Mechanistic Stoichiometry of Yeast Mitochondrial Oxidative Phosphorylation†

V. Fitton, M. Rigoulet,* R. Ouhabi, and B. Guérin

Institut de Biochimie Cellulaire du CNRS, Université de Bordeaux II, 1 rue Camille Saint-Saëns, 33077 Bordeaux Cedex, France

Received January 4, 1994; Revised Manuscript Received April 19, 1994®

ABSTRACT: This study investigates the relationships between the efficiency of oxidative phosphorylation (ATP/O) and respiratory flux in yeast mitochondria. To manipulate the electron flux through the respiratory chain, different substrates leading to NAD(P)H were used. By testing the effect of ADP either on respiratory rate in the presence or absence of oligomycin or on the level of NAD(P)H, on one hand, and the effects of uncouplers on respiration, on the other, we distinguished several categories of substrates: those for which the low respiration rate was mainly controlled by dehydrogenase activities and others for which the respiration was high and controlled downstream from the dehydrogenases. By using these different substrates, we observed that the ATP/O ratio decreased irrespective of the proton-motive force when the electron flux increased, unlike the situation when the respiratory rate was modulated by addition of the respiratory inhibitor. This result suggests that the oxidative phosphorylation efficiency depends on the value of the flux crossing the proton pumps. This relationship between efficiency (ATP/O) and electron flux was linked to a main control upstream from the respiratory chain. Such changes in the ATP/O ratio at least involved changes in the stoichiometry ($H^+/2e^-$) of the respiratory chain. Indeed, in non-phosphorylating mitochondria, the ratio of stoichiometries at site 2+3 over site 3 varied according to the proton-motive force. This cannot be explained by a variation in proton leak alone but involved both (i) a variable stoichiometry ($H^+/2e^-$) in relation to the electron flux value and (ii) different relationships between the variation in stoichiometry and the flux value at each coupling site.

In mammalian mitochondria, electron transport through three distinct segments of the respiratory chain has long been known to provide the energy for ATP synthesis. Many studies to determine the stoichiometry of mitochondrial oxidative phosphorylation, using a wide range of techniques and avoiding several common systematic errors, have led to an increasing consensus in recent years. In isolated mammalian mitochondria, the estimated values of mechanistic $ATP/2e^-$ are close to 1, 0.5, and 1 at the three coupling sites, respectively (Pozzan et al., 1979; Hinkle, 1981; Stoner, 1987; Hinkle et al., 1991), even if slightly higher values have been reported (Lemaster, 1984; Beavis & Lenhinger, 1986).

Mitochondria isolated from *Saccharomyces cerevisiae* in the exponential growth phase have three main characteristics: (i) the lack of a phosphorylation site corresponding to coupling site 1 of animal mitochondria (Ohnishi et al., 1966; Matoon & Sherman, 1966; Kovac et al., 1968), (ii) the ability to oxidize exogenous NADH by a NADH dehydrogenase located toward the outer surface of the inner membrane (Von Jagow & Klingenberg, 1970), and (iii) the ability to oxidize lactate by directly using the third site span (Ohnishi et al., 1966). In previous studies, we have shown that the ATP/O ratio is about 1.5 with ethanol or succinate and 1 with substrates oxidized at site 3 (tetramethyl-*p*-phenylenediamine + ascorbate or lactate) when classical methods are used (Ouhabi et al., 1989). Moreover, with substrates donating their electrons to site 2 on the external side of the membrane, such as NADH or glycerol phosphate, the ATP/O ratio is about 1.25. Thus, ATP/O values seem to depend on the location of the dehydrogenase on either the outer or the inner face of the internal membrane (Ouhabi et al., 1989). Similar results have recently been reported in potato tuber mitochondria (Groen et al., 1992).

The wide agreement in the $ATP/2e^-$ values determined after correction for proton leakage seems to indicate that these ratios are the maximum mechanistic stoichiometries and that under these experimental conditions the H^+/O and H^+/ATP stoichiometries are both constant. However, such determinations have been made under steady-state conditions where the electron flux through each respiratory unit is maximum (state 3), even if the number of functional units is changed by using inhibitor titration.

In a previous study using an external NADH-regenerating system and the ability of yeast mitochondria to oxidize exogenous NADH, we showed that the value of ATP/O significantly increases when respiratory flux slows down in response to a limitation of substrate supply (Ouhabi et al., 1989). However, under these particular conditions, the ATP/O ratio measured at maximal respiratory rate was rather low (approximately 1).

The present study reevaluates the mechanistic stoichiometry of yeast mitochondrial oxidative phosphorylation when the electron supply to the respiratory chain is changed according to the different dehydrogenases involved, each working at a determined rate in a given steady state. The main observation is that the ATP/O ratio decreases irrespective of proton-motive force when the electron flux through each respiratory chain unit increases. Moreover, we show that the highest ATP/O values are linked to a high kinetic control of the oxidative activity upstream from the respiratory chain, at substrate supply or dehydrogenase activity levels. Therefore the values previously obtained in state 3 with either ethanol or succinate (Ouhabi et al., 1989) as substrates seem to be minimum. The change in the ATP/O ratio is a consequence of a change in the stoichiometry of at least one proton pump

† This work was supported by grants from Conseil Régional d'Aquitaine.

* To whom correspondence should be addressed. Telephone: 33-56-99-90-35. Fax: (33) 56-99-90-59.

® Abstract published in *Advance ACS Abstracts,* July 15, 1994.

0006-2960/94/0433-9692$04.50/0

Table 1: Respiratory Rates with Different Substrates and Their Stimulation[a]

substrate	JO state 4	JO_{+ADP} / JO_{state4}[b]	$JO_{+ADP,+oligo} / JO_{state4}$	$JO_{+KCl+val} / JO_{state4}$	ΔpH (mV) state 4	ΔpH (mV) +val, +KCl
β-hydroxybutyrate	6 ± 1	3.50 ± 0.50	1.68 ± 0.20	1.50 ± 0.20	13 ± 3	21 ± 4
pyruvate	11 ± 3	1.40 ± 0.30	1.32 ± 0.14	1.47 ± 0.17	53 ± 12	54 ± 2
malate	19 ± 4	1.47 ± 0.08	1.31 ± 0.16	1.35 ± 0.12	24 ± 5	42 ± 8
pyruvate + malate	60 ± 19	1.17 ± 0.10	0.90 ± 0.05	1.53 ± 0.04	36 ± 2	50 ± 1
2-oxoglutarate	62 ± 16	2.50 ± 0.12	2.00 ± 0.30	3.85 ± 1.10	41 ± 3	54 ± 3
isocitrate	78 ± 27	1.32 ± 0.08	0.88 ± 0.05	1.23 ± 0.09	37 ± 2	38 ± 6
ethanol	160 ± 34	2.70 ± 0.20	0.83 ± 0.10	3.00 ± 0.37	33 ± 2	45 ± 5

[a] Mitochondria (3 mg/mL, except 1 mg/mL for JO measurements with pyruvate + malate, isocitrate, 2-oxoglutarate, and ethanol) were incubated in basal medium as described in Materials and Methods, with 5 mM of each substrate except for ethanol (109 mM). ADP was used at 1 mM; oligomycin, 50 μg/(mg of protein). Valinomycin (when indicated) was added to 0.417 μg/(mg of protein). KCl concentration was 10 mM. JO_{state4} is the respiratory rate after substrate addition. [b] JO_{+ADP}, $JO_{+ADP,+oligo}$, and $JO_{+KCl,+val}$ correspond to different steady states after indicated additions, i.e., ADP, ADP + oligomycin, and KCl + valinomycin, respectively. JO values obtained with ADP + oligomycin were the same whether ADP was added before or after oligomycin. JO was expressed as natom of O per minute per milligram of protein. ΔpH was determined by distribution of [^{3}H]acetate after matrix volume determination. The data represent the mean ± SD of eight separate experiments performed with three different mitochondria preparations.

involved. The variation in the stoichiometry of the respiratory chain is confirmed by comparing the different relationships obtained by titration of the respiratory rate and $\Delta\tilde{\mu}_{H^+}$[1] at sites 2+3 and 3.

MATERIALS AND METHODS

Preparation of Mitochondria. Cells of diploid wild strain *Saccharomyces cerevisiae* (yeast foam) were grown aerobically at 28 °C in a complete medium [1% yeast extract (Difico), 0.1% KH_2PO_4, and 0.12% $MgSO_4$, pH 4.5] with 2% lactate as carbon source. The cells were harvested in logarithmic growth phase, and mitochondria were isolated from protoplasts as described by Guérin et al. (1979). Protein concentration was measured by the biuret method using bovine serum albumin as standard.

Determination of ATP/O. Mitochondrial oxygen consumption was measured at 27 °C in a 1.5-mL thermostatically controlled chamber equipped with a Clark oxygen electrode (Gilson) connected to a microcomputer giving an on-line display of rate values. Basal medium was as follows: 0.65 M mannitol, 0.36 mM EGTA, 3 mM Tris–P_i, 10 mM Tris–maleate (pH 6.7), 5 μM RbCl, and 0.1 μg/mL valinomycin. Substrate supply was either a saturating concentration of different substrates or the following lactate-regenerating system: basal medium supplemented with 5 mM pyruvate, 1 μg/(mg of protein) antimycin, 2 μmol/(mg of protein) α-cyanohydroxycinnamate in order to inhibit the pyruvate carrier (Mowbray, 1975), 1 mM NADH, and different amounts of D-lactate dehydrogenase from *Lactobacillus leichmannii* (EC 1.1.1.28). ATP was measured by two different methods: (i) $^{32}P_i$ incorporation in adenine nucleotides as previously described by Rigoulet and Guérin (1979) and (ii) glucose 6-phosphate formation in the presence of non-limiting amounts of hexokinase, 1 mM $MgCl_2$, and 10 mM glucose. Each of the techniques used to measure ATP could lead to misestimations of phosphorylation rate, i.e., either an underestimation due to ATPase contaminating activity ($^{32}P_i$ incorporation method) or an overestimation mainly due to adenylate kinase activity (glucose 6-phosphate method). However, whatever the method used for ATP estimation, the experimental points were distributed on the same straight line when phosphorylation and respiratory rates were titrated by antimycin, indicating that neither contaminating ATPase activity nor adenylate kinase activity significantly changed ATP synthesis rate estimation [not shown; but see Ouhabi et al. (1989)]. The O_2 concentration in the medium was determined with NADH quantitated spectrophotometrically and yeast mitochondria. The O_2 concentration of the standard incubation medium at 27 °C was 454 natom O/mL.[1]

The ATP/O ratio stoichiometries were determined from the average of oligomycin-sensitive phosphorylation rates vs respiratory rates.

Measurement of ΔpH and ΔΨ. Matrix space was determined by using [^{3}H]water and inner membrane impermeable [^{14}C]sucrose; ΔΨ and ΔpH, by distribution of ^{86}Rb and [^{3}H]-acetate, respectively (Rottenberg, 1979).

Mitochondrial NAD(P)H Fluorescence Measurement. Mitochondria (0.25 mg/mL) were suspended in the standard medium at 27 °C. NADH + NADPH fluorescence was monitored with a Kontron fluorimeter. The excitation wavelength was 340 nm, and the fluorescence emission was continuously collected at 450 nm.

First, mitochondria were incubated without any added substrate. This steady state gave the basic signal, *F*. Endogenous NADH, NADPH, and flavins can all contribute to this fluorescence signal, but it has been shown that the change in fluorescence due to substrate addition is essentially due to a change in NADH level (Estabrook, 1962). Thus addition of various substrates led to different steady states corresponding to state 4 of respiration. After state 4, a new steady state was reached by adding ADP (1 mM): this was state 3. Another case was the addition of oligomycin [50 μg/(mg of protein)] before the substrate; this corresponded to another steady state with ADP. Results were expressed as $\Delta F/F$, i.e., the relative variation of the NAD(P)H fluorescence signal (Koretsky & Balaban, 1987), with *F* corresponding to the basic signal.

RESULTS AND DISCUSSION

Relationships between ATP/O Ratio and Respiratory Rate. By using different substrates leading to matrix NAD(P)H formation (such as β-hydroxybutyrate, pyruvate, malate, isocitrate, malate + pyruvate, 2-oxoglutarate, or ethanol), we obtained very different respiratory steady states. In state 4, oxygen consumption varied from 6 natom O/[min·(mg of protein)] for β-hydroxybutyrate to 160 natom O/[min·(mg of protein)] for ethanol (Table 1).

With all the substrates used, the respiratory rate was stimulated by ADP addition even if the extent of this stimulation largely varied according to the substrate (Table

[1] Abbreviations: $\Delta\tilde{\mu}_{H^+}$ (expressed in mV), proton-motive force, i.e., proton electrochemical potentiel difference divided by the Faraday constant; CCCP, carbonyl cyanide *m*-chlorophenylhydrazone; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid; natom O/mL, nanomoles of atom O per milliliter.

Table 2. Variation of NAD(P)H Fluorescence Signals Induced by Different Substrates[a]

substrate	$\Delta F/F$ (%)		
	state 4	state 3	+ADP, +oligo
pyruvate	2 ± 0.40	7 ± 1.00	5 ± 1.00
malate	11 ± 4.00	13 ± 5.00	19 ± 4.00
isocitrate	9 ± 2.00	31 ± 7.00	33 ± 7.00
pyruvate + malate	14 ± 2.00	17 ± 3.00	16 ± 2.00
2-oxoglutarate	2 ± 0.05	11 ± 2.00	8 ± 1.00
β-hydroxybutyrate	6 ± 4.00	9 ± 4.00	9 ± 3.00
ethanol	41 ± 5.00	15 ± 7.00	41 ± 4.00

[a] Mitochondria (0.25 mg/mL) were incubated in basal medium as described in Materials and Methods with a respiratory substrate. State 3 of respiration was obtained by adding ADP (1 mM). Oligomycin was used at 50 μg/(mg of protein). The data represent the mean ± SD of eight different experiments performed with three different mitochondria preparations.

1). However, this stimulation was totally oligomycin-sensitive only with ethanol, isocitrate, and pyruvate + malate as substrates. As expected, the respiratory rate in the presence of pyruvate + malate was higher than the sum of the rates with pyruvate or malate alone. For β-hydroxybutyrate and 2-oxoglutarate, oligomycin had only a partial effect on the stimulation by ADP. With pyruvate and malate, oligomycin did not significantly change this stimulation.

Addition of a protonophoric uncoupler such as CCCP stimulated state 4 respiration only with ethanol [not shown; but see Rigoulet et al. (1987)]. In contrast, addition of valinomycin in the presence of 10 mM KCl increased the respiratory rate with all of the substrates used (Table 1). This discrepancy between the effect of CCCP and that of valinomycin + KCl could be explained in two ways which are not exclusive. (i) CCCP depresses the transmembrane ΔpH, which is directly or indirectly responsible for the mitochondrial distribution of respiratory substrates (Palmieri et al., 1970). Since respiration appears to be controlled by electron delivery, a decrease in ΔpH may diminish the substrate transport and consequently the dehydrogenase activity. In contrast, valinomycin + KCl induced a large decrease in $\Delta\Psi$ but no change or an increase in ΔpH with all of the substrates tested (Table 1). (ii) In mammalian mitochondria, a low concentration of valinomycin (nanomolar range) in the presence of K^+ induces a slight oxidation of NADH, a reduction of cytochrome *c*, and a stimulation of the respiratory rate, which may be explained by stimulation of the electron flow between NADH and cytochrome *c* rather than by a decrease in $\Delta\tilde{\mu}_{H^+}$ (Quinlan & Halestrap, 1986). Such a stimulation in electron flow seemed to intervene in yeast mitochondria since the NADH level was lower with valinomycin + K^+ than with CCCP (not shown). Thus, the stimulation of the respiratory rate by valinomycin + K^+ observed in yeast mitochondria with all of the substrates is due partly to a stimulatory effect on the respiratory chain in addition to a $\Delta\tilde{\mu}_{H^+}$ decrease.

On isolated mitochondria, it is generally found that the state 4–state 3 transition corresponds to a decrease in both $\Delta\tilde{\mu}_{H^+}$ and the level of NAD(P)H (Koretsky & Balaban, 1987). Such a phenomenon was observed when ethanol was used as substrate (Table 2). With other substrates leading to NADH formation, the state 4–state 3 transition is linked to an increase in NAD(P)H level (Table 2). Regarding these substrates, three cases are to be noted: (i) With pyruvate, malate, and β-hydroxybutyrate, state 4 respiration was very low and ADP stimulation was partially or totally insensitive to oligomycin; therefore, the respiration rate on these substrates was mainly controlled by dehydrogenase activities. (ii) With pyruvate + malate and isocitrate, the dehydrogenases were activated by ADP as shown by the increase in NAD(P)H (Table 2) but without any respiration increase in the presence of oligomycin; this suggests a control located downstream from the dehydrogenases as for ethanol. (iii) With 2-oxoglutarate, the oxidation rate is tightly controlled by substrate-level phosphorylation (Rigoulet et al., 1985). Nevertheless, these results indicate an ADP-dependent dehydrogenase regulation in yeast mitochondria as previously reported in mammalian mitochondria (Hansford, 1980).

Figure 1A shows that the ATP/O ratio was about 1.5 when the respiratory rate was higher than 100 natom O/[min·(mg of protein)]. Below this value, ATP/O increased and reached 2.5 when pyruvate or β-hydroxybutyrate was the substrate. In contrast, the proton-motive force was nearly constant whatever the respiratory rate (Figure 1B). It should be noted that the flux through the ATP synthase is clearly dependent on respiratory rate rather than being dependent only on the $\Delta\tilde{\mu}_{H^+}$. If there was no misestimation in the phosphorylation rate (see Materials and Methods for the precautions taken in determining the oligomycin-sensitive phosphorylation rate), two possible systematic errors must be considered. The first concerns the diffusion of O_2 into the cell, which can be a source of error particularly at a low respiratory rate. Since (i) the use of a large amount of protein (as much as 5 mg/mL) did not change the ATP/O ratio measured with malate, pyruvate, or β-hydroxybutyrate and (ii) at the low respiratory rate obtained with ethanol plus myxothiazol (comparable to that observed with malate or pyruvate) the ATP/O ratio was similar to that measured with ethanol alone (1.5), we avoided the possibility of overestimating the ATP/O ratio by underestimating oxygen consumption (not shown). The second source of error could be the absence of correction for a proton leak in state 3. Such a correction has been made by some authors [see, for instance, Hinkle et al. (1991)], assuming that the rate of proton leak and slip is controlled only by the electrochemical proton gradient. However, although other parameters play a part in slip control, i.e., the value of the flux through each respiratory chain unit (Ouhabi et al., 1989, 1991), such a correction is not valid. In any case, the relationship between the proton conductance and $\Delta\tilde{\mu}_{H^+}$ obtained in yeast mitochondria indicates that the proton conductance is low for the $\Delta\tilde{\mu}_{H^+}$ value of state 3, and that it increases only when $\Delta\tilde{\mu}_{H^+}$ is higher than 160 mV (Ouhabi et al., 1991).

Nevertheless, we think there are three proton fluxes during oxidative phosphorylation: (i) proton efflux catalyzed by the respiratory chain, $J_{H^+out} = n\,JO$, where *n* is the stoichiometry H^+/O; (ii) proton influx through ATPsynthase, $J_{H^+p} = n'J_{ATP}$, where *n'* is the stoichiometry H^+/ATP linked to ATP synthesis *per se* and the transport processes; and (iii) proton influx through the proton leakage, $J_{H^+L} = L_{H^+}\Delta\tilde{\mu}_{H^+}$, where L_{H^+} is the conductance of the membrane for the protons.

In our conception, an eventual slip of the proton pumps (Pietrobon et al., 1983) will be part of a change in the stoichiometries *n* or *n'*. Therefore, J_{H^+out} would correspond to the net flux of H^+ excreted by the respiratory chain.

Under steady state,

$$J_{H^+out} = J_{H^+p} + J_{H^+L}$$

$$nJ_O = n'J_{ATP} + L_{H^+}\Delta\tilde{\mu}_{H^+}$$

and

$$\frac{ATP}{O} = \frac{J_{ATP}}{J_O} = \frac{J_{H^+p}}{J_{H^+out}}\frac{n}{n'} = \frac{n}{n'}\frac{J_{H^+p}}{J_{H^+p} + J_{H^+L}}$$

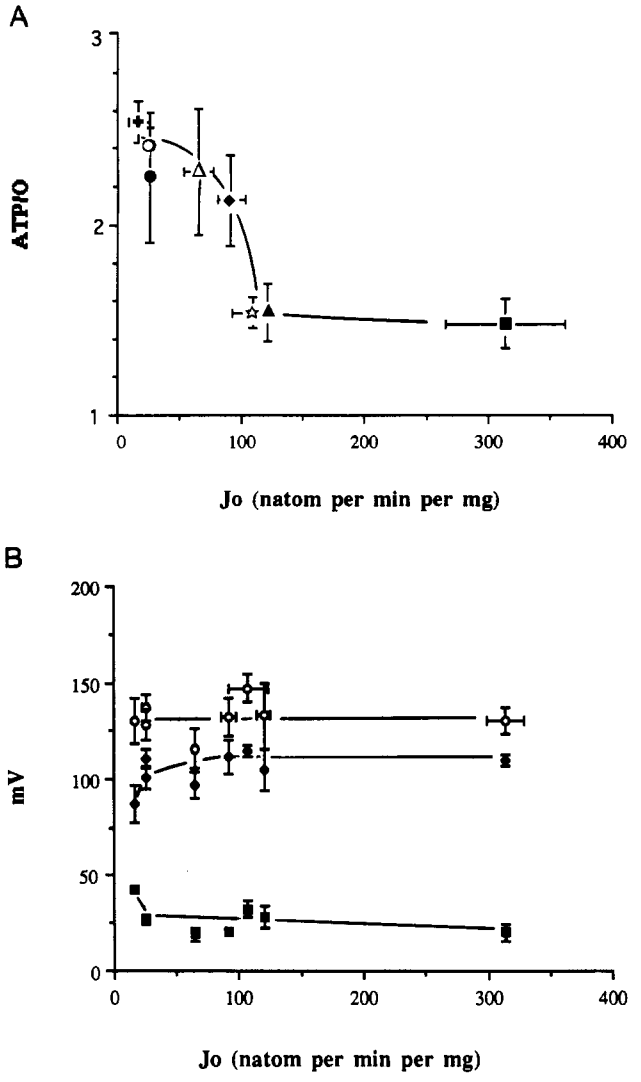

FIGURE 1: Dependence of ATP/O and proton-motive force on respiratory rate at different steady states obtained with various substrates. Mitochondria were incubated in the following basal medium: 0.65 M mannitol, 0.36 mM EGTA, 10 mM Tris–maleate, 3 mM Tris–P_i, 0.1 μg/mL valinomycin, and 5 μM RbCl, supplemented with 70 units/mL hexokinase, 10 mM glucose, 1 mM $MgCl_2$, and a respiratory substrate. Phosphorylation was induced by adding 1 mM ADP. (A) Phosphorylation and oxidation rates (mitochondria were 3 mg/mL for pyruvate, β-hydroxybutyrate, and malate as substrates because of the low respiratory rate and 1 mg/mL for pyruvate + malate, isocitrate, 2-oxoglutarate, succinate, and ethanol). The substrates were pyruvate (+), β-hydroxybutyrate (○), malate (●), pyruvate + malate (△), isocitrate (◆), 2-oxoglutarate (☆), succinate (▲), and ethanol (■). (B) $\Delta\Psi$ (◆), ΔpH (■), and $\Delta\tilde{\mu}_{H^+}$ (○) were determined as described in Materials and Methods. As the respiratory rates increased, the substrates used were pyruvate, β-hydroxybutyrate, malate, pyruvate + malate, isocitrate, 2-oxoglutarate, succinate, and ethanol. The values presented are from at least five different experiments performed with three different mitochondria preparations.

Two hypotheses may be considered: (1) If $J_{H^+L} \ll J_{H^+p}$, the ATP/O value is close to n/n'. (2) If J_{H^+L} is not small in relation to J_{H^+p}, then the lower the difference between J_{H^+p} and J_{H^+L}, the lower the ATP/O ratio.

Since the proton-motive force was constant, the increase in the ATP/O ratio when respiratory rate decreased can be explained only by a change in the mechanistic stoichiometry of at least one of the proton pumps involved. Of course, this explanation is only valid if the proton leak depends exclusively on the magnitude of $\Delta\tilde{\mu}_{H^+}$ for a given membrane, as is

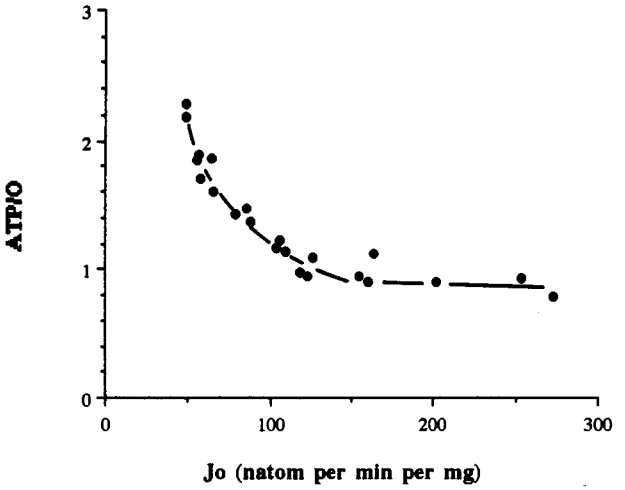

FIGURE 2: Dependence of ATP/O on respiratory rate at the cytochrome *c* oxidase level. Mitochondria were incubated in a lactate-regenerating system as described in Materials and Methods with different concentrations of D-lactate dehydrogenase. ATP synthesis was initiated by addition of 1 mM ADP; after acid extraction, ATP was measured by $^{32}P_i$ incorporation in adenine nucleotides.

generally accepted (Pietrobon et al., 1981; Beavis & Lehninger, 1986; Brand & Murphy, 1987; Zolkiewska et al., 1989; Hafner & Brand, 1991; Hinkle et al., 1991). However, as was recently proposed, if the proton leak is also dependent on the respiratory rate (Garlid et al., 1989, 1993), a change in the proton pump stoichiometry is not a prerequisite to explain our results: indeed, in such a hypothesis, a change either in proton leak or in proton pump stoichiometries may have the same consequences on ATP/O.

In these experiments the electron flow was modulated by acting on the reduction level of internal NAD^+ with different substrates. Another method to control the electron flow through the respiratory chain is to use an electron donor generating system. In the following experiments, mitochondria in the presence of antimycin were incubated with a lactate-generating system at different concentrations of NAD^+-dependent D-lactate dehydrogenase (see Materials and Methods). Figure 2 shows that the ATP/O ratio decreased when the electron flux through the cytochrome *c* oxidase complex increased. This result confirms and extends the previous report in which the ATP/O value was studied as a function of NADH delivery (Ouhabi et al., 1989).

Generally, the ATP/O ratio has been measured at maximal electron flux; to avoid membrane uncoupling, respiratory chain inhibitor titration has been used (Tsou & van Dam, 1969; Beavis & Lenhinger, 1986). Indeed, it has been shown that a low concentration of uncoupler depresses the phosphorylation rate at all rates of respiration but has little effect on the slope of the linear relationship between respiratory and phosphorylation rates when the latter are made to vary with respiratory chain inhibitors. Consequently, the effect of endogenous uncoupler can be largely avoided by determining the ATP/O or ATP/$2e^-$ stoichiometry from the slope of this relationship. We have developed this approach with yeast mitochondria in previous studies (Ouhabi et al., 1989). By using antimycin or cyanide as inhibitors, we have shown that with each substrate tested, i.e., ethanol, succinate, and lactate, the experimental points were distributed on the same straight line intercepting the axis at the origin. Thus, for the substrates giving the lower ATP/O ratio, the slope of the titration curve is equal to the value obtained at state 3 and is reported in this study (Figure 1A). This fact indicates that the decrease in the ATP/O ratio when the fluxes increased in the absence of

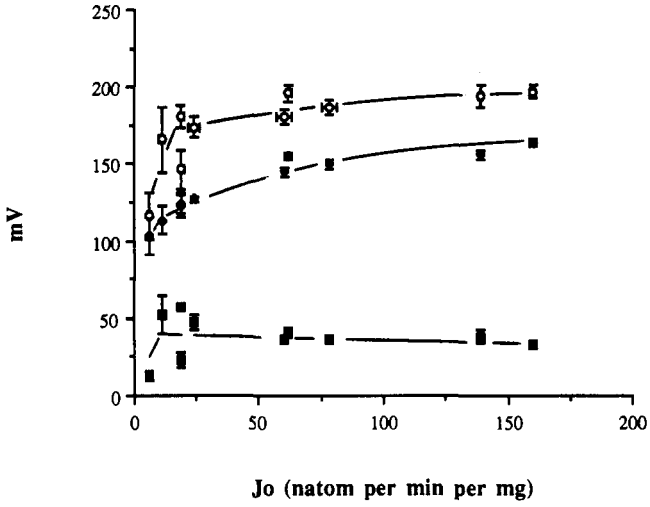

FIGURE 3: Proton-motive force in state 4 of respiration with different substrates. Mitochondria (3 mg/mL) were incubated in basal medium (0.65 M mannitol, 0.36 mM EGTA, 10 mM Tris–maleate, 3 mM Tris–P_i, 0.1 μg/mL valinomycin, and 5 μM RbCl) supplemented with oligomycin [50 μg/(mg of protein)], in the presence of a respiratory substrate. ΔΨ (◆), ΔpH (■), and $\Delta\tilde{\mu}_{H^+}$ (○) were determined as described in Materials and Methods. As the respiratory rates increased, the substrates used were β-hydroxybutyrate, pyruvate, malate, pyruvate + ADP, malate + ADP, pyruvate + malate, 2-oxoglutarate, isocitrate, succinate, and ethanol. The values presented are from at least five different experiments performed with three different mitochondria preparations.

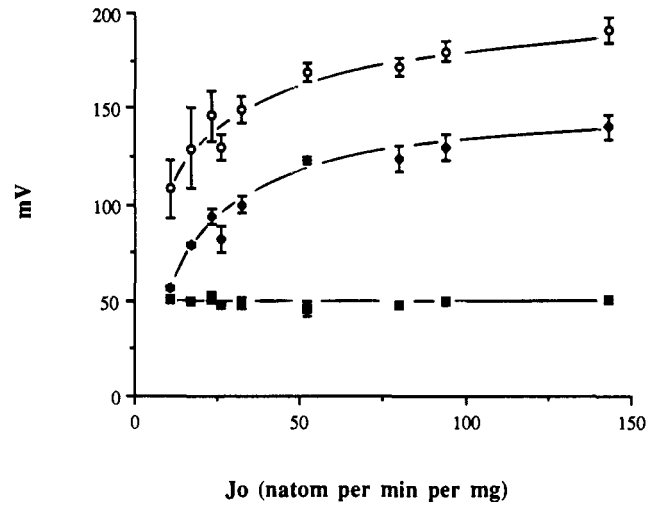

FIGURE 4: Proton-motive force in state 4 of respiration determined by varying respiratory rate with the lactate-regenerating system. Mitochondria (3 mg/mL) were incubated in the basal medium supplemented with the lactate-regenerating system. ΔΨ (◆), ΔpH (■) and $\Delta\tilde{\mu}_{H^+}$ (○) were determined as described in Materials and Methods.

inhibitor was not due to an endogenous uncoupling. As expected, this kind of titration with the other substrates used in this work, i.e., pyruvate, malate, pyruvate + malate, β-hydroxybutyrate, isocitrate, and 2-oxoglutarate, also gives a straight line crossing the axis at the origin (not shown). Thus, under steady states in which the electrons flowing through each respiratory chain unit are changed by modulating either the external (Ouhabi et al., 1989) or the internal (this work) NADH level at constant $\Delta\tilde{\mu}_{H^+}$, the oxidative phosphorylations in yeast mitochondria behave just like a thermal machine; i.e., the efficiency decreases as the flux increases.

Relationships between $\Delta\tilde{\mu}_{H^+}$ and Respiratory Rate in Non-Phosphorylating Mitochondria. Until now, no perfect method has been described to measure directly proton pump efficiency (i.e., $H^+/2e^-$ or H^+/ATP) at high flux and force. However, an indirect approach consists in measuring the response of either respiratory or ATP hydrolysis rates to a change in proton-motive force (Pietrobon et al., 1981, 1983; Brown, 1989). A non-ohmic dependence between passive proton permeability and proton-motive force has been observed in many kinds of mitochondria (Nicholls, 1974; Krishnamoorthy & Hinkle, 1984; O'Shea et al., 1984; Brown & Brand, 1986; Zoratti et al., 1986; Brown, 1989), including yeast mitochondria (Ouhabi et al., 1991). Yet, whatever the particular relationship between proton conductance and proton-motive force, if the $H^+/2e^-$ stoichiometry at each coupling site of the respiratory chain is constant, the following may be expected in non-phosphorylating mitochondria: (i) First, the respiratory rate versus proton-motive force titration must be the same if the coupling sites involved do not change; this must be so whatever the means used for modulating the flux, unless these means change the passive proton conductance of the membrane. In a previous paper, we showed that at a given respiratory rate obtained with the same substrate, i.e., external NADH, $\Delta\tilde{\mu}_{H^+}$ is greater when the electron supply is a limiting process than when the respiratory chain activity is modulated by inhibitor titration (Ouhabi et al., 1991). (ii) Second, from the titrations of electron flux and proton-motive force at each coupling site, the ratio between the electron fluxes is constant and equal to the ratio of the $H^+/2e^-$ stoichiometries of the coupling sites involved, this being so at each proton-motive force value. To test this hypothesis, we established the relationships between proton-motive force and respiratory rate for both systems corresponding to either site 2+3 or site 3 alone.

Figure 3 represents the dependence of $\Delta\tilde{\mu}_{H^+}$ on respiratory rate in non-phosphorylating conditions obtained either in the absence of ADP or in the presence of ADP plus oligomycin for substrates giving electrons to site 2. It is clear from these results that $\Delta\tilde{\mu}_{H^+}$ increases more rapidly at lower respiratory rates. Such a nonlinear dependence between respiratory rate and proton-motive force has often been observed in different kinds of mitochondria (Nicholls, 1974; Krishnamoorthy & Hinkle, 1984; O'Shea et al., 1984; Brown & Brand, 1986; Zoratti et al., 1986; Brown, 1989). In a previous study, we proposed that in yeast mitochondria such a relationship is a consequence of both processes: a non-ohmic proton conductance of the inner membrane and a saturation of redox proton pump slip when the respiratory rate increases (Ouhabi et al., 1991).

Figure 4 shows the dependence between either ΔpH, ΔΨ, or $\Delta\tilde{\mu}_{H^+}$ and respiratory rate in state 4 with lactate as substrate when the flux was titrated by changes in the concentration of enzyme-providing lactate. Thus, this figure presents the titration of respiratory rate and the components of $\Delta\tilde{\mu}_{H^+}$ at site 3. The variation in $\Delta\tilde{\mu}_{H^+}$ as a function of respiratory rate corresponded to a decrease in ΔΨ, with ΔpH remaining constant.

As previously stated by Brown (1989), in a steady state in non-phosphorylating mitochondria,

$$J_{H+L} = nJ_{2e^-}$$

where n is the stoichiometry $H^+/2e^-$, and

$$J_{H+L} = L_{H^+}\Delta\tilde{\mu}_{H^+}$$

If different pumps are used to generate $\Delta\tilde{\mu}_{H^+}$, i.e., site 2+3 (JO_s) or site 3 alone (JO_l), comparing the respiratory rate at

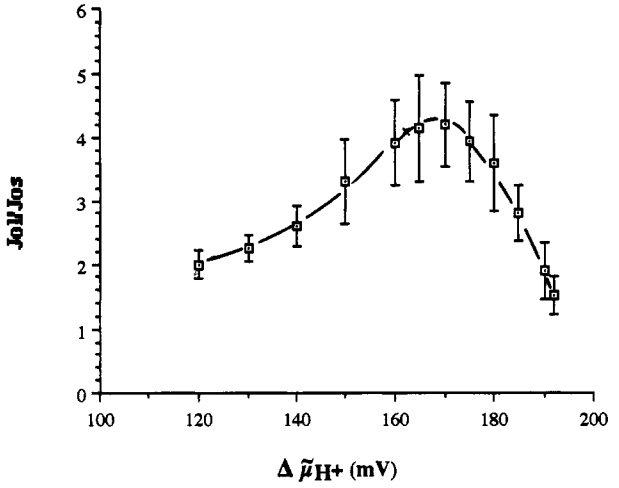

FIGURE 5: Ratio between respiratory rates with the lactate-regenerating system (JO_l) and substrates (JO_s) as a function of proton-motive force. The values of JO_l and JO_s were from Figures 3 and 4, respectively, and the ratios between the two respiratory rates were calculated at the same $\Delta\tilde{\mu}_{H^+}$.

any given value of $\Delta\tilde{\mu}_{H^+}$ gives

$$n_s JO_s = L_{H^+}\Delta\tilde{\mu}_{H^+} = n_l JO_l$$

where n_s is the stoichiometry $H^+/2e^-$ of site 2+3, and n_l, the stoichiometry of site 3. Consequently, $n_s/n_l = JO_l/JO_s$; thus, the JO_l/JO_s ratio for all values of $\Delta\tilde{\mu}_{H^+}$ represents the ratio between the $H^+/2e^-$ stoichiometries (site 2+3 versus site 3).

Therefore, Figures 3 and 4 make it possible to calculate the ratio JO_l/JO_s for each $\Delta\tilde{\mu}_{H^+}$. Figure 5 shows that this ratio is not constant; it increases from 2 to 4 and then decreases to 1.5 as $\Delta\tilde{\mu}_{H^+}$ increases. These results strongly suggest the existence of a variable stoichiometry at the level of the proton pump. As a working hypothesis, we propose that, at low $\Delta\tilde{\mu}_{H^+}$ and low respiratory rate, the $H^+/2e^-$ stoichiometry of either site 2+3 or site 3 is high. In contrast, at high $\Delta\tilde{\mu}_{H^+}$ and high respiratory rate, the stoichiometries are low. Between these two situations, the evolution of either site 2+3 or site 3 alone is different: a decrease in the stoichiometry of site 3 alone is faster than that of site 2+3.

CONCLUSION

In yeast mitochondria, we have used different substrates to show that the electron supply to the respiratory chain in state 4 or 3 changes as a function of the various dehydrogenases involved, each working at a determined rate in a given steady state. Thus, in the absence of inhibitor, the relationships between size of flux and efficiency of proton pumps may be studied. The main observation is that the ATP/O ratio decreases irrespective of the proton-motive force as the electron flux through each respiratory unit increases. It has already been proposed that the stoichiometry of the proton pumps of mammalian mitochondria decreases as $\Delta\tilde{\mu}_{H^+}$ increases (Pietrobon et al., 1981, 1983; Zoratti et al., 1986; Murphy & Brand, 1988). The present experiments show that the size of the flux also plays a part in determining the degree of coupling of the proton pump. Even if such a flux-yield dependence is a general feature of the behavior of a working engine, not all mitochondrial proton pumps are necessarily concerned. However, the comparison of the different relationships obtained by titration of respiration in state 4 and $\Delta\tilde{\mu}_{H^+}$ at sites 2+3 and 3 shows that the ratio of the $H^+/2e^-$ stoichiometries of the coupling sites involved is not constant; therefore, at least one of these stoichiometries varies.

The wide agreement regarding ATP/$2e^-$ values in mammalian mitochondria after correction for proton leakage seems to indicate that these ratios are maximum mechanistic stoichiometries. In fact, these values represent the maximum phenomenological stoichiometries at highest flux. However, when the respiratory flux is lowered as a consequence of a high kinetic control of the oxidative activity upstream from the respiratory chain, then these stoichiometries may increase considerably.

ACKNOWLEDGMENT

The authors wish to thank Mrs. O. Bunoust for her excellent technical assistance and Dr. R. Cooke for his contribution to the editing of the manuscript.

REFERENCES

Balaban, R. S. (1990) *Am. J. Physiol. 258*, C377–C389.
Beavis, A. D., & Lenhinger, A. L. (1986) *Eur. J. Biochem. 158*, 315–322.
Brand, M. D., & Murphy, M. P. (1987) *Biol. Rev. 62*, 141–193.
Brown, G. C. (1989) *J. Biol. Chem. 264*, 14704–14709.
Brown, G. C., & Brand, M. D. (1986) *Biochem. J. 234*, 75–81.
Estabrook, R. W. (1962) *Anal. Biochem. 4*, 231–245.
Garlid, K. D., Beavis, A. D., & Ratkje, S. K. (1989) *Biochim. Biophys. Acta 976*, 109–120.
Garlid, K. D., Semrad, C., & Zinchenko, V. (1993) in *Modern Trends in Biothermokinetics* (Schuster, S., et al., Eds.) pp 287–293, Plenum Press, New York.
Groen, B. H., van Mil, H. G. J., Berden, J. A., & van Dam, K. (1992) *Biochim. Biophys. Acta 1140*, 37–44.
Guérin, B., Labbe, P., & Somlo, M. (1979) *Methods Enzymol. 55*, 149–159.
Hafner, R. P., & Brand, M. D. (1991) *Biochem. J. 275*, 75–80.
Hansford, R. G. (1980) in *Current Topics in Bioenergetics* (Sanadi, D. R., Ed.) pp 217–278, Academic Press, New York.
Hinkle, P. C. (1981) in *Chemiosmotic Proton Circuits in Biological Membranes* (Skulachev, V. P., & Hinkle, P. C., Eds.) pp 49–58, Addison-Wesley Publishing Co., Reading, MA.
Hinkle, P. C., Kumar, M. A., Resetar, A., & Harris, D. L. (1991) *Biochemistry 30*, 3576–3582.
Kacser, H., & Burns, J. A. (1973) in *Control of Biological Processes* (Davies, D. D., Ed.) pp 65–104, Cambridge University Press, London.
Koretsky, A., & Balaban, R. S. (1987) *Biochim. Biophys. Acta 893*, 398–408.
Kovac, L., Bednarova, H., & Greksak, M. (1968) *Biochim. Biophys. Acta 153*, 32–42.
Krishnamoorthy, G., & Hinkle, P. C. (1984) *Biochemistry 23*, 1640–1645.
Lemaster, J. J. (1984) *J. Biol. Chem. 259*, 13123–13130.
Matoon, J. R., & Sherman, F. (1966) *J. Biol. Chem. 241*, 4330–4338.
Mazat, J. P., Jean-Bart, E., Rigoulet, M., & Guérin, B. (1986) *Biochim. Biophys. Acta 849*, 7–15.
Mowbray, J. (1975) *Biochem. J. 148*, 41–47.
Murphy, M. P., & Brand, M. D. (1988) *Eur. J. Biochem. 173*, 637–644.
Nicholls, D. G. (1974) *Eur. J. Biochem. 50*, 305–315.
Ohnishi, T., Kawaguchi, K., & Hagihara, B. (1966) *J. Biol. Chem. 241*, 1797–1806.
O'Shea, P. S., Petrone, G., Casey, R. P., & Azzi, A. (1984) *Biochem. J. 219*, 719–726.
Ouhabi, R. (1992) Ph.D. Thesis, University of Bordeaux.
Ouhabi, R., Rigoulet, M., & Guérin, B. (1989) *FEBS Lett. 254*, 199–202.
Ouhabi, R., Rigoulet, M., Lavie, J. L., & Guérin, B. (1991) *Biochim. Biophys. Acta 1060*, 293–298.
Palmieri, F., Quagliariello, E., & Kingenberg, M. (1970) *Eur. J. Biochem. 17*, 230–238.

Pietrobon, D., Azzone, G. F., & Walz, D. (1981) *Eur. J. Biochem. 117*, 389–394.
Pietrobon, D., Zoratti, M., & Azzone, G. F. (1983) *Biochim. Biophys. Acta 723*, 317–321.
Pozzan, T., Miconi, V., Di Virgilio, F., & Azzone, G. F. (1979) *J. Biol. Chem. 254*, 10200–10205.
Quinlan, P. T., & Halestrap, A. P. (1986) *Biochem. J. 236*, 789–800.
Rigoulet, M., & Guérin, B. (1979) *FEBS Lett. 102*, 18–22.
Rigoulet, M., Velours, J., & Guérin, B. (1985) *Eur. J. Biochem. 153*, 601–607.
Rigoulet, M., Guérin, B., & Denis, M. (1987) *Eur. J. Biochem. 168*, 275–279.
Rottenberg, H. (1979) *Methods Enzymol. 55*, 547–569.
Stoner, C. D. (1987) *J. Biol. Chem. 262*, 10445–10453.
Tsou, C. S., & van Dam, K. (1969) *Biochim. Biophys. Acta 172*, 174–176.
von Jagow, G., & Klingenberg, M. (1970) *Eur. J. Biochem. 12*, 583–592.
Zolkiewska, A., Zablocka, B., Duszynski, J., & Wojtczak, L. (1989) *Arch. Biochem. Biophys. 275*, 580–590.
Zoratti, M., Favaron, M., Pietrobon, D., & Azzone, G. F. (1986) *Biochemistry 25*, 760–767.