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SHORT COMMUNICATION

ROLES OF BUFFERING CAPACITY AND PENTOSE PHOSPHATE PATHWAY ACTIVITY IN THE GAS GLAND OF THE GULF TOADFISH *OPSANUS BETA*

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The teleost gas gland is truly remarkable in its abilities to secrete gases into the swim bladder of physoclistous fish. The physiological and metabolic adaptations of this tissue have been elegantly summarized in a recent review article by Pelster and Scheid (1992). There are two key contributors to the function of the gland. First, a specialized metabolism of the swim bladder, involving copious and simultaneous production of lactate and CO_2 from anaerobic glycolysis and the pentose phosphate pathway (also known as the hexose monophosphate shunt), respectively, contributes to gas exchange through pH and salting-out effects on the oxygen-carrying capacity of the blood. Second, a countercurrent multiplier system (i.e. a rete mirabile) enables gas tensions to be elevated further by back diffusion. Several features of metabolism and acid–base physiology remain unclear. First, despite the remarkable ability of this tissue to produce acid, it is not clear if or how intracellular pH (pHi) is regulated. Since ultimately the blood must be acidified, one would predict that the pHi of the tissue would be well regulated *via* high rates of membrane exchange of protons and/or high tissue buffering capacity. Second, although the functioning of the pentose phosphate pathway has been strongly inferred from measurements of enzyme activities (Boström *et al.* 1972; Pelster and Scheid, 1991), and from measurements of enhanced rates of CO_2 excretion relative to the rates of oxygen uptake (Pelster *et al.* 1989), direct evidence for the existence of the shunt is lacking. Lastly, although the pentose phosphate pathway *is* expected to produce CO_2, and thus contribute to the acidification of blood entering the gland, the pathway may have a different primary, or perhaps a dual, role, namely to maintain high tissue levels of NADPH for protection against oxygen radical damage to cells (Pelster and Scheid, 1992). The composition of the gas stored in the swim bladder can approach pure oxygen in some

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species, so it is not surprising that the teleost gas gland contains substantial levels of the enzymes catalase, superoxide dismutase and glutathione peroxidase, which scavenge deleterious radicals of oxygen and related harmful compounds (Morris and Albright, 1984). Noteworthy is glutathione peroxidase, which requires a constant supply of NADPH (presumably from the shunt) to maintain glutathione in a reduced state. Reduced glutathione is then used in a variety of oxygen radical detoxification mechanisms. If the pentose phosphate pathway has a role in oxygen detoxification, one would predict that flux rates through the pathway would increase with increased oxygen levels.

In order to address these three facets of the metabolism of the gas gland, we have conducted *in vitro* experiments with the gas gland of the gulf toadfish, *Opsanus beta*. Our key findings are (1) that the tissue has an extremely high non-bicarbonate buffering capacity; (2) that the tissue has an active pentose phosphate pathway (as demonstrated by relative $^{14}CO_2$ production rates from C1- *versus* C6-labelled glucose); and (3) that flux through the pathway is enhanced under hyperoxic conditions.

Gulf toadfish, *Opsanus beta* (Goode and Bean), were collected by roller trawl from Biscayne Bay, Florida, in July and August 1990 for metabolic flux experiments and enzyme activity measurements and in November 1990 for buffering capacity measurements. Fish were held for no more than 1 week in the laboratory, without feeding, in flowing sea water, at 24–28°C and with a salinity of 33‰, under a natural photoperiod. Fish were anaesthetized with MS-222 for 5min at $0.5\,g\,l^{-1}$ in sea water buffered with $NaHCO_3$. The swim bladder was removed through a ventral incision and opened from the dorsal side, and the gas gland and surrounding layer of epithelial tissue was then gently peeled away from the ventral floor of the bladder. Detailed drawings of the toadfish swim bladder and gas gland appear in Fänge and Wittenberg (1958). For measurements of enzyme activities and buffering capacity, glands were placed in 1ml plastic cryotubes and frozen at −80°C. Enzyme activities and buffering capacities were measured within 30 days of freezing. The frozen glands for buffering capacity measurements were transported on dry ice to London, Ontario. The total non-bicarbonate buffer capacity of the gas gland was determined by acid titration as described by Cameron and Kormanik (1982). Individual gas glands were ground to a fine powder with a mortar and pestle under liquid nitrogen and then suspended in 3ml of 0.9% NaCl. The tissue homogenate was titrated to pH8.0 with $1mol\,l^{-1}$ NaOH, allowed to stabilize and then back titrated to pH6.8 with $0.02mol\,l^{-1}$ HCl at 25°C under a nitrogen atmosphere (to prevent absorption of atmospheric CO_2 by the sample, which would affect the buffering). The slope of the line relating pH with μmol HCl added was taken as an estimate of the total non-bicarbonate buffer capacity in $\mu molpHunit^{-1}\,g^{-1}$ (or slykes).

For radiotracer experiments, the gas gland was transferred to a glass slide and carefully sliced approximately in half with a scalpel such that each half had part of the rete and surrounding epithelial layer. Both halves were weighed (range was approximately 25–100mg per half) and placed in 0.95ml of toadfish Hanks salts solution, plus $3\,mmol\,l^{-1}$ $NaHCO_3$, $1mmol\,l^{-1}$ $CaCl_2$ and $1mmol\,l^{-1}$ glucose, in the bottom of a 20ml glass scintillation vial, as in prior studies of toadfish hepatocytes (Walsh, 1989). After a 15min preincubation period, 50 μl of saline containing 0.5 μCi of either C1- or C6-labelled [^{14}C]glucose (Amersham, Arlington Heights, IL) was added to the flask.

Labelled CO_2 can be produced from C1-labelled glucose by both the pentose phosphate pathway and aerobic glycolysis, whereas C6-labelled glucose gives rise to $^{14}CO_2$ only *via* glycolysis. Therefore, the ratio of CO_2 production from C1- *versus* C6-labelled glucose gives the relative activity of pentose phosphate pathway activity. All experiments were paired in that half of the gland from each fish received C1 label and the other half received C6 label on a random basis, without regard for left or right or for slight differences in weights of the half glands. The flasks were immediately sealed with rubber stoppers containing centre wells with circles of 2.4cm diameter Whatman GF/C filters, and the tissues were incubated with gentle shaking for 2h. Chemical controls (i.e. as above without tissues) were run in parallel daily. For hyperoxic incubations, saline solutions were vigorously gassed with 100% O_2 for 1h prior to bicarbonate addition and pH adjustment and, after capping the vials, the gas space was purged with 100% oxygen for 30s. No loss of $^{14}CO_2$ by this purging was detected. Typical oxygen partial pressures were 73.3kPa (550mmHg) as determined with a Radiometer oxygen electrode and blood gas analyser. At the end of the incubation, 0.2ml of hyamine hydroxide solution (Sigma, St Louis, MO) was injected through the stopper onto the filter paper, and 0.1ml of 70 % perchloric acid was injected into the saline. The samples were shaken for 1.5h, and the filters were removed and counted for ^{14}C activity in 10ml of Ecolume (ICN) in a Tracor Analytic Betatrac liquid scintillation counter. Counts were converted to *total* CO_2 production rates ($\mu mol\,g^{-1}\,tissue\,h^{-1}$) using the specific activity of glucose in the flask (i.e. $disintsmin^{-1}$ of [^{14}C]glucose added divided by 1 μmol total glucose).

For measurements of enzyme activities, glands were homogenized in their cryotubes in 0.5ml of $50mmol\,l^{-1}$ Hepes, pH7.5, using a Brinkman polytron. Samples were then centrifuged for 1min at 13000 ***g*** in a Fisher 235B microcentrifuge in a 4˚C room. 10–50 μl of these supernatants (or 1:10 dilutions in the case of LDH) was used directly in enzyme assays. The following assays were performed, initially omitting the last listed item in each assay as a control. All assays were performed in a 1ml volume of Hepes, pH7.5, at 24±0.2˚C and the change in absorbance was followed using an LKB Ultrospec (connected to a Kipp and Zonen chart recorder) at 340nm [micromolar extinction coefficient for NAD(P)H=6.22] unless noted.

Glucose-6-phosphate dehydrogenase (E.C. 1.1.1.49, G6PDH): $0.4mmol\,l^{-1}$ $NADP^+$, $7\,mmol\,l^{-1}$ $MgCl_2$, $1mmol\,l^{-1}$ glucose 6-phosphate.

6-Phosphogluconate dehydrogenase (E.C. 1.1.1.44, 6PGDH): $0.8mmol\,l^{-1}$ $NADP^+$, $20mmol\,l^{-1}$ $MgCl_2$, $3mmol\,l^{-1}$ 6-phosphogluconate.

Transaldolase (E.C. 2.2.1.2, TA): $0.12mmol\,l^{-1}$ NADH, $10mmol\,l^{-1}$ EDTA, $0.8mmol\,l^{-1}$ erythrose 4-phosphate, 0.6 units of alpha-glycerophosphate dehydrogenase, 9 units of triosephosphate isomerase, $2.7mmol\,l^{-1}$ fructose 6-phosphate.

Transketolase (E.C. 2.2.1.1, TK): $20mmol\,l^{-1}$ K_2HPO_4 (in addition to Hepes), $100mmol\,l^{-1}$ KCl, $5mmol\,l^{-1}$ $MgCl_2$, $0.12mmol\,l^{-1}$ NADH, $0.2mmol\,l^{-1}$ thiaminepyrophosphate (=cocarboxylase), $0.6mmol\,l^{-1}$ erythrose 4-phosphate, 0.6 units of alpha-glycerophosphate dehydrogenase, 9 units of triosephosphate isomerase, $1.0mmol\,l^{-1}$ xylulose 5-phosphate.

Phosphoglucose isomerase (E.C. 5.3.1.9, PGI): $0.39mmol\,l^{-1}$ $NADP^+$, $6.8mmol\,l^{-1}$

$MgCl_2$, 0.5 units of glucose-6-phosphate dehydrogenase, 1.4mmol l^{-1} fructose 6-phosphate.

Phosphofructokinase (E.C. 2.7.1.11, PFK): 0.12mmol l^{-1} NADH, 10mmol l^{-1} $MgCl_2$, 50mmol l^{-1} KCl, 2mmol l^{-1} ATP, 1 unit of alpha-glycerophosphate dehydrogenase, 5 units of aldolase, 5 units of triosephosphate isomerase, 5mmol l^{-1} fructose 6-phosphate.

Glyceraldehyde-3-phosphate dehydrogenase (E.C. 1.2.1.12, G3PDH): 0.12mmol l^{-1} NADH, 2mmol l^{-1} $MgSO_4$, 0.9mmol l^{-1} EDTA, 1mmol l^{-1} ATP, 10 units of phosphoglycerate kinase, 6mmol l^{-1} glycerate 3-phosphate.

Lactate dehydrogenase (E.C. 1.1.1.2, LDH): 0.12mmol l^{-1} NADH, 0.5mmol l^{-1} pyruvate.

Malate dehydrogenase (E.C. 1.1.1.37, MDH): 0.12mmol l^{-1} NADH, 0.5mmol l^{-1} oxaloacetate.

Citrate synthase (E.C. 4.1.3.7, CS): pH8.0, 0.1mmol l^{-1} 5,5′-dithiobis-(2-nitrobenzoic acid), 0.3mmol l^{-1} acetylcoenzyme A, 0.5mmol l^{-1} oxaloacetate. (Read at 412nm, micromolar extinction coefficient=13.6.)

All chemicals were reagent grade and biochemicals were purchased from Sigma (St Louis, MO).

Non-bicarbonate buffer capacity in toadfish gas gland was -105.4 ± 9.4mmol pHunit^{-1} kg^{-1} wetmass (mean ± S.E., N=6). Compared to the buffer capacity of other tissues estimated in a similar fashion, that of the toadfish gas gland is 2–3 times that of most teleost skeletal muscle and is of comparable magnitude to that of tuna skeletal muscle (Castellini and Somero, 1981). It is of interest to note, however, that, despite the comparable buffer capacity, toadfish gas gland LDH activity is only one-tenth that reported for tuna muscle, indicating that the gas gland's ability to produce copious amounts of lactate may be facilitated by an enhanced capacity to buffer the acidic end-products. This enhanced ability of the gas gland to buffer acid, and presumably to maintain intracellular pH despite continuous acid production, would explain the observation by Pelster and Scheid (1991) that the glycolytic enzymes of eel gas gland show no special adaptation to function in an acidic milieu.

Enzyme activities are listed by metabolic pathway in Table 1 and they generally agree well with prior studies (Boström *et al.* 1972; Ewart and Driedzic, 1990; Pelster and Scheid, 1991). There are substantial activities of two of the four enzymes of the pentose phosphate pathway (G6PDH and 6PGDH), but much lower activities of the remaining two enzymes (TA and TK). These low activities suggest that the shunt is not simply a bypass, but yields pentose sugars for general RNA synthesis, without rejoining glycolysis at the level of glyceraldehyde 3-phosphate to any appreciable extent. The gas gland has a substantial glycolytic activity, as indicated by activity of the enzymes PGI, PFK and G3PDH, but the activity appears to be largely anaerobic as LDH values are as high as those found in toadfish skeletal muscle (Walsh *et al.* 1989), and MDH and CS values are rather low. Interestingly, LDH in this tissue appears to be able to use NADPH as a cofactor at rates comparable to measured rates of shunt enzyme activities (Table 1). The NADPH used in this study was checked for NADH contamination by using purified rabbit M_4 LDH and no activity was noted with NADPH. However, given that the gas

Table 1. *Activities of selected enzymes in the gas gland of the gulf toadfish* Opsanus beta

Metabolic pathway Enzyme	Activity (μmol min^{-1} g^{-1} wetmass)
Pentose phosphate pathway	
G6PDH	2.22±0.29
6PGDH	1.89±0.12
TA	0.29±0.04
TK	0.10±0.02
Glycolysis	
PGI	29.15±1.82
PFK	23.96±2.78
G3PDH	29.97±3.52
LDH (NADH)	173.80±18.4
LDH (NADPH)	2.28±0.20
Tricarboxylic acid cycle	
MDH	9.69±1.09
CS	0.14±0.05

Values are mean ± S.E. (N=6).

Table 2. In vitro *rates of total CO_2 production in gas glands of the gulf toadfish* Opsanus beta *from C1- or C6-labelled glucose*

Experimental treatment	CO_2 production rate (μmol g^{-1} wetmass h^{-1})
Normoxia	
C1-labelled glucose	0.118±0.029
C6-labelled glucose	0.034±0.006
C1/C6 ratio	3.370±0.427*
Hyperoxia	
C1-labelled glucose	0.234±0.043
C6-labelled glucose	0.041±0.010
C1/C6 ratio	6.357±0.823*

Values are mean ± S.E. (N=5).

*Significantly different (P<0.05, Student's t-test). Note that ratios were calculated for each fish, then averaged.

gland enzyme assays were performed with crude homogenates, the possibility of a novel form of LDH should be considered as preliminary.

Overall rates of glucose oxidation are comparable to those in prior studies with hepatocytes (Walsh *et al.* 1985), and the ratio of CO_2 production from C1- *versus* C6-labelled glucose is significantly greater than 1 (Table 2), indicating substantial flux through the pentose phosphate pathway. A typical value for this ratio in a teleost liver is 11.2 (Walsh *et al.* 1985), which is considered a relatively high pentose phosphate pathway activity. The high pentose phosphate pathway activity measured directly corroborates the previous suggestion by Pelster *et al.* (1989) of a highly active pentose

phosphate pathway in teleost gas gland. Generation of CO_2 from C1-labelled glucose and the ratio of CO_2 production from C1-labelled *versus* C6-labelled glucose virtually double under hyperoxic conditions (Table 2), indicating an increase in flux through the pentose phosphate pathway. This observation strongly suggests that an additional role for this pathway in the teleost swim bladder is one of assisting in the protection of tissues from damage by oxygen radicals. Thus, further study of the gas secretion properties of the gas gland must continue to include as part of their experimental design careful control of oxygen partial pressures. Additionally, comparative studies which address both aspects of gas gland metabolism are strongly indicated.

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