RESPIRATORY CONTROL IN CYTOCHROME OXIDASE

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SUMMARY: Vesicles of cytochrome oxidase, generated by dilution of the oxidase with a 15-fold excess of lipid by the Hinkle-Racker method, showed a respiratory control index of greater than 5 in presence of the combination of valinomycin and nigericin. Uncouplers were found to be ineffective in releasing respiratory control in the absence of valinomycin. Valinomycin titration in the presence of excess nigericin gave approximately a one to one stoichiometry with cytochrome oxidase. We propose that coupling of electron transfer to valinomycin K^+ transport in cytochrome oxidase vesicles is a molecular event; the insensitivity of respiratory control to uncouplers is a consequence of the absence of the systems other than cytochrome oxidase which are required for the action of uncouplers.

INTRODUCTION

Hinkle and Racker have shown that respiratory control is demonstrable in membrane vesicles generated by diluting purified cytochrome oxidase with a 15-fold excess of phospholipid (1,2). The rate of oxidation of reduced cytochrome c by molecular oxygen was found to be stimulated by valinomycin in combination either with nigericin or with an uncoupler such as FCCP or 1799. These experiments have been explicitly represented as dramatic proof of a major postulate of the chemiosmotic theory, the generation of a proton gradient by an asymmetrically aligned respiratory complex (1-3). We have confirmed the essential findings of Hinkle and Racker but have investigated more closely the requirements for release of respiratory control. The data presented in this paper strongly suggest that respiratory control in membranous vesicles of cytochrome oxidase is a localized molecular phenomenon and not a membrane phenomenon as implicit in the chemiosmotic model.

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METHODS

Protein concentrations were estimated by the method of Lowry *et al.* (4) using bovine serum albumin as standard. Heme a was estimated by the pyridine hemochromogen difference spectral method of Williams ($E_{587-620}$ = 21.7) (5).

Cytochrome oxidase was prepared by the method of Yonetani (6) employing two "final fractionation steps."

Mitochondrial lipids were extracted by the acetone treatment of Fleischer and Fleischer (7). This method was used in preference to chloroform-methanol extraction which solubilizes a significant amount of protein along with phospholipid.

Assay of cytochrome oxidase respiration and the effect of uncouplers

Oxygen uptake was measured with a Clark oxygen electrode at 30°C. The assay medium (4 ml) contained 2.4 mg of cytochrome c, 200 µmoles of Tris-ascorbate (pH 6.8) and 200 µmoles of potassium phosphate buffer, pH 7.4. Readings of oxygen consumption were taken for 2 minutes prior to the addition of the sample. Cytochrome oxidase vesicles (25-100 µl) were added next and readings were taken for 2 to 5 minutes depending on the rate. Finally, uncouplers in ethanol were added. Corrections were made for autoxidation of ascorbate in the absence of cytochrome oxidase.

Reconstitution of cytochrome oxidase vesicles

Cytochrome oxidase vesicles were prepared as described by Racker (2). A suspension of phospholipid was prepared by sonication in a medium 50 mM in potassium phosphate (pH 7.4) containing 2% cholate. The final concentration of phospholipid was 40 µmoles per ml. The above phospholipid suspension was mixed with a 0.3% suspension of cytochrome oxidase in the proportion v/v of 3 to 2. The mixture was dialyzed for 3 hours in the cold (4°) against a liter of 50 mM potassium phosphate (pH 7.4) prior to the measurement of respiration.

RESULTS AND DISCUSSION

Table I shows that neither valinomycin nor nigericin alone was capable of releasing respiratory control. The combination of the two ionophores gave

TABLE I. Effect of uncouplers on the rate of respiration of cytochrome oxidase vesicles.

Additions	Rate of respiration in μmoles O per min per mg protein
None	1.5
Valinomycin (5 μl, 1 mg/ml)	2.0
Nigericin (5 μl, 1 mg/ml)	3.0
Valinomycin + Nigericin	11.3
DNP (2×10^{-4} M)	1.5
DNP (1×10^{-3} M)	2.1
FCCP (2×10^{-7} M)	2.2
FCCP (1×10^{-6} M)	3.9
mClCCP (1×10^{-6} M)	2.5
S_{13} (1×10^{-8} M)	3.3
DNP (2×10^{-4} M) + Valinomycin	10.3
FCCP (2×10^{-7} M) + Valinomycin	8.5
mClCCP (1×10^{-6} M) + Valinomycin	8.3
S_{13} (10^{-8} M) + Valinomycin	8.4

Abbreviations: DNP = dinitrophenol, FCCP = carbonylcyanide-p-trifluoromethoxyphenylhydrazone, mClCCP = m-chlorophenylhydrazone, and S_{13} = 5-chloro-3-tert-butyl-2′-chloro-4′-nitrosalicylanilide.

a 7-fold stimulation. Classical uncouplers worked to some degree but required a much higher concentration than that found to uncouple mitochondria or submitochondrial particles (8-10). Addition of valinomycin greatly potentiated the uncoupler release of respiratory control. The data suggest that uncouplers are effective in dissipating the pH gradient generated by valinomycin-induced K^+ transport (1), as shown by their ability fully to replace nigericin, but are ineffective in replacing the valinomycin K^+ complex which is the electrogenic component. This is of interest since uncouplers can uncouple all energy trans-

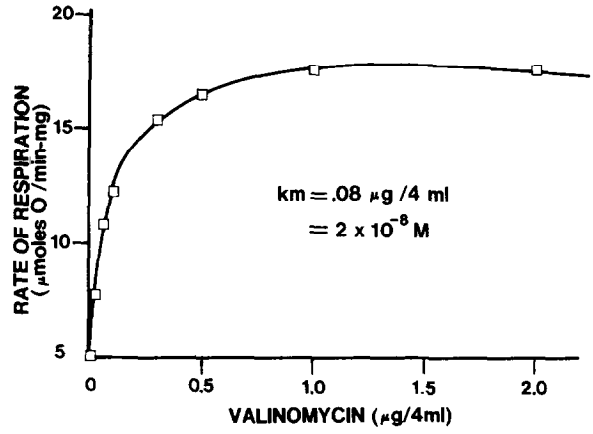

Figure 1. Concentration of valinomycin required to release respiratory control. To cytochrome oxidase vesicles (12 μg/ml) suspended in the phosphate-ascorbate reaction mixture given in methods, nigericin (0.5 μg/ml) was added. Respiration was then started with addition of cytochrome c. After 1 minute, valinomycin was added and the increased rate of oxidation was determined. The rate of oxidation with no addition of valinomycin was about 5 μmoles O/min-mg.

duction found in the mitochondrial inner membrane (8-10) in the absence of ionophores such as valinomycin.

In the presence of excess nigericin, the concentration of valinomycin required to give half-maximal stimulation was about 2×10^{-8} M (see Figure 1). Since the concentration of cytochrome oxidase was 6×10^{-8} M (assuming a molecular weight of 200,000 for a heme content of 10 mμmoles/mg), release of respiratory control appears, therefore, to require a close association of one valinomycin K^+ complex for each molecule of energized cytochrome oxidase.

CONCLUSION

Respiratory control is manifested in cytochrome oxidase only when the cytochrome oxidase complex is diluted out in membranous form with a large excess of lipid (2,11). Protein-protein interaction would be diminished in this case to almost zero. The high lipid to protein ratio in cytochrome oxidase is in sharp contrast to the corresponding ratio in the mitochondrial inner membrane which is 30 times less. This isolation of cytochrome oxidase from other respiratory enzymes and from the proteins concerned in ATP synthesis leads to two major

consequences. Electron transfer from cytochrome c to oxygen can be coupled stoichiometrically to valinomycin mediated K^+ transport with $2K^+$ ions moving inward per oxygen atom (1,3). Since there is approximately one valinomycin per cytochrome oxidase molecule, this coupling appears to be a localized event and not a secondary response to a membrane potential generated by electron transfer. Lombardi *et al.*, in studies of valinomycin induced Rb^+ transport into *E. Coli* vesicles arrived at a similar conclusion (12). Secondly, when electron transfer can no longer be coupled to the phosphorylation system, uncouplers become ineffective in releasing respiratory control. This suggests that the site of action of uncouplers is in the protein and such a conclusion is in agreement with the results of recent work in our laboratory on lysolecithin treated submitochondrial particles (13,14). Disruption of the lipid continuum by lysolecithin selectively reduces the release of respiratory control by the valinomycin-nigericin combination but does not affect release by uncoupler. Hatefi *et al.* have demonstrated specific binding sites in mitochondrial proteins for uncouplers (15), and this demonstration is in line with our own conclusions of the essentiality of certain complexes (missing in cytochrome oxidase vesicles) for the action of uncoupler.

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