Respiration-driven proton translocation in *Paracoccus denitrificans*: role of the 'permeant' ion

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Scholes & Mitchell (1970) demonstrated that the addition of pulses of O₂, as air-saturated KCl, to weakly-buffered anoxic suspensions of Micrococcus (now Paracoccus) denitrificans elicited the vectorial ejection of H⁺ into the bulk aqueous phase external to the organisms, where they could be detected with a sensitive glass electrode. Yet, in the absence of valinomycin or the SCN- ion, the half-time of H⁺ translocation was very much greater than the half-time of O₂ reduction; the extrapolated \rightarrow H⁺/O ratio was also significantly less than that observed when appropriate concentrations of valinomycin or SCNwere present. It was proposed that, in the absence of such compounds, a large transmembrane potential was built up by the translocation of a small fraction of the pumped protons, and that inhibition of this bulk-to-bulk transmembrane potential by the transmembrane electrophoretic co- or counter-transport of 'permeant' ions allowed measurement of the true stoichiometry of respiration-driven H⁺ translocation.

Gould & Cramer (1977), working with Escherichia coli, challenged this explanation of the role of valinomycin and SCN⁻ by demonstrating (in the absence of 'permeant' ions) that at high cell/O₂ ratios, when the calculated membrane potential was energetically insignificant, the measured \rightarrow H⁺/O ratio did not remotely attain its limiting stoichiometric value. Further, the stoichiometry of H⁺ ejection following the addition of a second oxygen pulse immediately after the first was unchanged. We have therefore reinvestigated the role of so-called 'permeant' ions in *P. denitrificans*, as part of a general study of the pathway of H⁺ transfer in membrane energy-coupling processes (Kell, 1979; Kell & Morris, 1981a; Kell *et al.*, 1981).

P. denitrificans N.C.I.B. 8944 was grown and maintained as described previously (McCarthy *et al.*, 1981). Mid-exponentialphase cultures were washed three times and resuspended at approx. 3mg dry weight/ml in a 6ml reaction mixture containing 150 mM-KCl/0.25 mM-glycylglycine, pH 6.5 plus 80 μ g of carbonic anhydrase/ml. The potentiometric system was as described by Kell & Morris (1981b), and O₂ pulses were delivered as air-saturated KCl in the usual way (Scholes & Mitchell, 1970). The following results were obtained: (1) The number of measurable H⁺ ions translocated across the bacterial membrane, Δ H⁺, increased linearly with the size of the O₂ pulse from 4.7 to 47 ng-atom of O. (2) More than 90% of the observed H⁺ had been pumped across the bacterial membrane in that they were not observed when carbonyl cyanide *p*-trifluoromethoxy-phenylhydrazone (FCCP, 2µM) was present. (3) This pattern

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was unchanged when the cell concentration was decreased by a factor of 3 or when NaCl or choline chloride were substituted for the KCl. (4) The \rightarrow H⁺/O ratio obtained when 100 mM-KSCN was added increased from approx. 2 to approx. 7.5; the relative kinetics of the pH traces was similar to those obtained by Scholes & Mitchell (1970). (5) Low concentrations (10- $60 \mu M$) of sodium tetraphenylborate increased the observed \rightarrow H⁺/Oratio approx. 2-fold. This could not have been caused by electrophoretic movement of tetraphenylborate from inner to outer bulk aqueous phases, since (a) the number of 'extra' H^+ ions observed was greater than the number of free intracellular tetraphenylborate ions, and (b) the number of 'extra' H^+ ions observed in the bulk phase at a given tetraphenylborate concentration was independent of the size of the oxygen pulse when this was varied between 14 and 42 ng-atoms. The appearance of these 'extra' protons was again sensitive to FCCP.

It may be concluded that, in the absence of compounds such as SCN-, protons translocated during electron transport pass directly to membrane-located sinks such as the ATPase (Hanselmann, 1974) via a non-bulk-phase pathway. The residual H⁺ ions observable in the bulk phase under these conditions are thus not used for membrane energy coupling. Proton ejection into the bulk phase external to the organisms may be stimulated both by blocking formation of the transmembrane field (electrophoretic mechanism) and by directly interfering with the function of membrane-located proteinaceous devices which normally act to channel pumped H⁺ ions to their membrane-located energetic sinks (non-electrophoretic mechanism). It is concluded from such measurements of respiration-driven H⁺ translocation that a non-osmotic mode of protonmotive energy coupling is exploited by P. denitrificans, in accordance with a previously outlined model (Kell, 1979; Kell & Morris 1981a; Kell et al., 1981).

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Characterization of succinate dehydrogenase from Micrococcus lysodeikticus

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Succinate dehydrogenase is an integral part of the respiratory chain of *Micrococcus lysodeikticus* (Pollock *et al.*, 1971). A specific antiserum to this enzyme has been produced in rabbits

after separating the enzyme by a crossed immunoelectrophoresis technique (B. Crowe & P. Owen unpublished work). The enzyme was then precipitated selectively from an extract of *M. lysodeikticus* membranes in 4% Triton X-100. The complex contained four polypeptides, of M_r 72000, 30000, 17000 and 15000. By using cells grown on radioactively labelled riboflavin it was found that the largest subunit contains covalently bound flavin. The precipitated complex also contained a *b*-type cytochrome with maximum absorption (reduced – oxidized at 77K) at 556 nm.



Fig. 1. E.s.r. spectra, measured at 12 K, of succinate dehydrogenase-antibody precipitate from M. lysodeikticus membranes: (a) oxidized with 0.1 mm-K₃Fe(CN)₆; (b) reduced with 20 mmsuccinate for 5 min at 20°C (the signal at g = 1.93 is strongly saturated at this temperature); (c) reduced with 3 mm-Na₂S₂O₄+0.03 mm-Methyl Viologen (the signal at g = 2.0 in this spectrum is the reduced Methyl Viologen radical)

Conditions of measurement: microwave power 20mW; frequency 9.18 GHz; modulation amplitude 0.1 mT.

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The iron-sulphur clusters in the precipitated succinate dehydrogenase were examined by low-temperature e.s.r. (electron-spin-resonance) spectroscopy. They were found to be equivalent to those in succinate dehydrogenase of eukaryotic mitochondria (Ohnishi, 1979) and purple photosynthetic bacteria (Yoch & Carithers, 1979). In the oxidized state a signal at g = 2.01 was detected (Fig. 1a), consistent with the $[4Fe-4S]^{3+}$ cluster S-3. On treatment with succinate the enzyme was incompletely reduced, probably because of its particulate nature. A spectrum at g = 2.03, 1.93 appeared (Fig. 1b), with a temperature-dependence indicative of the [2Fe-2S] cluster S-1. On further reduction with Na₂S₂O₄ and Methyl Viologen the g = 2.03, 1.93 spectrum increased in intensity (Fig. 1c).

The dependence of the e.s.r. signals of succinate dehydrogenase on redox potential was investigated in *M. lysodeikticus* membrane preparations. The g = 2.01 signal (centre S-3) showed a midpoint potential of $10 \pm 15 \,\mathrm{mV}$ (versus the standard hydrogen electrode at pH 7.0). Reduction of the g = 2.03, 1.93 signal occurred in two stages, at 50 mV and $-295 \,\mathrm{mV}$, corresponding to centres S-1 and S-2. As with other succinate dehydrogenases, the amplitude of the second increase in the g =1.93 signal (S-2) was considerably less than the first (S-1), if allowance was made for microwave power saturation (Beinert *et al.*, 1977; Albracht, 1980). Because of the relative potentials of centres S-1 and S-3, it was possible to observe the enzyme with both centres in their paramagnetic states. Power-saturation studies then indicated a spin-spin interaction between the two clusters.

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Ca²⁺-dependent ATPase of rat mammary-gland Golgi vesicles: influence of Ca²⁺ and Mg²⁺ on the formation and breakdown of a phospho-enzyme intermediate

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We have reported that incubation of rat mammary-gland Golgi vesicles with $[\gamma^{32}P]ATP$ leads to the transient phosphorylation of a membrane protein of apparent molecular weight 65000 (West & Clegg, 1981). This phosphorylated protein has some properties in common with phospho-enzyme intermediates of a number of ATP-dependent enzymes (Yamamoto & Tonomura, 1968; Mackinose, 1969; Bastide *et al.*, 1973; Katz & Blostein, 1975). On the basis of these properties, we have hypothesized that this Golgi-vesicle phosphoprotein is a phospho-enzyme intermediate of the Ca²⁺-translocating ATPase that occurs in the Golgi-vesicle membrane (West, 1981). However, in comparison with the Ca²⁺-dependent ATPase of the sarcoplasmic reticulum, which exemplifies the properties of well-characterized Ca²⁺-translocating enzymes, the response of the

Golgi-vesicle phosphoprotein to Ca²⁺ ions is atypical. Specifically, we have shown that removal of Ca²⁺ with EGTA led to a 3-fold increase, relative to controls without EGTA, in the amount of ³²P incorporated into the 65 000-mol.wt. Golgi-vesicle protein after 20s incubation with $[\gamma^{-32}P|ATP]$. Conversely, the addition of 100 μ M-Ca²⁺ caused a halving of the ³²P incorporation (West & Clegg, 1981).

The Ca²⁺-dependence of the sarcoplasmic-reticulum ATPase arises from the requirement for saturation of its Ca²⁺-binding sites before it can react with ATP to form a phospho-enzyme intermediate; consequently, removal of Ca²⁺ by using EGTA prevents phospho-enzyme formation (Hasselbach, 1974; Mac-Lennan & Holland, 1975). Clearly, if our hypothesis concerning the involvement of the 65000-mol.wt. Golgi-vesicle phosphoprotein in Ca²⁺-translocation is to retain credibility, we must further propose that the mechanism of ATP-dependent Ca²⁺ transport by the mammary-gland Golgi membrane differs from that demonstrated in the sarcoplasmic-reticulum system. In