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Participation of a proton-translocating plasma membrane ATPase, acid phosphatase and alkaline phosphatase in ATP degradation by *Aspergillus niger* extracts

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Abstract

Extracts of *A. niger* could catalyze sequential hydrolysis of the three phosphate moieties of the ATP molecule optimally at pH 2 and at pH 8. At pH 2 the hydrolysis was effected by an ATPase followed by acid phosphatase while at pH 8 alkaline phosphatase was the only involved enzyme. Separation of these three phosphate-hydrolyzing enzymes was achieved by Sephadex G-100 column chromatography. The *A. niger* ATPase seems to have two unique features. First, it was easily solubilized in distilled water and second it had optimum activity at pH 2. The activity of this enzyme was not affected on addition of Na^+, K^+ or Ca^{2+} to the assay reaction mixture. It was neither inhibited by sodium azide nor by potassium nitrate but inhibited by orthovanadate, DES, DCCD, Mg^{2+} and P_i. The substrate concentration–activity relationship was of the hyperbolic type. The enzyme had high specificity for ATP, was inert with ADP and its activity with GTP represented about 6% only of that obtained with equimolar amount of ATP.

Keywords: ATPase; Acid phosphatase; Alkaline phosphatase; Phosphatase; ATP degradation; Ribonucleotide; (*A. niger*)

1. Introduction

To our knowledge, the pathways for ATP degradation have been demonstrated in few biological systems non of which belongs to a mold. These systems are human intestine [1], extracts of tobacco leaves [2] and of sweet potato [3] as well as extracts of *Azotobacter vinelandii* [4]. The enzymes involved in phosphate hydrolysis from ATP were characterized as alkaline phosphatase in human intestine, acid phosphatase in tobacco leaves and sweet potato while nothing has been mentioned about the nature of the *Azotobacter enzyme*(s).

The aim of the present investigation was to demonstrate the pathway(s) of ATP degradation by extracts of an *A. niger* strain and to determine the nature of the participating phosphate-hydrolyzing enzymes. Extracts of this strain had been already proved to contain a purine nucleoside hydrolase which was active on adenosine [5,6] as well as acid and alkaline phosphatases that could dephosphorylate AMP [7,8].

2. Materials and methods

2.1. Materials

ATP, ADP, AMP, adenosine and adenine were products of Sigma. Phenyl disodium orthophosphate (ph.ph) was a product of BDH. GTP and Sephadex G-100 were from Aldrich. Ammonium vanadate and sodium azide were products of Fluka while DCCD and diethylstilbestrol were from Merck. All the ribonucleotides were disodium salts.

2.2. Methods

Preparation of A. niger extracts

The 3–4-day-old mats grown (at 28°C) on liquid Czapek-Dox's medium were harvested by filtration, washed

Abbreviations: AMP, adenosine 5′-monophosphate; ADP, adenosine 5′-diphosphate; ATP, adenosine 5′-triphosphate; GTP, guanosine 5′-triphosphate; ph.ph, phenyl disodium orthophosphate; P_i, inorganic phosphate; DCCD, *N,N′*-dicyclohexylcarbiimide; DES, diethylstilbestrol.

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thoroughly with distilled water, blotted dry with absorbent paper then ground with cold sand and extracted with cold distilled water. The slurry so obtained was centrifuged at 5500 rpm for 4 min. and the supernatant was used as the crude enzyme preparation. The pH of the extracts was 4.0 to 4.2. Dialysis of the extracts was made against 200-fold its volume of distilled water for 3 h at about 7°C.

2.3. Colorimetric determinations

Inorganic phosphate was measured as described by Ames [9]. Reducing compounds were determined (as ribose) by the method described by Ashwell [10]. Protein was estimated by the method of Sutherland et al. [11] and NH_3 by Nessler's reagent.

2.4. Chromatographic separation and identification of ATP, ADP, AMP, adenosine and adenine

Ascending paper chromatography technique, using 3MM (46 × 57 cm) filter papers and two solvent systems, was used. The solvent systems were: solvent I consisted of n-butanol/acetone/acetic acid (glacial)/ammonia (5%)/water; 45:15:10:10:20 [12] and solvent II consisted of isobutyrate/conc. NH_4OH/water; 66:1:33 [4]. The R_f values of the ultraviolet located spots were determined and compared with those of authentic samples.

2.5. Chromatographic identification of ribose

Ribose was chromatographically identified using ascending technique, Whatman No. 1 filter paper and two solvent systems: n-propanol/ethyl acetate/water, 70:10:20 and isopropanol/water, 160:40 [12]. The spots were located on the dried chromatograms after spraying with aniline oxalate.

2.6. Separation of the ATPase, the acid phosphatase and the alkaline phosphatase using Sephadex G-100 column chromatography

Steps of separation

Acetone fractionation. To the crude extracts half volume of cold acetone (−5°C) was added and the precipitate formed was separated by centrifugation at 12000 rpm for 10 min at −20°C and discarded. To the supernatant twice its volume of cold acetone was added and the precipitated protein was separated by centrifugation under the same conditions. The precipitate was then dissolved in 15 ml of cold 0.02 M sodium citrate buffer at pH 6.

Sephadex G-100 column chromatography. A Sephadex G-100 column (2 cm × 46 cm) was washed with 0.02 M citrate buffer at pH 6 then loaded with 10 ml of the acetone fraction. Protein was eluted by addition of the same buffer. Fractions of 5 ml each were collected at room temperature (22°C) at flow rates of about 23 ml/h. At the end of the fractionation, the activity of each fraction was tested with ATP, ADP, AMP, and ph.ph at pH 2, pH 4 and pH 8. The buffers used were KCl-HCl, citric acid-sodium citrate and Tris-HCl, respectively. The concentration of the buffer was 80 mM.

Specific activity was expressed as μmol P_i liberated from 5 μmol substrate/mg protein/20 min at 40°C.

The assay reaction mixture, if not mentioned otherwise, contained (in 1 ml) 5 μmol substrate, 80 μmol buffer, 2.2–2.5 mg extract protein or 30–65 μg protein of the separated enzymes.

3. Results

3.1. Extents of ATP degradation by A. niger extracts

Fig. 1 shows that extracts of *A. niger* could catalyze phosphate hydrolysis from ATP, ADP, AMP and ph.ph at different pH values. From the pH activity profiles it could be suggested that the extracts contain three phosphate hydrolyzing enzymes. The first which was optimally active on ATP at pH 2 can be considered as an ATPase and the other two are the acid and the alkaline phosphatases. The acid phosphatase which had an optimum at pH 4 seems to be active on ADP and AMP while the alkaline phosphatase, the optimum of which was at pH 8, was active on ATP, ADP and AMP.

Fig. 2 demonstrates the extents of ATP degradation at the three pH optima. These results show that almost all the phosphate content of the ATP molecule had been released by the end of the incubation period when the pH of the

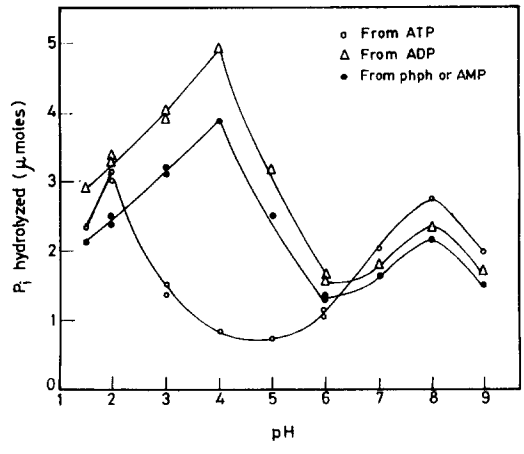

Fig. 1. Pattern of P_i hydrolysis from ATP, ADP, AMP and ph.ph by *A. niger* extracts at different pH values. Reaction mixture contained: substrate, 5 mM; extract protein, 2.52 mg; buffer, 80 mM (KCl-HCl pH 2; phthalic acid-potassium phthalate pH 2–3, citric acid-potassium citrate pH 3–6 and Tris-HCl pH 6–9); total vol., 1 ml; temp., 40°C and incubation period 20 min. * pH 1.5 was adjusted by addition of HCl.

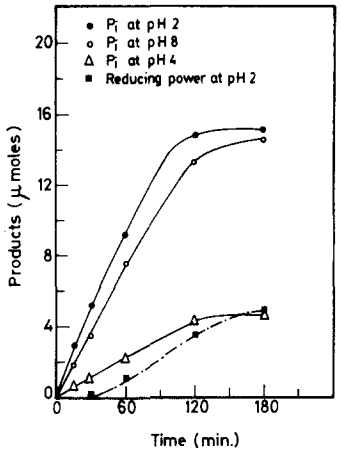

Fig. 2. Extents of ATP degradation at pH 2, pH 4 and pH 8. Reaction mixture contained: substrate, 5 mM; extract protein, 2.23 mg; buffer, 80 mM (KCl-HCl pH 2, citrate pH 4 and Tris-HCl pH 8) vol., 1 ml; temp., 40°C.

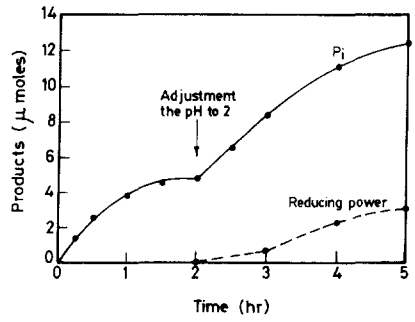

Fig. 3. Effect of increasing the hydrogen ion concentration on ATP hydrolysis at pH 4. Reaction mixture contained: ATP, 5 mM; extract protein, 10.2 mg; citrate buffer at pH 4, 80 mM and 2 drops of 0.1 M HCl to adjust the pH to 2; vol., 1 ml; temp., 40°C.

reaction mixture was either 2 or 8. In addition, at pH 2 a reducing power appeared in the reaction mixture. The amount of this reducing power reached by the end of the incubation period to almost the total amount that resides in the ATP molecule. On the other hand, at pH 4 about less than one third of the three phosphate moieties of ATP was hydrolyzed.

The extents of ADP degradation were also investigated at the three pH optima. The data obtained showed that when 5 μmol ADP were incubated with 2.20 mg extract protein and 80 mM buffer (in 1 ml vol.) for 3 h at 40°C; 9.35, 9.62 and 9.17 μmol P_i and 4.48, 4.73 and 0.0 μmol ribose were liberated when the pH was 2, 4 or 8, respectively. This shows that almost complete hydrolysis of the phosphate content of the ADP molecule occurred at the three pH values including pH 4.

In order to find out an explanation for incomplete hydrolysis of the three phosphate moieties of ATP at pH 4 (Fig. 2) in spite of the finding that this pH represented an optimum for complete phosphate hydrolysis from ADP (preceding results) and AMP [7], the pathways for ATP degradation were investigated.

3.2. Pathways of ATP and ADP degradation by A. niger extracts

Chromatographic analysis of the reaction mixture during the progresses of the reactions of ATP degradation at pH 2 revealed results presented in Table 1. Ribose was also chromatographically detected in this reaction mixture at the end of the incubation period. When similar analysis was carried out on analogous reaction mixtures containing ATP as substrate but adjusted at pH 8 or pH 4, the same sequence of intermediates appeared when the pH was 8, however, the end product in this case was adenosine. On the other hand, when the pH was 4, ADP was the only detected product.

Chromatographic analysis of reaction mixtures that contained ADP as substrate showed that ADP was degraded to adenine plus ribose via the intermediate formation of AMP then adenosine when the pH was either 2 or 4. However, when the pH was 8 ADP was degraded to AMP then adenosine which was the end product under this condition. The *N*-glycosidic bond of adenosine could not be hydrolytically cleaved at pH 8 because the purine nucleoside hydrolase of the extracts was not active above pH 6 [5].

From these data it appears that enzymatic hydrolysis of the total phosphate content of the ATP and ADP molecules took place in a step-wise manner.

Table 1
Chromatographic identification of the intermediates and the products formed during ATP degradation at pH 2

Compounds identified in the reaction mixture	R_f values of compounds identified at							
	zero time		30 min		60 min		180 min	
	S I	S II	S I	S II	S I	S II	S I	S II
ATP	0.10	0.15	0.09	0.14	–	–	–	–
ADP	–	–	0.15	0.20	0.15	0.21	–	–
AMP	–	–	–	–	0.26	0.44	–	–
Adenosine	–	–	–	–	0.47	0.75	–	–
Adenine	–	–	–	–	–	–	0.56	0.88

S = solvent.
Reaction mixture contained: substrate (ATP), 5 μmol; extract protein, 1.7 mg; KCl-HCl buffer pH 2, 80 μmol; total volume, 1 ml; time, as indicated; temp., 40°C.

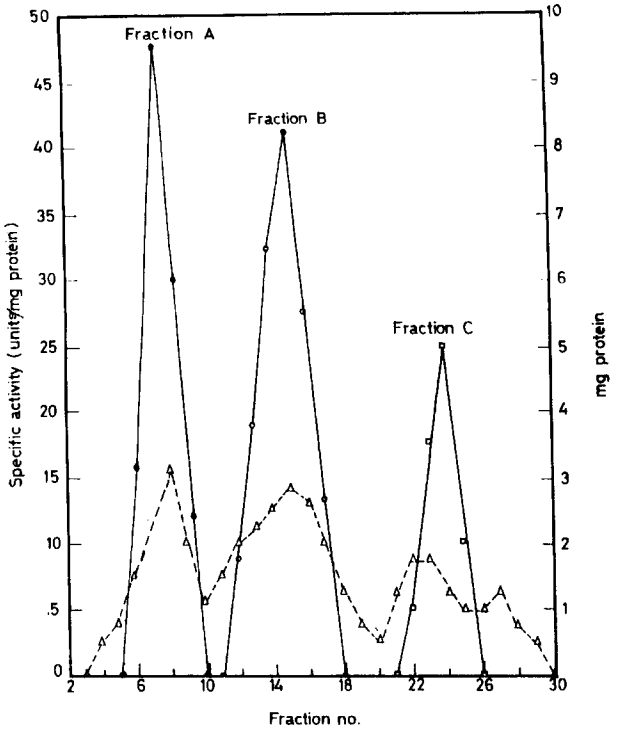

Fig. 4. Elution diagram of the three phosphate hydrolyzing enzymes. (△) protein, (•) specific activity of the ATPase with ATP at pH 2, (○) specific activity of the acid phosphatase with ph.ph at pH 4, (□) specific activity of the alkaline phosphatase with ph.ph at pH 8.

Cessation of ATP degradation at the ADP level when the pH of the reaction mixture was 4 (inspite of the finding that this pH represented an optimum for ADP degradation when it was used as substrate) was suggested to be due to inability of the ADP formed from ATP to be released from the surface of the enzyme that catalyzed its formation (presumably an ATPase) at this hydrogen ion concentration. Hence, it could not be subjected to the action of the acid phosphatase of the extracts. However, at pH 2 such release could occur. Results of Fig. 3 may support this suggestion as lowering the pH of the reaction mixture from 4 to 2, after phosphate hydrolysis from ATP had stopped, resulted in liberation of almost the remaining two phosphate moieties.

Table 3
Substrate specificities of fractions A, B and C

Substrate	Product (μmol P_i) using		
	fraction A	fraction B	fraction C
ATP	4.55	–	14.82
ADP	–	9.43	9.57
AMP	–	5.10	4.85
ph.ph	–	4.97	4.83

Reaction mixture contained: substrate, 5 mM; buffer (at pH 2 with F(A), at pH 4 with F(B) and at pH 8 with F(C)) 80 mM; protein, 60 μg; incubation period, 3 h at 40°C; volume, 1 ml.
F = fraction.

3.3. Nature of the phosphate-hydrolyzing enzymes that participate in ATP degradation

From the preceding data, it was suggested that the extracts contained an ATPase, an acid phosphatase and an alkaline phosphatase and that dephosphorylation of ATP to adenosine could be effected, at pH 2, by the combined actions of the ATPase and the acid phosphatase. However, at pH 8, this dephosphorylation process could be achieved by the action of the alkaline phosphatase alone. Conclusive evidences that substantiate this suggestion are provided in Table 2, Fig. 4 and Table 3. Table 2 shows, qualitatively, separation of the three enzymes, Fig. 4 demonstrates their elution diagrams expressed as specific activities and Table 3 presents substrate specificity of each fraction on using a long incubation period.

3.4. Partial characterization of the A. niger ATPase

Kinetic studies on the separated ATPase (fraction A, Fig. 4) revealed the following:

The activity was optimum at pH 2 (Fig. 5). Neither Na^+ nor K^+ had a stimulatory effect on the enzyme activity as replacing (on molar bases) KCl-HCl buffer by NaCl-HCl buffer, phthalate buffer or even HCl was almost without effect. Addition of the divalent cation Ca^{2+} (as $CaCl_2$) to the reaction mixture at a concentration of 20 mM had no effect. On the other hand Mg^{2+} when added

Table 2
Separation of the three phosphate hydrolyzing activities by Sephadex G-100 column

Substrate	Activity in fraction number								
	6–9 at pH			12–17 at pH			22–25 at pH		
	2	4	8	2	4	8	2	4	8
ATP	+ve	+ve	−ve	−ve	−ve	−ve	−ve	−ve	+ve
ph.ph	−ve	−ve	−ve	+ve	+ve	−ve	−ve	−ve	+ve
ADP or AMP	−ve	−ve	−ve	+ve	+ve	−ve	−ve	−ve	+ve

Reaction mixture contained: substrate, 5 μmol; buffer, 80 μmol; (KCl-HCl pH 2, citrate pH 4 and Tris-HCl pH 8); protein, 0.2 ml; total volume, 1 ml; time, 30 min; temp., 40°C.

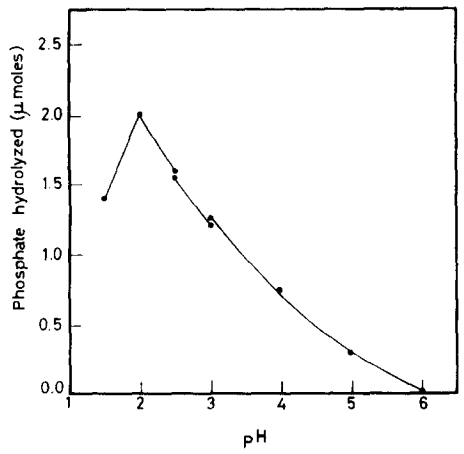

Fig. 5. Determination of the optimum pH for the *A. niger* ATPase Reaction mixture contained: ATP, 5 mM; protein, 62 μg; buffer, 80 mM; vol., 1 ml; time 20 min and temp. 40°C. The buffers used are KCl-HCl (pH 2–2.5), phthalic acid-potassium phthalate (pH 2.5–3) and citric acid-potassium citrate (pH 3–6). HCl was used to adjust the pH to 1.5.

Table 4
Effect of the inhibitors of the ATPases on the *A. niger* ATPase

Inhibitor added	Relative activity % ($\frac{+\text{inhibitor}}{-\text{inhibitor}}$) at inhibitor concentration of		
	1 mM	10 mM	20 mM
Potassium nitrate	100	100	100
Sodium azide	100	100	100
Ammonium vanadate	40	0.0	0.0
DCCD	10	0.0	0.0
Diethylstilbestrol	55	26	0.0
Sodium fluoride	0.0	0.0	0.0

Reaction mixture contained: ATP, 5 μmol; KCl-HCl, buffer pH 2, 80 μmol; protein, 60 μg; ±inhibitor; vol., 1 ml; temp., 40°C; time, 20 min.

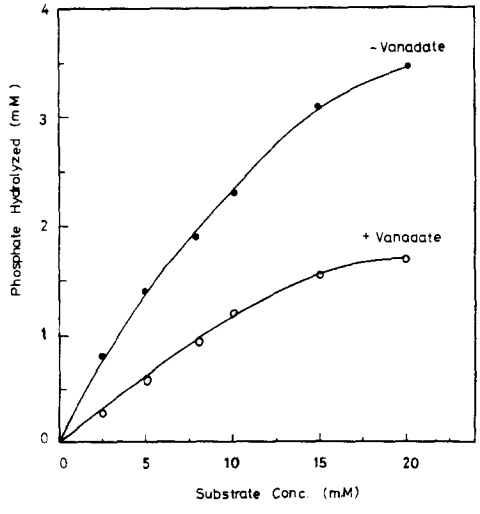

Fig. 6. Substrate concentration–activity relationship of the ATPase in presence and absence of orthovanadate. Reaction mixture contained: protein, 9.0 μg; KCl-HCl buffer at pH 2, 80 mM; substrate, as indicated, ±5 mM ammonium vanadate; vol., 1 ml; time 20 min at 40°C.

(as $MgCl_2$) at a concentration of 5 and 20 mM caused inhibition of activity that reached about 80 and 100%, respectively. Product inhibition was not recorded when ADP was added at a concentration of 20 mM (four times that of the substrate) while P_i when added at the same concentration resulted in about 27% inhibition. The enzyme activity with GTP was found (after 2 h incubation) to represent about 6% only of that obtained with equimolar amount of ATP. Testing the effects of some of the specific inhibitors of the mitochondrial ATPase, the plasma membrane ATPase and the vacuolar ATPase on the *A. niger* ATPase revealed results presented in Table 4. Increasing the amount of KNO_3 to 150 mM did not cause any inhibition of activity. Investigating the effect of substrate concentration on the enzyme activity revealed a hyperbolic rather than a sigmoid relationship (Fig. 6). The same figure also shows that inhibition of the enzyme activity by vanadate was of the non-competitive type.

4. Discussion

The results presented here show that *A. niger* extracts could catalyze hydrolysis of the three phosphate moieties of the ATP molecule in a step-wise manner at either the very acidic or the alkaline pH. This hydrolysis was effected by an ATPase followed by an acid phosphatase when the pH of the reaction mixture was 2. However, when it was 8 one enzyme which is the alkaline phosphatase catalyzed successive hydrolysis of the three phosphate groups. From these results it appears that: (1) the *A. niger* alkaline phosphatase resembles the human intestine alkaline phosphatase [1] in catalyzing the sequential release of the three phosphates from ATP. (2) The *A. niger* acid phosphatase differs from the acid phosphatase of tobacco leaves [2] and of sweet potato [3] for being inert with ATP. (3) The pathways demonstrated in *A. niger* extracts are partially different from the pathway found in extracts of *Azotobacter vinelandii* [4] in which the AMP formed due to sequential release of phosphate from ATP was degraded to adenine + ribose-5-phosphate by AMP nucleosidase. The formed adenine was further deaminated to hypoxanthine. In this connection, *A. niger* extracts were found to contain aminohydrolase activity with only cytidine out of the tested purine and pyrimidine bases, their ribonucleosides and ribonucleosides monophosphate [7].

With regard to the *A. niger* ATPase, it can be more likely classified (according to Goffeau and Slayman [13], Bowman et al. [14] and Dschida and Bowman [15]) as a proton-translocating plasma membrane ATPase. This classification is based on its following properties: it was insensitive to azide which is a specific inhibitor of the mitochondrial ATPase and insensitive to KNO_3 which is an inhibitor of vacuolar ATPase. It did not seem to be composed of more than one subunit (the mitochondrial and the vacuolar ATPases consist of more than one polypep-

tide chain). It was inhibited by orthovanadate and diethylstilbestrol which are specific inhibitors of cation-translocating plasma membrane ATPases. Its inhibition by vanadate was of the non-competitive type. It was not activated by Na^+, K^+ or Ca^{2+} but activated by protons. It had high specificity for ATP and its activity with GTP represented about 6% only of that obtained with ATP. These results also indicate that the *A. niger* ATPase preparation was free from contaminating mitochondrial or vacuolar ATPases as the activity was not affected on addition of the specific potent inhibitors of these enzymes. This could be attributed to the mild method used for extraction and/or to the low pH of the extracts (pH 4) at which, according to Schneider et al. [16], mitochondria aggregate and can be removed by low speed centrifugation.

Concerning the effect of Mg^{2+} on ATPase activity, Goffeau and Slayman [13] reported that there is general agreement that Mg^{2+} is the physiological cofactor of the ATPase and that it seems clear that the real substrate for the plasma membrane ATPase is the Mg^{2+}-ATP complex. On the contrary, inhibition by Mg^{2+} has been recorded for the *A. niger* ATPase (during the present study), for myosin ATPase [17], for the plasma membrane ATPase of *Saccharomyces cereviciae* [18] and of *Neurospora crassa* [19] as well as for the F_1 moiety of *Phycomyces blakesleeanus* ATPase [20].

Of almost the unique features of the *A. niger* ATPase are its low pH optimum (pH 2) and its easy solubilization by just grinding the mats with washed cold sand and extracting with cold distilled water. As for its optimum pH, it seems to be quite logical that a proton-translocating ATPase is activated by protons, as, if H^+ were an obligatory participant in the transport cycle, it would be expected to exert a sizable stimulation on the ATPase activity. Thus, this result in particular points to proton being the primary transported cation. However, this interpretation is not consistent with what have been reported for the mitochondrial and the proton-translocating fungal plasma membrane ATPases, as, the first type has an optimum at pH 8–9 while the optimum of the second type is at pH 5–7. Moreover, these two optima were used to differentiate these two types of ATPases [13]. Recently, Sakler and Pick [21] also reported a sharp optimum at pH 6 for a plasma membrane H^+-ATPase from the extremely acidophilic algae *Dunaliella acidophila*. Furthermore, exposure to acid pH led to partial inactivation of the plasma membrane H^+-ATPase of *Schizosaccharomyces pombe* [22] and of *Saccharomyces cereviciae* [23].

Easy solubilization of the *A. niger* ATPase compared to plasma membrane ATPase of other organisms could be due to different cell wall structure and/or weak bonding of the ATPase with other cell membrane components.

As for the biological functions of the *A. niger* ATPase, it can be suggested that one of these functions might be accelerating H^+-efflux whenever the intracellular pH falls below the normal physiological range. In support of this are data obtained (not cited) which showed that the pH of the extracts was about 4.0–4.2 at the 3rd to 4th day of mycelial growth while the pH of the growth medium at that time reached to 1.9–2.2. The pH of the medium at the zero time of growth was 5–5.5. This H^+ pumping could be accompanied not only by a movement of anions out of the cell but also by an influx of certain cations or sugars. Regulation of the cytoplasmic pH by H^+-ATPase of *Streptococcus faecalis* has been reported by Kobayashi et al. [24].

Based on the properties of the *A. niger* ATPase, it can be stated that in spite of existing similarity in kinetic properties of fungal plasma membrane proton-translocating ATPases of yeast, *N. crassa* and some plants [13] yet the *A. niger* ATPase exhibited few remarkably different properties. In this connection, the membrane-bound ATPase of *Mycoplasma gallisepticum* has been reported to have properties which are not consistent with any known ATPase [25].

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