Purification and Some Properties of Inorganic Pyrophosphatase from Human Erythrocytes*

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SUMMARY

Inorganic pyrophosphatase was purified 1800-fold from human erythrocytes by a procedure involving complete hemolysis of the cells, removal of hemoglobin, ammonium sulfate fractionation, diethylaminoethyl cellulose column chromatography, and gel filtration. Magnesium chloride and 2-mercaptoethanol stabilized the activity.

The procedure yielded an enzyme with an optimal pH of 7.7, and an apparent Michaelis constant of $9.7 \times 10^{-6}$ M. Magnesium was essential for activity with an optimal $Mg^2+$ $PP_i$ ratio of 1. These data suggest that the natural substrate for the erythrocyte pyrophosphatase is $MgPP_i^\circ$. The enzyme showed very strict specificity for inorganic pyrophosphate. Adenosine triphosphate, adenosine diphosphate, inosine triphosphate, and inorganic tripolyphosphate were not acted upon in the presence of $Mg^2+$ or $Zn^2+$ as activator. Moreover, unlike the mitochondrial and the microsomal pyrophosphatases, the erythrocyte enzyme showed no $PP_i$-glucose transphosphorylase activity.

The Arrhenius plot of the erythrocyte pyrophosphatase exhibited a transition at 29° with an activation energy of 8,560 cal per mole above this temperature and 12,900 cal per mole below it. The activity was inhibited by $p$-hydroxymercuribenzoate, but was much less sensitive to alloxan, 8,560 cal per mole above this temperature and 12,900 cal per mole below it. The activity was inhibited by $p$-hydroxymercuribenzoate, but was much less sensitive to alloxan, N-ethylmaleimide, and iodoacetamide. In the erythrocyte, inorganic pyrophosphatase probably plays a role in nicotinamide adenine dinucleotide formation by hydrolyzing the $PP_i$ produced in its three synthetic steps.

Inorganic pyrophosphate is produced in several metabolic reactions, e.g. amino acid activation, nucleic acid polymerization, and the biosynthesis of several nucleotides. Inorganic pyrophosphatase (pyrophosphate phosphohydrolase, EC 3.6.1.1) shifts such reactions toward the synthetic products by hydrolyzing the pyrophosphate formed (1, 2). Moreover, the orthophosphate produced controls several biochemical processes, e.g.

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the assay system described previously. The initial velocity was linearly proportional to the amount of enzyme up to 3.0 μmoles of PPi, hydrolyzed in 10 min. Each assay was performed in duplicate or triplicate with a reproducibility of ±5%. Specific activity is reported as micromoles of orthophosphate produced per 10 min per mg of protein.

**Protein Determination**—Protein was determined by the biuret method (13). For accurate measurements of samples containing hemoglobin, two blanks were essential, one for the alkalized per 10 min per mg of protein.

**Activity**

The activity is reported as micromoles of orthophosphate produced in 10 min. Each assay was performed in duplicate or triplicate with a reproducibility of ~5%. Specific enzyme preparations, the protein was measured by the method of Warburg and Christian as described by Layne (14). This method was not suitable in the presence of hemoglobin, since it gave nearly double the true value of the protein in the hemolyte.

**Polyacrylamide Disc Electrophoresis**—The procedure of Davis (15) was used in the disc gel electrophoresis of the enzyme preparation.

**Conductivity Measurements**—The conductivity of the chromatographic fractions and the dialyzed solutions was measured with a Radiometer conductivity meter (type DCM 2) at 0° with the aid of a syringe-type bridge.

**RESULTS**

**Purification of Inorganic Pyrophosphatase**

All steps were performed at 0-4°C. The procedure is summarized in Table I.

**Whole Hemolysate**—The erythrocytes (about 840 ml of packed cells) were washed four times with equal volumes of buffered saline (0.15 m NaCl-10 mM MgCl₂-1 mM EDTA-10 mM Tris-Cl, pH 7.1). The cells were sedimented at 2,500 × g for 10 min and finally hemolyzed by the addition of an equal volume of water. The hemolysate was dialyzed (36 to 40 hours) against two changes of 5 to 10 volumes of MgCl₂-EDTA solution (1 mM MgCl₂ and 0.2 mM EDTA, pH 6.5). The dialyzed hemolysate was brought up to 3 times the packed cell volume by addition of the MgCl₂-EDTA solution described previously, and the stroma were sedimented by centrifugation at 14,600 × g for 50 min. The stromal sediment was washed once with an equal volume of the MgCl₂-EDTA solution. The washing was combined with the stroma-free hemolysate.

**Removal of Hemoglobin**—For each 100 ml of the original packed cell volume, 25 g of semi-dry DEAE-cellulose were added to the stroma-free hemolysate, and the mixture was thoroughly stirred for 1 hour. The DEAE-cellulose was collected on a Buchner funnel and washed with MgCl₂-EDTA solution until the filtrate was almost colorless. The latter was then transferred to a beaker and the enzyme eluted with a volume of salt solution (0.25 m NaCl-2 mM MgCl₂-0.2 mM EDTA-1 mM 2-mercaptoethanol-1 mM Tris-Cl, pH 7.1) equal to the packed cell volume. After 30 min of stirring, the mixture was filtered and the cellulose cake was washed with 100 ml of the same solution. The filtrate at this stage was brownish yellow.

**Ammonium Sulfate Fractionation**—The enzyme solution from the previous step was made 80% saturated with ammonium sulfate (35.1 g/100 ml) and stirred for 1 hour. The precipitated protein was centrifuged at 14,000 × g for 15 min. The supernatant fluid was then made 80% saturated with ammonium sulfate (17.5 g/100 ml) and stirred for 1 hour. The precipitated protein was centrifuged down, and the supernatant solution was discarded.

**DEAE-cellulose Chromatography**—The brownish yellow precipitate was dissolved in a minimal volume of Tris-Cl buffer (2 mM MgCl₂-1 mM 2-mercaptoethanol-0.2 mM EDTA-1 mM Tris-Cl, pH 7.1) and dialyzed (16 to 24 hours) against four changes (1 liter each) of the same buffer. Insoluble proteins were removed by centrifugation. The conductivity of the dialyzed solution should not exceed twice that of the buffer. The enzyme solution was transferred to a DEAE-cellulose column (2.4 × 45 cm) which was packed under gentle pressure. Elution was carried out by a sodium chloride gradient fed into the column from two identical cylinders. The reservoir cylinder contained 500 ml of 0.5 m NaCl, and the mixing cylinder contained 500 ml of a mixture of 2 mM MgCl₂-10 mM 2-mercaptoethanol-10 mM Tris-Cl buffer, pH 7.4. Fractions (10.4 to 10.6 ml each) were collected, and the eight tubes (approximately 84 ml) containing the highest enzymatic activity were pooled. This fractionation step was critically dependent on the presence of 2-mercaptoethanol for enzymatic stability. In other steps, mercaptoethanol was used as a precaution.

**Gel Filtration**—The enzyme in the pooled active fraction from the DEAE-cellulose column was precipitated by 85% ammonium sulfate saturation, and the precipitate was dissolved in a minimal volume of Tris-Cl buffer (composition is as described in the previous step). The volume was about one-fifteenth the column bed volume. The enzyme was then chromatographed on Sephadex G-100 column (4.4 × 54 cm) which was equilibrated with the elution fluid before the sample was introduced. The elution fluid contained 9 mM MgCl₂, 1 mM 2-mercaptoethanol, 0.2 mM EDTA, and 1 mM Tris-Cl, pH 7.1. Fractions (10.4 to 10.6 ml each) were collected, and the three tubes with maximal pyrophosphatase activity (approximately 32 ml) were pooled and stored as shown below. The enzyme preparation at this stage was colorless. Analysis of a sample with disc electrophoresis revealed two protein bands with the activity manifested in one only.

**Enzyme Storage and Stability**—The enzyme can be stored at 0-4°C for at least a month without appreciable loss of activity when suspended in 85% ammonium sulfate-saturated solution in the presence of 2-mercaptoethanol (10 mM) and MgCl₂ (10 mM). Routinely, each batch was distributed into aliquots of a few milliliters each. When needed, the enzyme was sedi-
mented by centrifugation, and the precipitate dissolved in an appropriate volume of Tris-Cl buffer (3 mM MgCl₂-3 mM 2-mercaptoethanol-2 mM Tris-Cl, pH 7.1). When this solution was allowed to stand at 0–4°C overnight, the enzyme regained almost all of its original activity. Except where otherwise stated (see for example Table II), this reactivation procedure was adopted routinely.

The pH of the storage medium was not critical between 6.0 and 8.8. Beyond this pH range, however, the activity diminished rapidly on standing at 0–4°C.

Properties of Enzyme

Effect of pH on Pyrophosphatase Rate—When a Mg²⁺:PPi ratio of 1 was used, the enzyme had an optimal pH of 7.7 (Fig. 1). Raising this ratio to 3.8 shifted the optimal pH to 7.4 and lowered the enzymatic activity. The bearing of these observations on the nature of the substrate of inorganic pyrophosphatase is discussed below.

Effect of Divalent Cations—In the complete absence of divalent cations the enzyme showed no activity. Among four cations tested (Table II), Mg²⁺ showed the highest activation effect, whereas Ca²⁺ was completely inert. The maximal activity obtained with Mn²⁺ or Co²⁺ was less than 0.1 that with Mg²⁺. As indicated in Fig. 2, the optimal Mg²⁺:PPi ratio was about 1 when PPi was used at 2 mM concentration. This suggests that the cation combines stoichiometrically with the pyrophosphate. Although some inhibition was observed with higher concentrations of Mg²⁺, about 70% of the activity was retained with Mg²⁺:PPi ratios as high as 50.

Activity as Function of Substrate Concentration—When the optimal Mg²⁺:PPi ratio was used, a linear Lineweaver-Burk plot (Fig. 3) was obtained which gave an apparent Michaelis constant of $9.7 \times 10^{-6} \text{ M}$ and a maximal velocity of 705 μmoles of Pi released per mg of protein per 10 min at pH 7.7 and 30°C. As indicated above, the optimal Mg²⁺:PPi ratio was about 1 when PPi was used at 2 mM concentration. However, this ratio was found to increase progressively as the concentration of PPi was diminished (see “Discussion”). The optimal Mg²⁺:PPi ratio was determined for each of the seven concentrations reported in Fig. 3.

Specificity of Enzyme—Beside the natural substrate, several pyrophosphate compounds were tested for hydrolytic activity with Mg²⁺ as well as Zn²⁺ as activating cations. Zn²⁺ was employed because Schlesinger and Coon (16) have found that it activates the hydrolysis of ATP by yeast pyrophosphatase. The erythrocyte enzyme showed no hydrolytic activity with the following compounds: tripolyphosphate, ADP, ATP, and ITP, with either Mg²⁺ or Zn²⁺ as activator at pH 7.7. It was also of interest to test the enzyme for possible pyrophosphate-glucose phosphotransferase activity. Such an activity has been shown.
in liver mitochondria (17) and microsomes (18, 19). The purified erythrocyte enzyme, as well as a crystalline yeast inorganic pyrophosphatase preparation (Worthington), failed to show any detectable phosphotransferase activity when Rafter's (17) assay conditions were used at pH 7.7.

**Activation Energy**—The effect of temperature on the enzyme activity is illustrated in Fig. 4. The graph is biphasic with a sharp transition at 29°. The activation energy is 8,560 cal per mole above the transition temperature and 12,900 cal per mole below it.

**Fig. 3.** Lineweaver-Burk plot of the reciprocal of initial pyrophosphatase velocity against the reciprocal of PP$_i$ molar concentration. The optimal Mg$^{2+}$:PP$_i$ ratio was predetermined for each point. The buffer was 33.3 mM Tris-Cl, pH 7.7, and the temperature was 30°.

**Fig. 4.** Arrhenius plot of the inorganic pyrophosphatase activity. The reaction mixture contained 2.0 mM PP$_i$, 2.3 mM MgCl$_2$, and 33.3 mM Tris-Cl, pH 7.7.

### Table III

**Sensitivity of erythrocyte pyrophosphatase to sulfhydryl inhibitors**

The reaction mixture contained 10 mM Tris-Cl, pH 7.0, 2.0 mM MgCl$_2$, 0.2 mM EDTA, 56.8 µg of protein, and the inhibitor at the specified concentration in a total volume of 1 ml. After preparing the mixture at 0°, it was incubated at 30° for 5 min, then rechilled. Aliquots (0.1 ml each) were then assayed for pyrophosphatase activity at pH 7.7 as described under "Experimental Procedure."

<table>
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<th>Inhibitor</th>
<th>Concentration (mM)</th>
<th>Specific Activity (µmol P/mg protein/10 min)</th>
<th>Inhibition (%)</th>
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<td></td>
</tr>
<tr>
<td>p-Hydroxymercuribenzoate</td>
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<tr>
<td>N-Ethylmaleimide</td>
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**Inhibition by Sulfhydryl-binding Agents**—The sensitivity of erythrocyte pyrophosphatase activity to four sulfhydryl inhibitors is summarized in Table III. p-Hydroxymercuribenzoate was the most effective inhibitor. The enzyme was much less sensitive to alloxan, iodoacetamide, and N-ethylmaleimide under the conditions described in Table III.

### DISCUSSION

The most notable characteristic of human erythrocyte pyrophosphatase is its strict specificity for inorganic pyrophosphate. Thus, whereas the yeast enzyme hydrolyzes also certain nucleoside di- and triphosphates in presence of Zn$^{2+}$ (16), and the *Escherichia coli* pyrophosphatase cleaves tri- and tetrapolyphosphates (4), the erythrocyte enzyme does not act on any of these substrates. Moreover, we have not been able to detect any PP$_i$-glucose phosphotransferase activity with the erythrocyte or the yeast pyrophosphatases. This is in contrast to the mitochondrial (17) and the microsomal (18, 19) enzymes.

The optimal pH and Mg$^{2+}$:PP$_i$ ratio provide useful information for identifying the natural substrate of the erythrocyte pyrophosphatase. As indicated above, the enzyme requires 0.2 to 0.3 mM excess Mg$^{2+}$ for concentrations of pyrophosphate ranging from 0.08 mM to 2.0 mM. The favorable effect of a slight excess of Mg$^{2+}$ is probably caused by minimizing the dissociation of the pyrophosphate-magnesium complex to the free pyrophosphate anion which is inhibitory (20). Taking into consideration the requirement for stoichiometric amounts of Mg$^{2+}$ and the optimal pH of 7.7, it would appear that the natural substrate of human erythrocyte pyrophosphatase is MgPP$_i$$^+$, the predominant species under optimal conditions of enzyme action. Based upon the stability constants reported by Lambert and Watters (21), the free pyrophosphate and the dimag-
nesium pyrophosphate would constitute less than 5% of the total pyrophosphate under these conditions. However, since about 70% of the maximal activity persisted over a wide range of Mg²⁺:PPᵢ ratios above 1, it seems that the compound Mg₃PP₆ can also be acted upon by the enzyme, although at a lower rate.

The biphasic nature of the Arrhenius plot with a transition at 29° is of special interest. The yeast enzyme shows a similar plot with an activation energy of 15,300 cal per mole below 20° and 9,500 calories per mole above it (3). A sharp conformational change of the enzyme at the transition temperature could explain the observed discontinuity. Dixon and Webb (22) have reviewed the few cases which exhibit this phenomenon.

Although alloxan, N-ethylmaleimide, and iodoacetamide were not efficient inhibitors of the pyrophosphatase activity, the enzyme is probably sulfhydryl-dependent, as indicated by its stabilization by 2-mercaptoethanol and its inhibition by p-dimethylaminobenzaldehyde. The noticeable difference in efficacy of the four sulfhydryl inhibitors tested might be due to the nature of the environment around the essential thiol group of this enzyme.

Finally, one might inquire about the physiological role of inorganic pyrophosphatase in the red blood cell. As the erythrocyte matures, it loses many of its synthetic activities and the enzymes involved therein. However, it retains the power to synthesize NAD⁺, an essential nucleotide for the glycolytic pathway. Preiss and Handler (23) presented evidence for a three-step mechanism of NAD⁺ synthesis in the erythrocyte. Inorganic pyrophosphate was a by-product of each of these steps. Thus, the pyrophosphatase can play a role in NAD⁺ production by shifting the equilibrium of these reactions in favor of nucleotide synthesis.

REFERENCES
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