Electrochemically driven respiration in mitochondria and *Paracoccus denitrificans*

The coupling of the electrochemistry of horse heart cytochrome *c* with respiration in mitochondria and a model thereof, *Paracoccus denitrificans*

J.O.D. Coleman, H.A.O. Hill+, N.J. Walton+ and F.R. Whatley

School of Botany, South Parks Road, Oxford, OX1 3RA and +*Inorganic Chemistry Laboratory, South Parks Road, Oxford, OX1 3QR, England*

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By exploiting the rapid, direct electrochemistry of horse heart cytochomre *c* at a modified gold electrode it has been possible to couple the electrode reaction with respiration in rat liver mitochondria and in protoplasts of *Paracoccus denitrificans*, but not in protoplasts from *E. coli*. Oxidation of endogenous and exogenous sources of reducing equivalents via cytochrome *c* is also observed.

Cytochrome c *Cytochrome oxidase* *NADH* *Rat liver mitochondria*
Paracoccus denitrificans Escherichia coli *Electrochemistry*

1. INTRODUCTION

The electron transfer reactions associated with respiration and their connections with oxidative phosphorylation are still subjects of intense study, even debate [1,2]. There have been many investigations of the rates of electron transfer between isolated components of the respiratory chain, within mitochondria and sub-mitochondria, and in intact cells and organisms [3]. Of the redox proteins involved, or implicated, cytochrome *c* has been the object of the most intensive examination of the relationship between its structure and function. Recently cytochrome *c* has been shown to undergo well-defined electron transfer reactions at surface-modified gold electrodes [4], thus allowing its redox state to be controlled directly by the applied potential and eliminating the need for chemical reductants, oxidants or mediators in the study of its rôle in biological electron transfer. We have previously exploited [5,6] this direct electrochemistry in the study of homogeneous electron-transfer reactions of cytochrome *c* with purified redox enzymes. We now describe the coupling of the electrode reactions of cytochrome *c* with respiration in rat liver mitochondria and *Paracoccus denitrificans*.

2. EXPERIMENTAL

Rat liver mitochondria were isolated from adult rats as in [7]. The reaction medium contained 25 mM sucrose, 0.1 M KCl, 10 mM $MgCl_2$, 1 mM EDTA, 10 mM KH_2PO_4 and 10 mM TES (*N*-tris[hydroxymethyl]methyl-2-aminoethane sulphonic acid) at pH 7.2.

Protoplasts (type I) of *Paracoccus denitrificans* were prepared as in [8]. The reaction medium contained 0.2 M sucrose, 0.15 M KCl and 20 mM Tris–acetate, at pH 7.0.

Protoplasts of *E. coli* were prepared from harvested cells grown on glucose as in [9]. The reaction medium contained 0.3 M sucrose, 0.15 M KCl, 3 mM $MgCl_2$ and 10 mM potassium phos-

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phate, at pH 7.0.

Horse heart cytochrome *c* obtained from Sigma was purified [10] and used in all experiments at 0.7 mM. Rapid electrochemical reduction and reoxidation were observed in all the reaction media used.

The experimental cell consisted of a cylindrical working compartment (8 mm diam.) with separate side-arms for the reference (Radiometer type K401 SCE, $E° = 247$ mV *vs* NHE at 20°C) and counter (platinum gauze) electrodes. A gold foil working electrode (area 1.9 cm^2) was attached to the inside of the glass cell with epoxy cement. An additional removable disc-shaped gold foil electrode (area 0.39 cm^2) sealed the top of the compartment. There is a central unstirred capillary access port. The whole cell was clamped against the O-ring of a Clark-type oxygen electrode (Rank Brothers, Botisham, Cambridge) which forms the base. The chamber volume was 490 μl. This was stirred magnetically with a 7 mm PTFE-coated magnetic stirrer bar, giving a short half-life in 3 min for bulk electrolysis due to the high surface-area to volume ratio.

A conventional 3 electrode potentiostat (Oxford Electrodes) was used throughout. Experiments were conducted at room temperature (20°C).

Before each experiment the gold working electrodes were polished lightly with a 0.3 μm alumina/water slurry on cotton wool, rinsed thoroughly and then stirred with a solution of 2 mM bis(4-pyridyl) disulphide (Aldrithiol-4 from Aldrich, recrystallised at low temperature from acetone/hexane) in appropriate reaction medium for ~5 min to modify the surface irreversibly [11,12]. The modified electrodes were thoroughly rinsed with reaction medium to give a surface which was stable and long-lived (>1 h), even in the presence of the biological materials.

3. RESULTS AND DISCUSSION

Fig. 1 shows simultaneous measurements of the amount of dioxygen present and the current flowing in the system: cytochrome *c* plus rat liver mitochondria. When the potential is stepped from +395 mV to +95 mV *vs* NHE a large reduction current is observed which tends to approach a steady state current. (In the presence of cytochrome *c* alone the current shows a first-order

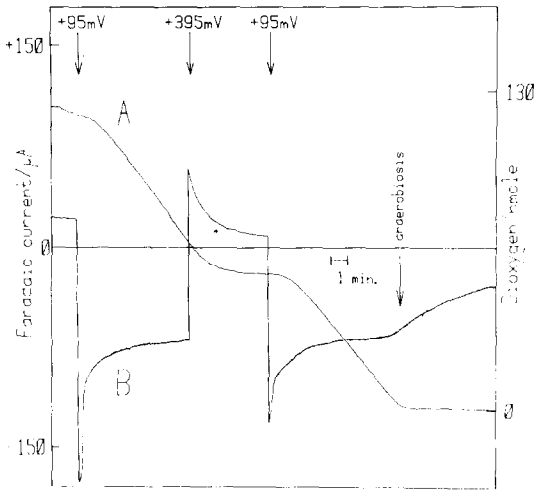

Fig. 1. Amount of dioxygen (A) and Faradaic current (B) as functions of time for 0.7 mM horse heart cytochrome *c* plus 2.0 mg protein/ml rat liver mitochondria in appropriate reaction medium. Potentials *vs* NHE of the working electrode as indicated (+95 mV reducing, +395 mV oxidising).

type decay, as expected [13] for a bulk electrolysis.) Simultaneously, the rate of oxygen consumption increases to a steady value. The same effect is observed with protoplasts of *Paracoccus denitrificans*, although in this case the increase is superimposed on an endogenous rate of respiration. We may equate the constant rate of dioxygen consumption with that of the steady state turnover of cytochrome *c* at the electrode; from this correlation it can be calculated that 4.0 electrons are used per dioxygen molecule consumed, as expected if cytochrome oxidase acts as the intermediary between electron transfer and dioxygen consumption. If the potential of the electrode is returned to +395 mV then, as fig. 1 shows, respiration very quickly ceases as reduced cytochrome *c* is rapidly depleted from the solution both by cytochrome oxidase and by the anode. A subsequent imposition of a reducing potential again brings about 'electrochemical' respiration. Further evidence for rapid turnover of electrochemically produced cytochrome(II)*c* by the in situ cytochrome oxidase is the correspondence between the onset of anaerobiosis and the time of transition of steady state reduction current to first-order decay. We presume that cytochrome oxidase can then no longer accept reducing equivalents and the system becomes fully

reduced. This too is shown in fig. 1. The same effect is produced in an aerobic solution if potassium cyanide, a well-known inhibitor of cytochrome oxidase, is introduced (not shown).

The experiment was repeated except that protoplasts of *E. coli* were used in place of those of *P. denitrificans*. No increae above the slow endogenous rate of oxygen consumption was observed. This inability to reduce oxygen is consistent with the absence of cytochrome *c* from the respiratory chain of *E. coli* [14]. It can be concluded that the electrochemically reduced cytochrome *c* is not simply a redox-active mediator with $E^\circ = +260$ mV but that it interacts only with those enzyme components which are themselves involved in the reduction and oxidation of endogenous cytochrome *c*. Thus cytochrome aa_3 in rat liver mitochondria and *Paracoccus denitrificans* can be driven by this electrochemically powered system because in both cases cytochrome *c* is a component of their respiratory chains. In contrast cytochrome *c* is not involved in terminal oxidation in *E. coli*.

In *Paracoccus denitrificans* protoplasts a basal rate of respiration is always present due to oxidation of endogenous substrates. Electrochemical oxidation of these endogenous reducing equivalents is shown in fig. 2(a). We propose that in such an aerobic system the flow of endogenous electrons is partitioned between cytochrome oxidase/O_2 and cytochrome *c* which is oxidised at the anode. The partition may be controlled by the relative affinities for, and accessibilities of exogenous cytochrome (III)*c* and cytochrome aa_3 to reduced cytochrome *c* bound to the membrane. There is further evidence for this hypothesis in fig. 2(a) where the oxidation current can be seen to increase at the onset of anaerobiosis. Presumably, cytochrome oxidase can no longer utilise electrons, which are diverted to react with exogenous cytochrome *c*. Addition of exogenous succinate to the same anaerobic solution (fig. 2(b)) gives another increase in current which represents reduction of cytochrome *c* by succinate *via* the low potential part of the respiratory chain.

Rapid reduction of cytochrome *c* by NADH in the presence of mitochondria is observed as shown in fig. 3. The outer mitochondrial membrane flavo-cytochrome b_5 enzyme is the likely catalyst. Fig. 3 shows that cytochrome *c* reduced by the first aliquot of NADH is reoxidised both by cytochrome oxidase and at the electrode. However, when the solution is exhausted of dioxygen reoxidation can take place only at the electrode. Initial steady state oxidation currents after each aliquot are not limited by the concentration of cytochrome *c* and most probably correspond to maximal enzyme turnover.

In conclusion, we have shown that, through the use of direct electrochemical reduction or oxidation of cytochrome *c*, it is possible to probe the biological domains responsible for respiration. In-

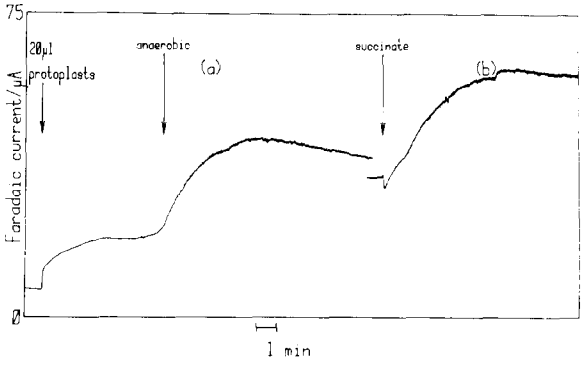

Fig. 2. Oxidation current at +395 mV *vs* NHE *vs* time for 0.7 mM horse heart cytochrome *c* +4.6 mg protein/ml protoplasts of *Paracoccus denitrificans* in appropriate reaction medium: (a) initially aerobic solution; (b) addition of succinate (final conc. 40 mM) to solution (a) after 3/4 h.

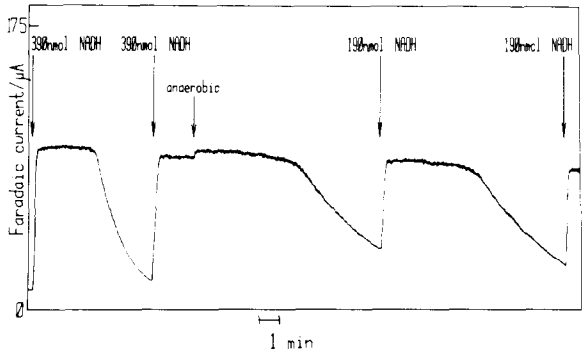

Fig. 3. Oxidation current at +395 mV *vs* NHE against time for solution containing 0.7 mM horse heart cytochrome *c* + 1.9 mg protein/ml rat liver mitochondria in appropriate reaction medium. Aliquots of NADH added as indicated.

gress of the electrons presumably takes place at complex IV; egress at complex III. It should be feasible, using this system, to investigate possible sites of cytochrome *c* oxidation on the cytochrome oxidase complex, as well as effects of applied potential and consequentially the ratio of oxidised to reduced cytochrome *c* on the proposed coupling at this site [15].

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REFERENCES

[1] Wikström, M., Krab, K. and Saraste, M. (1981) Cytochrome Oxidase: a Synthesis, Academic Press, London, New York.
[2] Storey, B.T., Lee, C.-p. and Wikström, M. (1981) Trends Biochem. Sci. 6, 166–170.
[3] Cass, A.E.G. (1982) in: Inorganic Biochemistry, vol. 3 (Hill, H.A.O., sen. rep.), pp. 183–267, The Royal Society of Chemistry, London; and references therein.
[4] Eddowes, M.J. and Hill, H.A.O. (1982) in: Electrochemical and Spectrochemical Studies of Biological Redox Components (Kadish, K.M. ed) ACS Chemistry Series 201, American Chemical Society, Washington DC; and references therein.
[5] Hill, H.A.O., Walton, N.J. and Higgins, I.J. (1981) FEBS Lett. 126, 282–284.
[6] Hill, H.A.O. and Walton, N.J. (1982) J. Am. Chem. Soc. 104, 6515–6519.
[7] Weinbach, E.C. (1961) Anal. Biochem. 2, 335–343.
[8] Alefounder, P.R. and Ferguson, S.J. (1980) Biochem. J. 192, 231–240.
[9] Garland, P.B., Downie, J.A. and Haddock, B.A. (1975) Biochem. J. 152, 547–559.
[10] Brautigan, D.L., Ferguson-Miller, S. and Margoliash, E. (1978) Methods Enzymol. 53, 128–164.
[11] Taniguchi, I., Toyosawa, K., Yamaguchi, H. and Yasukouchi, K. (1982) J. Chem. Soc. Chem. Commun. 1032–1033.
[12] Taniguchi, I., Toyosawa, K., Yamaguchi, H., and Yasukouchi, K. (1982) J. Electroanal. Chem. 140, 187–193.
[13] Bard, A.J. and Faulkner, L.R. (1980) in: Electrochemical Methods: Fundamentals and Applications, pp. 370–428, Wiley, New York.
[14] Jones, C.W. (1977) in: Society for General Microbiology symp. 27 (Haddock, B.A. and Hamilton, W.A. eds) pp. 23–57; and references therein.
[15] Wikström, M. and Pentilla, T. (1982) FEBS Lett. 144, 183–189.