Effect of Heat and Nucleotides on Human Erythrocyte Inorganic Pyrophosphatase.* (32015)

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We have previously shown that the activity of erythrocyte inorganic pyrophosphatase is inversely proportional to cell age(1). This cytoplasmic enzyme affords the means of regulating the intracellular concentration of pyrophosphate which is formed as a by-product in many biosynthetic reactions. In contrast to other enzymes, little is known about the properties of pyrophosphatase from human erythrocytes. This report is concerned with studies on the thermal inactivation of the enzyme and on the effects of nucleotides on the enzyme reaction.

Materials and methods. Adult blood was collected in sequestrene and the erythrocytes washed 3 times with 20 volumes of ice-cold 0.15 M NaCl solution by centrifugation at 1500 rpm for 5 minutes. The supernatant and buffy coat were removed by suction. Lysates were prepared by diluting a 50% cell suspension in ice-cold distilled water. Enzyme activity was measured in reaction mixtures containing in 3.5 ml: 1.6×10^{-2} M neutralized $Na_4P_2O_7$; 1.6×10^{-2} M $MgCl_2$; 5×10^{-2} M tris-HCl buffer, pH 7.4 and 0.5 ml of hemolysate. After 30 minutes incubation at 37°C the reaction was stopped with 1.5 ml of 10% trichloroacetic acid. The amount of orthophosphate liberated was estimated by the method of Fiske and SubbaRow(2). Enzyme and substrate controls were run with each assay. Specific activity was expressed as micromoles of orthophosphate formed at 37°C in 30 minutes per mg hemoglobin(1).

Thermal inactivation of pyrophosphatase was investigated under several conditions using a thermostatically controlled water-bath constant to ±0.5°C. The remaining activity was subsequently measured by the standard assay procedure at 37°C.

The effects of nucleotides on the enzyme reaction and their specificity as substrates for pyrophosphatase were studied on stroma-free hemolysates. These were obtained by centrifugation at 105,000 × *g* for 60 minutes with Model L Spinco ultracentrifuge. In these experiments equivalent concentrations of the compound under investigation were added to the controls. Further details are indicated on Table II.

Results. Thermal inactivation. Incubation of a 5% aqueous lysate with or without 5×10^{-2} M tris-HCl (pH 7.4) at 37°C over a 45-minute period revealed an exponential loss of activity with first-order kinetics. Under these conditions 50% of the pyrophosphatase activity was destroyed during the first 21 minutes of incubation (Fig. 1). Addition of neutralized cysteine at a final concentration of 2×10^{-3} M protected the enzyme against heat inactivation. The enzyme was also protected when incubation was carried out in the presence of 1.2×10^{-2} M $MgCl_2$ or 1.2×10^{-2} M neutralized pyrophosphate; in both cases 90% of the initial activity remained after 45 minutes at 37°C. Ca^{2+}, Mn^{2+} and Zn^{2+} did not protect pyrophosphatase against heat inactivation.

Incubation at 50°C resulted in a more rapid loss of activity, and cysteine, $MgCl_2$ or pyrophosphate protected less effectively than at 37°C (Fig. 1). Addition of cysteine or $MgCl_2$ to samples preincubated at 37°C or 50°C did not restore activity. The inactivation in all cases was irreversible; no recovery of activity was noted after incubation at 4°C or 25°C for up to 10 days. Removal of erythrocyte stroma from the enzyme preparations did not alter the inactivation patterns noted.

Since proteins are usually more stable in concentrated solution, the effects of enzyme concentration on thermostability were investigated. For this purpose serial aqueous dilutions were made from a 50% erythrocyte suspension in 0.15 M NaCl, and aliquots pre-

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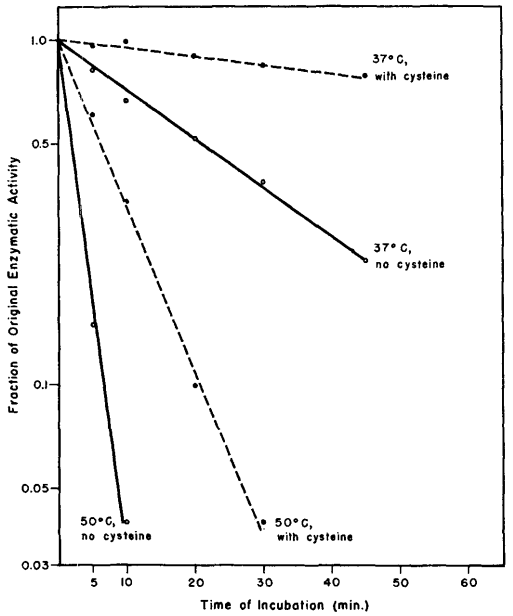

FIG. 1. Effect of heat on erythrocyte pyrophosphatase in presence of tris and cysteine. Five percent lysates were prepared in water, in 50 mM tris-HCl (pH 7.4) and in 2 mM neutralized cysteine. Replicate 0.5 ml aliquots of each preparation were incubated at 37° and 50° for times indicated and then stored at 4°. One set of controls for each preparation was placed at 50° for times specified and another set was kept at 4°. Activity was measured as indicated in text and results related to controls. Specific activity of original specimen was 0.80.

incubated at 37°C and 48°C for different lengths of time. The results indicate that thermostability of erythrocyte pyrophosphatase was directly related to its concentration (Table I). A possible protective effect of NaCl was excluded since the same results were obtained when NaCl was added to the various enzyme dilutions to a final concentration of 0.15 M. The addition of hemoglobin to dilute enzyme preparations had no stabilizing effect. When concentrated enzyme preparations were heat-inactivated and then mixed with very dilute lysates, no protection against heat inactivation was noted. These observations rule out the presence of protecting substances which dissociate by dilution of the enzyme preparations.

Effects of nucleotides. When the possible protective effect of adenosine triphosphate against heat inactivation was tested, marked inhibition of pyrophosphatase activity was noted. This observation led to the investigation of the effects of this and other nucleotides on the enzyme reaction and to the simultaneous study of their specificity as substrates. Stroma-free enzyme preparations were used in these experiments to assure the absence of any membrane-associated nucleotidase activity. Of the nucleotides tested, only the triphosphates inhibited the pyrophosphatase reaction and with the exception of inosine triphosphate, none served as substrate; while adenosine mono-and diphosphate were without effect (Table II).

Discussion. The results of these studies indicates that pyrophosphatase from human erythrocytes is a relatively heat-labile enzyme and that it is stabilized by its substrate and its metallic activator. These observations resemble some of those described for the enzyme from rabbbits with induced reticulocytosis(3). A protective effect by Mg^{2+} against thermal inactivation has been reported for several inorganic pyrophosphatases of microbial origin(4-6). Bloch-Frankenthal(7) has suggested that the actual substrate for the enzyme is the complex anion $(MgP_2O_7)^{2-}$. The protection noted with Mg^{2+} and pyrophosphate indicates binding to the enzyme of one component of this complex, even in the absence of the other. This interpretation is in accord with the recent reports on metal binding to the enzyme(8) and on the binding of pyrophosphate(9). The protection ob-

TABLE I. Effect of Enzyme Concentration on Thermostability of Pyrophosphatase Activity.

	Percent activity remaining			
	Incubation at 37°	Incubation at 48°		
Dilution	30 min	10 min	20 min	30 min
Undiluted	92	71	46	36
1:5	82	38	33	8
1:10	62	31	25	5
1:20	56			
1:25	40			
1:50	30			
1:100	29			

A 50% suspension in 0.15 M NaCl was prepared with thrice washed erythrocytes. The indicated dilutions were made in distilled water; replicate 0.5 ml aliquots were incubated at 37° and 48° for the times specified and then stored at 4°. Controls were kept at 4°. Activity was measured as indicated in text and related to controls. "Undiluted" sample was assayed as 10% aq. lysate. Specific activity of original specimen was 0.73.

TABLE II. Specificity of Nucleotides as Inhibitors and as Substrates of Erythrocyte Inorganic Pyrophosphatase.

Compound	% Inhibition	Relative rates as substrate
$Na_4P_2O_7$	0	1.00
Adenosine	—10	—
AMP	—13	0
ADP	—11	0
ATP	43	.02
CTP	60	.05
GTP	48	.03
ITP	22	.26
UTP	48	.05

For inhibition studies, replicate 0.5 ml of stroma-free lysate were diluted with 1 ml of 0.05 M tris-HCl (pH 7.4) containing 0.016 M neutralized nucleotide, followed by addition of 1 ml of 0.016 M neutralized $Na_4P_2O_7$ in tris buffer and 1 ml of 0.032 M $MgCl_2$. After incubation at 37° for 30 min, Pi formed was determined as indicated in text and the results related to those obtained without inhibitor. For substrate specificity studies, procedure was the same as indicated above, except that $Na_4P_2O_7$ was replaced by 1 ml of 0.05 M tris-HCl (pH 7.4) and that 1 ml of 0.016 M $MgCl_2$ was used. Rates were related to that obtained with $Na_4P_2O_7$. Specific activity with $Na_4P_2O_7$ was 0.75.

served with cysteine is compatible with the suggestions that pyrophosphatase is a sulfhydryl enzyme(10) and that heat inactivation causes intramolecular disulfide formation of essential SH-groups(3).

The increased thermolability noted with dilute enzyme preparations differs from the results obtained with an alkaline phosphatase from human cell cultures(11), where the fraction of residual activity was independent of enzyme concentration. Since no ionic effects could be demonstrated, the mixing experiments described rule out the presence of protecting substances in the concentrated enzyme preparations. However, these mixing experiments could not exclude the possibility of a stabilizer that is tightly bound to its enzyme, but such a relation of a protecting factor to an enzyme would be difficult to distinguish operationally from a change in the enzyme molecule proper.

Our results on the effects of nucleotides on the reaction of erythrocyte pyrophosphatase differ from those reported for the enzyme from rat-liver mitochondria(12), which was inhibited by all 3 adenine nucleotides but not by guanosine triphosphate. Our observations on the specificity of nucleotide triphosphates as substrates for stroma-free enzyme preparations resemble only qualitatively those reported previously(13,14). Although Liakopoulou and Alivisatos(14) demonstrated that inosine triphosphate was the best substrate for their stroma-free hemolysates, the authors did not test their preparations for inorganic pyrophosphatase activity. Preliminary heat inactivation studies have shown that the loss of pyrophosphatase activity parallels the loss of activity against inosine triphosphate, suggesting that both activities may reside in the same protein.

Summary. Human erythrocyte inorganic pyrophosphatase rapidly lost activity by incubation above 37°C. The enzyme was protected against heat inactivation by pyrophosphate, magnesium and cysteine. Dilute enzyme preparations were more labile than concentrated ones. The reaction catalyzed by pyrophosphatase was inhibited by nucleotide triphosphates. Inosine triphosphate served as substrate for stroma-free hemolysates.

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