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Kinetic limitations in the overall reaction of mitochondrial oxidative phosphorylation accounting for flux-dependent changes in the apparent $\Delta G^{ex}_{P}/\Delta\tilde{\mu}H^{+}$ ratio

Wolfgang Kunz, Frank Norbert Gellerich, Lorenz Schild and Peter Schönfeld

Institut für Biochemie, Medizinische Akademie Magdeburg, Leipziger Str. 44, Magdeburg 3090, GDR

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Changes in J_o, $\Delta\tilde{\mu}H^{+}$ and ΔG^{ex}_{P} were investigated as a function of load. The flux control coefficients, particularly those of the adenine nucleotide translocator and H^{+}-ATPase at the maximum rate of oxidative phosphorylation were seen to strongly depend on the phosphate concentration accounting in common for the highest share in flux control. There was no unique relationship observed between J_P and $\Delta\tilde{\mu}H^{+}$ in load-controlled, well coupled systems, but J_P was found to depend on $\Delta\tilde{\mu}H^{+}$ at excessive load and increasing proton leakage. All the results presented can be elucidated on the grounds of delocalized chemiosmotic coupling.

Proton electrochemical gradient; Phosphorylation potential; Adenine nucleotide translocator; H^{+}-ATPase; Flux control coefficient; Delocalized chemiosmotic coupling

1. INTRODUCTION

Relations between fluxes and driving forces can be affected by kinetic parameters. The extent to which kinetics are capable of causing deviations from the postulated unique relationships depends on the number of steps involved in the overall reaction considered and their kinetic properties as well as on the flux rate. This paper provides evidence that the adenine nucleotide translocator and H^{+}-ATPase exert the most pronounced limiting effect on the rate of load-dependent transformation of energy by mitochondria oxidizing succinate and, thus, influence unique flow-force relationships near to and at the maximum rate of respiration.

Correspondence address: W. Kunz, Institut für Biochemie, Medizinische Akademie Magdeburg, Leipziger Str. 44, Magdeburg 3090, GDR

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2. MATERIALS AND METHODS

Rat liver mitochondria were isolated as in [1]. The first derivative of the rate of oxygen consumption (J_o) and changes in the transmembrane potential ($\Delta\psi$) were measured at 25°C in the same closed, thermostated and magnetically stirred vessel with a volume variable from 5 ml to 1 ml, equipped with a Clark-type and an ion-sensitive electrode. Measurements of ΔpH were always carried out in the same vessel in which J_o and $\Delta\psi$ were determined. The potassium-free incubation medium contained in mM: 90 sucrose, 60 tricine, 5 $MgCl_2$, 0.5 Na_2-EDTA, 15 glucose, 40 nicotinamide, 5 ATP, 10 succinic acid, 0.001 rotenone, and either 10 sodium phosphate and 53 Tris or 3 sodium phosphate and 60 Tris, pH 7.4. Changes in the medium are given in the legends of the figures. For determination of extramitochondrial adenine nucleotides and G-6-P, 1 ml samples were withdrawn from the incubation mixture and quickly filtered through glass fibre filters (Millipore Type AP 25) for separation of the mitochondria into 4 ml of a phenol/chloroform/isoamyl alcohol mixture (38:24:1) (v/v) saturated with water plus 0.6 ml of 66 mM EDTA for quenching and extraction [2]. Extramitochondrial phosphorylation potentials were calculated using a computer program [3] taking into account the standard phosphorylation potentials published in [4]. The rates of phosphorylation (J_P) were estimated from the G-6-P formation. The $\Delta\psi$ was determined by means of a dibenzyl dimethyl ammonium (DDA^{+}) sensitive electrode as described in [5]. The ΔpH was calculated from the distribution of [^{14}C]DMO (2 kBq/ml) according to [6]. The

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mitochondrial matrix volume was determined in the same way using [^{3}H]H_2O as a marker for the total water and [^{14}C]sucrose for estimation of the extramitochondrial water volume. Phosphate was determined according to [7] from quenched samples, only in experiments in which the phosphate concentration was varied. In the other experiments the extramitochondrial phosphate concentration was calculated from the initial phosphate content in the medium and the G-6-P formed. The flux control coefficients [8,9] of various enzymes on hexokinase-stimulated maximum rate of mitochondrial respiration were calculated as described in [10].

3. RESULTS AND DISCUSSION

Fig.1 demonstrates how load-dependent changes in J_o and $\Delta\psi$ were measured. The traces clearly reveal the transition and the stationary states, the latter only being used to determine $\Delta\psi$ and J_o values.

It should be emphasized that, as soon as the maximum rate of respiration was reached, another load increase by enhancement of the added activity of hexokinase did not influence J_o, nor did it exert any effect on the corresponding $\Delta\psi$, despite a marked decrease in the extramitochondrial phosphorylation potential.

In addition to recording of traces, our experimental setup permitted samples to be withdrawn from the vessel to determine either the associated distribution of ions or the contents of extramitochondrial ATP, ADP, AMP and G-6-P, as applicable, after quick separation and quenching.

In fig.2A, respiration rates at different loads are plotted versus the corresponding $\Delta\psi$ for a number of separate experiments. The data points are seen to roughly reflect steep dependences of the rate of oxidation on the magnitude of $\Delta\psi$. However, two pronounced phosphate-dependent deviations from the unique relationships claimed were noted. It is evident that, with 3 mM phosphate, the magnitude of $\Delta\psi$ at comparable J_o was somewhat higher than in the presence of 10 mM phosphate, and for 10 mM phosphate both the maximum rate of respiration and the minimum $\Delta\psi$ exceeded those measured in the presence of 3 mM phosphate.

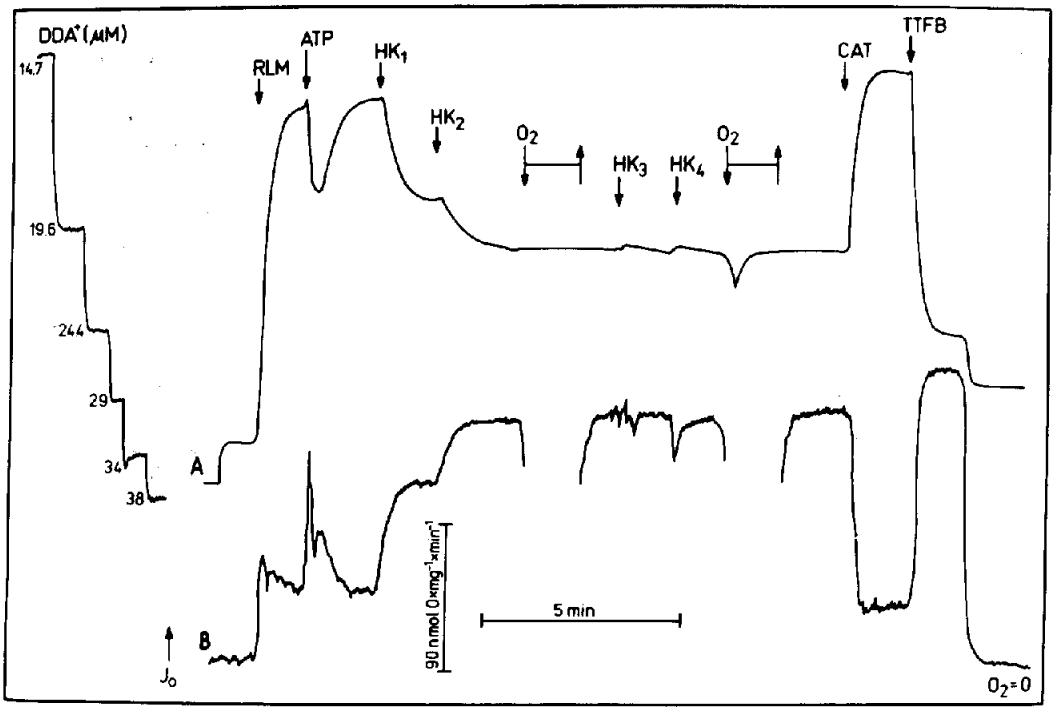

Fig.1. Changes in emf of the DDA^+ electrode reflecting changes in $\Delta\psi$ (A) and in J_o (B) at increasing load. Additions: 0.81 mg/ml mitochondrial protein; ATP, 0.95 mM; HK_1, 0.16 U/mg, HK_2–HK_4, 0.32 U/mg mitochondrial protein yeast hexokinase; CAT, 5.9 nmol/mg carboxyatractyloside; TTFB, 0.48 μM; ↓---↑, oxygen admission; P_i in the medium, 10 mM.

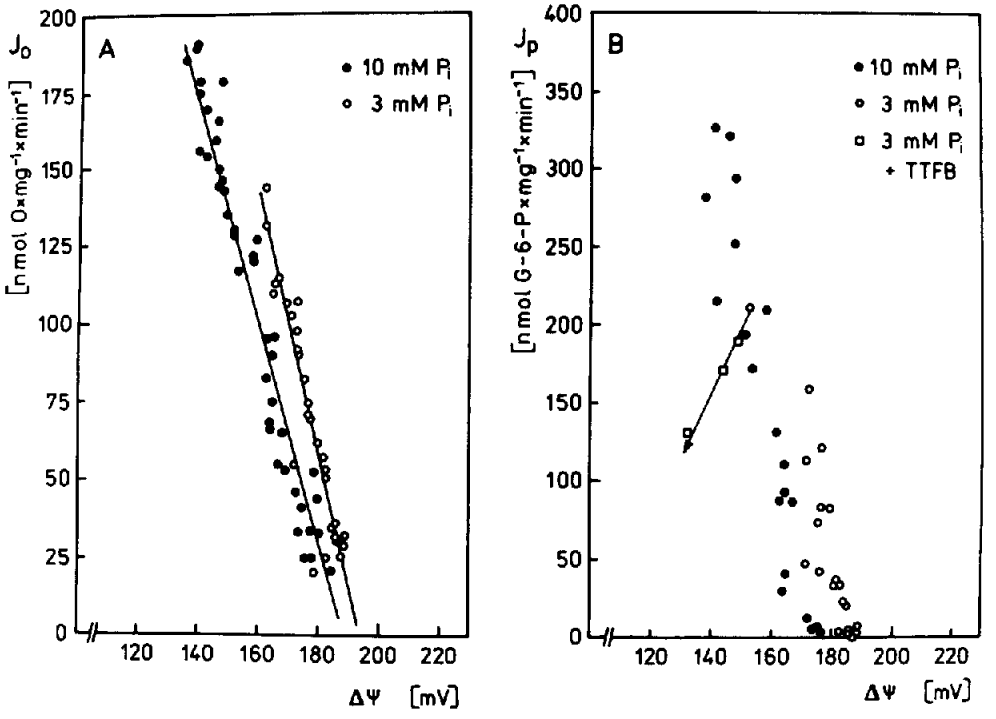

Fig.2. Rates of mitochondrial respiration (A) and phosphorylation (B) versus $\Delta\psi$. (Circles) $\Delta\psi$ was varied by 0–0.4 U/mg mitochondrial protein hexokinase. (Squares) $\Delta\psi$ was varied by TTFB titration in the presence of 0.4 U/mg mitochondrial protein hexokinase and 1 mM ATP.

Fig.2B depicts the phosphorylation rate (J_P) against $\Delta\psi$. When, in well coupled mitochondria, $\Delta\psi$ was varied by load enhancements through increasing activities of added yeast hexokinase, then J_P increased with diminishing $\Delta\psi$. In contrast, additional stepwise drainage of protons by TTFB titration in the presence of an excess of hexokinase caused the J_P to become linearly smaller with decreasing $\Delta\psi$. This finding demonstrates that the relationships between J_P and $\Delta\psi$ depend on the experimental conditions employed. Force flow relations as predicted by delocalized chemiosmotic coupling were clearly detected by variation of the driving force $\Delta\tilde{\mu}H^+$ for phosphorylation on the input side, but not by variations on the output side.

In fig.3 we studied the phosphate dependency of the well coupled succinate oxidizing system working in the maximum rate in greater detail. J_o is seen to rise with increasing [P_i] in a hyperbolic manner, whereas $\Delta\tilde{\mu}H^+$ calculated from $\Delta\psi$ and ΔpH goes down. The inset reveals that a virtually linear relation existed between the rate of oxidation and the electrochemical potential of protons. Since the magnitude of $\Delta\tilde{\mu}H^+$ was high in the maximum active state even at low phosphate concentrations, the availability of phosphate is supposed to limit its utilization rather than noticeably limiting its build up. This is in line with the observation reported earlier [11] that phosphate additions to a suspension of coupled mitochondria immediately resulted in oxidation-reduction cycles of nicotinamide nucleotides along with short changes in the rate of oxidative phosphorylation. In the light of this observation, it was reasonable to assume that, under these conditions, the ability of the proton-translocation ATPase to form ATP is controlled by the actual phosphate activity in the matrix compartment. In order to verify experimentally whether or not this assumption was correct, we determined the contribution of different steps to the control of oxidative phosphorylation by titration with inhibitors in the presence of 3 mM or 10 mM phosphate in the medium (table 1). The maximum rate of oxidative phosphorylation was

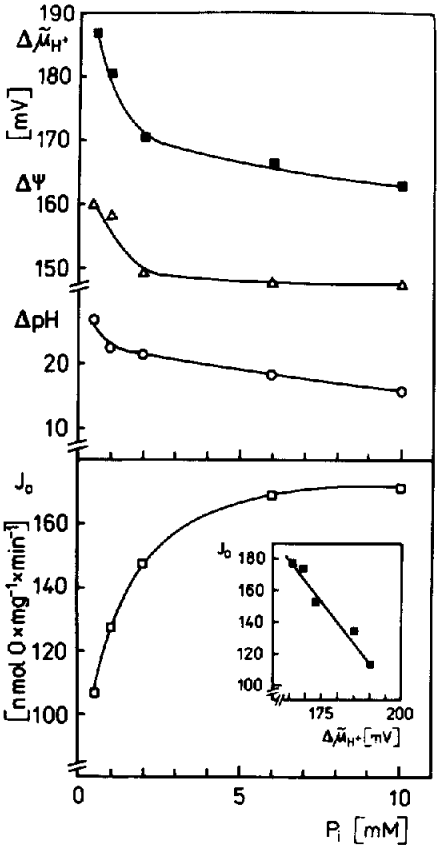

Fig.3. Respiration rate and parameters of the proton electrochemical gradient versus phosphate concentration. Active state respiration was adjusted by addition of 240 μM ADP. Decrease in phosphate concentration was compensated for by Tris.

ensured by addition of an excess of hexokinase. The highest amounts of control were then found to be exerted by H^+-ATPase as well as the ATP/ADP translocator which were directly or indirectly involved in the utilization of $\Delta\tilde{\mu}H^+$ due to an extramitochondrial decrease in the ATP/ADP ratio. Moreover, it is demonstrated that the flux control coefficients strongly depended on the phosphate concentration, in the case of H^+-ATPase increasing at lower phosphate levels. This finding is in line with data published recently [15], but in contrast with others [16]. Similarly, earlier investigations in our laboratory [17] had not revealed any contribution of H^+-ATPase to rate control, probably because of the high phosphate concentrations in the medium. In addition, the lag phase of oligomycin inhibition [14] was neglected. Contribution of H^+-ATPase to the rate control in the state of maximum oxidative phosphorylation means that the rate of ATP synthesis as a function of the net thermodynamic forces across the

Table 1

Flux control coefficients of various steps in the state of maximum oxidative phosphorylation at different phosphate concentrations with 10 mM succinate plus 1 μM rotenone

Enzyme	$C_i^{J_o}$						Inhibitor	Type of inhibition	Inhibition constant
	3 mM P_i			10 mM P_i					
H^+-ATPase	0.41	± 0.05	(5)	0.17	± 0.06	(5)	oligomycin	irreversible	I_{max} = 283 pmol/mg
ATP/ADP translocator	0.19	± 0.07	(5)	0.45	± 0.09	(5)	carboxyatractyloside	irreversible	I_{max} = 300 pmol/mg
Dicarboxylate translocator	0.12	± 0.01	(2)	0.14	± 0.06	(3)	phenylsuccinate	competitive	K_I = 710 μM K_M = 1.2 mM [12]
Cytochrome oxidase	0.10	± 0.03	(3)	0.13	± 0.03	(3)	azide	non-competitive	K_I = 100 μM [13]
P_i translocator	0.002	± 0.004	(3)	0.002	± 0.003	(3)	mersalyl	irreversible	I_{max} = 7.8 nmol/mg
$\Sigma C_i^{J_o}$	0.822	± 0.164		0.892	± 0.24				

Mitochondria (0.34–0.81 mg mitochondrial protein/ml) were incubated in the presence of an excess of yeast hexokinase (>3 U/mg protein) and about 5 mM ATP. With the exception of oligomycin, inhibitors were added step by step after stationary rates of respiration had been reached. Because of the time dependence of oligomycin inhibition [14], samples were preincubated for 5 min with different amounts of oligomycin and started by addition of hexokinase plus ATP. The values represent means ± SE of separate experiments as given in parentheses

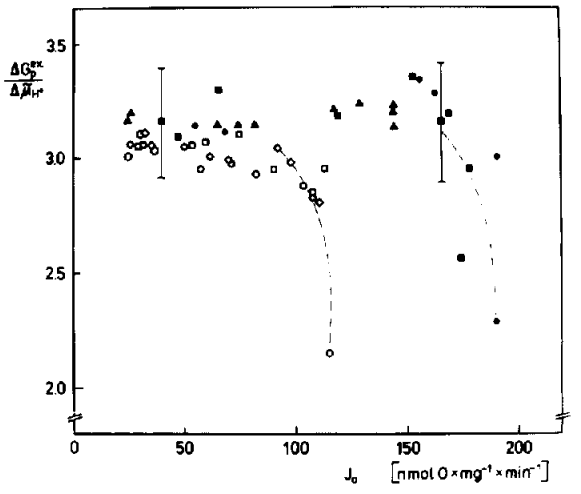

Fig.4. Relationships between $\Delta G_P^{ex}/\Delta\tilde{\mu}H^+$ ratios and respiration rates at increasing load adjusted by yeast hexokinase. (Closed symbols) with 10 mM P_i; (open symbols) with 3 mM P_i.

H^+-ATPase complex is not in equilibrium. It follows that the value of the $\Delta G_P^{ex}/\Delta\tilde{\mu}H^+$ ratio is liable to drop sharply if the ΔG_P^{ex} value corresponding to the maximum rate of oxidative phosphorylation undergoes a further decline due to an elevated extramitochondrial load or, in other words, if the demand for ATP utilization exceeds the capacity of ATP formation. This is clear from fig.4. It is shown that, within the experimental error, the $\Delta G_P^{ex}/\Delta\tilde{\mu}H^+$ ratio remained constant, being somewhat higher than 3 in the intermediate states with 3 mM or 10 mM phosphate in the medium, but decreasing earlier at lower than at higher phosphate concentrations.

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