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A CALCIUM ION-DEPENDENT ATP PYROPHOSPHOHYDROLASE IN *PHYSARUM POLYCEPHALUM*

MASARU KAWAMURA and KEI NAGANO

Biological Laboratory, Jichi Medical School, Tochigi Pref. (Japan)

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Summary

An activity of ATP pyrophosphohydrolase (EC 3.6.1.8) was found in the soluble fraction of the plasmodium of *Physarum polycephalum*. The products of the enzyme reaction were inorganic pyrophosphate and 5'-AMP in equimolar quantities. The enzyme had a pronounced requirement for Ca^{2+} with high specificity. Mg^{2+} was not an essential cofactor but stimulated the enzyme activity about 2.5-fold of the control. The enzyme hydrolyzed ITP, GTP and β,γ-methylene ATP at a limited rate. Among inhibitors tested, 3 mM caffeine reduced the activity to about 75% of the control. The enzyme had a broad pH optimum around pH = 7.0 and the K_m for ATP was 2.0 mM. An Arrhenius plot showed a break at about 18°C and the calculated activation energies were 6.7 and 11.4 kcal/mol above and below the transition temperature, respectively. Disc electrophoresis in dodecyl sulfate and gel filtration on Sephadex G-200 gave apparent molecular weights of 56 000 and 240 000, respectively, suggesting that the native enzyme was built up from 4 polypeptide chains. The possible role of the enzyme in vivo was discussed.

Introduction

The plasmodium of the acellular slime mold, *Physarum polycephalum*, is a unique material for the investigation of the ATP-driven contractile system [1,2]. In the course of study on the mode of ATP utilization by the plasmodium, we observed a strong apyrase-type ATPase activity in the supernatant of the homogenate. The activity, however, abruptly disappeared at a certain stage of the enzyme purification. After some examination the activity was found to be due not to a single enzymatic entity but the combined action of two enzymes: a pyrophosphohydrolase and a pyrophosphatase. The former, ATP

pyrophosphohydrolase (EC 3.6.1.8), was partially purified as much as 1700-fold over the original extract in terms of specific activity.

The procedure of purification and several properties of the enzyme are the main subjects of this paper. A possible role of the enzyme as a regulator in the cellular dynamics of the plasmodium is discussed.

Materials and Methods

Organism

Plasmodia of *P. polycephalum* were cultured in a plastic vessel by the method of Hatano and Oosawa [3].

Enzyme preparation

Plasmodia which crept up the inside walls of the vessel were collected and directly dropped into about 10 vol. of cold acetone (−20 °C) and stirred vigorously for 10 min at room temperature. The acetone treatment was repeated at least twice, after which the material was dried and stored in a desiccator at −20 °C.

To the dry plasmodial powder, cold water was added in the ratio of 20 ml to 1 g of powder. The pH was adjusted to 8.0 by a few drops of 1 M Tris. Extraction was carried out under gentle stirring for 30 min at 4 °C and the extract was centrifuged at 5000 × *g* for 15 min. The pellet was discarded and the yellow supernatant was centrifuged at 150 000 × *g* for 2 h. 3 vol. of cold ethanol (−20 °C) was added to the clear supernatant fraction under gentle stirring. The precipitate was gathered by centrifugation at 5000 × *g* for 10 min and suspended in a small volume of 0.05 M Tris/HCl buffer, pH 7.6. The suspension was centrifuged at 20 000 × *g* for 30 min. Then, the supernatant was applied to a Sephadex G-100 column and eluted with the same buffer. A 2.5 × 30 cm column was used. Peaks of pyrophosphohydrolase activity were pooled and stored at 4 °C.

For further purification, DEAE-Sephadex A-25 chromatography was used. The sample was loaded onto a DEAE-Sephadex column 1.5 × 25 cm. Elution was performed by a linear gradient system consisting of 100 ml of 0.05 M Tris/HCl, pH 7.6 and 100 ml of the same buffer containing 1 M NaCl. After dialysis against 0.05 M Tris/HCl buffer (pH 7.6), the pyrophosphohydrolase-containing fractions from the column were concentrated by ultrafiltration and stored at 4 °C.

Enzyme assays

The assay for pyrophosphate (PP_i) liberated from ATP depended on the hydrolysis of PP_i to orthophosphate (P_i) by inorganic pyrophosphatase [4] which was free from activities for ATP, ADP and 5′-AMP. Inorganic pyrophosphatase was partially inhibited by Ca^{2+}, so it was necessary to add an excess of inorganic pyrophosphatase to the assay mixtures (see Results).

The crude extract of plasmodia contained endogenous inorganic pyrophosphatase, but this was separated from ATP pyrophosphohydrolase by Sephadex G-100 chromatography. Unless otherwise stated, the reaction mixtures (1.0 ml) contained 0.05 M Tris/HCl buffer (pH 7.6), 0.005 M $MgCl_2$,

0.001 M $CaCl_2$, 0.003 M (sodium)ATP, 5 μg of inorganic pyrophosphatase and the enzyme fraction from the Sephadex G-100 column (corresponding to 10–20 μg of protein). After 10 min at 25 °C, 1.0 ml of $HClO_4$ (8%) was added, and P_i was estimated using 1.0 ml aliquots by the method of Fiske and SubbaRow [5]. Absorbance was determined at 660 nm after standing for 15 min at room temperature. When inorganic pyrophosphatase was omitted, the absorbance of the sample at 660 nm was the same as that of the sample to which ATP pyrophosphohydrolase was appended after $HClO_4$ addition.

In some experiments, pyrophosphohydrolase activity was measured as the amount of 5′-AMP produced from ATP. After the reaction had been stopped by $HClO_4$, the mixture was neutralized with KOH, and after removal of potassium perchlorate by centrifugation, the supernatant was applied to a column of Dowex 1 (Cl^- form). The elution was performed with a continuous pH gradient system, which separated ATP, ADP, 5′-AMP and cyclic AMP without any overlapping.

Polyacrylamide gel electrophoresis

Disc electrophoresis was carried out at room temperature in glass tubes (7 mm outer diameter) as described by Davis [6] using 1.2 ml of a 7.5% separating gel, pH 9.4, and running at 2.5 mA per tube. Stacking gel was not employed. Bromophenol blue was the tracking dye. Protein in the gel was detected by Coomassie blue staining [7].

Electrophoresis in the presence of sodium dodecyl sulfate was performed according to the method of Weber and Osborn [7]. The gels consisted of 7.5% acrylamide, 0.2% *N,N′*-methylene-bis-acrylamide, 0.1% sodium dodecyl sulfate and 0.1 M phosphate buffer (pH 7.2). The gels were stained by amidoblack 10B and destained electrophoretically after boiling the gels for 1 h.

Molecular weight estimation

The molecular weight was estimated following Weber and Osborn [7] for disc electrophoresis and following Andrews [8] for Sephadex column chromatography.

Protein assay

Protein content was determined after Lowry et al. [9] with bovine serum albumin as the standard.

Reagents

γ-Globulin was kindly supplied by Dr A. Gotoh. Other standard proteins and yeast inorganic pyrophosphatase were products of Boehringer Corp. ATP was from Kyowa Hakko, and other nucleotides were from Sigma. β,γ-Methylene ATP was purchased from Miles Inc. All other chemicals and solvents were of reagent grade or better qualities.

Results

Enzyme purification

Fig. 1 shows the profile of absorbance at 280 nm and the distribution of

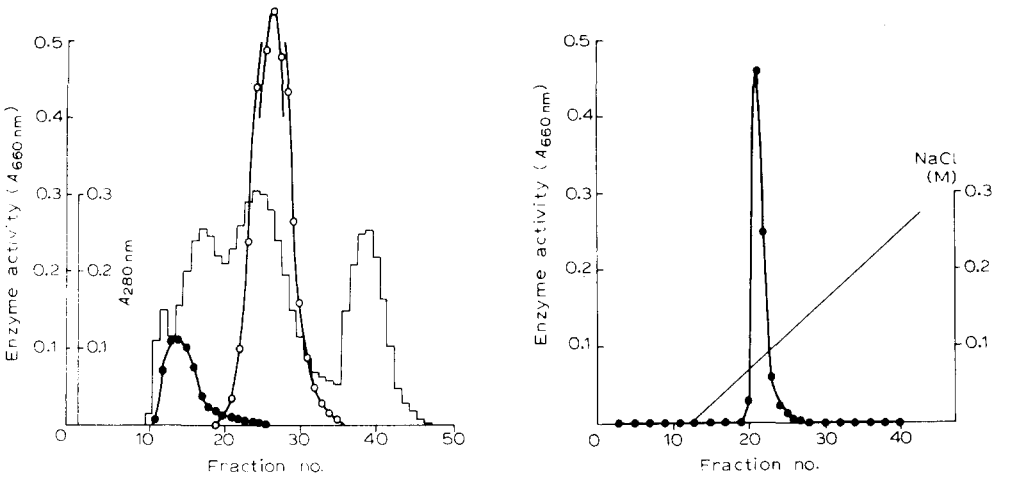


Fig. 1. Separation of ATP pyrophosphohydrolase and inorganic pyrophosphatase by Sephadex G-100 gel filtration. The gel was equilibrated with 0.05 M Tris/HCl, pH 7.6. Each fraction of 2.9 ml was analyzed for enzymic activities. ATP pyrophosphohydrolase activity was measured by addition of excess amount of yeast inorganic pyrophosphatase as described in Materials and Methods. Inorganic pyrophosphatase activity was determined in a medium containing 0.05 M Tris/HCl buffer (pH 7.6), 0.005 M $MgCl_2$, 3 mM sodium pyrophosphate in a final volume of 1 ml. After incubation at 25°C for 10 min, the phosphate released was determined. ●———●, ATP pyrophosphohydrolase activity, A_{660nm} (arbitrary units); ○———○, plasmodial inorganic pyrophosphatase activity, A_{660nm} (arbitrary units). Histogram shows absorbance at 280 nm.

Fig. 2. Column chromatogram on DEAE-Sephadex A-25 of the enzyme fraction obtained by Sephadex G-100 chromatography. 5 ml of the enzyme fraction (1 mg protein) was subjected to linear-gradient elution through a DEAE-Sephadex column. Each fraction of 2.9 ml was analyzed for enzymic activity. The assay conditions were the same as in Fig. 1. ●———●, ATP pyrophosphohydrolase activity, A_{660nm} (arbitrary units).

enzyme activities in fractions from a Sephadex G-100 column. The plasmodial inorganic pyrophosphatase, which was extracted together with ATP pyrophosphohydrolase, was separated from the latter enzyme at this purification step. The fractions containing pyrophosphohydrolase activity were pooled and placed on a DEAE-Sephadex column. The result of DEAE-Sephadex chromatography is shown in Fig. 2. Pyrophosphohydrolase, which eluted from the column at about 0.08 M NaCl, was dialysed against 0.05 M Tris/HCl buffer (pH 7.6) and concentrated by ultrafiltration. The specific activity increased about 80–100-fold over that of the original supernatant obtained by ethanol treatment. Recoveries of protein and ATP pyrophosphohydrolase activity throughout the purification are summarized in Table I. To check the purity of the enzyme, the electrophoresis on polyacrylamide gel was carried out. Fig. 3 shows electrophoretic pattern and its densitometric profile of the peak fraction from DEAE-Sephadex column. The purified enzyme exhibited only one main band and appeared homogeneous on the polyacrylamide gel.

Reaction products and their stoichiometry

With the partially purified enzyme preparation, approx. 2 mol of P_i were formed per mol of ATP only in the presence of added inorganic pyrophosphatase (Fig. 4). When the inorganic pyrophosphatase was omitted, no produc-

TABLE I

PURIFICATION OF PLASMODIAL ATP PYROPHOSPHOHYDROLASE

The starting material was 10 g of acetone-dried, powdered plasmodia. The specific activity of the homogenate of the plasmodium was around 0.02 μmol/mg/min.

Procedure	Total protein (mg)	Total activity (μmol/h)	Spec. act. (μmol/mg/min)	Recovery (%)
Ethanol precipitation	150	4350	0.48	100
Sephadex G-100	6.6	3300	8.33	76
DEAE Sephadex	1.0	2080	34.6	48

TABLE II

SUBSTRATE SPECIFICITY OF PLASMODIAL ATP PYROPHOSPHOHYDROLASE

The concentration of each substrate is indicated in parenthesis. β,γ-Methylene ATPase activity was assayed for 5'-AMP by the use of a Dowex column and the others were assayed for P_i with inorganic pyrophosphatase added as described in Materials and Methods.

Substrate (mM)	Relative activity (%)
ATP (3.0)	(100)
ITP (2.7)	33
GTP (2.8)	21
ADP (2.6)	3
CTP (2.3)	0
UTP (2.2)	0
p-Nitrophenylphosphate (3.0)	0
β,γ-Methylene ATP (3.0)	12

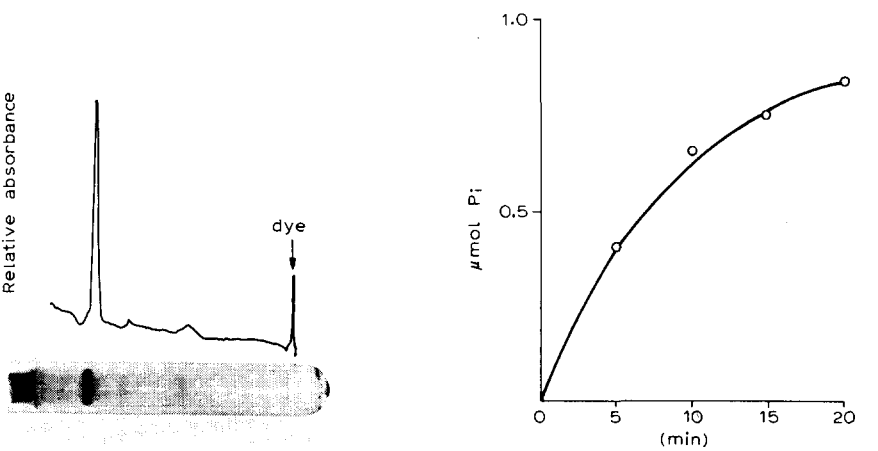


Fig. 3. Polyacrylamide gel electrophoretic pattern and the densitometric profile of the peak fraction of ATP pyrophosphohydrolase from DEAE-Sephadex column. About 5 μg of protein was applied to the gel. Electrophoresis was carried out on 7.5% gel at 2.5 mA/tube. Bromophenol blue was used as a tracking dye. The gel was stained by Coomassie blue in 45% methanol and 10% acetic acid overnight and destained by several changes of 7% acetic acid.

Fig. 4. Time course of the ATP pyrophosphohydrolase reaction. The activity of the enzyme was estimated by conversion of PP_i to P_i by added inorganic pyrophosphatase as described in Materials and Methods. 0.5 μmol of ATP were added to each reaction mixture of 1.0 ml.

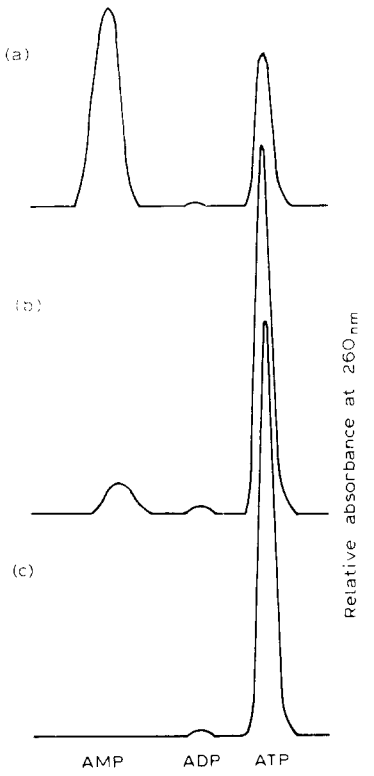


Fig. 5. Column chromatography of the reaction products with Dowex-1 (Cl^- form) resin. After incubation of the reaction mixture without inorganic pyrophosphatase, the products were separated with a Dowex column as described in Materials and Methods. The ADP peak is due to a slight contamination in the ATP used. (a) complete system; (b) Sr^{2+} substituted for Ca^{2+} in equimolar concentration; (c) without Ca^{2+}. Possible contamination of a trace amount of Ca^{2+} was chelated by EGTA (1 mM).

tion of P_i was detected by the method of Fiske and SubbaRow, in spite of an extensive conversion of ATP to 5′-AMP (cf. Fig. 5a). These results made us expect that PP_i was the original form of the phosphate released from ATP.

It was confirmed also by ion-exchange chromatography (data not shown) that the produced nucleotide was 5′-AMP and not cyclic AMP. 5′-AMP and PP_i (calculated as a half amount of P_i) were produced in equimolar quantities. In a typical experiment where 1.74 μmol of P_i was estimated after inorganic pyrophosphatase treatment of the reaction product, chromatography of the same sample yielded 0.89 μmol of 5′-AMP.

No cyclic AMP phosphodiesterase activity was detected in the partially purified enzyme preparation nor any non-specific phosphatase activity (with *p*-nitrophenylphosphate as substrate).

From these observations the enzyme was concluded to be an ATP pyrophosphohydrolase.

Ca^{2+} dependency

That the ATP pyrophosphohydrolase activity was dependent on the concentration of Ca^{2+} was shown in Fig. 6. Up to 1 mM concentration of Ca^{2+}, the

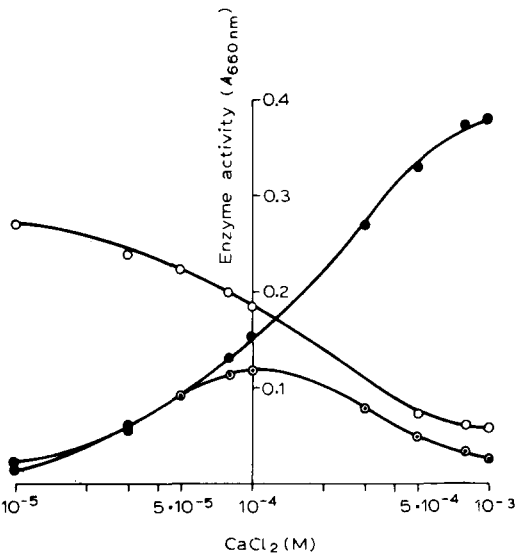


Fig. 6. Influence of Ca^{2+} on ATP pyrophosphohydrolase and on inorganic pyrophosphatase. Inorganic pyrophosphatase activity was measured using 1 μg enzyme in a medium of 1 ml containing 3 mM sodium pyrophosphate, 0.05 M Tris/HCl, pH 7.6, 0.005 M $MgCl_2$, and varied concentration of $CaCl_2$. For the assay of ATP pyrophosphohydrolase activity, 3 mM sodium ATP was used as substrate and inorganic pyrophosphatase (10 μg or 0.5 μg) was added to the reaction medium. Other conditions were the same as that for inorganic pyrophosphatase measurement. ○———○, inorganic pyrophosphatase activity; ●———●, ATP pyrophosphohydrolase activity observed in the presence of 10 μg inorganic pyrophosphatase; ⊙———⊙, ATP pyrophosphohydrolase activity in the presence of 0.5 μg inorganic pyrophosphatase.

ion stimulated the enzyme activity with saturation kinetics, provided that an excess amount of inorganic pyrophosphatase (10 μg) was added. On the other hand, inorganic pyrophosphatase activity was decreased with increasing Ca^{2+} concentration until about 80% inhibition observed at 1 mM Ca^{2+}. Under insufficient amount of inorganic pyrophosphatase in the assay system, therefore, an apparent maximum appeared on the ATP pyrophosphohydrolase/Ca^{2+} concentration plot since, at higher concentrations of Ca^{2+}, inorganic pyrophosphatase was rate limiting (Fig. 6). The apparent optimal concentration varied, as is easily seen from inspection of Fig. 6, with the levels of inorganic pyrophosphatase added.

Therefore, it was necessary to add an excess amount of inorganic pyrophosphatase to the assay system in order to obtain correct ATP pyrophosphohydrolase activity. The level of inorganic pyrophosphatase to be added was checked at 1 mM Ca^{2+}, the highest concentration in our routine assays (Fig. 7). 5 μg of inorganic pyrophosphatase, which was the amount of the enzyme added in our routine assay, was found to be sufficient.

Matumura and Hatano [10] have reported the presence of Ca^{2+}-dependent ATPase activity in *Physarum* which exhibited a sharp peak at about 10^{-5} M Ca^{2+}. Our "ATPase activity", that is, pyrophosphohydrolase plus pyrophosphatase, also shows a Ca^{2+}-dependent peak (Fig. 1). However, the apparent optimal concentration of Ca^{2+} (10^{-4} M) does not coincide with their ATPase activity.

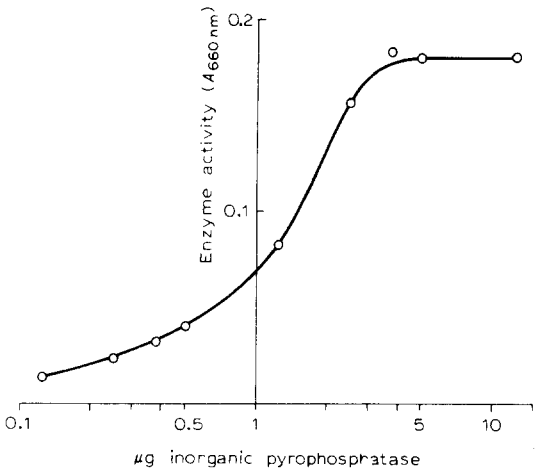


Fig. 7. Correlation of apparent ATP pyrophosphohydrolase activity and amount of inorganic pyrophosphatase added. Incubation medium was the same as described in Materials and Methods, except inorganic pyrophosphatase, which was varied.

Ca^{2+} dependency of the pyrophosphohydrolase was directly shown without using inorganic pyrophosphatase (Fig. 5). In the presence of added Ca^{2+}, chromatography of the reaction products on Dowex-1 column yielded a large amount of 5′-AMP that could be accounted for by the disappearance of ATP. On the other hand, when not Ca^{2+} but ethyleneglycol bis-(β-aminoethyl ether)-*N,N′*-tetraacetate (EGTA), a chelator with high affinity for Ca^{2+}, was added to the reaction mixture, no peak was eluted in the 5′-AMP area, and ATP remained completely unhydrolyzed (Fig. 5c).

Whether any other divalent cations could substitute for Ca^{2+} or not was tested in the same way as above. Among divalent cations tested, only Sr^{2+} could substitute for Ca^{2+} and a low level of pyrophosphohydrolase activity (about one tenth of Ca^{2+}) was observed in the presence of added Sr^{2+} (Fig. 5b). Other cations (Cd^{2+}, Mn^{2+}, Ba^{2+}, Co^{2+} and Cu^{2+}) were completely inactive. That plasmodial pyrophosphohydrolase is highly specific for Ca^{2+} might be important in the physiological significance of the enzyme.

Effect of Mg^{2+}

Inorganic pyrophosphatase has a pronounced requirement for Mg^{2+}, so this cation must be included in our routine assay system. For the ATP pyrophosphohydrolase activity itself, however, it was shown in two ways that Mg^{2+} was not an essential requirement.

In the first procedure, the products of the ATP pyrophosphohydrolase reaction were separated with Dowex-1 chromatography. Even in the absence of added Mg^{2+}, more than 40% of the activity remained (Fig. 8).

The second way involved the addition of inorganic pyrophosphatase to the reaction mixture after the pyrophosphohydrolase reaction had been stopped. For termination of the pyrophosphohydrolase reaction, the incubated reaction mixture was kept in a boiling water bath for 1.5 min. After cooling, the mixture was again incubated with inorganic pyrophosphatase for 12 min at

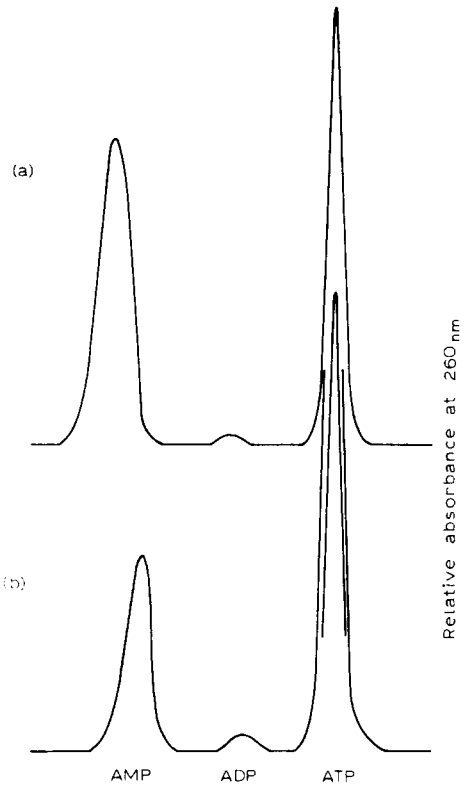


Fig. 8. Effect of Mg^{2+} on ATP pyrophosphohydrolase activity. The assay conditions were the same as described in the legend for Fig. 5. (a) 3 mM Mg^{2+} added; (b) no Mg^{2+}.

25 °C. The pyrophosphohydrolase reaction was performed under varied concentrations of Mg^{2+}, but the pyrophosphatase reaction was performed under constant Mg^{2+} concentration by adding a suitable quantity of Mg^{2+} before the

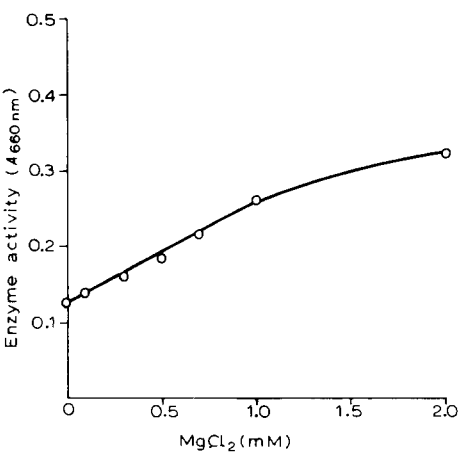


Fig. 9. Effect of Mg^{2+} on ATP pyrophosphohydrolase activity. The enzyme was assayed as described in the text.

second incubation. The results were shown in Fig. 9, and were in good agreement with those obtained by Dowex-1 chromatography. The optimal concentration for Mg^{2+} was about 5 mM.

Specificity of pyrophosphohydrolase

In addition to ATP, plasmodial pyrophosphohydrolase hydrolyzed ITP and GTP to some extent, while CTP and UTP were completely ineffective as substrates (Table II). As the table shows, the enzyme nearly failed to hydrolyze ADP. It was interesting that 5'-AMP was formed by the enzyme from the artificial substrate β,γ-methylene ATP. This result was in accordance with the fact that the enzyme cleaves ATP at the position between α and β phosphate.

Effect of pH and of other compounds

The enzyme had a broad pH optimum around 7.0 (Fig. 10). Buffers used in the pH range examined were: pH 4.0—7.5, 2-(*N*-morpholino) ethane sulfonic acid; and for pH 7.0—9.0, Tris/HCl. The activity was the same at pH 7.0 for the two buffers.

Caffeine, which is an inhibitor of calcium transport in sarcoplasmic reticulum [11] and which causes fragmentation of plasmodium [12], had only a limited effect on pyrophosphohydrolase. 75% of the orginal enzyme activity remained on the addition of 3 mM caffeine. Neither ouabain nor oligomycin were effective as inhibitors of the enzyme. We have been unable to detect any inhibition of mersalyl at 10 μM. Lecithin was neither inhibitory nor stimulatory. Similarly, cyclic AMP, an activator of ATP pyrophosphohydrolase of a cellular slime mold *Dictyosterium discoidium* [13], did not show any effect on our plasmodial enzyme.

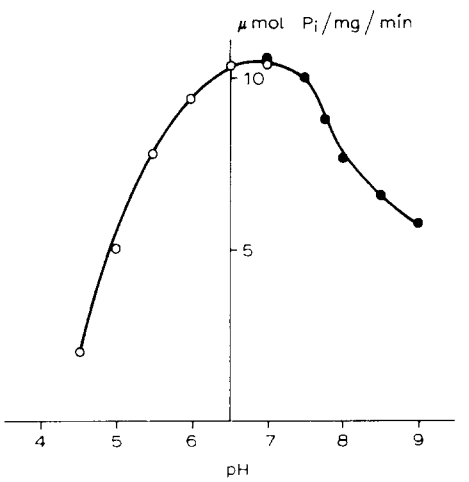


Fig. 10. pH optimum of ATP pyrophosphohydrolase. The incubation mixtures were preapred in duplicates, one with 5 μg and the other with 10 μg inorganic pyrophosphatase. No difference in P_i was observed between these paired incubations at every pH tested, suggesting that inorganic pyrophosphatase was not rate limiting over the whole range of pH values tested. Other conditions were the same as described in Materials and Methods except that the pH was varied. ○———○, 2-(*N*-morpholino)-ethane sulfonic acid; ●———●, Tris/HCl.

K_m for ATP

The initial rate of the enzyme reaction was measured at varied ATP concentrations. Fig. 11 shows a Lineweaver-Burk plot of the initial rate, which gave a straight line over the range of ATP concentration examined. The K_m calculated from the straight line was 2.0 mM, which was much higher than the values reported for several ATP-splitting enzymes ($\sim 10^{-4}$ M).

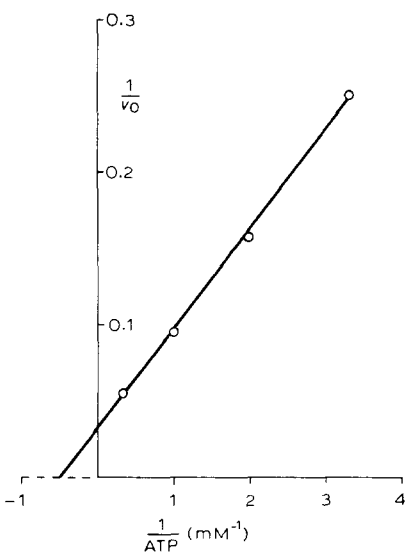
Temperature dependence of ATP pyrophosphohydrolase

Arrhenius plot for ATP pyrophosphohydrolase activity resolved itself into two straight lines with a transition point at about 18°C (Fig. 12). The energies of activation calculated from the slopes were about 6.7 and 11.4 kcal/mol above and below the transition temperature, respectively.

In order to assure that inorganic pyrophosphatase was not rate limiting, an experiment similar to Fig. 12 was performed where the inorganic pyrophosphatase concentration was twice as much as the routine assays. The result was identical to that of Fig. 12 over the whole range of temperatures tested, indicating that the break in the Arrhenius plot was not an apparent one but reflected the ATP pyrophosphohydrolase activity itself.

Molecular weight estimation of ATP pyrophosphohydrolase

The molecular weight of the purified enzyme was estimated by gel filtration on a Sephadex G-200 column and by dodecyl sulfate polyacrylamide gel electrophoresis (Figs 13 and 14). Upon application to Sephadex G-200, the enzyme eluted at a region corresponding to a mol. wt of 240 000, while the



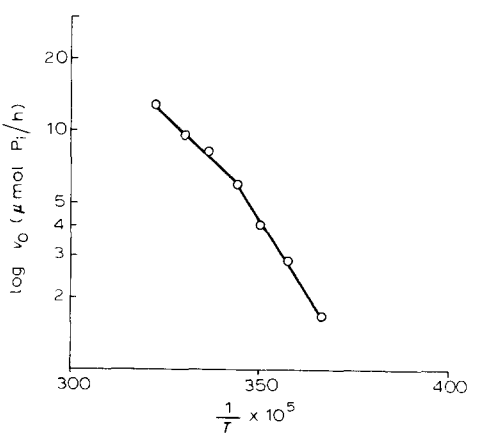
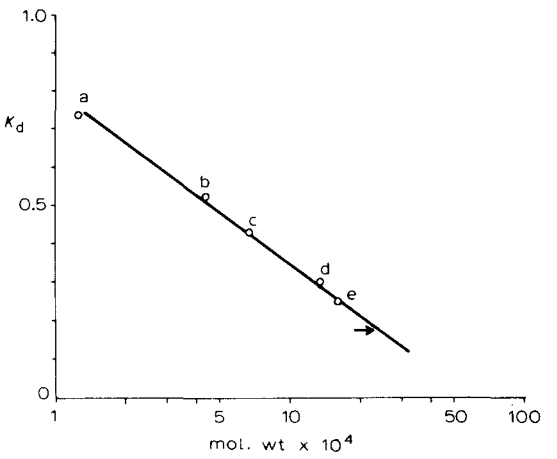


Fig. 11. Lineweaver-Burk plot of ATP pyrophosphohydrolase reaction. Assay conditions were the same as described in Materials and Methods except that the concentration of ATP was varied.

Fig. 12. Arrhenius plot for ATP pyrophosphohydrolase activity. The incubation media were prepared in duplicates, one with 5 μg and the other with 10 μg inorganic pyrophosphatase. No difference in P_i was observed between the paired tubes over the whole range of temperatures tested.



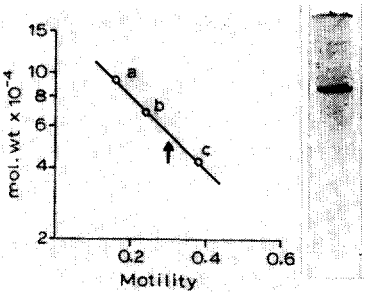


Fig. 13. Estimation of molecular weight of ATP pyrophosphohydrolase by gel filtration of Sephadex G-200. The column size was 1.1 X 80 cm and elution was performed with 0.05 M Tris/HCl buffer, pH 7.6. (a) cytochrome *c* (mol. wt. 13 500); (b) ovalbumin (43 000); (c) serum albumin (67 000); (d) serum albumin dimer (134 000); (e) γ-globulin (160 000). The arrow indicates the K_d value of ATP pyrophosphohydrolase.

Fig. 14. Estimation of molecular weight of ATP pyrophosphohydrolase using 7.5% polyacrylamide gel containing 0.1% sodium dodecyl sulfate. About 10 μg of standard proteins and the purified enzyme treated with 1% sodium dodecyl sulfate and with 5% β-mercaptoethanol were subjected to electrophoresis at pH 7.2. A current of 6 mA/tube was supplied for 6 h at room temperature. Coomassie blue was used as tracking dye. The gel was stained by amido black 10B and destained electrophoretically. The photograph shows the electrophoretic pattern of the purified enzyme sample. (a) phosphorylase a (mol. wt. 94 000); (b) alkaline phosphatase I (69 000); (c) ovalbumin (43 000). The arrow indicates the mobility of the ATP pyrophosphohydrolase.

molecular weight obtained in dodecyl sulfate gels where samples were fully reduced and dissociated was 56 000. These results suggested that the enzyme ATP pyrophosphohydrolase was made up of four similar, if not identical, polypeptide subunits.

Discussion

The mechanisms operating in the streaming protoplasm of the plasmodium has been suggested to be similar to those of muscular contraction [1,2]. Vesicles similar to the endoplasmic reticulum of the striated muscle were found to accumulate Ca^{2+} in the plasmodium [14]. This finding prompted us to study whether plasmodium would contain Ca^{2+}-transport ATPase. In studying plasmodial enzymes along this line of investigation, Ca^{2+}-stimulated ATP hydrolysis by crude extract from the plasmodium was observed by us and at the same time by Matsumura and Hatano [10]. During the course of the purification of the enzyme, however, it became evident that the enzyme was of quite a different character both in localization and in mechanism of ATP hydrolysis from the Ca^{2+}-transport ATPase of the striated muscle. In fact, the hydrolysis of ATP by a crude extract of the plasmodium was found to be catalyzed by two distinct enzymes, one being ATP pyrophosphohydrolase and the other, inorganic pyrophosphatase, as reported in the Results.

The possibility was excluded that ATP was hydrolyzed to 5′-AMP and P_i

by other enzymes such as ATP diphosphohydrolase (apyrase) or the adenylate cyclase/phosphodiesterase/inorganic pyrophosphatase system.

The plasmodial pyrophosphohydrolase was a soluble enzyme rather than a constituent of membranous structures. The possibility that acetone treatment might have solubilized the enzyme which was originally bound to the membrane was also ruled out by the fact that the enzyme was extracted in the soluble fraction even without acetone treatment. The fact that the enzyme activity was not affected by lecithin, along with the stability against acetone treatment, suggested that the enzyme was not a lipoprotein.

The function of the pyrophosphohydrolase in the economy of the plasmodium is not understood yet. It is, however, to be noted that the ATP level in the plasmodium must be extremely low if the enzyme is kept fully active, that is, if the free Ca^{2+} level in the plasmodium is high enough and available to the enzyme all the times. This means, in turn, that Ca^{2+} regulates the ATP level in the plasmodium by regulating the ATP pyrophosphohydrolase activity.

The protoplasmic streaming has been suggested to be regulated by low concentrations of Ca^{2+} [12] and ATP level [15] in the plasmodium. It is tempting to suppose that the pyrophosphohydrolase described here has a role in this regulating mechanism. The K_m for Ca^{2+} of the enzyme may be too high to be directly involved in the regulation (cf. Fig. 1). However, we have still little information about intraplasmodial compartmentation or uneven distribution of Ca^{2+} as well as the enzymes participating in the regulation. On the other hand, other physiological roles are quite possible for the pyrophosphohydrolase. Further study seems to be required for conclusion.

Finally, we should describe an additional result that we could not provide any evidence for the presence of ouabain-sensitive Na^+,K^+-activated ATPase in the plasmodium.

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