ENERGY-LINKED ALTERATION OF MITOCHONDRIAL PERMEABILITY TO ANIONS

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Received April 7, 1969

Evidence is presented which indicates that a massive uptake of KCl and osmotic swelling which occurs when respiring mitochondria are treated with valinomycin is the result of a transient alkalization of the membrane. Energy-linked contraction and KCl extrusion follow the initial swelling phase spontaneously. The data appear to be consistent with the predictions of Mitchell's chemiosmotic coupling hypothesis.

Isolated mitochondria are impermeable to Cl^- at neutral pH (1-3). Azzi and Azzone (3) have established, however, that considerable permeability to this anion develops as the pH is increased, and at pH 8 passive uptake of both K^+ and Cl^- occurs, provided the membrane is made permeable to K^+ by the addition of valinomycin. We have recently noted that, in the presence of respiration, valinomycin (or gramicidin) induces a massive uptake of both K^+ and Cl^- and osmotic swelling of beef heart mitochondria which is characterized by expansion of the matrix in electron micrographs (4). This swelling and Cl^- uptake occur at pH 7 and below but the process is accompanied by extensive transient pH shifts which are linked to respiration and K^+ movement. The present communication examines this respiration and valinomycin dependent Cl^- uptake and presents evidence that the altered permeability to anions is the result of an energy-linked alkalization of either the membrane itself or of the matrix compartment of the mitochondrion.

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Results - Beef heart mitochondria prepared by Nagarse treatment in the presence of EGTA (5,6) (Ca^{++} content less than 5 mumoles/mg) do not swell or accumulate ions when they respire in a lightly buffered medium of 100mM KCl. The effect of the addition of valinomycin (2.5×10^{-7}M) to such a system is shown in Fig. 1. The antibiotic induces a rapid extrusion of H^+ into the medium which reverses spontaneously to re-establish the initial pH. The cycle of pH changes is complete in about 10 sec. and during this period the trace of absorbance at 546 mu indicates that rapid and extensive swelling occurs. Swelling ceases at about the time the original pH is re-established. Respiration is activated by the addition of valinomycin and, if respiration is allow to continue, the mitochondria spontaneously begin an energy-linked contraction and ion extrusion (Fig. 1A). During the contraction phase respiration continues at a high rate and the glass electrode trace shows no indication of H^+ movements. Contraction ceases at anaerobiosis and there is usually a slight swelling at this point followed by a long refractory period in which no further volume changes occur. At anaerobiosis the pH trace shows an alkaline shift corresponding to the uptake of about 27 mumoles of H^+ per mg of protein (Fig. 1A). If the pH is increased by addition of KOH during this period of anaerobiosis, passive swelling and KCl uptake occur as expected (3).

Addition of an uncoupler of oxidative phosphorylation such as m-chlorocarbonylcyanidephenylhydrazone (CCP) or of antimycin before the addition of valinomycin causes the same alkaline shift as observed at anaerobiosis and abolishes the effect of valinomycin on both the pH cycle and the swelling cycle (Fig. 1B). Increasing the pH to 8.4 with a pulse of KOH again results in rapid, passive, swelling of the mitochondria, indicating that increased permeability to K^+ in the presence of valinomycin and permeability to Cl^- induced by high pH are sufficient to support passive osmotic swelling in this medium.

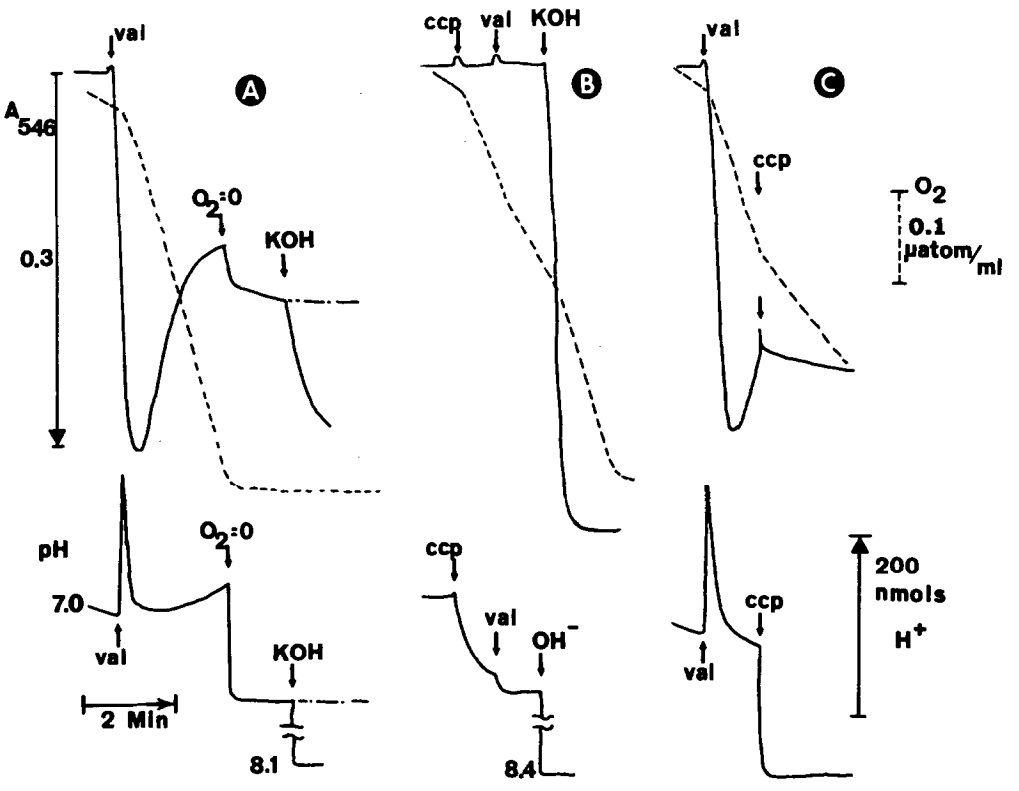

Fig. 1 - Effect of valinomycin on heart mitochondria suspended in 0.10M KCl. Rotenone-treated mitochondria (5 mg protein) prepared by Nagarse treatment in a medium of sucrose-Tris-EGTA (5,6) were added to 8 ml of 0.1M KCl containing 2mM Tris succinate at pH 7.0 in a plexiglass cuvette equipped with a magnetic stirrer. Swelling and contraction were followed by changes in absorbance at 546 mu with an Eppendorf photometer, oxygen uptake with a Clark electrode, and pH with a Thomas 4858 combination electrode. At the indicated points valinomycin (2.5×10^{-7}M) m-Cl-carbonyl-cyanidephenylhydrazone (CCP, 5×10^{-7}M), or KOH (sufficient 1M to bring the pH to 8.1 or 8.3) were added. In Part A the dashed traces show the traces obtained in the absence of the pulse of KOH to pH 8.1.

The contraction phase of the reaction is also abolished by addition of either CCP or antimycin (Fig. 1C). At neutral pH there is no further contraction and little swelling after disruption of the supply of energy with either of these reagents. Both antimycin and CCP result in a somewhat greater alkaline shift (30 mumoles/mg) if added to swollen mitochondria in the process of energy-linked contraction (Fig. 1C). Under these conditions CCP in a concentration which normally activates respiration

inhibits the valinomycin-dependent high rate of respiration (compare Fig. 1C and 1B).

The energy requirement for both the initial swelling at neutral pH and the ensuing contraction can be met by all respiratory substrates tested and by exogenous ATP. In each case both phases of the reaction are inhibited by appropriate respiratory inhibitors and the ATP-dependent reactions are oligomycin sensitive.

The concentration of valinomycin necessary to initiate the pH cycle is about 3 orders of magnitude less than that required for optimal swelling and K^+Cl^- uptake. The valinomycin-induced swelling is strongly inhibited by sucrose, Tris Cl, and by any addition which increases the osmotic pressure on the mitochondrion. Concentrations of KCl over 100mM show identical pH cycles but the swelling phase decreases with increasing KCl concentration. High concentrations of KCl also strongly inhibit the contraction phase of the cycle. When the reaction is carried out in 25 mM KCl, the hypotonically swollen mitochondria show a pH cycle similar to that shown in Fig. 1, extensive additional swelling, and complete reversal to the original volume during the contraction cycle.

A similar cycle but with much less energy-linked contraction can be induced in 100mM NaCl by gramicidin. In 100mM NH_4Cl at pH 7.0 respiration is activated by valinomycin but no pH cycle or swelling occur. Swelling also does not occur in this medium at elevated pH until an uncoupler is added.

Studies with $^{36}Cl^-$ verify that an uptake of Cl^- in line with an osmotic swelling has occurred in the swelling phase of Fig. 1 and that Cl^- is extruded during the contraction phase. Other non-permeant anions, such as Br^- and I^-, will replace Cl^- in these reactions, but addition of a permeant anion, such as acetate or phosphate, causes reversal of the contraction phase and additional energy-linked swelling.

Discussion - When valinomycin-treated mitochondria are suspended in a KCl medium at elevated pH the particles swell osmotically and appear to be permeable to both K^+ and Cl^- (3). The present study suggests that a similar situation can be induced at neutral pH and below when respiring mitochondria suspended in KCl are treated with excess valinomycin. Under these conditions energy-linked K^+ uptake is stimulated (7) and the particles would be expected to be permeable to K^+ since valinomycin has been shown to function as a carrier for this cation in a number of natural and artificial membrane systems (7). The close relationship between the observed rapid swelling and reversible cycle of H^+ extrusion and uptake suggests that extensive alkalinization of either the inner membrane or the matrix space as the result of either metabolic separation of H^+ and OH^- or a K^+ for H^+ exchange reaction may alter the membrane in the same way as an externally applied pH increase. Mitchell et al. (8) have reported a similar pH cycle on addition of O_2 to valinomycin-treated mitochondria in a medium of 150mM KCl and noted that the bromthymol blue indicator shows a corresponding shift in the direction of internal alkalinization. It appears possible that the membrane altered by the high pH effectively collapses and admits H^+ (or loss of OH^-) as well as additional K^+ and considerable Cl^-. Osmotic swelling would then result as a consequence of net ion uptake. This spontaneous neutralization of the excessive pH gradient would now re-establish a membrane with little permeability to Cl^- from the outside, but with excess K^+ and Cl^- in the matrix compartment. This condition is quite analogous to the salt loaded mitochondrial preparations described by Azzi and Azzone (3) and, as they have documented, ion extrusion and mitochondrial contraction ensue when a source of energy is added. Under the conditions of the present study, since there is no interruption in the supply of energy, the contraction and ion extrusion proceed spontaneously.

During the extrusion of ions there is little net pH change indicated

by the glass electrode. It appears possible therefore that, if a respiration-dependent H^+ extruding reaction (8,9) were functioning, a net osmotic contraction could be obtained by the action of an exchange diffusion carrier which exchanges external H^+ for internal K^+ (8,9). The entering H^+ would react with the internal OH^- and Cl^- would move out passively to compensate for the net exit of positive charge with K^+. Such a reaction sequence would provide a net, respiration-linked efflux of ions and osmotic contraction without the need for additional assumptions such as the existance of a separate, outwardly directed ion pump, reorientation of the membrane, or a contractile process. At anaerobiosis the uptake of H^+ (or OH^- release) would indicate the possibility of further changes in membrane pH (and permeability) and would provide a rationale for the observed refractory period in which little in the way of ion fluxes or volume changes can be detected (Fig. 1A). A permeant anion such as acetate which enters the matrix, presumably as acetic acid would neutralize the internal OH^- and result in net ion uptake and swelling under the same circumstances. This is the result obtained experimentally. NH_4^+ entering either as the cation as the result of valinomycin treatment or as NH_3 would encounter a high concentration of OH^- and no build up of cations would be expected. Uncouplers would discharge the pH gradient (8,9) and permit osmotic swelling due to $NH_4^+ + Cl^-$ uptake in analogy to the situation in chloroplasts reported by Crofts (10).

It should be noted that, while the present observations can probably be explained by a number of schemes which are currently under consideration to account for energy-linked mitochondrial ion movements, the results appear quite compatible with the chemiosmotic hypothesis of Mitchell (8,9). The only additional assumption is that metabolically generated OH^- is able to alter the membrane and control permeability reversibly in a manner similar to an externally imposed pH increase. A more complete discussion of these experiments will be presented for publication elsewhere.

Acknowledgements

These studies were supported by USPHS grant HE09364 and by a grant-in-aid from the American Heart Association. I thank Drs. C. D. Stoner and C. T. Settlemire for helpful discussions.

References

(1) Chappell, J. B., and Crofts, A. R., in J. M. Tager, S. Papa, E. Quagliariello, and E. C. Slater, (Editors), Regulation of Metabolic Processes in Mitochondria, Elsevier, New York, 1966, p. 293.

(2) Brierley, G. P., Settlemire, C. T., and Knight, V. A., Arch. Biochem. Biophys., 126, 276 (1968).

(3) Azzi, A. and Azzone, G. F., Biochim. Biophys. Acta, 135, 444 (1967).

(4) Hunter, G. R., Kamashima, Y., and Brierley, G. P., Biochim. Biophys. Acta, in press.

(5) Hatefi, Y., Jurtshuk, P. J., and Haavik, A. G., Arch. Biochem. Biophys., 94, 148 (1961).

(6) Settlemire, C. T., Hunter, G. R., and Brierley, G. P., Biochim. Biophys. Acta, 162, 487 (1968).

(7) Pressman, B. C., Federation Proc., 27, 1283 (1968).

(8) Mitchell, P., Moyle, J., and Smith, L., European J. Biochem., 4, 9 (1968).

(9) Mitchell, P., Chemiosmotic Coupling in Oxidative and Photosynthetic Phosphorylation, Glynn Research, Bodmin, Cornwall, 1966.

(10) Crofts, A. R., J. Biol. Chem., 242, 3352 (1967).