CALCIUM UPTAKE BY CYTOCHROME OXIDASE VESICLES

R. N. ROSIER* and T. E. GUNTER+

Department of Radiation Biology and Biophysics, School of Medicine and Dentistry, University of Rochester, Rochester, NY 14642, USA

Received 11 September 1979
Revised version received 12 November 1979

1. Introduction

Cytochrome oxidase vesicles (COV) are well known for their properties of respiratory control and energy-dependent pH gradient and membrane potential formation [1,2]. This generation of protonmotive force in COV has been used to drive the formation of ATP in a reconstituted system of oxidative phosphorylation [3–5]. In addition, energized COV in the presence of valinomycin have been used to study potassium uptake driven by a membrane potential which was generated by electron transport [1,6].

Study of ion transport in COV has been limited by the sensitivity of available ion-specific electrodes. However, the recent aggregation technique [7] for the separation of proteoliposomes from suspending media allows quantitation of very small amounts of ion uptake in vesicles when radioactively-labelled ions are used. Using this separatory technique, energy-dependent uptake of Ca^{2+} in COV has been observed and is reported here. While specific interaction of Ca^{2+} with cytochrome oxidase has been noted in the past [8–11], and a subfractionated preparation of cytochrome oxidase has been found to possess specific Ca^{2+} ionophoric properties [12–15], transport of Ca^{2+} in an energy-dependent manner, mediated by cytochrome oxidase has not been reported. Fry and Green [15] have described increased passive permeability to Ca^{2+} in liposomes reconstituted with subfractionated preparations of cytochrome oxidase, particularly subunit I. Whether the Ca^{2+} transporting properties of cytochrome oxidase have any relationship to Ca^{2+} transport in mitochondria remains to be determined, but the possibility that cytochrome oxidase may be involved in the coupling of mitochondrial ion transport to oxidation cannot be excluded.

In addition, if the COV system is to be fully utilized as a system for studying Ca^{2+} uptake into proteoliposomes through additional mechanisms for facilitating the transport of Ca^{2+}, then the Ca^{2+} transport properties of the COV themselves must be fully delineated.

2. Materials and methods

Cytochrome oxidase was prepared from beef heart and purified as in [16,17]. COV were made by the cholate dialysis technique [1,2]. Acetone-extracted asolectin [18] was sonicated to clarity at 40 mg phospholipid/ml in 40 mM K_2SO_4, 10 mM *N*-2-hydroxyethyl piperazine-*N'*-2-ethane sulfonic acid (HEPES) (pH 7.0) in the presence of 1% sodium cholate. Cytochrome oxidase was then added to final conc. 0.25–0.63 mg/ml, and cytochrome *c* added at this time to the appropriate concentration when indicated. The suspension was dialyzed at 4°C to remove the detergent. Respiratory control ratios were measured polarographically using described techniques [2] except that carbonyl cyanide *m*-chlorophenyl hydrazone (CCCP) was used as the uncoupling agent. Ca^{2+} uptake was measured as follows. Samples were prepared in 1.7 ml final vol. 40 mM K_2SO_4, 10 mM HEPES (pH 7.0) containing 12 mg COV phospholipid (for non-energized controls) or this amount of COV plus 125 μmol Tris–ascorbate (pH 7.0) and 6 mg cytochrome *c* (for energized samples). The uptake reaction was initiated by the addition of 0.1 ml 10 mM $CaCl_2$ containing 5–10 μCi $^{45}Ca^{2+}$. Samples

+ To whom correspondence should be addressed

* Present address: Department of Orthopedics, University of Iowa, College of Medicine, Iowa City, IA, USA

were incubated at room temperature with continuous agitation in a shaker bath to ensure adequate oxygenation for the appropriate amount of time. Then, following the aggregation technique [7], 0.2 ml protamine (0.8 mg) was added to precipitate the COV and the sample immediately filtered through a 0.45 μm Millipore filter using a vacuum-assisted, modified Millipore filtration device. After being washed with 10 ml suspension buffer, the filters were dried and counted using standard liquid scintillation techniques to quantitate the uptake of Ca^{2+}. Control samples filtered at $t = 0$ were always used so that external binding of calcium to the filters and COV could be subtracted.

3. Results and discussion

It has been established that in the presence of oxidizable substrate and cytochrome *c*, cytochrome oxidase will generate an internally negative membrane potential in COV [1,19–21]. Ca^{2+}, being a divalent cation, could be expected to be accumulated in COV in response to this membrane potential given an appropriate transport system conferring Ca^{2+} permeability on the COV membrane. As can be seen from fig.1, COV made by standard techniques with purified cytochrome oxidase accumulate Ca^{2+} when energized, despite the lack of any additional mediator of Ca^{2+} permeability. Internal volumes of the COV suspensions as measured using trapped [^{14}C]sucrose gave ~1 μl/mg phospholipid. Assuming that internal and external binding are equal, total maximum uptakes in COV showed accumulation of Ca^{2+} to internal concentrations of 5–9-times the external concentration of Ca^{2+}. Since most of the cytochrome oxidase in these vesicles is associated with a small fraction (~20%) of the phospholipid vesicles [19], the actual internal concentrations of accumulated Ca^{2+} in energized COV may be as much as 5-times greater than these estimates representing a very significant Ca^{2+} transport capability. Non-energized COV (incubated in the absence of ascorbate, cytochrome *c*, or both) accumulate only a small amount of Ca^{2+}, presumably due to passive equilibration. Maximum total uptakes into energized COV were obtained after ~40 min incubation.

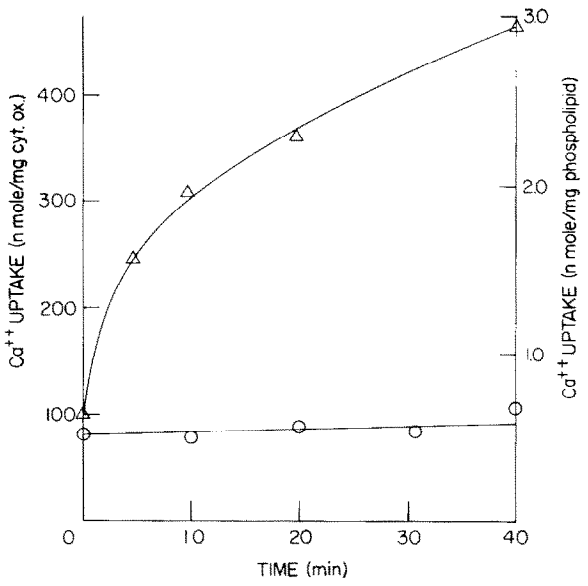

Fig.1. COV were made and assayed for calcium uptake as in section 2. The cytochrome oxidase concentration in the vesicle suspension prior to dialysis was 0.25 mg/ml except that cytochrome *c* was added at the time of vesicle formation. (△) represent COV energized as in section 2. (○) represent non-energized COV (no ascorbate or cytochrome *c*). $^{45}Ca^{2+}$ is added in both cases at $t = 0$.

The effects of various inhibitors on the rate of Ca^{2+} accumulation by COV were studied. KCN at 3 mM almost completely inhibited both respiratory activity and Ca^{2+} uptake (99%). This is consistent with the results obtained in the non-energized case and indicates dependence of uptake on electron transport. Agents which break down the proton gradient developed in COV [6], such as CCCP or the combination of nigericin, valinomycin and K^+ were found to be potent inhibitors of Ca^{2+} accumulation (≤96%). Ruthenium red, an inhibitor of Ca^{2+} uptake in mitochondria [22–24], did not appreciably affect Ca^{2+} uptake in COV (<6%). Ca^{2+} accumulation in COV appears to be electrophoretic and dependent on formation of an internally negative membrane potential. This implies the presence of a mediator of Ca^{2+} permeability, which may be a component of the cytochrome oxidase complex, a phospholipid in the asolectin, or a protein contaminant of the cytochrome oxidase preparation.

When COV are formed in the presence of cytochrome *c*, the cytochrome oxidase assumes a random bidirectional orientation in the phospholipid vesicle membranes [17]. COV can then be energized with phenazinium methyl sulfate (PMS) and ascorbate if external cytochrome *c* is first removed from the suspension by Sephadex gel filtration. Energization in this manner sets up an internally positive membrane

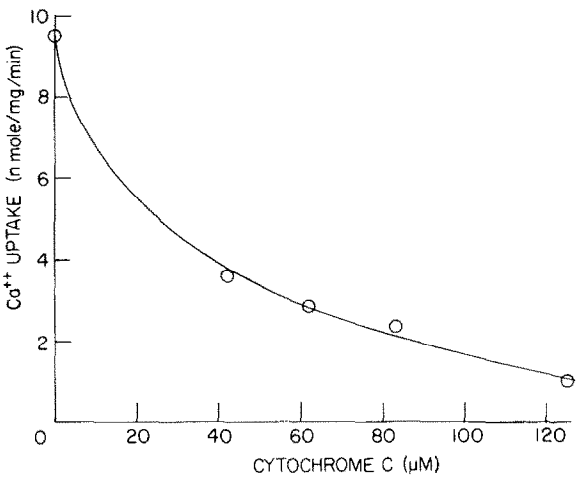

Fig.2. COV were made and assayed for calcium uptake as previously described. The concentration of cytochrome oxidase prior to dialysis was 0.25 mg/ml, and the indicated amounts of cytochrome *c* were added before dialysis. External cytochrome *c* was removed from the suspensions by passage through a Sephadex G-150 column equilibrated with 150 mM KCl, 10 mM HEPES (pH 7.0). The uptake rates shown represent the net uptake rates after subtracting uptake in non-energized controls, and were determined at 20 min incubation.

potential, analogous to similar experiments with submitochondrial particles [25–27]. Figure 2 shows the effect of varying amounts of cytochrome *c*, included at the time of vesicle formation, on Ca^{2+} transport rates in COV energized in the conventional manner with external cytochrome *c* and ascorbate. The cytochrome *c*, by inducing bidirectional orientation of cytochrome oxidase and thereby decreasing the amount of active enzyme under these conditions of energization, markedly decreases the rate of Ca^{2+} transport into COV. At the higher cytochrome *c* concentrations shown, some of the inhibition may be due to direct inhibitory effects of cytochrome *c* on the respiratory acivity of cytochrome oxidase [17]. When energized with ascorbate and PMS, (reverse energization) COV accumulated less Ca^{2+} than non-energized controls as would be expected, since formation of an internally positive potential would be inhibitory to electrogenic Ca^{2+} influx.

Several lines of evidence indicate that the Ca^{2+} transport is mediated by some component (probably a protein) of cytochrome oxidase. Ca^{2+} uptake is markedly inhibited (>95%) by a brief period of bath sonication after the proteins are added to the detergent-solubilized phospholipids. Although the amount of sonication used in these experiments was too little to measurably alter the oxidation rate, cytochrome oxidase is known to be somewhat sensitive to sonication [17,28]. A protein carrier would more likely be sensitive to mild sonication than would a phospholipid. In addition, more extensive sonication of the phospholipids prior to addition of cytochrome oxidase had no observable effect. The use of cytochrome oxidase preparations of varying degrees of purity as judged by SDS–polyacrylamide gel electrophoresis showed the more highly purified fractions to be slightly less effective (25% less uptake) than the more contaminated fractions, even though the respiratory control ratios (uncoupled oxidation rates/oxidation rates without uncoupler) were higher in the purified preparations. This may indicate that either the Ca^{2+} transporting component is a contaminant peptide or that the purification procedure is detrimental to the Ca^{2+} transporting properties of a portion of the cytochrome oxidase complex itself. In view of the finding in [15] that Ca^{2+} permeability of liposomes is greatly increased by purified subunit I of the oxidase, the latter possibility seems more likely.

As would be expected from an electrogenic model of Ca^{2+} uptake in COV, addition of a known electrogenic Ca^{2+} ionophore, the Ca^{2+}–ligand of in [29], greatly enhances the rate of Ca^{2+} uptake in COV. The protonmotive force of the COV is derived from electron transport by cytochrome oxidase, which then drives Ca^{2+} uptake over a mediator whether it is a protein subunit of the cytochrome oxidase, a contaminating protein present in the cytochrome oxidase preparation, or a specific Ca^{2+} ionophore. The coupling of the Ca^{2+} transport to electron transport is not tight, since the addition of Ca^{2+} to an oxidizing suspension of COV does not measurably affect the oxidation rate. The average rates of O_2 consumption in these suspensions were ~3000 nmol . mg protein^{-1} . min^{-1}, as compared to the Ca^{2+} uptake rates shown in the preceding figures.

The above experiments have demonstrated that Ca^{2+} uptake in COV is directly dependent upon electron transport by cytochrome oxidase and formation of an appropriately oriented membrane potential. The transport appears to occur by an electrogenic mechanism probably of the simple uniport type. While most of the data presented would also fit a K^+/Ca^{2+} antiport model, COV made and assayed in sodium salts in the absence of potassium showed no difference in Ca^{2+} transport rates.

There appears to be a specific mediator of Ca^{2+} permeability in COV which allows Ca^{2+} uptake under appropriate conditions of energization. The sensitivity of the mechanism to sonication of the oxidase and the dependence of the Ca^{2+} uptake rate on the particular fraction of cytochrome oxidase used, suggest that the mediator is a protein associated with the cytochrome oxidase and not a phospholipid component of the asolectin. The finding of a subfraction of cytochrome oxidase with Ca^{2+} ionophoric activity (the ionophore transfer component) supports the concept that the Ca^{2+} transport mediator is inherent in the cytochrome oxidase complex itself [15]. In the light of numerous recent reports indicating that cytochrome oxidase may operate under certain conditions as a proton pump powered by electron transport [11,30–33], it is conceivable that components of the cytochrome oxidase complex might possess ionophoric specificity for other cations (such as Ca^{2+} or K^{+}) and act as ion pumps for these cations under certain conditions.

The relationship between Ca^{2+} transport mediated by COV and Ca^{2+} transport by mitochondria is not clear. The lack of effect of ruthenium red on the Ca^{2+} uptake in COV may indicate either that modification of the Ca^{2+} transport mediator has occurred or that Ca^{2+} transport in COV is mediated by an entirely different, perhaps less specific transport system. It is possible that the Ca^{2+} transport system of cytochrome oxidase is related to the separate Ca^{2+} efflux mechanism of mitochondria proposed in [34,35]. Understanding of the relation of Ca^{2+} transport in COV to mitochondrial Ca^{2+} transport will depend on further elucidation of the specific components involved in this process.

Acknowledgements

The authors wish to thank Dr W. Simon for the generous gift of his synthesized Ca^{2+} ionophore, and to thank D. Tucker, I. Jain and S. Meerdink for technical assistance. They also wish to acknowledge the helpful suggestions of Dr Chris Miller of Brandeis University. This paper is based on work performed under contract with the US Department of Energy at the University of Rochester Department of Radiation Biology and Biophysics and has been assigned report no. UR-3490-1727 and was supported in part by NIH grant no. AM-20359-01.

References

[1] Hinkle, P. C., Kim, J. J. and Racker, E. (1972) J. Biol. Chem. 247, 1338–1339.
[2] Racker, E. (1972) J. Membr. Biol. 10, 221–235.
[3] Kagawa, Y., Kandrach, A. and Racker, E. (1973) J. Biol. Chem. 248, 676–684.
[4] Kagawa, Y. (1972) Biochim. Biophys. Acta 265, 297–338.
[5] Racker, E. and Kandrach, A. (1971) J. Biol. Chem. 246, 7069–7070.
[6] Racker, E. (1974) in: Dynamics of Energy Transducing Membranes (Ernster, L. et al. eds) pp. 269–281, Elsevier/North-Holland, Amsterdam, New York.
[7] Rosier, R. N., Gunter, T. E., Tucker, D. A. and Gunter, K. K. (1979) Anal. Biochem. 96, 384–390.
[8] Saris, N. E. L., Wikstrom, M. K. F. and Saari, H. T. (1975) in: Calcium Transport in Contraction and Secretion (Carafoli, E. ed) pp. 95–101, Elsevier/North-Holland, Amsterdam, New York.
[9] Wikstrom, M. K. F. (1974) Ann. NY Acad. Sci. 227, 146–157.
[10] Wikstrom, M. K. F., Harmon, H. J., Ingledew, W. J. and Chance, B. (1976) FEBS Lett. 65, 259–277.
[11] Wikstrom, M. K. F. and Saari, H. T. (1977) Biochim. Biophys. Acta 462, 347–361.
[12] Kessler, R. J., Blondin, G. A., VandeZande, H., Haworth, R. A. and Green, D. E. (1977) Proc. Natl. Acad. Sci. USA 74, 3662–3666.
[13] Blondin, G. A., Kessler, R. J. and Green. D. E. (1977) Proc. Natl. Acad. Sci. USA 74, 3667–3671.
[14] Fry, M., Vandezande, H. and Green, D. E. (1978) Proc. Natl. Acad. Sci. USA 75, 5908–5911.
[15] Fry, M. and Green, D. E. (1979) Proc. Natl. Acad. Sci. USA 76, 2664–2668.
[16] Kuhoyama, M., Yong, F. C. and King, T. E. (1972) J. Biol. Chem. 247, 6375–6383.
[17] Carroll, R. C. and Racker, E. (1977) J. Biol. Chem. 252, 6981–6990.
[18] Kagawa, Y. and Racker, E. (1971) J. Biol. Chem. 246, 5477–5487.
[19] Drachev, L. E., Jasaitis, A. A., Kaulen, A. D., Kon'rashin, A. A., Chu, L. V., Semenov, A. Y., Severina, I. I. and Skulachev, V. P. (1976) J. Biol. Chem. 251, 7072–7076.
[20] Drachev, L. A., Jasaitis, A. A., Kaulen, A. D., Kondrashin, A. A., Liberman, E. A., Nemecek, I. B., Ostroumov, S. A., Semenov, A. Y. and Skulachev, V. P. (1974) Nature 249, 321–324.
[21] Skulachev, V. P. (1974) Ann. NY Acad. Sci. 227, 188–202.
[22] Bygrave, F. L. (1977) Curr. Top. Bioenerg. 6, 259–318.
[23] Vasington, F. D., Gazzotti, P., Tiozzo, R. and Carafoli, E. (1972) Biochim. Biophys. Acta 467, 12–18.
[24] Luthra, R. and Olson, M. S. (1977) FEBS Lett. 81, 142–146.
[25] Gunter, K. K., Rosier, R. N., Tucker, D. A. and Gunter, T. E. (1977) in: Calcium Binding Proteins and Calcium Function (Wasserman, R. N. et al. eds) pp. 498–500, Elsevier/North-Holland, Amsterdam, New York.

[26] Sorgato, M. C. and Ferguson, S. J. (1978) FEBS Lett. 90, 178–182.
[27] Gunter, T. E., Rosier, R. N., Tucker, D. A. and Gunter, K. K. (1978) Ann. NY Acad. Sci. 307, 246–247.
[28] Racker, E. (1973) Biochem. Biophys. Res. Commun 55, 224–230.
[29] Vuilleumier, P., Gazzotti, P., Carafoli, E. and Simon, W. (1977) Biochim. Biophys. Acta 467, 12–18.
[30] Sigel, E. and Carafoli, E. (1978) Eur. J. Biochem. 89, 119–123.
[31] Wikstrom, M. K. F. (1977) Nature 266, 271–273.
[32] Wikstrom, M. K. F. (1977) in: Mechanisms of Proton and Calcium Pumps, (Azzone, G. F. ed) pp. 215–226, Elsevier/North-Holland, Amsterdam, New York.
[33] Krab, K. and Wikstrom, M. (1978) Biochim. Biophys. Acta 504, 200–214.
[34] Puskin, J. S., Gunter, T. E., Gunter K. K. and Russell, P. R. (1976) Biochemistry 15, 3834–3842.
[35] Crompton, M., Kunzi, M. and Carafoli, E. (1977) Eur. J. Biochem. 79, 549–558.