The Role of the Membrane-Bound Hydrogenase in the Energy-Conserving Oxidation of Molecular Hydrogen by *Escherichia coli*

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H₂-dependent reduction of fumarate and nitrate by spheroplasts from *Escherichia coli* is coupled to the translocation of protons across the cytoplasmic membrane. The $\rightarrow\text{H}^+/2\text{e}^-$ stoichiometry (g-ions of H⁺ translocated ÷ mol of H₂ added) is approx. 2 with fumarate and approx. 4 with nitrate as electron acceptor. This proton translocation is dependent on H₂ and a terminal electron acceptor and is not observed in the presence of the protonophore carbonyl cyanide *m*-chlorophenylhydrazone and the respiratory inhibitor 2-*n*-heptyl-4-hydroxyquinoline *N*-oxide. H₂-dependent reduction of menadione and ubiquinone-1 is coupled to a protonophore-sensitive, but 2-*n*-heptyl-4-hydroxyquinoline *N*-oxide-insensitive, proton translocation with $\rightarrow\text{H}^+/2\text{e}^-$ stoichiometry of approx. 2. H₂-dependent reduction of Benzyl Viologen (BV⁺⁺) to its radical (BV⁺) liberates protons at the periplasmic aspect of the cytoplasmic membrane according to the reaction:

It is concluded that the effective proton translocation observed in the H₂-oxidizing segment of the anaerobic respiratory chain of *Escherichia coli* arises as a direct and inevitable consequence of transmembranous electron transfer between protolytic reactions that are spatially separated by a membrane of low proton-permeability.

Membrane-bound hydrogenases (EC class 1.12) are involved in the energy-conserving oxidation of molecular H₂ by several species of bacteria (Aleem, 1977; Thauer *et al.*, 1977; Schlegel & Schneider, 1978), including *Escherichia coli* (Macy *et al.*, 1976; Bernhard & Gottschalk, 1978*a,b*; Yamamoto & Ishimoto, 1978). The physiological oxidant of hydrogen in *E. coli* is probably fumarate (Macy *et al.*, 1976; Bernhard & Gottschalk, 1978*a,b*). Nitrate and O₂ are also effective electron acceptors (Jones, 1979*b*), but their presence during growth represses hydrogenase synthesis (Cole & Wimpenny, 1966).

The chemiosmotic hypothesis of Mitchell (1966, 1976) predicts that energy-conserving electron transport is obligatorily coupled to the stoichiometric translocation of protons across the coupling membrane, which, in bacteria, is the cytoplasmic mem-

brane. Proton translocation has been demonstrated to be driven by both aerobic (Lawford & Haddock, 1973) and anaerobic (Brice *et al.*, 1974; Garland *et al.*, 1975; Garland & Jones, 1977; Gutowski & Rosenberg, 1977) respiration by *E. coli*. Measurement of the quenching of atebirin fluorescence has shown that H₂-dependent reduction of fumarate is proton-translocating (Jones, 1979*b*). To elucidate the mechanism of this H₂-dependent proton translocation, it is essential to measure the $\rightarrow\text{H}^+/2\text{e}^-$ stoichiometry and to identify the sites of interaction of the membrane-bound respiratory carriers with electrons and protons (Jones *et al.*, 1978). A preliminary report of part of this work has been published (Jones, 1980).

Experimental

Growth of Escherichia coli

An overnight batch culture was made by inoculating 0.5 ml of preliminary overnight culture (Jones & Garland, 1977) of *E. coli* A1002 into a 500 ml stoppered bottle containing 450 ml of the medium described by Cohen & Rickenberg (1956) supple-

Abbreviations used: BV⁺⁺, oxidized species of Benzyl Viologen; BV⁺, radical species of Benzyl Viologen; $\rightarrow\text{H}^+/2\text{e}^-$, no. of protons translocated per pair of electrons flowing through the respiratory chain; $\Delta\text{H}^+/\text{H}_2$, g-ions of H⁺ released ÷ mol of H₂ oxidized; $\Delta\text{e}^-/\text{H}_2$, mol of BV⁺⁺ reduced to BV⁺ ÷ mol of H₂ oxidized; $\Delta\psi$, membrane potential.

mented with peptone (0.5%, w/v), sodium fumarate (0.5%, w/v), glycerol (0.4%, w/v), 1 mM-MgCl₂, L-isoleucine (20 µg/ml), L-valine (20 µg/ml), L-methionine (20 µg/ml), 1 µM-(NH₄)₆Mo₇O₂₄ and 1 µM-K₂SeO₄ (all expressed as final concentrations) adjusted to pH 6.4. The culture was incubated without stirring at 37°C for 12–16 h.

Cells were harvested as described previously (Jones & Garland, 1977) and starved by resuspension in 100 ml of the medium of Cohen & Rickenberg (1956) and vigorous shaking in air for 2 h. The starved cells were reharvested and washed as described previously (Jones & Garland, 1977). Spheroplasts were prepared as described by Garland *et al.* (1975).

Measurement of respiratory-driven proton translocation

The apparatus for measurement of respiratory-driven proton translocation has been described by Lawford & Garland (1972). Additions were made as indicated in the legends to Figs. 1 and 2. The solubility of H₂ in water at 25°C was calculated to be 0.78 mM from the value of α given by Umbreit *et al.* (1964).

Protein determination

Protein concentration was assayed by the method of Lowry *et al.* (1951), with bovine serum albumin (fraction V; BDH Chemicals Ltd, Poole, Dorset, U.K.) as standard.

Materials

2-n-Heptyl-4-hydroxyquinoline *N*-oxide, carbonyl cyanide *m*-chlorophenylhydrazone, menadione and valinomycin were purchased from Sigma (London) Chemical Co., Kingston upon Thames, Surrey KT2 7BH, U.K. Bacteriological peptone was purchased from Hopkin and Williams, Romford, Essex RM1 1HA, U.K. H₂ gas (99.99% pure) was purchased from BDH Chemicals, Poole, Dorset, U.K. Ubiquinone-1 was a generous gift from Hoffman-La Roche and Co., CH-4002 Basle, Switzerland, and also from Eisai Co., Koishikawa 4, Bunkyo, Tokyo, Japan. All other reagents were of the highest available purity.

Results

Hydrogen-dependent proton translocation

When a rapid burst of respiration was initiated by the addition of H₂-saturated water to spheroplasts of *E. coli* incubated anaerobically in medium containing either sodium fumarate (Fig. 1*a*) or KNO₃ (Fig. 1*d*), a transient acidification of the medium was observed. This phase of the pH change was complete in 4–8 s and was followed by a slower decay to the initial pH with a half-time of 20–40 s. That the

Fig. 1. Hydrogen-dependent proton translocation by spheroplasts from *Escherichia coli*

Spheroplasts made from *E. coli* de-repressed for hydrogenase synthesis were incubated anaerobically to a concentration of 2.6 mg of protein/ml in 1 ml of 300 mM-sucrose/150 mM-KCl/3 mM-MgCl₂/1.5 mM-glycylglycine, pH 7.0, at 25°C in the electrode chamber. Added electron acceptors were: 10 mM-sodium fumarate (*a*, *b* and *c*), 10 mM-KNO₃ (*d*) or no acceptor (*e*). Further additions were: valinomycin (5 µg/ml) (*a*, *b*, *c*, *d* and *e*), carbonyl cyanide *m*-chlorophenylhydrazone (15 µM) (*b*) or 2-n-heptyl-4-hydroxyquinoline *N*-oxide (5 µg/ml) (*c*). pH changes were recorded in response to the addition of H₂-saturated (H₂) or N₂-saturated (N₂) water, the volume (µl) of which is given in parentheses. Calibration was made by addition of 5 µl of N₂-saturated 5 mM-HCl/150 mM-KCl (vertical bar).

observed pH changes were dependent on H₂ was shown by the absence of any pH change when N₂-saturated water substituted for H₂-saturated water (Fig. 1*a*). The H₂-dependent pH change was not observed in the presence of the protonophore carbonyl cyanide *m*-chlorophenylhydrazone (Fig. 1*b*) or the respiratory inhibitor 2-n-heptyl-4-hydroxyquinoline *N*-oxide (Fig. 1*c*). Furthermore, acidification of the medium was not observed in the absence of an electron acceptor (Fig. 1*e*). The peak of acidification was corrected for the decay as described by Scholes & Mitchell (1970) and was

Table 1. $\rightarrow H^+/2e^-$ stoichiometries for the oxidation of hydrogen by spheroplasts from *Escherichia coli*. Experimental conditions were as described in the legends to Figs. 1 and 2. Corrected $\rightarrow H^+/2e^-$ stoichiometries were measured as described by Scholes & Mitchell (1970). The s.d. for each spheroplast preparation was always less than 10% of the mean. The mean value for each spheroplast preparation was taken and used to calculate the mean $\pm$ s.d. for several spheroplast preparations. The numbers of spheroplast preparations used, each from a separate overnight culture, are given in parentheses.

Acceptor	$\rightarrow H^+/2e^-$
Fumarate	1.85 ± 0.15 (7)
Nitrate	3.31 ± 0.29 (4)
Menadione	1.67 ± 0.33 (8)
Ubiquinone-1	1.81 (2)

found to be directly proportional to the amount of H_2 added over the range of initiating H_2 concentration of 1.56 – $11.7 \mu M$. These observations indicate that the pH changes were due to proton translocation coupled to the oxidation of H_2 .

Corrected $\rightarrow H^+/2e^-$ stoichiometries were 1.85 and 3.31 with fumarate and nitrate as respective terminal oxidants (Table 1). If $\rightarrow H^+/2e^-$ stoichiometries in *E. coli* can be equated with integral even numbers (Cox & Haddock, 1978; Garland & Jones, 1977), these values can be considered as 2 and 4 respectively.

Site of proton release by hydrogenase

Setting aside for the moment any consideration of proton translocation, hydrogenase catalyses the scalar reaction:

Since the enzyme is membrane-bound, these protons can be released at the periplasmic or cytoplasmic aspect of the membrane, an important consideration in understanding how proton translocation is effected by the oxidation of H_2 . The appropriate experiment to investigate this problem is to measure pH changes in the external medium when a small pulse of H_2 -saturated water is added to spheroplasts incubated anaerobically in the presence of an electron acceptor. Benzyl Viologen (BV^{++}) was chosen as electron acceptor for several reasons: the Benzyl Viologen radical species (BV^+) is intensely coloured, so that reduction to this species can be followed spectrophotometrically in parallel experiments (Jones & Garland, 1977, 1978); it accepts only electrons, no protons being involved in its reduction; it is reduced directly by hydrogenase at

the cytoplasmic aspect of the cytoplasmic membrane (Bernhard & Gottschalk, 1978b; Jones, 1979a). Although BV^{++} is unable readily to cross the cytoplasmic membrane, BV^+ is able to do so (Jones *et al.*, 1976; Jones & Garland, 1977). To load the spheroplasts with BV^{++} , the spheroplasts were incubated in buffer containing $1 \text{ mM-}BV^{++}$ to which was added sufficient $25 \text{ mM-}Na_2S_2O_4/10 \text{ mM-KOH}$ to produce an intense purple colour. After 15 min incubation, the medium was aerated by vigorous stirring until the colour had disappeared. Benzyl Viologen is thus trapped within the spheroplasts as intracellular BV^+ is oxidized to non-permeant BV^{++} . The medium was then made once again anaerobic by addition of $25 \text{ mM-}Na_2S_2O_4/10 \text{ mM-KOH}$ to give A_{600} approx. 0.2.

When a small amount (1.56 – $20 \mu M-H_2$) of H_2 -saturated water was added to spheroplasts so loaded with BV^{++} , an acidification of the medium was observed. This pH change was not followed by a decay back to the baseline and neither the rate nor the extent was affected by carbonyl cyanide *m*-chlorophenylhydrazone. The $\Delta H^+/H_2$ ratio was 1.95 ± 0.12 (mean $\pm$ s.d. for six determinations), very close to the value of 2 predicted by eqn. (1). Since the full rate and extent of pH changes in the cytoplasm can only be detected in the external bulk phase in the presence of a protonophore (Garland & Jones, 1977; Jones & Garland, 1978), it can be concluded that the stoichiometric release of protons of eqn. (1) occurs at the periplasmic aspect of the cytoplasmic membrane. Parallel spectrophotometric experiments showed that the rate of reduction of BV^{++} to BV^+ occurred at a similar rate to the pH changes within the limits set by the need to do parallel experiments (i.e. possible differences in mixing time, the different buffer used and different rates of leakage of air into the system causing autoxidation of BV^+). The $\Delta e^-/H_2$ stoichiometry was 2.11 ± 0.11 (mean $\pm$ s.d. for six determinations), very close to the value of 2 predicted from equation 1. Carbonyl cyanide *m*-chlorophenylhydrazone affected neither the rate nor the extent of BV^{++} reduction to BV^+ . These observations confirm that the pH changes observed were due to the stoichiometric release of the protons of eqn. (1).

Hydrogen-dependent proton translocation with other non-physiological electron acceptors

Menadione, an analogue of the endogenous menaquinone-8, and ubiquinone-1, an analogue of the endogenous ubiquinone-8, were tested for their ability to function as electron acceptors in H_2 -dependent proton translocation. When a small pulse of H_2 -saturated water was added to spheroplasts incubated anaerobically in medium containing menadione a transient acidification followed by a slower decay back to the initial pH value was

Fig. 2. *Hydrogen-plus-menadione-dependent proton translocation by spheroplasts from Escherichia coli*. Experimental conditions were as in Fig. 1. Menadione (1 mM) and valinomycin (5 μ g/ml) were present in traces (a) and (b); 2-n-heptyl-4-hydroxyquinoline *N*-oxide (5 μ g/ml) was present in trace (b). Additions and calibration were as in Fig. 1.

observed (Fig. 2a), characteristic of respiratory-driven proton translocation. This proton translocation showed one remarkable difference from that observed with either fumarate or nitrate (Fig. 1): although abolished by carbonyl cyanide *m*-chlorophenylhydrazone, it was unaffected by 2-n-heptyl-4-hydroxyquinoline *N*-oxide at concentrations that completely inhibited H_2 -dependent proton translocation with fumarate as electron acceptor (Fig. 2b). The $\rightarrow H^+/2e^-$ stoichiometry was 1.67 (Table 1). H_2 -dependent reduction of ubiquinone-1 was also coupled to carbonyl cyanide *m*-chlorophenylhydrazone-sensitive 2-n-heptyl-4-hydroxyquinoline *N*-oxide-insensitive proton translocation of $\rightarrow H^+/2e^-$ stoichiometry 1.81 (Table 1). The inability of 2-n-heptyl-4-hydroxyquinoline *N*-oxide at the concentration used (2 μ g/mg of protein) to abolish H_2 -dependent proton translocation with menadione or ubiquinone-1 as electron acceptor shows that its effect when fumarate is acceptor is due to inhibition of electron transfer and not to a secondary uncoupling effect (Wikström, 1978).

Discussion

Although much controversy exists over $\rightarrow H^+/2e^-$ stoichiometries in mitochondria (Brand, 1977), there is strong evidence that in *E. coli* the $\rightarrow H^+/2e^-$ stoichiometry is 4 for oxidation of formate and NADH and 2 for oxidation of succinate or ubiquinol, with either nitrate or O_2 as terminal electron

acceptor (Garland *et al.*, 1975; Garland & Jones, 1977; Cox & Haddock, 1978). $\rightarrow H^+/2e^-$ stoichiometries for H_2 -dependent reduction of fumarate and nitrate approximate to 2 and 4 respectively, reflecting the difference in midpoint potentials of the fumarate-succinate ($E_0 = +30$ mV) and nitrate-nitrite ($E_0 = +420$ mV) couples (Kroger, 1977).

Previous measurements of $\rightarrow H^+/2e^-$ stoichiometries for fumarate reduction by *E. coli* with endogenous substrates gave values of much less than 2 (Brice *et al.*, 1974; Gutowski & Rosenberg, 1977). These values have been considered an underestimate due to the fact that fumarate transport is rate-limiting and the peak of acidification had been reached and begun to decay before all the added fumarate had been reduced (Gutowski & Rosenberg, 1977). Using inside-out membrane vesicles from *E. coli*, in which transport of fumarate is not necessary for its reduction, Miki & Wilson (1978) measured an $\rightarrow H^+/2e^-$ stoichiometry of almost 2 for the fumarate-dependent oxidation of L- α -glycerophosphate. In the experiments shown in Fig. 1, the spheroplasts were incubated in 10 mM-sodium fumarate and probably therefore contained sufficient fumarate for its transport not to be rate-limiting in subsequent bursts of H_2 oxidation.

Role of hydrogenase in proton translocation

In order to reduce BV^{++} at the cytoplasmic aspect of the cytoplasmic membrane and to liberate protons at the periplasmic aspect, hydrogenase must have access to the bulk phase on either side of the membrane (Scheme 1a). The evidence that hydrogenase reduces BV^{++} directly is as follows: purified hydrogenase from *E. coli* reduces BV^{++} (Bernhard & Gottschalk, 1978b; Adams & Hall, 1979); H_2 -dependent BV^{++} reduction is independent of functional cytochromes (Jones, 1979a); the BV^{++}/BV^+ couple has a midpoint potential of -350 mV (Homer *et al.*, 1960), so that its reductant must be of low potential. The midpoint potential of the $H_2/2e^-$ couple is -420 mV (Kroger, 1977), whereas those of menaquinone, ubiquinone and fumarate reductase are too high to be expected to be able to reduce BV^{++} . Scheme 1(b) also predicts that H_2 -dependent reduction of BV^{++} is electrogenic. However, BV^+ is a permeant cation (Jones *et al.*, 1976; Jones & Garland, 1977) and is therefore able to collapse any $\Delta\psi$ that would be generated. It is inevitable in the experiments described in the present paper that there is always some BV^+ present in the electrode chamber because anaerobic conditions must be maintained.

Scheme 1(b) shows the proposed mechanism for H_2 -dependent proton translocation with menadione as electron acceptor. The exact site of menadione reduction by H_2 is unknown, but the observations that the reaction is 2-n-heptyl-4-hydroxyquinoline

Scheme 1. Proposed mechanism for the pH changes associated with hydrogen-dependent reduction of (a) Benzyl Viologen, (b) menadione, (c) fumarate and (d) nitrate by spheroplasts from *Escherichia coli*

See the text for details. Abbreviations: MD, menadione; MDH_2 , menadiol; MK, menaquinone; UQ, ubiquinone; UQH_2 , ubiquinol; *cyt. b*, cytochrome *b*; FR, fumarate reductase.

N-oxide-insensitive and that purified hydrogenase will reduce menadione (Bernhard & Gottschalk, 1978b) suggest that menadione is reduced directly by hydrogenase. Brookman *et al.* (1979) have reported that addition of menadione as a pulsed oxidant to cells of *E. coli* incubated in medium containing mannitol, an NADH-linked substrate, gave rise to proton translocation. However, since this effect was not observed in a mutant lacking adenosine triphosphatase, these authors concluded that proton translocation was driven, not by NADH dehydrogenase, but by hydrolysis of ATP synthesized at the substrate level. However, in the experiments of Fig. 2, H_2 was added as a pulsed reductant, so it is more likely that the associated proton translocation is due to H_2 -dependent reduction of menadione rather than to ATP hydrolysis. Menadione is considered to be an analogue of menaquinone and will reconstitute respiratory-driven active transport in menaquinone-deficient mutants of *Bacillus subtilis* (Bisschop *et al.*, 1975), but it is difficult to be certain just how closely menadione mimics the endogenous menaquinone in *E. coli*. Other menaquinone analogues, menaquinone-1 and menaquinone-5, are also reduced by purified hydrogenase (Bernhard & Gottschalk, 1978b), so it might be argued that endogenous menaquinone is reduced directly by hydrogenase. The observation that ubiquinone-1 reduction by H_2 is also 2-n-heptyl-4-hydroxyquinoline *N*-oxide-insensitive suggests

that this acceptor is also reduced directly by hydrogenase.

Scheme 1(c) shows the proposed pathway for H_2 -dependent fumarate reduction. It differs from the linear pathway proposed by Bernhard & Gottschalk (1978b) in that their scheme shows a *b*-type cytochrome and a 2-n-heptyl-4-hydroxyquinoline *N*-oxide-sensitive site between hydrogenase and menaquinone. The evidence given in the present paper suggests that the 2-n-heptyl-4-hydroxyquinoline *N*-oxide-sensitive site is on the fumarate side of menaquinone. The site of fumarate reduction is known to be at the inner face of the cytoplasmic membrane (Jones & Garland, 1977). In this scheme the function of fumarate reductase is to reoxidize menaquinol. A *b*-type cytochrome is included in the scheme because it has been claimed that, although functional cytochromes are not necessary for reduction of fumarate (Singh & Bragg, 1975), they are required for fumarate-dependent active transport of amino acids (Singh & Bragg, 1976). Furthermore, fumarate reductase appears to co-purify with a *b*-type cytochrome (Dickie & Weiner, 1979).

Scheme 1(d) shows a model for the translocation of protons coupled to H_2 -dependent nitrate reduction. In addition to the two protons translocated by H_2 -dependent reduction of ubiquinone, two more are translocated by the ubiquinol–nitrate pathway (Garland & Jones, 1977).

H_2 -dependent proton translocation (Scheme 1b–

d) is effected by a direct chemiosmotic mechanism (Mitchell, 1972). There is no evidence for a conformational proton pump of the type proposed for mitochondrial respiratory systems (e.g. Wikström, 1978). In fact, in the schemes proposed in the present paper protons are not actually translocated. The translocated species is the electron. Protolytic reactions connected by an electron-translocating limb of the respiratory chain give rise to what is observed as an effective proton translocation (Scheme 1*b*). A similar mechanism of proton translocation has been demonstrated for the ubiquinol-nitrate respiratory pathway of *E. coli* (Garland & Jones, 1977; Jones & Garland, 1978; Jones *et al.*, 1978) and the formate-fumarate pathway of *Vibrio succinogenes* (Kroger, 1977, 1978).

One outstanding feature of Scheme 1(*b*) is that it supposes that one enzyme, hydrogenase, is able to effect proton translocation. Hydrogenase may be of interest in considerations of the evolution of proton pumps (Raven & Smith, 1976), not only because it is possibly the simplest respiratory proton-translocating system, but also because, at the beginning of life, the atmosphere of this planet contained a larger proportion of H₂ than it does today.

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