CONTINUOUS MONITORING OF THE ELECTRICAL POTENTIAL ACROSS ENERGY-TRANSDUCING MEMBRANES USING ION-SELECTIVE ELECTRODES

Application to submitochondrial particles and chromatophores

D. B. KELL and P. JOHN
Botany School, University of Oxford, South Parks Road, Oxford OX1 3RA

and

M. C. SORGATO and S. J. FERGUSON
Department of Biochemistry, University of Oxford, South Parks Road, Oxford, OX1 3QU, England

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1. Introduction

It is now widely believed that a primary result of electron transport in the energy-transducing membranes of mitochondria, chloroplasts and bacteria is the vectorial translocation of protons, leading to the generation of a transmembrane electrochemical proton gradient, the protonmotive force Δp [1].

Δp is composed of both a chemical component ΔpH and an electrical component $\Delta\psi$ according to the relationship:

$$\Delta p = \Delta\psi - \frac{RT}{F}\,\Delta\mathrm{pH} \qquad (1)$$

and the methods employed for determining the components of Δp have recently been critically reviewed [2]. A favoured method of determining the membrane potential, $\Delta\psi$, generated across energy-transducing membranes is to follow the distribution of an appropriately charged permeant ion between the suspending medium and the lumen of the membrane vesicle, with the assumption that the ion equilibrates across the membrane according to the Nernst equation. Thus for anion uptake by vesicles in which electron transport causes the lumen to become positively charged with respect to the medium [2];

$$\Delta\psi = \frac{2.3\,RT}{nF}\log\frac{[A^{n-}]_{in}}{[A^{n-}]_{out}} \qquad (2)$$

We report here that commercially available anion-selective electrodes are sufficiently sensitive for the real-time determination of $\Delta\psi$ in bovine heart submitochondrial particles and in chromatophores from *Rhodospirillum rubrum*. The same value for $\Delta\psi$ was obtained from either the uptake of thiocyanate (SCN^-) or of NO_3^- into respiring submitochondrial particles. The SCN^- electrode has also been used to estimate $\Delta\psi$ as a function of the respiration rate in submitochondrial particles. We believe that the present method possesses a number of advantages over other methods currently available for the measurement of $\Delta\psi$ [2].

2. Materials and methods

Ion-selective electrode measurements were made in

Abbreviation: FCCP, carbonylcyanide *p*-trifluoromethoxyphenylhydrazone

Address correspondence to: D. B. Kell, Botany School, South Parks Road, Oxford, OX1 3RA, England

a thermostatted 25 ml beaker using an Orion Series 94-58 solid state SCN^- electrode or an Orion Series 93-07 liquid membrane NO_3^- electrode. The potential developed by the SCN^- or NO_3^- electrode was measured against a double junction reference electrode (model 90-02, Orion Research, MSE, Manor Royal, Crawley, Sussex) using a pH meter (Pye Unicam model 290 Mk II) set on the mV mode and connected in parallel with a 100 Ω variable resistor to a Servoscribe chart recorder, essentialy as described [3]. The 5 ml reaction mixtures, which are given in the legends to the figures, were stirred with a magnetic bar, and both the stirrer speed and the variable resistor were adjusted so that the noise level did not exceed 1% of a full scale deflection. Where appropriate, a jet of oxygen, saturated with water at 30°C, was blown at the surface of the reaction mixture. The bridging solution in the double junction reference electrode was 10% KF. Electrodes were soaked in a 0.1 M solution of the ion to be sensed for at least 1 h before use.

Measurement of the extent of ion uptake together with estimates of the internal volume enclosed by the vesicles, namely 1.3 μl/mg protein for submitochondrial particles (unpublished observations) and 50 μl/mg bacteriochlorophyll for chromatophores [4], enabled $\Delta\psi$ to be calculated from eq. (2).

Mg–ATP submitochondrial particles were prepared as described [5], except that all chlorides were replaced by the corresponding acetate salts. The preparation of *R. rubrum* chromatophores [4], the flow dialysis measurements [4], oxygen uptake [5] and protein determinations [5] are detailed elsewhere.

3. Results

Figure 1 shows that the response of the Orion SCN^- electrode is linear rather than Nernstian at the low concentrations of SCN^- that are used as probes for $\Delta\psi$. The $t_{\frac{1}{2}}$ for the response of the electrode is near the limit of response of the chart recorder (1–2 s). On addition of a respiratory substrate, SCN^- is taken up into the lumen of submitochondrial particles with pseudo-first order kinetics, $t_{\frac{1}{2}}$ about 45 s (cf. [6,7]), until a steady state is attained (fig.1). On addition of 0.2 mM ADP, the steady state uptake of SCN^- decreases transiently (for less than 1 min) while the ADP is phosphorylated. All the SCN^- taken

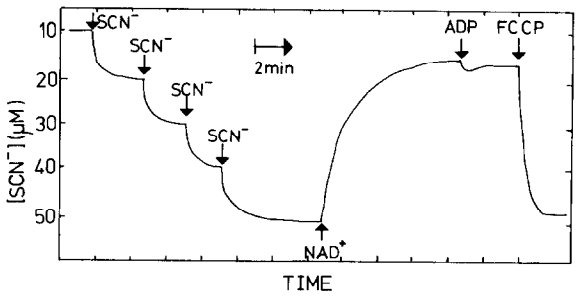

Fig.1. Energy-dependent uptake of SCN^- by bovine heart submitochondrial particles monitored with an ion-sensitive electrode. Submitochondrial particles (18.8 mg protein) were incubated in 5 ml reaction medium containing 10 mM P_i–Tris, pH 7.3, 5 mM magnesium acetate, 1% ethanol, 0.2 mg alcohol dehydrogenase and 10 μM KSCN. The temperature was 23°C. The free SCN^- concentration was monitored potentiometrically as described in section 2. Calibrating additions of KSCN were made as indicated by the arrows. NAD^+ (0.2 mM), ADP (0.2 mM) and FCCP (2.5 μM) were added as indicated. The electrode response after addition of FCCP did not quite return to the level observed before adding NAD^+. This effect was due to a slight interference with electrode by NAD^+ which was also seen when NAD^+ was added to the particles after FCCP.

up is released upon addition of 2.5 μM FCCP as uncoupler.

Figure 2 shows the effect of increasing rotenone concentrations on $\Delta\psi$, obtained both from experiments similar to that shown in fig.1, and from a parallel assay of $S^{14}CN^-$ uptake using flow dialysis [4]. Also plotted are the results of parallel experiments in which the steady state rate of oxygen uptake was determined. (Rates of oxygen uptake were linear only after a pre-incubation with rotenone (cf. [8].) Except at the highest respiratory rates, the relationship between the respiratory rate and $\Delta\psi$ is essentially ohmic, as found, for example, in rat liver mitochondria [9], and as suggested [8] for submitochondrial particles. The results using the potentiometric assay of SCN^- uptake are very similar to those obtained with the flow dialysis method (fig.2).

When NO_3^- is used as the permeant ion [10], the respiration-driven uptake into submitochondrial particles (fig.3), monitored with the NO_3^- electrode, again displays pseudo-first order kinetics, with $t_{\frac{1}{2}}$ approx. 2 min, almost 3-fold that for SCN^- uptake under the same conditions. Nevertheless, $\Delta\psi$ calcu-

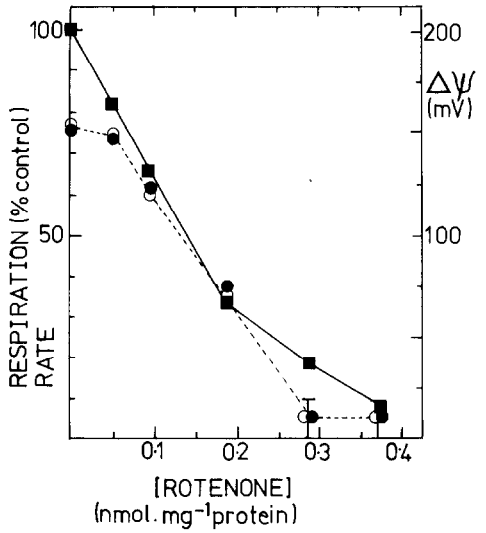

Fig.2. The relationship between the respiratory rate and the membrane potential of bovine heart submitochondrial particles. $\Delta\psi$ was measured from the extent of SCN^- uptake which was monitored either by the SCN^- electrode (●–●–●) or by the flow dialysis method (○–○–○). When the electrode was used the reaction mixture was as described in the legend to fig.1 except that 12.5 mg submitochondrial particle protein were used. For flow dialysis measurements the upper chamber of the flow dialysis cell contained in total vol. 1 ml, submitochondrial particles (6.0 mg protein), 10 mM P_i–Tris, pH 7.3, 5 mM magnesium acetate, 1% ethanol, 0.05 mg alcohol dehydrogenase, 0.2 mM NAD^+ and 20 μM $KS^{14}CN$ (60 $mCi.mmol^{-1}$). At the two lowest respiratory rates the uptake of SCN^- was undetectable, and the limit of detection is indicated on the figure. Respiration was monitored using an oxygen electrode with 3 ml reaction mixture which contained: submitochondrial particles (2.1 mg protein), 10 mM P_i–Tris, pH 7.3, 5 mM magnesium acetate, 1% ethanol, 0.1 mg alcohol dehydrogenase and 50 μM KSCN. Respiration was initiated by the addition of 0.2 mM NAD^+. Respiration rates (■–■–■) are plotted as a percentage of the rate in the absence of rotenone, which was 366 natom O/min/mg protein. In all experiments the submitochondrial particles were preincubated for 2 min with the appropriate concentration of rotenone. The temperature throughout was 23°C.

lated from the extent of NO_3^- uptake (fig.3) is between 145 mV and 150 mV, the same as that calculated from the extent of SCN^- uptake (fig.1,2). The use of eq. (2) assumes that anions passively equilibrate with an electrical potential across the membrane, and thus the steady-state level of accumulation of an anion should be independent of the nature of the anion. Our finding that measurement of

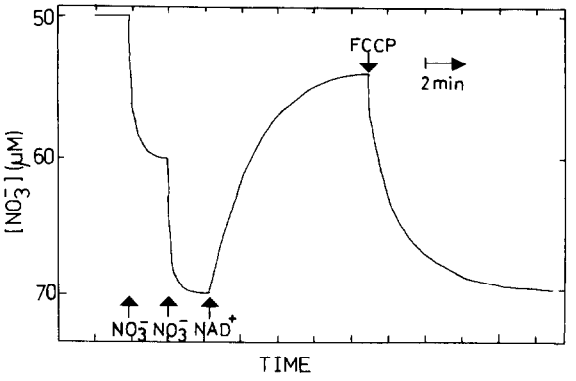

Fig.3. Energy-dependent uptake of NO_3^- by bovine heart submitochondrial particles monitored with a NO_3^- electrode. Submitochondrial particles (5.9 mg protein) were incubated in 5 ml reaction medium containing 10 mM P_i–Tris, 5 mM magnesium acetate, 1% ethanol, 0.2 mg alcohol dehydrogenase and 50 μM KNO_3. The temperature was 23°C. The free nitrate concentration was monitored with a NO_3^- electrode as described in section 2. KNO_3 (10 μM), NAD^+ (0.2 mM) and FCCP (2.5 μM) were added as indicated by the arrows.

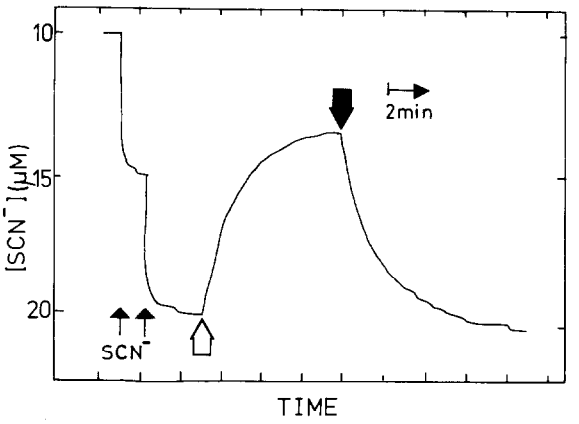

Fig.4. Light-induced uptake of SCN^- by *Rhodospirillum rubrum* chromatophores monitored with a NO_3^- selective electrode. *R. rubrum* chromatophores (0.63 mg bacteriochlorophyll) were incubated in 5 ml reaction medium containing 10 mM P_i–Tris, pH 8.0, 5 mM magnesium acetate, 20 mM sucrose, 0.2 mM sodium succinate and 10 μM KSCN. Free SCN^- concentration was monitored with a NO_3^- electrode as described in section 2, and calibrating additions of KSCN were made as indicated. At the open arrow, the illumination (a 300 W projector, filtered with a Kodak Cinemoid Deep Orange filter and 5 cm of water) was turned on. It was turned off at the full arrow. The (saturating) light intensity at the centre of the reaction vessel was approx. 3×10^5 erg $cm^{-2}s^{-1}$. When the light was off, the reaction mixture was covered with aluminium foil. The temperature throughout was 27°C.

the uptake of either SCN^- or of NO_3^- gives the same values for $\Delta\psi$ thus provides important support for use of eq. (2).

Figure 4 illustrates the versatility of the electrodes, and their general applicability to the study of energy-transducing membrane vesicle preparations. Figure 4 shows the use of the Orion NO_3^- electrode to sense the SCN^- ion (to which it is sensitive in the absence of NO_3^-) in a reaction mixture containing *R. rubrum* chromatophores. A marked, light-induced, pseudo-first order uptake of SCN^- is observed (fig.4). This uptake corresponds to a $\Delta\psi$ of about 100 mV (cf. [4,11]). The NO_3^- electrode, unlike the SCN^- electrode, is insensitive to light and so can be used to determine light-dependent uptake of either NO_3^- or SCN^-. Indeed, a considerable number of anions may be sensed by liquid membrane electrodes of this kind [12–15]. NO_3^- does not interfere with the SCN^- electrode and so this electrode can be used to follow SCN^- uptake in the presence of NO_3^-.

4. Discussion

The use of ion-selective electrodes in biochemical studies has been widely reported, as testified by the current interest in poteniometric methods of analysis [16–19]. In studies of bioenergetics, ammonium- [20,21] and potassium-selective electrodes [22–25] have been used previously, as well as the electrodes sensitive to lipophilic ions [26], the latter of which are unfortunately not commercially available. Papa et al. [24] found that a SCN^- electrode had too slow a response for their kinetic studies. Possibly the Orion electrodes that we used in the present work do not suffer from this disadvantage (fig.1,3,4). Neither electrode was significantly interfered with by lipophilic ionophores (valinomycin, nigericin), by electron transport inhibitors (antimycin, rotenone) or by low concentrations of uncouplers (FCCP, 1799). Millimolar concentrations of succinate, however, did interfere with the electrodes when they were sensing micromolar concentrations of NO_3^- or SCN^-.

We believe that these electrodes possess a number of clear advantages over methods currently used for the monitoring of transmembrane electrical potentials in microscopic systems:

1. Quantitative probing of bulk transmembrane electrical potentials by following ion distribution is a method which possesses a sound theoretical basis, in contrast, for example, to the use of fluorescence probes, for which the basis of response to $\Delta\psi$ remains unclear.

2. The electrodes can sense low concentrations of ions, and avoid the use of radioisotopes of high specific activity (cf. [4]).

3. The electrodes are constructed of biologically inert materials.

4. A direct readout in real time is provided.

5. A large number of ions may be sensed.

6. The method is non-destructive, allowing further analysis of samples.

7. The size and high sensitivity of the electrodes is such that only small amounts of biological material are required.

Electrodes should also be of particular value in extending studies on the relationship between the rate of electron flow and the magnitude of $\Delta\psi$ [8,9,27–29], as titrations with an uncoupler or electron transport inhibitor can be made on a single sample.

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References

[1] Boyer, P. D., Chance, B., Ernster, L., Mitchell, P., Racker, E. and Slater, E. C. (1977) Ann. Rev. Biochem. 46, 955–1026.

[2] Rottenberg, H. (1975) J. Bioenerg. 7, 61–74.
[3] John, P. (1977) J. Gen. Microbiol. 98, 231–238.
[4] Kell, D. B., Ferguson, S. J. and John, P. (1978) Biochim. Biophys. Acta in press.
[5] Ferguson, S. J. and Sorgato, M. C. (1977) Biochem. J. 168, 299–303.
[6] Mitchell, P. and Moyle, J. (1969) Eur. J. Biochem. 9, 149–155.
[7] Lehninger, A. L. (1974) Proc. Natl. Acad. Sci. USA 73, 125–130.
[8] Hinkle, P. C., Tu, Y. L. and Kim, J. J. (1975) in: Molecular Aspects of Membrane Phenomena (Kaback, H. R., Neurath, H., Radda, G. K., Schwyzer, R. and Wiley, W. R. eds) pp. 222–232, Springer-Verlag, Berlin, Heidelberg, New York.
[9] Nicholls, D. G. (1974) Eur. J. Biochem. 50, 305–315.
[10] Montal, M., Chance, B. and Lee, C-P. (1969) J. Membrane Biol. 2, 201–234.
[11] Schuldiner, S., Padan, E., Rottenberg, H., Gromet-Elhanan, Z. and Avron, M. (1974) FEBS Lett. 49, 174–177.
[12] Coetzee, C. J. and Freiser, H. (1968) Anal. Chem. 40, 2071.
[13] Coetzee, C. J. and Freiser, H. (1969) Anal. Chem. 41, 1128–1130.
[14] Reinsfelder, R. E. and Schultz, F. A. (1973) Anal. Chim. Acta 65, 425–435.
[15] Jyo, A., Torikai, M. and Ishibashi, N. (1974) Bull. Chem. Soc. Jap. 47, 2862–2868.
[16] Moody, G. J. and Thomas, J. D. R. (1971) Selective ion-sensitive electrodes, Merrow, Watford, England.
[17] Koryta, J. (1975) Ion-selective electrodes, Cambridge University Press, Cambridge.
[18] Bailey, P. L. (1976) Analysis with ion-selective electrodes. Heyden, London.
[19] Lakshminarayaniah, N. (1976) Membrane Electrodes, Academic Press, New York.
[20] Rottenberg, H. and Grunwald, T. (1972) Eur. J. Biochem. 25, 71–74.
[21] Rottenberg, H. and Lee, C-P. (1975) Biochemistry 14, 2675–2680.
[22] Jackson, J. B., Crofts, A. R. and Von Stedingk, L-V. (1968) Eur. J. Biochem. 6, 41–54.
[23] Mitchell, P. and Moyle, J. (1969) Eur. J. Biochem. 7, 471–484.
[24] Papa, S., Guerrieri, F., Simone, S., Lorusso, M. and Larosa, D. (1973) Biochim. Biophys. Acta 292, 20–38.
[25] Collins, S. H. and Hamilton, W. A. (1976) J. Bacteriol. 126, 1224–1231.
[26] Grinius, L. L., Jasaitis, A. A., Kadziauskas, Y. P., Liberman, E. A., Skulachev, V. P., Topali, V. P., Tsofina, F. M. and Vladimirova, M. A. (1970) Biochim. Biophys. Acta 216, 1–12.
[27] Nicholls, D. G. (1977) Eur. J. Biochem. 77, 349–356.
[28] Baccarini-Melandri, A., Casadio, R. and Melandri, B. A. (1977) Eur. J. Biochem. 78, 389–402.
[29] Johnson, R. N. and Hansford, R. G. (1977) Biochem. J. 164, 305–322.