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Metabolic responses to anoxia and freezing by the freeze tolerant marine mussel *Geukensia demissus*

Kenneth B. Storey*, Thomas A. Churchill[1]

Institute of Biochemistry and Department of Biology, Carleton University Ottawa, Ontario, Canada K1S 5B6

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Abstract

The effects of 2 or 12 h freezing at −6 °C or anoxia exposure at 5 °C on the metabolic responses of gill, mantle, adductor muscle, and hepatopancreas were characterized in the freeze tolerant, intertidal ribbed mussel *Geukensia demissus* (Dillwyn). In general, freezing and anoxia elicited different metabolic responses and these also differed in a tissue-specific manner. Fermentative end products accumulated in all tissues except gill under both stresses. In adductor muscle, lactate was produced in both cases. In mantle and hepatopancreas, only succinate accumulated in anoxia, correlated with an opposite decrease in aspartate, and changes in glycolytic intermediates were consistent with anoxia-induced metabolic arrest. During freezing, however, both lactate and succinate accumulated (as well as alanine in hepatopancreas) with net end product accumulation 3–4-fold greater than during anoxia and changes in glycolytic intermediates were consistent with the derivation of these products from carbohydrate catabolism. Other differences in response to freezing versus anoxia included the accumulation of glucose in all tissues and a reduction of energy charge in mantle during freezing, but not in anoxia. Taurine and glycine comprised about 80–85% of the total free amino acid pools in all tissues; in gill levels of both amino acids fell significantly during freezing or anoxia exposures whereas in mantle a significant decrease in these amino acids occurred during anoxia only. The data show both a broader range of metabolic responses, and greater net changes in metabolite levels during freezing compared with anoxia. This suggests (1) that metabolic responses to freezing include both cryoprotective and ischemia-protective changes, and (2) that tissue mechanisms of metabolic rate depression are rapidly induced during anoxia exposure but develop more slowly during freezing.

Keywords: Anaerobiosis; Energetics; Freeze tolerance; Glycolytic regulation; Marine mollusc cold hardiness

* Corresponding author.

[1] Present address: Academic Department of Surgery, Royal Free Hospital School of Medicine, London NW3 2QG, UK.

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1. Introduction

The intertidal zone is a rigorous environment for animal life and to survive there animals must be able to endure wide variations in oxygen and water availability, salinity, and temperature. At high latitudes, temperature variation can include subzero exposures during the winter months and this has led to the development of freeze tolerance in many species. The characteristics of freeze tolerance in marine invertebrates, including the effects on survival of different subzero temperatures, duration of freezing, freezing rates, and percent body ice accumulated, have been described for several species of mussels, snails, and barnacles (for review Aarset, 1982; Murphy, 1983). Hemolymph ice nucleator proteins, that induce ice formation in extracellular fluid spaces, have been identified in both bivalves and gastropods (Aunaas, 1982; Hayes & Loomis, 1985; Madison et al., 1991) but intertidal species do not accumulate the high concentrations of low molecular weight cryoprotectants that commonly characterize freeze tolerance in terrestrial species (Storey & Storey, 1988). However, although specific colligative cryoprotectants are lacking, the normally high osmolality of body fluids would help to limit cell volume reduction during freezing and it has been shown that acclimation to higher salinities (which elevates intracellular ion and free amino acids pools) improves freeze tolerance (Williams, 1970; Murphy, 1979, 1983). Furthermore, pre-exposure to anaerobic conditions also enhances survival during subsequent freezing exposures possibly due to one or more characteristics of anaerobiosis including elevated levels of end products and Ca^{2+} (these have been shown to have membrane stabilizing effects), metabolic arrest, or a reduced potential for damage by oxygen free radicals when freezing begins from an anoxic, versus aerobic, state (Murphy, 1983; Storey & Storey, 1988; Loomis, 1987).

Various characteristics of marine mollusc freeze tolerance have been analyzed using as the model animal the ribbed mussel, *Geukensia demissus demissus* (Dillwyn), a sessile species that lives high in the intertidal zone (Murphy & Pierce, 1975; Murphy, 1977a,b). Mussels showed very limited supercooling, freezing beginning only about 1 °C below the freezing point of body fluids. Fifty percent mortality after 12 h freezing exposure occurred at about −13 °C for mussels previously acclimated to 0 °C in full strength seawater. Acclimation temperature, salinity, and season all influenced freeze tolerance and animals were less tolerant of freezing (50% mortality occurred at higher subzero temperatures) when mussels were acclimated to higher temperatures or lower salinities or when they were collected in the summer (Murphy & Pierce, 1975). Freeze tolerance was improved by prior anoxia exposure (which included elevated levels of alanine and Ca^{2+}) and Ca^{2+} was further shown to have a major effect on freezing survival in this species (Murphy, 1977a,b). Thus, the percentage of 15 °C-acclimated mussels that survived freezing at −10 °C for 12 h rose from less than 20% in low Ca^{2+} (3 mM) seawater to 60–70% within 6 h after transfer to high Ca^{2+} seawater (9.6 mM) concomitant with a rise in blood Ca^{2+} levels from 4.2 to 8.5 mM (Murphy, 1977b).

Apart from these studies, very little is known about the metabolic responses of marine molluscs to freezing. Freezing places two fundamental stresses on cells: (1) osmotic stress results because water is sequestered in extracellular ice crystals, leading to cell volume decrease and an increase in the ionic strength and osmolality of remaining

intracellular fluids, and (2) ischemia is imposed because freezing cuts off supplies of oxygen and exogenous substrates to cells. Marine intertidal molluscs have well-developed mechanisms for dealing with these stresses in other forms – mechanisms of cell volume regulation are in place to deal with wide variations in seawater salinity and anoxia tolerance supports survival during daily aerial exposures at low tide (Gilles, 1979; Storey, 1992). These pre-existing capacities may have facilitated the development of freeze tolerance in various species. The present study compares and contrasts the metabolic responses to freezing versus anoxia exposures in *G. demissus* to assess the extent to which metabolic changes seen during freezing can be attributed to freezing-induced ischemia and anaerobic metabolism. Because tissue-specific patterns of anaerobic metabolism are seen in bivalves (de Zwaan, 1983; Korycan & Storey, 1983), we chose to assess the effects of freezing and anoxia on metabolism in four tissues to identify both general and tissue-specific patterns of metabolic response to the two stresses.

2. Materials and Methods

2.1. Animals and chemicals

All chemicals and biochemicals were purchased from Sigma Chemical Co., St. Louis, MO, or Boehringer Mannheim Corp., Montreal, PQ. Ribbed mussels *G. demissus* (about 3 inches shell length) were obtained from the Marine Biological Laboratory, Woods Hole, MA in mid-October. Mussels were acclimated in large aerated aquaria filled with artificial seawater (1000 Fathoms brand commercial aquarium mix; specific gravity = 1.040) for at least 4 wk prior to experimentation. Most mussels were held at 5 °C but a small group was acclimated at 20 °C.

2.2. Animal experiments

Control mussels were sampled immediately after removal from the 5 °C aquaria. Before either freezing or anoxia exposure, mussels were gently perturbed while still in their aquaria so that they closed their valves. A rubber band was then secured around each mussel to hold the valves shut and then the animal was removed from the seawater. Hence, for both freezing and anoxia exposures, mussels were held out of water. For freezing exposure, a YSI telethermometer probe was slipped into a secure position under the rubber band and against the shell. Mussels were then placed in an incubator set to −6.0 °C and were allowed to cool in air at a rate of about 1.0 °C/min (the rate varied slightly depending on body size). Telethermometer output was to a chart recorder and the surface temperature of mussels was monitored as they cooled to their supercooling points. The initiation of ice formation (nucleation) was recorded as an instantaneous jump in temperature (exotherm) due to the heat release of crystallization. After freezing for either 2 or 12 h (timed from the exotherm), mussels were either dissected immediately for tissue sampling or used for the determination of percentage of body water that was ice, followed by monitoring recovery. Ice content was determined by calorimetry. For this, mussels were melted in a small volume of water in an

insulated container and the change in water temperature was recorded; ice content was then calculated as described by Lee & Lewis (1985). For recovery, mussels were returned to 5 °C seawater for 24 h and survival was assessed both by the presence of the normal valve closure response to handling and by contractile responses by adductor muscle and mantle in response to probing during subsequent dissection. Initial assessments of percent body ice and survival after freezing at −6.0 °C were conducted for animals acclimated to both 5 °C and 20 °C whereas subsequent metabolic studies used only mussels acclimated to 5 °C.

For anoxia exposure, mussels were placed in a jar (without water) in an atmosphere of N_2 gas (prior to adding mussels the jars were flushed with N_2 gas for 30 min). Jars were then sealed except for two syringe needle ports in the lid, one used to continuously gass the jar with N_2 throughout the anoxic exposure and one used to vent the gas. Jars were placed in an incubator at 5 °C and were sampled after 2 or 12 h of anoxia exposure. To assess recovery after anoxia, some mussels were returned to the aerated aquaria and survival was assessed after 24 h as described above for frozen mussels.

2.3. Tissue sampling and extraction

After experimental exposure to freezing or anoxia, mussels were quickly opened and tissues were dissected out, blotted to remove extraneous ice or water, and then immediately frozen in liquid nitrogen. Tissue samples were then stored at −80 °C. Perchloric acid extracts of frozen tissue samples were made as described by Churchill & Storey (1989). Aliquots of the well-mixed homogenate were removed for glycogen determination; glycogen was measured as glucose released after enzymatic hydrolysis with amyloglucosidase (Keppler & Decker, 1974). Pyruvate, PEP, arginine phosphate, ATP, ADP, and AMP were measured immediately in neutralized extracts and the remainder of the extract was then frozen at −80 °C for the subsequent assay of other metabolites. Metabolites were assayed spectrofluorometrically as described by Lowry & Passonneau (1972) except for succinate which was measured by the method of Williamson & Corkey (1969). Arginine phosphate and arginine were assayed using modifications of the procedures for creatine phosphate and creatine (Lowry & Passonneau, 1972) by substituting arginine kinase for creatine kinase and altering assay pH to 8.0. In separate assays, lactate was assayed using either D- or L-lactate dehydrogenase; only L-lactate was detected. Free amino acids were quantified using a Waters HPLC after precolumn derivatization with ortho-phthalaldehyde using the standard procedure provided by Waters Corp.

2.4. Tissue water content

Tissue water contents and dry masses were determined from initial and final weight measurements of tissue samples that were dried to a constant weight at 80 °C for 72 h. All data are expressed per g dry weight to correct for changes in tissue water content due to extracellular ice formation. Data are presented as means ± SEM, $n = 4$ mussels in each group. Statistical analysis was performed using one-way analysis of variance followed by the Dunnett's test (two-tailed).

3. Results

3.1. Survival, percent ice, and tissue water contents

Initial tests analyzed survival of the two stresses. Mussels showed 100% recovery after 2 h or 12 h of freezing at −6 °C or anoxia exposure at 5 °C. The 12 h exposure time was chosen to represent the normal maximum aerial exposure that could occur during the tidal cycle and has been used previously in characterizing the effects of temperature, salinity and season on freeze tolerance in this species (Murphy & Pierce, 1975). The shorter 2-h exposure was chosen to highlight differences between short term, transitional responses, particularly with respect to glycolysis and cellular energetics. Both 5- and 20 °C-acclimated mussels endured freezing and reached equivalent maximal ice contents after 12 h at −6 °C; values for ice, determined by calorimetry, were 55.5 ± 1.0% and 52 ± 1.5%, respectively (both $n = 6$) of total body water. However, the 5 °C-acclimated mussels were slower to accumulate ice and at the 2 h time point had only 39.5 ± 2.5% ice compared with the significantly higher value ($p < 0.01$) of 54.2 ± 1.2% ice for the 20 °C-acclimated animals (both $n = 6$).

The effect of anoxia or freezing exposure on the water content of tissues was determined from measurements of wet and dry masses. There was little or no effect of either stress on the tissue percent water. Overall, mean water contents were 91.7 ± 0.8, 80.5 ± 1.1, 91.1 ± 0.9, and 88.1 ± 0.9% of the wet masses for gill, adductor muscle, mantle, and hepatopancreas, respectively.

3.2. Anaerobic end product accumulation

Fig. 1 shows the effects of 2- and 12-h freezing and anoxia exposures on the levels of L-lactate, L-alanine, and succinate (three potential products of anaerobic metabolism) as well as L-aspartate (a fermentative substrate in marine molluscs) in gill and adductor muscle of mussels. Fig. 2 shows the comparable results for mantle and hepatopancreas. In gill, there were no significant changes in the levels of any of the four metabolites under either stress (Fig. 1a). In adductor muscle, however, lactate accumulated under both stresses, increasing by 2.6- and 2.2-fold after 12 h of anoxia or freezing, respectively (Fig. 1b). Mantle showed different metabolic responses to anoxia versus freezing (Fig. 2a). During anoxia in mantle succinate levels increased by 2-fold whereas aspartate decreased by 50%; the net decrease in aspartate (53 μmol/g dry weight) more than accounted for the net accumulation of succinate (36 μmol/g dry weight). During freezing, however, mantle accumulated lactate and succinate but no change in aspartate was seen. Lactate in mantle rose by 4- and 5.4-fold after 2 and 12 h of freezing, respectively, whereas succinate had risen by 4.3- and 3.1-fold at the same times. In hepatopancreas, an inverse relationship between succinate accumulation and aspartate depletion (net μmol/g dry weight) was again seen during anoxia; net changes after 12 h anoxia were 17 and 14 μmol/g dry weight, respectively (Fig. 2b). However, as also occurred in mantle, lactate and succinate again accumulated with no change in aspartate and, in addition, alanine levels also increased in frozen hepatopancreas. Changes after 12 h freezing were a 3.0-fold increase in lactate, a 3.4-fold increase in succinate, and a 40% rise in alanine.

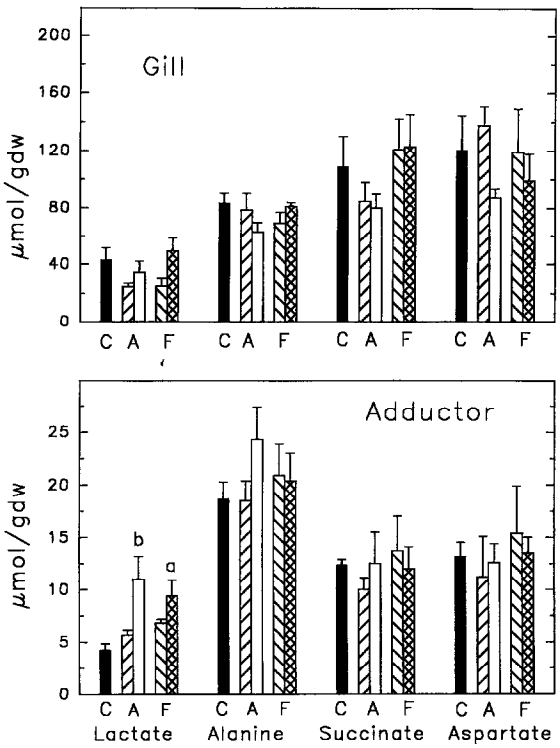

Fig. 1. Effects of anoxia and freezing exposures on the levels of L-lactate, L-alanine, succinate, and L-aspartate in gill and adductor muscle of the mussel, *G. demissus*. Data are means ± SEM, $n = 4$. Bars denote: (solid), control; (rising right), 2 h anoxia; (open), 12 h anoxia; (rising left), 2 h frozen; (crosshatched), 12 h frozen. Abbreviations are: (C) control, (A) anoxic, (F) frozen. For comparative purposes, control values for gill and adductor muscle, quantified as µmol/g wet weight, were 3.46 ± 0.66 and 0.86 ± 0.12 µmol/g wet weight for L-lactate, 6.7 ± 0.5 and 3.8 ± 0.3 µmol/g wet weight for L-alanine, 7.5 ± 1.7 and 2.5 ± 0.2 µmol/g wet weight for succinate, and 9.8 ± 1.9 and 2.7 ± 0.3 µmol/g wet weight for L-aspartate, respectively. a,b, Significantly different from the corresponding control value using the Dunnett's test, $p < 0.05$, $p < 0.01$, respectively.

The results for lactate deserve a further comment. The identification of L-lactate in this mollusc is unusual for other studies have shown that molluscs generally possess D-lactate dehydrogenase (LDH). We confirmed this unusual result in two ways. Firstly, perchloric acid extracts of tissues were incubated under the assay conditions for lactate in the presence of either L-LDH or D-LDH; a significant change in optical density was noted only in the presence of L-LDH. Secondly, we prepared tissue extracts for enzyme assay (in 20 mM imidazole buffer, pH 7.0) and then assayed these for LDH activity in the reverse direction (20 mM Tris buffer, pH 9, 2 mM NAD, 10 mM lactate) and found enzyme activity only with L-lactate as the substrate.

3.3. Regulation of glycolysis

Table 1 shows the effect of anoxia and freezing exposures on the levels of glucose in mussel tissues. Glucose levels in all four tissues were unaffected by anoxia exposure

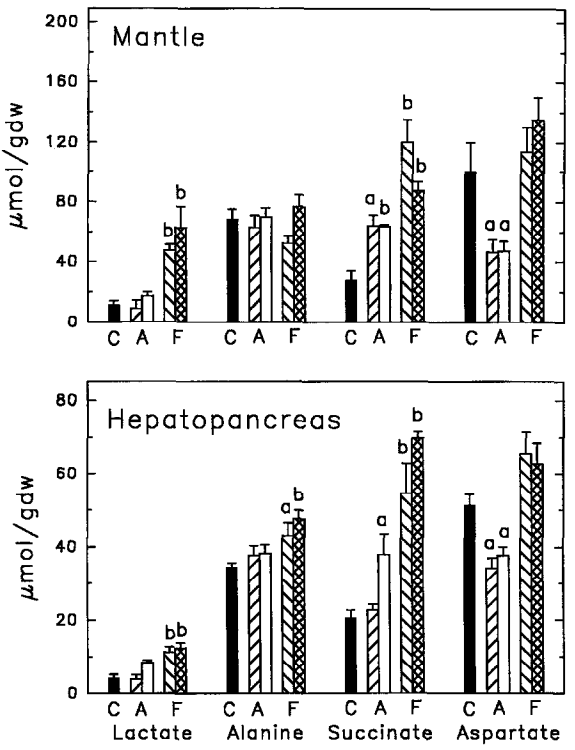

Fig. 2. Effects of anoxia and freezing exposures on the levels of L-lactate, L-alanine, succinate, and L-aspartate in mantle and hepatopancreas of the mussel, *G. demissus*. Other information as in Fig. 1. For comparative purposes, control values for mantle and hepatopancreas, quantified as μmol/g wet weight, were 1.18 ± 0.24, and 0.58 ± 0.11 for L-lactate, 6.9 ± 0.7 and 4.8 ± 0.2 μmol/g wet weight for L-alanine, 4.7 ± 1.1 and 3.7 ± 0.8 μmol/g wet weight, for succinate, and 6.5 ± 0.3 and 7.2 ± 0.5 μmol/g wet weight for L-aspartate, respectively.

but freezing led to elevated glucose in all cases. Both adductor muscle and mantle showed increased glucose after 2 h freezing exposure and after 12 h glucose had risen

Table 1
Effect of anoxia and freezing exposures on glucose in *G. demissus* tissues

	Gill	Adductor	Mantle	Hepatopancreas
	μmol/g dry weight			
Control	7.60 ± 1.21	1.76 ± 0.21	5.02 ± 1.23	3.53 ± 0.61
2 h Anoxic	5.22 ± 0.67	2.16 ± 0.25	5.30 ± 0.51	2.53 ± 0.44
12 h Anoxic	7.35 ± 0.68	2.60 ± 0.45	4.80 ± 0.49	3.22 ± 0.44
2 h Frozen	8.78 ± 1.78	3.23 ± 0.27[a]	9.82 ± 0.74[a]	4.65 ± 0.53
12 h Frozen	18.3 ± 2.07[a]	3.63 ± 0.26[a]	13.8 ± 1.63[a]	9.71 ± 0.40[a]

Data are means ± SEM, $n = 4$. [a] Significantly different from the corresponding control value by the Dunnett's test, $p < 0.01$.

by 2.4-, 2.1-, 2.8-, 2.8-fold in gill, adductor, mantle, and hepatopancreas, respectively. Glycogen contents of the tissues varied widely being lowest in adductor muscle and 17-fold higher in mantle but glycogen contents were unaltered by either stress in three organs. Overall mean values were 1.6 ± 0.2, 27.0 ± 1.5, and 10.7 ± 0.3 mmol/g dry weight for adductor, mantle, and hepatopancreas, respectively. Both stresses, however, stimulated a decrease in gill glycogen which fell significantly from 8.8 ± 1.1 mmol/g dry weight in controls to 3.6 ± 0.4 and 3.2 ± 0.4 in 2 and 12 h anoxic animals and to 4.7 ± 0.8 and 7.6 ± 0.6 mmol/g dry weight in 2 and 12 h frozen animals ($p < 0.05$).

Levels of glycolytic intermediates in mantle, hepatopancreas and adductor muscle are shown in Table 2. Glycolytic intermediates in gill were also quantified but no changes in the concentration of any intermediate occurred as a result of either anoxia or freezing (data not shown). In anoxic mantle, levels of fructose-6-phosphate (F6P) rose significantly by 2 h and by 12 h levels of glucose-1-phosphate (G1P) and glucose-6-phosphate (G6P) had also increased significantly. Levels of each of the three hexose phosphates were 3–5-fold higher in 12 h anoxic mantle than in controls. Levels of phosphoenolpyruvate (PEP) and pyruvate also changed in mantle during anoxia, PEP increasing and pyruvate decreasing. After 12 h of anoxia exposure PEP had increased by 2.7-fold whereas pyruvate had decreased to only 23% of the control value (Table 2). During freezing, mantle showed no changes in the levels of hexose phosphates, fructose-1,6-bisphosphate ($F1,6P_2$), or the triose phosphates. However, PEP and pyruvate changed significantly; PEP was elevated at both sampling times whereas pyruvate decreased after 2 h but returned to control values by 12 h. In hepatopancreas, glycolytic intermediates showed no changes from control values during anoxia but levels of all intermediates, except $F1,6P_2$, changed during freezing exposure. Levels of G1P and G6P increased by 19- and 12-fold, respectively, after 12 h freezing whereas F6P content dropped by 50%. Combined dihydroxyacetonephosphate + glyceraldehyde-3-phosphate (DHAP + GAP) levels rose significantly after 2 h freezing, as did PEP and pyruvate. However, only PEP remained elevated after 12 h freezing exposure. In adductor muscle, anoxia exposure led to an increase in F6P and $F1,6P_2$ after 2 h and these had increased further after 12 h, accompanied by a rise in G6P levels. However, triose intermediates of glycolysis were not affected by anoxia exposure. During freezing exposure, G6P and F6P in muscle also increased, reaching higher levels than in anoxic tissue, whereas G1P content was sharply decreased. Again, triose intermediates were not altered during freezing exposure.

3.4. Energy status

Adenylates and phosphagen (arginine phosphate) were quantified in all tissues. Levels of arginine phosphate did not change in any of the tissues under either of the experimental stresses; mean levels of arginine phosphate (combining data for all five conditions) were 16.8 ± 1.3, 16.9 ± 2.0, 69.2 ± 12.7, and 45.1 ± 4.2 μmol/g dry weight ($n = 20$) in gill, adductor muscle, mantle, and hepatopancreas, respectively. Similarly, adenylate energy status was unperturbed by anoxia or freezing exposures in adductor muscle or hepatopancreas. Overall mean values for ATP, ADP and AMP were 33.4 ± 2.4, 17.0 ± 0.7, and 3.7 ± 0.2 μmol/g dry weight in adductor ($n = 20$) and

Table 2
Effect of anoxia and freezing on the levels of glycolytic intermediates in adductor muscle, mantle, and hepatopancreas of the mussel, *G. demissus*

	G1P	G6P	F6P	$F1,6P_2$	DHAP + GAP	PEP	Pyruvate
	μmol/g dry weight						
Mantle:							
Control	0.40 ± 0.14	0.65 ± 0.21	0.53 ± 0.18	0.09 ± 0.02	1.07 ± 0.38	0.89 ± 0.59	3.52 ± 0.34
2 h Anoxic	n.d.	1.25 ± 0.24	1.32 ± 0.22[a]	0.07 ± 0.04	1.10 ± 0.07	1.18 ± 0.46	0.87 ± 0.28[b]
12 h Anoxic	1.29 ± 0.55[a]	3.46 ± 0.44[a]	1.31 ± 0.25[a]	0.08 ± 0.03	1.07 ± 0.24	2.41 ± 0.62[a]	0.82 ± 0.09[b]
2 h Frozen	0.25 ± 0.07	0.87 ± 0.25	0.16 ± 0.04	0.15 ± 0.04	1.06 ± 0.13	3.99 ± 0.35[b]	2.00 ± 0.09[a]
12 h Frozen	0.42 ± 0.15	1.37 ± 0.64	0.18 ± 0.03	0.18 ± 0.06	1.06 ± 0.21	2.96 ± 0.34[a]	3.01 ± 0.81
Hepatopancreas:							
Control	0.09 ± 0.04	0.24 ± 0.11	0.37 ± 0.08	0.13 ± 0.02	0.74 ± 0.09	2.03 ± 0.46	0.86 ± 0.19
2 h Anoxic	0.13 ± 0.05	0.27 ± 0.19	0.40 ± 0.10	0.09 ± 0.02	0.60 ± 0.10	1.54 ± 0.21	1.25 ± 0.31
12 h Anoxic	0.26 ± 0.10	0.65 ± 0.17	0.41 ± 0.10	0.08 ± 0.01	0.90 ± 0.19	1.57 ± 0.36	1.80 ± 0.50
2 h Frozen	1.50 ± 0.08[b]	0.78 ± 0.19	0.11 ± 0.01[a]	0.08 ± 0.01	1.26 ± 0.09[a]	3.69 ± 0.41[b]	2.42 ± 0.64[a]
12 h Frozen	1.70 ± 0.07[b]	2.84 ± 0.23[b]	0.18 ± 0.03[a]	0.11 ± 0.03	1.00 ± 0.14	3.27 ± 0.33[a]	1.84 ± 0.44
Adductor muscle:							
Control	1.15 ± 0.25	0.26 ± 0.05	0.04 ± 0.01	0.05 ± 0.01	0.17 ± 0.02	0.41 ± 0.04	2.6 ± 0.4
2 h Anoxic	0.93 ± 0.36	0.21 ± 0.12	0.09 ± 0.01[a]	0.06 ± 0.01[a]	0.18 ± 0.02	0.61 ± 0.09	2.7 ± 0.1
12 h Anoxic	1.53 ± 0.39	0.93 ± 0.07[b]	0.13 ± 0.01[b]	0.08 ± 0.01[a]	0.16 ± 0.02	0.98 ± 0.23	2.8 ± 0.1
2 h Frozen	0.48 ± 0.30	1.14 ± 0.06[b]	0.11 ± 0.02[b]	0.04 ± 0.01	0.19 ± 0.03	0.51 ± 0.24	3.2 ± 0.2
12 h Frozen	0.13 ± 0.12[a]	1.93 ± 0.14[b]	0.28 ± 0.02[b]	0.03 ± 0.01	0.12 ± 0.04	0.72 ± 0.22	3.4 ± 0.3

Data are means ± SEM, $n = 4$. [a,b]Significantly different from the corresponding control values using the Dunnett's test, $p < 0.05$, $p < 0.01$, respectively. n.d. = not determined.

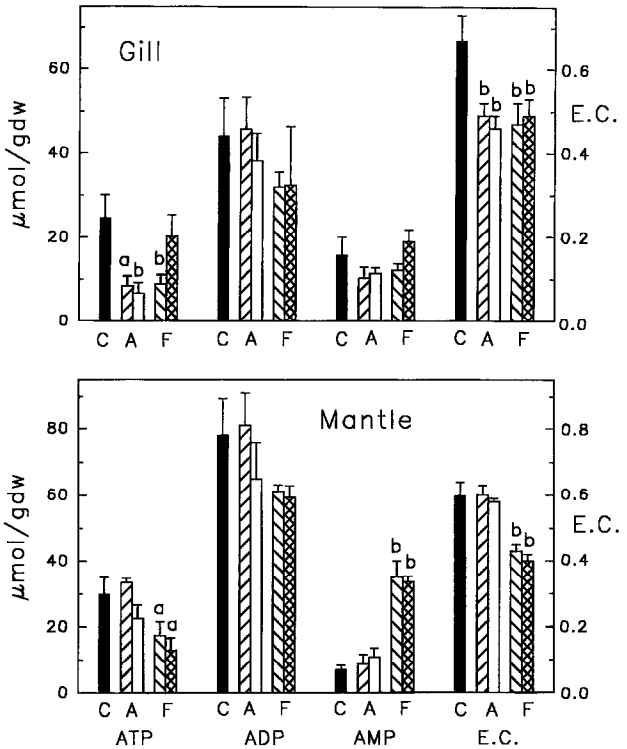

Fig. 3. Effects of anoxia and freezing exposures on ATP, ADP, and AMP levels and energy charge in gill and mantle of the mussel, *G. demissus*. Energy charge is defined as [ATP + 1/2 ADP]/[ATP + ADP + AMP]. Other information as in Fig. 1.

19.1 ± 1.4, 31.0 ± 4.9, and 14.3 ± 1.1 μmol/g dry weight ($n = 20$) in hepatopancreas. Mean energy charge, defined as [ATP + 1/2 ADP]/[ATP + ADP + AMP], was 0.77 ± 0.01 in adductor muscle and 0.53 ± 0.02 in hepatopancreas. Effects of anoxia and freezing exposures on adenylates and energy charge in gill and mantle are shown in Fig. 3. ATP levels in gill decreased significantly during anoxia as did energy charge which fell from 0.67 in controls to 0.46 in 12 h anoxic mussels. ATP was also significantly reduced in 2 h frozen gill and energy charge was significantly lower than control values in gills from both 2 and 12 h frozen mussels. In mantle, adenylates and energy charge were unaffected by anoxia exposure but during freezing ATP levels dropped by about one-half, AMP rose 5-fold, and energy charge dropped from 0.60 ± 0.04 in controls to 0.40 ± 0.02 in 12 h frozen animals.

3.5. *Amino acids*

Levels of glycine and taurine, the two major amino acids in *G. demissus* tissues are shown in Table 3 along with values for the total free amino acid pool. Generally, glycine comprised 15–25% of the total pool size and taurine made up 50–75% of the pool.

Table 3
Effect of 12 h anoxia or freezing exposures on the levels of glycine, taurine and total free amino acids in tissues of *G. demissus*

	Glycine	(%)	Taurine	(%)	Total AAs
	μmol/g dry weight				
Gill:					
Control	288 ± 46	(15)	1108 ± 115	(72)	1534 ± 171
Anoxic	169 ± 2[a]	(17)	762 ± 54[a]	(76)	1003 ± 59[a]
Frozen	163 ± 28[a]	(15)	538 ± 57[a]	(48)	1120 ± 37[a]
Adductor:					
Control	157 ± 49	(25)	382 ± 48	(61)	627 ± 123
Anoxic	153 ± 32	(25)	389 ± 32	(64)	605 ± 65
Frozen	132 ± 50	(17)	422 ± 110	(57)	776 ± 36
Mantle:					
Control	400 ± 31	(24)	928 ± 44	(55)	1696 ± 70
Anoxic	184 ± 11[a]	(20)	615 ± 31[a]	(66)	930 ± 61[a]
Frozen	242 ± 16[a]	(17)	873 ± 124	(63)	1393 ± 141
Hepatopancreas:					
Control	277 ± 37	(24)	664 ± 47	(57)	1158 ± 76
Anoxic	256 ± 33	(20)	827 ± 45	(65)	1275 ± 72
Frozen	250 ± 48	(19)	886 ± 115	(67)	1326 ± 119

Data are means ± SEM, $n = 3$; values in brackets show glycine or taurine values as percentages of the total pool size. Levels of other amino acids generally ranged between 1 and solidus 50 μmol/g dry weight.
[a]Significantly different from the corresponding control value, $p < 0.05$.

Together, these two amino acids accounted for 87, 86, 79, and 81% of the total free amino acid pool in control gill, adductor muscle, mantle, and hepatopancreas, respectively. Levels of other amino acids rarely exceeded 50 μmol/g dry weight, except for aspartate and alanine in some instances (Figs. 1 and 2). Again, apart from aspartate and alanine, the levels of amino acids showed no significant changes during either freezing or anoxia in adductor muscle and hepatopancreas. In mantle, a general decrease in the levels of all amino acids, including glycine and taurine, occurred during anoxia but only glycine and four minor amino acids decreased in mantle of freezing-exposed mussels. In gill, levels of glycine and taurine decreased under both stresses but among other amino acids, only seven decreased in anoxia and three during freezing.

4. Discussion

The freezing exposure used in the present experiments (up to 12 h at −6 °C) represents a natural condition that could readily occur during aerial exposure at winter low tides. Mussels readily survived this exposure, in agreement with previous studies that showed an LD_{50} value (for 12-h exposures) of −13 °C for winter-collected *G. demissus* acclimated to 4 °C (Murphy & Pierce, 1975), correlated with an ice content of adductor

muscle of about 80%. Thus, the freezing conditions used here were well within survivable limits and, with an extracellular ice content 52–55% of total body water, placed a substantial stress on cells that should elicit cryoprotective responses. Murphy & Pierce (1975) showed that a decrease in acclimation temperature pushed the 50% survival values to lower subzero temperatures and also increased the amount of body ice tolerated. Our present results for 20 °C versus 5 °C-acclimated mussels suggest that one reason for this effect of acclimation temperature on freezing survival may be an adaptive response that lowers the rate of ice accumulation in cold-acclimated animals. Thus, 20 °C-acclimated mussels had reached maximal ice content within 2 h at −6 °C (ice content did not change between 2 and 12 h) whereas 5 °C-acclimated mussels had reached only about 40% ice within this time. Obviously, then, the rate at which osmotic stress due to freezing was imposed on tissues was greater for the 20 °C mussels and this would reduce the time available for animals to implement cell volume regulatory and cryoprotective responses.

By its nature, extracellular freezing imposes an ischemic state on tissues; cells are cut off from extracellular supplies of oxygen and substrates for the duration of the freezing stress and must rely upon endogenous fuels and fermentative pathways for energy production (Storey & Storey, 1988). Presumably, therefore, at least some of the metabolic responses to freezing by freeze tolerant species should be responses that deal with ischemia/anoxia. Hence, it could be expected that the metabolic responses used by intertidal molluscs to deal with anoxia during aerial exposure (e.g. alternative pathways of fermentative ATP production, metabolic rate depression) might also be expressed as responses to freezing. The present study aimed to determine if this was true and, furthermore, by a comparison of freezing versus anoxia effects on intermediary metabolism, to differentiate between those metabolic responses to freezing that deal primarily with the energy stress due to oxygen lack and those that are primarily cryoprotective, addressing cell volume regulation and the stabilization of membranes and other macromolecules.

The metabolic responses to freezing and anoxia by tissues of *G. demissus* clearly differed; some general differences were exhibited by all tissues whereas others were tissue-specific. For example, all tissues showed a significant increase in glucose levels during freezing whereas glucose did not change in any tissue during anoxia. Elevated glucose suggests an increase in glycogenolysis during freezing to support fermentative metabolism and increased glycolytic activity was also indicated by changes in the levels of glycolytic intermediates and the accumulation of lactate in three tissues during freezing. Glycogen reserves in all tissues were very high (about 1000-fold greater than glucose levels) so it was not surprising that no significant changes in glycogen were seen in adductor muscle, mantle, and hepatopancreas despite the accumulation of glycolytic end products during freezing. However, gill glycogen decreased significantly during both freezing and anoxia and this result may merit further investigation as it could suggest a possible synthesis and/or export of carbohydrate products under these stresses. However, apart from glucose we found no synthesis of other common carbohydrate cryoprotectants such as glycerol, fructose, and sorbitol (data not shown) during freezing exposure in any tissue.

Mantle and hepatopancreas both showed distinct patterns of metabolic end prod-

uct accumulation under anoxia versus freezing. Both tissues accumulated only succinate as a metabolic end product in anoxia (lactate and alanine did not change) and this was matched by an opposite decrease in aspartate. The fermentation of aspartate to succinate, with ATP generated at the fumarate reductase reaction, is a common pathway of anaerobic energy production in molluscs (de Zwaan, 1983). During freezing, however, both tissues not only accumulated higher amounts of succinate than during anoxia but both also built up lactate and hepatopancreas also accumulated alanine. These results implicated anaerobic glycolysis as the main pathway for ATP production during freezing. Both lactate and alanine are directly produced from pyruvate whereas succinate accumulation, in the absence of aspartate utilization, implies that succinate was derived from glycolytic carbon using the phosphoenolpyruvate carboxykinase reaction to channel PEP into the pathway of succinate synthesis (de Zwaan, 1983). Thus, metabolic responses to the two stresses appear to differ qualitatively in mantle and hepatopancreas, the response to freezing apparently involving carbohydrate fermentation via glycolysis to lead to multiple glycolytic end products whereas the response to anoxia involved aspartate fermentation. Responses to the two stresses also differed quantitatively, the total end product accumulation being 3–4-fold higher at 110 and 70 μmol/g dry weight (net succinate + lactate + alanine) in mantle and hepatopancreas from frozen mussels versus only 36 and 17 μmol/g dry weight in anoxic mussels, respectively (Fig. 2). This indicates a substantially higher mean rate of anaerobic glycolysis in some tissues of frozen mussels, particularly when the difference in body temperatures (11 °C lower for frozen mussels) is also considered. One reason for this may be that end products could be accumulated during freezing, not just to meet ATP demands, but also for cryoprotective purposes. Thus, even though the net accumulation of these products would have little colligative impact on cell volume regulation during freezing, Loomis et al. (1989) have demonstrated that anaerobic end products including lactate, succinate and alanine are effective in protecting either membrane bilayer structure or enzyme activity from freezing damage. Alternately, the demand for fermentative ATP production during freezing might be enhanced. In support of this, the data of Fig. 3 show that mantle underwent a significant energy stress during freezing since both ATP levels and energy charge decreased in mantle of frozen mussels but were unaltered in anoxic animals. One reason for a greater ATP demand during freezing might be to support the implementation of other cryoprotective or cell volume regulatory measures that are energy-expensive. Oppositely, higher rates of fermentative metabolism during freezing might suggest that mechanisms of metabolic arrest that are typically induced when oxygen tension falls below a critical level may, for some reason, be less effectively implemented when oxygen limitation develops due to ischemia (restriction of O_2 delivery by ice formation) rather than due to anoxia (lack of environmental O_2).

Changes in the levels of glycolytic intermediates in mantle and hepatopancreas also showed that the two tissues responded differently to freezing versus anoxia. In mantle anoxia led to an accumulation of F6P (and other hexose phosphates) without a change in F1,6P_2. Similarly, PEP levels rose whereas pyruvate levels fell during anoxia. These paired changes in the substrates (increases) and products (decrease or no change) of the two regulatory enzymes of glycolysis, phosphofructokinase (PFK) and pyruvate

kinase (PK), are indicative of inhibitory blocks on glycolytic flux at both of these loci during anoxia. This is a common feature of anoxia-induced metabolic arrest in marine molluscs and is regulated by protein kinase-mediated phosphorylation of the two enzymes to create less active enzyme forms (Storey, 1992). During freezing changes in PEP and pyruvate levels still indicated inhibitory control on PK. However, comparable changes in F6P and $F1,6P_2$ that would indicate inhibition of PFK were lacking; indeed, a trend indicating the opposite response was indicated, although due to high SEM values the changes were not significant. These data are consistent with the end product data for mantle. Thus, the absence of inhibitory control on mantle PFK during freezing is consistent with a higher net flux through glycolysis and the greater net accumulation of end products derived from carbohydrates during freezing, compared with anoxia. The maintenance of the inhibitory block at PK, however, is consistent with the proposal, discussed above, that succinate is probably derived from glycolytic carbon during freezing. Glycolytic intermediates in hepatopancreas also showed different responses under the two stresses. No significant changes in these compounds occurred during anoxia whereas during freezing levels of G1P and G6P were greatly increased, consistent with the rise in glucose levels. However, F6P levels fell, suggesting an activation of PFK whereas levels of all the triose intermediates rose. Again, this is consistent with a higher overall end product accumulation in hepatopancreas of freezing-exposed mussels and the probable derivation of succinate from glycolytic carbon rather than from aspartate.

Metabolic responses by adductor muscle and gill were both quantitatively and qualitatively different from those of mantle and hepatopancreas. Glycolysis ending with lactate accumulation appeared to be the sole mode of energy production in both anoxic and frozen adductor muscle. There was no evidence of the possible production of other pyruvate-utilizing end products; alanine did not accumulate and neither arginine phosphate nor arginine levels changed in anoxia or freezing suggesting that octopine would not be accumulated. The glycolytic nature of muscle metabolism was further supported by the rise in G6P and F6P levels under both stresses. However, the patterns of change in glycolytic intermediates in muscle suggested different regulatory loci under anoxia versus freezing stresses. During freezing, the rise in F6P with no change in $F1,6P_2$ levels indicates that an inhibitory block on glycolytic flux was exerted at the PFK locus; this is consistent with the higher levels of both F6P and G6P, as well as the accumulation of glucose, seen in adductor from frozen versus anoxic mussels. During anoxia, however, both hexose phosphates and $F1,6P_2$ were elevated suggesting that PFK was not regulating glycolytic flux under this condition.

In gill, metabolic responses appeared to be very limited under either stress. No changes in metabolic end products or glycolytic intermediates were seen (Fig. 1, Table 2) although the reduced ATP content and energy charge indicated that the tissue experienced energy stress under both anoxia and freezing (Fig. 3). The major effect of freezing or anoxia exposure on gill was a decrease in the total amino acid pool of 25–35%, resulting primarily from decreases in glycine and taurine levels. Mantle also showed a significant decrease in the free amino acid pool during anoxia but not during freezing. These results were unexpected for changes in glycine and taurine levels are generally in response to osmotic stresses on cells and no osmotic stress should have

occurred during anoxia because of shell valve closure. Furthermore, the effect of extracellular ice formation (because it draws water out of cells) should be a passive elevation of the levels of intracellular osmolytes during freezing rather than their loss. However, the fact that gill showed the same amino acid loss under both anoxia and freezing indicates that the changes may represent natural adjustments made by this tissue during shell valve closure. Additional studies are needed to elucidate the reasons for these tissue-specific responses by the free amino acid pools.

In summary, then, the data show that each tissue of the mussel *G. demissus* has specific and different metabolic responses to anoxia and freezing. In general, metabolic arrest and well-known patterns of fermentative metabolism were elicited by anoxia exposure whereas freezing exposure led to an overall higher accumulation of end products, which might have cryoprotective consequences, and imposed a greater energy stress on tissues.

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