Purification and Initial Characterization of Intrinsic Membrane-bound Alkaline Phosphatase from Chicken Epiphyseal Cartilage*

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Alkaline phosphatase has been purified from microsomes of chicken epiphyseal cartilage by first selectively extracting certain adventitious proteins with 0.25 M trichloroacetate. The membrane-bound enzyme was then solubilized by 1% cholate in buffered 33% saturated ammonium sulfate and purified by column chromatography on Bio-Gel A-5m, extraction with 1-butanol, and ion exchange chromatography on DEAE-Bio-Gel A. The purified alkaline phosphatase from the cartilage membrane had a subunit molecular weight of 53,000 and a holoenzyme weight of 207,000-220,000, indicating a tetramer.

The pH optima for p-nitrophenolphosphate, ATP, and pyrophosphate hydrolysis were 10.3, 9.0, and 8.5, respectively. Values of $V_{max}$ (in micromoles/min/mg) were 220, 3.1, and 1.88, respectively. Substrate inhibition was pronounced at values of pH below 8.5. Inhibition of $p$-nitrophenolphosphate hydrolysis at pH 16.3 showed that phosphate and arsenate were competitive inhibitors ($K_i = 1.88$ and 0.15 mm, respectively) and levamisole was an uncompetitive inhibitor ($K_i = 0.32$ mm), while $t$-phenylalanine and ZnCl$_2$ were mixed inhibitors ($K_i = 15.8$ and 0.02 mm, respectively). Inhibition by preincubation in 1 mM EDTA was reversible by readdition of 0.25 mM MgCl$_2$ and 20 mM ZnCl$_2$.

The data indicate that this membrane-bound alkaline phosphatase from chicken epiphyseal cartilage is a Zn$^{2+}$ and possibly Mg$^{2+}$-containing enzyme. While the subunit molecular weight and kinetic properties of the enzyme are quite typical of vertebrate alkaline phosphatases, the tightness of binding to the membrane lipids, the extreme sensitivity to substrate inhibition, and the tetrameric conformation of the holoenzyme are unusual.

Alkaline phosphatase, at high levels of activity, has long been associated with calcifying tissues; however, its role in the calcification process remains unclear. Recently, it was discovered that alkaline phosphatase is a major enzymatic activity in extracellular matrix vesicles (1-4) which are known to be closely associated with the initial calcification of epiphyseal cartilage (5, 6). Matrix vesicles isolated from crude collagenase-digested epiphyseal cartilage can accumulate $^{45}$Ca from metastable calcium/phosphate solutions (7-10). Such preparations, however, usually require either elevated pH (7), Ca $\times$ P, ion products (7, 8, 10), and/or the addition of high energy phosphate substrates (7-10) to support calcification. This calcification can be inhibited by compounds which inhibit alkaline phosphatase (7, 10).

Alkaline phosphatase in matrix vesicles appears to be very tightly associated with the membrane as an intrinsic membrane component. It has been isolated previously from matrix vesicles and chondrocytes that were obtained by crude-collagenase digestion of epiphyseal cartilage (11). The extremely low subunit molecular weight ($M_s = 18,000$) and the instability of the enzyme following treatment with metal ion chelators, however, suggests that the enzyme had been altered by proteases known to contaminate the crude collagenase (12, 13). While enzyme activity is not destroyed by treatment of the tissue with proteases, it is likely that this nevertheless damages the non-catalytic portion of the protein. This may affect characteristics critical to its function in calcification.

Alkaline phosphatase has been isolated from rabbit fracture-callus cartilage which had not been treated with proteases (14). Two antigenically distinct forms were obtained. However, because of the method employed (i.e. extraction of whole tissue), it was impossible to ascertain the subcellular source of the two forms. While one may have been derived from chondrocytes and the other from matrix vesicles, it is equally possible that one form may be a soluble and the other, a membrane-associated form of the enzyme. This was not explored.

Recently, we found that microsomes isolated from chicken epiphyseal cartilage by non-protease-dependent methods not only were able to support rapid mineralization using physiological synthetic cartilage lymph but also were enriched 5-fold in alkaline phosphatase (15). Mineralization of this fraction was significantly retarded by inhibitors of this enzyme (15, 16). We now report the isolation and initial characterization of this alkaline phosphatase, an integral protein of the microsomal membranes of chicken epiphyseal cartilage. This work represents the first successful isolation of the membrane-bound enzyme from mineralizing cartilage, without resort to the use of proteases. The enzyme, unlike that isolated from protease-treated tissue, has a significantly higher subunit molecular weight and different holoenzyme structure (17). The catalytic activity (pH optima, kinetic behavior, response to inhibitors, etc.) are, however, similar to that of other bone alkaline phosphatases (14, 18, 19). The enzyme is unusual in that it is a tetramer, and displays physical characteristics typical of an intrinsic membrane protein.

MATERIALS AND METHODS

Purification of Alkaline Phosphatase

All steps prior to the chromatography columns were performed at 0-4 °C unless otherwise noted. The ammonium sulfate solution was saturated at room temperature and all per cent saturations are relative to this. Chromatographic columns were operated at room temperature. Protein concentrations were estimated by the procedure of Lowry et al. (20).
Isolation of Microsomes—Slices of epiphyseal cartilage (100-120 g wet weight) from the proximal end of the metatarsus of 8- to 10-week-old broiler-strain chickens were obtained by a published procedure (21). The slices were suspended in 175 ml of an isolation medium composed of 10% (w/v) sucrose, 50 mM Tris-HCl (pH 8.0) and 2.5 mM MgCl₂, and homogenized for 6 min with a Tekmar Tissuemizer equipped with a type SDT-180EN probe. Differential centrifugation was employed as previously described (13). The pellet from the 85,000 x g centrifugation was resuspended in the isolation medium with the aid of a Potter-Elvehjem homogenizer and adjusted to a total volume of 15 ml.

Solubilization—To the microsomal pellet suspension was added, at room temperature and with vigorous stirring, 0.11 volume of a solution containing 5% Triton X-100 and 2.5 mM MgCl₂. The suspension was then centrifuged at 250,000 x g for 30 min. The supernatant was discarded and the pellet was resuspended to a total volume of 10 ml with isolation medium.

The phosphatase was extracted by dropwise addition of 0.5 volume of saturated ammonium sulfate followed by 0.08 volume of 20% (w/v) potassium cholate (pH 8). The mixture was stirred for 15 min and then centrifuged at 85,000 x g for 60 min. The pellets were discarded and the supernatants were saved. The enzyme can be frozen overnight at this stage, with no apparent loss of activity.

Gel Filtration—The cholate-soluble material was precipitated by the addition of an equal volume of saturated ammonium sulfate. It was stirred in an ice bath for 15 min and then centrifuged at 120,000 x g for 60 min. The supernatants were removed by puncturing the bottoms of the tubes. The coagulum was dissolved in isolation medium with the aid of a Potter-Elvehjem homogenizer and adjusted to a total volume of 15 ml with isolation medium.

Results

Membrane-bound Epiphyseal Cartilage Alkaline Phosphatase

| Purification step | Protein | Activity a | Specific activity | Activity yield | Purification |
|-------------------|---------|------------|------------------|----------------|-------------|
| Microsomes        | 112     | 841        | 7.51             | 100            | 1.0         |
| Trichloroacetate  | 63      | 620        | 9.84             | 74             | 1.3         |
| Cholate-soluble   | 16      | 435        | 27.2             | 52             | 3.6         |
| Bio-Gel A-5m      | 2.7     | 187        | 69.4             | 22             | 9.2         |
| DEAE-Bio-Gel A    | 0.56    | 124        | 222              | 15             | 29.6        |

*P-Nitrophenylphosphorylation at pH 10.3* (see "Materials and Methods").

Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis

This was performed in slab gels (130 x 200 x 1.5 mm) essentially as described by O'Farrell (24), using gels containing 12.5% acrylamide. Samples from each of the various purification steps were lyophilized and dissolved in 50 μl of sample buffer. The cholate-soluble and Bio-Gel A-5m fractions were diluted overnight against 100 μl of sample buffer to remove excess salt. Electrophoresis was carried out at 35 mA until the trailing dye (bromphenol blue) was within 5 cm of the bottom. The gel was stained for protein by the method of Blakesley and Boezi (25).

Nondissociative Polyacrylamide Gel Electrophoresis

A discontinuous system, essentially as described by Davis (26) was used. Samples of purified alkaline phosphatase (10 μg of protein) were applied in the buffer with which they were eluted from the DEAE-Bio-Gel A column. Other proteins (hemoglobin, catalase, and aldolase) were dissolved in 50 mM Tris-HCl (pH 8.0). The running buffer was 0.192 mM glycine, 35 mM Tris. Electrophoresis was carried out using the same type of slab gels, tracking dye (bromphenol blue), and protein staining as described above.

Assay of Column Fractions

Column fractions were assayed in 0.7 ml pNPP, 1.125 ml 2-amino-2-methyl-1-propanol buffer (pH 10.0) at 37 °C. Assays were started by addition of enzyme and absorbance at 410 nm was monitored continuously with a GCA/McPherson model EU-701 spectrophotometer.

Sucrose Density Gradient Centrifugation

Purified alkaline phosphatase was layered onto a continuous sucrose-trichloroacetate gradient (5-20% w/v, dissolved in 50 mM Tris-HCl buffer, pH 8.0, containing 0.1 M NaCl and 2.5 mM MgCl₂). Centrifugation was carried out for 12 h at 40,000 rpm using a SW 50.1 rotor. Standards (bovine serum albumin, catalase, and lactic acid dehydrogenase) were run on parallel gradients. Fractions of 0.1 ml were collected and assayed by standard procedures for catalase (27) or lactic acid dehydrogenase (28) activity. The bovine serum albumin peak was detected by protein concentration. Sedimentation data were analyzed by the method of Martin and Ames (29).

Chemicals

L-carnosine, L-phenylalanine, p-nitrophenol (25 mM standard solution), cholic acid, acrylamide, N,N,N',N'-tetramethylethylenediamine, N,N',N'-tetramethylethylenediamine, and 2-amino-2-methyl-1-propanol were obtained from Sigma; pNPP was from Technicon; Bio-Gel A-5m and DEAE-Bio-Gel A were products of Bio-Rad; AcaA4 was obtained from LKB Products; and [32P]PP was purchased from New England Nuclear. All other chemicals used were of reagent grade and were supplied by Fisher.

RESULTS

Purification of Membrane-bound Alkaline Phosphatase—Table 1 summarizes a typical preparation of membrane-bound alkaline phosphatase from 91 g (wet weight) of chicken epiphyseal cartilage. An approximately 30-fold purification from

1 The abbreviation used is: pNPP, p-nitrophenylphosphate.
the microsomal pellet was achieved. Recovery of activity from the microsomal pellet ranged from 15-25%. The fact that only a 30-fold purification was required to achieve homogeneity suggests that a substantial proportion of the protein in the microsomal pellet was alkaline phosphatase. Since the microsomal pellet itself provided a 5-fold enrichment of alkaline phosphatase activity relative to the supernatant from the 600 × g centrifugation (15), the total purification from the tissue was greater than 150-fold.

A typical elution profile from Bio-Gel A-5m is shown in Fig. 1. The hydrolytic activity did not correspond to any discrete peak with absorbance at 280 nm. A large degree of purification was obtained at this stage, however. Extraction with 1-butanol could not precede this step as the enzyme was then inactivated. In addition, it was critical that cholate be included in the eluting buffer to prevent irreversible aggregation of the enzyme and concomitant loss of activity.

Ion exchange chromatography on DEAE-Bio-Gel A (Fig. 2) resulted in the removal of the final contaminating proteins. Dilution of the 1-butanol-extracted aqueous phase was necessary in order for the enzyme activity to bind to the column. The inability of the enzyme to bind appeared to be caused by the presence of the saturating levels of butanol in the aqueous phase.

The results of sodium dodecyl sulfate-polyacrylamide gel electrophoresis of aliquots from each of the purification steps are shown in Fig. 3. There was no obvious reduction in the number of protein bands following extraction of microsomes with 0.25 M trichloroacetate; however, incorporation of this step was found to be crucial. Its omission resulted in the presence of several contaminating proteins after the DEAE-Bio-Gel A step and these could not be removed by varying the conditions for elution from the columns or by inclusion of a concanavalin A-Sepharose chromatography step.

**Physical Characterization**—Calibration of the sodium dodecyl sulfate-polyacrylamide gel electrophoresis yielded a molecular weight (M₀) for the subunits of this alkaline phosphatase of 53,000, a value near the range reported for other vertebrate alkaline phosphatases (M₀ = 55,000–70,000) (17). The molecular weight of the holoenzyme was estimated to be 207,000 by sucrose-density gradient ultracentrifugation, and 220,000 by gel filtration on AcA34 (data not shown). These findings suggest that the enzyme exists as a tetramer in its native configuration.

Electrophoresis of the enzyme under nondissociative conditions was also performed using a graded series of acrylamide concentrations. When these results were analyzed through the use of Ferguson plots (30), an apparent molecular weight of approximately 80,000 was obtained (Fig. 4). The low molecular weight observed by this method may be caused by a high charge density for the enzyme and/or the loss of metal ions.
Membrane-bound Epiphyseal Cartilage Alkaline Phosphatase

The effects of various inhibitors of pNPP hydrolysis are summarized in Table II. Sodium phosphate was a competitive inhibitor (K_i = 1.88 mM, Fig. 8) as was sodium arsenate, a...
Fig. 7. Effect of pH on kinetic parameters of cartilage alkaline phosphatase. Kinetic parameters were estimated by a non-linear least squares fitting procedure as described under "Materials and Methods." A, log (V_max/K_m) as a function of pH. V_max values are in micromoles min^(-1) mg^(-1); K_m values are in millimolar concentrations except where marked. •---•, p-nitrophenylphosphate hydrolysis; ○---○, pyrophosphate hydrolysis (K_m in micromolar concentrations); ▲---▲, ATP hydrolysis. All values for ATP have been increased by 1 to permit separation from pyrophosphate values. B, Log V_max as a function of pH. Values of V_max are in micromoles min^(-1) mg^(-1). To avoid overlap, as above, values of log V_max + 1 are plotted for pyrophosphate and ATP. C, effect of pH on -log K_m. All K_m values are in molar units. For clarity, to prevent overlap with data for other substrates, -log K_m + 2 was plotted for pyrophosphate.

| Reversible inhibitors of membrane-bound alkaline phosphatase from chicken epiphyseal cartilage | K_m (mM) | Type of inhibition |
|---|---|---|
| Sodium phosphate | 1.88 | Competitive |
| Sodium arsenate | 0.15 | Competitive |
| L-Tetramisole | 0.32 | Uncompetitive |
| L-Phenylalanine | 15.8 | Mixed* |
| Zinc chloride | 0.019 | Mixed* |

* Inhibition of p-nitrophenolphosphate hydrolysis at pH 10.3 (see "Materials and Methods"). * Mixed uncompetitive and noncompetitive inhibition.

structural analog of P_i. It was a considerably more potent inhibitor than P_i (K_i = 0.15 mM; Fig. 9). Levamisole (L-tetramisole) was an uncompetitive inhibitor (Fig. 10). Plots of 1/V_max versus [levamisole] were linear and yielded a K_i of 0.32 mM. Inhibition by L-phenylalanine was of mixed non- and uncompetitive type. A plot of 1/V_max versus [phenylalanine] was also linear and extrapolated to a K_i of 15.8 mM. The most
This paper describes the isolation and characterization of a membrane-bound alkaline phosphatase from cartilage alkaline phosphatase enzyme.

**Results**

**A.** Line-averaged plots of the hydrolysis data. Levamisole concentrations were: 0 mM; 0.25 mM; 0.50 mM; 1.0 mM. **B.** Plot of the reciprocal of the apparent $V_{max}$ as a function of Levamisole concentration. The line is drawn using linear least squares fit.

**Discussion**

This paper describes the isolation and characterization of a membrane-bound alkaline phosphatase from cartilage. The enzyme is tightly bound and can only be solubilized by treatment of the membranes with detergents. Since neither treatment with high ionic strength salt solutions nor the chaotrope, trichloroacetate, solubilizes it, this enzyme is properly classified as an intrinsic membrane protein.

The cartilage alkaline phosphatase which we describe here is a tetramer composed of apparently identical subunits with a $M_r$ of 53,000. While this subunit molecular weight is similar to that reported for other vertebrate alkaline phosphatases, the oligomeric structure is significantly different in that the typical structure is a dimer. A tetrameric alkaline phosphatase from pig kidney with subunits of $M_r = 39,000$ has been described by Wachsmuth and Hiwada (33). However, Ramaswamy and Butterworth later reported this enzyme to be a dimer with a total molecular weight of about 185,000 (34). While it is possible that aggregation of our cartilage enzyme may have occurred, as is known to happen with the placental enzyme (35), this appears unlikely considering the low protein concentrations employed (less than 50 $\mu$g/ml). Further, 0.1 mM NaCl was included in the buffers during determinations of molecular weights by the hydrodynamic method to minimize the possibility of aggregation. In an experiment where the salt was omitted, alkaline phosphatase activity eluted from a Bio-Gel A-15m column in several distinct peaks corresponding approximately to multiples of the tetrameric enzyme, the largest appearing to be of 16 subunits. In the presence of 0.1 mM NaCl the enzyme activity eluted as a single symmetrical peak corresponding to the tetrameric configuration.

As noted in the introduction, we feel that several characteristics of matrix vesicles isolated from cartilage indicate that they have been damaged by this protease treatment. These include the necessity of high Ca$^2+$ for the enzyme to be a dimer with a total molecular weight of about 53,000 units. The enzyme is not greatly different from that of enzymes isolated from other vertebrate sources, in agreement with findings of Arsenis et al. (14). In several respects the phosphatase I from rabbit cartilage reported by Arsenis et al. (14) is similar to the membrane-bound enzyme which we have isolated from chicken cartilage. Assuming a dimeric structure for their enzyme (no subunit determination was reported), a $M_r$ of 63,000 would be obtained for the subunits of their enzyme. This would be in reasonable agreement with the value reported here, the small difference perhaps being a species variation. Substrate specificities of both enzymes are similar in that both are active toward $p$-nitrophenyl phosphate. The enzyme from chicken cartilage was fully active in the absence of exogenous Mg$^{2+}$ ions.

This membrane-bound enzyme showed kinetics quite typical of other alkaline phosphatases. For example, the pH optimum varied with the type of substrate, ranging from pH 10.3 for pNPP to about 8.5 for PP$_i$. The enzyme was fully active in the absence of exogenous Mg$^{2+}$ ions.

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**Conclusion**

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We observed obvious inhibition at pH 8.5 when PP₂ concentrations exceeded 100 μM. By contrast, Fortuna et al. (31, 32) saw inhibition only at concentrations above 10 mM, and Arsenis et al. (14) routinely assayed pyrophosphatase activity using 2 mM PP₂ at pH 8.5. Under these conditions, our enzyme showed barely detectable activity. This difference may be of physiological significance since PP₂ is a known inhibitor of hydroxyapatite crystal growth (36).

In conclusion, because of the apparent close association between the membrane-bound cartilage alkaline phosphatase and the mineralization induced by matrix vesicles (11, 14, 18), any studies of the physical properties of this enzyme must be done on an enzyme that is fully preserved during the isolation procedure. Unfortunately, in the previously reported matrix vesicle alkaline phosphatases (11, 14, 18), the proteases present in the crude collagenases required for release of the matrix vesicles from which the enzyme was isolated make it almost certain that these had undergone some degree of proteolysis. That this is indeed true is suggested by differences we now report in the molecular weight, stability, and kinetic behavior of this membrane-bound cartilage enzyme.

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