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COUPLED MITOCHONDRIA FROM OYSTER GILL TISSUE*

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SUMMARY

Mitochondria showing coupled (ADP-substrate dependent), KCN sensitive respiration were prepared from oyster *(Crassostrea virginica)* gill tissue. We found no evidence for an alternate respiratory chain. Pyruvate and proline were metabolized very slowly compared to glutamate, 2-oxoglutarate, succinate and malate. The aminotransferases for aspartate, alanine and ornithine plus an ADP-activated glutamate dehydrogenase were found in the mitochondria.

Key words: mitochondria, oysters, amino acids, gills, respiration.

INTRODUCTION

Most of the enzymes of the tricarboxylic acid cycle and the respiratory chain have been detected in homogenates of oyster and other bivalve tissues (Black, 1962a,b; de Zwaan, 1977). Although most investigators have presented no evidence for an alternate electron transport system in oyster tissue, most have worked with extracts of respiratory particles and have not attempted to prepare coupled mitochondria. Recently, we developed a simple procedure for the preparation of coupled mitochondria from ribbed mussel *(Modiolus demissus)* gill tissue (Burcham et al., in press). The gill mitochondria show respiratory control indices (Chance and Williams, 1956; Estabrook, 1967) between 3 and 7 and the predicted coupling ratios for the substrates tested. Oxygen consumption by these mitochondria was blocked by rotenone, antimycin A and azide in the predicted manner. KCl at fairly high concentrations (~ 80 mM) stimulated respiration and raised the respiratory control indices (RCI) for the substrates tested. Very high KCl concentrations (~ 150 mM) did

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not lower the respiratory rate but did lower the RCI. High concentrations of KCl apparently also improved the respiratory performance of squid heart mitochondria (Mommsen and Hochachka, 1981). Overall, it was concluded that from the physiological viewpoint, the respiratory chain in the mitochondria from the gills of this estuarine bivalve was similar to the respiratory chain in vertebrate tissues. There was no evidence for an alternate electron transport system as suggested by Zaba (1983) for mitochondria from sea mussel *(Mytilus edulis)* tissues or for an abridged electron transport system as found in the mitochondria from some helminth parasites (see Saz, 1981; Rodrick et al., 1982). The data on ribbed mussel gill mitochondria were in agreement with earlier work on the nature of the electron transport systems in free living invertebrates (see references in Burcham et al., in press).

In this paper, we report preliminary results on a procedure to prepare coupled mitochondria from gill tissues of the American oyster, *Crassostrea virginica*. The procedure avoids the use of D-mannitol or high concentrations of KCl in the isolation medium to alleviate possible problems with D-mannitol oxidase that has been found in mitochondria from some molluscs (Vorhaben et al., 1980) or with KCl loading of mitochondria in the cold that can cause sustained state 3 respiration in some mitochondria (Amoore and Bartley, 1958; Burcham et al., in press).

MATERIALS AND METHODS

Oysters were obtained from Northeast Marine Environmental Laboratory (Monument Beach, MA) or from Dr. Edwin Cake (Gulf Coast Laboratory, Ocean Springs, MS) and held in the laboratory at 22°C in tanks of aerated 25‰ artificial sea water (Jungle Salts, Jungle Laboratories, Sanford, FL). Except where indicated all chemicals were purchased from Sigma Chemical Co. (St. Louis, MO). All amino acids were the L-isomers. Enzyme grade sucrose was obtained from Schartz-Mann (Orangeburg, NY). Defatted serum albumin was prepared from fraction V (Sigma) using the modified procedure of Chen (see Burcham et al., in press).

Isolation of mitochondria

Gill tissue was homogenized in a buffered isolation medium (pH 7.5) containing 0.4 M sucrose, 20 mM potassium HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid), 1mM potassium EGTA (ethyleneglycol-bis(β-aminoethylether)N,N'-tetracetic acid) and 0.5% defatted BSA using Ultraturrax Model T45/N (Tekmar, Cincinnati, OH). This isolation procedure essentially involves filtration of the homogenate through Miracloth (Calbiochem, Irvine, CA), followed by differential centrifugation of the filtrate at 1500 $\times$ g for 8 min and 9000 $\times$ g for 15 min to separate the mitochondria in the 9000 $\times$ g pellet. The mitochondria were then washed and resuspended in the isolation medium as described previously (Burcham et al., in press).

Oxygen consumption

Oxygen consumption by the mitochondria was determined by mixing the resuspended mitochondria in respiration buffer (see below) then measuring change in oxygen content with a Clark type oxygen electrode (Yellow Springs Instrument Co., Yellow Springs, OH). The electrode was calibrated as previously described (Burcham et al., in press; Estabrook, 1967). All measurements were made at 25°C. Respiration was measured in solution containing 1 ml of the resuspended mitochondria, 0.05 ml of 0.2 M KH_2PO_4, 0.05 ml of 50 mM substrate, and 1.4 ml of a solution containing 0.13 M sucrose, 150 mM KCl, 20 mM potassium HEPES (pH 7.5), 1 mM EGTA, and 5 mg/ml defatted BSA. The resulting reaction mixture (less ADP) contained 238 mM sucrose, 90 mM KCl, 20 mM potassium HEPES (pH 7.5), 1 mM EGTA, 5 mg/ml defatted BSA, 4 mM KH_2PO_4, 1 mM substrate, and mitochondria in 2.5 ml.

Enzyme assays

Aspartate aminotransferase (E.C. 2.6.1.1) was assayed spectrophotometrically in the oxaloacetate forming direction at pH 8.3 in a reaction mixture containing 20 mM aspartate, 10 mM 2-oxoglutarate, 70 μM NADH, 4 U of malate dehydrogenase, 100 mM Tris-HCl and enzyme in 2 ml. Alanine aminotransferase (E.C. 2.6.1.2) was assayed spectrophotometrically in the pyruvate forming direction at pH 8.3 in a reaction mixture containing 20 mM alanine, 10 mM 2-oxoglutarate, 70 μM NADH, 4 U lactate dehydrogenase, 50 mM Tris-HCl and enzyme in 2 ml. Ornithine aminotransferase (E.C. 2.6.1.13) was assayed colorimetrically using O-aminobenzaldehyde to determine the formation of Δ^1,-pyrroline-5-carboxylate as described previously in a 1.4 ml reaction mixture (pH 7.5) containing 50 mM Tris–HCl, 5 mM L-ornithine, 4 mM 2-oxoglutarate and enzyme. Glutamate dehydrogenase (E.C. 1.4.1.3) was assayed spectrophotometrically according to the procedure of Reiss et al. (1977) at pH 8.5 in a 3 ml mixture containing 300 μmol of Tris-HCl, 25 μmol of 2-oxoglutarate, 170 μmol ammonium acetate, 0.075 μmol NADH and 2.5 μmol of ADP. All enzyme measurements were made at 22°C. Protein was measured by the biuret procedure of King (1967) and standardized with a sucrose–BSA solution.

RESULTS AND DISCUSSION

Mitochondrial respiration

Initial experiments on *C. virginica* gill mitochondria indicated that the mitochondria contained considerable amounts of an endogenous substrate whose state 3 respiration was not sensitive to rotenone (10 nmol/mg protein) but was 100% inhibited by antimycin A at a concentration of one nmol/mg protein. This pattern of inhibition of oxygen consumption and P:O ratio (Table 1, experiment A, no substrate added) is characteristic of succinate as a substrate. Since oysters and some

TABLE I

Oxygen consumption by oyster gill mitochondria[a].

	nmol O_2 per min per mg protein		Coupling ratios	
Experiment A				
Substrate	State 3	State 4	RCI	P:O
No substrate	4.2 ± 0.1	2.2 ± 0.2	1.9 ± 0.1	1.5 ± 0.2
Pyruvate	4.5 ± 1.0	1.8 ± 0.2	2.5 ± 0.3	2.2 ± 0.1
Malate	7.7 ± 0.3	2.6 ± 0.2	3.0 ± 0.1	2.5 ± 0.1
Malate + pyruvate	9.0 ± 0.9	2.7 ± 0.2	3.4 ± 0.1	2.7 ± 0.3
Succinate	8.2 ± 0.5	3.8 ± 0.3	2.2 ± 0.1	1.8 ± 0.2
Glutamate	6.7 ± 0.1	2.1 ± 0.2	3.3 ± 0.1	2.7 ± 0.1
Experiment B				
Substrate	State 3	State 4	RCI	P:O
Succinate	6.9 ± 0.3	2.8 ± 0.4	2.4 ± 0.1	1.9 ± 0.2
Malate	6.0 ± 0.2	1.7 ± 0.4	3.7 ± 0.2	2.7 ± 0.3
2-Oxoglutarate	9.1 ± 2.8	0.9 ± 0.2	9.9 ± 0.4	3.0 ± 0.1
Glutamate	6.6 ± 0.4	0.6 ± 0.2	11.8 ± 0.4	3.0 ± 0.1

[a]Assays were performed as described in Fig. 1. All substrates were 1 mM. State 3 was the respiratory rate produced by addition of 200 nmol of ADP and State 4 was the respiratory rate following utilization of the ADP. Data represent averages and SEM where $n = 4$ for Experiment A and $n = 3$ for Experiment B.

other bivalves can tolerate long periods of anoxia with a concomitant rise in tissue succinate (Hammen, 1969; de Zwaan, 1977), these initial mitochondrial preparations probably contained endogenous succinate. This condition was partially alleviated by maintaining the animals in air-saturated sea water for 24 h prior to isolating gill mitochondria. The mitochondria had increased RCI's and higher state 3 respiratory rates when allowed to respire in the presence of 90 mM KCl. Therefore, 90 mM KCl was included in all the oxygen consumption experiments of this report. Some KCl may be required for proper mitochondrial respiration in a fashion similar to the squid heart and ribbed mussel gill mitochondria (Mommsen and Hochachka, 1981; Burcham et al., in press).

Figure 1, showing the oxygen consumption traces with 2-oxoglutarate and glutamate as substrates, demonstrates the typical responses of *C. virginica* gill mitochondria to ADP in the presence of inorganic phosphate and NADH generating substrates. These results demonstrated well defined transitions from state 3 to state 4 respiration allowing direct measurements of RCI's and P:O ratios. In addition, respiration with glutamate as the substrate was completely blocked by the addition of freshly prepared potassium cyanide suggesting the absence of a major cyanide insensitive respiratory chain in oyster gill mitochondria, in contrast to that suggested by Zaba (1983) for *M. edulis* gill mitochondria.

Of the substrates tested (glutamate, malate, 2-oxoglutarate, pyruvate, and suc-

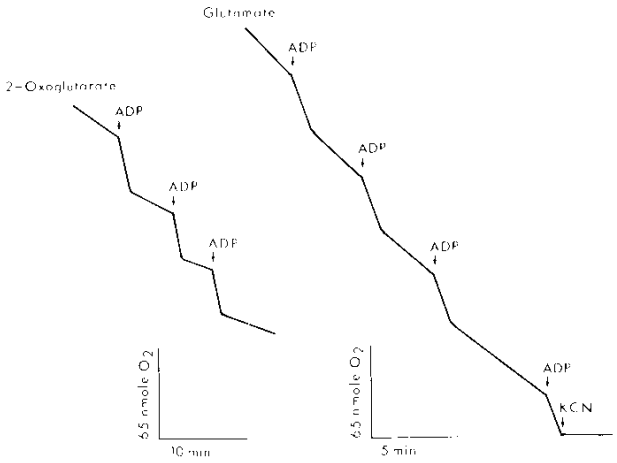

Fig. 1. Oxygen consumption by oyster gill mitochondria. Reaction mixture contains 1 mM substrates and 5 mg mitochondrial protein in 2.5 ml of respiration solution (see Materials and Methods). ADP was added in 200 nmol increments, and KCN was added to 1 mM final concentration where indicated.

cinate), most produced state 3 respiratory rates which were significantly higher than the state 3 rates due to the presence of endogenous substrates (Table I, experiment A). Although slight increases in RCI's and P:O ratios occurred in the presence of pyruvate, this substrate failed to generate a significant increase in state 3 rates over that of endogenous substrates and no differences were observed for RCI's and P:O ratios between malate and the combination of malate plus pyruvate (Table I, experiment A). These results indicate only negligible oxidation of exogenously added pyruvate by oyster gill mitochondria which has also been observed for ribbed mussel gill mitochondria (Burcham et al., in press) and probably for squid heart mitochondria (Mommsen and Hochachka, 1981). In this regard, pyruvate was not required to stimulate respiration with glutamate as has been reported for squid heart mitochondria (Mommsen and Hochachka, 1981).

Since several factors of the mitochondria isolation procedure (metabolic state of the animals, homogenization and filtering techniques, and centrifugation and resuspension steps) can directly influence the physiological functionality of isolated mitochondria, day-to-day variability among mitochondria preparations can be expected from the same group of animals. Table I depicts two different preparations which were isolated during the same week in identical fashion. The major differences between experiments A and B (Table I) for all substrates is reflected in the state 4 oxygen consumption rates. This was particularly evident in the case of glutamate, and accounts for the 3-fold difference in observed RCI's, because there is no significant difference between either the state 3 rates or P:O ratios of both ex-

periments (Table I). However, with malate and succinate as substrates the differences in state 4 respiration are offset by proportional differences in state 3 rates, thus resulting in comparable RCI's between experiments A and B (Table I). The data of Table I (experiment B and in data not shown) indicate that in the best gill mitochondria preparations, glutamate and 2-oxoglutarate give the highest respiratory control with 2-oxoglutarate giving the fastest state 3 rate. In data not shown L-proline would not stimulate respiration or alter the RCI and P:O from that due to endogenous substrate.

These mitochondria appear to respire in a 'normal' fashion. We find no evidence for an alternate, KCN insensitive, respiratory chain as suggested by Zaba (1983). These mitochondria apparently lack or have a very low level of proline oxidase. Proline oxidase has been found in mitochondria from squid heart (Mommsen and Hochachka, 1981) and from ribbed mussel gill mitochondria (Burcham et al., in press) but not in mitochondria from the hepatopancreas of sea mussels (Zaba et al., 1978). The strong stimulation of oxygen consumption with glutamate as a substrate in the absence of pyruvate indicated the probable presence of an active glutamate dehydrogenase (GDH). Low levels of GDH have been reported in oyster tissue (Wickes and Morgan, 1976).

Mitochondrial transaminases and glutamate dehydrogenase

The enzymes listed in Table II were assayed as part of our continuing study on the regulation of amino acid metabolism in estuarine bivalves. Previous studies with the aminotransferases from other molluscan species indicate that most tissues have isozymes of aspartate aminotransferase and alanine aminotransferase in both the cytosol and mitochondria (Sollock et al., 1979; Chambers et al., 1975; Addink and Veenhof, 1975; Johnson et al., 1972; Johnson and Utter, 1973; K.T. Paynter, R.J. Hoffmann, L.L. Ellis and S.H. Bishop, submitted for publication). The relative ac-

TABLE II

Aminotransferase and glutamate dehydrogenase activities in oyster gill mitochondria[a].

Enzyme	Activity (nmol per min per mg mitochondrial protein)
Ornithine aminotransferase	73
Alanine aminotransferase	35
Aspartate aminotransferase	85
Glutamate dehydrogenase (no ADP)	2.5
Glutamate dehydrogenase (with ADP)	40

[a]Assay conditions and preparation of the mitochondria are described in the text. Isolated mitochondria were broken by sonicating an ice cold suspension three times for 20 sec each with a Branson Sonifier (Model S75) at a power setting of 7. The debris was removed by centrifugation (15,000 × *g* for 20 min) and the supernatant fluid used as the source of the enzymes. The activity values represent the average of three separate determinations.

tivity levels of the alanine and aspartate aminotransferases in the oyster gill mitochondria (Table II) are within the general range reported by Chambers et al. (1975) for mitochondria from whole body homogenates of American oysters. In data not shown, it appears that oyster gills have electrophoretically distinct mitochondrial and cytosolic aspartate and alanine aminotransferases. On the other hand, the ornithine aminotransferase (Campbell et al., 1972; Mommsen and Hochachka, 1981) and the glutamate dehydrogenase (Addink and Veenhof, 1975; Mommsen and Hochachka, 1981; Reiss et al., 1977; Storey et al., 1978) seem to be mitochondrial, and activation of the glutamate dehydrogenase by ADP is similar to that reported for this activity in other molluscan tissues (Addink and Veenhof, 1975; Friedl, 1979; Reiss et al., 1977; Sollock et al., 1979; Storey et al., 1978). This 16-fold activation of the glutamate dehydrogenase activity with the addition of ADP (Table II) means that the enzyme would be very responsive to changes in ADP levels and that the estimation of oyster tissue glutamate dehydrogenase levels by Wickes and Morgan (1976) is a considerable underestimation of the physiologically important glutamate dehydrogenase activity in oyster tissues. It would appear that glutamate can be metabolized by the mitochondria directly through the glutamate dehydrogenase pathway without transamination with pyruvate or oxaloacetate even though both the alanine and aspartate aminotransferases are present in the mitochondria.

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