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PURIFICATION AND KINETIC PROPERTIES OF HUMAN ERYTHROCYTE Mg^{2+}-DEPENDENT INORGANIC PYROPHOSPHATASE

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Summary

Inorganic pyrophosphatase (pyrophosphate phosphohydrolase, EC 3.6.1.1) from human erythrocyte hemolysates has been purified up to 10000-fold.

The purified enzyme is homogenous and has a specific activity of 79.75 μmol PP_i hydrolysed · min^{-1} · mg^{-1} at pH 8 and 37°C.

It was confirmed that it is a dimer with a molecular weight of 42000, composed of two identical protomers.

From kinetic studies, it is proposed that human erythrocyte inorganic pyrophosphatase activity depends on free Mg^{2+} concentration in different ways. This ion constitutes part of the substrate (the $Mg \cdot PP_i$ complex; $K_m = 1.4 \cdot 10^{-4}$ M) and probably acts as an allosteric activator (kinetic activation constant: $K_a^{Mg^{2+}} = 7.5 \cdot 10^{-4}$ M).

Equilibrium binding studies performed in the absence of PP_i showed 4 binding sites for Mg^{2+}, all having the same high affinity (dissociation constant: $K_d^{Mg^{2+}} = 4 \cdot 10^{-6}$ M).

Since the concentration of free Mg^{2+} in red blood cells is very low and may vary with the oxygenation state, it is likely that in vivo erythrocyte pyrophosphatase activity is regulated.

Introduction

Inorganic pyrophosphate (PP_i) plays a central role in the bioenergetics of living cells. Biosynthetic reactions which produce pyrophosphate (polysaccharides, proteins, DNA and RNA synthesis) are subject to a "thermodynamic pull" [1—3] due to the subsequent complete and irreversible hydrolysis of pyro-

Abbreviations: $Mg^{2+} \cdot PP_i$, magnesium · pyrophosphate complex in the molar ratio 1 : 1; P-rib-PP, phosphoribosyl-pyrophosphate.

phosphate by widely distributed pyrophosphatases (standard free energy of hydrolysis: $\Delta G^\circ = -23.56$ kJ · mol^{-1} of PP_i in the absence of divalent cations [4]).

On the other hand it has been shown that PP_i can act as an energetic donor in transphosphorylation reactions [5—7] and several authors have recently claimed that its concentration in cells is low but not negligible [8—10]. Moreover, Guyn et al. [11] had demonstrated that the PP_i-hydrolysis reaction is not close to equilibrium in vivo.

These results prompted us to search for a possible control of pyrophosphatase activity, which should be able to modify intracellular PP_i concentration. Amongst the different enzymes which catalyse pyrophosphate hydrolysis in human tissues (alkaline and acid phosphatases, microsomal glucose 6-phosphatase, nucleotide pyrophosphatase and inorganic pyrophosphatase) we choose to study the inorganic pyrophosphatase which is the most specific, the most widespread and the most active of these enzymes.

The present work describes an improved purification of this enzyme from human erythrocytes, based on the method of Pynes and Younathan [12]. A study of its kinetic properties has also been undertaken. For this, the main difficulty lies in the determination of the exact concentration of the true substrate ($Mg^{2+} \cdot PP_i$) of the enzyme. This was calculated for each assay condition using a computer program.

Materials and Methods

[^{32}P]Pyrophosphate (specific activity 5400 Ci · mol^{-1}) was supplied by New England Nuclear Corporation, Boston, Mass., U.S.A.

Tris base, metal salts and reagents, all analytical grade, were purchased from Merck, Darmstadt, G.F.R., and marker proteins from Boehringer Mannheim GmbH, Mannheim, G.F.R.

Hexamethylenediamine-Sepharose was obtained according to the method of Cuatrecasas [13]. Pyrophosphate was bound to the hexamethylenediamine-Sepharose by the following procedure: 45 ml of decanted hexamethylenediamine-Sepharose 4-B were mixed with 100 ml of sodium pyrophosphate 100 mM at pH 6.7. A solution of 500 mg of carbodiimide dissolved in a minimum volume of water was added over 5 min. The mixture was kept at 23°C for 18 h with gentle stirring. Quantitation of the amount of PP_i coupled to hexamethylenediamine-Sepharose was determined by the use of $^{32}PP_i$. The average PP_i concentration obtained for decanted gel was 4 mM.

The radioactivity was measured with an end-window Geiger tube (Tracerlab ICN model SC 510). All tests were made in duplicate, and counting was corrected for background.

Magnesium assays were carried out with an atomic absorption spectrophotometer Unicam SP 90 with a sensitivity as low as 10^{-5} M.

Pyrophosphatase assay. Enzymatic hydrolysis of PP_i was assayed using the method described previously [14]. Each assay was performed in duplicate with a reproducibility of 1%. The hydrolysis of PP_i, for the lowest concentrations of substrate, was in no case greater than 20%.

One enzyme unit is defined as the amount necessary to hydrolyse 1 μmol

pyrophosphate per min at 37°C. Specific activity is expressed in μmol pyrophosphate hydrolysed/min (at 37°C) per mg protein.

Protein determination. Proteins were determined in presence of mercaptoethanol according to the method of Geiger and Bessman [15].

Equilibrium dialysis technique. For binding experiments with Mg^{2+}, the purified enzyme solution was treated for 2 h with 10 mM EDTA, subsequently passed through a Sephadex G-25 column and then dialysed for 4 h against 2 mM Tris buffer (pH 8)/1 mM mercaptoethanol. Double-distilled water and ultrapure Tris base were used and all glassware was cleaned with EDTA to avoid contamination by magnesium or other metals. The membranes were cut from cellulose dialysis tubing pretreated as recommended by Waygood et al. [16].

Calculations of substrate concentrations. When Mg^{2+} and PP_i were mixed in water, it was demonstrated that several complexes were present and that the concentration of each complex depended on pH and on total magnesium and pyrophosphate concentrations.

$$\begin{array}{ccccc} PP_i^{4-} & \rightleftharpoons & Mg \cdot PP_i^{2-} & \rightleftharpoons & Mg \cdot PP_i^{0} \\ \Updownarrow & & \Updownarrow & & \\ HPP^{3-} & \rightleftharpoons & HMg \cdot PP_i^{-} & & \\ \Updownarrow & & & & \\ H_2PP_i^{2-} & & & & \end{array}$$

Conservation equations in Mg^{2+} and PP_i can be written since the molar enzyme concentration is lower than those of the metal or pyrophosphate. With the 5 values of the association complex constants calculated by Lambert and Watters [17] at fixed pH and ionic strength, 7 equations with 7 unknowns are obtained.

At high pH, however, the protonated species are virtually nonexistent and can be neglected. At pH 8.2, it is then possible to use the simplified system of 4 equations proposed by Josse [18]:

$$PP_i^{4-} \underset{K_{Mg^{2+} \cdot PP}}{\overset{Mg^{2+}}{\rightleftharpoons}} Mg \cdot PP_i^{2-} \underset{K_{Mg_2 \cdot PP}}{\overset{Mg^{2+}}{\rightleftharpoons}} Mg_2 \cdot PP_i$$

$$K_{Mg \cdot PP_i^{2-}} = \frac{Mg \cdot PP_i^{2-}}{(Mg^{2+})(PP_i^{4-})} = 10^{5.41}\ M^{-1}$$

$$K_{Mg_2 \cdot PP_i} = \frac{Mg_2 \cdot PP_i}{(Mg^{2+})(Mg \cdot PP_i^{2-})} = 10^{2.34}\ M^{-1}$$

Conservation equations:

$$Mg^{2+}\,(\text{total}) = Mg^{2+} + 2(Mg_2 \cdot PP_i) + Mg \cdot PP_i^{2-}$$

$$PP_i^{4-}\,(\text{total}) = PP_i^{4-} + (Mg_2 \cdot PP_i) + Mg \cdot PP_i^{2-}$$

This system can be resolved through a third order polynomial, the valid positive root of which expresses the concentration of $Mg \cdot PP_i$ *, true substrate of the erythrocyte pyrophosphatase.

* All the values of $Mg \cdot PP_i$ complex, free Mg^{2+} and PP_i ions given in the text and the figures are thus calculated through the system of four equations.

Results and Discussion

Enzyme purification. Affinity chromatography improved the purification process described by Pynes and Younathan [12] and allowed the use of smaller quantities of human blood, freshly collected on heparin. The procedure is summarized in Table I. The first 3 purification steps were performed according to the process of Pynes and Younathan [12]. The enzyme was desalted by means of Sephadex G-25, and was then eluted from a Sephadex G-100 column (100 × 2.5 cm) equilibrated with buffer 1: 2 mM $MgCl_2$/0.2 mM EDTA/5 mM mercaptoethanol/1 mM Tris · HCl (pH 8.2). Active fractions were collected and the final purification was made on the affinity chromatography column (40 × 1 cm) (Fig. 1). Fractions of highest enzymatic activity were pooled and the total volume was concentrated to 2 ml by ultrafiltration and desalted by filtration on Sephadex G-25. At this step the preparation is apparently homogeneous after polyacrylamide gel electrophoresis (Fig. 2).

Enzyme properties. It was confirmed that the stability of the erythrocyte pyrophosphatase depends on the presence of reduced thiols groups and Mg^{2+}. The optimal pH of the enzyme (approx. pH 8) was also verified. The variation of the maximum velocity with pH (Fig. 3) is compatible with the intervention of at least two amino acids of different pK_a. One of these has pK_a 6 (probably histidine) and the other has pK_a 9.3 (probably tyrosine). The molecular weight of the dimer enzyme (42000) was determined using electrophoresis on polyacrylamide gradient slabs with marker proteins. For the monomer, a molecular weight of 20000 was obtained using SDS-treated proteins. These results are in good agreement with those of Fisher et al. [20].

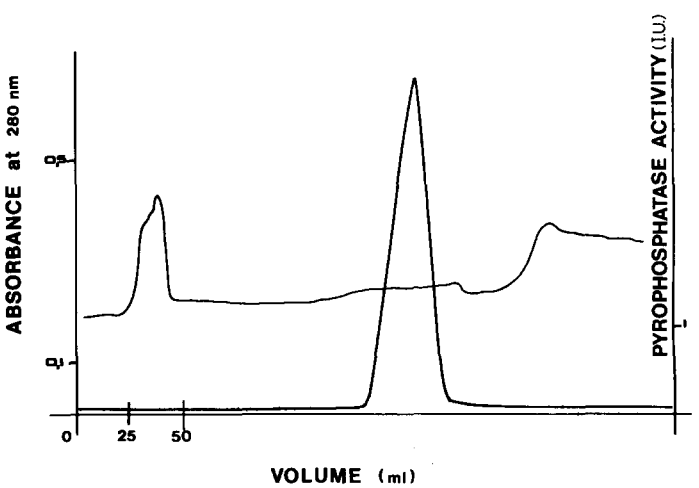


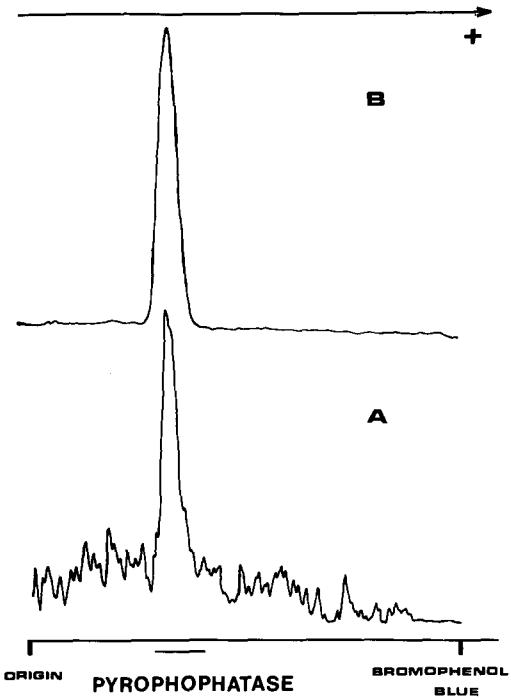
Fig. 1. Elution recording of affinity column. Elution by buffer 1: 2 mM $MgCl_2$/0.2 mM EDTA/5 mM mercaptoethanol/1 mM Tris · HCl, pH 8.2, till the first 50 ml, then elution by linear gradient of pyrophosphate constituted by: 150 ml of buffer 1 in the mixing cylinder and 150 ml of buffer 2: 25 mM PP_i/10 mM $MgCl_2$/0.2 mM EDTA/5 mM 2-mercaptoethanol/1 mM Tris · HCl, pH 8.2, in the reservoir cylinder. The absorbance at 280 nm is shown by the upper trace. The lower curve illustrates the pyrophosphatase activity.

TABLE I

PURIFICATION OF HUMAN ERYTHROCYTE INORGANIC PYROPHOSPHATASE

Purification was started with 100 ml of washed packed red cells obtained from a blood bank with a specific activity depressed by a factor of 1.5 during conservation.

Fraction		Total activity (μmol $PP_i \cdot min^{-1} \cdot mg^{-1}$)	Specific activity (μmol $PP_i \cdot min^{-1} \cdot mg^{-1}$)	Recovery (%)	Purification
I	Whole hemolysate	266	0.008	100	
II	Hemoglobin-free eluate	238	0.722	89	90.2
III	Ammonium sulfate fractionation	172	2.28	64	285
IV	Gel filtration eluate (G-100)	133	9.0	50	1125
V	*Sepharose eluate*	127.6	79.75	48	9968



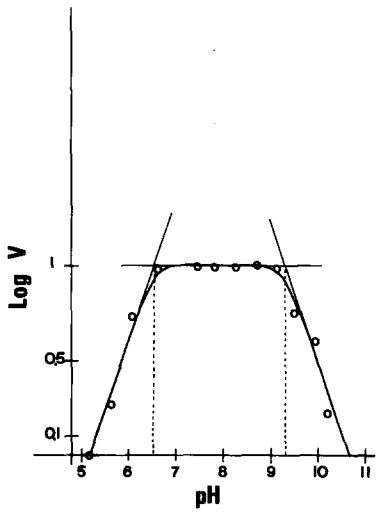


Fig. 2. Electrophoretograms of erythrocyte inorganic pyrophosphatase on polyacrylamide gels performed with a Gilfor linear transport spectrophotometric system. A, amido-black staining; B, specific staining. Specific staining on polyacrylamide disc gels was obtained using the technique described by Tonio and Kornberg [19].

Fig. 3. Influence of pH on the maximum velocity of the human erythrocyte inorganic pyrophosphatase with free Mg^{2+} concentration 0.350 mM.

Kinetic studies. A study was made using a constant purified erythrocyte inorganic pyrophosphate concentration at different PP_i and Mg^{2+} concentrations. In each case, $Mg^{2+} \cdot PP_i$ and free Mg^{2+} concentrations were calculated. Fig. 4 shows that the initial velocity of the reaction depends not only on substrate concentration but also on free Mg^{2+} concentration. At various free Mg^{2+} concentrations, Michaelis-Menten plots are hyperbolic. The reciprocal plots are linear and give nearly the same K_m, $1.4 \cdot 10^{-4}$ M, for the substrate $Mg^{2+} \cdot PP_i$, but different maximal velocities. This activation of pyrophosphatase activity by Mg^{2+} might be explained by an allosteric phenomenon as suggested by secondary plots of the slopes a and of the intercepts V of the Lineweaver plots, versus $1/Mg^{2+}$ which give markedly concave upward curves. Accordingly, the V versus free Mg^{2+} concentration is sigmoidal and the corresponding Hill plot gives a Hill coefficient, $n_H = 2.4$ and an activation constant, $K_a^{Mg^{2+}} = 7.5 \cdot 10^{-4}$ M.

To complete these kinetic data, Mg^{2+}-binding studies were performed.

Equilibrium dialysis studies. The direct curve of the concentration of Mg^{2+} bound to the dimer enzyme against the concentration of free Mg^{2+} (Fig. 5A) is clearly sigmoidal with a saturation phenomenon at high concentrations of free Mg^{2+}. The corresponding Scatchard plot (Fig. 5B) is upwards convex and

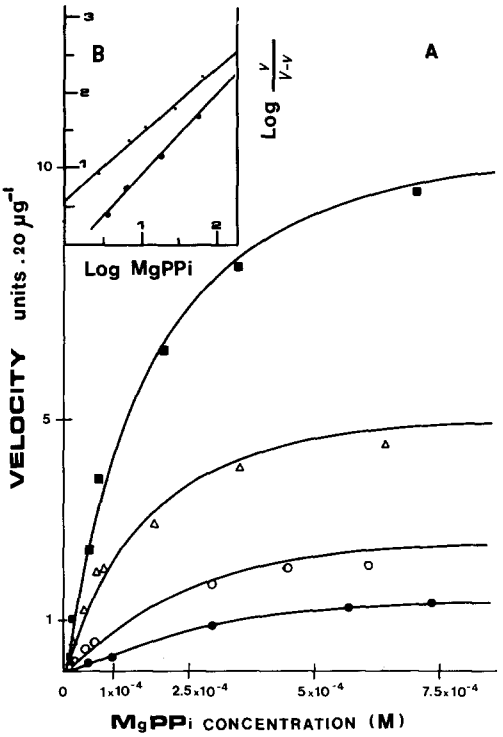


Fig. 4. A. Michaelis-Menten plot of the human erythrocyte inorganic pyrophosphatase activity at various free Mg^{2+} concentrations. ●———●, Mg^{2+} = 0.350 mM, V = 1.43 µmol $PP_i \cdot min^{-1}$; ○———○, Mg^{2+} = 0.505 mM, V = 2.5 µmol $PP_i \cdot min^{-1}$; △———△, Mg^{2+} = 0.670 mM, V = 5 µmol $PP_i \cdot min^{-1}$; ■———■, Mg^{2+} = 1.800 mM, V = 10 µmol $PP_i \cdot min^{-1}$. B. Hill coefficient n_H obtained with free Mg^{2+} concentrations. ●———●, Mg^{2+} = 0.350 mM, n_H = 0.98; ■———■, Mg^{2+} = 1.800 mM, n_H = 1.12.

gives a stoichiometry of n = 4.2 mol Mg^{2+} bound per mol of dimer enzyme and an apparent dissociation constant near saturation: $K_d^{Mg^{2+}} = 4 \cdot 10^{-6}$ M. Since, during the kinetic studies, we have never observed modifications of the enzyme properties by dilution, it seems improbable that the sigmoidal shape of the curve in Fig. 5A reflects a dissociation of the dimer into two monomers. It could therefore be assumed that Mg^{2+} binds cooperatively to 4 sites of the enzyme dimer.

Since experimental conditions are different, we cannot compare the dissociation constant ($K_d^{Mg^{2+}} = 4 \cdot 10^{-6}$ M) obtained in presence of free Mg^{2+} only and that $K_a^{Mg^{2+}} = 7.5 \cdot 10^{-4}$ M given by kinetic studies carried out in the presence of both Mg^{2+} and PP_i species. However, in both kinetic and binding studies, cooperativity is evident and it could reasonably be suggested that Mg^{2+} acts as an allosteric activator, as previously reported by Rapoport et al. [21] for bakers yeast pyrophosphatase.

If hydrolytic splitting to P_i is the only way to remove PP_i from a cell, this regulation of Mg^{2+}-dependent pyrophosphatases might be important since PP_i can intervene in biosynthetic equilibria of the cell either directly [1—3,22] or indirectly as an energy donor through transphosphorylase reactions [5—7].

In order to ascertain whether the regulation of the erythrocyte pyrophosphatase may have physiological consequences, it is necessary to estimate the

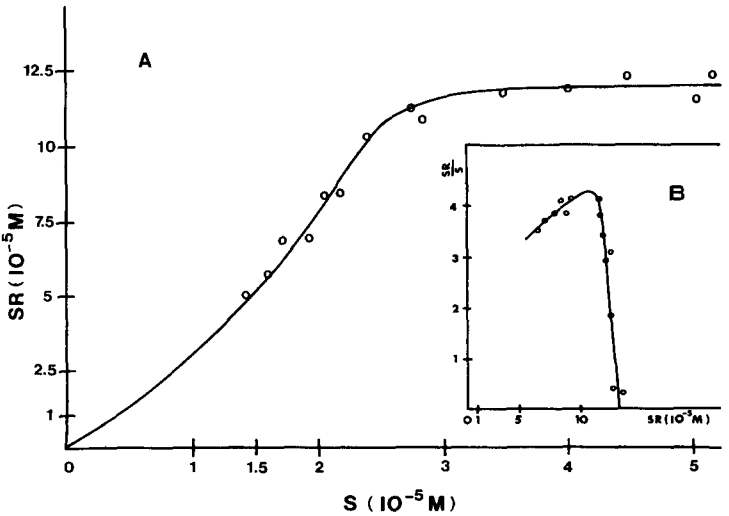


Fig. 5. A. Experiment of equilibrium dialysis study with a solution of erythrocyte inorganic pyrophosphatase $3.15 \cdot 10^{-5}$ M. In a typical experiment, 0.3 ml purified Mg^{2+}-free enzyme solution in 2 mM Tris/1 mM mercaptoethanol, pH 8, (approx. 1.2 mg · ml^{-1} protein) was dialysed against 0.3 ml $MgCl_2$ of different concentrations in the same buffer, 8 h at 20—22°C by gentle rotation of the cells. 0.2-ml aliquots of solution were then removed from both compartments for atomic absorption spectrophotometric assay of magnesium. B. Scatchard plot representation of the same experimental data. (S, free Mg^{2+} concentration; SR, bound Mg^{2+} concentration).

ratio between the PP_i hydrolysis and the PP_i liberation rates.

In human red cells where most of the anabolic and catabolic pathways have been lost before maturity, PP_i hydrolysis at the intracellular pH 7.36 is mainly the consequence of the inorganic pyrophosphatase activity which is, for adults: 0.72 ± 0.02 μmol PP_i hydrolysed · h^{-1} · mg^{-1} of hemoglobin from our assays.

PP_i production (essentially derived from mono- [23] and dinucleotide [24] synthesis) could be estimated at 0.6—1 μmol PP_i liberated · h^{-1} · mg^{-1} of hemoglobin. Therefore, the ratio between maximal pyrophosphatase activity and PP_i production-rate should be less than 10. On the other hand, the total Mg^{2+} concentration in the human erythrocyte is 3—4 mM. Yet this Mg^{2+} is largely chelated [25], mainly by 2,3-diphosphoglycerate (4.6 mM) and ATP (1.5 mM). Upon oxygenation, which induces liberation of 2,3-diphosphoglycerate and ATP from hemoglobin, there is a considerable decrease in free Mg^{2+} concentration, from 1.9 mM in deoxygenated to 0.57 mM in oxygenated red blood cells [26]. In vitro, such a variation has been shown to decrease pyrophosphatase activity by a factor of 10. Although the rate of hydrolysis in vivo might differ, it is likely that human erythrocyte inorganic pyrophosphatase is physiologically regulated by the intracellular concentration of free Mg^{2+}, which depends on the hemoglobin oxygenation state.

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