Vol. 82, No. 3, 1978 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
June 14, 1978 Pages 913-920

INORGANIC PYROPHOSPHATASE AND NUCLEOSIDE DIPHOSPHATASE IN THE PARASITIC PROTOZOON, *ENTAMOEBA HISTOLYTICA**

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Received April 25,1978

SUMMARY: *Entamoeba histolytica* contains two acid pyrophosphatases. One is an inorganic pyrophosphatase with a relatively high K_m ($\simeq$ 1 mM) and no cation requirement. The other is a nucleoside diphosphatase with a relatively low K_m ($\simeq$ 50 µM) and Ca^{2+} requirement. No Mg^{2+} dependent neutral or alkaline inorganic pyrophosphatase is present. The pyrophosphatases are localized in subcellular particles, display structure-linked latency and are tightly bound to membranes.

In the parasitic protozoon, *Entamoeba histolytica*, in contrast to most eukaryotic organisms, inorganic pyrophosphate (PP_i) plays a significant role in carbohydrate metabolism, replacing ATP in several reactions (1-3). It has been suggested that it is the utilization of PP_i during carbohydrate metabolism that prevents its accumulation (1), a necessity for maintenance of the anabolic prosesses (4). It has also been stated that no inorganic pyrophosphatase is present in this organism (1).

In a study of the subcellular distribution of hydrolases in *E. histolytica* we observed the release of orthophosphate (P_i) from a variety of pyrophosphates. In this paper we present evidence that the organism contains at least two organelle associated acid pyrophosphatases, one hydrolyzing inorganic and the other organic pyrophosphates, and that it has no Mg^{2+} requiring alkaline inorganic pyrophosphatase.

MATERIALS AND METHODS

Entamoeba histolytica (strain NIH:200) was cultured axenically in TPS-1 medium (5; North American Biologicals, Inc., Miami, Florida). After 72-96 hrs growth at 37°C, the cells were harvested and washed twice

*Results of this work were presented in part at the VII Seminario Sobre Amibiasis, Mexico, D.F., Mexico, November 3-5, 1977 [proceedings to be published as a supplement to Arch. Invest. Med. (México)] and at the Annual Meeting of the American Society of Biological Chemists, Atlanta, Ga., June 4-8, 1978.

0006-291X/78/0823-0913$01.00/0

by centrifugation in ice cold buffered sucrose (10 mM Tris.HCl, pH 7.4; 15 mM KCl; 225 mM sucrose). The washed amoebae were suspended in buffered sucrose and homogenized with a Dounce glass homogenizer (5-10 strokes with a B pestle at 0°C). A PNS[a] fraction was obtained by sedimenting the unbroken cells and nuclei in a refrigerated centrifuge (Sorvall Model RC-2B equipped with an SS 34 rotor; 10 ml tubes, 2500 rpm for 4 min).

The assay system for inorganic pyrophosphatase contained 5 mM Na pyrophosphate or 5 mM linear Na tripolyphosphate, 75 mM acetate buffer, pH 5.0, 0.2% (w/v) Triton X-100 and enzyme sample to a final volume of 250 µl. After 15 min at 30°C, 50 µl of 8% (w/v) sulfosalicylic acid containing 5 mM $CuSO_4$ (6) was added. The assay system for nucleoside diphosphatase contained 1 mM TPP[b] or 1 mM nucleoside diphosphate (ADP, GDP, UDP), 4 mM Ca acetate, 75 mM acetate buffer, pH 5.5, 0.2% (w/v) Triton X-100 and enzyme in a final volume of 1.0 ml. After 15 min at 30°C, the reaction was stopped by the addition of 200 µl of 8% (w/v) sulfosalicylic acid. In both assays, P_i was determined in the supernatant solution (7) after the precipitate was removed by centrifugation at 4°C. Acid phosphatase was assayed at 30°C with p-nitrophenol phosphate as substrate (8). Alcohol dehydrogenase was assayed with isopropanol as a substrate (9). Protein was determined by an automated Lowry technique (10). Units of enzyme activity are defined as the amount of enzyme necessary to form one µmol of product in 1 min under the assay conditions.

RESULTS

Properties of the pyrophosphatases. Whole homogenates of *E. histolytica* exhibit pyrophosphatase activities against PP_i and linear tripolyphosphate as well as organic pyrophosphates (TPP, ADP, UDP, GDP) (Table 1). Hydrolysis of PP_i and tripolyphosphate follows typical Michaelis-Menten kinetics up to at least 8 mM substrate with relatively high K_m values (Figure 1). In contrast, hydrolysis of TPP and nucleoside diphosphates exhibits a much lower K_m value with pronounced substrate inhibition at concentrations above 750 µM (Table 1 and Figure 1).

The optimal pH values for hydrolysis of PP_i and TPP are in the acid range, pH 5.0 for PP_i and 5.5 for TPP. The pH-activity curves suggest that the negligible activities observed at and above pH 7 are not due to separate enzymes but represent the activities of the acid enzymes. The hydrolysis of PP_i is unaffected by Mg^{2+} (at pH 5.0 and 8.0) and by other divalent cations (Ca, Co, Fe, Cu, Cd) whereas hydrolysis of TPP and the nucleoside diphosphates is markedly stimulated by Ca^{2+} but not

[a] PNS: postnuclear supernatant

[b] TPP: thiamine pyrophosphate

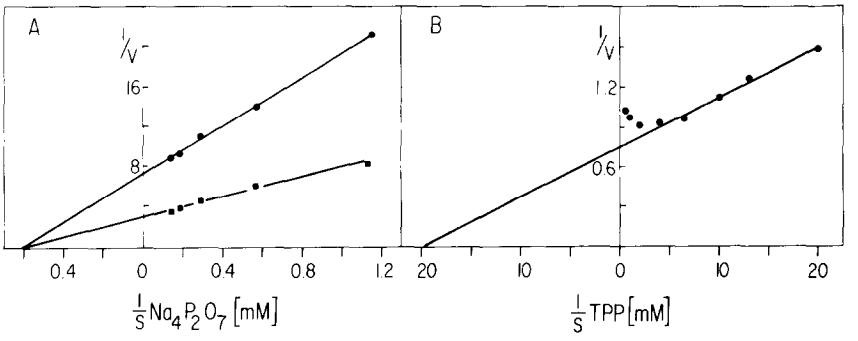

Figure 1. Lineweaver-Burk plots of *E. histolytica* pyrophosphatases. A: Inorganic pyrophosphatase activity of a PNS fraction with Na pyrophosphate as substrate. Assay in the presence of 250 mM sucrose without (●—●) and with (■—■) 0.01 % Triton X-100. An enzyme preparation as used in "B" gave similar results. B: Nucleoside diphosphatase activity with TPP of a preparation as described in footnote *b* to Table 1.

TABLE 1. PHOSPHATASE ACTIVITIES IN *E. HISTOLYTICA*

Substrate	Specific activity mU/mg protein[a]	K_m (mM)[b]
Na pyrophosphate	189 ± 14 (6)	1.25
Na tripolyphosphate	93	2.85
TPP	203 ± 9 (5)	0.05
ADP	140	0.068
UDP	144	--
GDP	145	0.064
p-Nitrophenol phosphate	200 ± 20 (10)	2.20[c]

[a] Assays performed on cell homogenates. Mean values ± S.D. (number of experiments).

[b] Experiments performed on material sedimented from a PNS fraction treated with 0.2 % (w/v) Triton X-100 by centrifugation at 19,000 rpm for 60 min in the Sorvall RC-2B centrifuge. The sediments contained about 90 % of the pyrophosphatase activities and 20-30 % of the protein of the PNS fractions.

[c] Experiment performed on PNS fractions.

by other divalent cations (Mg, Co, Fe, Cu, Cd). Thus we included 4 mM Ca^{2+} in the standard assay system. If this is omitted, hydrolysis of TPP, ADP and GDP is 4 to 7 times slower and that of UDP is not detectable. The hydrolysis of PP_i and of TPP in the absence of Ca^{2+} are unaffected by EDTA (5 mM), cysteine (5 mM), cyanide (5 mM) or tartrate

(10 mM), but are markedly inhibited by F^- (500 μM). Hydrolysis of p-nitrophenol phosphate is also inhibited by F^-.

The activity against PP_i and linear tripolyphosphate is heat stable, the activity against TPP and ADP is markedly heat sensitive and that against p-nitrophenol phosphate has an intermediate sensitivity (Figure 2).

Our findings strongly suggest that the observed hydrolysis of pyrophosphates in *E. histolytica* is due to two distinct enzymes. These are an inorganic pyrophosphatase acting on PP_i and tripolyphosphate and a nucleoside diphosphatase acting on the organic pyrophosphates. No Mg^{2+} dependent alkaline inorganic pyrophosphatase was found, but the presence of an acid phosphatase is confirmed (11).

Subcellular localization of the pyrophosphatases. The structure-bound latency of all phosphatases in osmotically protected PNS fractions (Table 2) suggests that they are localized in membrane bounded subcellular organelles. The latency of inorganic pyrophosphatase does not change if the PP_i concentration in the medium is varied from 1 to 8 mM showing that substrate does not penetrate into the organelles (12). Treatment with Triton X-100 and sonication, but not freezing and thawing, abolish the latency of the phosphatases. The low non-latent inorganic pyrophosphatase activity observed in osmotically protected PNS fractions has the same K_m value as the total activity observed after detergent treatment (Figure 1), thus the non-latent activity is likely to represent the enzyme present in damaged particles and not another enzyme species.

Large percentages of the phosphatase activities in PNS fractions are sedimentable, further suggesting their connection with organelles (Table 2). If PNS fractions are centrifuged in discontinuous sucrose gradients alcohol dehydrogenase, the marker enzyme for the nonsedimentable portion of the cytoplasm (9,13), remains in the sample zone (Figure 3). Of the phosphatases only small amounts remain in this zone. Most of the activities sediment onto the cushion and a small fraction (3 to 10 per-

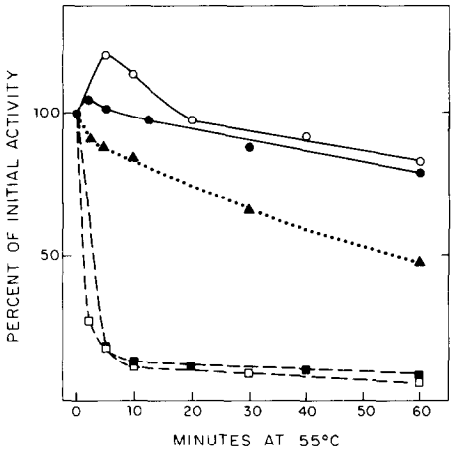

Figure 2. Influence of heat treatment on phosphatases of *E. histolytica*. A PNS fraction was incubated for varying times at 55°C, then assayed under optimal conditions. Substrates: PP_i (O—O), tripolyphosphate (●—●), ADP (■--■), TPP (□--□), and p-nitrophenol phosphate (▲•••▲).

TABLE 2. SEDIMENTABILITY AND LATENCY OF ENZYME ACTIVITIES IN *E. HISTOLYTICA*

Enzyme	Sedimentability[a]		Latency[b]		
	control	frozen and thawed (10X)	control	frozen and thawed (10X)	sonicated[c]
Inorganic pyrophosphatase[d]	92	91	73 ± 8 (6)	60	0
Nucleoside diphosphatase[e]	90	91	77 ± 6 (5)	73	3
Acid phosphatase	85	83	82 ± 8 (9)	75	-
Alcohol dehydrogenase	5	4	0	0	-
Protein	27	36	-	-	-

[a] Activity sedimented by centrifugation from a PNS fraction at 39,000 rpm for 60 min (Beckman model L-2 ultracentrifuge) expressed as percent of the sum of activities recovered in the sediment and supernatant fraction. Recoveries were between 87 and 108 percent.

[b] Latency is defined as the difference between activities measured in osmotically protected PNS fractions in the absence (free activity) and in the presence of Triton X-100 (total activity) expressed as percentage of total activity. For controls mean values ± S.D. (number of experiments) are given.

[c] A PNS fraction was sonicated with a Branson Sonifier cell disruptor (model W-350) for 6 min (pulsed treatment at 50% duty cycle with output set at 3).

[d] Assayed with a Na pyrophosphate as substrate.

[e] Assayed with TPP as substrate.

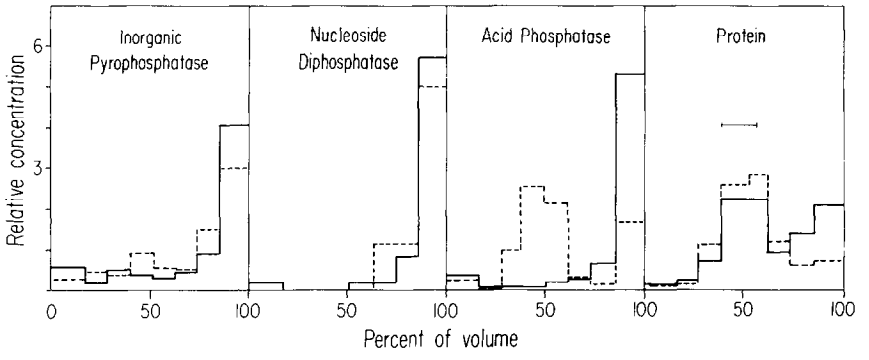

Figure 3. Discontinuous sucrose gradient centrifugation of PNS fractions of *E. histolytica*: untreated (——), Triton X-100 (0.6 mg/mg protein) treated (– – –). Bar inserted in the histogram for protein designates the position of the sample layer. Each 5 ml cellulose nitrate centrifuge tube contained (from the bottom) 500 µl 2 M sucrose, 900 µl 750 mM sucrose, 900 µl 625 mM sucrose, 1000 µl sample in 500 mM sucrose, 900 µl 325 mM sucrose, 900 µl 250 mM sucrose. The tubes were centrifuged in an ultracentrifuge (Beckman model L-2 equipped with a SW 39 swinging bucket rotor; 39,000 rpm for 60 min). Fractions were collected by tube slicing (14). Graphs give normalized distribution histograms of enzyme activities as a function of the collected volume (radial distance increases from left to right). Ordinate is concentration in fraction relative to concentration corresponding to uniform distribution throughout gradient. Percentage recoveries (control and Triton-treated) were for inorganic pyrophosphatase: 95 and 107; nucleoside diphosphatase: 88 and 76; acid phosphatase: 76 and 83; protein: 119 and 99, respectively.

cent) rises to the top of the gradient. The hydrolases in this zone are also latent suggesting the presence of organelles of low density. Freezing and thawing do not affect the sedimentability of the phosphatases as expected from the lack of changes in their latency. Treatment with Triton X-100, which damages the organelles as shown by the loss of enzyme latency, releases only the acid phosphatase from the particles and decreases only slightly the sedimentability of the pyrophosphatases (Table 2 and Figure 3). Thus these enzymes remain associated with certain larger structures, possibly with the membranes of the particles.

The results are thus consistent with the assumption that both pyrophosphatases of *E. histolytica* are localized in membrane bounded organelles, most likely tightly bound to the inner aspect of the limiting membrane.

DISCUSSION

Our findings show that in *E. histolytica* there are at least two hydrolases that are able to split pyrophosphate bonds and that these are localized in membrane-limited organelles. One is an acid inorganic pyrophosphatase with no cation requirement and the other a Ca^{2+}-stimulated acid nucleoside diphosphatase. A TPP-hydrolyzing enzyme was demonstrated by histochemical techniques in membrane bounded organelles of *E. histolytica* (15). No evidence was found for the existence of a cytoplasmic Mg^{2+} dependent alkaline inorganic pyrophosphatase that is regarded to be the main enzyme responsible for keeping PP_i levels low in most prokaryotic and eukaryotic cell types.

The inorganic pyrophosphatase of *E. histolytica* resembles the lysosomal pyrophosphatase of mammalian cells (16). The nucleoside diphosphatase is closer to similar enzymes found in mammalian microsomes (17) but differs from them in its acid pH optimum and strict Ca^{2+} requirement. A significant characteristic of the *E. histolytica* pyrophosphatases is their intimate binding to membranes, in contrast to the mammalian enzymes which are easily solubilized (16,18). Further work is needed to clarify the nature of the organelle that contains these enzymes.

The kinetic properties and subcellular localization of the inorganic pyrophosphatase suggest that this enzyme may not play a major role in the regulation of PP_i levels in *E. histolytica*, in agreement with the peculiar carbohydrate metabolism of this organism. Nevertheless the activity present is likely to contribute to this regulation, albeit to an extent not yet known. The functional role of the nucleoside diphosphatase is not yet clear. In other cells this enzyme is thought to be involved in the removal of nucleoside diphosphates formed during synthesis of polysaccharides and glycoproteins (19).

ACKNOWLEDGEMENTS

We thank Dr. Louis S. Diamond (Bethesda, Md.) for the *E. histolytica* strain and Mr. Donald Fishel and Miss Nancy Dick for technical assistance. Figure 2 is reproduced by permission from Arch. Invest. Med. (México) (in press). This research was supported by grant PCM 76-16657 from the National Science Foundation.

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