RUTHENIUM RED AS A CARRIER OF ELECTRONS BETWEEN EXTERNAL NADH AND CYTOCHROME C IN RAT LIVER MITOCHONDRIA

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Received February 9,1976

Summary: Exogenous NADH is oxidized by rat liver mitochondria when ruthenium red (2μM) is present in the medium. The oxidation is insensitive to rotenone and antimycin A, but sensitive to KCN, and is markedly reduced in a saline medium or in the presence of divalent cations. The same effect of ruthenium red can be seen in mitochondria depleted of the outer membrane. This suggests that ruthenium red acts as a mediator of electrons between NADH and cytochrome c. This is also supported by the observation that ruthenium red can replace N,N,N^1,N^1-tetramethyl-p-phenylenediamine (TMPD) in the oxidation of ascorbate by mitochondria. The effect of ruthenium red is abolished by $LaCl_3$.

INTRODUCTION

Ruthenium red, a mucopolysaccharides stain, has been recently used as an inhibitor of a number of Ca-transport systems (1-4). In mitochondria, low concentrations of ruthenium red inhibit the energy-dependent Ca transport and the binding of Ca to the membranes, without affecting mitochondrial respiration or energy transfer (1,2); however, high concentrations of the stain inhibit mitochondrial respiration (2). We have investigated the effect of ruthenium red on the mitochondrial respiratory activity, and the results are reported in this paper. Exogenous NADH, which is not oxidized by intact rat liver mitochondria, can be oxidized in the presence of ruthenium red in an antimycin A-insensitive process. It is suggested that ruthenium red acts by transferring electrons from external NADH directly to cytochrome c. In agreement with this suggestion, it has been found that ruthenium red substitutes TMPD in the oxidation of ascorbate by cytochrome c. An intermembrane shuttle of electrons mediated by ruthenium red has been ruled out, since the effect of the dye is seen also in mitochondrial preparations from which the outer membrane has been removed. The possible relationship between the effect of ruthenium red as mediator of electrons to cytochrome c, and its ability to inhibit Ca^{2+} transport are discussed.

Methods and Materials: Liver mitochondria were prepared by a conventional method from rats fasted for 12 hrs. The isolation medium contained: 220mM Mannitol, 70mM Sucrose, 10mM Tris-Cl pH 7.4 and 0.5mM Na-EDTA. The protein of the suspensions was measured with a biuret method. Oxygen consumption was measured polarographically with a Clark electrode. Mitochondria depleted of the outer membrane were obtained according to Schnaitman et al (5). The NADH solution at pH 8.0 was prepared fresh every day. Ruthenium red was purchased from Johnson Matthey Chemicals Ltd (London), and it was further purified according to Luft (6).

Results and Discussion: The respiratory rate of intact rat liver mitochondria in the presence of NADH is low, due to the well-known impermeability of the inner mitochondrial membrane to the substrate (7). However, when 4 μM ruthenium red is added to the medium, a large increase in the rate of respiration is observed. Under these conditions, the respiration is insensitive to the respiratory inhibitors rotenone and antimycin A, but it is still sensitive to KCN (Fig.1A). Oxidation of NADH by liver mitochondria in the presence of ruthenium red could also be demonstrated upon repeated additions of small amounts of NADH to media already containing ruthenium red (Fig.1B). The stimulation of NADH oxidation as a function of the concentration of added ruthenium red was also studied and found to reach half maximal rate at 1.5μM ruthenium red. The addition of 10mM $MgCl_2$ or 1mM $CaCl_2$, or the substitution of the Mannitol-Sucrose medium with a saline medium (120mM KCl), markedly reduced the effect of ruthenium red. A possible explanation for the latter observations is the interference of salts and divalent cations with the binding of the stain to the membrane. On the basis of these data, two possible explanations can be offered: a) ruthenium red is acting as an electron mediator from external NADH to cytochrome c, or b) it promotes an intermembrane shuttle of electrons between the NADH-cyt.b_5 reductase located in the outer membrane, and cytochrome c located on the outer face of the inner membrane. The results reported in Fig.2A apparently rule out the second possibility, since ruthenium red stimulates the oxidation of NADH also in mitochondria depleted of the outer membrane. That ruthenium red can act as an electron donor to cytochrome c is also shown in the experiment of Fig.2B,where ruthenium red is used instead of TMPD as an electron mediator in the oxidation of ascorbate by oxidized cytochrome c. In fact, ruthenium red is even more efficient than TMPD, since the concentrations required to induce maximal rates of oxidation of ascorbate are lower than those of TMPD. Finally, Fig.3 shows that $LaCl_3$, another inhibitor of the transport of Ca^{2+} in mitochondria (8), inhibits the ruthenium-red-stimulated NADH oxidation. The concentration of $LaCl_3$, at which half maximal inhibition is achieved,

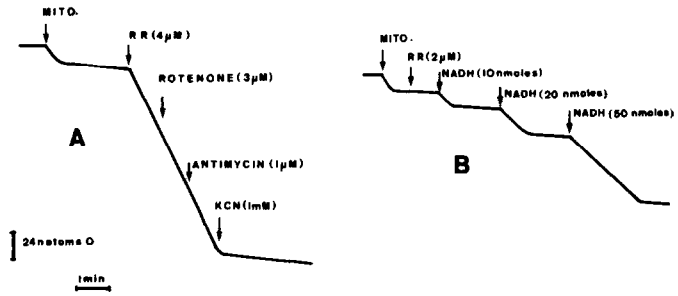

Fig.1: A) The effect of ruthenium red (RR) on the NADH-oxidation by mitochondria. The medium contained 220mM Mannitol, 70mM Sucrose and 10mM Tris-HCl, pH 7.4. 1mg of mitochondrial protein was added to a final volume of 2ml. B) The oxidation of externally added NADH by mitochondria, preincubated with ruthenium red. The medium was the same as in Fig.1A except that rotenone (2μM) was present from the beginning.

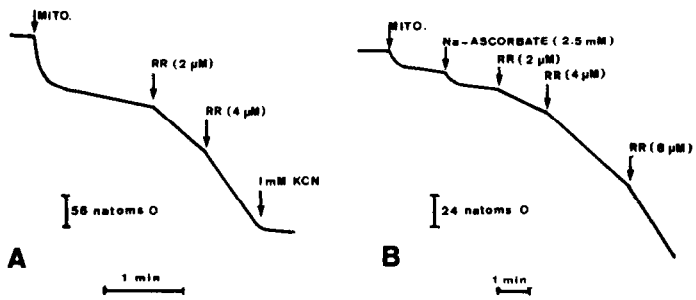

Fig.2: A) Ruthenium-red-induced oxidation of NADH by outer-membrane-depleted mitochondria. The medium was as reported in Fig.1B but contained 1mM NADH. Protein content was 1mg per ml. B) The effect of ruthenium red on the oxidation of ascorbate by mitochondria. The medium was the same as in Fig.1A, containing 1mg of protein per 2ml.

is approximately 70μM, which is very similar to the concentration required to inhibit the binding of ruthenium red to the mitochondrial membrane (9). Therefore, the stimulation of the oxidation of NADH by ruthenium red requires the binding of the dye to the mitochondrial membrane. Since electrons are apparently fed by ruthenium red to cytochrome c, it is tempting to speculate that the binding site for ruthenium red could be cytochrome c, or a site in the membrane located in close proximity to it, perhaps a site that binds Ca^{2+}. The observations reported in this paper, and the known ability of ruthenium red to inhibit the transport of Ca^{2+} in mitochondria, could then be related to both the interesting suggestion by

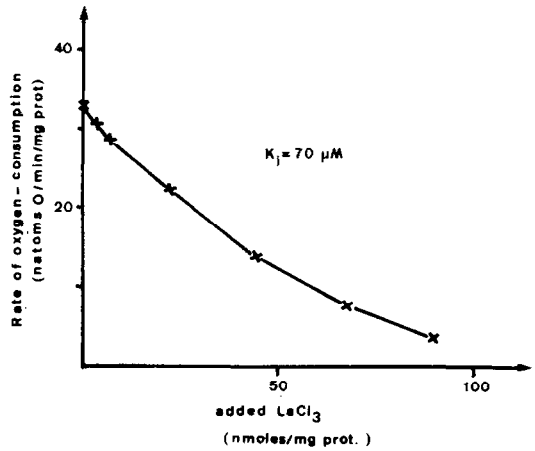

Fig.3: Inhibition of the ruthenium-red-dependent NADH-oxidation by $LaCl_3$. The medium was the same as in Fig.1A but with 3.6mg of mitochondrial protein in a total volume of 2ml. Rotenone (2µM), antimycin A (0.7µM) and ruthenium red (2µM) were present from the beginning.

Margoliash et al (10) that cytochrome c could function as a Ca^{2+} carrier in the mitochondrial membrane, and to the suggestion by Azzi et al (11) that Ca^{2+} could mediate the interaction of cytochrome c with the membrane environment.

Acknowledgment: The research was carried out with the help of financial assistance from the Swiss Nationalfonds (Grant No. 3.1720.73).

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