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ENERGY-LINKED H^+ EFFLUX AND UNCOUPLER-INDUCED H^+ INFLUX IN SUBMITOCHONDRIAL PARTICLES FROM SKELETAL MUSCLE

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SUMMARY

Submitochondrial particles (SMP) from rabbit skeletal muscle mitochondria (RMM) show an energy-linked efflux of H^+ in the suspending medium with either succinate or L-3-glycerolphosphate as substrates. Uncoupler not only reverses the energy-linked H^+ efflux, but also induces a non-energy-linked H^+ uptake. Intact RMM also show energy-linked H^+ efflux under the same conditions used to energize SMP. Skeletal muscle SMP are open membrane fragments incapable of sustaining a transmembrane gradient, in contrast to SMP from beef heart, which may be closed vesicles and which show energy-linked H^+ uptake. The difference of direction in energy-linked H^+ movements in the two types of SMP, both of which retain the capacity for energy coupling, strongly supports the concept that this H^+ movement is secondary to the energy conservation process, which is postulated to occur in the membrane and to be shielded from the suspending medium.

Submitochondrial particles derived from rabbit skeletal muscle mitochondria by disruption with the French pressure cell oxidize succinate, L-3-glycerolphosphate and NADH with conservation of energy, as demonstrated by the criteria of respiratory control and uncoupler-induced oxidation of cytochrome b (1). L-3-glycerolphosphate and NADH are both impermeable to the inner mitochondrial membrane, and their respective dehydrogenases are located on opposite sides of the membrane (2-4). This means that submitochondrial particles from skeletal muscle are effectively open membrane fragments which retain the energy conservation capacity of the intact inner membrane but cannot maintain transmembrane electrochemical gradients (1,5). Submitochondrial particles derived from beef heart mitochondria by

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sonication not only retain the capacity to conserve energy (6,7), they also can utilize energy to take up H^+ from the medium. The direction of H^+ uptake is opposite to the energy-linked H^+ release observed with intact beef heart mitochondria (8-14). The direction of H^+ movement in the two beef heart systems is the one predicted by the chemiosmotic theory of energy conservation (8,15,16), if, as originally suggested by Lee and Ernster (7), submitochondrial particles are sealed vesicles of inner membrane with functional permeability barriers, but with the membrane orientation "inside-out" relative to the intact mitochondria. These beef heart particles should be capable of maintaining between the outside and inside aqueous phases a transmembrane H^+ gradient whose electrochemical potential would be given by the Nernst formulation (16). But the direction of H^+ movement could be equally well accounted for by a movement of H^+ into the membrane: the gradient would then exist between membrane and medium, and the electrochemical potential between the phases would be given by the Donnan formulation (17).

The preparation of submitochondrial particles from skeletal muscle which cannot sustain a transmembrane H^+ gradient provides a useful test system for analyzing these H^+ movements. Three questions arise. 1) Can open membrane fragments of the inner membrane utilize the energy of substrate oxidation to drive a net observable H^+ movement between medium and membrane? 2) In what direction do the H^+ move? 3) Does the membrane orientation of the substrate dehydrogenase influence the direction of H^+ movement? In this paper, we report the results of experiments designed to answer these questions.

MATERIALS AND METHODS

All reagents were of the purest grade available commercially and were used directly. The uncoupler of oxidative phosphorylation, 1799 (bis-hexafluoroacetonylacetone), was the generous gift of Dr. Peter G. Heytler of E. I. duPont de Nemours Co.

Skeletal muscle mitochondria (RMM) were isolated from the hind leg muscles of rabbits by the procedure previously described (1). The final mitochondrial pellet was resuspended in 0.25 M sucrose/10 mM Tris-acetate, pH 7.4, at 15-30 mg protein/ml. Submitochondrial particles (SMP) were pre-

pared from RMM by hypotonic treatment, followed by disruption in a French pressure cell as previously described (1). The final pellet was resuspended in 0.25 M sucrose/5 mM Tris-acetate, pH 7.4, at 5-10 mg protein/ml. Protein was determined by the method of Miller (18).

Movements of H^+ were measured as pH changes in the suspending medium of RMM and SMP upon oxygenation of the anaerobic suspension containing catalase with H_2O_2, following the procedure of Papa et al. (10,13). The reaction medium was 0.25 M sucrose containing catalase at 0.3 mg/ml at pH 7.5. No additional buffer capacity was added beyond that provided by the substrates. Succinate is a relatively poor buffer at pH 7.5, and so pH changes were readily observable; L-3-glycerolphosphate is a good buffer at this pH which reduced the sensitivity of the measurement. pH changes were measured in a stirred reaction volume of 1.5 ml with a Leeds and Northrup glass pH electrode (LN 117145) and reference electrode (LN 117147) connected to an Orion 701A pH meter. Stability of 0.001 pH unit was readily attained with electrical shielding and was not perturbed by stirring, since the fiber junction of the reference electrode is constructed to be insensitive to perturbations of pressure. A blanket of N_2 was maintained over the reaction mixture to maintain anaerobiosis in the absence of added O_2. The reaction was started by addition of an aliquot of 1.5% H_2O_2 in deionized water to the anaerobic suspension of RMM or SMP containing substrate, which caused no perturbation of pH. Both RMM and SMP were treated with oligomycin (0.4 µg/ml protein) 40-60 min prior to the start of the experiment.

RESULTS AND DISCUSSION

A comparison of energy-linked H^+ movements observed with RMM and SMP with succinate or glycerolphosphate as substrate is shown in Figure 1. With both RMM and SMP, H^+ efflux is observed; the transition to anaerobiosis results in H^+ uptake. The H^+ efflux from RMM reaches a true steady state, but that from SMP continues to increase slowly, after an initial rapid rate, during the entire period of O_2 availability. Maximal H^+ efflux from SMP was 30 ng ion/mg protein with both substrates. The effect of the uncoupler 1799 upon energy-linked H^+ efflux from SMP with both succinate and L-3-glycerolphosphate as substrates is shown in Figure 2. Uncoupler induces H^+ uptake, whose extent is greater than that of the original H^+ release. This is in contrast to the H^+ uptake resulting from anaerobiosis which is less than the original efflux (Fig. 1). Uncoupler has two effects with these SMP: it not only reverses energy-linked H^+ efflux, it also induces a non-energy-linked H^+ uptake which can be observed on its addition after anaerobiosis.

The only added anion present in the experiments shown in Figure 2 was the substrate anion. The effect of added anions on H^+ efflux in these SMP is

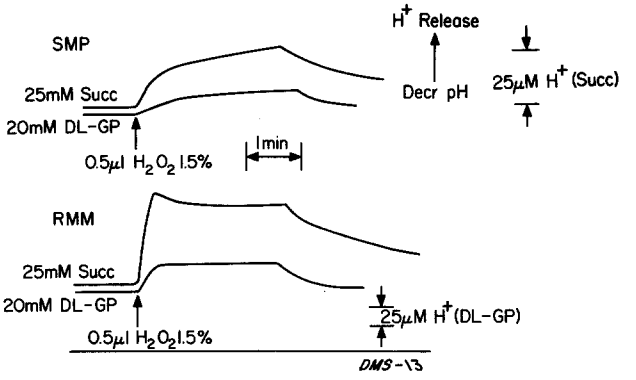

Fig. 1: Efflux of H^+ from SMP and RMM energized with succinate (K^+ salt) or L-3-glycerolphosphate added as the disodium salt of the DL form (DL-GP). SMP (0.9 mg protein/ml) or RMM (1.0 mg protein/ml) were incubated in 0.25 M sucrose containing 0.3 mg catalase/ml and the indicated substrate at pH 7.5 and 25°C, as described in Materials and Methods. The reaction was initiated by addition of H_2O_2, as shown by the arrows. Note the higher sensitivity for a given efflux of H^+ with succinate as compared to DL-glycerol-phosphate.

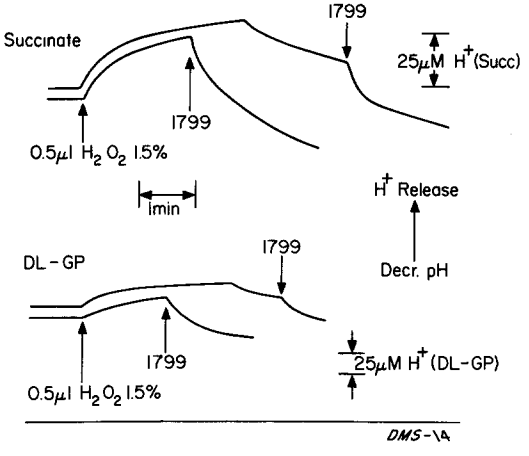

Fig. 2: Uncoupler (1799)-induced uptake of H^+ in SMP energized with succinate or L-3-glycerolphosphate. Conditions were as in the experiment of Fig. 1; 25 mM substrate was used. Final concentration of 1799 was 3 μM. The top trace of each pair shows the proton uptake which occurs on anaerobiosis, prior to addition of uncoupler. SMP at 0.9 mg protein/ml.

shown in Fig. 3; in addition to the substrate succinate, Cl^- (Fig. 3b) and SCN^- (Fig. 3c) were present as the K^+ salts. Compared with the control, 12 mM Cl^- increased the extent of H^+ efflux 20%, and 15 mM SCN^- increased it 40%; 12 mM NO_3^- gave precisely the same result as 12 mM Cl^-. With Cl^-,

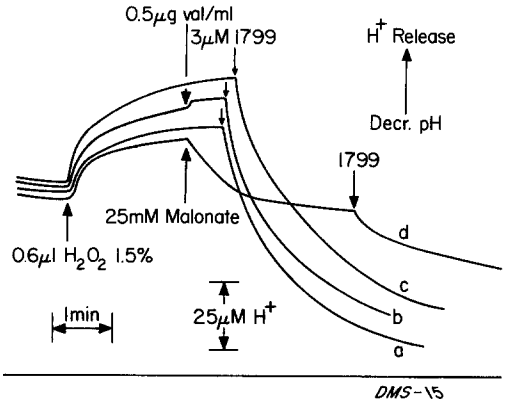

Fig. 3: Effect of KCl, KSCN, valinomycin and malonate on H^+ efflux from SMP energized with succinate. Conditions as in the experiment of Fig. 1. a) Control. b) 12 mM KCl in medium. Valinomycin (val) was added at the point shown. c) 15 mM KSCN in medium. d) 25 mM malonate added at point shown. Sensitivity scale for H^+ movement does not apply to this trace after malonate addition because of buffering capacity added by the malonate. SMP at 0.9 mg protein/ml.

there was a slight increase in efflux upon addition of valinomycin (Fig. 3b). The rate and extent of H^+ uptake upon addition of uncoupler was nearly the same for the control and the two anions. This uptake includes both the reversal of energy-linked efflux which can be accomplished by addition of an inhibitor such as malonate and a separate effect of uncoupler (Fig. 3d).

Neither Cl^- nor SCN^- had any effect on the energy-linked H^+ efflux from RMM (Fig. 4) under the same conditions used with SMP (Fig. 3). Valinomycin induced a rapid release of H^+, followed by a slower uptake (Fig. 4a). As with SMP, uncoupler induces H^+ uptake whose extent exceeds that of the original efflux and is due to a direct effect as well as to abolition of energy-linked functions.

The three questions concerning H^+ movements in skeletal muscle submitochondrial particles can be answered as follows. 1) Energy-linked H^+ movement occurs between membrane and medium in these membrane fragments. 2) The direction of H^+ movement is efflux from the membrane, the same direction observed with intact mitochondria under the same conditions.

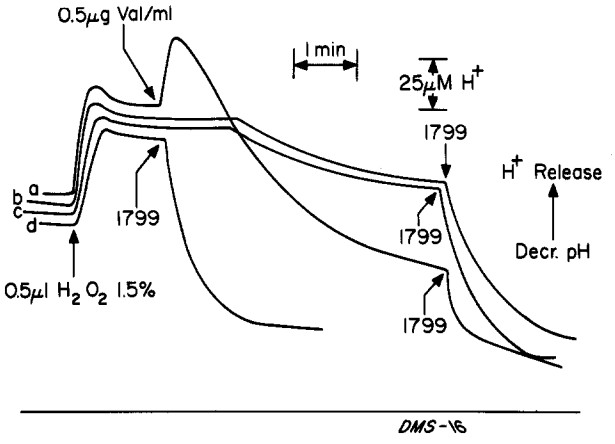

Fig. 4: Efflux of H^+ from RMM under conditions comparable to those of the experiment in Fig. 3. a) 12 mM KCl in the medium. Valinomycin (val) added at the point shown. b) Control. c) 15 mM KSCN in the medium. d) Uncoupler (3 μM 1799) added before the transition to anaerobiosis. Conditions otherwise as in the experiment of Fig. 1. RMM at 1.1 mg protein/ml.

3) The membrane orientation of substrate dehydrogenase does not effect the direction of H^+ movement: the same direction and extent is observed with succinate and L-3-glycerolphosphate, whose dehydrogenases are on opposite sides of the inner mitochondrial membrane (2-4).

The observation that energy-linked H^+ movement in SMP from rabbit skeletal muscle have a direction opposite to that observed in beef heart submitochondrial particles supports the suggestion that the latter are sealed vesicles (7) which have the capacity for transmembrane ion transport (19-21). The former cannot sustain such transport, since they have lost the permeability barriers characteristic of the inner mitochondrial membrane (1,5). We propose that the first reaction of energy-linked cation transport by the inner mitochondrial membrane is cation uptake by the membrane on its outer face in exchange for H^+. The process stops at this point in rabbit skeletal muscle SMP. The process occurs in beef heart SMP inside the vesicles and can continue under the proper conditions to give a net uptake of H^+ from the medium by ion translocation. This model would explain why our results with Cl^-, NO_3^-, SCN^- and valinomycin are in such marked contrast to those of Papa

et al. (10,13). They showed that H^+ uptake in beef heart submitochondrial particles was low in the presence of succinate alone or succinate plus Cl^-, but was markedly increased by valinomycin or by NO_3^- or SCN^- in the absence of valinomycin: just the effects to be expected if ion translocation were occurring.

The difference in direction of energy-linked H^+ movement in the two different types of SMP strongly supports the concept that ion movements between membrane and suspending medium are secondary to the energy conservation process. If this is so, what then is the primary process? There is much experimental support and a general consensus that H^+ is a key actor in this drama (22). One should look within the membrane for this primary process which would be shielded from the suspending medium (23). Indeed, intramembrane H^+ transfer, as detected by the fluorescent probe quinacrine, can be observed in beef heart submitochondrial particles not to equilibrate with the suspending medium (24,25). An examination of intramembrane H^+ transfer in skeletal muscle SMP has been carried out using this probe and will be the subject of a future report.

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