STEADY STATE SPECTRA OF CYTOCHROME *c* OXIDASE IN RECONSTITUTED VESICLES

J. M. WRIGGLESWORTH
Department of Biochemistry, Chelsea College, University of London, Manresa Road, London SW3 6LX, England

and

P. NICHOLLS
Department of Biological Sciences, Brock University, St. Catherines, Ontario L2S 3A1, Canada

Received 4 May 1978

1. Introduction

Cytochrome *c* oxidase can be isolated from other components of the mitochondrial respiratory chain and reincorporated into phospholipid vesicles [1]. Reconstituted vesicles have the ability, under suitable conditions, to generate a membrane potential [2,3] and undergo respiration-dependent exchange of protons for potassium ions [4]. The spectrum of oxidised cytochrome *c* oxidase incorporated into phospholipid vesicles is also sensitive to externally imposed pH gradients across the vesicle membrane [5].

One characteristic of the reconstituted system that it shares with mitochondria is that turnover of the enzyme is stimulated by uncouplers of oxidative phosphorylation [4,6]. In mitochondria, respiratory control is associated with distinct energy-dependent changes in the redox and spectral properties of cytochrome *c* oxidase [7,8] and the demonstration of control of turnover in the model system suggests that similar changes may also be taking place. However, steady-state spectral studies on cytochrome *c* oxidase in reconstituted vesicles have not yet been reported. Such studies are complicated by the relatively low concentrations of incorporated enzyme necessary for respiratory control and also by the spectral presence of cytochrome *c* which has to be added, as an impermeable reductant, to impose an asymmetry to the respiring system. We describe here experimental procedures by which visible spectroscopic changes of cytochrome *c* oxidase can be monitored under controlled and uncoupled conditions when the enzyme is incorporated into phospholipid vesicles. Steady-state difference spectra show a 'cross-over' point lying between cytochromes *c* and *a* upon the addition of uncoupler. The spectra also indicate the presence of a compound with an $A_{430\,nm}$ max in the controlled steady state.

Abbreviations: FCCP, carbonylcyanide *p*-trifluoromethoxy phenylhydrazone; TMPD, tetramethyl-*p*-phenylenediamine

2. Materials and methods

Cytochrome *c* oxidase was prepared and incorporated into phospholipid (asolectin) vesicles by sonication as in [1]. The final phospholipid:protein weight ratio was ~20:1. Oxygen uptake was monitored at 30°C using a Clark type oxygen electrode in a closed cuvette. Spectra were obtained at room temperature with an Aminco DW-2 spectrophotometer using 1 ml cuvettes. Other experimental details are given in the figure legends.

3. Results and discussion

3.1. *Respiratory control*

From table 1 it can be seen that oxygen uptake by the reconstituted vesicles can be stimulated by the

Table 1
Effect of TMPD on the respiratory control ratio of reconstituted vesicles at different cytochrome *c* concentrations

TMPD (μM)	Cytochrome *c* (μM)	Respiratory rate (μM O_2 . min^{-1})		Respiratory control ratio
		Control	Uncoupled	
175	12	38	108	2.8
175	1.2	13	31	2.4
175	0	1.6	1.7	1.1
0	2.3	7.0	8.2	1.2

Conditions of assay were 4.3 ml 50 mM potassium phosphate, pH 7.4, at 30°C containing 7 mM sodium ascorbate and oxidase vesicles to final aa_3 conc. 12 nM. The uncoupled sample contained, in addition, 120 ng. ml^{-1} valinomycin and 12 μM FCCP

addition of valinomycin and FCCP when externally added cytochrome *c*, ascorbate and TMPD are used together as reductants. Decreasing the concentration of cytochrome *c* from 12–1.2 μM has only a slight effect on the degree of stimulation when TMPD is present. In the absence of TMPD, the respiratory control ratio of vesicles respiring using ascorbate plus a low concentration of cytochrome *c* is decreased to 1.1. Similarly, vesicles respiring using ascorbate plus TMPD alone have a respiratory control ratio close to unity.

Reconstituted vesicles produced by sonication [1] have a random (50:50) orientation of cytochrome *c* oxidase across the membrane. Vectorial reactions of the enzyme in either orientation can be studied using appropriate permeable and impermeable reagents. Oxidation of ascorbate by externally added cytochrome *c* results in an internal alkalinity change in the vesicles and the turnover of the enzyme can be stimulated by ionophores (except when respiratory rates are low). Turnover of the oxidase using ascorbate plus TMPD in the absence of added cytochrome *c* is not stimulated by ionophores since TMPD, being membrane permeable, can react with the enzyme in either orientation. The present results show that, in the presence of TMPD, even relatively low concentrations of cytochrome *c* are sufficient to maintain functional asymmetry. Presumably the turnover of those cytochrome *c* oxidase molecules with their cytochrome *c* reaction sites exposed to the external medium is at a high enough rate to produce asymmetric charge transfer across the vesicle membrane. Spectral studies on cytochrome *c* oxidase in reconstituted systems under controlled and stimulated conditions can therefore be made at low cytochrome *c* concentrations in the presence of TMPD and ascorbate.

3.2. *Spectra of cytochrome c oxidase under steady state conditions*

Figure 1 presents the steady state difference spectrum of reconstituted vesicles under controlled and uncoupled conditions. A 'cross-over' response between cytochromes *c* and *a* is produced on the addition of uncoupler. The steady state level of reduced cytochrome *c* at 550 nm is greater in the non-stimulated state whilst spectral changes both at 444 nm and 605 nm show that the steady state level of reduced cytochromes *a* and a_3 is less. This can be more clearly seen in fig.2 where a difference spectrum of controlled minus uncoupled steady states is presented. A peak at 550 nm corresponding to an increased steady state concentration of cytochrome *c* and a trough at 605 nm corresponding to a decreased concentration of reduced cytochrome *a* can be seen.

The apparent standard redox potential of one heme component of cytochrome *c* oxidase was reported to become more negative on energisation of the mitochondrial membrane [9]; an electrical membrane potential across the mitochondrial membrane has been shown to decrease the measured mid point potentials of both cytochromes *a* and a_3 [7]. The present results are consistent with these findings. A higher concentration of oxidised cytochrome *a* and a_3 and a lower concentration of oxidised cytochrome *c*

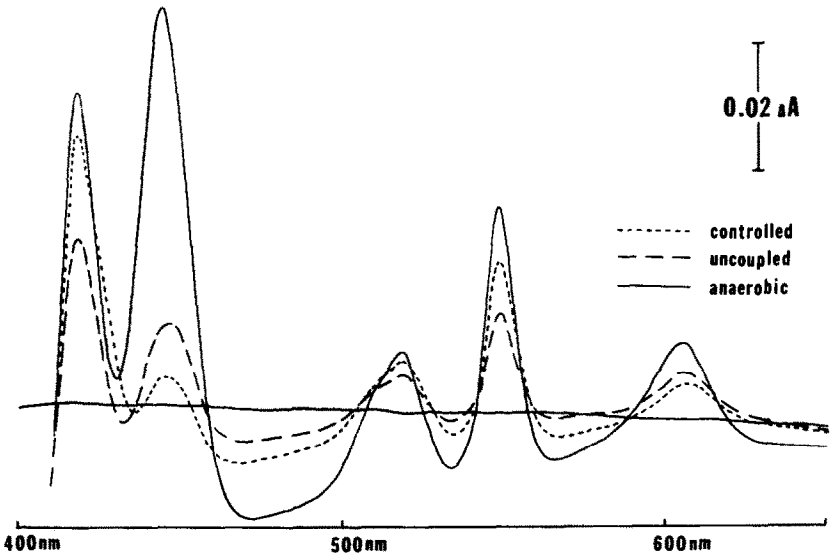

Fig.1. Steady-state difference spectra of reconstituted vesicles under controlled and 'uncoupled' conditions. Reconstituted vesicles of cytochrome *c* oxidase, 0.2 ml, were suspended in total 2 ml 50 mM potassium phosphate, pH 7.5, containing 2 μM cytochrome *c* at 25°C. Difference spectra (steady state minus oxidised) were measured after the addition of potassium ascorbate (10 mM) and TMPD (150 μM) to the sample cuvette. The controlled (dotted line) and uncoupled (dashed line) steady states are shown. Valinomycin (50 ng. ml^{-1}) plus nigericin (50 ng. ml^{-1}) were added to produce maximal respiration rates. The anaerobic difference spectrum (solid line) was measured 1 min after anaerobiosis.

can be seen spectroscopically during the controlled steady state in the reconstituted vesicles. Flux through the system could decrease when the heme components of the oxidase become more difficult to reduce in the controlled state. This provides a basis for a simple equilibrium type of control as suggested for mitochondria [7].

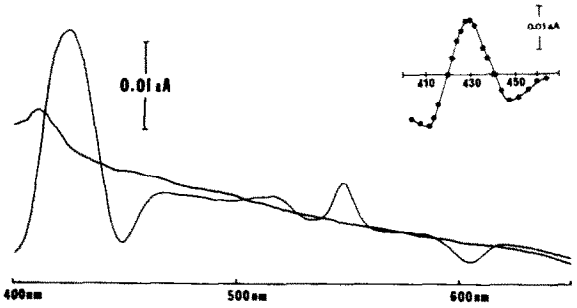

Fig.2. Steady-state difference spectrum of controlled minus 'uncoupled' reconstituted vesicles of cytochrome *c* oxidase. Conditions of measurement as in fig.1. Inset: Calculated spectrum in the Soret region after subtraction of the contribution of reduced cytochrome *c* to the absorption in the controlled steady state.

3.3. *'Low-spin' form of the enzyme*

The steady-state difference spectra shown in fig.1,2 also show that there is an increased absorbance between 420 nm and 430 nm in the controlled ('energised') state. The contribution of reduced cytochrome *c* in this region of the spectrum can be calculated from its A_{550} value and subtracted. The resulting spectrum (fig.2 inset) shows distinct differences from that of the normal 'high-spin' form of ferric cytochrome oxidase. There are troughs at 411 nm and 447 nm, and a peak at 430 nm. The spectrum is more similar in pattern to that obtained after adding ATP, or cyanide, to ferric cytochrome oxidase in pigeon heart mitochondria [10] and also is similar in effect to the spectral shift induced by an electrical diffusion potential in ferric cytochrome aa_3 incorporated into phospholipid vesicles [5]. These spectroscopic changes in ferric aa_3 are characteristic of a high → low spin state transition and the present results indicate for the first

time that stabilisation of this form of the ferric enzyme may be occurring in the controlled steady state. The corresponding steady state results with mitochondria are difficult to obtain because of the contribution of the cytochromes *b* in the spectra. We conclude that reconstituted vesicles can provide a useful system for spectroscopic studies of the energy dependent properties of the oxidase molecule.

Acknowledgments

We would like to thank Ms Freda Nicholls for technical assistance and Ms Virginia Hildebrandt for cytochrome *c* oxidase preparations. This work was supported by Canadian NRC grant A-0412 to P.N. J.M.W. acknowledges a travel grant from the Wellcome Trust.

References

[1] Wrigglesworth, J. M. (1978) in: Proc. 11th FEBS Meet., 1977, vol. 45, symp. A4 (Jorgensen, P. L. and Moody, A. J.) pp. 95–103, Pergamon Press.
[2] Jasaitis, A. A., Nemecek, I. B., Severinal, I. I., Skulachev, V. A. and Smirnova, S. M. (1972) Biochim. Biophys. Acta 275, 485–490.
[3] Gutweniger, H., Massari, S., Beltrame, M., Colnna, R., Veronese, P. and Ziche, B. (1977) Biochim. Biophys. Acta 459, 216–224.
[4] Hinkle, P. C., Kim, J. J. and Racker, E. (1972) J. Biol. Chem. 247, 1338–1339.
[5] Wikstrom, M. K. F. and Saari, H. T. (1977) Biochim. Biophys. Acta 462, 347–361.
[6] Hunter, D. R. and Capaldi, R. A. (1974) Biochem. Biophys. Res. Commun. 56, 623–628.
[7] Hinkle, P. and Mitchell, P. (1970) Bioenergetics 1, 45–60.
[8] Wikstrom, M. K. F., Harmon, H. J. Ingledew, W. J. and Chance, B. (1976) FEBS Lett. 65, 259–277.
[9] Wilson, D. F. and Dutton, P. L. (1970) Arch. Biochem. Biophys. 136, 583–584.
[10] Wilson, D. F., Erecinska, M. and Nicholls, P. (1972) FEBS Lett. 20, 61–65.