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SOLUTE EFFECTS ON MITOCHONDRIAL RESPIRATION: THE KINETICS OF PROLINE OXIDATION BY MITOCHONDRIA FROM THE VENTRICLE OF THE MARINE CLAM *MERCENARIA MERCENARIA*

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Abstract—1. The effects of osmolarity on the maximal velocity (V_{max}) and apparent Michaelis constant (K_m) for the oxidation of proline by both intact and sonicated mitochondria from the ventricle of the marine clam *Mercenaria mercenaria* have been determined.

2. Compared to the isoosmotic state (1040 mOsm/l), the rate of oxidation at any concentration of proline by intact mitochondria is greater in the hypoosmotic state and lower in the hyperosmotic state.

3. Maximal rates of proline oxidation at low osmolarities equal the maximal rates of electron transport over the range 500 to 700 mOsm/l.

4. The response of the apparent Michaelis constant for proline to the osmolarity of the medium may be due to changes in the inner mitochondrial membrane. Such changes were monitored by measuring the rate of NADH oxidation by intact mitochondria.

5. It is suggested that volume changes in mitochondria during the early stages of osmotic stress may be responsible for adjustments in intracellular concentration of certain amino acids observed during volume regulation in marine bivalves.

INTRODUCTION

During the early stages of hyper- (Baginski and Pierce, 1975) and hypoosmotic stress (Pierce and Greenberg, 1976) in many marine bivalves, cell volume changes are quickly restored by changing amino acid levels in the cytoplasm. Some amino acids in the tissues of these animals are not oxidized but appear to be present solely as innocuous osmolytes e.g. taurine (Yancey *et al.*, 1982) while others are important as substrates for oxidative metabolism (Ballantyne and Storey, 1983). Amino acids serving as energy sources are catabolized at higher rates during osmotic stress resulting in lower levels of these amino acids in the tissues (Bartenberger and Pierce, 1976). The pathway of catabolism of these amino acids and the mechanism of control by osmolarity are not well understood (Burcham *et al.*, 1980). As the sites for the oxidation of amino acids, mitochondria may be involved in the regulation of some amino acid levels during osmotic stress in marine bivalves.

Oxygen consumption by isolated gill tissue of *Mercenaria mercenaria* has been shown to decline with increasing external osmolarity (van Winkle, 1968). van Winkle speculated that these changes could be due to changing osmotic properties of mitochondria of the gill. As membrane-bound organelles, mitochondria would be susceptible to the osmotic effects experienced during the early stages of volume regulation. The swelling of mitochondria is known to affect their oxidative properties (Chappell and Perry, 1954; Atsmon and Davis, 1967; Campbell *et al.*, 1975; Matlib and Srere, 1976; Holtzman *et al.*, 1978). Volume changes in mitochondria have been implicated in the regulation of the oxidation of glutamine at the level of the glutamine transporter in rat kidney mitochondria (Kovacevik *et al.*, 1980).

The present study was carried out to determine whether the observed effects of osmolarity on the respiration of whole tissues of *Mercenaria mercenaria* (van Winkle, 1968) are paralleled by changes in mitochondrial oxidation of substrates and whether these changes can be related to the processes of volume regulation. Proline is a major substrate in the ventricle of *Mercenaria mercenaria* (Ballantyne and Storey, 1983). It may function both as an osmolyte during certain stages of volume regulation in marine bivalves (Baginski and Pierce, 1977; Pierce and Greenberg, 1972; Strange and Crowe, 1979) and as an energy source for the increased metabolic energy requirements during osmotic stress. To determine the role of the inner mitochondrial membrane in these processes both intact and sonically disrupted mitochondria were used.

MATERIALS AND METHODS

Animals

Quahogs, *Mercenaria mercenaria*, were obtained from a local supplier of seafood and held in aerated, recirculated artificial seawater (1040 mOsm/l) for periods up to 3 weeks.

Isolation of mitochondria

Mitochondria with high respiratory control ratios (RCR = 6–10 based on the state 3 to state 4 transition) were obtained from the hearts of the clams as previously described (Ballantyne and Storey, 1983).

Oxidative phosphorylation

Oxygen uptake of intact and disrupted mitochondria was measured in a 0.71 ml cell (using a Clark-type electrode) with temperature held constant at 15°C. The assay medium consisted of 10 mM HEPES (pH 7.20 adjusted at 15°C), 8.6 mM KH_2PO_4, 0.86% bovine serum albumin (essentially fatty acid free), 57 mM sucrose (sucrose inhibits mitochondrial swelling; Brierley, 1976) and was therefore kept at a low level), 0.14 mM ethylene diamine tetraacetic acid (EDTA), 0.29 mM ethylene bis β-aminoethyl ether–*N,N,N',N'* tetraacetic acid (EGTA) and varying amounts of KCl. KCl was used as the osmolyte because other nonionic solutes e.g. sucrose or mannitol inhibit mitochondrial volume changes. The lack of effect of increasing KCl concentration on the V_{max} for the oxidation of proline by disrupted mitochondria suggests that an osmotic effect of KCl rather than an effect due to increasing ionic strength is responsible for the effects described here. ADP (1.4 mM) was added to both the intact and sonicated mitochondria for the determination of maximal rates.

Sonication of mitochondria

Freshly isolated mitochondria suspended in ice-cold isolation medium were sonicated with a 20 sec pulse of a Kontes micro ultrasonicator set at maximum power.

Determination of apparent Michaelis constant and V_{max}

The state 3 rates minus the state 2 rates (as defined by Chance and Williams, 1956) were used as the rates of proline oxidation. Increasing amounts of proline were added sequentially to the same aliquot of mitochondria in the reaction vessel to generate a concentration vs rate relationship. Linear rates for each addition were observed in all cases confirming the absence of significant product inhibition over the time course of the assays. The volume of additions of substrate did not alter the concentrations of the other constituents to a significant extent. The resulting rates and substrate concentrations were plotted using Lineweaver–Burk plots to obtain the apparent K_m and V_{max} at each concentration of KCl.

Protein concentration

Protein concentration was determined using the method of Gornall *et al.* (1949).

Chemicals

All chemicals and biochemicals were of the highest purity available.

RESULTS

A comparison of the effects of hyper-, hypo- and isoosmotic solutions on mitochondria from the clam ventricle shows that lower osmolarities result in higher rates of oxidation of proline while higher osmolarities cause a lower rate of proline oxidation at a given concentration of proline (Fig. 1). Osmolarity has little effect on the maximal rate of oxidation of proline by sonicated mitochondria. By contrast intact mitochondria show a peak in the maximal rate of oxidation over the range 500 to 700 mOsm/l (Fig. 2). The maximal rate of the electron transport chain is demonstrated in sonically disrupted mitochondria oxidizing NADH (Fig. 2). Increasing osmolarity results in slowly declining rates of electron transport. A comparison of the rates of maximal

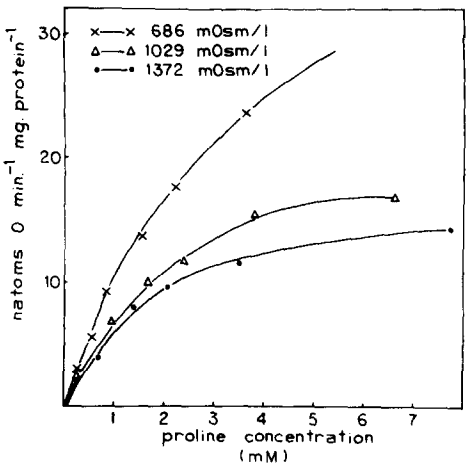

Fig. 1. The effects of osmolarity on the relationship between substrate concentration and the rate of proline oxidation by intact mitochondria from the ventricle of *Mercenaria mercenaria*. The data presented are the results of a representative experiment on the same batch of mitochondria. The protein concentration was 1.27 mg protein per ml. ADP concentration was 1.4 mM. Osmolarity was varied with KCl. Other constituents were as described in Materials and Methods.

oxidation of proline over the range 500 to 700 mOsm/l shows that proline oxidation can proceed as fast as the rate of electron transport in this range.

Increasing osmolarity has little effect on the apparent K_m for proline in disrupted mitochondria over the entire range tested. In the intact mitochondria the apparent K_m reaches a peak value of about 10 mM at 500 mOsm/l (Fig. 3). Above this osmolarity the apparent K_m declines sharply to 2.1 mM at 1000 mOsm/l and remains at this level as osmolarity increases further. Figure 3 also shows the effect of increasing osmolarity on the permeability of the mitochondria as measured by the rate of oxidation of exogenous NADH by intact mitochondria. Intact mitochondria

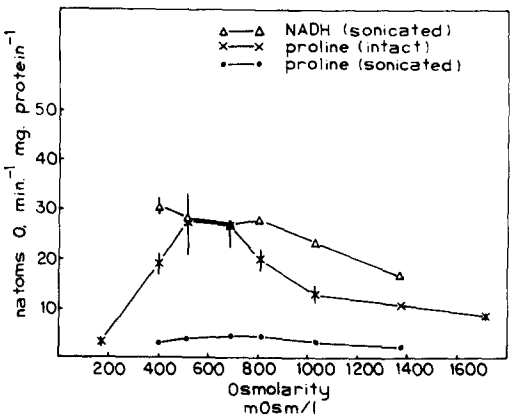

Fig. 2. The effects of osmolarity on the maximal rates of proline oxidation by intact and disrupted mitochondria from the ventricle of *M. mercenaria*. Maximal rates of proline oxidation were determined using Lineweaver–Burk plots as described in Materials and Methods. NADH oxidation was measured at 1.4 mM NADH (saturating under all conditions used). Results are means ± SEM for determinations on three preparations. Where no error bars appear SEM is less than the size of the symbol. Assay conditions were as described in Materials and Methods.

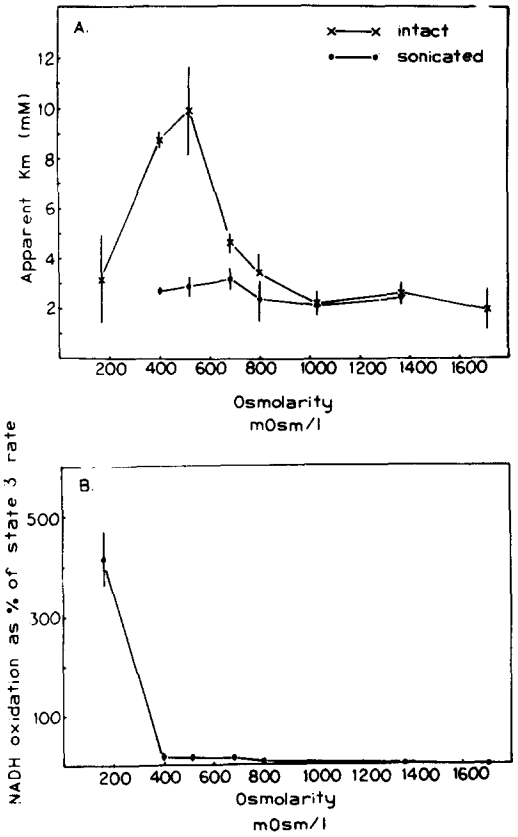

Fig. 3. (A) The effects of osmolarity on the apparent Michaelis constant for the oxidation of proline by intact and sonically disrupted mitochondria from the ventricle of *Mercenaria mercenaria*. (B) The effect of osmolarity on the stimulation of respiration by NADH in intact mitochondria. Results are means ± SEM for three determinations. Where no error bars appear the SEM is smaller than the size of the symbol. Assay conditions were as described in Materials and Methods.

are impermeable to NADH. In the clam mitochondria in the high range of osmolarities down to 500 mOsm/l the rate of oxidation of added NADH is minimal (less than 5% of the state 3 rate). As the osmolarity decreases further (500 down to 200 mOsm/l), the oxidation of NADH increases to 450% of the state 3 rate suggesting complete lysis of the mitochondria.

DISCUSSION

The results obtained here support the speculation of van Winkle (1968) that osmotic effects on mitochondria may explain the higher rates of oxygen consumption by isolated tissues of *Mercenaria mercenaria* in dilute seawater. Osmotic effects on mitochondria may regulate proline oxidation at the level of the proline transporter in the heart of *Mercenaria mercenaria*. Such effects may be involved in the control of the intracellular levels of this and other amino acids during osmotic stress. Indirect evidence for this hypothesis has been obtained using intact and sonicated mitochondria. In the first place, the apparent Michaelis constant and maximal velocity for the oxidation of proline are constant over a wide range of osmolarities in the disrupted mitochondria. This contrasts with the situation in the intact mitochondria where both the K_m and V_{max} rise then fall with increasing osmolarity. In the second place, this effect in intact mitochondria is paralleled by changes in the mitochondrial permeability to NADH implying some stretching of the inner mitochondrial membrane at low osmolarities. This stretching may increase the activity of the proline transporter in the inner mitochondrial membrane. In an analogous situation in rat kidney, the transport of glutamine is altered by mitochondrial swelling (Kovacevik *et al.*, 1980).

Mitochondria from the ventricle of *M. mercenaria* have the ability to oxidize proline at the maximal rate permitted by the electron transport chain over the range 500–700 mOsm/l. The rate of proline oxidation is therefore limited only by the rate of electron transport in this range. The site of control of metabolic flux in mitochondria however, can shift under changing conditions (Tager *et al.*, 1983). In the isoosmotic and hyperosmotic states proline transport may be rate limiting in clam heart mitochondria. Hoek and Njogu (1980) have reported proline transport is not rate limiting in rat liver mitochondria but little is known of the properties of this transporter or its regulation in any species or tissue (LaNoue and Schoolwerth, 1979).

Changing osmolarity in the clam heart does not affect the general permeability (non-specific) of these mitochondria except at osmolarities below 400 mOsm/l although some stretching must occur above this value. As the inner mitochondrial membrane stretches, the configuration of the proline transporter may change, resulting in altered kinetics of transport. Below 400 mOsm/l the mitochondria burst.

The higher apparent K_m observed in low osmolarity would prevent saturation of the transporter during periods when high rates of oxidation of amino acids are required. In early stages of hypoosmotic stress proline from other tissues may be passed to tissues capable of its oxidation such as the heart. A transporter which is saturated only at very high concentrations would be most effective in dealing with this situation. This condition is analogous to the role of liver glucokinase in vertebrates (high K_m for glucose) in dealing with boluses of dietary glucose.

In a euryhaline osmoconforming bivalve the role of short term swelling of mitochondria in the regulation of amino acid oxidation may be crucial to the control of the concentration of those amino acids that can be oxidized during hypoosmotic stress. Among the aerobic tissues the capacity to oxidize amino acids varies as do the types of amino acids oxidized. The mitochondria of the hepatopancreas of *Mercenaria mercenaria* are incapable of oxidizing proline for example (Ballantyne and Storey, 1984). Oxidizable amino acids must presumably be transferred to tissues with the capacity for their oxidation. The selective role of tissues in the oxidation of amino acids has already been proposed in a euryhaline crab (Vincent-Marique and Gilles, 1970) and a bivalve (Henry and Mangum, 1980).

The osmotic properties of mitochondria can account for some of the observed changes in amino acid levels in marine bivalves during osmotic stress. In the early stages of osmotic stress shrinkage or swelling

of the cells will be accompanied by corresponding changes in the mitochondria. These changes critically alter the conformation of an amino acid transporter resulting in different kinetics of amino acid oxidation. At high osmolarity the net effect will be a lower rate of oxidation at a given level of amino acid while at lower osmolarities higher rates of oxidation will be observed. These rates may even approach the maximal rates allowed by electron transport.

Matrical K^+ equilibrates slowly with cytoplasmic K^+ resulting in K^+ levels in the matrix similar to those in the cytoplasm (Brierley, 1976). The osmotic effects described here would occur only in the eariy stages of osmotic stress before K^+ and other osmolytes equilibrate across the inner mitochondrial membrane. The time for the attainment of this equilibrium may therefore determine the time taken to attain volume regulation using mitochondrial mechanisms.

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