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ADP PHOSPHORYLATION AND GLUTAMATE OXIDATION IN MITOCHONDRIA ISOLATED FROM DICTYOSTELIUM DISCOIDEUM AMOEBAE[1]

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SUMMARY. Mitochondria have been isolated from D. discoideum amoebae in which respiration is coupled to ADP phosphorylation. P:O ratios and respiratory control ratios have been obtained for a number of metabolites. In rat liver mitochondria, glutamate is oxidized almost exclusively by a respiration-dependent cyclic transamination pathway, in which glutamate is converted to aspartate. When D. discoideum amoebae are incubated with glutamate alone, aspartate does not accumulate appreciably. Furthermore, when the mitochondria are incubated with glutamate plus malonate at a concentration sufficient to inhibit respiration, their utilization of glutamate is depressed only slightly. Thus, it appears that glutamate oxidation within the mitochondria of D. discoideum amoebae does not, for the most part, proceed by the cyclic transamination pathway.

INTRODUCTION. The cellular slime mold D. discoideum undergoes a starvation-induced morphogenesis in which unicellular amoebae aggregate and then differentiate into two cell types: stalk cells and spore cells. The respiratory activity of the cells during their differentiation may be related to accompanying changes in the morphology of their mitochondria. In the early stages of the developmental sequence, the mitochondria develop inclusions and change in shape (1,2). During this same period, the rate of cellular oxygen consumption begins to decline (3). During the later developmental stages, the outer membranes of the prespore cell mitochondria seem to disappear (1). In the final stages of development the mitochondria of the prespore cells become rounder and more crenated (4,5) and the rate of oxygen consumption declines still further (3). To date, any detailed study of the relationship between the observed changes in the morphological and functional

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characteristics of D. discoideum mitochondria has been precluded by the lack of a method for obtaining functionally intact mitochondria. This paper describes a procedure for the isolation of D. discoideum amoebae mitochondria in which electron transport and oxidative phosphorylation are coupled.

Previous workers have demonstrated that there is a dramatic decrease in both cellular protein content and amino acid pool size during development (6 - 8). During this same period, there is also a seven-fold increase in the rate of glutamate oxidation by the cells (9). Since mitochondria of D. discoideum contain glutamate dehydrogenase activity (10) as well as alanine and aspartate transaminase activities (11), the first step in the intramitochondrial oxidation of glutamate could be either an oxidative deamination or a transamination reaction. A preliminary investigation of the pathways of glutamate oxidation in mitochondria isolated from D. discoideum amoebae is also described here.

EXPERIMENTAL. Isolation of Mitochondria. D. discoideum, strain NC-4 (ATCC 24697), was grown in association with Aerobacter aerogenes as previously described (12). Amoebae were harvested from thirty 100-mm petri dishes with cold distilled water and washed free of bacteria by centrifugation (12). Functional mitochondria were isolated by a modification of the Waring Blender homogenization procedure employed by Sussman and Rayner (13) with axenic strains of D. discoideum. Amoebae were resuspended in 50 ml of cold STEB (0.38 M sucrose, 20 mM Tris-HCℓ, 1 mM EGTA, 0.2% defatted bovine serum albumin, pH 8.0, in glass distilled water) and homogenized in a Waring Blender at low speed for 20 sec. The homogenate was centrifuged at 2,000 × g for 15 min. The resulting supernatant fraction was decanted and recentrifuged twice more under the same conditions. The thrice-centrifuged 2,000 × g supernatant fraction was centrifuged at 10,000 × g for 5 min. The resulting pellet was resuspended in 20 ml of STEB and recentrifuged under the same conditions. The pelleted material was resuspended in 0.5 ml of cold STEB and used immediately. The protein content of the suspension was measured by the method of Lowry et al. (14).

Measurement of Mitochondrial Respiratory Control and P:O Ratios. The rate of oxygen uptake at 22°C was monitored with a Clark-type polarographic oxygen electrode equipped with a Yellow Springs Instruments power supply and a Leeds-Northrup recorder. The incubation mixtures contained 10 mM putative respiratory substrate, 0.35 M sucrose, 10 mM potassium phosphate, 5 mM $MgCℓ_2$, 20 mM KCℓ, 20 mM Tris-HCℓ, 2 mM EGTA, and 0.2% defatted bovine serum albumin, pH 7.4, in glass distilled water (Incubation Medium) and 0.1 ml of mitochondrial suspension in a total volume of 3.0 ml. At the appropriate times, 0.5 umol of ADP was added to the solution.

Malonate Inhibition of Intramitochondrial Glutamate Oxidation. Mitochondria were incubated under the conditions described above, with glutamate serving as the respiratory substrate. Various incubation mixtures also

contained malonate (10 mM) or malonate plus either oxaloacetate or malate (each at 10 mM). The rates of oxygen consumption were again monitored with the oxygen electrode.

Glutamate Utilization by Isolated Mitochondria. Mitochondria were incubated under the conditions described above with glutamate alone and with glutamate plus either oxaloacetate or pyruvate (each at 10 mM). Some of the incubation mixtures also contained malonate (10 mM) or malonate plus malate (10 mM). The incubations were initiated by the addition of 0.1 ml of the mitochondrial suspension. Each incubation was carried out at 22°C for 30 min and was terminated by the addition of 0.3 ml of 35% (w/v) perchloric acid. Precipitated protein was removed by centrifugation at 10,000g for 10 min. Each supernatant fraction was neutralized by the addition of 1.6 mmol of KOH, and the precipitated $KClO_4$ was removed by centrifugation at 2,000g for 5 min. The resulting supernatant fractions were assayed for glutamate and either alanine or aspartate.

Assays for Glutamate, Alanine and Aspartate. The three amino acids were determined enzymatically. Alanine was measured by coupling the alanine transaminase and lactate dehydrogenase reactions. With both enzymes and NADH in excess, the rate of NADH oxidation is proportional to the amount of alanine added (15). Aspartate was measured in a similar manner, by coupling the aspartate transaminase and malate dehydrogenase reactions, and monitoring the oxidation of NADH (16). Glutamate was determined by monitoring the reduction of NAD^+ in the presence of glutamate dehydrogenase (17). For each of the three assay procedures, the quantity of amino acid present was determined by comparison with a concomitantly prepared standard curve.

RESULTS AND DISCUSSION. The respiration rate of mitochondria prepared by the procedure described here was low in the absence of added respiratory substrates [500 nmol $O_2 h^{-1}$ (mg mitochondrial protein)$^{-1}$], as would be expected for intact mitochondria (18). Respiratory control was demonstrated with glutamate, NADH, and succinate, as well as with alanine, oxaloacetate, 2-oxoglutarate, and pyruvate when malate (0.001 M) was added to the incubation mixture (Table 1). As would be expected for intact mitochondria, the addition of NAD^+ to incubation mixtures containing each of the respiratory substrates did not stimulate mitochondrial respiration. The ability of D. discoideum mitochondria to utilize NADH as a respiratory substrate is a property shared in common with the mitochondria of a number of microorganisms (18). The respiratory control and P:O ratios obtained with most of the respiratory substrates examined were relatively low compared to values obtained for mammalian mitochondria (19). However, the values reported here are comparable to those obtained with some yeast and protozoa (18).

TABLE 1: Respiratory control and P:O ratios for mitochondria isolated from D. discoideum amoebae

Respiratory Substrate (10 mM)	Respiratory Control Ratio	P:O Ratio
glutamate	2.33 - 3.74[1] (2.70;10)[2]	2.07 - 3.45 (2.65;8)
NADH	1.36 - 1.40 (1.38;2)	1.61 - 1.89 (1.75;2)
succinate	1.04 - 1.20 (1.12;11)	0.62 - 0.76 (0.70;6)
alanine[3]	1.45 - 2.37 (2.05;4)	2.77 - 3.13 (2.94;4)
oxaloacetate[3]	1.55 - 1.85 (1.69;3)	2.08 - 2.17 (2.13;3)
2-oxo-glutarate[3]	1.29 - 1.55 (1.38;4)	1.53 - 2.04 (1.84;4)
pyruvate[3]	1.94 - 2.82 (2.23;6)	2.17 - 5.55 (3.48;6)

[1] Range of values obtained.

[2] Mean value and number of trials.

[3] 0.001 M malate added to incubation mixture.

Malonate (10 mM) inhibited oxygen uptake by mitochondria incubated with glutamate (Table 2); at a higher malonate concentration (20 mM), the inhibition of oxygen uptake was the same. Presumably, the observed respiratory inhibition resulted from an inhibition of the succinate dehydrogenase reaction, as is the case for other systems. Again, as in other systems, substrates that replenish oxaloacetate tended to alleviate the malonate inhibition of oxygen uptake (Table 2).

When amoebae that have been grown in association with bacteria are harvested, they contain previously ingested bacteria. It was necessary, therefore, to verify that any contaminating bacteria were removed during the isolation procedure described here. This was accomplished in two ways: 1. Cultured A. aerogenes cells were harvested and subjected to the mitochondrial isolation procedure. By the end of the procedure, no detectable

TABLE 2: The effect of malonate on oxygen uptake by mitochondria isolated from D. discoideum amoebae[1]

Additions (10 mM)	Q O_2 [2] %
None	100
Malonate	30 ± 2
Malonate and Malate	86 ± 0
Malonate and Oxaloacetate	57 ± 1

[1] Mitochondria were incubated with glutamate (10 mM). One incubation also contained malonate, while others contained malonate plus either malate or oxaloacetate.

[2] The maximal rate of oxygen uptake was obtained with glutamate alone (Q O_2 = 100%). Each value is the mean (± S.E.) of three replicates. Details of the incubations and measurements are presented in the Experimental section.

material remained. 2. A. aerogenes cells contain ATP-stimulated, AMP-insensitive glutamate dehydrogenase activity, while D. discoideum mitochondrial glutamate dehydrogenase activity is stimulated by AMP and inhibited by ATP (10). When the glutamate dehydrogenase activity in an extract of a mitochondrial preparation was examined for its sensitivity to adenylates, no bacterial glutamate dehydrogenase activity could be detected on this basis.

Isolated D. discoideum mitochondria contain both alanine transaminase and aspartate transaminase activities; the addition of glutamate and either oxaloacetate or pyruvate to the mitochondria resulted in the disappearance of glutamate and the concomitant formation of aspartate or alanine, respectively (Table 3). Intramitochondrial glutamate oxidation in D. discoideum amoebae mitochondria could begin with an oxidative deamination reaction or a simple transamination reaction. Alternatively, glutamate oxidation could proceed by a respiration-dependent cyclic transamination pathway in which glutamate is converted to aspartate (Fig. 1). In rat liver mitochondria, glutamate is oxidized almost exclusively by the cyclic transamination pathway (20-22); the addition of malonate greatly suppresses this pathway. When

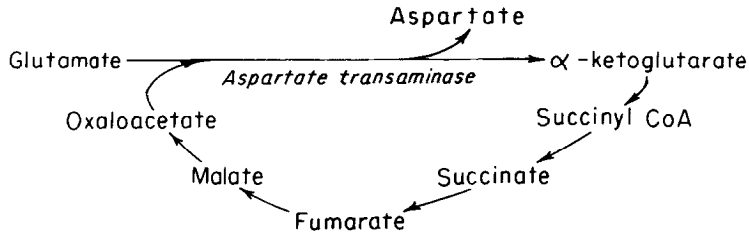

Fig. 1. Cyclic transamination pathway for glutamate oxidation.

mitochondria isolated from D. discoideum amoebae were incubated with glutamate, only about 20% of the glutamate that is utilized was recovered as aspartate (Table 3). Furthermore, when the mitochondria were incubated with glutamate plus malonate, glutamate utilization was depressed only slightly (Table 3). These results suggest that the cyclic transamination pathway accounts for no more than about 20% of the glutamate that is oxidized by D. discoideum amoebae mitochondria.

TABLE 3: Glutamate utilization by mitochondria isolated from D. discoideum amoebae[1]

Additions (10 mM)	Glutamate Utilization	Aspartate Formation	Alanine Formation
	umol		
None	2.6 ± 0.3	0.5 ± 0.1	Not measured
Malonate	2.0 ± 0.2	0.5 ± 0.1	" "
Malonate and Malate	3.0 ± 0.2	2.0 ± 0.2	" "
Oxaloacetate	4.5 ± 1.0	4.0 ± 1.0	" "
Pyruvate	6.0 ± 1.8	0.5 ± 0.3	4.5 1.1

[1] Mitochondria were incubated for 30 min with 10 mM glutamate. Each value is the mean (± S.E.) of three replicates. Mitochondrial protein (mg) was the same for each of the replicates. Alanine was measured only in the pyruvate incubations. Details of the incubation procedure are presented in the Experimental section, as are details of the assays for glutamate, aspartate and alanine.

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