UNCOUPLER-INSENSITIVE RESPIRATORY CONTROL OF α-KETOGLUTARATE OXIDATION BY PLANT MITOCHONDRIA[1]

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Summary: When applied singly or in combination, and in appropriate concentrations, the uncouplers 2,4 dinitrophenol, dicumarol, and arsenate reduced ADP:O ratios of α-ketoglutarate oxidation by isolated wheat mitochondria but failed to stimulate State 4[2] oxidation rates unless small amounts of ATP were also present. The conventional lack of respiratory control of α-ketoglutarate oxidation in the presence of 2,4 dinitrophenol was observed with rat liver mitochondria in the absence of ATP. The evidence suggests that an uncoupler-insensitive ADP-effector site controls α-ketoglutarate oxidation in plant mitochondria.

Although several workers have stressed the fundamental similarities of animal and plant mitochondria, the same investigators (2,3,4) have also found important differences. One phenomenon frequently observed in studies of uncoupling agents and plant mitochondria is that uncouplers seldom give maximal stimulation of State 4 rates in the absence of ATP (2,5,6). Ikuma and Bonner (2) observed such effects of 2,4-dinitrophenol (DNP) on malate oxidation, and of carbonyl cyanide m-chlorophenylhydrazine on succinate oxidation. Wiskich et al (6) observed respiratory control of α-ketoglutarate oxidation in plant mitochondria in the presence of DNP. This control was attributed to the substrate level phosphorylation. In contrast to the plant system, animal mitochondria do not show respiratory control of α-ketoglutarate oxidation in the presence of DNP (7).

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[2] The conventions of B. Chance (1) are used to denote steady-state conditions of oxidative phosphorylation.

We compared the respiratory control of α-ketoglutarate oxidation in plant and animal mitochondria in the presence of uncouplers. In the plant system, control of oxidation by ADP was insensitive to uncouplers of substrate level and respiratory chain phosphorylations. The animal system showed typical lack of respiratory control in the presence of DNP.

MATERIALS AND METHODS

Shoots (sprouts) of wheat, Triticum aestivum var. 31 ms, were a source of mitochondria. The seeds were surface-sterilized in 1% sodium hypochlorite and germinated in rolls of moist paper toweling in the dark at 29 C. The 2 1/2 day-old shoots were harvested and immediately chilled.

Shoots were ground lightly with a mortar and pestle for 45 seconds in the proportion of 1 g shoots : 2 ml of grinding buffer consisting of 0.5 M sucrose, 0.067 M potassium phosphate (pH 7.5), 0.1% BSA, and 1 mM EDTA. The homogenate was diluted five fold with grinding buffer, and strained through two layers of nylon fabric (40 μ mesh). The homogenate was centrifuged in a Sorvall RC-2B with SS-34 head, with a maximum force of 10,000 x g. The supernatant liquid was decanted, then centrifuged with a maximum force of 40,000 x g. The force-time integrals for the centrifugations were 10,000 x g x min. and 40,000 x g x min, respectively. The mitochondrial pellet obtained in the second centrifugation was suspended in pH 7.5 buffer containing 0.3 M mannitol, 0.01 M potassium phosphate and 0.01 M Tris. The grinding and centrifugation was completed in less than fifteen minutes.

Rat livers were sliced, then homogenized in an equal weight of grinding buffer with two passes of a smooth glass-teflon homogenizer which was driven at approximately 2,000 rpm. The homogenate was diluted to the equivalent of 0.1 g liver per ml grinding buffer, and was forced through two layers of nylon. The procedures for centrifugation, suspension, and assay of rat liver mitochondria were identical to those used for wheat shoot mitochondria. All preparative procedures were carried out at 0 to 4 C.

Oxygen uptake was determined at 29 C in a sealed YSI glass-Lucite cell with a Clark oxygen electrode. Assays were made with mitochondria suspended in a total volume of 3 ml. The assay medium contained 0.3 M mannitol, 0.01 M potassium phosphate (pH 7.5), 0.01 M Tris-HCl (pH 7.5), 0.005 M $MgCl_2$, 0.01 M KCl, and 0.1% BSA. Substrates and inhibitors were added during the assay as concentrated solutions. The sequence of additions and final concentrations in uncoupler titrations was as follows: mitochondria, 10 mM α-ketoglutarate, 5 mM malonate, uncoupler, and 0.05 mM ADP. The ADP was added two minutes after uncoupler. The ADP concentration of the stock solution was determined spectrophotometrically at 259 mμ, on the basis of the millimolar extinction coefficient of 15.4. The ADP solution was also standardized with the coupled lactate dehydrogenase, pyruvate kinase assay (8). The enzymatic assay indicated that 99% of the 259 mμ absorbance was due to ADP.

Nitrogen was determined by digestion and direct nesslerization (9) of 5% trichloroacetic acid precipitates of the mitochondrial suspensions.

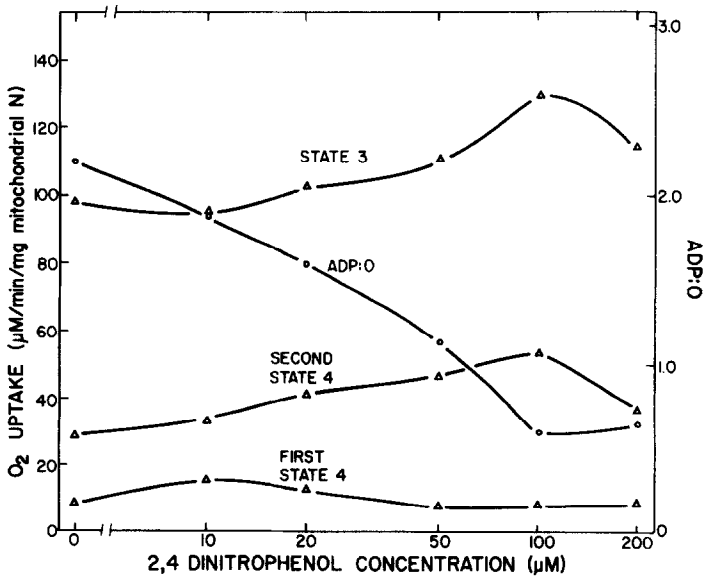

Figure 1. Effect of 2,4 dinitrophenol on α-ketoglutarate oxidation and phosphorylation by wheat mitochondria.

RESULTS AND DISCUSSION

Figure 1 shows the control by ADP of α-ketoglutarate oxidation in wheat shoot mitochondria as affected by DNP. In Figure 2, similar results with dicumarol are shown. The ADP:O ratios decreased to low values between 50 and 100 μM DNP, and between 30 and 50 μM dicumarol. Maximal stimulation of second State 4 rates was observed at 100 μM DNP, and 30 μM dicumarol, but only negligible effects on the first State 4 were observed at any uncoupler concentration. At all uncoupler concentrations, State 4 to State 3 transitions could be induced repeatedly until oxygen was exhausted.

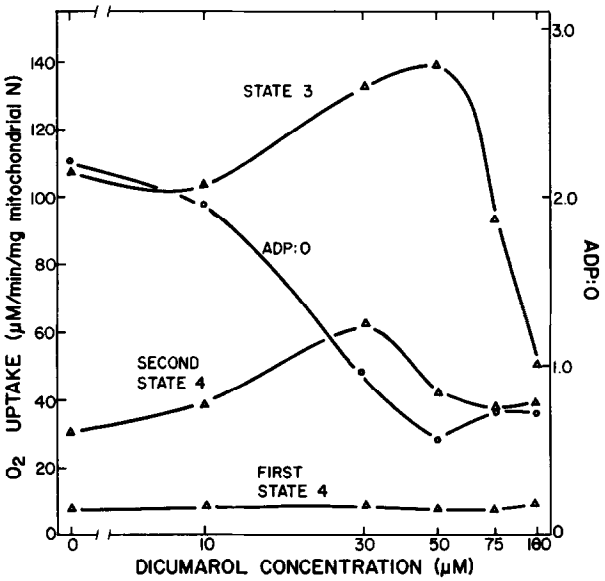

Figure 2. Effects of dicumarol on α-ketoglutarate oxidation and phosphorylation by wheat mitochondria.

Oxidation of α-ketoglutarate by rat liver mitochondria was uncoupled at a DNP concentration of 40 μM, as indicated by stimulations of the first State 4 rates (Figure 3). State 4 and State 3 rates could not be distinguished above 20 μM DNP.

If the DNP-insensitive ADP control site in wheat mitochondria is the substrate level phosphorylation connected with α-ketoglutarate oxidation, arsenate should relieve ADP control when applied in conjunction with DNP

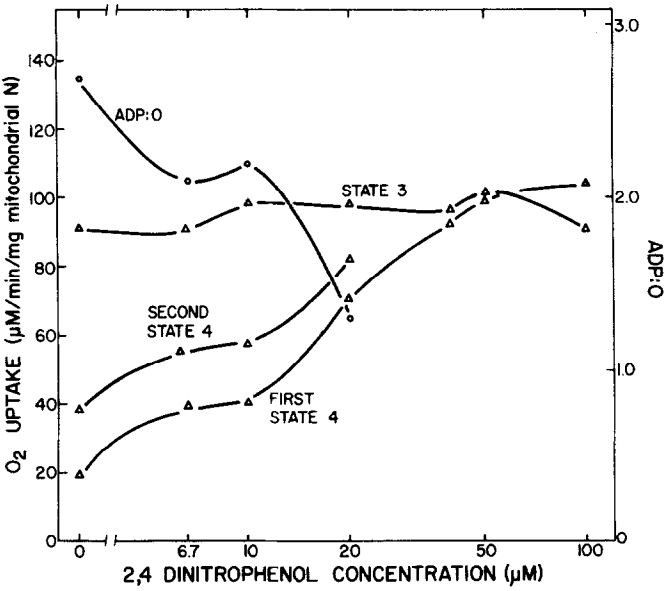

Figure 3. Effect of 2,4 dinitrophenol on α-ketoglutarate oxidation and phosphorylation by rat liver mitochondria.

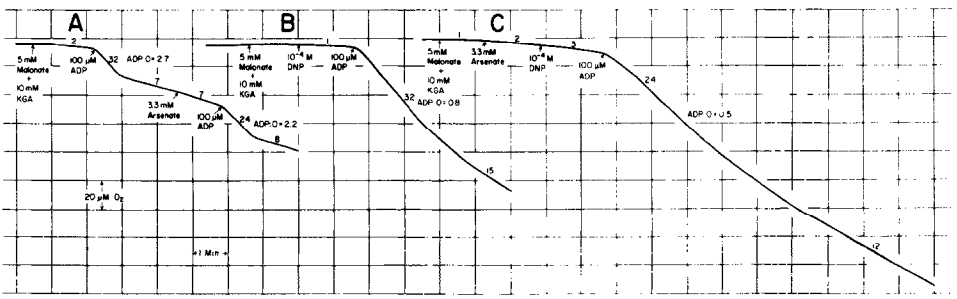

Figure 4. Effect of arsenate and 2,4 dinitrophenol on α-ketoglutarate oxidation and phosphorylation by wheat mitochondria. Additions of substrates and inhibitors to final concentrations given are indicated on traces. Numbers along traces are oxygen uptake in μM/min. Each assay contained 193 μg mitochondrial nitrogen in 3 ml. Oxygen concentration at time of α-ketoglutarate addition was 240 μM.

(10,11). We found that 3.3 mM arsenate reduced the ADP:O ratio of α-ketoglutarate oxidation from 2.7 to 2.2, while inhibiting the State 3

less than 25% (Figure 4, trace A). Higher concentrations of arsenate severely inhibited State 3 rates. In the presence of 100 μM DNP, 3.3 mM arsenate resulted in lower ADP:O values than did DNP alone (Figure 4, traces B and C). In spite of the significant effects of arsenate on the DNP-insensitive phosphorylation, the first State 4 rates of α-ketoglutarate oxidation were not appreciably stimulated by the combination of arsenate and DNP (Figure 4, trace C).

Chappell and Greville (7) showed the existence of an ADP requirement in DNP-treated rat liver mitochondria oxidizing glutamate or α-ketoglutarate, but oligomycin was required to demonstrate the effect. It was concluded that DNP stimulated an oligomycin-sensitive ATPase in this system, and that endogenous ADP was cycled by the substrate level phosphorylation and the ATPase when DNP was present, but not when oligomycin was also present.

Since DNP stimulates ATPase activity in wheat mitochondria prepared by methods similar to those used here (12), the ADP requirement for uncoupled α-ketoglutarate oxidation by wheat mitochondria must reflect a compartmentation or control phenomenon distinct from the animal system. Furthermore, the arsenate effects observed here are difficult to reconcile with an hypothesis of ADP control by the phosphorylation associated with the succinate thiokinase reaction.

The present results appear to be at variance with the current understanding of ADP control of mitochondrial functions. The discrepancy is of considerable interest in view of Ikuma and Bonner's (2) observations that in mung bean mitochondria, malate and succinate State 4 oxidations, which do not involve substrate level ADP control, are stimulated by uncouplers only when ATP is present. While the ATP effect could be a direct one, as has been recently observed with succinic dehydrogenase of rat brain mitochondria (13), an interpretation more consistent with our data is that uncouplers act in the plant system in the presence of suitable amounts of ATP by stimulating ATPase, thus supplying ADP to an uncoupler-insensitive

control site. Since the nature of this control site can not be deduced from the available evidence, we are investigating the problem in detail.

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