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L-3-GLYCEROL PHOSPHATE OXIDATION WITH ENERGY COUPLING IN SUBMITOCHONDRIAL PARTICLES FROM SKELETAL MUSCLE MITOCHONDRIA

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SUMMARY

Submitochondrial particles (SMP) from rabbit skeletal muscle mitochondria (RMM) oxidize NADH, succinate and L-3-glycerol phosphate (L-3-GP); NADH is not oxidized by RMM unless cholate is added. Ferricyanide has complete access to succinic and L-3-GP dehydrogenases in SMP, but access only to L-3-GP dehydrogenase in RMM. Energy conservation in SMP was demonstrated by the criteria of respiratory control and uncoupler-induced oxidation of cytochrome b with all three substrates. L-3-GP dehydrogenase is on the outer, while NADH and succinic dehydrogenases are on the inner side of the RMM inner membrane which is impermeable to NADH, L-3-GP and ferricyanide. Since SMP are effectively permeable to these three substrates, they must be open membrane fragments capable of conserving energy in the absence of transmembrane electrochemical potentials.

Submitochondrial particles derived from beef heart mitochondria by sonication in the presence of Mg^{+2} and ATP (1) or EDTA (2) retain the energy conservation capacity of the intact inner mitochondrial membrane. These particles show an energy-linked uptake of protons induced by respiration with substrate (3-7), a reaction now considered central to the mechanism of energy conservation (8). The direction of proton uptake is opposite to that observed upon energization of intact mitochondria (3-5,8). This result is predicted by the chemiosmotic theory of energy conservation (3), if submitochondrial particles are, as originally suggested by Lee and Ernster (2), sealed vesicles whose membranes are "inside-out" and still retain the permeability barriers characteristic of the inner mitochondrial membrane. Retention of these barriers by the membrane is required to maintain between two discrete aqueous phases the transmembrane gradients and/or potentials which are central to the chemiosmotic hypothesis of energy conservation. We have

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previously categorized this hypothesis as a transmembrane hypothesis for energy-linked ion movements in the mitochondrial membrane (9,10). An alternate hypothesis suggested was the intramembrane hypothesis (9,10) which postulates that ion movements occur between suspending medium and the membrane itself. This hypothesis, and the localized or microchemiosmotic model of Williams (11), agree precisely in defining the requirements for energy conservation as two phases rather than three; transmembrane permeability barriers do not enter into this formulation. Williams (11) clearly summarizes the differences between the two models, which revolve around the crucial point of barrier requirements and their consequences, particularly as regards membrane charge and the mode of vectorial ion transport (11,12).

We sought an experimental test for the retention or loss of the permeability barriers of the membrane in submitochondrial particles by utilizing skeletal muscle mitochondria. These have two dehydrogenases located on opposite sides of the inner mitochondrial membrane which are linked to the respiratory chain: NADH dehydrogenase on the inner or matrix side, and L-3-glycerol phosphate (L-3-GP) dehydrogenase on the outer side (13). The inner membrane has been shown to be impermeable to both substrates (13-15). The transmembrane or chemiosmotic theory would predict that skeletal muscle submitochondrial particles, which show energy coupling, would oxidize NADH but not L-3-GP if "inside-out"; if "right-side-to", they would oxidize L-3-GP but not NADH. The intramembrane or localized hypothesis predicts that these particles could oxidize both NADH and L-3-GP with energy conservation, since they need not be sealed vesicles with a membrane permeability barrier. This paper reports the preparation of submitochondrial particles from skeletal muscle which oxidize both substrates with energy conservation.

MATERIALS AND METHODS

All reagents were of the best grade available commercially and were used without further purification. The uncoupler of oxidative phosphorylation, bis(hexafluoroacetonyl) acetone (designated 1799), was generously provided by Dr. Peter G. Heytler of E.I. duPont de Nemours Co.

Rabbit skeletal muscle mitochondria (RMM) were isolated from the hind legs of rabbits at 4°C. Excised muscle was placed in 0.15 M KCl buffered with 20 mM Tris-Cl at pH 7.4. Connective tissue was removed and the muscle (40-50 g) was minced. The mince was homogenized for 25 sec with an inverted Polytron in a Blendor jar (16) containing 700 ml of medium consisting of 120 mM KCl, 50 mM Tris-Cl, pH 7.4, 3 mM $MgSO_4$, and 1 mM EGTA. The homogenate was filtered through 2, then 4 layers of bleached cheesecloth, and centrifuged at 700 x g for 15 min. The supernatant was decanted through 8 layers of cheesecloth, then stirred for 5 min with Nagarse (Enzyme Development Corp., New York; 1 mg/g tissue). The digest was diluted 1:1 with medium containing 0.8% BSA, then centrifuged at 9500 x g for 10 min. The pellet was resuspended in 50 ml of the BSA medium, followed by centrifugation at 800 x g for 10 min. The supernatant was centrifuged at 7700 x g for 10 min, the pellet washed, resuspended in medium containing 0.25 M sucrose/10 mM Tris-Cl, pH 7.4, centrifuged at 7700 x g for 10 min, then resuspended in 0.25 M sucrose/10 mM Tris-Cl, pH 7.4, to give 15-30 mg protein/ml.

Submitochondrial particles (SMP) were prepared from RMM by hypotonic treatment (20 min; 2-3 mg protein/ml) in medium containing 20 mM mannitol, 5.0 mM sucrose, 2.0 mM $MgSO_4$, 0.1 mM EDTA, 2.5 mg/ml BSA and 5 mM MOPS, pH 7.4, followed by disruption in a French pressure cell at 4600 psi. The suspension was centrifuged at 8500 x g for 20 min; the pellet was discarded and the supernatant centrifuged at 100,000 x g for 30 min. This pellet (SMP) was resuspended in 250 mM sucrose/10 mM Tris-Cl to give 5-10 mg protein/ml.

The respiratory activities of RMM and SMP were determined polarographically. Spectrophotometric determination of cytochromes and ferricyanide reduction was carried out with a DW-2A dual wavelength spectrophotometer (American Instrument Co., Silver Spring, MD). Protein was determined by the method of Miller (17).

RESULTS AND DISCUSSION

A comparison of NADH oxidation by RMM and SMP is shown in Fig. 1. There is no oxidation of NADH by RMM in the absence of cholate, but addition of cholate induces oxidation (Fig. 1A). At 0.4% cholate, the NADH oxidation rate is maximal, and there is no stimulation by added uncoupler. NADH is readily oxidized by SMP (Fig. 1B), and the rate is stimulated by uncoupler. There is no stimulation of the uncoupled oxidation rate with SMP by added cholate. The rates obtained with cholate-treated RMM and uncoupled SMP are nearly the same; near identity of rates was found consistently with these preparations. The NADH dehydrogenase on the matrix side of the inner membrane of RMM becomes accessible to its substrate in SMP, in which respect these SMP resemble beef heart EDTA particles (2).

The rates of L-3-GP, succinate and NADH oxidation by SMP are compared in Fig. 2. The uncoupled rates with L-3-GP and succinate are consistently

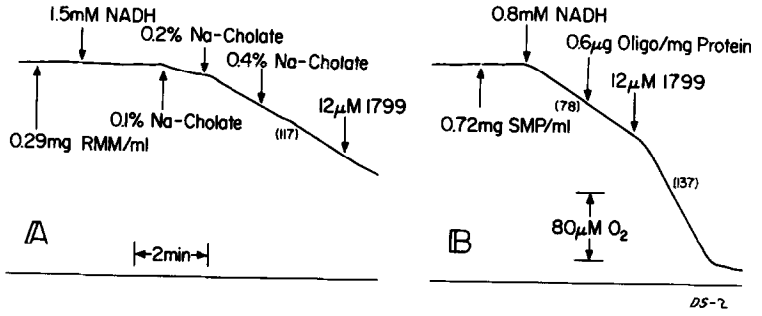

Fig. 1: Oxygen uptake at 25°C by intact skeletal mitochondria (RMM) and submitochondrial particles with NADH as oxidizable substrate. Assay medium contained 0.25 M sucrose with 10 mM Tris-HCl, pH 7.4. Protein concentrations are shown at beginning of the traces. Numbers in parentheses give rates as nmol O_2/min-mg protein. A. Effect of cholate addition on NADH oxidation in RMM. B. NADH oxidation by SMP.

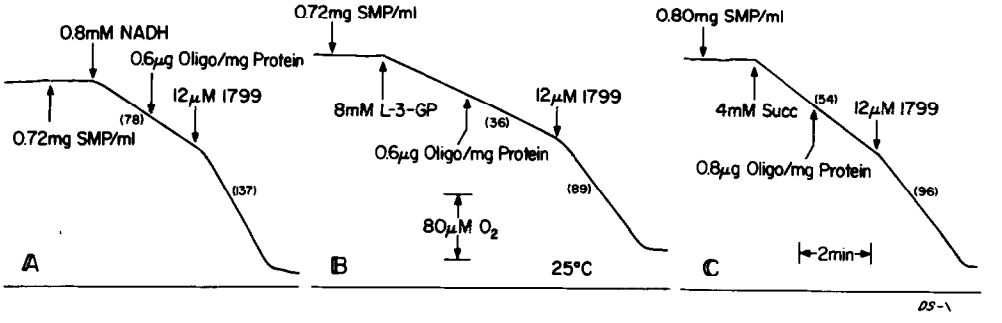

Fig. 2: Oxygen uptake by SMP at 25°C with NADH, L-3-GP and succinate as substrates. Rates expressed as nmol O_2/min-mg protein. Respiratory control ratios (uncoupled/oligomycin) are 1.7, 2.4 and 1.7 for NADH, L-3-GP and succinate, respectively. Assay medium as in Fig. 1.

about 30% less than that observed with NADH and again very similar to those observed in RMM. Energy conservation is observed with all three substrates by the criterion of respiratory control, as defined by uncoupler stimulation of respiratory rate in oligomycin-treated submitochondrial particles, a general criterion developed by Lee and Ernster (2). These SMP have not been exposed to high pH, as have EDTA particles, and so are coupled as prepared: oligomycin does not inhibit the initial rate, but uncoupler stimulates it. These SMP also show energy conservation by the criterion of uncoupler-induced oxidation of cytochrome b (18) as shown in Fig. 3. This experiment also

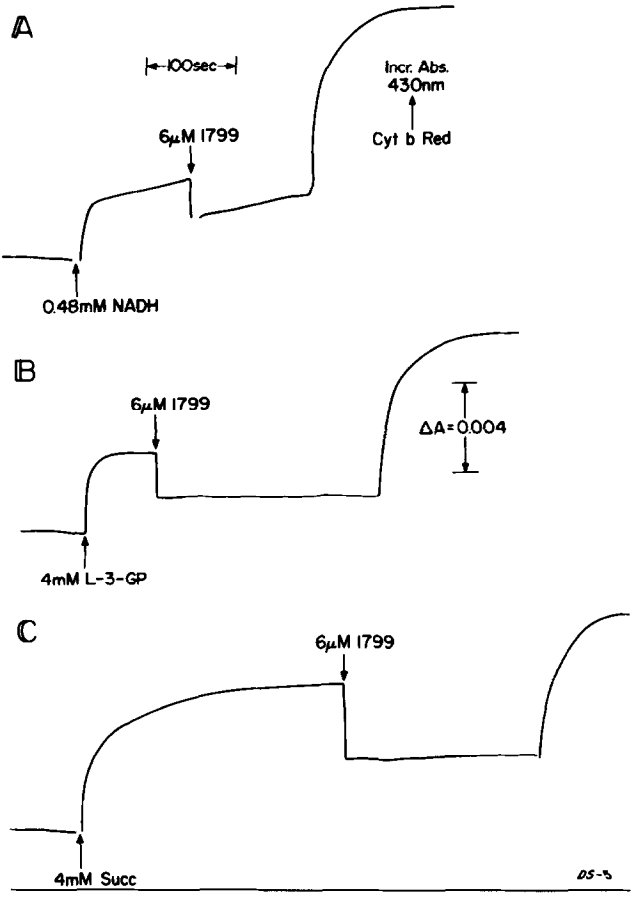

Fig. 3: Uncoupler-induced reoxidation of cytochrome b measured at 430-410 nm. Reaction mixture contained 0.5 ml assay medium, 0.16 mg SMP protein/ml and 2.0 μg oligomycin/mg SMP protein. Light path was 0.5 cm. Reaction was started with addition of NADH, L-3-GP or succinate. After the steady state redox level of cytochrome b was attained, uncoupler (I799) was added, causing a shift in the redox level toward oxidation (downward deflection of trace). The rapid rate of O_2 consumption induced by uncoupler causes anaerobiosis in a few minutes, with resultant reduction of cytochrome b (upward deflection of trace). The same quantity of cytochrome b is reduced in anaerobiosis by each of the three substrates.

shows that each substrate reduced the same pool of cytochrome b in anaerobiosis. Cytochrome c is also completely and rapidly reduced in anaerobiosis by each of the three substrates. Were this a mixed population of "inside-out" and "right-side-to" sealed vesicles, NADH and L-3-GP could each reduce only part of the cytochrome b and c pools; both substrates would be required for complete reduction.

Succinate-supported reduction of ferricyanide is inhibited by antimycin A in RMM, but L-3-GP-supported reduction is not, as demonstrated by Klingenberg and Bucholz (15) who did not examine this reaction in SMP. We find in SMP that ferricyanide reduction by both succinate and L-3-GP is not inhibited by antimycin A (Fig. 4); the reduction rates with both substrates are similar. Malonate inhibits ferricyanide reduction by succinate but not by L-3-GP (Fig. 4), showing that two different dehydrogenases are operative. Ferricyanide has access to succinic dehydrogenase in SMP, but not in RMM where its reduction is mediated by cytochrome c, since ferricyanide is impermeable to the inner mitochondrial membrane (15). The reduction of ferricyanide by L-3-GP is mediated by L-3-GP dehydrogenase, to which ferricyanide has access in RMM. Ferricyanide has equal access to both dehydrogenases in SMP, showing that, in contrast to RMM, SMP have no permeability barrier to this acceptor.

SMP prepared by high pressure disruption of skeletal muscle mitochondria retain the capacity for energy conservation, but lose the permeability barriers of the intact inner mitochondrial membrane toward NADH, L-3-GP and ferricyanide. If SMP were truly sealed vesicles, one would have to postulate either the creation of channels or carriers for L-3-GP and ferricyanide or the complete inversion of L-3-GP dehydrogenase orientation in the membrane during SMP preparation. These postulates would render invalid all other criteria for judging the "sidedness" of SMP. We conclude that these SMP lack "sidedness" and so are not tightly sealed vesicles, but are open membrane fragments. Similar conclusions from experiments with other preparations of submitochondrial particles have been drawn by Komai et al. (19) and Azzone et al. (20). Energy coupling in open membrane fragments is consistent with the localized (11) or intramembrane theory (10) but not with the chemiosmotic theory (3). We do not claim that all submitochondrial particles are devoid of functional sidedness: Fleischer et al. (21) report that "inside-out" submitochondrial vesicles with low sucrose permeability can be

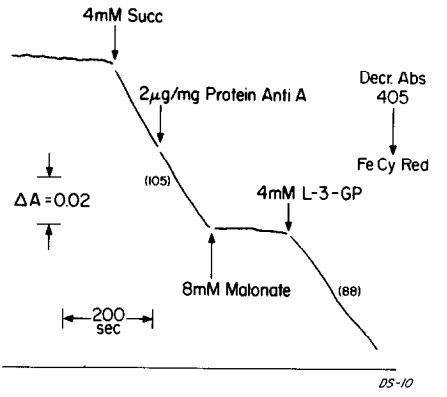

Fig. 4: Ferricyanide reduction by SMP at 25°C with succinate and L-3-GP as substrates, measured at 405-436 nm. Reaction mixture contained 0.5 ml, as in Fig. 3, 0.33 mg SMP protein/ml, 0.8 mM ferricyanide and 1.0 mM KCN. Numbers in parentheses are rates of ferricyanide reduction expressed as nmol/min-mg protein.

prepared from beef heart mitochondria by nitrogen decompression at 2000 psi. We do propose, however, that the results obtained with skeletal muscle SMP show that energy conservation in the mitochondrial membrane involves chemical and/or conformational reactions of membrane proteins (22-24) - consistent with the concept of a "Membrane Bohr Effect" (25,26) - and does not require the formation and maintenance of transmembrane electrochemical potentials.

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