Respiration-Driven Proton Translocation by Yeast Mitochondria with Differing Efficiencies of Oxidative Phosphorylation

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Measurements were made of the stoichiometry of proton translocation coupled to respiration in mitochondria from *Candida utilis* where the number of functional energy-conservation sites between intramitochondrial NADH and oxygen was one in a mutant with a novel oxidase (Downie & Garland, 1972), two in sulphate-deficient cells (Haddock & Garland, 1971) or three in glycerol-limited cells (Light & Garland, 1971). The stoichiometries of protons translocated per atom of oxygen utilized (i.e. $\rightarrow H^+/2e^-$ ratio; Mitchell, 1966) were close to 2.0, 4.0 and 6.0 respectively. Thus by using the same substrate (intramitochondrial NADH) and oxygen throughout, the $\rightarrow H^+/2e^-$ ratio is shown to be 2.0 per energy-conservation site when the number of such sites is varied from one to three.

The chemiosmotic hypothesis of Mitchell (1966) proposes, *inter alia*, that each span of mitochondrial respiratory carriers and enzymes covering a so-called energy-conservation site (Lehninger, 1966) is so arranged that $2H^+$ are translocated across the mitochondrial inner membrane for each pair of reducing equivalents transferred across that span. Evidence in favour of this value of 2.0 for the ratio of protons translocated to reducing-equivalent pairs transferred (i.e. $\rightarrow H^+/2e^-$ ratio) has come mainly from one type of experiment. In this, the length of the respiratory chain under study has been altered either by changing the oxidant [e.g. oxygen or ferricyanide (Mitchell & Moyle, 1967)] or ubiquinone analogues (Lawford & Garland, 1972).

In the present paper we describe an independent method for measurement of the $\rightarrow H^+/2e^-$ ratio per energy-conservation site. The same substrate (intramitochondrial NADH) and oxidant (oxygen) is used throughout, but the number of energy-conservation sites is varied from one to three by using mitochondria from variants of *Candida utilis* with modified respiratory chains. We conclude that the $\rightarrow H^+/2e^-$ ratio is 2.0 per energy-conservation site.

Materials and Methods

Growth of yeasts

C. utilis (strain 193 from the National Collection of Yeast cultures, Nutfield, Surrey, U.K.) was grown in continuous culture under conditions of glycerol limitation (Light & Garland, 1971) or sulphate limitation (Haddock & Garland, 1971). A variant of *C. utilis* that does not require copper and has a cyanide-insensitive terminal oxidase (Downie & Garland, 1972, 1973) was grown in continuous cul-

ture in a copper-extracted medium (Light, 1972). Under these conditions the mitochondrial concentration of spectroscopically detectable cytochrome $a+a_3$ falls to less than 1% of the normal, copper-supplemented value (Downie & Garland, 1973). *Saccharomyces carlsbergensis* (N.C.Y.C. 745) was grown in the defined medium of Oltmanns *et al.* (1969) containing 1% (w/v) glucose and harvested at the late exponential phase of growth after 12–16 h.

Preparation of mitochondria

Harvested and washed cells (Light & Garland, 1971) were converted into protoplasts and mitochondria isolated as described by Kovac *et al.* (1972).

Analytical methods

Protein was determined by the method of Lowry *et al.* (1951) with bovine albumin as standard. Measurements of respiration-driven proton translocation were made with the apparatus described by Lawford & Garland (1972) in 1.0 ml of anaerobic 0.6 M-mannitol, 0.15 M-KCl, 3.0 mM-2-(*N*-morpholino)ethanesulphonic acid, pH 5.5, containing 9–10 mg of mitochondrial protein and the substrates and other reagents indicated in the Results section. Polarographic measurements of P/O ratios were performed as described by Chance & Williams (1956) by using the experimental conditions of Light & Garland (1971).

Reagents

Antimycin A was purchased from Sigma (London) Chemical Co., Ltd., Kingston-upon-Thames, Surrey, U.K. Carbonyl cyanide *m*-chlorophenylhydrazine

and valinomycin were from Calbiochem Ltd., London W.1, U.K., piericidin A was a kind gift from Professor K. Folkers, and ubiquinone₀, ubiquinone₁ and ubiquinone₂ were gifts from Hoffman-La Roche, Basle, Switzerland, or Merck, Sharpe and Dohme Research Laboratory, Rahway, N.J., U.S.A. The disodium salt of NADH was purchased from Boehringer Corp. (London) Ltd., London W.5, U.K. Standard solutions of HCl for calibration of pH-electrode responses were obtained from British Drug Houses Ltd., Poole, Dorset, U.K., and diluted to 50 mM or 5 mM before use. Bovine albumin (fraction V) was from British Drug Houses Ltd.

Results

Conditions required for the observation of respiration-driven proton translocation

Kovac *et al.* (1972) observed that the proton permeability of *Saccharomyces cerevisiae* mitochondria was markedly pH-dependent, increasing with pH. In preliminary experiments we found that *C. utilis* mitochondria exhibited a continuous output of protons when suspended in anaerobic medium (0.6 M-mannitol, 0.15 M-KCl, 3.0 mM-2-(*N*-morpholino)ethanesulphonic acid) at pH 6.0, 6.5 and 7.0, but exhibited a continuous uptake of protons at pH 5.0 and 4.5. This drift in the pH of the medium complicated measurements of proton translocation in response to pulses of air-saturated 0.15 M-KCl. The drift is minimal at pH 5.5, and all subsequent experiments with *C. utilis* mitochondria were made in medium at this pH, with readjustment with 50 mM-KOH or 50 mM-HCl in 0.15 M-KCl at the start of any incubation. Suspensions of mitochondria from *S. carlsbergensis* also showed a similar pH-dependent pH drift, and measurements of respiratory-driven proton translocation were also made at pH 5.5. The cause of these drifts is not known, but they may be due to contamination of the mitochondrial preparation by intact protoplasts.

Proton translocation in response to oxygen pulses in *S. carlsbergensis* mitochondria

Fig. 1 shows pH recordings of an anaerobic suspension of *S. carlsbergensis* mitochondria supplemented with the potassium salts of pyruvate (3.0 mM) and L-malate (3.0 mM). The upper trace (a) shows the response to small additions of air-saturated 0.15 M-KCl in the absence of valinomycin, and the lower trace (b) shows the response in the presence of valinomycin (1 μ g/ml). The $\rightarrow$ H⁺/O ratio was increased by valinomycin from a mean of 2.7 to a mean of 3.8. The rate of acidification after a pulse of air-saturated KCl was perceptibly faster in the presence of valinomycin, although a more quantita-

tive analysis is precluded by the response time of the mixing and measuring system, which is about 3 s for completion (Lawford & Garland, 1972). The subsequent decay in the pH value back to its previous value proceeded with a half-life of 35 s in the absence of valinomycin and 10 s in its presence. Measurements of the respiratory-driven pH change were taken from the peak value, and correction was not made for the simultaneous onset of decay. The error in the observed peak value was shown by analogue computer simulation (H. Lawford & J. Kernohan, personal communication) to cause an approx. 10% underestimate of the true value for this set of conditions. Acidification resulting from the addition of air-saturated KCl was abolished by antimycin A (0.7 μ g/ml) or carbonyl cyanide *m*-chlorophenylhydrazine

Fig. 1. Recordings of the pH of suspensions of *S. carlsbergensis* mitochondria

The apparatus was as described by Lawford & Garland (1972). Mitochondria (9.2 mg of protein) were added to 1.0 ml of N₂-saturated 150 mM-KCl, 0.6 M-mannitol, 3.0 mM-2-(*N*-morpholino)ethanesulphonic acid, pH 5.5, at 30°C, containing 5 mM-pyruvate and 5 mM-L-malate as their potassium salts. Valinomycin (1 μ g/ml) was present in the experiment of the lower trace (b), and absent in the upper trace (a). Small additions of 0.15 M-KCl (air-saturated at 30°C) were made, after 10 min preincubation, by injection with a microsyringe. The times of these injections are indicated by arrows against which the amount of oxygen (ng-atoms) added is shown. Acidification of the bulk phase sensed by the electrode is shown as an upward deflection. Reading from left to right the peaks of acidification correspond to $\rightarrow$ H⁺/O ratios of 2.6, 2.9 and 2.4 in (a) and 3.8, 3.8, 4.2 and 3.8 in (b).

Table 1. $\rightarrow H^+/O$ ratios for *C. utilis* mitochondria

Measurements of the pH of suspensions of mitochondria (9–10 mg/ml) were made as for Fig. 1, with valinomycin (1 μ g/ml) and substrates and inhibitors as indicated below. The pulses of air-saturated KCl covered a range of oxygen additions of 4.7–18.8 ng-atoms. The values shown are the means $\pm$ s.d. with the number of observations in parentheses. n.t., not tested.

Substrates and inhibitors	$\rightarrow H^+/O$ ratios for mitochondria from <i>C. utilis</i> cells that were:		
	Normal glycerol-limited	Normal sulphate-limited	Variant copper-deficient
Pyruvate (3 mM), L-malate (3 mM)	5.8 $\pm$ 0.3 (17)	3.1 $\pm$ 0.5 (23)	2.0 $\pm$ 0.2 (41)
Pyruvate (3 mM), L-malate (3 mM), piericidin A (1.3 μ M)	3.7 $\pm$ 0.1 (5)	2.5 $\pm$ 0.2 (16)	<0.3
Pyruvate (3 mM), L-malate (3 mM), antimycin A (0.7 μ g/ml)	<0.3	<0.3	2.0 $\pm$ 0.15 (28)
DL-glycerol 3-phosphate (3 mM), piericidin A (1.3 μ M)	3.6 $\pm$ 0.3 (13)	2.5 $\pm$ 0.5 (25)	<0.3
DL-glycerol 3-phosphate (3 mM), antimycin A (0.7 μ g/ml)	<0.3	<0.3	<0.3
NADH (0.5 mM), piericidin A (1.3 μ M)	2.6 $\pm$ 0.2 (15)	n.t.	n.t.
NADH (0.5 mM), piericidin A (1.3 μ M), antimycin A (0.7 μ g/ml)	<0.3	n.t.	n.t.

(1 μ M), and unchanged by substituting potassium succinate (3.0 mM) for pyruvate and L-malate. In the presence of antimycin A (0.7 μ g/ml), *NNN'*-tetramethylene-*p*-phenylenediamine (50 μ M) and L-ascorbate (5.0 mM), the $\rightarrow H^+/O$ values in response to addition of 10, 20 and 30 μ l of air-saturated KCl under conditions otherwise similar to those of the lower recording of Fig. 1 were 1.85, 1.85 and 2.04 respectively.

These observations are consistent with the number and location of energy-conservation sites in mitochondria from *S. carlsbergensis* harvested in the late exponential growth phase where site 1 is missing (Ohnishi, 1970; Mackler & Haynes, 1973). The absence of site 1 in our preparations was confirmed polarographically.

Proton translocation in response to oxygen pulses in C. utilis mitochondria

Table 1 summarizes the results of measurements of $\rightarrow H^+/O$ ratios made in experiments similar to those of Fig. 1 but with *C. utilis* mitochondria in the presence of valinomycin. The half-time for the decay of acidification in response to pulses of air-saturated 0.15 M-KCl was about 10 s for mitochondria from glycerol-limited normal cells, 4 s from sulphate-limited normal cells, and 7 s from copper-deficient variant cells. The $\rightarrow H^+/O$ ratios observed for the mitochondria from sulphate-limited cells are likely

to be underestimated by 30%. It can be concluded from the results in Table 1 that: (1) the $\rightarrow H^+/O$ ratio for intramitochondrial NADH_i oxidation is approx. 6.0 for mitochondria from glycerol-limited normal cells (which have all three energy-conservation sites), is between 3.0 and 4.0 for mitochondria from sulphate-limited normal cells (which have two energy-conservation sites) and is 2.0 for mitochondria from copper-deficient variant cells (which have only one energy-conservation site). In this last situation proton translocation, like respiration (Downie & Garland, 1973), is insensitive to antimycin A. (2) The $\rightarrow H^+/O$ ratio for glycerol 3-phosphate oxidation approximates to 4.0 in mitochondria from glycerol-limited cells, is rather lower in mitochondria from normal sulphate-limited cells and approaches zero with mitochondria from copper-deficient variant cells.

The $\rightarrow H^+/O$ ratio for the oxidation of added NADH by mitochondria from glycerol-limited normal cells is 1.0 unit lower than that for glycerol 3-phosphate oxidation. If it is assumed that the site of interaction between added NADH (with one H⁺) and the respiratory chain is on the outer surface of the inner mitochondrial membrane (von Jagow & Klingenberg, 1970), then irrespective of whether NADH interacted at that surface with a hydrogen or an electron carrier, the anticipated $\rightarrow H^+/O$ ratio would be 1 unit less than that predicted for the transfer of two reducing equivalents across two energy-conservation sites. This appears to be the case.

Fig. 2. Recordings of the pH of suspensions of *C. utilis* mitochondria in response to additions of ubiquinone₂

The experimental conditions are as for Fig. 1(b), with the further addition of antimycin A (0.7 μ g) and KCN (1 μ mol), and the use of 11.5 mg of mitochondrial protein. In the lower trace (b) 1 μ M-carbonyl cyanide *m*-chlorophenylhydrazone was also present. Ubiquinone₂ (UQ₂) in methanol was added by injection with a microsyringe, and the times of addition are indicated by arrows. The numbers in parentheses are the amounts of ubiquinone₂ (nmol) added on each occasion. The peaks of acidification in the upper trace (a) reading from left to right correspond to $\rightarrow$ H⁺/2e⁻ ratios of 1.2, 1.1, 1.1 and 1.0. The addition of methanol alone was without effect on the pH.

Proton translocation associated with the oxidation of intramitochondrial NADH by added ubiquinone homologues and duroquinone

When ubiquinone₂ replaced oxygen as the oxidant for intramitochondrial NADH in antimycin A-treated anaerobic mitochondria from glycerol-limited normal cells, proton translocation with an $\rightarrow$ H⁺/2e⁻ ratio of 1.1 was observed, and this was replaced by alkalization if carbonyl cyanide *m*-chlorophenylhydrazone was added (Fig. 2). Proton translocation associated with ubiquinone₂ reduction by intramitochondrial NADH was abolished by 2 μ M-piericidin A. Similar experiments performed with ubiquinone₀ gave $\rightarrow$ H⁺/2e⁻ ratios of 0.2, and with ubiquinone₁ 0.2. Nor did duroquinone (25–50 μ M) support proton translocation in these experiments. It would appear from these results that mitochondria from glycerol-limited *C. utilis* possess a piericidin-sensitive proton-translocating NADH-ubiquinone₂ reductase, as do ox heart and rat liver mitochondria

(Lawford & Garland, 1972). A more-detailed investigation was not made; the high rates of piericidin A-insensitive, non-proton-translocating routes of NADH oxidation by ubiquinone₀, ubiquinone₁ and duroquinone complicated study of the proton-translocating pathway to an unacceptable extent.

Discussion

Kovac *et al.* (1972) reported that the $\rightarrow$ H⁺/O ratio for *S. cerevisiae* mitochondria approximated to 4.0 irrespective of whether the substrate was intramitochondrial NADH (generated from pyruvate with L-malate, or from isocitrate oxidation), or succinate. This observation was in keeping with the absence of site 1 energy conservation in mitochondria from *S. cerevisiae* harvested in the late exponential growth phase (Mackler & Haynes, 1973). Our own observations extend this finding to *S. carlsbergensis* mitochondria from cells also harvested in the late exponen-

tial growth phase. Aeration of such cells under conditions of starvation results in the appearance of site 1 (Ohnishi, 1970) and, although values for $\rightarrow\text{H}^+/\text{O}$ have not been reported, they would presumably remain at 4.0 with substrates such as glycerol 3-phosphate or succinate and rise to 6.0 with intramitochondrial NADH.

Our finding that the $\rightarrow\text{H}^+/\text{O}$ ratios approximate to 2.0, 4.0 and 6.0 for intramitochondrial NADH oxidation by *C. utilis* mitochondria possessing respectively one, two or three energy-conservation sites provides further evidence in support of the proposal (Mitchell, 1966) that the $\rightarrow\text{H}^+/2\text{e}^-$ ratio is 2.0 for each energy-conservation site. We were unable to observe proton translocation coupled to the reduction of added ubiquinone₁ or ubiquinone₀ by intramitochondrial NADH, and it seems likely that the non-proton-translocating route of NADH reduction of added ubiquinone predominates over the proton-translocating route (Lawford & Garland, 1972). This disadvantage of *C. utilis* mitochondria is more than overcome by the availability of a variant with a highly active proton-translocating NADH oxidase utilizing only the first energy-conservation site. Studies of the kinetics of proton translocation and oxidoreduction by this segment of the respiratory chain should be facilitated by this particular variant.

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