Properties of Inorganic Pyrophosphatase of Pig Scapula Cartilage

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The properties of a highly purified inorganic pyrophosphatase (pyrophosphate phosphohydrolase; EC 3.6.1.1) from pig scapula cartilage were studied. The enzyme had a molecular weight of 66000 and a pH optimum of 7–8. It was markedly activated by magnesium, but not, or only to a much smaller degree, by other metal ions. PP_i was the only substrate found and had a K_m value of $11\ \mu\text{M}$. The enzyme was not inhibited by phosphate and other inhibitors of alkaline phosphatase such as CN^- , amino acids and theophylline; it was slightly inhibited by tartrate, formaldehyde and ammonium molybdate and strongly inhibited by F^- , Ca^{2+} and other metal ions. The properties of the enzyme in the presence of concentrations of PP_i present in plasma ($3.5\ \mu\text{M}$) were similar to those found at higher (2 mM) concentrations of PP_i . The diphosphonates ethane-1-hydroxy-1,1-diphosphonate and dichloromethylenediphosphonate inhibited the enzyme in the presence of low PP_i concentrations. The characteristics of this enzyme are therefore similar to pyrophosphatases from other sources, such as from yeast and erythrocytes, and do not support a specific role of this enzyme in the calcification process.

In the preceding paper (Felix & Fleisch, 1975) we described the purification of an inorganic pyrophosphatase (pyrophosphate phosphohydrolase; EC 3.6.1.1) from the ossifying zone of pig scapula cartilage. The enzyme preparation obtained was of high specific activity and behaved heterogeneously on ion-exchange chromatography. In the present paper we describe some properties of fractions DE-II-SE, DE-III-SE and DE-IV-SE. Some studies were made both at optimum substrate concentration and at plasma concentrations of PP_i (Russell *et al.*, 1971), in view of the suggestion that this enzyme might be of importance in the calcification process because it destroys PP_i (Alcock & Shils, 1969), a substance known to inhibit calcium phosphate precipitation (Fleisch & Neuman, 1961).

Experimental

Materials

The reagents used were obtained from the following sources: *p*-hydroxymercuribenzoate, *N*-ethylmaleimide, phenylmethanesulphonyl fluoride and phosphocreatinine were from Sigma Chemical Co., St. Louis, Mo., U.S.A.; glucose 1-phosphate, glucose 6-phosphate, ADP, UTP, NAD^+ , NADP^+ , yeast alcohol dehydrogenase and glucose 6-phosphate dehydrogenase were from Boehringer, Mannheim, W. Germany; ATP, AMP and phosphoenolpyruvate were from Calbiochem, Los Angeles, Calif., U.S.A.; thiamine pyrophosphate was from Hoffmann-La Roche A.G., Basle, Switzerland; cytochrome *c* was from Mann Research Laboratories, Orangeburg, N.Y., U.S.A.; egg albumin and bovine γ -globulin

were from Fluka A.G., Buchs, Switzerland; ethane-1-hydroxy-1,1-diphosphonate, dichloromethylenediphosphonate and *n*-pentanemonophosphonate were from the Procter and Gamble Company, Cincinnati, Ohio, U.S.A.; hydroxyapatite Bio-Gel HTP was from Bio-Rad Laboratories, Richmond, Calif., U.S.A. The tripolyphosphate and trimetaphosphate, chromatographically pure, were kindly supplied from G. Berg, Rochester, N.Y., U.S.A. Iodoacetic acid was three times recrystallized from hot chloroform. All the other reagents were analytical-grade purchased from E. Merck A.G., Darmstadt, W. Germany. ^{32}P -labelled sodium pyrophosphate was obtained from New England Nuclear Corp., Dreieichenhain, W. Germany. It was stored frozen and diluted with unlabelled PP_i to the desired specific radioactivity immediately before use.

Enzyme. The inorganic pyrophosphatase (pyrophosphate phosphohydrolase; EC 3.6.1.1) was purified as described in the preceding paper by DEAE-cellulose and Sephadex G-150 chromatography (Felix & Fleisch, 1975). The three fractions DE-II-SE, DE-III-SE and DE-IV-SE had the following specific activities: 104, 97 and 168 units/mg respectively [see Felix & Fleisch (1975) for definition of units].

For the determination of the pH optima and the activation by magnesium, each of the fractions DE-II-SE, DE-III-SE and DE-IV-SE was rechromatographed on a DEAE-cellulose column, pH 7.5. Only the tubes in the middle of the peaks were collected. This resulted in homogeneous fractions $\text{DE}^2\text{-II-SE}$, $\text{DE}^2\text{-III-SE}$ and $\text{DE}^2\text{-IV-SE}$ each of which was eluted as a single peak of pyrophosphatase

activity when they were rechromatographed on a DEAE-cellulose column, pH 7.5.

Methods

Pyrophosphatase assay. Pyrophosphatase activity was assayed either at high or low substrate concentration. High substrate concentration: the enzyme was incubated for 15 min at 37°C in 0.5 ml of a medium containing 2 mM-PP_i, 5 mM-MgCl₂ and 0.1 M-Tris-HCl, pH 7.5. P_i release was determined by the method of Wöltgens & Ahsmann (1970). For the determination of K_m the P_i was assayed by the more sensitive Malachite Green method (Richterich, 1971). P_i release was linearly proportional to the amount of enzyme and to time under the conditions used.

Low substrate concentration: the enzyme was incubated in 0.5 ml of a mixture containing 3.5 μ M-[³²P]PP_i, 0.6 mM-MgCl₂, 0.05 M-Tris-HCl, 0.15 M-NaCl and other additions as described. The reaction was stopped by adding 0.1 ml of a solution containing unlabelled 0.01 M-P_i and 0.005 M-PP_i followed by 0.6 ml of 2.7 M-HCl, 3.35% (w/v) ammonium molybdate, at 0°C; 100 μ l was taken for total radioactivity determination (P_i and PP_i). PP_i and P_i were then separated by the method modified by Hall (1963); 1.1 ml of a 4:1 (v/v) 2-methylpropan-1-ol-light petroleum (b.p. 80–100°C) mixture was added, shaken and the phases were separated by rapid centrifugation. Then 100 μ l of the 2-methylpropan-1-ol-light petroleum phase (containing P_i) and 100 μ l of the aqueous phase (containing PP_i) were put on a planchette and counted for radioactivity on a methane flow counter (Frieske & Hoepfner, Erlangen-Bruck, W. Germany). Hydrolysis of PP_i was proportional to time and to the amount of enzyme added.

Assay for other substrates. The incubation medium contained generally 2 mM substrate, 5 mM-MgCl₂, 0.1 M-Tris-HCl, pH 7.5, in a final volume of 0.5 ml. Where indicated, 4 mM-ZnSO₄ was substituted for MgCl₂. After incubation at 37°C for 15 min P_i release was determined by the method of Wöltgens & Ahsmann (1970). NAD⁺ was determined by using a 0.5 M-yeast alcohol dehydrogenase assay system (ethanol in 0.1 M-Tris-HCl at pH 10.1).

Inorganic pyrophosphate-glucose phosphotransferase activity. It was assayed by the method of Nordlie & Arion (1964) and at pH 7.5 in the absence or the presence of 5 mM-MgCl₂.

All assays were done in duplicate or quadruplicate.

Molecular-weight determination. It was performed by gel filtration by the method of Whitaker (1963). Cytochrome *c* (mol.wt. 12400), egg albumin (mol.wt. 45000), bovine serum albumin (mol.wt. 71000) and bovine γ -globulin (mol.wt. 205000) were used as markers. The above-mentioned values are the apparent molecular weights determined by gel filtration (Andrews, 1965).

Affinity to apatite. Apatite was suspended in 3 ml of crude enzyme extract containing 1.4 units of pyrophosphatase activity/ml, 0.01 M-Tris-HCl, pH 7.5, 0.01 M-MgCl₂ and 0.01 M-2-mercaptoethanol. The suspension was left for 1 h at 0°C, then centrifuged and the supernatant was assayed for enzyme activity. The pellet was resuspended either in a solution containing 1 M-NaCl, 0.01 M-Tris-HCl, pH 7.5, 0.01 M-MgCl₂, 0.01 M-2-mercaptoethanol or in 1 M-sodium phosphate, pH 7.5, and centrifuged after 20 min at 0°C. The NaCl supernatant was assayed directly, phosphate was removed by dialysis and the dialysis residue assayed.

Results

Molecular weight

The same amount of enzyme activity from fractions DE-II-SE, DE-III-SE and DE-IV-SE was applied together on to a Sephadex G-150 column. Only a single sharp peak with no shoulder was eluted. The three fractions therefore had similar molecular weights, values of 65000 and 67000 being estimated from two different runs.

pH optima

For each of the fractions DE²-II-SE, DE²-III-SE and DE²-IV-SE the pH optimum was determined at 2 mM-PP_i and at various MgCl₂ concentrations. Fig. 1 represents the results obtained for fraction DE²-IV-SE. The pH optimum lay between 7.0 and 8.0. At low magnesium concentrations Tris-maleate gave lower values than Tris-HCl and exhibited another pH optimum. Fractions DE²-II-SE and DE²-III-SE gave similar results.

Effect of magnesium

For each fraction DE²-II-SE, DE²-III-SE and DE²-IV-SE the activation by Mg²⁺ at different PP_i concentrations was studied. Fig. 2 represents the results for fraction DE²-II-SE. The activity increased steeply until the ratio Mg²⁺/PP_i reached about 1. Thereafter the slope of the curve decreased. Optimal activities were observed at a Mg²⁺/PP_i ratio between 1.5 and 4; the lower the PP_i concentration the higher was the optimal Mg²⁺/PP_i ratio. A slight inhibition was obtained at high Mg²⁺ concentrations. For fractions DE²-III-SE and DE²-IV-SE very similar results were obtained.

Substrate specificity

Glucose 1-phosphate, glucose 6-phosphate, *p*-nitrophenyl phosphate, ATP (in the presence or absence of 4 mM-ZnSO₄), ADP, UTP, NAD⁺, thiamine pyrophosphate, phosphoenolpyruvate,

phosphocreatine and PPP_i and meta- PPP_i (in the presence of 5 or 30mM- $MgCl_2$) were tested as substrates. Negligible activity (less than 2%) could be detected when fractions DE-II-SE, DE-III-SE and DE-IV-SE were used as the enzyme source. No pyrophosphate-glucose phosphotransferase activity (Nordlie & Arion, 1964) was found.

Effect of metal ions

Several metal ions were tested for their ability to substitute for Mg^{2+} in activating the enzyme. As shown in Table 1, no other ion could replace Mg^{2+} to a comparable extent. Be^{2+} , Mn^{2+} , Fe^{2+} , Co^{2+} and Zn^{2+} enhanced the activity to some extent, but not more than 10% of the activation induced by Mg^{2+} . The maximum activation was achieved at lower concentrations than with Mg^{2+} .

The effect of metal ions in the presence of the optimal Mg^{2+} concentration is presented in Fig. 3. All metal ions tested, especially Ca^{2+} , inhibited the enzyme reaction. Those which had shown a small

Fig. 1. Determination of the pH optimum

The activity of fraction DE²-IV-SE was determined as a function of pH at various $MgCl_2$ concentrations. The activities are expressed as a percentage of the value obtained in 0.1M-Tris-maleate (pH7.5)–10mM- $MgCl_2$; 0.017 unit of enzyme was added to 0.5ml of incubation medium containing 2mM- PP_i and 0.1M of either Tris-maleate (●), Tris-HCl (■) or acetate (▲). —, 10mM- Mg^{2+} ; ----, 4mM- Mg^{2+} ; ····, 1.7mM- Mg^{2+} .

Fig. 2. Determination of the activation by Mg^{2+}

The activity of fraction DE²-II-SE was determined as a function of the $MgCl_2$ concentration at various PP_i concentrations. The activity is expressed as a percentage of the value obtained in 1 mM- PP_i –3.2mM- $MgCl_2$; 0.015 unit of enzyme was added to 0.5 ml of incubation medium containing either 0.5mM- (○), 1.0mM- (▲), 2.0mM- (■) or 4mM- (●) PP_i , 0.1M-Tris-HCl, pH7.5, and increasing amounts of $MgCl_2$.

Table 1. Effect of metal ions on pyrophosphatase activity

Fraction DE-IV-SE (0.014 unit) was added to 0.5ml of incubation medium containing 2mM- PP_i , 0.1M-Tris-HCl, pH7.5, 0.2mM- $MgCl_2$ (added with the enzyme solution, where it stabilizes the enzyme) and various concentrations of metal ions. The activity is expressed as percentage of that found at 5mM- $MgCl_2$. Except in the presence of $BaCl_2$ no precipitation was observed. At 0.2mM- $MgCl_2$ no activity was found. The individual values are given.

Metal salt	Concn. (mM)	...	Relative activity at different metal ion concentrations (%)			
			0.5	1.0	2.5	5
$BeSO_4$			3.8/3.3	8.0/9.2/9.6/9.3	0/0	0/0
$CaCl_2$			–0.2/–0.3	–0.4/–0.4/0/0	–0.2/–0.2	0/0
$BaCl_2$			—	Ppt.	—	Ppt.
$MnCl_2$			5.6/5.6	4.0/4.0	5.1/4.6	2.1/2.0
$(NH_4)_2Fe(SO_4)_2$			4.3/4.3	7.1/6.4	0/0	—
$CoSO_4$			6.0/6.0	8.4/8.4	3.6/4.3	—
$NiSO_4$			0/0	0/0	0/0	—
$CuSO_4$			0/0	0/0	0/0	—
$ZnSO_4$			4.2/4.1	9.1/9.4	0/0	0/0
$CdSO_4$			—	0/0	—	0/0

Fig. 3. Effect of metal ions in the presence of optimal Mg^{2+} concentration

Fraction DE-IV-SE (0.014 unit) was added to 0.5 ml of incubation medium containing 2 mM- PP_i , 5.2 mM- $MgCl_2$, 0.1 M-Tris-HCl, pH 7.5, and 0.1, 1.0 or 5 mM of the metal ions as chloride, sulphate or acetate salts. The activity is expressed as percentage of the value found in the absence of these metal ions. At 5.0 mM- $CoSO_4$ and at $BaCl_2$ concentrations higher than 0.1 mM, precipitation occurred.

activation in the absence of Mg^{2+} showed a smaller degree of inhibition.

Effect of P_i , phosphonates and other inhibitors

The effect of P_i was measured in an incubation medium containing 2 mM- PP_i , 5 mM- $MgCl_2$, 0.1 M-Tris-HCl, pH 7.5. P_i at 0.5, 1.0, 1.5, 2.0 and 2.5 mM- P_i had no effect on enzyme activity.

The two diphosphonates, ethane-1-hydroxy-1,1-diphosphonate and dichloromethylenediphosphonate

Table 2. Effect of phosphonates and other inhibitors

Fraction DE-IV-SE (0.012–0.014 unit) was added to 0.5 ml of incubation medium containing 2 mM- PP_i , 5.2 mM- $MgCl_2$ and 0.1 M-Tris-HCl, pH 7.5. Additional compounds were dissolved in water and adjusted to pH 7.5. Since phosphonates bind Mg^{2+} , a further amount of $MgCl_2$ equal to the molarity of the phosphonate was added. The activity was expressed as a percentage of the value found in the absence of the inhibitors. Some turbidity was observed in the presence of 0.1 and 1.0 mM-ethane-1-hydroxy-1,1-diphosphonate, dichloromethylenediphosphonate and *n*-pentanemonophosphonate.

Compound	Concn. (mm)	Relative activity (%)		
		0.1	1.0	10
Ethane-1-hydroxy-1,1-diphosphonate	...	97	94	30†
Dichloromethylenediphosphonate		99	102	55†
<i>n</i> -Pentanemonophosphonate		85	60†	17†
Tartrate		—	93	38†
Sodium fluoride		21†	2†	0†
Formaldehyde		—	90*	68†
Ammonium molybdate		—	89	60†

* Significance of difference from control, $P < 0.01$.

† Significance of difference from control, $P < 0.005$.

‡ Significance of difference from control, $P < 0.001$.

ate, substances with structural similarities to PP_i , had no significant effect at 0.1 and 1 mM, but inhibited at concentrations of 10 mM (Table 2). In contrast, *n*-pentanemonophosphonate showed a stronger inhibition than the diphosphonates.

Tartrate, formaldehyde and ammonium molybdate gave a moderate inhibition, whereas at 10 mM-NaF inhibition was complete (Table 2). There was no inhibition with L-phenylalanine, L-histidine, imidazole, L-homoarginine, L-tryptophan, succinate and maleate at 1 or 10 mM, potassium cyanide and theophylline at 0.1, 1 or 5 mM or borate at 1, 10 or 100 mM.

Michaelis constant

When enzyme activity was measured by using 0.01–0.2 mM- PP_i , a K_m of 11 μM was found by graphical estimation (Fig. 4).

Inhibition by thiol-group-binding agents and other inactivating compounds

The sensitivity of the enzyme to these compounds is summarized in Table 3. *p*-Hydroxymercuribenzoate, iodoacetate and *N*-ethylmaleimide were strong inhibitors. Maleic anhydride, succinic anhydride and phenylmethanesulphonyl fluoride inhibited to some degree.

Fig. 4. Determination of K_m

Either 2.2 munits (●) or 1.5 munits (■) of fraction DE-IV-SE was added to 0.5 ml of medium containing 0.01–0.2 mM- PP_i , 0.1 M-Tris-HCl, pH 7.5, and $MgCl_2$ so that the Mg^{2+}/PP_i ratio was 10, so that all the PP_i would be in the form of $MgPP_i$, the probable substrate. After incubation for 3 min at 37°C the reaction was stopped by the addition of 2.5 ml of Malachite Green reagent. P_i release was determined by the Malachite Green method (Richterich, 1971).

Affinity to apatite

When various amounts of apatite were suspended in 3 ml of crude enzyme extract containing 1.4 units of pyrophosphatase activity/ml different amounts of enzyme activity were bound, namely 15, 60 or 240 mg of apatite bound 20, 70 or 100% of the activity respectively. This bound enzyme could not be released by 1 M-NaCl, but was released by 1 M-sodium phosphate solution (see under 'Methods').

Properties of the enzyme at physiological concentrations of PP_i and Mg^{2+}

The enzyme activity was also measured at physiological concentrations of PP_i (3.5 μM) and Mg^{2+} (0.6 mM). The pH optimum was at 8.5. Compared with the optimum measured at higher pyrophosphate concentrations (see Fig. 1), the optimum had shifted 0.5–1 pH unit towards the alkaline region.

At 3.5 μM - PP_i the optimal Mg^{2+} concentration was 1 mM, and increasing concentrations up to 10 mM produced no inhibition. In the absence of Mg^{2+} no activity was found.

Phosphate at concentrations as high as 4 mM did not inhibit, but strong inhibition was induced by Ca^{2+} at concentrations as low as 0.4 mM (Table 4). In the presence of physiological concentrations of both P_i and Ca^{2+} , the reaction was totally inhibited.

The diphosphonates ethane-1-hydroxy-1,1-diphosphonate and dichloromethylenediphosphonate in-

Table 3. Sensitivity of pyrophosphatase to thiol-group-binding agents and other inactivators

About 0.4 unit of enzyme was preincubated at room temperature in 200 μl of mixture containing one of the following sets of components: 0.1 M-Tris-HCl, pH 7.5, 0.01 M- $MgCl_2$ and *p*-hydroxymercuribenzoate, iodoacetate or *N*-ethylmaleimide (preincubation time 30 min); or 0.1 M-borate, pH 8.5, 0.01 M- $MgCl_2$, 0.01 M-2-mercaptoethanol and maleic anhydride or succinic anhydride (preincubation time 30 min); or 0.1 M-Tris-HCl, pH 7.5, 0.01 M- $MgCl_2$, 0.01 M-2-mercaptoethanol and phenylmethanesulphonyl fluoride (preincubation time 3 h). Phenylmethanesulphonyl fluoride was dissolved in propan-2-ol and added to the buffer to give 5% (v/v) propan-2-ol in buffer. After the preincubation time 10 μl of the mixture was assayed for enzyme activity in 0.5 ml of medium containing 2 mM- PP_i , 5 mM- $MgCl_2$, 0.1 M-Tris-HCl, pH 7.5. The activity was expressed as percentage of the mixture which had been incubated in a buffer without inactivator.

Inhibitor	Concentration (mM)	Relative activity (%)
<i>p</i> -Hydroxymercuribenzoate	1	88
	5	13
Iodoacetic acid	10	28
	30	9
<i>N</i> -Ethylmaleimide	10	48
	30	25
Maleic anhydride	25	75
Succinic anhydride	25	53
Phenylmethanesulphonyl fluoride	1	58

Table 4. Pyrophosphatase activity at physiological concentration of PP_i and Mg^{2+}

Enzyme (fraction DE-III-SE; 0.5 munit) was added to 0.5 ml of incubation medium containing 3.5 μM - $[^{32}P]PP_i$, 0.6 mM- Mg^{2+} , 0.1 M-Tris-HCl, pH 7.4, 0.15 M-NaCl and the ions tested. The reaction was stopped after 4 min incubation at 37°C. The relative activity is expressed as percentage of the control.

$[P_i]$ (mM)	$[Ca^{2+}]$ (mM)	Relative activity (%)
0.4–4.0	—	100–90
—	0.4–2.5	4–2
0.5–3.0	1.0 or 1.6	0–2

hibited the enzyme reaction to some degree in the concentration region 0.05–0.5 mM, the effect of the former being stronger than that of the latter (Table 5).

Discussion

The results presented in this paper give evidence for the similarity between pyrophosphatases from cartilage and other tissues (Table 6) with respect to molecular weight, pH optimum, Michaelis constant,

Table 5. *Effect of ethane-1-hydroxy-1,1-diphosphonate and dichloromethylenediphosphonate at 3.5 μ M-PP_i*

Fraction DE-III-SE (0.5munit) was added to 0.5ml of incubation medium containing 3.5 μ M-[³²P]PP_i, 0.6mM-MgCl₂, 0.1 M-Tris-HCl, pH 7.4, 0.15M-NaCl and either ethane-1-hydroxy-1,1-diphosphonate or dichloromethylenediphosphonate. Incubation time was 4 min at 37°C and the activity is expressed as a percentage of the value found in the absence of phosphonates.

Diphosphonate	Concn. (mM)	...	Relative activity (%)			
			0.01	0.05	0.1	0.5
Ethane-1-hydroxy-1,1-diphosphonate			83	49*	34*	10*
Dichloromethylenediphosphonate			105	100	94	48*

* Significance of difference from control, $P < 0.001$.

substrate specificity, activation by Mg²⁺, effect of other metal ions especially inhibition by Ca²⁺ and the effect of cysteine. This pyrophosphatase of pig scapula cartilage resembles most closely those from rat costal cartilage, human erythrocytes and yeast.

All three fractions DE-II-SE, DE-III-SE and DE-IV-SE had the same molecular weight, pH optimum, dependence on Mg²⁺ and specificity towards PP_i as substrate. No pyrophosphate-glucose phosphotransferase activity, typical of microsomal pyrophosphatase (Nordlie & Arion, 1964), was found. These observations suggest that the different fractions eluted from the DEAE-cellulose column (Felix & Fleisch, 1975) may be isoenzymes.

The inhibition of the cartilage pyrophosphatase by Ca²⁺ could be explained by a stronger binding of CaPP_i, which is not hydrolysed, than MgPP_i to the enzyme, as was found for the yeast enzyme (Moe & Butler, 1972b). Other metal ions might act in the same way, or could act by binding directly to the activator site (Ridlington & Butler, 1972; Cooperman & Chiu, 1973a; Rapoport *et al.*, 1973; Höhne & Rapoport, 1973).

When tested in the presence of 2mM-PP_i as substrate the diphosphonates ethane-1-hydroxy-1,1-diphosphonate and dichloromethylenediphosphonate inhibited only at high concentrations. This inhibition was not due to complexing of magnesium, since the diphosphonates were added as a magnesium complex. Negi *et al.* (1972) found competitive inhibition by methanediphosphonate at high inhibitor concentrations. In contrast, Cooperman & Chiu (1973a) and Sperow *et al.* (1973) could not find such an inhibition as long as magnesium was present in excess. However, the former authors did find competitive inhibition by hydroxymethanediphosphonate (K_i 120 μ M). We found a relatively strong inhibition by *n*-pentanemonophosphonate. This might be compared with the described effects of diphosphonates, which have been shown to inhibit both mineralization (Fleisch *et al.*, 1970) and bone resorption (Russell *et al.*, 1970).

Inhibitors of the alkaline phosphatase (Table 4), such as P_i (Fernley & Walker, 1967), amino acids (Fishman & Sie, 1971), imidazole (Brunel & Cathala, 1972), CN⁻ (Fishman *et al.*, 1962) and theophylline (Fawaz & Tejirian, 1972) did not inhibit. The weak inhibition by amino acids and by tartrate is probably due to binding of magnesium. The strongest inhibitor was F⁻ which has no effect on alkaline phosphatase but inhibits some acid phosphatases (Reiner *et al.*, 1955; Chersi *et al.*, 1966).

The thiol-group-binding agents inactivated strongly. Maleic anhydride and succinic anhydride, which react with protein amino groups, and phenyl-methanesulphonyl fluoride which can be used as a substitute for di-isopropyl phosphorofluoridate (Fahrney & Gold, 1963) also inactivated to some degree. Thus no inactivation specific for only one amino acid residue could be demonstrated. Cooperman & Chiu (1973b) showed rapid inactivation of yeast inorganic pyrophosphatase by 0.02M-maleic anhydride which they interpreted as dissociation of the enzyme into subunits rather than modification of essential lysine residues.

The pyrophosphatase was bound to apatite and was eluted with 1M-sodium phosphate but not with 1M-NaCl. This fact might explain the results of Alcock & Shils (1969), who found a decrease in pyrophosphatase in cartilage after calcification had occurred. Indeed, it is possible that the enzyme is still present, but was not measurable in this study since it is bound to the apatite.

In view of the suggestion that inorganic pyrophosphatase acts as a calcification enzyme its properties were investigated at substrate and ion concentrations similar to those of plasma, assuming that it would have to work extracellularly. Indeed the pyrophosphatase activity of alkaline phosphatases show greatly different characteristics at low PP_i concentrations than at optimum PP_i concentrations (Tenenhouse & Rasmussen, 1968; Felix & Fleisch, 1974). This seems not to be the case for inorganic pyrophosphatase. Thus the pH optimum was only slightly shifted, and,

Table 6. Comparison with inorganic pyrophosphatases of other sources

Where PP_i is given as being the substrate, it is not excluded that other substrates are hydrolysed to a small degree.

Source	Mol.wt.	pH optimum	Optimal Mg/ PP_i ratio	Substrate	Partially activated by	Inhibition by metal ions	Inhibition by F^-	K_m (μM)	Cysteine
Pig scapula cartilage	66 000	7.5	≥ 1.5	PP_i	Be^{2+} , Mn^{2+} , Fe^{2+} , Co^{2+} , Zn^{2+}	Yes	Yes	11	Essential
Rat costal cartilage*	—	6.8	5	—	Zn^{2+} , Mn^{2+} , Co^{2+}	Yes	Yes	—	—
Rat liver†	68 000	8.0	1.5–4	Not only PP_i	—	Yes	Yes	56–86	Essential
Human erythrocytes‡	—	7.7	1	PP_i	Mn^{2+} , Co^{2+}	—	—	9.7	Essential
Yeast§	70 000	7.0	1.5	PP_i	Mn^{2+} , Co^{2+}	Yes	—	16.6	Not essential
<i>Escherichia coli</i>	118 000	9.1	1	PP_i	Mn^{2+} , Zn^{2+} , Co^{2+}	—	Yes	5	Not essential
<i>Bacillus subtilis</i> ¶	68 000	9.0	0.5	PP_i	Mn^{2+} , Ca^{2+} , Zn^{2+} , Co^{2+}	—	Weak	130	Not essential

* Alcock & Shils (1969); Alcock (1972).

† Irie *et al.* (1970).

‡ Pynes & Younathan (1967).

§ Schachman (1952); Hansen *et al.* (1972); Kunitz (1952); Moe & Butler (1972a,b); Ridlington & Butler (1972); Rapoport *et al.* (1972); Cooperman & Chiu (1973a,b).

|| Wong *et al.* (1970); Josse (1966a,b); Burton & Josse (1970).

¶ Tono & Kornberg (1967).

as with 2mM- PP_i , a strong inhibition by Ca^{2+} , but none by P_i , was found. Ethane-1-hydroxy-1,1-diphosphonate and dichloromethylenediphosphonate also inhibited, although now at lower concentrations, indicating that they probably compete with PP_i at the active site.

These results make it less likely that the role of the enzyme is to promote calcification by destroying the calcification inhibitor PP_i . Indeed, the calcification is occurring extracellularly and the enzyme is unlikely to work there, first because it is probably intracellular and secondly because it is strongly inhibited by calcium concentrations, such as those observed at the calcification site (Howell *et al.*, 1968). One possibility might, however, be that the pyrophosphatase is present in the matrix vesicles which are thought to be the initial site of calcification and which are claimed to originate from the cells (Anderson, 1969). The various characteristics described make it more likely that the inorganic pyrophosphatase of calcifying cartilage is not different from the inorganic pyrophosphatases in other organs and that it has the same function, namely to facilitate the pyrophosphorolytic reaction in biosynthesis by removing PP_i (Kornberg, 1962). This is consistent with the finding of Kuhlman (1965), who observed the highest inorganic pyrophosphatase activity in cartilage in the proliferating zone, where cell division but not calcification occurs, whereas alkaline phosphatase activity is highest in the hypertrophic zone and in the calcified zone of the cartilage.

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