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Survival of the intertidal pulmonate *Onchidium tumidium* during short term and long term anoxic stress

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**Abstract** *Onchidium tumidium* showed a triphasic response to anoxia. Twelve hours of anoxic exposure had no effect on the glycogen content in *O. tumidium*. However, there were significant increases in the alanine, lactate and succinate contents in the anoxic individuals. These were accompanied by a significant decrease in the ATP content. These results suggest that *O. tumidium* survived the first 12 h of anoxic exposure without increasing the glycolytic flux to compensate for the lower efficiency of ATP production through anaerobic pathways. Indeed, the fructose-2,6-bisphosphate (F-2,6-P₂) content and the percentage of phosphofructokinase (PFK) associated with the subcellular particles remain unchanged in *O. tumidium* exposed to 12 h of anoxia. Hence, a reduction in the metabolic rate of these individuals might have occurred during such a period of anoxia. In contrast, in between 12 and 24 h of anoxic exposure, the glycogen content *O. tumidium* decreased significantly, and levelled off thereafter. A significant increase in the percentage of PFK associated with the subcellular particles was observed in individuals exposed to 24 h of anoxia. In addition, the F-2,6-P₂ content of these anoxic individuals increased significantly. Taken together, these two mechanisms could activate PFK and lead to a greater glycolytic flux. Beyond 24 h of anoxic exposure, survival of *O. tumidium* must have required considerable suppression of metabolism as accumulation of end products and depletions of glycogen and ATP had reached constant levels.

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**Introduction**

*Onchidium tumidium* is a slug-like pulmonate commonly found in the brackish mangrove swamps in Singapore. It leads an amphibious life on the mud flats in the intertidal zone and comes out of its burrow only during ebb tides, which do not last longer than 12 h. During this period, it grazes on the surface algae. As the tide rises, it burrows into the mud. Careful observations of *O. tumidium* in its natural habitat reveal that it grazes on the surface algae only on sunny occasions. Hence, it is exposed to 6–12 h of environmental hypoxia once or twice a day. However, on cool rainy days, it stays in the burrow and abstains from feeding, and the duration of its hypoxic exposure can increase to 24–48 h.

When confronted with hypoxic stress, *Onchidium tumidium* depends mainly on the formation of succinate, rather than lactate and opines, for the maintenance of redox and the production of ATP (Ip et al. 1993). Pyruvate kinase from *O. tumidium* exposed to 24 h of anoxia exhibits a decrease in affinity to phosphoenolpyruvate to facilitate the formation of succinate through phosphoenolpyruvate carboxykinase (Ip et al. 1993). The pathway leading to succinate formation would yield 5 mol of ATP per mole of glucose-1-phosphate. Although this is energetically more efficient than the lactate/opine pathways, the efficiency of ATP production under anoxia would definitely be lower than that under normoxia (36 to 38 mol of ATP per mole of substrate).

The present study was therefore undertaken to elucidate whether there was an increase in the degradation of glycogen in *Onchidium tumidium* exposed to anoxia in order to compensate for the decrease in the supply of ATP. It was hypothesized that different strategies might be adopted by *O. tumidium* to survive short term (< 12 h) and long term (> 24 h) anoxia. Working on the land snail, *Otala lactea* (Pulmonata), Churchill and Storey (1989) observed that short term (2 h) exposure to...
anoxia activated glycolysis. Their crossover analyses indicated regulatory control at the phosphofructokinase (PFK), aldolase, and pyruvate kinase (PK) loci. During prolonged anoxia (14 h), a glycolytic rate depression, which involved the inhibition of PFK and PK in the foot muscle and the inhibition of PFK in the hepatopancreas, was observed. It has been shown that covalent modification by phosphorylation produces a less active form of PFK in some molluscs (Storey 1984; Michaelidis and Storey 1991) and that anoxia altered the kinetic properties of PK in O. tumidium (Ip et al. 1993). Thus, the effects of normoxia or anoxia on the kinetic properties of PFK from O. tumidium were also examined.

It was subsequently discovered that, unlike other molluscs studied, the kinetic properties of PFK from Onchidium tumidium were unaltered by 48 h of anoxic exposure. Hence, it was necessary to examine the possible regulation of PFK activity through changes in fructose-2, 6-bisphosphate (F-2,6-P_2) content, as well as the association of this enzyme with subcellular particles in O. tumidium exposed to anoxia.

### Materials and methods

#### Collection and maintenance of specimens

Onchidium tumidium were collected from the mud flats of the mangrove swamp at Mandai, Singapore. They were maintained under normoxic conditions in the laboratory at 25 °C in 50% (15‰ salinity) seawater (SW) in small aquaria. The SW level in the aquarium was kept below 3 mm, and the specimens were covered with one layer of cotton cloth. Water was changed daily, and no attempt was made to feed the specimens. Experiments were performed after 5 d of acclimatization to the laboratory conditions.

#### Exposure of specimens to anoxic conditions

Groups of 15 Onchidium tumidium were placed under a layer of cotton cloth in 250 ml conical flasks containing 20 ml of 50% SW, and maintained at 25 °C. Each flask was flushed with N_2 for 30 min before the introduction of O. tumidium. This was followed by flushing with N_2 for 10 min, before the flask was sealed for a period of 12, 24, or 48 h. At the end of the specific experimental period, the O. tumidium were immediately freeze-clamped in liquid N_2, and stored at −80 °C until further analyses. Individuals kept in normoxic conditions in the aquaria were sampled directly as controls for comparison.

#### Determination of glycogen content

Each frozen sample was ground to a powder and digested in 1 ml of 30% KOH in a boiling water bath for 30 min. The glycogen was extracted according to the method of Good et al. (1933). Glycogen was determined by the combined methods of Bergmeyer et al. (1974) and Roehrig and Allred (1974). Glycospyl units were released from glycogen by incubating 0.1 ml of sample in 10 mM acetic buffer (pH 4.5) with 10 IU amylglucosidase (from Rhizopus genus mold, Sigma Chemical Co., USA) at 55 °C for 10 min. The glycogen content was expressed as μmol glycosyl units g⁻¹ wet weight.

#### Determination of alanine, lactate, succinate and ATP contents

Each frozen sample was ground to a powder and 7 vol (w/v) of ice-cold 0.6 M perchloric acid added to it. The sample was homogenized three times with an Ultra Turrax homogenizer (Janke and Kunkel GmbH and Co., Germany) at 24000 rpm for 20 s each with 10 s off intervals. The homogenized sample was then centrifuged at 10000 × g for 15 min. The supernatant fluid obtained was titrated with K_2CO_3 (5 M) to pH 6.5–7.0 for the quantitative analyses of alanine, lactate, succinate and ATP.

Alanine was assayed following the procedure of Williamson DH (1974). Lactate was determined according to Gutmann and Wahlefeld (1974). Succinate was assayed according to the method of Williamson JR (1974).

ATP was assayed following the method of Trautschold et al. (1985) with slight modifications. The assay medium contained in a final volume of 1.35 ml: 50 mM triethanolamine (pH 7.5), 6.7 mM MgCl_2, 0.33 mM NADP⁺, 50 mM glucose, 0.62 IU glucose-6-phosphate dehydrogenase (from Leuconostoc mesenteroides, Type XXIII; Sigma Chemical Co., USA), and 0.2 ml of sample. The reaction was initiated by the addition of 2.4 IU hexokinase (from baker's yeast, Type III; Sigma Chemical Co., USA). The change in absorption was used to estimate the ATP content in the sample. Results were expressed as pmol g⁻¹ wet weight.

#### Determination of F-2,6-P_2 content

Each frozen sample was ground to a powder, and 10 vol (w/v) of 50 mM NaOH added to it. The sample was homogenized three times with an Ultra Turrax homogenizer at 17000 rpm for 20 s with 10 s off intervals. The homogenate was heated to 80 °C for 5 min. After cooling, it was centrifuged at 1500 × g for 15 min at 4 °C. The supernatant fluid was removed and assayed for F-2,6-P_2 following the method of Van Schaftingen (1984). The PPI-PFK used in the coupled assay was obtained from Sigma Chemical Co., USA. Results were expressed as pmol g⁻¹ wet weight.

#### Preparation of samples for PFK assay

Each frozen sample was ground to a powder under liquid N_2. The powdered sample was weighed and homogenized three times in 5 vol (w/v) of ice-cold extraction buffer containing 50 mM imidazole-HCl (pH 7.8), 100 mM NaF, 5 mM EDTA, 5 mM EGTA, 0.1 mM PMSP, 30 mM 2-mercaptoethanol, and 40% v/v glycerol using an Ultra Turrax homogenizer at 24000 rpm for 20 s with 10 s off intervals. The homogenized sample was centrifuged at 25000 × g for 20 min at 4 °C. After centrifugation, 0.75 ml of the supernatant fluid was removed and passed through a 10 ml column of Sephadex G-25 Superfine (Pharmacia LKB, Sweden) equilibrated in a suspension buffer containing 40 mM imidazole-HCl (pH 7.8), 5 mM EDTA, 15 mM 2-mercaptoethanol, and 20% glycerol in order to remove low molecular weight metabolites (Helmerhorst and Stokes 1980). The column was centrifuged at 2950 × g for 2 min in a Kokusan H-103N refrigerated centrifuge (Kokusan Enshinko Co., Tokyo, Japan). The filtrate was used as the source of enzyme for subsequent assays.

#### PFK assay

PFK activity was determined according to the method of Michaelidis and Storey (1991). The enzyme activity was measured...