

The role of free amino acids in cellular osmoregulation in the freshwater bivalve *Ligumia subrostrata* (Say)

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Free amino acids constitute 11% of the cellular solute in *Ligumia subrostrata* acclimated in pond water but less than 1% of blood total solute. Arginine, glutamate, histidine, and alanine account for 60% of the cellular free amino acid concentration. Tissue free amino acid concentrations increase when the animals are exposed to dehydration and anoxic stress. Free amino acids contributing most to the elevated tissue amino acid concentrations are glutamate, alanine, threonine, and serine. These specific free amino acids play an important role in cellular volume regulation.

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Les acides aminés libres constituent 11% du soluté cellulaire, mais moins de 1% du soluté sanguin, chez des *Ligumia subrostrata* acclimatés à de l'eau d'étang. 60% de la concentration cellulaire d'acides aminés est formé d'arginine, de glutamate, d'histidine et d'alanine. Les concentrations d'acides aminés libres dans les tissus augmentent lorsque l'animal subit une déshydratation ou lorsqu'il est soumis à une anoxie. Le glutamate, l'alanine, la thréonine et la sérine sont les acides aminés libres qui contribuent le plus aux concentrations élevées d'acides aminés dans les tissus: ils jouent un rôle important dans le contrôle du volume cellulaire.

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Introduction

Marine and brackish-water bivalves use free amino acids for intracellular osmoregulation (Simpson *et al.* 1959). Freshwater bivalves maintain significantly lower body-fluid solute concentrations, but free amino acids constitute up to 20% of the intracellular solute (Potts 1958). In addition, intracellular free amino acids increase severalfold when freshwater bivalves are acclimated to dilute seawater (Potts 1958; Bedford 1973).

Salinity stress is not common in freshwater bivalves; however, anoxia and occasional dehydration are more probable forms of environmental stress (Dietz 1974). In this study we used the latter forms of stress to induce an osmotic imbalance to quantitate the changes and identify the contribution of 15 free amino acids in cellular osmoregulation.

Materials and Methods

Male *Ligumia subrostrata* (Pelecypoda: Unionidae) were collected from a pond near Baton Rouge from November 1974 to July 1975. The animals were accli-

mated to artificial pond water (0.5 mM NaCl, 0.4 mM CaCl₂, 0.2 mM NaHCO₃, 0.05 mM KCl) at 21-24 °C for 3 to 5 days before use. After acclimation the animals were either sampled or transferred to containers for dehydration or anoxic stress. Animals subjected to dehydration were placed on an open tray (22-25 °C) at 45-55% relative humidity for 4 to 5 days. Other animals were transferred to a desiccator jar and supported above water in a N₂ atmosphere (P_O₂ < 0.2 torr (1 torr = 1 mm Hg)). The atmosphere was replaced two or three times daily by 5-min flushing with N₂ gas. CO₂ absorbant was placed in the container.

Blood was obtained by cardiac puncture (Fyhn and Costlow 1975) and mixed immediately with 60% sulfosalicylic acid to obtain a final acid concentration of 10%. The samples were refrigerated (4 °C) overnight, centrifuged 10 min at 8000 g and the supernatant transferred to a polyethylene vial for amino acid analysis.

The entire soft tissue was dissected from the shell, transferred to a container, and frozen (-20 °C). The frozen tissue was transferred to a freeze dryer (-50 °C) and dried to constant weight (48 h). The tissue was ground with a tissue mill and 100 mg tissue extracted in 10 ml 10% sulfosalicylic acid for 48 h (4 °C). Each extract was centrifuged 5 min at 8000 g and the supernatant used for amino acid analysis.

Free amino acid concentrations in the extracts were determined with a Beckman amino acid analyzer. Samples from three or more animals are expressed as mean ± one SEM. Data obtained from one or two animals are listed separately and not averaged. Differences between controls acclimated to pond water and stressed animals

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TABLE 1. Free amino acid concentrations in *Ligumia subrostrata* acclimated to pond water

Amino acid	Blood, nM/ml	Tissue	
		nM/g dry	Calculated, ^a nM/ml ICF
Aspartate	27 ± 10(4)	1702 ± 149(6)	436 ± 37(6)
Threonine	59 ± 13(4)	1093 ± 221(6)	280 ± 57(6)
Serine	83 ± 7(4)	1678 ± 93(6)	430 ± 24(6)
Glutamate	54 ± 16(4)	3055 ± 304(6)	783 ± 77(6)
Proline	14 ± 1(4)	T ^b	T
Glycine	12 ± 4(4)	1236 ± 156(6)	317 ± 40(6)
Alanine	55 ± 7(4)	2046 ± 158(6)	524 ± 39(6)
Valine	14 ± 2(4)	635 ± 66(6)	162 ± 19(6)
Isoleucine	8 ± 1(4)	188 ± 24(6)	48 ± 6(6)
Leucine	13 ± 1(4)	335 ± 19(6)	86 ± 6(6)
Tyrosine	3	541 ± 87(3)	139 ± 24(3)
Phenylalanine	8 ± 1(3)	616 ± 193(5)	158 ± 48(5)
Histidine	26 ± 1(4)	2527 ± 185(6)	648 ± 48(6)
Lysine	15 ± 4(4)	1385 ± 122(6)	355 ± 32(6)
Arginine	14 ± 4(4)	6533 ± 960(6)	1675 ± 247(6)

^aBased on 3.90 ml ICF/g dry tissue.^bT = trace.

were determined using the Student *t*-test and were considered significant if $P < 0.05$.

Results

Total blood-solute concentration in *L. subrostrata* is about 53 mosM. The total free amino acid contribution to blood solute in mussels acclimated to pond water is small (0.4 mM). The most abundant blood free amino acids are serine, threonine, alanine, and glutamate (Table 1).

Free amino acid content in tissues is 23.6 μ M/g dry tissue. Using an intracellular fluid volume of 3.90 ml/g dry tissue (Murphy and Dietz 1976), the calculated intracellular fluid (ICF) concentration of total free amino acids is 6.1 mM. Cellular osmoticity is probably equal to the blood. The amino acid contribution to total cellular solute is about 11%. The most abundant cellular free amino acids are arginine, glutamate, histidine, alanine, aspartate, and serine. A trace of proline was detected in tissues from animals acclimated to pond water but could not be quantitated.

L. subrostrata dehydrate when they are removed from water and are exposed to air (relative humidity 45–55%). Table 2 shows the effect of moderate dehydration (17% loss of tissue weight) on free amino acid content. Total tissue free amino acid content increased 64% relative to mussels acclimated to pond water (38.7 μ M/g dry tissue). Ten free amino acids increased signifi-

cantly. The amino acids contributing most to the elevated tissue content are glutamate, alanine, threonine, serine, and tyrosine. Tissue arginine content decreased slightly.

During dehydration, water is not lost equally from extracellular and intracellular fluid compartments; cell volume is conserved. From previous studies (Dietz 1974), when these animals lose 17% of the tissue weight the predicted increase in blood Na concentration is 20% above normal. The observed blood Na concentration increased 29%. Thus, of the 1.5 g H₂O/g dry tissue lost from an animal during dehydration 0.98 ml was from the extracellular fluid compartment and 0.52 ml was from the intracellular compartment. We therefore used the value of 3.38 ml/g dry tissue as the intracellular fluid volume to calculate the intracellular free amino acid concentrations in Table 2. The free amino acid concentration increased 88% over the controls acclimated to pond water, leading to a significant contribution to intracellular solute.

Total blood free amino acid concentration increased 16% above mussels acclimated to pond water. Although most of the individual free amino acids tended to increase in concentration, only phenylalanine increased significantly. Free amino acid contribution to total blood solute remains negligible (0.47 mM free amino acids, 65 mosM, respectively).

Exposure of *L. subrostrata* to a N₂ atmosphere

for 3 days caused a 54% increase in total free amino acid content (36.3 μ M/g dry tissue). Animals were in a water-saturated atmosphere, which minimized weight loss. The concentration of free amino acids increased (10.5 μ M/g dry tissue) and free amino acids increased significantly with animals acclimated to pond water.

TAB1

Am

Aspa
Thre
Serin
Gluta
Proli
Glyci
Alani
Valin
Isoleu
Leuci
Tyros
Pheny
Histic
Lysin
ArginNor
Bas

TA

A

As
Th
Ser
Glu
Pro
Gly
Ala
Val
Iso
Leu
Tyr
Phe
His
Lys
ArgNo
0.01
B

TABLE 2. Free amino acid concentrations in *Ligumia subrostrata* subjected to dehydration

Amino acid	Blood, nM/ml	Tissue	
		nM/g dry	Calculated, ^a nM/ml ICF
Aspartate	13 ± 1(3)	2216 ± 225(5)	656 ± 66(5)
Threonine	21	3073 ± 319(5)**	909 ± 94(5)
Serine	87 ± 47(3)	2709 ± 317(5)**	802 ± 94(5)
Glutamate	94 ± 51(3)	7473 ± 767(5)**	2211 ± 227(5)
Proline	28 ± 8(3)	1362 ± 402(5)	403 ± 119(5)
Glycine	4 ± 1(3)	1333 ± 212(5)	394 ± 63(5)
Alanine	69 ± 11(3)	4695 ± 1041(5)*	1389 ± 308(5)
Valine	18 ± 2(3)	871 ± 43(5)*	258 ± 13(5)
Isoleucine	9 ± 2(3)	302 ± 39(5)*	89 ± 12(5)
Leucine	15 ± 4(3)	519 ± 50(5)**	153 ± 15(5)
Tyrosine	34 ± 13(3)	2027 ± 300(5)**	600 ± 89(5)
Phenylalanine	22 ± 1(3)	1257 ± 180(5)*	372 ± 53(5)
Histidine	24 ± 6(3)	3392 ± 470(5)	1004 ± 139(5)
Lysine	24 ± 10(3)	2228 ± 228(5)*	659 ± 67(5)
Arginine	6 ± 2(3)	5248 ± 488(5)	1553 ± 144(5)

NOTE: Significantly different from pond-water animals: **P* < 0.05, ***P* < 0.01.
^aBased on 3.38 ml ICF/g dry tissue.

TABLE 3. Free amino acid concentrations in *Ligumia subrostrata* subjected to N₂ atmosphere

Amino acid	Blood, nM/ml	Tissue	
		nM/g dry	Calculated, ^a nM/ml ICF
Aspartate	13, 13	2050, 1798	526, 461
Threonine	5, 9	3536, 3509**	907, 900
Serine	11, 14	2834, 2857**	727, 733
Glutamate	10, 12	6540, 5526*	1677, 1417
Proline	T	1164, 1436**	298, 368
Glycine	5, 7	988, 1213	253, 311
Alanine	13, 13	4603, 4541**	1180, 1164
Valine	8, 6	951, 1099	243, 282
Isoleucine	1, 1	411, 300*	105, 77
Leucine	3, 2	703, 482**	180, 124
Tyrosine	7, 6	1041, 1212	267, 311
Phenylalanine	9, 8	1510, 1378	387, 353
Histidine	15, 14	3283, 3328	842, 853
Lysine	6	1963, 2460	503, 631
Arginine	5, 2	4824, 5058	1237, 1297

NOTE: Significantly different from pond-water animals: **P* < 0.05; ***P* < 0.01.
^aBased on 3.90 ml ICF/g dry tissue.

for 3 days caused a 54% increase in tissue free amino acid content (36.3 μM/g dry tissue). The animals were in a water-saturated N₂ environment, which minimized dehydration to 5% weight loss. The concentration of all but two free amino acids increased (Table 3). Seven free amino acids increased significantly when compared with animals acclimated to pond water.

Cellular arginine concentration declined slightly. It is not known if there are shifts in fluid compartment volumes owing to anoxia. Since dehydration was minimal, we have expressed the intracellular free amino acid concentrations assuming 3.90 ml H₂O/g dry tissue. The free amino acids contributing most to the elevated tissue amino acid concentration are alanine, glutamate,

threonine, and serine. These are the same amino acids that increased when the animals were desiccated.

Total blood free amino acids declined significantly in the anoxic animals (0.11 mM). The specific amino acids present in low concentrations in control animal blood decreased about 50% during anoxia. The specific amino acids that normally are present in larger amounts (threonine, serine, glutamate, and alanine) were reduced to 10–30% of control concentrations.

Discussion

Intracellular free amino acid concentrations increase when *L. subrostrata* are subjected to dehydration. The differential changes in amino acids suggest the presence of specific regulatory mechanisms. Of the 15 amino acids measured, three accounted for 60% of the elevated free amino acid concentration (glutamate, alanine, and threonine). The source of amino acids is unknown but must be endogenous since the animals were not feeding.

The elevated free amino acids contribute significantly to cellular volume regulation. When mussels lose 17% of their fresh tissue weight there is a 12-mosM elevation in blood total solute, from 53 to 65 mosM (Dietz 1974). Intracellular solute concentration must also increase 12 mosM. The increase in cellular free amino acid concentration is about 5 mosM. The remaining intracellular solute increase is primarily due to water loss from the cells. Thus, if there were no solute losses from the cells, contraction of the intracellular fluid volume by 0.52 ml/g dry tissue (3.90 to 3.38 ml/g dry tissue) would increase the cell solute concentration by 8 mosM. Some cellular Cl⁻ ion losses have been noted that reflect the increasing cellular organic anion concentration (Dietz 1974).

The brackish-water clam *Mya arenaria* also uses glutamate and alanine in cell volume regulation (Virkar and Webb 1970) as does *Rangia cuneata* (Allen 1961). In addition, marine bivalves have the ability to synthesize taurine for intracellular volume regulation (Gilles 1972; Bricteux-Grégoire *et al.* 1964). Although *L. subrostrata* does not have detectable taurine, Bedford (1973) reported taurine present in the freshwater mussel *Hyridella menziesi*. However, Bedford noted that glutamate and alanine con-

When *L. subrostrata* are dehydrated in air, oxygen consumption decreases 80% but they continue to use oxygen (Dietz 1974). However, these animals will tolerate forced anoxia. When subjected to N₂ in a water-saturated atmosphere, intracellular free amino acid concentrations increase 50%. Three amino acids are primarily responsible for this elevation: alanine, glutamate, and threonine. Free amino acids probably have an important role in cell volume regulation when these mussels are subjected to anoxic stress. Under anoxia, blood Ca⁺⁺ concentration increases fivefold (Dietz 1974). The Ca⁺⁺ is probably mobilized from the shell as metabolic acids are neutralized. To maintain intracellular fluid isosmotic with the blood, there must be a concomitant increase in cell solutes. The source of cellular solute is, in part, free amino acids. Since the animals were not feeding the free amino acids were produced endogenously either from decreased use or increased production. Some of the increase in cellular free amino acids (0.3 mM) may have been from the blood, accounting for the marked decline in extracellular free amino acid concentration. Probably little change in the distribution of body water occurs. Evidence for this is indirect because the compartment volumes have not been measured under anoxic conditions. However, blood Na⁺ and Cl⁻ concentrations do not change when *L. subrostrata* is in a N₂ atmosphere (Dietz 1974).

Although glutamate and alanine play an important role in cellular osmoregulation, these particular amino acids have another function associated with anaerobic metabolism. Hochachka and Mustafa (Hochachka and Mustafa 1972, 1973; Mustafa and Hochachka 1973) have demonstrated that facultative anaerobes obtain considerable substrate-level phosphorylation by coupling glycolysis with amino acid catabolism. Alanine is an end product of glycolysis and glutamate is deaminated to α -ketoglutarate which can enter the Krebs' cycle. Glutamate (and proline) may be produced from arginine and would account for the reduction in cellular arginine content.

Thus, free amino acids play a similar role in cellular volume regulation and metabolism in freshwater mussels as previously noted in marine bivalves. These adaptations are

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