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Anoxic brain function: molecular mechanisms of metabolic depression

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An examination of the kinetic parameters of phosphofructokinase, pyruvate kinase and glycogen phosphorylase, and the cellular concentration of fructose 2,6-bisphosphate during anoxia in the turtle *Pseudemys scripta* showed that the total activity of glycogen phosphorylase, and the phosphofructokinase inhibition constants for citrate and ATP were decreased in anoxic turtle brain. These results suggest that the ability of turtle brain to survive extended periods of anoxia is the result of metabolic rate depression regulated, at the molecular level, by enzyme inactivation through anoxia-induced covalent modification.

Brain metabolism; Metabolic depression; Covalent modification; (*Pseudemys scripta*)

1. INTRODUCTION

The superior ability of the brains of certain lower vertebrates to survive extended periods of anoxia markedly contrasts with the results found for mammalian brains. In mammalian brain, degenerative changes occur within seconds when the organ is deprived of oxygen, and damage is irreversible in a matter of minutes [1]. In contrast, the brains of freshwater turtles can survive up to 6 months of anoxia during hibernation at 3°C [2,3]. Measurements made during the first 24 h of anoxia show that ionic and electrical integrity is maintained [4,5] even though the whole-body metabolic rate, including that of the brain [4], declines to 15% of aerobic values within 1 h of anoxia [6]. The key to long-term anoxia survival is the ability to depress metabolic activity in response to limiting ATP availability [5–9]. Thus, the organs of facultative anaerobes do not show a Pasteur effect, the typical response to anoxia by mammalian organs such as brain [1,10].

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Anoxia-tolerant marine molluscs utilize three molecular mechanisms to depress glycolytic rate during anaerobiosis: (i) covalent modification of regulatory enzymes, by protein phosphorylation or dephosphorylation, to produce less active enzyme forms [11]; (ii) reduction in the level of fructose 2,6-bisphosphate, to help restrict carbohydrate availability for anabolic purposes [12]; and (iii) decrease in the binding of enzymes to particulate matter to reduce flux through structured glycolytic complexes [13]. The present study examines the universality of these mechanisms in controlling metabolic flux in anoxic turtle brain tissue.

2. EXPERIMENTAL

Anoxia was imposed by submerging turtles in a sealed tank of water (18°C) that had been previously bubbled with gas (95% N_2/5% CO_2) for 12 h. After 5 h individuals were removed and decapitated. The brain was excised from the skull in less than 20 s and immediately frozen in liquid N_2. Frozen brain was stored at −80°C until use. Brain samples from control turtles were obtained similarly. For analysis of enzyme properties, extracts of brain were made in buffer containing EDTA, EGTA and NaF as in [9]. Glycogen phosphorylase activity was measured in aliquots of the well-mixed homogenate; phosphofructokinase (PFK) and pyruvate kinase (PK) were measured in the 12000 × *g* supernatant after a desalting step

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through a Sephadex G-25 column equilibrated in assay buffer. Enzymes were assayed at 22°C. Conditions for glycogen phosphorylase were as before [9]. PFK and PK activities were measured in 50 mM imidazole-HCl buffer, pH 7.0, containing 50 mM KCl, 5 mM $MgCl_2$, 0.2 mM NADH, and the appropriate coupling enzymes in excess [9]. For the determination of kinetic constants, concentrations of non-varied substrates were 0.5 mM ATP and 8 mM fructose 6-phosphate (4 mM for K_a determinations) for PFK, those for PK being 0.6 mM phospho*enol*pyruvate and 2 mM ADP. Extraction and quantification of fructose 2,6-bisphosphate and lactate were performed as described [9]. All data are presented as means ± SE (n = 5).

3. RESULTS

Previous studies have shown that, after 5 h of anoxia, *P. scripta* have established a stable, depressed state as documented by both whole-body calorimetry [3,6] and analysis of brain metabolism [5]. The data in table 1 show that the kinetic properties of turtle brain PK remained unchanged in anoxic animals, however, both glycogen phosphorylase and PFK were altered in response to anoxia.

Phosphorylase *a* activity in the brains of turtles, submerged in N_2-bubbled water for 5 h, was reduced to 70% of that in aerobic controls. This decrease was due to a significant reduction in both the percentage of phosphorylase in the active *a* form and the total activity of the enzyme (table 1). This reduction occurred despite the obvious dependence on glycolysis for ATP production in anoxic brain, demonstrated by an increase in brain lactate content from 3.9 ± 0.7 in control to 20.5 ± 1.1 μmol/g wet wt after 5 h of anoxia.

Brain PFK also showed substantial changes in its kinetic properties as a result of whole-animal anoxia: the $S_{0.5}$ for fructose 6-phosphate increased, the I_{50} values for two inhibitors, ATP and citrate decreased, and the K_a for AMP increased (table 1). However, the concentration of the PFK activator, fructose 2,6-bisphosphate, did not change during anoxia (control = 10.7 ± 2.9, anoxic = 11.5 ± 0.9 nmol/g wet wt).

4. DISCUSSION

The extensive regulatory mechanisms affecting PFK activity reflect its rate-controlling role in glycolysis. For example, the Pasteur effect is primarily the result of the release of metabolic con-

Table 1

Effect of anoxia on properties of glycogen phosphorylase, phosphofructokinase and pyruvate kinase from turtle brain

	Aerobic control	Anoxia (5 h)	
Glycogen phosphorylase			
Activity, forms $a+b$ (IU/g)	6.0 ± 0.12	5.4 ± 0.38	$P < 0.1$
% form *a*	40 ± 2.4	31 ± 3.9	$P < 0.1$
Phosphofructokinase			
$S_{0.5}$ fructose 6-P (mM)	6.7 ± 0.31	7.5 ± 0.47	$P < 0.1$
Hill coefficient	5.7 ± 0.43	5.6 ± 0.55	NS
K_m (Mg·ATP) (μM)	20 ± 2.1	21 ± 1.3	NS
I_{50} (Mg·ATP) (mM)	0.96 ± 0.18	0.54 ± 0.16	$P < 0.1$
I_{50} (Mg·citrate) (mM)	0.61 ± 0.09	0.21 ± 0.03	$P < 0.005$
K_a (AMP) (μM)	27 ± 4.3	35 ± 2.7	$P < 0.1$
K_a (fructose 2,6-P_2) (μM)	0.32 ± 0.08	0.44 ± 0.10	NS
K_a (P_i) (mM)	0.21 ± 0.05	0.27 ± 0.03	NS
V_{max} (IU/g wet wt)	9.9 ± 0.9	10.6 ± 0.4	NS
Pyruvate kinase			
K_m (phospho*enol*pyruvate) (μM)	77 ± 13	82 ± 10	NS
K_m (Mg·ADP) (mM)	173 ± 16	190 ± 22	NS
I_{50} (Mg·ATP) (mM)	11.6 ± 0.9	13.7 ± 0.7	$P < 0.1$
V_{max} (IU/g wet wt)	162 ± 6	187 ± 3	$P < 0.01$

NS, not significantly different as determined by the one-tailed Student's t-test. Turtle brain PK was not affected by fructose 1,6-bisphosphate or L-alanine

trol at the PFK locus [12]. That control over PFK should be the primary means of glycolytic rate depression (and the absence of a Pasteur effect) in facultative anaerobes is not surprising. Covalent modification is perhaps the simplest method to achieve this control, since a single enzyme modification: (i) alters the kinetic constants for a variety of effectors, reducing enzyme activity at physiological levels of substrates and effectors; (ii) changes binding interactions with subcellular structures [8,14–16]; and (iii) influences enzyme polymerization [17]. The activity state of the enzyme (and thus the glycolytic rate) can, therefore, be controlled without major changes in either the rate of protein synthesis/degradation or the levels of metabolite effectors. Indeed, levels of ATP, AMP [4], and fructose 2,6-bisphosphate in anoxic turtle brain are virtually equivalent to control values. Both these latter processes are implicated in the control of enzyme activity in other systems [18].

The present study indicates that glycolytic rate depression and the control of ATP supply in the brain of a facultative anaerobe are controlled (at least partially) by covalent modification of key regulatory enzymes. However, long-term homeostasis in the hypometabolic state is possible only when the rates of all metabolic processes are balanced. Critical changes in anoxic mammalian brain result from the degeneration of ion gradients across the cell membrane that occur when ATP-driven ion pumps are incapable of counterbalancing ion movement through membrane ion channels in the absence of an ATP supply from oxidative phosphorylation [1,19,20]. The facultative anaerobe also faces this problem and thus the maintenance of homeostasis in a hypometabolic state must be supported by a reduction in the number, or activity, of membrane ion channels [19,20]. A prime candidate for the reversible regulatory control of ion channel activity is covalent modification. Thus, a coordinated depression of all cellular functions during anaerobiosis may be achieved through anoxia-induced covalent modifications acting on selected proteins and enzymes.

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