the rat incisor must be distinct species or slightly degraded products of the α-phosphophoryn lacking the antigenic epitope of the antibody prepared.

The phosphophoryns (PPs) are major noncollagenous protein components of dentin (Dimuzio & Veis, 1978a,b). They are highly anionic proteins in which aspartic acid and serine residues, combined, account for about 80% of the total amino acid content. Most of the seryl residues are phosphorylated (Dimuzio & Veis, 1978a; Richardson et al., 1978). It has been suggested that the phosphophoryns are involved in the process of dentin mineralization. Typical arguments used to support this suggestion are that they bind calcium ions strongly (Lee et al., 1977; Kuboki et al., 1979; Cookson et al., 1980; Zanetti et al., 1981), that they can induce the formation of hydroxyapatite from supersaturated calcium phosphate solutions (Nawrot et al., 1976), and that they are absent from the dentin of patients with Dentinogenesis Imperfecta, type II, a mineralization disorder (Takagi & Veis, 1983).

Previous autoradiographic (Weinstock & Leblond, 1973) and biosynthetic studies (Dimuzio & Veis, 1978b; Maier et al., 1983) have shown that the phosphophoryns are synthesized by the odontoblasts and are deposited in the dentin matrix in the immediate vicinity of the mineralization front at the dentin–predentin junction. The secretory pathway for the phosphophoryns is different from that of the collagen (Weinstock & Leblond, 1973; Dimuzio & Veis, 1978b). Most surprisingly phosphophoryns have not been found in the predentin (Carmichael et al., 1975; Jontell & Linde, 1983). Thus, the intracellular secretory pathway and the route by which the phosphophoryns reach the mineralization front are important pathways, but as yet unknown.

The rat incisor system has been the most studied, but it is complicated by the presence of several phosphoproteins (Dimuzio & Veis, 1978a; Butler et al., 1981). Moreover, the radioautographic studies of Weinstock & Leblond (1973) and similar studies of Cho & Garant (1984) are difficult to interpret because the radiolabeled amino acids used as markers are not sufficiently specific to the phosphophoryns when the relative amounts of the other proteins are taken into account. As the first step in clarifying the intracellular secretory route for the phosphophoryns of the rat incisor, we have prepared phosphophoryn antibodies. Since, as noted above, there are two or more phosphophoryns in the rat incisor, our efforts have been focused on the preparation of a monospecific antibody. This report describes the methods of antibody production and detection for anti-α-phosphophoryn and the proof of its specificity.

Materials and Methods

Demineralization. The protocol for the isolation and purification of rat dentin phosphophoryn is described in Figure 1. Frozen heads from Sprague-Dawley rats were purchased from Pel-Freeze, Inc. The method used to extract and clean the teeth is essentially that of Butler et al. (1981). After extraction, the teeth were washed with 15% NaCl containing 2.5 mM benzamidine hydrochloride, 50 mM ε-amino-*n*-caproic acid, 0.5 mM *N*-ethylmaleimide, and 0.3 mM phenylmethanesulfonyl fluoride as protease inhibitors. They were then stirred in 4 M guanidine hydrochloride containing protease inhibitors at 4–5 °C for 24 h. The incisors were demineralized with 0.5 M ethylenediaminetetraacetic acid (EDTA) containing 50 mM tris(hydroxymethyl)aminomethane hydrochloride (Tris-HCl), pH 8.0, and protease inhibitors in Spectrapor no. 2 membrane tubing (molecular weight cutoff 12 000–14 000). Total demineralization time was approximately 2 weeks. After demineralization, the contents of the dialysis tubings were pooled and centrifuged at 10 000 rpm in a Sorvall RC-2B centrifuge using an SS-34 rotor for 30 min at 4 °C. The soluble dentin extract was concentrated and dialyzed against distilled H_2O in an Amicon Diaflow cell using a PM-10 membrane (molecular weight cutoff 10 000). The solution was then lyophilized (fraction I).

$CaCl_2$ Precipitation. Initial separation of PP from total EDTA extract was attained by using the $CaCl_2$ precipitation

† This work was supported by Grant DE-01374 from the National Institute of Dental Research. This work represents studies included in a Doctoral Dissertation presented by T.-G.T. to the Graduate School of Northwestern University, Evanston, IL, Aug 1984.
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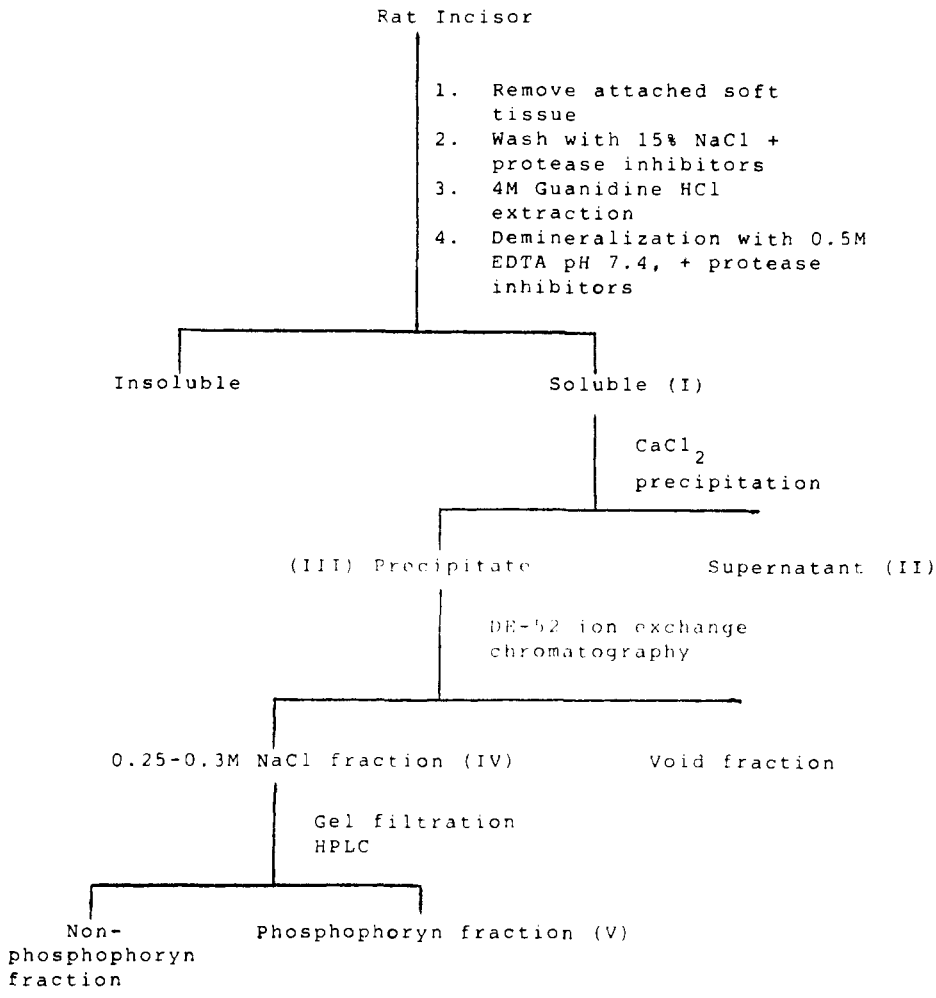

FIGURE 1: Flow diagram for the isolation of rat incisor dentin phosphophoryn. The Roman numerals denote the fraction designations used in the text.

method of Kuboki et al. (1979) as modified by Butler et al. (1981). The EDTA extract was dissolved in a small quantity of 20 mM Tris-HCl, pH 8.0. Solid $CaCl_2$ was added to the solution until the final concentration of 1 M $CaCl_2$ was reached. The solution was then allowed to stand overnight in the cold room. The precipitate was collected by centrifugation. The resulting pellet was redissolved in 0.5 M EDTA, dialyzed against distilled H_2O, and lyophilized (fraction III). The supernatant from the $CaCl_2$ precipitation procedure was also dialyzed and lyophilized (fraction II).

DEAE-cellulose Chromatography. One hundred milligrams of the $CaCl_2$ precipitate was dissolved in 10 mL of 50 mM Tris buffer, pH 8.0. The solution was pumped onto a DE-52 column (Whatman) 2.6 × 15 cm. Fractions of 7.5 mL were collected, and the absorbance at 230 nm was monitored. The column was eluted with 200 mL of 50 mM Tris-HCl buffer before a linear gradient of NaCl (from 0 to 0.5 M over a total volume of 1000 mL) was begun. Conductivity was measured in every fifth fraction to determine NaCl concentration. The fractions eluting between 0.25 and 0.3 M NaCl, which were found to contain a high phosphorus content in the phosphate assay, were pooled, dialyzed against several changes of distilled H_2O in Spectrapor no. 2 tubing, and lyophilized (fraction IV).

High-Performance Liquid Chromatography (HPLC). Five hundred milligrams of the lyophilized phosphophoryn-containing fraction from DE-52 chromatography was dissolved in 1 mL of 4 M guanidine hydrochloride buffer. After the fraction was filtered through a Millex-HA (0.45 μm) filter, 100 μL of the sample was loaded onto a TSK-Gel G4000SW column (Varian) previously equilibrated with the 4 M guanidine running buffer. Fractions of 0.9 mL were collected, and the absorbance at 230 nm was monitored continuously by using a Waters Model 450 variable wavelength detector. The 4 M guanidine hydrochloride buffer was pumped at a flow rate of 0.5 mL/min. Phosphate assays were employed to determine the phosphate content of each fraction. The fractions that had a high phosphate content were pooled and dialyzed against distilled H_2O in an Amicon Diaflow cell using a PM-10 membrane. After lyophilization, the sample (fraction V) was stored in a −20 °C freezer until use.

Phosphate Assay. The ultramicro method of total phosphorus determination as described by Kirkpatrick et al. (1971) was used to determine the relative phosphate content of the chromatography fractions. About 100 μL of sample was first hydrolyzed with 1.5 mL of digestion mixture at 225 °C for 180 min. Strong acids were used to hydrolyze organic phosphate to phosphoric acid. After the sample was cooled in the hood, 0.85 mL of ammonium molybdate reagent was added to the remaining wet ash to form a phosphomolybdate complex. This complex was further reduced by adding 0.05 mL of aminonaphtholsulfonic acid reagent and boiling in a water bath for 10 min. The absorbance of the product was measured at 830 nm.

Polyacrylamide Gel Electrophoresis. Gradient (5–15%) polyacrylamide gel electrophoresis (PAGE) in the presence of sodium dodecyl sulfate (SDS), as described by Laemmli (1970) and modified by Butler et al. (1981), was used. Differential staining of phosphophoryn using 1-ethyl-2-[3-(1-

ethylnaphtho[1,2-*d*]thiazolin-2-ylidene)-2-methyl-1-propenyl]naphtho[1,2-*d*]thiazolium bromide (Stains-all) on polyacrylamide gels was done essentially as described by Green et al. (1973). The Stains-all stained gels were then restained with silver according to the method of Wray et al. (1981).

When the gels were stained by the silver method prior to Stains-all, PPs did not stain well. On the other hand, the initial staining with Stains-all increased the sensitivity of subsequent silver staining toward PPs. The latter sequence, however, also produced a less desirable background, but it picked up all trace contaminants.

Amino Acid Analyses. The analyses were carried out on a JEOL JL6AH automatic amino acid analyzer. Protein samples were hydrolyzed in 6 N HCl at 110 °C for 22 h in sealed tubes. The analyses were performed by using a single-column technique.

Preparation of Anti-PP Antibody. Three New Zealand White rabbits, 4–6 lb, were used in this study. The initial attempt at producing anti-PP antibody was carried out by immunizing the rabbits with homogenized polyacrylamide gel which contained the major PP bands. To immunize three rabbits, 1 mg fraction III was run in a 5–15% gradient SDS–PAGE. After the completion of the electrophoresis, the SDS was removed by washing in 25% (v/v) isopropyl alcohol for 2 h with three changes of the solution. Sections (1 mm wide) from two sides of the gel were cut and stained with Stains-all for 30 min. The stained pieces of gel were then placed next to the remaining gel. The portion in the central, unstained gel that corresponded to the PP position was cut, homogenized, and emulsified with incomplete Freund adjuvant and injected into the rabbits subcutaneously. The remaining unstained gel was stained to verify that the proper components had been sliced out.

The first immunogen injection of homogenized gel corresponded to 200 μg of PP. After 4 months and 8 times of equivalent repeated biweekly injections, the titer of the antisera was still very low. Consequently, the immunization method was changed. Purified PP, fraction V, was dissolved in 2 mL of distilled H_2O and then emulsified with an equal volume of incomplete Freunds adjuvant. Emulsion (1.3 mL), which contained 1 mg of phosphophoryn, was injected subcutaneously in multiple sites along the back of each rabbit. The immunization was repeated every 2 weeks (Table I). Blood (50 mL) was collected 7–10 days after the booster injections from each rabbit by puncturing the ear artery with an 18-gauge needle. Anti-PP antibody was isolated from the blood by initial ammonium sulfate precipitation followed by affinity chromatography with purified phosphophoryn as ligand. Guanidine hydrochloride (4 M) was used to elute antibody from the column.

Detection and Characterization of Anti-PP Antibody. Double immunodiffusion in two dimensions was performed as described by Ouchterlony (1968). Agarose (1%) was made in the veronal acetate buffer. The antigen was dissolved in phosphate-buffered saline (PBS). The concentrations of antigen used were 1 mg/mL and 100, 10, and 1 μg/mL. Antigen and antibody solutions (10 μL each) were applied to the wells punched in the cast agarose gel and were allowed to diffuse in the gel overnight in the cold room.

The indirect enzyme-linked immunosorbant assay (ELISA) used in this study was essentially as described by Engvall (1971). The HPLC-purified rat dentin PP was first dissolved in coating buffer and was then serially diluted. A total of 200 μL of each diluted antigen solution was applied to the wells of 96-well microtiter plates (Immulon II, Dynateck). After a 2-h incubation period, the wells were sequentially incubated with 1% bovine serum albumin (BSA) in PBS, anti-PP antibody, peroxidase-conjugated goat anti-rabbit antibody, and *o*-phenylenediamine. The resulting color was measured in situ by using a Titerteck Multiskan (Flow Laboratories, Inc., Model 310C).

Inhibition ELISA was performed under a procedure similar to the indirect ELISA described above except that the anti-PP antibody was preincubated with different concentrations of inhibitors before it was allowed to react with the antigen coating the plastic wells.

Affinity Chromatography. Affinity columns were prepared by coupling the desired protein to CNBr-activated Sepharose 4B. The ligand was coupled to washed CNBr–Sepharose by incubation in 0.1 M $NaHCO_3$–0.5 M NaCl, pH 8.3, coupling buffer for 2 h at room temperature or overnight in the cold room. The gel–ligand mixture was continuously agitated. At the end of the coupling period the gel was collected by low-speed centrifugation. Excess activated groups were blocked by reacting the ligand-linked gel, after several washings with coupling buffer, with 4% (v/v) ethanolamine (Kodak), pH 8.3. After 2 h at room temperature the ligand-linked gel was washed sequentially in 0.1 M Tris–HCl–0.5 M NaCl, pH 8.3, in 0.1 M sodium acetate–0.5 M NaCl, pH 4.5, and finally in PBS with 0.2% (w/v) sodium azide. The gels were packed in columns and stored at 4 °C.

About 5 mL of gel was used in each column. The columns were washed with PBS until a stable 280-nm base line was attained. For collection of the anti-PP, Fraction V PP was linked to the Sepharose. A solution of 2 mL of ammonium sulfate precipitated antibody was applied in PBS and incubated at room temperature on the column for 2 h. The column was then eluted with PBS until A_{280} again reached base-line value. The antibody bound so strongly that decreasing the eluant pH to 2.0 did not elute the bound protein. The anti-PP antibody was stripped from the column with 4.0 M guanidine hydrochloride in PBS. Immediately after elution with 4.0 M guanidine hydrochloride the column was washed back to PBS plus azide. Although harsh, the guanidine hydrochloride wash did not destroy the column. This affinity column was stored at 4 °C and was used repeatedly without apparent loss of activity.

The anti-PP eluted from the PP-linked column was used as the ligand for construction of a PP-binding column. The same conditions were used. This column too was found to be stable and was used repeatedly. The column was immediately cleared of 4 M guanidine hydrochloride after use.

RESULTS

Characterization of Rat Incisor Dentin Phosphophoryns. Demineralization of the incisors with EDTA results in a soluble and an insoluble fraction. From previous experiments it is known that the insoluble fraction contains collagen and a small amount of protein tightly bound to the collagen (Dimuzio & Veis, 1978a). This fraction was discarded after centrifugation. The amino acid composition of soluble fraction I is shown in Table I. The fraction is rich in aspartic acid, serine, and phosphoserine. These residues comprise more than 60% of the amino acid total, indicating the presence of a high proportion of PP in the total EDTA extract.

When fraction I was run in a 5–15% SDS–polyacrylamide slab gel, two Stains-all bands were seen migrating between the M_r 92 000 and M_r 66 000 standards (Figure 2b), corresponding to the location of rat dentin PPs reported previously (Butler et al., 1981; Dimuzio & Veis, 1978b). However, in contrast to their findings, we observed only two PP bands in

Table I: Amino Acid Compositions of Fractions Obtained during the Purification of Rat Incisor Dentin Phosphophoryn[a]

amino acid	fraction no.[b] I	II	III	IV	V
lysine	25	38	14	11	10
histidine	17	19	11	9	10
arginine	18	35	6	7	7
aspartic acid	252	142	348	317	356
threonine	32	49	17	16	13
serine/phosphoserine	350	146	469	524	495
glutamic acid	91	143	54	50	47
proline	30	76	10	7	9
glycine	66	108	34	34	31
alanine	42	80	14	13	13
half-cystine	4	12	4	0	T[c]
valine	19	35	4	4	3
methionine	4	8	0	0	0
isoleucine	6	11	2	1	3
leucine	26	54	6	4	4
tyrosine	10	21	4	3	2
phenylalanine	10	22	2	2	2

[a] Expressed as residues per 1000 total amino acid residues. [b] Fractions as designated in Figure 1. [c] T = trace.

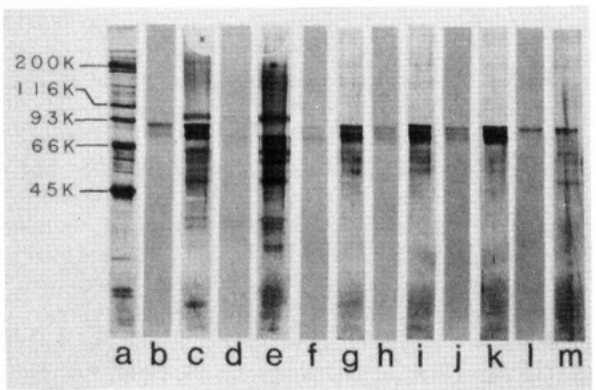

FIGURE 2: $NaDodSO_4$ gradient polyacrylamide gel electrophoresis of rat dentin phosphophoryn at various stages of purification (Stains-all and double stained). Lanes b, d, f, h, j, and i are gels stained with Stains-all only. Lanes a, c, e, g, i, k, and m are the same gels stained with silver after Stains-all staining. Lane a, standards consisting of myosin (M_r 200 000), β-galactosidase (M_r 116 250), phosphorylase B (M_r 92 500), bovine serum albumin (M_r 67 000), and ovalbumin (M_r 45 000). Lanes b and c, fraction I. Lanes d and e, fraction II. Lanes f and g, fraction III. Lanes h and i, fraction IV. Lanes j and k, fraction V. Lanes l and m, fractions eluted from an anti-PP antibody affinity column.

that region. Two to three faint Stains-all bands were also present below the M_r 45 000 standard. The constituents of these bands are not known. The PPs do not stain with Coomassie blue and only faintly with silver staining. However, if silver staining was performed after a gel had been treated with Stains-all, the PP bands became very prominent (Figure 2c). The doubly stained gel is very sensitive and reveals 10 or more bands that are not found with Stains-all. One of the two more prominent of these bands migrates between the 116 000 and 92 000 standards. The other band is located at the same position as bovine serum albumin.

The terminology used by Dimuzio & Veis (1978a,b) to describe the two PPs is adopted in this study. The α-PP denotes the slow moving species and β-PP, the fast species, as seen in the 5–15% gradient SDS–PAGE (Figure 2b,d).

Fraction II yields three faint bands with Stains-all staining following slab gel electrophoresis (Figure 2d). One of these bands migrates to the position of the β-PP. A slow-moving band was observed just above the M_r 93 000 standard, corresponding to the position of the lower phosphorylated phosphoprotein (LP) reported by Butler et al. (1981). When the gel was doubly stained (Figure 2e), the distribution of electrophoretically separated components was identical with that of fraction I with the exception that α-PP was absent from fraction II. The amino acid analysis of fraction II shows a relatively large proportion of aspartic acid, serine/phosphoserine, glutamic acid, and glycine (Table I). But the former two amino acids are significantly lower in concentration than in fraction I, indicating the removal of most PPs from this fraction.

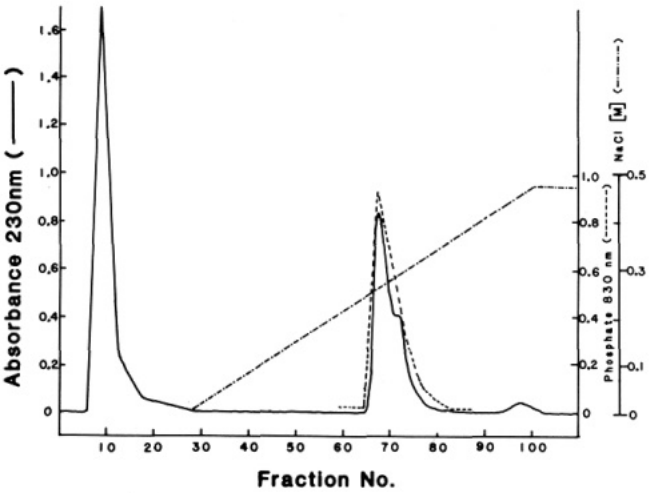

FIGURE 3: DEAE-cellulose chromatography of fraction III (calcium chloride precipitate). A total of 50 mg of sample was applied to a DE-52 (Whatman) column. (·–·) indicates the molarity of the sodium chloride gradient. (- - -) denotes the results of phosphate assay. Only the positive findings for organic phosphate are shown.

The $CaCl_2$ precipitate (fraction III) gives two Stains-all bands corresponding to α- and β-PPs (Figure 2f). In the doubly stained gel, the number of the non-PP bands was significantly less than in both fractions I and II (Figure 2g). The amino acid composition of fraction III is shown in Table I. The aspartic acid and serine/phosphoserine constitue more than 80% of the amino acid total. The quantities of other amino acids are much less than the corresponding amounts in fraction I.

Anion-exchange chromatography resolved fraction III into two main pools (Figure 3). After sample application, the DE-52 column was first washed with 0.5 M Tris buffer, pH 8.0. Unbound material eluted in the void peak. This fraction was determined previously to be composed of EDTA and a small amount of low molecular weight protein (Dimuzio & Veis, 1978a). The phosphate assay did not show any organic phosphate to be present in the void peak. When the column was eluted with a steep gradient of NaCl, a characteristic biphasic peak of PPs was eluted from 0.25 to 0.3 M NaCl concentration (fraction IV). The phosphate assay confirmed the presence of organic phosphate in this fraction. The amino acid analysis of fraction IV (Table I) shows an increase in the amount of aspartic acid plus serine and phosphoserine. The quantities of other amino acids either decreased or remained the same.

When fraction IV was run in a gradient slab gel, one additional new Stains-all staining band was found between the α-PP and β-PP positions (Figure 2h). Several runs of DE-52 chromatography were subsequently performed with different batches of sample. The PP fractions from all these runs consistently yielded the three PP bands in the gradient SDS–PAGE. The possibility that this middle Stains-all staining band arose as an heterogeneity artifact caused by pooling the samples is minimal. For the ease of discussion, this middle

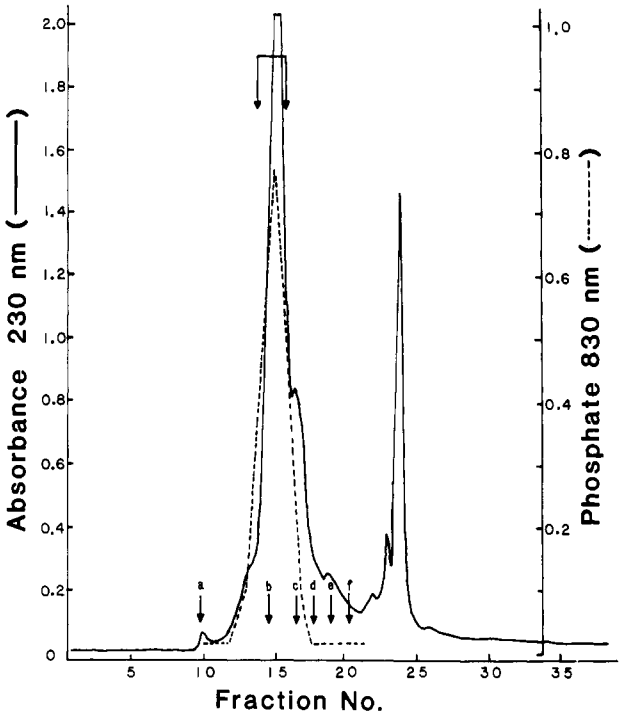

FIGURE 4: Gel filtration HPLC of phosphophoryn fraction from DE-52 chromatography (fraction V) on a TSK G4000SW column. Dotted line indicates the results of phosphate assay. Only the positive findings for organic phosphate are denoted. Downward arrows mark the elution positions of molecular weight standards. They are (a) blue dextran (M_r 2000000), (b) aldolase (M_r 158000), (c) bovine serum albumin (M_r 67000), (d) ovalbumin (M_r 43000), (e) chymotrypsinogen A (M_r 25000), and (f) ribonuclease A (M_r 13700). Double downward arrow indicates the fractions collected.

PP band will be named m-PP. The distribution of non-PP components in fraction IV is similar to that in fraction III (Figure 2i).

Fraction IV was rechromatographed on a gel filtration column under dissociative conditions. Prior to a run, the TSK G4000SW column was calibrated for molecular weight analysis with globular protein standards. The plot of log molecular weight vs. partition coefficient was linear in the range of standards examined.

The elution profile of fraction IV on the TSK G4000SW column is shown in Figure 4. The major asymmetric peak which eluted between the M_r 156000 and M_r 43000 standards is of particular interest. The location of PPs, as determined by the phosphate assay, was found mainly in this peak. The apparent molecular weight of rat dentin PPs determined by this HPLC system is 130000, higher than any previously reported values (Jontell & Linde, 1977; Dimuzio & Veis, 1978a,b; Jontell et al., 1982; Butler et al., 1983). However, the TSK G4000SW column is not capable of separating α- and β-PPs; the molecular weight determined thus represents the average of these PPs. A small peak eluting along the descending slope of the major peak has an apparent molecular weight of 67000, corresponding to the elution position of the bovine serum albumin standard (Figure 4). That this small peak represents rat serum albumin was not tested.

Purified rat incisor dentin PPs (fraction V) contain aspartic acid and serine/phosphoserine as more than 85% of the amino acid total (Table I). Small quantities of nonpolar residues are present. When compared to the highly phosphorylated phosphoprotein (HP) and moderately phosphorylated phosphoprotein (MP) reported by Butler et al. (1983), it was found that fraction V had a smaller content of aspartic acid and serine/phosphoserine than the HP. Other amino acid residues

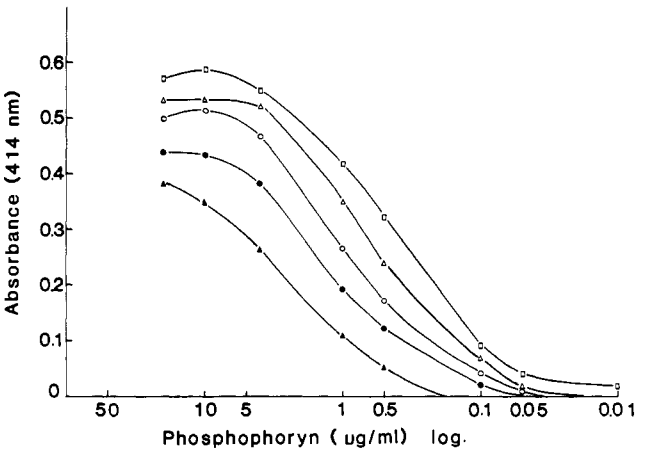

FIGURE 5: ELISA, titration of rat dentin PPs, and affinity purified anti-α-PP antibody. Antigen: purified rat dentin PP (fraction V). Antibody: affinity-purified antibody obtained after three booster injections of 1 mg each of purified PPs. The product of the enzyme reaction was measured at 414 nm and plotted vs. the concentration of PPs. A family of titration curves were generated varying in accordance with dilutions of antibody. Antibody dilutions: open square, 1:200; open triangle, 1:400; open circle, 1:800; closed circle, 1:1600; closed triangle, 1:3200.

are slightly higher in fraction V than in the HP. In comparison with MP, however, the opposite relations were found. Since fraction V is composed of α-, β-, and m-PPs (HP and MP as used by Butler and co-workers), this result agees well with their data. On a gradient SDS–PAGE, fraction V yields three Stains-all bands identical with those found in fraction IV (Figure 2j). When the gel is doubly stained (Figure 2k), fraction V is seen to be essentially free of non-PP components.

Characterization of the Anti-PP Antibody. The initial attempt of producing anti-PP antibody by the injection of homogenized gel, from the region corresponding to α-, m- and β-PP (M_r 85K–90K), did not give sera of reasonable titer. The immunization method was subsequently changed to incorporate the use of a large dose of purified PP, fraction V (lanes j and k of Figure 2). The ELISA test of the affinity-purified antibody obtained from the rabbits after three injections using this method is shown in Figure 5. The products of the peroxidase reaction were measured at 414 nm and plotted vs. the concentration of PP used to coat the plate. A family of similar curves was generated when different dilutions of antibody were used. Both a decrease in PP concentration and an increase in antibody dilution resulted in a smaller amount of bound enzyme and therefore of the nascent color.

Two control experiments were used in the ELISA assay. The first involved the use of preimmune serum in place of the specific anti-PP antibody. The second used rat serum albumin to coat a microtiter plate instead of purified rat dentin PPs. The results from both controls were negative. The color yields monitored at 414 nm were below 0.1 absorbance unit and irregular in distribution. Due to the difficulty of plotting such data, the results are not shown here.

The inhibition ELISA procedure was used to check the cross-interaction between rat and bovine dentin PPs and also to determine the presence of antibody against contaminating serum albumin. A standard inhibition curve was obtained by using various amounts of PP to inhibit the reaction between 20 μg/mL of PP and 1:400 diluted specific antibody. The result is plotted as percent inhibition vs. concentration of the inhibitor (Figure 6). The concentration of PP needed to inhibit 50% of the reaction between the given amount of antigen and antibody is 8 μg/mL. That such a standard inhibition curve can be established is also an indication of the

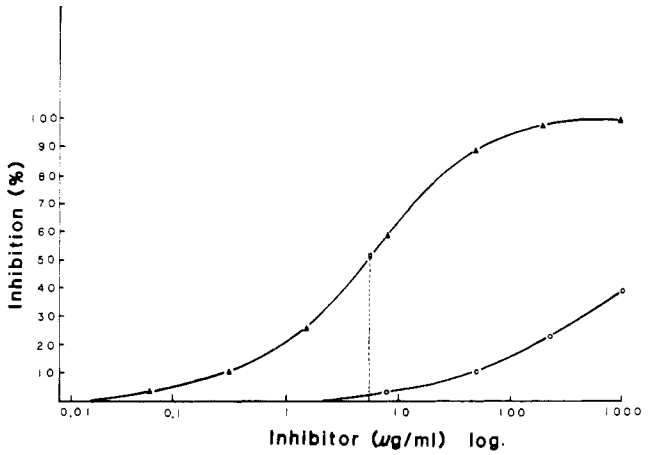

FIGURE 6: Inhibition of the binding of anti-α-PP antibody to microtiter plate coated with rat dentin PPs by rat dentin PPs and bovine dentin PP. Triangles represent the standard curve obtained by using rat dentin PPs as the inhibitors. A rectangle in the middle of the standard inhibition curve marks 50% inhibition. Circles denote the inhibition obtained by using bovine dentin PP.

presence of a specific antibody.

When bovine dentin PP was used as an inhibitor, a much decreased inhibition was observed (Figure 6). At a concentration of 1 mg/mL, bovine dentin PP inhibited the reaction between rat dentin PP and anti-rat dentin PP antibody by 34%. No inhibition was found even at a concentration as high as 1 mg/mL when rat serum albumin was used as an inhibitor, indicating that our antibody preparation was free of anti-rat serum albumin antibody.

The principle of the ELISA and inhibition ELISA is based on the specificity of antigen–antibody interactions. However, these tests are not capable of identifying the number of antigen–antibody species in the system. Even if some antibodies are directed against impurities, these tests will only show the compound results of all the antigen–antibody reactions. Therefore, the results of the ELISA and inhibition ELISA indicate the presence of a specific antibody only if the antigen used for these tests is pure. Moreover, if the immunogen contains more than one component as in the case of the rat dentin PPs, separate tests must be performed in order to determine the reactivity of antibody toward each component. To circumvent these problems, an affinity column was made by using anti-PP antibody as ligand. The $CaCl_2$ precipitate (fraction III) was then applied to the column. After the unbound materials were removed, the column was eluted with 4 M guanidine–PBS buffer. The eluted materials were dialyzed, lyophilized, and run in a gradient SDS–PAGE. As shown in lane 1 of Figure 2, the eluted antigen yielded only one band, exactly corresponding to α-PP. Since the rabbits were immunized with fraction V, which as shown in lanes j and k of Figure 2 contained a mixture of all three Stains-all staining proteins, α-, β-, and m-PP, it is clear that the antibody we have isolated is directed to an epitope present only in α-PP.

The double-stain procedure is extremely sensitive. For example, Coomassie Blue staining of a more heavily loaded gel of the molecular weight standard proteins yields bands only for the identified proteins of lane a of Figure 2. Silver staining alone brings out several additional bands, but the combined Stains-all–Silver stain brings out all of the many minor constituents and is perhaps 100 times as sensitive as the Stains-all alone. The final very purified preparation depicted in lanes l and m of Figure 2 shows in m traces of bands in very low concentration not visualized in the Stains-all lane l. These may be degradation products of α-PP which contain the PP epitope. Bands were seen in lanes c and e of Figure 2, combination stained, but not revealed with Stains-all alone. Before the possible relationship of these minor constituents to α-PP were recognized, an attempt had been made to remove antibodies to non-PP impurities by passing the crude antibody preparation over an affinity column that contained fraction II, the $CaCl_2$ supernatant (Figure 2, lane d) as ligand. The exclusion chromatography showed total absorption of the α-PP antibody; the flow-through fraction did not give any reaction in an ELISA test. These data appear to confirm the conclusion that the $CaCl_2$ supernatant contains some epitope-containing sequences of α-PP that are not precipitable with $CaCl_2$.

Discussion

In order to produce a specific antibody, it is mandatory to have a specific antigen. The antigen used for immunization need not be pure. However, a pure antigen is required for the subsequent isolation and determination of the presence of such a specific antibody. Due to the highly anionic nature of rat dentin PP, it has been reported that various noncollagenous components (NCP) may bind to PPs and be copurified with them (Linde et al., 1980; Butler et al., 1983). Our approach to separate PP from other NCPs involved the initial $CaCl_2$ precipitation of the total EDTA extract followed by DEAE-cellulose chromatography and, finally, gel filtration chromatography under dissociative conditions. Two criteria were used to judge the purity of PP in various stages of isolation: amino acid composition and the electrophoretic pattern in 5–15% gradient SDS–PAGE. After electrophoresis the gels were sequentially stained with Stains-all and silver. Since PPs do not stain with Coomassie blue or silver alone, Stains-all was used to detect PPs. The sensitivity of Stains-all toward PPs is higher than Alcian blue, another dye that is frequently used. In our hands, Stains-all can normally reveal 2 μg of PPs whereas 20 μg or more is required with Alcian blue staining of similar gels (Butler et al., 1981, 1983). To detect the presence of non-PP impurities, the silver-staining method was used. Since we are interested in isolating pure PPs, a sensitive impurity detection was needed. In comparison with Coomassie blue, the silver staining method is 10–40 times more sensitive (Poehling & Neuhoff, 1981).

Electrophoresis of fraction II in a gradient SDS–PAGE yields two Stains-all bands in the top one-third of the gel (Figure 2d). The slower moving, M_r 110000 component has been characterized by Linde et al. (1980) and Butler et al. (1981, 1983) as LP. In odontoblast organ culture it has been shown that the quantity of LP decreases with time in a pulse–chase experiment, indicating degradation of this PP (Dimuzio et al., 1985). The presence of β-PP in fraction II, however, was not reported previously. This may indicate a relatively lower affinity of β-PP in binding calcium ions. Obviously β-PP is not totally precipitated by 1.0 M $CaCl_2$.

Using an identical gel electrophoresis method, Butler & co-workers (1983) have reported that the $CaCl_2$ precipitate gave at least three Alcian blue-staining bands migrating with apparent molecular wieghts between 83000 and 95000. We have consistently observed only α- and β-PP at the same region. However, when fraction III was chromatographed on a DE-52 column, the resulting PP fraction yielded three Stains-all bands in slab gel electrophoresis. The additional band (m-PP) was located between the α- and β-PP. Thus, it is most likely that the m-PP is a degradation product of the higher molecular weight α-PP.

The PP isolated by our preparative protocol, and used for immunization, was judged to be reasonably pure on the basis of the amino acid composition (Table I) and gel electrophoresis

(Figure 2j,k). Since our primary interest was to localize the total PPs in rat incisor, no attempt was made to isolate the different PPs at this stage.

The relative molecular weight of the purified rat dentin PPs determined by gel filtration HPLC in a TSK G4000SW column is 130 000. This value is much higher than the previously reported molecular weight for the rat dentin PPs. Jontell et al. (1983) have shown that the apparent molecular weight of HP (α-PP plus m-PP) determined by gel filtration in a Sepharose CL-6B column under dissociative conditions is 67 000. They found that HP cochromatographed with bovine serum albumin. However, we were able to separate PPs from the M_r 67 000 component by using the TSK G4000SW column (Figure 4).

The finding that rat dentin PP and its corresponding antibody did not form a precipitable complex in immunodiffusion led us to employ the ELISA method for antibody detection. This method utilizes antigen supported by a solid phase and therefore does not depend on the solubility of the antigen–antibody complex. The positive results from the indirect ELISA and inhibition ELISA constitute the basic evidence for the presence of a specific antibody.

Since the ELISA tests do not differentiate different antigen–antibody species in the system, the conclusive evidence for the presence of specific antibody was obtained from the affinity chromatography separation experiment. However, this experiment gave two unexpected results. First, it was found that whereas the mixture of three PPs was used for immunization, the rabbit only produced antibody against α-PP. Second, the attempt of removing presumably contaminating antibodies by immunoadsorption through an affinity column made with the $CaCl_2$ supernatant resulted in the total removal of all antibodies.

Together, these results indicate that the three PPs found in the region of M_r 83 000–95 000 in the slab gel electrophoresis are structurally distinct. They do not share a common antigenic determinant site. Also, the lower molecular weight bands found in slab gel electrophoresis may be the degradation products of α-PP. Butler et al. (1983) have shown two different amino-terminal sequences for HP (α-PP plus m-PP). The sequences of β-PP and LP were not determined, but they suggested that the primary structures of these PPs are different on the basis of the difference in amino acid compositions. Our finding that the antibody only reacts with α-PP further defines the structural differences between these PPs.

The finding that bovine dentin PP cross reacts with anti-rat α-PP antibody to a small extent is quite interesting. This is an indication that bovine dentin PP may share some structural similarity with rat α-PP. Further elucidation of this structural similarity may shed some light in understanding the importance of certain structural domains of PP molecules in the process of dentin mineralization.

In summary, we have, in this study, presented a method for producing anti-rat dentin PP antibody. A protocol was designed to detect and characterize the presence of specific antibody. We are now in the position that enables us to utilize this antibody for localization study at the light and electron microscopic levels.

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