EVIDENCE FOR THE EXISTENCE OF AN α-GLYCEROPHOSPHATE OXIDASE SYSTEM WITH THREE PHOSPHORYLATION SITES AND SENSITIVE TO ROTENONE AND PIERICIDIN A

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1. Introduction

The oxidation of α-glycerophosphate by mitochondria isolated from mammalian [1–3] and insect [4–5] muscle involves a specific α-glycerophosphate dehydrogenase [EC 1.1.99.5]; the site of entry of the electrons from α-glycerophosphate into the respiratory chain system is at the (ubiquinone-cytochrome *b*) level. Like the succinate oxidase system, both α-glycerophosphate and succinate are not NAD^+-linked substrates, the oxidation being insensitive to rotenone, amytal and piericidin A [1–3]. The free energy involved in oxidizing one molecule of α-glycerophosphate or succinate is coupled to the synthesis of only two molecules of ATP [1–3].

This paper reports an unusual situation in which the oxidation of α-glycerophosphate is associated with three coupling sites, sensitive to rotenone and piericidin A and also to an excess of the uncoupler, *p*-trifluoromethoxycarbonylcyanidephenylhydrazone (FCCP). This is the first observation of such an α-glycerophosphate oxidase system in mitochondria. The first phosphorylation step is located on the substrate side of the ubiquinone-cytochrome *b* of the respiratory chain system.

2. Experimental procedure

Mitochondria of the *Longissimus dorsi* muscle of ox or Pietrain pig were isolated by modifying the procedure of Makinen and Lee [6]. 1–2 mg Nagarse crystalline *Bacillus subtilis* proteinase was used instead of 5 mg per g wet weight of tissue and homogenization was carried out with a Thomas teflon-pestle glass homogenizer in place of an Ultra-Turrax. In addition, the mitochondrial-containing 14,000 *g* suspension was first centrifuged at 1000 *g* for 10 min and the supernatant from this was then recentrifuged at 7000 *g* for 10 min.

Oxygen uptake was measured polarographically with a Clark oxygen electrode at 25°. Protein was determined by Folin-phenol reagent [7] using bovine serum albumin as standard. The ADP/O ratio and the respiratory control index, RCI, were calculated polarographically [8].

Oligomycin and rotenone and the sodium salt of ADP, DL-α-glycerophosphate, L-malate and succinate were obtained from Sigma; sodium ascorbate and tetramethyl-*p*-phenylenediamine (TMPD) dihydrochloride from B.D.H.; all other reagents were of AnalaR grade.

3. Results and discussion

Mitochondria isolated from the *L. dorsi* of both the ox and the Pietrain pig oxidize α-glycerophosphate with an ADP/O ratio of 3, one higher than the value expected [1–5]. This signifies that in these mitochondria there are three phosporylation sites associated with α-glycerophosphate oxidation.

Fig. 1 illustrates a typical oxygen electrode tracing showing the stimulation of respiration by ADP on

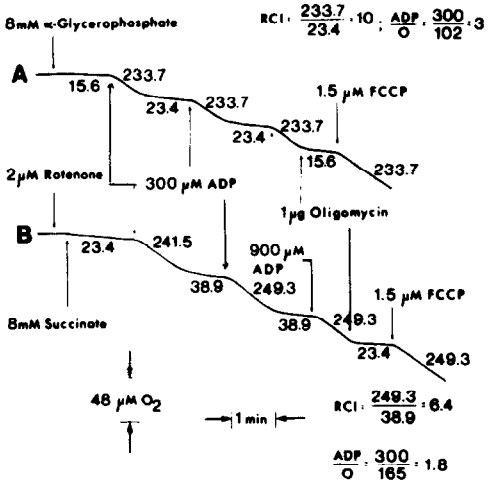

Fig. 1. Polarographic tracings showing the oxidation of α-glycerophosphate and succinate in the mitochondria isolated from the *L. dorsi* of Pietrain pig. All respiratory activities, expressed as natoms of O per min per mg protein, adjacent to the electrode traces, were measured in a 2.50 ml reaction vessel at 25°. Reaction medium (mM): EDTA, 1.0; KCl, 30.0; $MgCl_2$, 6.0; sucrose, 75.0 and KH_2PO_4, 20.0. Final pH 6.9. Total protein, 0.77 mg.

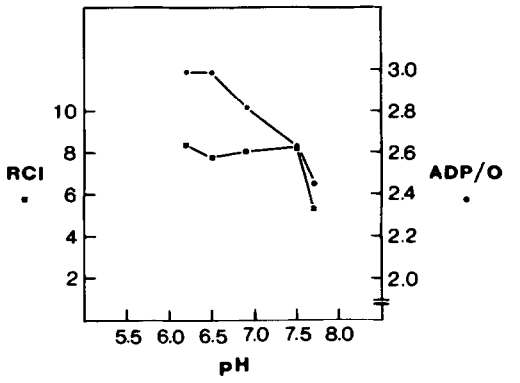

Fig. 3. Effect of pH on the ADP/O ratio and the RCI of α-glycerophosphate oxidation by the mitochondria of *L. dorsi* (Pietrain pig). Experimental procedure as described in fig. 1 using 1 mg mitochondrial protein. The data was obtained from an average of four cycles of state 3 to state 4 transitions.

α-glycerophosphate oxidation (A). The classical state 3 to state 4 transition [8] could be repeated several times giving an ADP/O ratio close to 3, and a RCI of about 10. The state 3 respiratory rate was inhibited

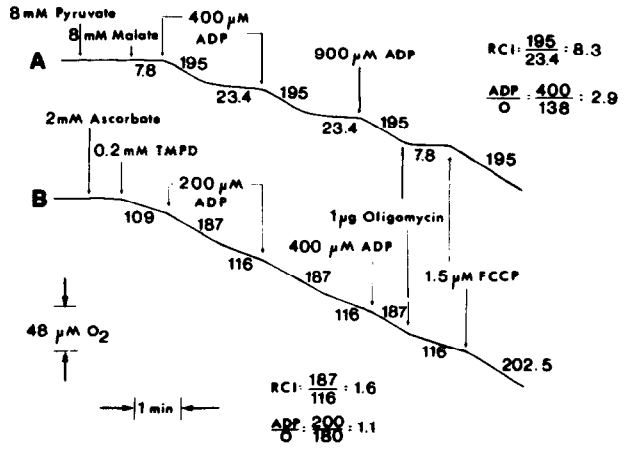

Fig. 2. Polarographic tracings illustrating the mitochondrial oxidation of pyruvate *plus* malate and ascorbate *plus* TMPD from *L. dorsi* (Pietrain pig). Experimental procedure as described in fig. 1 except that in the ascorbate *plus* TMPD experiment (B), antimycin A (0.2 μg per mg protein) was added to the reaction medium. Total protein: A, 0.77 mg; B, 0.77 mg. Final pH 7.20.

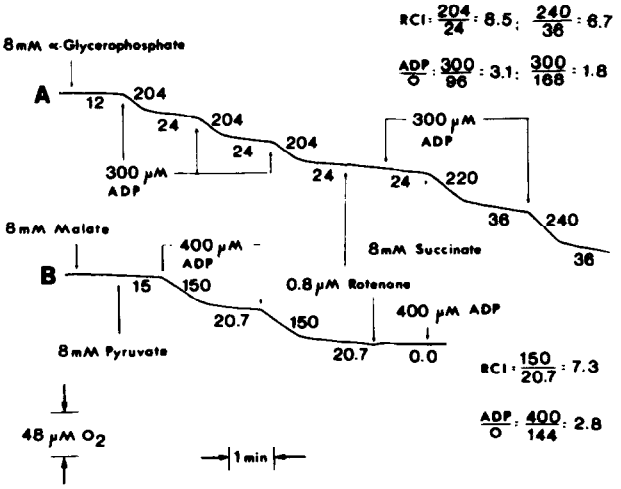

Fig. 4. Polarographic traces demonstrating the inhibition of rotenone on the oxidation of α-glycerophosphate and malate *plus* pyruvate in the mitochondria from the *L. dorsi* (Pietrain pig). Experimental details as described in fig. 1 except the total protein in (A) and (B) were 1.0 and 1.16 mg respectively.

by oligomycin and could be relieved by FCCP. Succinate oxidation on the other hand (B) gave an ADP/O ratio of 1.8, a value very close to the theoretical value of 2.0 [1–5], and a RCI of 6.4.

In order to provide further evidence that the mitochondrial preparation was functioning "normally" and giving the expected ADP/O ratio with other substrates, malate *plus* pyruvate and ascorbate *plus* TMPD were used. The NAD^+-linked pyruvate *plus* malate and the ascorbate *plus* TMPD oxidation should give an ADP/O ratio of about 3 and 1 respectively. Fig. 2 clearly shows that pyruvate *plus* malate (A) and ascorbate *plus* TMPD (B) involved three and one coupling sites, as expected [1–3].

The effect of pH on the ADP/O ratio and the RCI of α-glycerophosphate oxidation by the mitochondria of *L. dorsi* (Pietrain pig) is illustrated in fig. 3. The ADP/O ratio was more sensitive to pH than the RCI in the pH range of 6.0 to 7.5. The ADP/O ratio decreased quite markedly at pH 7.7, falling to about 2.45 from the initial value of about 3 at pH 6.5. The pH effect on the ADP/O for α-glycerophosphate oxidation in the mitochondria from the Pietrain pig was clearly observed in experiments conducted at pH 7.5. After the first two cycles of state 3 to state 4 transition following the addition of 300 μM ADP, the value of the ADP/O ratio gradually decreased from 2.50 to 1.92 at the fifth cycle state 3 to state 4 transition. (The data at pH 7.5 in fig. 3 was the average of the first two cycles from two separate experiments). This interesting phenomenon was not further investigated in the present study.

Another novel feature of the α-glycerophosphate oxidation by the mitochondria from the *L. dorsi* is its sensitivity towards rotenone and piericidin A, inhibitors which normally block NAD^+-linked substrate oxidation [9]. The electrode traces in fig. 4 illustrate the effect of rotenone on α-glycerophosphate oxidation using the NAD^+-linked substrate, pyruvate *plus* malate as a control. The second control was succinate, the oxidation of which was unaffected by rotenone (A). Succinate oxidation gave an ADP/O ratio of about 1.8 as compared with 3.1 with α-glycerophosphate.

Fig. 5 shows the percentage of inhibition of the oxygen uptake of the state 3 respiratory rate against the various concentrations of piericidin A using α-glycerophosphate (B) and pyruvate *plus* malate (A), the latter acting as a control. Both systems were inhibited by piericidin A. For complete blockage, α-glycerophosphate required a slightly higher concentration of piericidin A as compared with malate *plus* pyruvate.

The oxidation of succinate which has to penetrate the inner mitochondrial membrane for access to its dehydrogenase is blocked by an excess of uncoupler [10–12]. With the mitochondria from the *L. dorsi* of ox, an excess of FCCP (11 μM) inhibited 80 and 68% of the state 3 respiratory rate of succinate and α-glycerophosphate respectively. Thus, α-glycerophosphate could be oxidized in the mitochondrial matrix unlike other α-glycerophosphate oxidation reported for insect [13] and mammalian mitochondria [12] in which the α-glycerophosphate dehydrogenase is located on the outside of the inner mitochondrial membrane.

In addition to the demonstration of rotenone and piericidin A sensitivity and the three coupling sites in α-glycerophosphate oxidation of the *L. dorsi* three other important features emerge. Firstly, that the first coupling site of α-glycerophosphate oxidation is located on the substrate side of ubiquinone-cytochrome *b*, as the segment of the respiratory chain

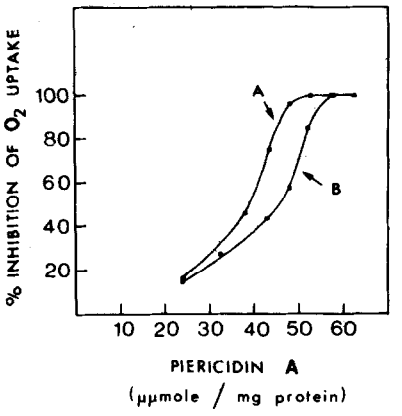

Fig. 5. Inhibition by piericidin A of the state 3 respiratory rate of α-glycerophosphate and malate *plus* pyruvate oxidation by the mitochondria of *L. dorsi* (Ox). Experimental conditions as described in fig. 1, using 1 mg mitochondrial protein and various concentrations of piericidin A to block the oxidation of α-glycerophosphate (B) and malate *plus* pyruvate (A). Piericidin A was added prior to ADP (300 μM for α-glycerophosphate experiment, 400 μM for malate *plus* pyruvate) to initiate the state 3 respiratory rate. The data was the average of two separate experiments.

system from ubiquinone-cytochrome *b* to oxygen involves two phosphorylation sites [1–3, 14]. Secondly, α-glycerophosphate is oxidized inside the inner mitochondrial membrane and a specific transporting system may be involved. Thirdly, the properties of this α-glycerophosphate oxidase system might be common to *L. dorsi* muscles of other species.

Further work is in progress at this laboratory concerning this system.

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