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Activities of enzymes of carbohydrate and energy metabolism of the intracellular stages of the microsporidian, *Nosema grylli*

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Summary

The activities of nine enzymes were investigated in intracellular stages of the microsporidian *Nosema grylli* from the fat body of the crickets *Gryllus bimaculatus* purified by centrifugation in Percoll density gradient. Phosphoglucomutase (EC 5.4.2.2), hexokinase (EC 2.7.1.1) and fructose 6-phosphate kinase (EC 2.7.1.11) were not detectable in stages. Glucose 6-phosphate dehydrogenase (EC 1.1.1.49), phosphoglucose isomerase (EC 5.3.1.9), 3-phosophoglycerate kinase (EC 2.7.2.3), pyruvate kinase (EC 2.7.1.40), glycerol 3-phosphate dehydrogenase (EC 1.1.1.8) and ATP-ase were detected with activities of 7 ± 1, 23 ± 2, 7 ± 1, 2 ± 0.3, 17 ± 3 and 17 ± 4 nmol/min×mg protein respectively. A comparison of these values with activities of enzymes of spores shows that the rate of carbohydrate catabolism in microsporidian meronts and early sporonts is lower than in spores. The observed results may be explained by the host-derived ATP dependence of microsporidian intracellular stages.

Key words: carbohydrate catabolism, enzymes, intracellular stages, microsporidia

Introduction

The microsporidia are a large group of obligate intracellular parasites. Their hosts are especially common among Arthropods. Recently several genera of microsporidiae have been described from patients with AIDS (Canning, 1994; Weber et al., 1994; Schwartz et al., 1996). Microsporidiae possess a number of features that point to their ancient origin (Vossbrinck et al., 1987; Philippe and Adoutte, 1995; Hashimoto, Hasegawa, 1996). Long evolution of parasites inside of host cell suggests the strong dependence of microsporidia on host metabolism. It was supposed that microsporidiae utilize host-derived ATP for energy supply since addition of ATP to the medium helped to maintain the integrity of *Nosema michaelis* sporoplasms (Weidner and Trager, 1973). In this context it is remarkable, that practically no information exists on metabolism of microsporidian intracellular stages. There have been few reports on the energy metabolism of microsporidian spores.

A high concentration of trehalose and trehalase activity were revealed in spores of *Nosema apis* (Vandermeer and Gochnauer, 1969, 1971) and *N. algerae* (Undeen et al., 1987; Undeen and Vander Meer, 1994). Considering that no nutrients stored in granules have been detected in spores it is likely that trehalose appears to be the main storage source of energy in spores. ATP may be obtained from the glucose catabolism via Embden-Meyerhof pathway. Starch gel-electrophoresis analysis with subsequent histochemical staining revealed the presence of phosphoglucose isomerase, an enzyme of glycolysis, in *Nosema heterosporum* spores (Hazard et al., 1981). Five enzymes of glycolysis: phosphoglucose isomerase, fructose 6-phosphate kinase, aldolase , 3-phosophoglycerate kinase and pyruvate kinase, were detected in spores of *Nosema grylli*. Side by side with extremely high PGI activities, hexokinase was not detected in spores (Dolgikh et al., 1997). It is likely that low activity of HK provides the low rate of energy metabolism for long-term survival of spores in the environment. Glycerol 3-phosphate dehydrogenase that was detected in *N. grylli* spores may reoxidize NADH generated by glyceraldehyde 3-phosphate dehydrogenase during glycolysis. The quantitative analysis of the trehalose-glucose balance in normal and germinated spores of *N. algerae* supports that glucose catabolism is occuring during normal spore germination (Undeen et al., 1987). It is very probable that oxidative metabolism is not involved in the energy supply of spores since

microsporidiae lack mitochondria and germinate in the absence of oxygen and in the presence of inhibitors of oxidative metabolism (Undeen, 1990).

The purpose of this study is to investigate activities of enzymes of carbohydrate and energy metabolism in *N. grylli* intracellular stages. These data will allow to compare rate of carbohydrate metabolism, especially of glycolysis, in spores and intracellular stages.

Material and Methods

Stages and spores

Purification of stages was performed according to Seleznev et al. (Seleznev et al., 1995) with slight modifications. A laboratory culture of crickets *Gryllus bimaculatus* was infected with the microsporidian *N. grylli*. Fat bodies of infected crickets were isolated and washed with 150 mM PBS (138 mM NaCl, 3 mM KCl, 1.5 mM KH_2PO_4, 8 mM Na_2HPO_4, pH 6.8). The organs were kept in PBS and homogenized in a glass homogenizer with a Teflon pestle. The homogenate was filtrated through syringe with cotton wad and through the filter paper for coarse deposits to be fixed in the syringe-mountable filter holder, and centrifuged in Eppendorf tubes at 120 g for 8 min . The upper layer of the pellet, consisting of meronts and early sporonts (Seleznev et al., 1995), was removed by aspiration, resuspended in PBS and centrifuged once again. The upper layer of pellet was layered over a 20% Percoll (v/v) in PBS (1 ml of Percoll was put in Eppendorf tube) and centrifuged at 800 g for 15 min in swing-out rotor. Percoll previously was precentrifugated in angle rotor at 17,000 g for 20 min. The white band containing meronts and early sporonts (Seleznev et al., 1995) was uptaken and washed out three times with PBS by centrifugation at 180 g for 6 min. The purified stages were resuspended in PBS with 0.1% Triton X-100, incubated for 10 min and centrifuged at 17,000 g for 10 min. The resultant supernatant was used for determination of enzyme activities. All operations were carried out at 4°C.

Purification and homogenization of spores was performed as described previously (Dolgikh et al., 1997).

Activities of enzymes

Activities of all enzymes were determined spectrophotometrically with an optical path of 10 mm at a wavelength of 340 nm and temperature 37° C. The compositions of the assay mixtures for glucose 6-phosphate dehydrogenase (G 6-PDH), phosphoglucomutase (PGM), hexokinase (HK), phosphoglucose isomerase (PGI), 3-phosphoglycerate kinase (PGK)(1st method) , pyruvate kinase (PK), glycerol 3-phosphate dehydrogenase (GPDH) were prepared as described previously (Dolgikh et al., 1997).

The reaction mixtures for other enzymes were as follows:

Fructose 6-phosphate kinase (6-phosphofructokinase) (PFK). 100 mM Tris-HCl (pH 7.8), 100 mM KCl, 5mM $MgSO_4$, 1 mM fructose 6-phosphate, 1 mM ATP, 1 mM phospho(enol)pyruvate, 0.2 mM NADH, 2 U/ml pyruvate kinase, 0.5 U/ml lactate dehydrogenase, the sample. Controls were performed without fructose 6-phosphate or the sample. In the case of detection of the spore enzyme, we increased the concentration of fructose 6-phosphate in 5 times, as high active PGI converted fructose 6-phosphate into glucose 6-phosphate in spores.

3-phosphoglycerate kinase (2nd method). 100 mM Tris-HCl (pH 7.8), 100 mM KCl, 5mM $MgSO_4$, 2 mM 3-phosphoglycerate, 1 mM ATP, 1 mM phospho(enol) pyruvate, 0.2 mM NADH, 2 U/ml pyruvate kinase, 0.5 U/ml lactate dehydrogenase, the sample. Controls were performed without 3-phosphoglycerate or the sample.

ATP-ase. 100 mM Tris-HCl (pH 7.8), 100 mM KCl, 5mM $MgSO_4$, 1 mM ATP, 1 mM phospho(enol)pyruvate, 0.2 mM NADH, 2 U/ml pyruvate kinase, 0.5 U/ml lactate dehydrogenase, the sample. Controls were performed without ATP or the sample.

Protein concentrations were determined by Bradford method (Bradford, 1976).

Percoll, substrates and enzymes were supplied by Sigma (St. Louis, MO) (Percoll, glucose 6-phosphate dehydrogenase, glyceraldehyde 3-phosphate dehydrogenase, lactate dehydrogenase, glucose 1-phosphate, fructose 6-phosphate, 3-phosphoglycerate, glycerol 3-phosphate, phospho(enol)pyruvate, Serva (Heidelberg, Germany) (glucose, ATP) and Reanal (Budapest, Hungary) (pyruvate kinase, glucose 6-phosphate, ADP, NADP, NAD, NADH).

Results and Discussion

The method employed for purification of intracellular stages enables to preserve their morphological integrity (Fig. 1). The incubation of purified stages with 0.1% Triton X-100 results in the rupture of microsporidian plasma membrane and the release of cytoplasm (Fig. 2). Small number of host nuclei occurs in the fraction of purified stages. However the extracts of microsporidian stages didn't seem to be contaminated with host enzymes because all tested enzymes (except ATP-ase) were localized in cytoplasm.

We tested activities of nine enzymes of carbohydrate and energy metabolism in purified microsporidian stages (Table 1). HK activity in stages , as well as in spores, was too low to be detected. In addition, PGM and PFK were not detectable in stages, whereas in spores both of them were revealed. PGI, which activity was very high in spores, reduced aproximately in 50 times in intracellular stages. In a similar manner, the activities of G 6-PDH, PGK and PK in stages reduced to half of its value in spores. Activ-

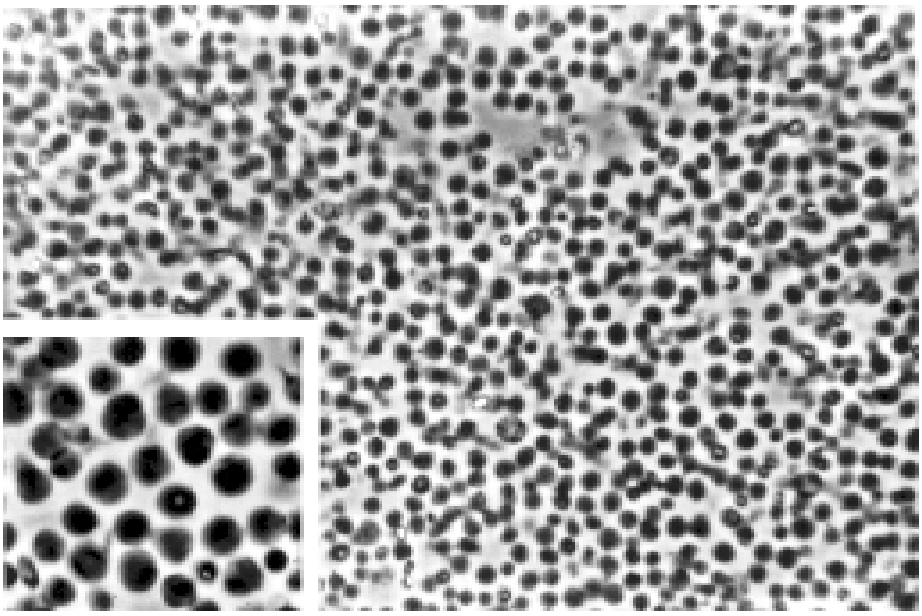

Fig. 1. Purified intracellular stages in PBS (×220 and ×600). Phase contrast.

ity of GPDH did not alter in stages in comparison with spores, whereas ATP-ase activity was higher in stages than in spores.

Loss of soluble enzymes seems not to occur in the course of purification because stages purified by the method used, preserved their morphological integrity (Seleznev et al., 1995). Besides, the activities of the tested enzymes in stages altered in a different way comparetively with spores. We used different solutions for preparation of extracts of spores (0.1 M Tris-HCl (pH 7.8), 0.3 M sucrose, 0.1% Triton X-100) and of stages (PBS, 0.1 % Triton X-100). However utilization of different solutions was not a reason of lower enzymes activities in stages. In some procedures of lysis of purified stages we replaced PBS and 0.1 % Triton X-100 by the solution for the preparation of the spore extracts. This replacement did not influence on activities of next tested enzymes: G 6-PDH, PGM, HK, PFK, PGI, PK. In a similar manner, the use of PBS for spores homogenization did not lead to decrease of G 6-PDH, PGI, PFK and PGK activities.

Lower activities of 6 enzymes in stages were not caused by action of proteases in these probes. SDS-PAGE did not reveal proteolysis of bands in samples from stages as well as from spores. Besides, PAGE with co-polymerized gelatine showed that protease activity was not higher in stages than in spores (unpublished data).

In the previous study we revealed low level of activities of carbohydrate and energy metabolism enzymes in *N. grylli* spores excluding PGI (Dolgikh et al., 1997). It was supposed that the low rate of energy metabolism provides a long-term survival of spores in the environment. However, the metabolically active intracellular stages (meronts and early sporonts) presumably require more energy for their development. As the other amitochondriate protists (McLaughlin, Aley, 1985; Jarroll et al., 1989; . Lindmark et al., 1989) microsporidiae seem to use anaerobic glycolysis for energy supply of their development. Hence the lower activities of G 6-PDH, PGM and enzymes of glycolysis in stages than in spores were unexpected. It is possible that there exists another mechanism for energy supply of microsporidian intracellular stages of development.

It is unlikely that microsporidiae do not use the oxidative metabolism as some other amitochondriate protists lacking a respiratory chain. As mentioned above, microsporidiae may be dependent on host-derived ATP in the course of intracellular development (Weidner and

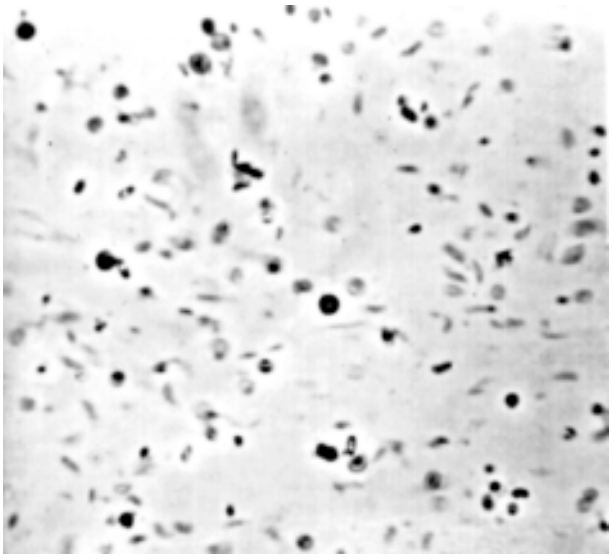

Fig. 2. The incubation of purified stages with 0.1% Triton X-100 results in the rupture of plasma membrane and release of cytoplasm (×220). Phase contrast.

Table 1. Activities of enzymes in microsporidian spores and in intracellular stages (mean ± SE).

Enzyme	Specific activities (nmole / min × mg protein)	
	Spores	Intracellular stages
Glucose 6-phosphate dehydrogenase	15 ± 1 *	7 ± 1 (8)[a]
Posphoglucomutase	7 ± 1 *	< 1 (4)
Hexokinase	< 0.2 *	< 1 (5)
Phosphoglucoisomerase	1549 ± 255*	23 ± 2 (9)
Fructose 6-phosphate kinase	10 ± 1*/13 ± 3[b] (2)	< 1 (5)
3-phosphoglycerate kinase (1st method)	16 ± 4*	7 ± 1 (5)
3-phosphoglycerate kinase (2nd method)	16 ± 2 (2)	8 ± 1 (4)
Pyruvate kinase	6 ± 1*	2 ± 0.3 (7)
Glycerol 3-phosphate dehydrogenase	16 ± 2*	17 ± 3 (5)
ATP-ase	7 ± 1 (3)	17 ± 4 (4)

* According to Dolgikh et al., 1997.

[a] The figures in brackets indicate the numbers of independent experiments carried out.

[b] PFK activity in spores determined by the method used in present study.

Trager, 1973). Some other intracellular parasites - *Rickettsia prowazekii* (Winkler, 1976; Plano and Winkler, 1989), *Plasmodium falciparum* (Kanaani and Ginsburg, 1989; Choi and Mikkelsen, 1990) and probably *Toxoplasma gondii* (Sorensen et al., 1997), possess the ATP/ADP carriers in their plasma membrane, which are similar to those found in mitochondrial membrane. Microsporidiae, the most ancient eukaryotic parasites, might also acquire such a mechanism for harvesting host's energy. The decrease of fat reserves in insect fat body after microsporidian infection may serve the circumstantial evidence that they depend on host energy metabolism (Canning, 1962; Darwish et al., 1989; Seleznev et al., 1996), since it cannot be explained by lipid utilization by the parasites, as microsporidiae lack mitochondria. However, only the revealing the adeninnucleotides exchange between the parasite and host cell as well as the direct evidence of occurrence of ATP/ADP carrier in parasite plasma membrane will prove that microsporidian intracellular stages use host-derived ATP.

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