Multiple relationships between rate of oxidative phosphorylation and $\Delta\tilde{\mu}H$ in rat liver mitochondria

Mario Zoratti and Valeria Petronilli

CNR Unit for the Physiology of Mitochondria and Institute of General Pathology, Via Loredan 16, 35131 Padova, Italy

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The relationship between rate of ATP synthesis and transmembrane electrochemical proton gradient has been determined in rat liver mitochondria oxidizing succinate, using the respiratory inhibitor malonate or the uncoupler FCCP to decrease $\Delta\tilde{\mu}H$ progressively. As previously reported [(1982) Eur. J. Biochem. 126, 443–451] two different relationships are obtained depending on the method used. Evidence is presented that this result is not due to underestimation of the $\Delta\tilde{\mu}H$ maintained by fast-respiring mitochondria, as recently suggested [(1985) FEBS Lett. 181, 323–327].

Oxidative phosphorylation *Electrochemical proton gradient* *(Rat liver mitochondria)* *Energy transduction* *Flow-force relationship*

1. INTRODUCTION

The chemiosmotic model predicts that the rate of ATP synthesis in energy-transducing organelles should be a function of the transmembrane electrochemical potential, $\Delta\tilde{\mu}H$. As long as the intrinsic kinetic properties of the ATP synthases and the output force, ΔG_p, are not modified, only one relationship should exist between steady-state phosphorylation rate and $\Delta\tilde{\mu}H$. Results at variance with this latter expectation have however been reported for rat liver [1] and *Heliantus tuberosus* [2] mitochondria and for *Rhodopseudomonas capsulata* chromatophores [3,4]. In these systems (but not in chloroplasts [5]) different J_{ATP} vs $\Delta\tilde{\mu}H$ relationships have been obtained depending on the method adopted to modulate these parameters. In particular, J_{ATP} can be extensively inhibited with only small decreases of $\Delta\tilde{\mu}H$ when electron flow is restricted, while larger decreases of $\Delta\tilde{\mu}H$ occur if protonophores are used (reviews [6–8]).

These results have been tentatively considered as evidence for either some form of direct linkage between redox and ATPase proton pumps [1] or the existence of 'localized coupling units' [8]. Alternatively, the steep relationships obtained via inhibition of electron flow might represent the actual, physiological dependence of J_{ATP} on $\Delta\tilde{\mu}H$, while the weaker dependence obtained with uncouplers would be an artefact due to erroneous $\Delta\tilde{\mu}H$ determinations [7]. Recently, Sorgato et al. [9] have reported that in beef heart submitochondrial particles the same J_{ATP} vs $\Delta\tilde{\mu}H$ relationship is obtained when either inhibiting respiration with malonate or uncoupling with FCCP. The authors suggest that the previous results with mitochondria may be due to an underestimation of $\Delta\tilde{\mu}H$ when fast-respiring organelles are recovered by centrifugation [1]. The mitochondria might become anaerobic as they aggregate to form the pellet, with consequent collapse of $\Delta\tilde{\mu}H$ and loss of probe

Abbreviations: $\Delta\tilde{\mu}H$, transmembrane electrochemical proton gradient; $\Delta\psi$, transmembrane electrical potential difference; J_{ATP}, rate of ATP synthesis; J_o, rate of oxygen consumption; TPMP, triphenylmethylphosphonium; DMO, 5,5-dimethyloxazolidine-2,4-dione; G6P, glucose 6-phosphate; Mops, 4-morpholinepropanesulfonic acid; FCCP, carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone; RLM, rat liver mitochondria

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from the matrix as the pellet forms. The onset of anaerobiosis would presumably occur sooner at higher rates of respiration; thus, $\Delta\tilde{\mu}H$ would be increasingly underestimated as the uncoupler concentration is increased.

Similar views had already been expressed (e.g. [10,11]) and rejected (e.g. [12,13]) without, however, conclusive experimental evidence being presented on either side. Since the centrifugation technique is commonly used, the point deserves attention on its own merits as well as within the context of flow-force correlations.

We have therefore reinvestigated the relationship(s) between J_{ATP} and $\Delta\tilde{\mu}H$ in rat liver mitochondria. $\Delta\psi$, the major component of $\Delta\tilde{\mu}H$ under our experimental conditions, has been determined by the probe distribution method, measuring the accumulation of TPMP both by the centrifugation technique and by means of a TPMP-sensitive electrode. The latter method allows one to avoid any anaerobiosis problems. The results indicate that (i) no significant underestimation of $\Delta\tilde{\mu}H$ occurs because of the onset of anaerobiosis during pellet formation; (ii) an underestimation may instead be caused by efflux of probe from the pellet after it has formed and (iii) a discrepancy indeed exists, in rat liver mitochondria, between the J_{ATP} vs $\Delta\tilde{\mu}H$ relationships obtained titrating with an uncoupler (FCCP) or a respiratory inhibitor (malonate).

2. MATERIALS AND METHODS

2.1. *Determination of the rate of ATP synthesis*

RLM were suspended in oxygen-saturated medium (see legend to fig.1) in an open, thermostatted vessel; mitochondrial protein concentration was 1 mg/ml. The medium contained a non-limiting amount of hexokinase (2.7 U/ml) and glucose, and malonate at a given concentration when desired. After a 2 min incubation 0.3 mM ADP was added, together with a given amount of FCCP when appropriate, and four 1-ml samples were withdrawn and quenched in perchloric acid over a 4 min period. Oxygen was repeatedly bubbled into the suspension during the sampling period. After centrifugation of the denatured protein and neutralization of an aliquot of the supernatant, the G6P content of the samples was determined by standard enzymatic methods. Rates of ATP synthesis were calculated from the slope of G6P vs time plots. The data were corrected for adenylate kinase activity by subtracting the rate of G6P formation (20 $nmol \cdot mg^{-1} \cdot min^{-1}$, constant during the incubation time) in incubations supplemented with excess oligomycin, antimycin and FCCP. TPMP uptake by mitochondria is accompanied by inactivation of the process of ATP synthesis and/or transport, the extent of which obviously depends on the TPMP/protein ratio. This leads to lowering of the phosphorylation rate and of the P/O ratio but does not affect the conclusions of the present study. In fact the same TPMP concentrations were present in J_{ATP} and $\Delta\psi$ assays during both the titrations with uncouplers and malonate.

2.2. *Determination of $\Delta\tilde{\mu}H$*

$\Delta\psi$ was determined from the distribution of [^{14}C]TPMP, using the centrifugation technique described [14], and by monitoring medium TPMP concentration in an open, thermostatted and stirred suspension of RLM with a TPMP-sensitive electrode. In the former type of experiments the time elapsing between start of the centrifuge and sample work-up (sampling and removal of supernatant and solubilization of the pellet) was 6–10 min. No correction for probe binding was applied in any case.

Fig.1 shows typical TPMP electrode traces and

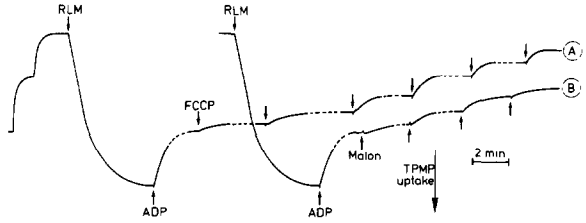

Fig.1. Typical TPMP electrode traces recorded during titrations with FCCP (A) or malonate (B). Medium composition: 0.18 M sucrose, 30 mM Tris/Mops, 5 mM P_i/Tris, 10 mM succinate/Tris, 4 mM $MgCl_2$, 5 mM glycerol, 0.5 mM EGTA, 4 μM rotenone, 15 mM glucose, 2.7 U/ml hexokinase. TPMP (added stepwise for calibration purposes, only 2 additions shown), 18 μM. RLM, 1 mg protein/ml. ADP, 0.3 mM. Temperature, 25°C. The medium was saturated with oxygen before adding the mitochondria. FCCP additions (trace A, ↓) sequentially: 4,6, 10, 20, 20, 20 pmol/mg. Malonate additions (trace B, ↑), sequentially: 0.5, 0.5, 1, 1 mM. Dashes replace portions of the traces disturbed by oxygen addition to the suspensions.

illustrates the procedure followed for electrode-based $\Delta\psi$ determinations. Stringent precautions were taken and controls carried out to avoid any danger of anaerobiosis. Oxygen was repeatedly bubbled through the suspension during the assay; the bubbling caused a disturbance of the electrode response (dashed segments), followed by a return to the previous level. None of the compounds used, including FCCP, affected electrode response at the concentrations used. Medium TPMP concentrations were calculated for each steady-state level from calibration plots of electrode output vs log(TPMP), determined before each individual titration. The slope of the plots was always very close to 60 mV/decade, with correlation coefficients usually 0.999. All $\Delta\psi$ determinations were carried out at least in duplicate, and averages are reported. Experimental conditions, including protein and TPMP concentrations, were the same for each set of determinations of J_{ATP} and $\Delta\psi$ (by the 2 methods), which were conducted in parallel. Data to be compared were obtained utilizing the same mitochondrial preparation.

ΔpH was assessed on the basis of DMO distribution using the centrifugation procedure. Under all conditions used in this work it was measured to be close to zero and not to vary within experimental scatter. We therefore present our results in terms of $\Delta\psi$ only.

RLM were prepared by a standard procedure in a sucrose-based medium [15]. Enzymes were purchased from Sigma, labelled compounds from Amersham. FCCP was used as a 2×10^{-6} or 2×10^{-5} M solution in ethanol. The TPMP-sensitive electrode was built using the body of a Radiometer F2112 selectrode. A radiometer K401 calomel electrode was used as reference. Output was fed to a Beckman ϕ 60 mVolmeter and then to a Linseis LS4 chart recorder.

3. RESULTS

Table 1 shows the results of experiments aimed at testing whether any serious underestimation of $\Delta\psi$ occurs because of the insurgence of anaerobiosis during centrifugation. If this artefact is to explain the divergence of the J_{ATP} vs $\Delta\bar{\mu}$H curves obtained with uncouplers and respiratory inhibitors, it must become progressively more important as the rate of respiration increases. The effect of an increase of the respiratory rate can be mimicked by either a decrease of the oxygen content or an increase in the concentration of mitochondrial protein in the suspension. In the experiments of table 1, RLM respiring at various rates were suspended in air-equilibrated or oxygen-saturated media, all other conditions being equal. Furthermore, the protein concentration was varied by a factor of up to 6. The effect of oxygen concentration changes was negligible in all cases, while an increase in the amount of mitochondrial protein reproducibly led to a slight increase in the calculated $\Delta\psi$. A similar apparent dependence of $\Delta\psi$ on the amount of protein in the assay was found in electrode-based measurements. Direct comparisons showed that the variations were somewhat smaller (approx. 2/3) when assessed by the electrode technique (not shown).

In table 2 a comparison is offered of the $\Delta\psi$

Table 1

Effect of variations in oxygen or protein concentration on $\Delta\psi$ values measured by the centrifugation technique

Conditions	J_o (ngatom $\cdot mg^{-1} \cdot min^{-1}$)	$\Delta\psi$ (mV)	
		$-O_2$	$+O_2$
+6.25 mM malonate[a]	9.7	166.2	168.6
+2.5 mM malonate[a]	13.9	185.4	183.5
State 4, 1 mg protein[b]	15.9	176.8	183.8
State 4, 3 mg protein[b]	15.9	193.8	191.5
State 4, 6 mg protein[b]	15.9	197.3	199.8
+10 pmol/mg FCCP[a]	38	170.3	166.5
+50 pmol/mg FCCP[a]	106	150.3	148.5
+100 pmol/mg FCCP[a]	192	114.8	111.1
State 3, 1 mg/ml[a]	110	167.0	169.3
State 3, 2 mg/ml[a]	110	168.8	171.1
State 3, 4 mg/ml[a]	110	175.4	174.2

Medium composition as in fig.1. Labels[a] and [b] distinguish 2 separate experiments. [a] Glucose and hexokinase were present only in the state 3 determinations and TPMP was 4 μM. [b] TPMP was 24 μM. The medium was either air-equilibrated or highly enriched with oxygen by prolonged bubbling of O_2 before suspension of the mitochondria. After 2 min of incubation, FCCP or ADP was added to the suspension, which was centrifuged after another 45 s. Protein concentration (unless otherwise specified); 2 mg/ml. Suspension volume was 1 ml in all cases. Temperature; 25°C.

Table 2

Comparison of $\Delta\psi$ values measured following TPMP distribution by the centrifugation method or with a TPMP-sensitive electrode

Conditions	J_o (ngatom $\cdot mg^{-1} \cdot min^{-1}$)	$\Delta\psi$ (mV)		$\Delta\Delta\psi$ (mV)
		Centrifugation	Electrode	
State 4[a]	16.1	172.0	188.2	16.2
State 3[a]	91	160.6	170.9	10.3
+ADP, +0.5 mM malonate [a]	49	156.6	167.4	10.8
+ADP, +2 mM malonate[a]	16.4	145.1	154.4	9.3
+ADP, +20 pmol mg FCCP[a]	100	144.6	158.9	14.3
+ADP, +60 pmol/mg FCCP[a]	115	128.6	135.1	6.5
State 3[b]	69	160.3	172.4	12.1
+20 pmol/mg FCCP[b]	60	150.4	169.4	19.0
+40 pmol/mg FCCP[b]	87	137.5	150.2	12.7
+80 pmol/mg FCCP[b]	148	107.6	113.9	6.3

Labels[a] and [b] distinguish 2 separate experiments. Medium composition: [a] as in fig.1, except for the omission of TPMP during J_o assays; [b] as in fig.1 minus hexokinase and glucose except for state 3 determinations, 24 μM TPMP. Protein concentration: [a]1 mg/ml; [b]2 mg/ml. The medium was oxygen-enriched. Temperature, 25°C. Procedures as in table 1 and text. The lower state 3 J_o in [b] was due, besides the different mitochondrial preparation, to TPMP uptake

values measured by the isotope distribution/centrifugation technique (in oxygen-supplemented media) with those determined using the TPMP electrode in parallel experiments under the same conditions. The electrode systematically yields somewhat higher $\Delta\psi$ values than the centrifugation method; the difference does not increase, but rather, if anything, decreases, as the respiratory rate increases (cf. also fig.3).

Experiments were performed to verify whether the discrepancy between electrode- and centrifugation-based $\Delta\psi$ values might be attributed to efflux of probe from the pellet after its formation. Suspensions of mitochondria were centrifuged together, and the work-up procedure (cf. [14]) was performed at variable times between ~3.5 and 45 min after starting the centrifuge. The $\Delta\psi$ values determined from the pellet and supernatant [^{14}C] TPMP content decreased in time as shown in fig.2. In a related experiment, the loss of radioactivity from a pellet (2 mg) of state 4 mitochondria was followed after replacing the supernatant with unlabelled medium. 7.8, 13.5, 18.5 and 22.5% of the radioactivity found in the pellet was lost after waiting a further 10, 20, 30 or 40 min, respectively.

Fig.3 shows plots of the rate of ATP synthesis, progressively decreased using either malonate or FCCP, vs the corresponding $\Delta\psi$ values, measured in parallel experiments by the electrode and the centrifugation techniques. Because of the systematic discrepancy between electrode- and centrifugation-based $\Delta\psi$ values, the 2 plots are shifted by some 10 mV with respect to one another, but they are similar. Regardless of the method used to follow TPMP distribution, the relationship between J_{ATP} and $\Delta\psi$ differs markedly depending on whether FCCP or malonate is used.

Fig.4 emphasizes the similarity between electrode- and centrifugation-based plots. Data from fig.3 and from another analogous experiment are nor-

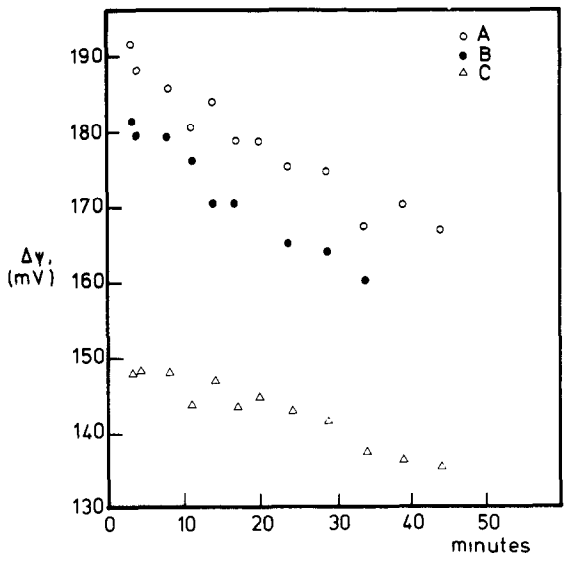

Fig.2. Dependence of $\Delta\psi$ (centrifugation) on the time elapsing before sample work-up. Medium composition as in fig.1, minus glucose and hexokinase. TPMP, 4 μM. Temperature, 25°C. Mitochondria were centrifuged for 1.5 min after a 1 min incubation. Suspension volume was 1 ml in all cases. Centrifuge start is taken as zero time. A (○); state 4 mitochondria, 6 mg protein. B (●); state 4 mitochondria, 2 mg protein. C (△); mitochondria (2 mg protein) + 50 pmol/mg FCCP.

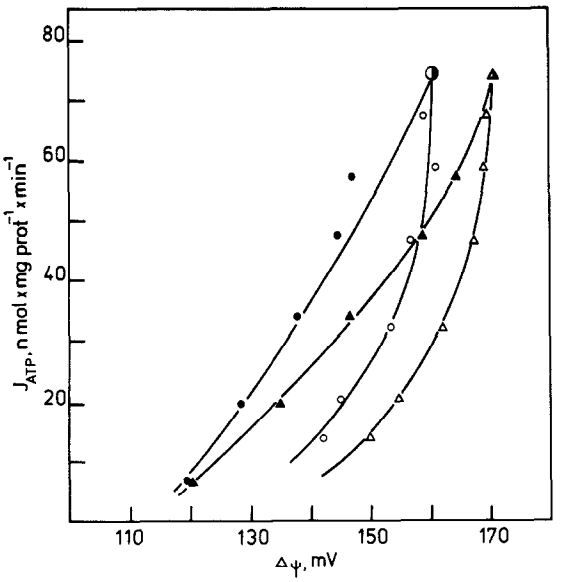

Fig.3. J_{ATP} vs $\Delta\psi$ relationships obtained in titrations with FCCP (▲,●) or malonate (△,○) determining $\Delta\psi$ by the centrifugation technique (○,●) or with a TPMP-sensitive electrode (▲),(△). Medium composition and conditions as for fig.1. FCCP was varied between 0 and 80 pmol/mg, malonate between 0 and 3 mM. In centrifugation experiments sample work-up was performed 7–10 min after centrifuge start. ΔpH was measured to be close to zero in all experiments.

malized by dividing J_{ATP} and $\Delta\psi$ by their values in the absence of either malonate or FCCP. Electrode- and centrifugation-based plots coincide, and both show a clear difference between FCCP- and malonate-based relationships. The normalized data are in excellent agreement with those obtained from several other centrifugation-based experiments (not shown).

4. DISCUSSION

The data presented here do not support the contention that $\Delta\bar{\mu}$H values determined by the labelled probe/centrifugation method might be underestimated because of the insurgence of anaerobiosis during the centrifugation step. In fact, increases of the medium oxygen or mitochondrial protein concentration, which would be expected to result in, respectively, higher and lower $\Delta\psi$ values, cause

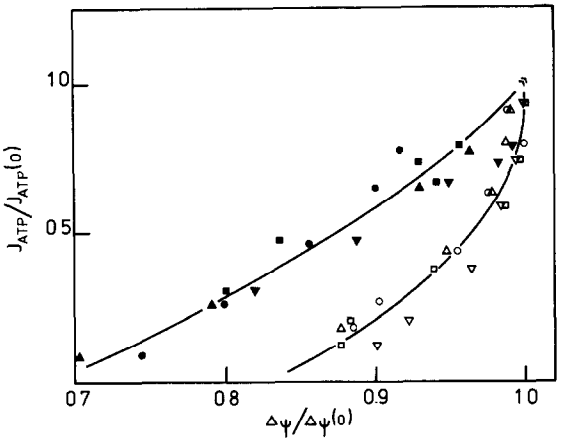

Fig.4. J_{ATP} vs $\Delta\psi$ relationships. Titrations with malonate and FCCP. Data from fig.3 (same symbols) and from another experiment [(▼) FCCP, electrode; (■) FCCP, centrifugation; (▽) malonate, electrode; (□) malonate, centrifugation]. The data were normalized dividing by the state 3 (no FCCP or malonate) J_{ATP} (74 or 96 nmol · mg^{-1} · min^{-1}) and (electrode: 170.9, 170.7 mV; centrifugation: 160.6, 153.4 mV).

respectively no significant variations and a slight increase in the measured $\Delta\psi$ (table 1). Most of the latter effect may well be due to an increased contribution of TPMP binding to the measured (uncorrected) $\Delta\psi$, and it points to an at least partial saturation of binding sites. This phenomenon would tend to mask any apparent $\Delta\psi$ decrease due to the increased rate of oxygen depletion. However, any such decrease could only have been very small because (i) variations in oxygen tension have no effect even at the highest protein concentrations and (ii) electrode-based measurements show smaller pseudo-increases of $\Delta\psi$ as the amount of protein is increased. The opposite result would be expected if a sizable efflux of TPMP from the matrix occurred during centrifugation. Slightly different rates of TPMP efflux from the pellet depending on its mass (cf. fig.2 and below) may account for the greater effects observed with the centrifugation technique.

The centrifugation technique does lead to systematically lower $\Delta\psi$ values than the electrode-based determinations, but this difference is (i) limited to an average of some 12 mV and (ii) independent of the rate of respiration (table 2 and fig.3). Thus, it cannot be ascribed to anaerobiosis during centrifugation. It is instead due, at least in part, to loss of probe after the pellet has formed, during the time needed for halting the centrifuge, sampling the supernatant etc. (cf. fig.2). This leads to a small, systematic underestimation, which can be minimized by reducing the time between pellet formation and work-up. As expected, probe loss occurs more slowly at lower TPMP accumulation ratios and/or when the pellet surface/volume ratio is smaller (higher amounts of protein); thus, electrode- and centrifugation-based $\Delta\psi$ values are expected to differ less as they decrease. This behavior can be recognized in fig.3 and in table 2. Whether TPMP efflux from the pellet can fully explain the discrepancy between the 2 methods is uncertain. The loss of probe could not be measured during the initial 3 min after pellet formation, during which it is expected to proceed most rapidly. Alternatively, some underestimation might arise in centrifugation experiments if part of the probe in the supernatant is actually bound or accumulated by light particles which escape pelletization.

Whatever its origin, the discrepancy between the $\Delta\psi$ values determined by the 2 methods cannot explain the difference between FCCP-based and inhibitor-based J_{ATP} vs $\Delta\tilde{\mu}H$ relationships, which persists when $\Delta\psi$ is measured by the electrode technique. This conclusion is in agreement with the results of the Bologna group [2–4], obtained utilizing either electrode-based probe distribution measurements [2] or electrochromic shift determinations [3,4] to evaluate $\Delta\psi$.

Mitochondria certainly become anaerobic as the pellet forms. Whether this results in a significant loss of probe will depend on (i) the time intervening between anaerobiosis and final compacting into the pellet, (ii) the time required for $\Delta\psi$ to collapse after anaerobiosis and (iii) the rate of probe efflux following $\Delta\psi$ decrease. The results of this work indicate that the relative magnitudes of these parameters are not such as to lead to significant TPMP losses from the matrix before pelletization. The driving force for probe efflux after anaerobiosis, i.e. its chemical gradient, is linked to the $\Delta\psi$ maintained under aerobic conditions. This would tend to counteract the effect of the higher rates of respiration often associated with low $\Delta\tilde{\mu}H$ values. The enormous (some 4 orders of magnitude) decrease in the matrix/medium interface occurring as the pellet forms is presumably instrumental in limiting (but not preventing, see above) subsequent losses of probe.

The results reported here indicate that beef heart submitochondrial particles and rat liver or plant mitochondria behave differently. The former apparently exhibit only one J_{ATP} vs $\Delta\tilde{\mu}H$ relationship [9] while the latter show several [1,2]; this paper). An analogous difference might exist between chloroplasts [5] and bacterial chromatophores [3,4]. Scrutiny of these variations from system to system could hopefully offer clues as to the reasons for the multiplicity of flow-force relationships.

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