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HYDROLYSIS OF NUCLEOSIDE DI- AND TRIPHOSPHATES BY CRYSTALLINE PREPARATIONS OF YEAST INORGANIC PYROPHOSPHATASE

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SUMMARY

1. Crystalline preparations of yeast inorganic pyrophosphatase catalyze phosphate liberation from ATP and ADP, as well as from pyrophosphate, in the presence of zinc ions.

2. A variety of other nucleoside di- and triphosphates are hydrolyzed in the presence of zinc ions and pyrophosphatase, and manganous and cobaltous ions can partially substitute for zinc in effecting ATP hydrolysis.

3. Although several lines of evidence suggest that a single protein possesses Mg^{++}-pyrophosphatase and Zn^{++}-ATPase activities, the possibility cannot be eliminated that a different protein, present in small amounts, accounts for the ATPase activity.

4. Evidence has been obtained that the hydrolysis of ATP occurs primarily between the β- and γ-phosphate groups to yield ADP, which is then converted to AMP.

Abbreviations used: ATP, adenosine triphosphate; ADP, adenosine diphosphate; AMP, adenosine 5′-phosphate; Tris, tris(hydroxymethyl)aminomethane; DPN, diphosphopyridine nucleotide.

* The experimental data in this paper are taken from a thesis submitted by M. J. SCHLESINGER in partial fulfillment of the requirements for the degree of Doctor of Philosophy in Biological Chemistry in the Rackham School of Graduate Studies of The University of Michigan.

INTRODUCTION

Yeast inorganic pyrophosphatase is generally believed to act on no other substrate than inorganic pyrophosphate. BAILEY AND WEBB[1] reached this conclusion in working with an extensively purified preparation of the enzyme and reported that the enzyme specifically required magnesium ions. In 1952 KUNITZ[2] described the isolation of crystalline pyrophosphatase from bakers' yeast and found that in the presence of Mg^{++} ions the enzyme did not catalyze the hydrolysis of such compounds as ADP, ATP, or thiamine pyrophosphate. He also observed that Co^{++} or Mn^{++} ions could substitute for Mg^{++} in pyrophosphate hydrolysis but that they were less effective in promoting catalytic activity.

In the course of studies in this laboratory on the ATP-dependent carboxylation of β-methylcrotonyl CoA[3] and on ATP degradation by pyruvic kinase in the presence of carbon dioxide, hydroxylamine, and zinc ions[4], preliminary evidence was obtained[5] that yeast inorganic pyrophosphatase may have wider specificity than generally recognized. The experiments outlined below indicate that crystalline preparations of inorganic pyrophosphatase attack certain organic pyrophosphate esters under conditions not previously tested, *i.e.*, with certain metals other than magnesium. Our findings suggest that pyrophosphatase may have a broader metabolic role than previously thought, and that considerable caution must be used in interpreting the effect of pyrophosphatase when added to crude enzyme systems containing divalent metal ions other than magnesium.

METHODS AND MATERIALS

Lyophilized, crystalline preparations of yeast inorganic pyrophosphatase, kindly provided by Dr. M. KUNITZ, were dissolved in deionized water and stored at 4°. A partially purified preparation of the enzyme, free of ATPase, was made from yeast according to the directions of HEPPEL AND HILMOE[6]. Protein concentrations were determined spectrophotometrically by the method of WARBURG AND CHRISTIAN[7]. The nucleoside di- and triphosphates, AMP, and DPN were commercial preparations.

In a typical assay for ATPase activity, 2 μmoles ATP were incubated with 4 μmoles zinc acetate, 100 μmoles Tris–maleate buffer, pH 6.0, and 10 μg crystalline pyrophosphatase in a final volume of 3.0 ml. The enzyme was omitted from a control tube, and the amount of phosphate found was subtracted from that in the complete tube. After incubation for 30 min at 38° (or 15 min when pyrophosphate served as substrate) the mixtures were deproteinized with trichloroacetic acid (10% final concentration), and aliquots were taken for phosphate determination[8]. Cysteine was occasionally added to the usual reagents[9] but with no apparent effect on color development. AMP was estimated according to the method of KALCKAR[10].

RESULTS

The liberation of phosphate from ATP in the assay system described above is dependent upon the presence of pyrophosphatase as well as zinc ions (Table I). Other experiments have shown that zinc chloride can replace zinc acetate and that the boiled enzyme is inactive. A lower, but significant, activity is obtained with cobaltous

and manganous ions, whereas magnesium and other metals tested are apparently without effect (Table II). The metal concentrations recorded are those at which the highest activity was found in the range $1.6 \cdot 10^{-3}$ to $1.0 \cdot 10^{-2}$ *M*. Various nucleoside

TABLE I

DEPENDENCY OF ATP HYDROLYSIS ON COMPONENTS OF ASSAY SYSTEM

Reaction mixture	*Phosphate formed (μmoles)*
Complete	1.80
No Zn^{++}	0.12
No enzyme	0.12
No ATP	0.00

TABLE II

METAL REQUIREMENT FOR ATP HYDROLYSIS

The reaction mixtures contained 5 μmoles ATP, 50 μg crystalline pyrophosphatase, and the following salts at the concentrations indicated: zinc acetate, cobalt chloride, manganese chloride, magnesium chloride, ferrous sulfate, ferric chloride, potassium aluminum sulfate, cupric sulfate, calcium chloride, and nickel acetate.

Metal added	*Molarity × 10^3*	*Phosphate formed (μmoles)*
Zn^{++}	3.3	4.20
Co^{++}	6.6	0.86
Mn^{++}	6.6	0.94
Ni^{++}	6.6	0.10
Mg^{++}	1.6	0.04
Fe^{++}	6.6	0.10
Fe^{+++}	3.3	0.01
Cu^{++}	3.3	0.06
Al^{+++}	6.6	0.01
Ca^{++}	3.3	0.00

TABLE III

HYDROLYSIS OF VARIOUS NUCLEOSIDE PHOSPHATES

50 μg of pyrophosphatase were employed with 4 μmoles of the triphosphates and 1 μmole of the diphosphates.

Substrate tested	*Phosphate formed (μmoles)*
Adenosine triphosphate	5.40
Guanosine triphosphate	3.62
Uridine triphosphate	3.58
Cytidine triphosphate	4.75
Inosine triphosphate	3.75
Adenosine diphosphate	0.72
Guanosine diphosphate	0.60
Uridine diphosphate	0.62
Cytidine diphosphate	0.46
Inosine diphosphate	0.38
Adenosine 5′-phosphate	0.00

di- and triphosphates were examined as possible substrates in the presence of zinc ions, and the crystalline pyrophosphatase preparation was found to have broad specificity, as shown in Table III. These compounds were not attacked, however, when Mg^{++} was substituted for Zn^{++} or enzyme was omitted. Additional experiments have shown that Co^{++} and Mn^{++} can substitute for Zn^{++} in ADP hydrolysis. DPN is apparently not attacked by pyrophosphatase and Zn^{++}, as judged by the finding that AMP is not liberated.

The pH optimum of the ATPase activity is about 6.0, as shown in Fig. 1, with appreciable activity in the broad range 5.2 to 8.0. KUNITZ[2] obtained pH optima of about 6.3 and 7.2 for Mg^{++}-activated pyrophosphatase, the different values being obtained at different metal–substrate ratios. Experiments at pH 6 with varying concentrations of substrate and metal ions have shown that the optimal Zn^{++}: ATP

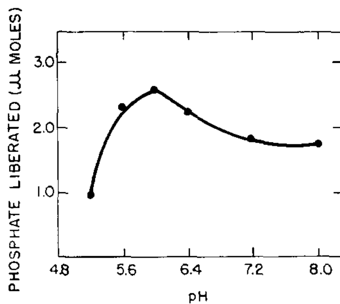

Fig. 1. Liberation of phosphate from ATP as a function of pH. The standard assay was employed with 2 μmoles ATP, 4 μmoles Zn^{++}, 50 μg crystalline enzyme, and 100 μmoles Tris–maleate buffer at the pH values indicated.

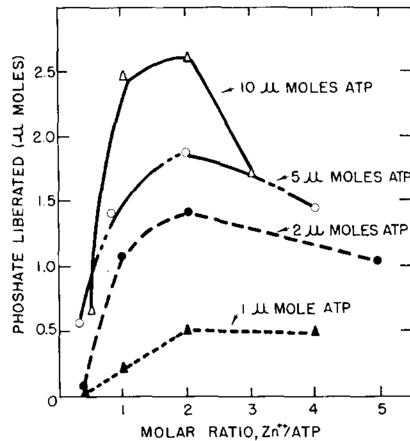

Fig. 2. Phosphate liberation from ATP as a function of the molar ratio of Zn^{++} to ATP. 10 μg pyrophosphatase were employed, with the ATP concentration at the four levels indicated.

ratio is 2.0 (Fig. 2). The corresponding values for Zn^{++}: ADP and Zn^{++}: pyrophosphate appear to be about 2.0 and 0.5, respectively. The relation of enzyme activity to substrate concentration is shown in Fig. 3; these and other data on the zinc-dependent reactions are complex and do not justify the calculation of precise K_m values. On the other hand, it is apparent that relatively low ATP and ADP concentrations are adequate to give significant reaction rates.

As expected from the general action of the enzyme under study, ADP furnishes equimolar amounts of AMP and phosphate (Expt. 1, Table IV). In Expt. 2 evidence is given that ATP undergoes cleavage primarily between the β- and γ-phosphate groups to yield ADP, which is then slowly converted to AMP. Paper chromatography of the reaction mixtures in isobutyric acid–water–concentrated ammonium hydroxide (66:33:1) also indicated that ADP is the initial product of ATP degradation.

Several attempts have been made to determine whether a single enzyme possesses Mg^{++}-pyrophosphatase activity and the Zn^{++}-dependent activities reported here. The data in Table V indicate that one relatively crude and four crystalline pyro-

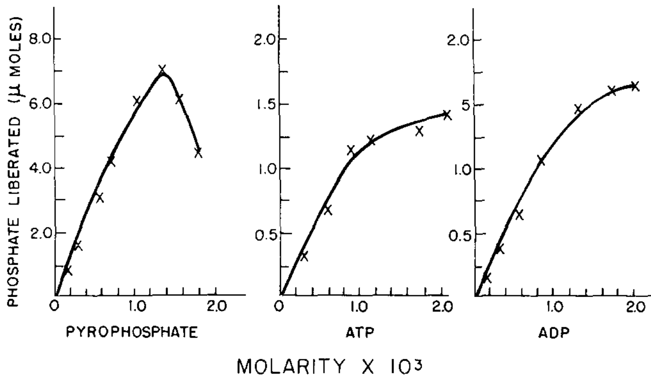

Fig. 3. Phosphate liberation from various substrates as a function of substrate concentration. The usual assay was modified in that 1 μg enzyme was employed with the zinc: pyrophosphate ratio 0.5; 10 μg enzyme with the zinc: ATP ratio 2.0; and 50 μg enzyme with the zinc: ADP ratio 2.0. Incubation, 20 min at 38° with ATP and ADP; 15 min with pyrophosphate.

TABLE IV

LIBERATION OF AMP AND PHOSPHATE BY HYDROLYSIS OF ATP OR ADP

In Expt. 1 the reaction mixture contained 4 μmoles ADP, 4 μmoles zinc acetate, 300 μmoles succinate buffer, pH 5.9, and 100 μg pyrophosphatase in a volume of 3.0 ml. The reaction was stopped by the addition of trichloroacetic acid. In Expt. 2 the reaction mixtures contained 4 μmoles ATP, 8 μmoles zinc acetate, 100 μmoles succinate buffer, pH 5.9, and 10 μg pyrophosphatase in a volume of 3.0 ml. At the intervals indicated individual tubes were heated to stop the reaction.

Expt.	*Substrate*	*Time (min)*	*AMP formed (μmoles)*	*Phosphate formed (μmoles)*
1	ADP	0	0	0
		30	1.59	1.64
2	ATP	10	0.08	1.28
		20	0.14	1.98
		30	0.22	3.32

TABLE V

PYROPHOSPHATASE, ATP-ASE, AND ADP-ASE ACTIVITY OF VARIOUS YEAST INORGANIC PYROPHOSPHATASE PREPARATIONS

The assays were carried out with limiting amounts of enzyme as described in the text, with substrates in these amounts: 50 μmoles Mg^{++} + 2 μmoles pyrophosphate; 4 μmoles Zn^{++} + 2 μmoles pyrophosphate, ATP, or ADP. Incubation, 30 min with ATP or ADP, 15 min with pyrophosphate.

Substrate	*Metal added*	*Specific activity of enzyme (μmoles phosphate liberated/10 μg protein)*				
		Partially purified preparation	*Crystalline preparations*			
			A	*B*	*C*	*D*
Pyrophosphate	Mg^{++}	$3.0 \cdot 10^{-2}$	54	64	85	110
Pyrophosphate	Zn^{++}	$2.3 \cdot 10^{-2}$	43	51	71	98
ATP	Zn^{++}	$1.6 \cdot 10^{-3}$	1.9	2.0	1.7	2.6
ADP	Zn^{++}	$0.2 \cdot 10^{-3}$	0.4	0.4	0.5	0.7

phosphatase preparations possess the same relative activity toward various substrates. In addition, attempts at partial denaturation by exposure to acetone or heating gave no indication for separate ATPase and pyrophosphatase activities (Table VI). One of the crystalline preparations (25 mg/ml in 0.01 *M* veronal buffer, pH 7.0) exhibited only one peak in the ultracentrifuge during 90 min at 59,000 rev./min at 4°*. After brief centrifugation to remove a trace of denatured protein, another crystalline preparation was submitted to zone electrophoresis in starch gel[11] in sodium borate buffer, pH 8.6, and found to give a single protein band about 1.5 cm from the origin**. A portion of the protein eluted from this band with 0.1 *M* Tris–maleate buffer, pH 6.0, had the same relative activity toward ATP (Zn^{++}) and pyrophosphate (Mg^{++}) as before electrophoresis.

TABLE VI

EFFECT OF PARTIAL DENATURATION ON PYROPHOSPHATASE AND ATP-ASE ACTIVITIES

The assays were carried out as indicated in the legend to Table V.

Attempted denaturation procedure	*% activity retained*		
	Mg^{++}-activated pyrophosphatase	*Zn^{++}-activated pyrophosphatase*	*Zn^{++}-activated ATPase*
Incubation with 20% acetone for 4 h at room temperature	100	100	100
Heating at 46° for 5 min	72	76	75
Heating at 46° for 5 min, then at 50° for 5 min	34	35	28
Same as above	18	22	25

DISCUSSION

The results presented indicate that crystalline preparations of yeast inorganic pyrophosphatase attack a number of organic pyrophosphate esters when Mn^{++}, Co^{++}, or Zn^{++} ions are substituted for Mg^{++}. Although the enzymic assays and physical studies described above suggest that a single protein possesses these various activities, it must be emphasized that two or more proteins might not be distinguishable by the methods so far employed.

The following turnover numbers, expressed as moles substrate hydrolyzed per 100,000 g protein per min under the conditions specified, have been calculated for preparation D from the data in Table V: pyrophosphate (Mg^{++}), $3.7 \cdot 10^4$; pyrophosphate (Zn^{++}), $3.3 \cdot 10^4$; ATP (Zn^{++}), $8.7 \cdot 10^2$; and ADP (Zn^{++}), $2.3 \cdot 10^2$. It should be pointed out that the turnover number would have been about 3 times as great if the optimal Zn^{++}: pyrophosphate ratio (0.5) had been used. Determined under slightly different conditions, the turnover number for magnesium-activated pyrophosphatase has been reported[2] as $6 \cdot 10^4$. The rate at which ATP and ADP are attacked and the apparent affinity of the enzyme for these substrates (*cf.* Fig. 3) indicate that the nucleoside pyrophosphatase activity of yeast pyrophosphatase preparations may be physiologically significant.

To our knowledge there have been no previous reports that the substitution of other metal ions for Mg^{++} extends the specificity of a purified inorganic pyrophosphatase. For example, the partially purified inorganic pyrophosphatase from pig

* Kindly carried out by Dr. W. D. BLOCK.
** Kindly carried out by Dr. R. L. HUNTER.

brain[12] has an absolute requirement for magnesium ions. Other investigators[2,6] have noted that the activity of pyrophosphatase is greatly influenced by the metal–substrate ratio, as shown also in the present work. Studies on the role of the metal have led to the proposal that magnesium pyrophosphate may serve as the substrate for erythrocyte[13] and rat brain[14] pyrophosphatases. In examining the exchange of [^{18}O]phosphate with water, catalyzed by yeast inorganic pyrophosphatase, COHN[15] has recently obtained evidence that magnesium phosphate may be a substrate in this exchange reaction.

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