Anaerobiosis, recovery from anoxia, and the role of strombine and alanopine in the oyster *Crassostrea virginica*

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Tissue-specific metabolism was monitored in gill, mantle, and adductor muscle of the oyster *Crassostrea virginica* over a time course of 96 h of anoxia followed by 48 h of recovery from anoxia. Succinate and alanine accumulated as products of anaerobic metabolism while aspartic acid was utilized as a substrate of anaerobiosis. The imino acids alanopine and strombine were not produced during anoxia. During aerobic recovery tissue levels of metabolites returned to control levels, succinate within 2 h in mantle and gill and 6 h in muscle, while restoration of alanine levels required about 24 h. Aspartate pools were restored in 4 to 6 h. Alanopine and strombine accumulated during the recovery period. By 2 h of recovery, alanopine content of mantle and gill had risen by 1.3 and 0.5 μmol/g, respectively, while in adductor muscle both alanopine and strombine accumulated with net increases of 2 and 2.7 μmol/g. Imino acid content declined after 6–12 h of recovery returning to control levels by 24 h. The roles of alanopine and strombine in the oyster are not as products of anaerobic metabolism but rather as products of glycolytic function during recovery. The increased metabolic rate associated with the return to aquatic conditions appears to require some glycolytic energy production to meet overall tissue energy requirements of recovery.

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Le métabolisme de trois tissus, la branchie, le manteau et le muscle adducteur, de l'huître *Crassostrea virginica*, a pu être suivi pendant une anoxie de 96 h suivie d'une période de récupération de 48 h. Durant l'anaérobiose, il y a accumulation de succinate et d'alanine, alors que l'acide aspartique sert de substrat. Il n'y a pas de production des imino-acides, alanopine et strombine. Durant la récupération, les métabolites retrouvent leurs niveaux normaux, en moins de 2 h dans le cas du succinate du manteau et de la branchie, en moins de 6 h pour le succinate musculaire et dans environ 24 h dans le cas de l'alanine. Les réserves d'aspartate retournent à la normale en 4–6 h. L'alanopine et la strombine s'accumulent durant la récupération. En 2 h, le contenu en alanopine augmente de 1,3 μmol/g dans le manteau, de 0,5 μmol/g dans la branchie et de 2 μmol/g dans le muscle. On note aussi dans le muscle une augmentation de strombine de l'ordre de 2,7 μmol/g. Après 6–12 h, les niveaux d'imino-acides décroissent pour atteindre des valeurs normales en 24 h. L'alanopine et la strombine sont des produits non pas du métabolisme anaérobique, mais plutôt de la fonction glycolytique durant la récupération. Le retour aux conditions aquatiques normales requiert un accroissement du taux métabolique, ce qui entraîne une production d'énergie glycolytique.

[Traduit par le journal]

Introduction

In 1976 Fields demonstrated the presence of a dehydrogenase requiring pyruvate and L-alanine as substrates in the adductor muscle of the oyster *Crassostrea gigas*, while Collicutt and Hochachka (1977) reported the anaerobic production of a compound (later identified as alanopine) from the anoxic catabolism of [^{14}C]glucose or [^{14}C]alanine in oyster ventricle. Alanopine dehydrogenase and strombine dehydrogenase

$$\text{pyruvate} + \text{L-alanine} + \text{NADH} + \text{H}^+ \rightleftharpoons \text{meso-alanopine} + \text{NAD}^+ + \text{H}_2\text{O}$$

$$\text{pyruvate} + \text{glycine} + \text{NADH} + \text{H}^+ \rightleftharpoons \text{D-strombine} + \text{NAD}^+ + \text{H}_2\text{O}$$

are now known to be widely distributed throughout marine invertebrate phyla (Dando *et al.* 1981; de Zwaan and Zurburg 1981; Barrett and Butterworth 1981; Ellington 1979; Storey 1983) with a function analogous to that of lactate dehydrogenase in vertebrate tissues and often replacing lactate dehydrogenase, or octopine dehydrogenase, as the sole or major cytosolic dehydrogenase in tissues. The roles of alanopine and strombine in marine invertebrates are currently under investigation. Particularly in species such as the oyster where lactate and octopine dehydrogenase activities are virtually absent, alanopine or strombine dehydrogenase must participate in a number of cellular functions. The enzymes provide redox balance for glycolytic energy production and may have roles in the survival of anoxia or other environmental stresses or in the support of muscular work.

Various aspects of metabolism have been well studied in the oyster (for review, see Hammen 1969) and the isolated ventricle has been used as a model system for investigating anaerobic metabolism (Collicutt and Hochachka 1977; Foreman and Ellington 1983). The present study examines tissue-specific metabolism in *Crassostrea virginica* during anoxia and recovery from

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anoxia placing particular emphasis on the role(s) of strombine and alanopine in anaerobiosis.

Materials and methods

Animals and chemicals

Oysters, *Crasssostrea virginica*, were purchased from a local seafood retailer and were held, without feeding, in an aerated, recirculating seawater tank at 18°C for at least 1 week before use.

All biochemicals were purchased from Sigma Chemical Co. or Boehringer Mannheim Corp. Alanopine and strombine dehydrogenases were prepared as previously described (Storey *et al.* 1982). Succinyl-CoA synthetase was a gift from Dr. W. Bridger, University of Alberta.

Anoxia and recovery

Control, aerobic animals were sampled directly from the seawater tank. To impose anoxia, animals were removed from the tank and placed in large jars which were then flushed with nitrogen gas for 20 min and tightly sealed. Anoxic animals were held at 20°C. At timed intervals during anoxia, groups of animals were sampled. Shells were quickly opened and tissues were dissected out, blotted, and frozen in liquid nitrogen. For recovery experiments, animals were held for 96 h under anoxic conditions and were then returned to the aerated seawater tank and sampled at time intervals. In all cases, tissues from two animals were pooled for each sample. Tissues were stored frozen at −80°C until use.

Sample preparation and metabolite assay

Neutralized perchloric acid extracts of tissues were prepared as described by Storey and Storey (1979) and were stored at −80°C until analysis. Metabolites were measured enzymatically, alanine and aspartate by the methods of Lowry and Passonneau (1972), alanopine and strombine as described by Storey *et al.* (1982), and succinate by the method of Williamson and Corkey (1969) with the substitution of the ATP-dependent succinyl-CoA synthetase from *E. coli*.

Measurement of enzyme activities

Fresh tissues were dissected out of aerobic animals, blotted, weighed, and homogenized in five volumes (w/v) 50 m*M* imidazole buffer, pH 7.0 containing 20 m*M* 2-mercaptoethanol. Homogenates were centrifuged at 27 000 × *g* for 20 min at 4°C. Supernatants were collected and dialyzed against homogenization buffer for 2 h.

All enzymes were assayed in 50 m*M* imidazole buffer, pH 7.0 at 23°C. Optimal assay conditions were 2 m*M* pyruvate and 0.1 m*M* NADH for lactate dehydrogenase with the addition of 200, 100, 25, or 50 m*M* L-alanine for alanopine dehydrogenase or 200, 200, 400, or 200 m*M* glycine for strombine dehydrogenase from phasic and catch adductor muscles, gill, and mantle, respectively.

Results

Anoxia

Figures 1–3 show the changes in metabolite levels in mantle, gill, and adductor muscle (phasic and catch portions combined) in *C. virginica* during 96 h of anoxia stress followed by 48 h of recovery from anoxia.

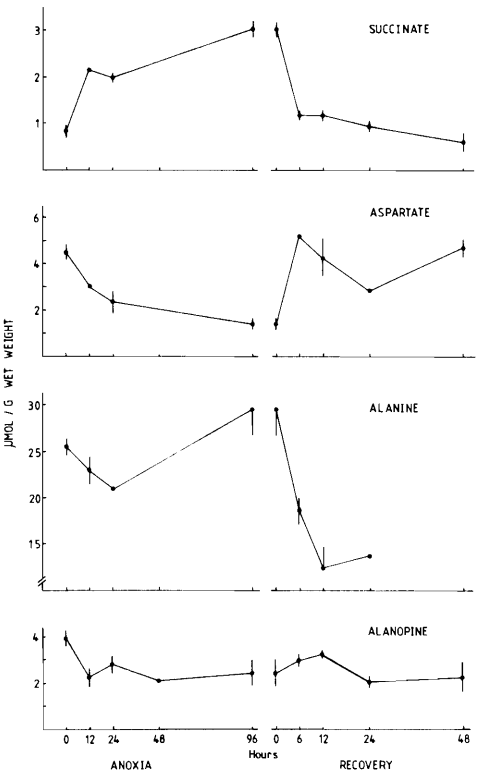

FIG. 1. Levels of some metabolites in the mantle of *C. virginica* during anoxia and recovery. Results are means ± SEM for $n = 3$ to 5 samples, two animals pooled per sample.

Alanine and succinate were end products of anaerobic metabolism. Succinate levels increased most rapidly during the first 24 h of anoxia with a total increase in succinate content in all tissues of about 2 μmol/g wet weight over the course of anoxia. Alanine content increased by about 4 μmol/g in mantle and adductor. A similar apparent increase in alanine content in gill was not significantly different from the value at time zero owing to high variation in individual alanine content of different animals. Aspartic acid was utilized as a substrate for anaerobiosis, levels decreasing by 2.5 to 3 μmol/g. Aspartate reserves were depleted after 24 h of anoxia in adductor muscle but aspartate content continued to decline throughout anoxia in gill and mantle. Alanopine levels in oyster tissues were not significantly altered during anoxia.

Recovery

Upon the return to aerated seawater, succinate content of the tissues was rapidly depleted. In gill and mantle succinate concentration had returned to control levels within 6 h, while 12 h was required in adductor muscle. Aspartate reserves were restored within 6 h; a slight overshoot in aspartate concentration was seen at 6 h with a decline towards control levels during the later stages of

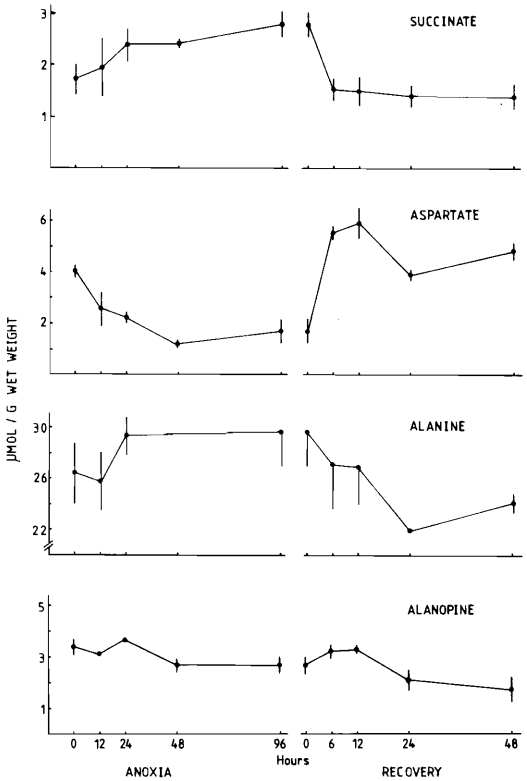

FIG. 2. Levels of some metabolites in the gill of *C. virginica* during anoxia and recovery. Results are means ± SEM for n = 3 to 5 samples, tissue from two animals pooled per sample.

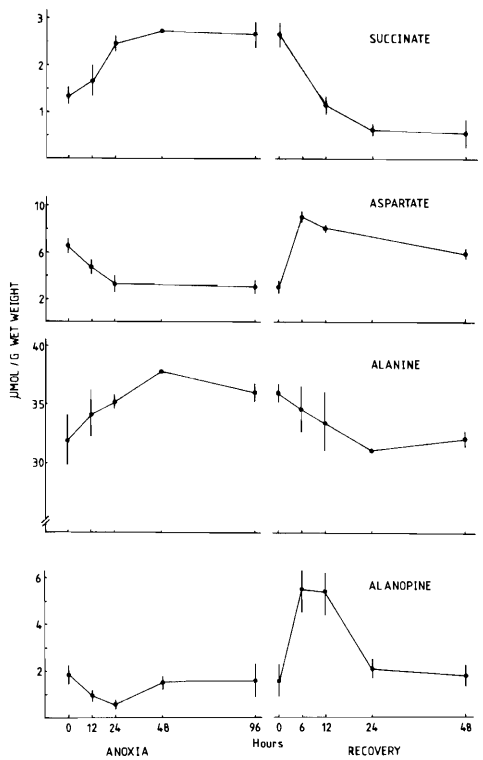

FIG. 3. Levels of some metabolites in the adductor muscle of *C. virginica* during anoxia and recovery. Results are means ± SEM for n = 3 to 5 samples, tissue from two animals pooled per sample. Both phasic and catch portions of the adductor muscle were pooled.

recovery. Restoration of alanine levels required a longer time; control levels of alanine were reestablished after 24 h in gill and adductor muscle, while final alanine concentration in mantle tissue was substantially less than that of controls. Recovery resulted in a rapid increase in alanopine content in all tissues, particularly in adductor muscle. Alanopine concentration in adductor muscle rose from 1.5 to 5.5 μmol/g within the first 6 h of recovery, remained elevated at 12 h, and then returned to control levels by 24 h. Gill and mantle showed similar but smaller (less than 1 μmol/g) increases in alanopine content over the same time period.

Detailed time course of recovery

To further investigate the role of alanopine and strombine in the recovery process in the oyster, a second experiment was performed in which oysters, after 96 h of anoxia, were monitored over a short time course of recovery between 1 and 12 h (Fig. 4). Neither alanopine or strombine were accumulated during anoxia in gill and mantle tissues but a small increase in both compounds (0.5 μmol/g alanopine and 1.0 μmol/g strombine) was found in the anoxic adductor muscle. During recovery there was a rapid accumulation of alanopine in all tissues. Alanopine content increased by 1.3 and 0.7 μmol/g in mantle and gill, respectively, the peak concentration occurring at 2 h of recovery with a return to control levels between 6 and 12 h. Minor accumulations of strombine were also seen in these tissues but alanopine was the major imino acid in gill and mantle with levels at least eight fold greater than those of strombine. In adductor muscle, however, alanopine and strombine were produced in similar amounts. After 2–4 h of recovery, alanopine content had increased by 2 μmol/g while strombine content rose from 1.3 to 4.0 μmol/g after 6 h of recovery. The levels of both imino acids declined as recovery progressed but neither had returned to control levels by 12 h.

Changes in the levels of succinate, alanine, and aspartate during recovery were also measured in this experiment with results similar to those seen in Figs. 1–3. However the detailed time course of recovery revealed that the catabolism of accumulated succinate in gill and mantle was largely complete (90%) within 1 h of recovery with control levels reestablished by 2 h of recovery. Succinate levels in adductor decreased at a slower rate to reach control levels after 6 h. Aspartate

TABLE 1. Activities of cytosolic dehydrogenases acting at the pyruvate branchpoint in *C. virginica*

Tissue	LDH	ADH/SDH		
		Alanine	Glycine	Ala/Gly
Gill	0.33±0.05	2.3±0.26	2.0±0.88	1.15
Mantle	0.44±0.07	2.6±0.54	1.8±0.92	1.44
Phasic adductor	0.19±0.06	19.9±4.6	18.4±4.4	1.08
Catch adductor	0.26±0.08	5.1±0.78	5.0±1.1	1.02

NOTE: Enzyme activities of lactate dehydrogenase (LDH) and of alanopine or strombine dehydrogenase (ADH/SDH) are given as means ± SEM for $n = 6$ animals. Activities are expressed as micromoles NADH utilized per minute per gram wet weight.

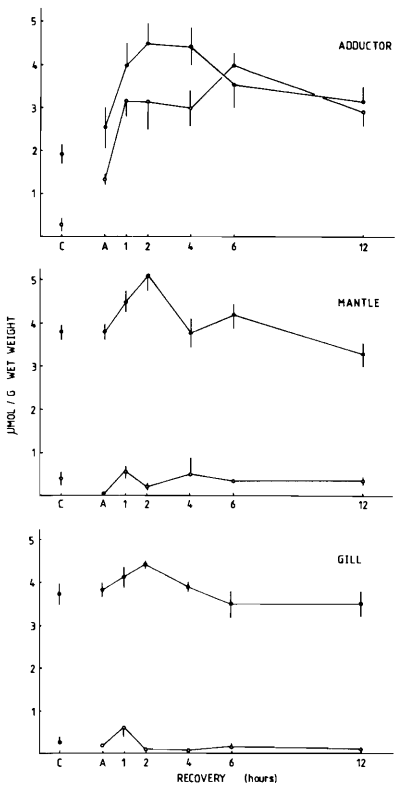

FIG. 4. Levels of alanopine and strombine in mantle, gill, and adductor muscle of *C. virginica* during recovery from anoxia stress. Results are means ± SEM for $n = 5$ samples, tissues from two animals pooled per sample.

concentration in all tissues were restored more slowly requiring 4–6 h to return to control levels. Alanine content, as noted previously, declined in a linear fashion over the 12 h of recovery but control levels were not restored by the end of the experimental time course.

Tissue enzyme activities

Table 1 shows the activities of cytosolic dehydrogenase acting at the pyruvate branch point in the tissues of *C. virginica*. Lactate dehydrogenase was present in low activities while octopine dehydrogenase was not detected. Imino acid dehydrogenase activities utilizing L-alanine or glycine as substrates were the major enzyme activities detected. Activities of alanopine dehydrogenase and strombine dehydrogenase (or a single enzyme catalyzing both activities) were highest in the phasic portion of the adductor muscle. At optimal substrate concentrations alanine-dependent activity was greater than or equal to that of glycine-dependent activity in all tissues, muscle tissues showing an alanine/glycine ratio of about 1.0 while soft tissues showed a higher ratio.

Discussion

Anaerobiosis in *C. virginica* resulted in the accumulation of succinate and alanine as end products as has been demonstrated for several other bivalve species (Stokes and Awapara 1968; de Zwaan 1977; Meinardus and Gäde 1981) including another oyster, *C. gigas* (Collicutt and Hochachka 1977). All three tissues showed a similar pattern of end-product accumulation and were also very similar in the amounts of the two end products accumulated. This contrasts with various other species which show tissue-specific differences in the quantities and time course of end-product accumulation (Zurburg and Kluytmans 1980; Zurburg and Ebberink 1981; Korycan and Storey 1983). Alanine production in oyster tissues exceeded that of succinate by twofold similar to the results reported by Collicutt and Hochachka (1977) for oyster ventricle. Succinate production over the full 96 h of anoxia could be quantitatively accounted for by the mobilization of tissue aspartate reserves while at least 50% of the amino groups needed for alanine synthesis could also be derived from this source. This inverse relationship between aspartate and succinate over the course of anoxia suggests that aspartate may be the sole precursor required for the anaerobic production of succinate in the oyster. Thus anaerobiosis would couple the oxidative breakdown of glycogen to form alanine with the reductive conversion

of aspartate into succinate, as first suggested by Collicutt and Hochachka (1977). The carboxylation of phosphoenolpyruvate (derived from glycogen) to form oxaloacetate (Hochachka and Mustafa 1972) is not necessary to account for succinate formation. However, as the possible formation of propionate and (or) acetate as additional end products was not determined in the present study, carboxylation of phosphoenolpyruvate cannot be ruled out as a mechanism functioning in the anaerobic production of energy in oysters.

Recovery from anoxia stress involved a rapid restoration of tissue concentrations of alanine, succinate, and aspartate to control levels. However, each compound showed a different time course for recovery, succinate levels were restored in 1–2 h in soft tissues, aspartate reserves were resynthesized in 4–12 h, while alanine required much longer. The different rates of succinate breakdown versus aspartate synthesis indicate that succinate cannot be directly reconverted to aspartate when forward functioning of the Krebs cycle is resumed; some or all of aspartate production must arise from a *de novo* synthesis of the amino acid. However alanine depletion during the period of aspartate resynthesis could account for the amino groups necessary for the conversion of oxaloacetate to aspartate. Similar time-dependent differences in the restoration of control levels of succinate, alanine, and aspartate after anoxia stress have been seen in *Mytilus edulis* (de Zwaan *et al.* 1983).

Alanopine and strombine are not products of anaerobic metabolism in the gill and mantle of the osyster and are only minor products of adductor muscle anaerobiosis in some cases (Fig. 3 vs. Fig. 4). The synthesis of alanine as an anaerobic end product, therefore, does not appear to potentiate redox regulation by the alanopine dehydrogenase reaction. However, imino acids were rapidly accumulated in all tissues during the early stages of recovery from anoxia. Both alanopine and strombine accumulated in adductor muscle while only alanopine was produced in gill and mantle. This tissue difference in imino acid(s) produced is likely largely due to the imino acid dehydrogenase complement of the individual tissues. Tissue-specific forms of the enzymes occur. Adductor muscles from several bivalve species are characterized by a strombine dehydrogenase which shows a high activity with either glycine or L-alanine as the amino acid substrate (Dando *et al.* 1981; de Zwaan and Zurburg 1981; Storey *et al.* 1982) while soft tissues show an alanopine dehydrogenase with a strong preference for L-alanine as its major substrate.

The production of imino acids during recovery from anoxia stress was first reported for *M. edulis* posterior adductor muscle (Zurburg *et al.* 1982; de Zwaan *et al.* 1983). Between 2 and 4 h of recovery (from 24 h anoxia) strombine content rose from 4 to 9 μmol/g wet weight and then subsequently declined to control levels after about 26 h (de Zwaan *et al.* 1983). *Crassostrea virginica* shows the same pattern of imino acid production during the recovery phase suggesting that this may be a general metabolic response amongst anoxia-tolerant intertidal bivalves. Our data demonstrate that this response occurs in at least three tissues, and possibly all tissues, of the animal, although it is most pronounced in adductor muscle. Another imino acid, octopine, plays a role in metabolic recovery from muscle work in various scallop species (Grieshaber 1978; Livingstone *et al.* 1981). In these species octopine accumulates not during muscle exercise (which is largely supported by the breakdown of arginine phosphate) but during the recovery period when energy reserves are being resynthesized. Although octopine is a metabolic end product of muscle work in various other bivalve species, a major role for imino acids in marine bivalves in general appears to be in the recovery from stress.

The reason for imino acid accumulation during recovery appears to be related to the energy requirements of the animal at this time. During anoxia, tissue ATP content is decreased in the oyster (Collicutt 1975). The metabolic demands of recovery include the restoration of ATP and arginine phosphate pools, the removal (oxidation, gluconeogenesis) of accumulated end products, and the resynthesis of aspartate and glycogen as well as the resumption of normal activities such as feeding. Although tissue reoxygenation occurs quite rapidly after anoxia (de Zwaan *et al.* 1983) aerobic metabolism cannot, apparently, fulfill the entire energy requirements of the recovery phase. Additional energy production, via glycolytic fermentation, is required with the subsequent accumulation of imino acids as products. The greater accumulation of alanopine and strombine in adductor muscle than in soft tissues is consistent with this theory. Adductor muscle has a much lower content of mitochondria than do tissues such as gill and mantle; an increased energy demand in this tissue is much more likely, therefore, to require a major contribution to energy production by glycolysis alone.

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