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Distribution of control of oxidative phosphorylation in mitochondria oxidizing NAD-linked substrates *

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The flux control distribution of the net rate of state 3 respiration was determined in heart and kidney mitochondria incubated with low concentrations of pyruvate (0.5 mM) or 2-oxoglutarate (1 mM), and in conditions that led to activation of NAD-linked dehydrogenases, i.e., high substrate or Ca^{2+} concentrations. Control of flux was exerted by the ATP/ADP carrier (flux control coefficient, $c_i = 0.37$) and Site 1 of the respiratory chain ($c_i = 0.28$) when dehydrogenase activity was low. Control of the process shifted to the ATP synthase ($c_i = 0.32$) and the P_i carrier ($C_i = 0.27$) when dehydrogenases were activated by high pyruvate and high Ca^{2+}. The changes in the control exerted by the ATP/ADP carrier and the ATP synthase were not due to changes in the transmembrane potential, nor to a modification of intramitochondrial ATP/ADP ratios. Applying the summation theorem of the control analysis, it was found that at low Ca^{2+} and pyruvate concentrations the dehydrogenases shared the control of state 3 respiration with other steps. The NAD-linked dehydrogenases did not exert any significant control at high Ca^{2+} or high pyruvate concentrations.

Introduction

Quantitative studies on the regulation of oxidative phosphorylation in mammalian isolated mitochondria [2–7] have shown that several steps are rate-limiting in the pathway, i.e., the flux control coefficients (c_i) of the ATP/ADP carrier, the ATP synthase, the cytochrome oxidase, the b-c_1 cytochrome complex and the dicarboxylate carrier varied from 0.1 to 0.4. However, most of these studies have been made with mitochondria incubated with saturating concentrations of succinate (5–20 mM) and in the presence of the Site 1 respiratory inhibitor, rotenone. In consequence, the contribution of the NAD-linked dehydrogenases to the control of oxidative phosphorylation was not ascertained. In the reports where NAD-dependent substrates were used [5,8], also saturating substrate concentrations (> 1 mM) were employed. Under these conditions it is possible that the control exerted by substrate dehydrogenation and transport on oxidative phosphorylation would be low. Since the cytosolic concentrations of pyruvate and 2-oxoglutarate in the working heart [9,10] and liver [11] are very likely below 1 mM, it is of relevance to determine flux control at concentrations of substrate that are in the physiological range.

It has been proposed that the activity of mitochondrial NAD-linked dehydrogenases may control oxidative phosphorylation, i.e., an activation of the dehydrogenases would raise NADH/NAD ratios, maximizing the redox driving force for ATP synthesis and flux without a decrease in the cytosolic phosphorylation potential, and the ATP/ADP ratio [12–14]. In this respect, it is known that variations in dehydrogenase activity are modulated by changes of mitochondrial matrix free Ca^{2+}, $[Ca^{2+}]_m$, that result from variations in extramitochondrial free $[Ca^{2+}]$ [13,15–17]. In fact, recent ^{31}P-NMR experiments showed that in heart, different work-loads produced changes in mitochondrial NAD reduction without a change in cytosolic free ADP, $[ADP]_c$ [18–20]. In contrast, determination of

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$[ADP]_c$ and cytosolic phosphorylation potential by conventional extraction techniques in the working heart [21,22] and ^{31}P-NMR studies in heart perfused with high pyruvate concentrations, or with the mitochondrial Ca^{2+} transport inhibitor, Ruthenium Red [23,24], showed a correlation between the rate of O_2 consumption and cardiac performance with $[ADP]_c$. Moreover, experiments with isolated liver [25] and heart [26] mitochondria incubated with low concentrations of 2-oxoglutarate or glutamate showed that an increased availability of Ca^{2+} resulted in an increase in flux with a negligible change in the NADH level [26]. It was suggested that Ca^{2+} could activate both the phosphorylating system (ATP/ADP carrier, P_i carrier, ATP synthase), and the 2-oxoglutarate dehydrogenase [26].

Therefore, in this work we examined the quantitative distribution of control of oxidative phosphorylation considering the contribution of the NAD-linked dehydrogenases to the overall process. This was studied through measurements of the rate of ADP-stimulated respiration at two different extramitochondrial Ca^{2+} concentrations (5 and 230 nM) in isolated mitochondria from rat heart and kidney incubated with low pyruvate (0.5 mM) and 2-oxoglutarate (1 mM) concentrations. The results indicated that both the NAD-linked dehydrogenases and the phosphorylating system participated in the control of oxidative phosphorylation.

Methods

Preparation and incubation of mitochondria. Heart mitochondria were prepared from Wistar rats weighing 150–220 g by a nagarse method [16]. Kidney mitochondria were prepared as described previously for liver mitochondria [27], except that the isolation medium contained 250 mM sucrose, 10 mM Hepes, 1 mM EGTA (pH 7.4) and 1% albumin.

Mitochondria were incubated in a standard medium that contained 120 mM KCl, 25 mM K-Mops, 5 mM potassium phosphate, 10 mM NaCl, 0.5 mM EGTA (pH 7.2) at 25°C. Ca^{2+}-EGTA buffers were prepared and used as described previously [6,16]. O_2 uptake was measured by means of a Clark-type O_2 electrode (Yellow Springs Instrument Co.). The solubility of O_2 in equilibrium with air was taken to be 417 ng atoms/ml at 25°C and 2240 m altitude.

Measurement of redox components. The percentage reduction of mitochondrial NAD was determined by differential absorbance of the mitochondrial suspension at 340–370 nm, under stirring and continuous gassing with 100% O_2 at 25°C using an Aminco DW2c dual wavelength spectrophotometer. Calibration of the absorbance signal was carried out as described elsewhere [26]. The percentage reduction of cytochrome *c* was determined by differential absorbance at 550–540 nm; the references for calibration were the minimal signal generated by addition of 12.5 μM antimycin + 0.25 μM CCCP in relation to the signal generated by addition of excess of sodium dithionite (full reduction). For the calculation of the redox potential of sites 1 + 2 of the respiratory chain midpoint potentials of −320 mV and +235 mV were assumed for NAD and cytochrome *c*, respectively [28].

Determination of the transmembrane potential. Mitochondria were incubated with 10 μM safranine in 2 ml of standard medium and the absorbance difference at 511–533 nm was followed by dual spectrophotometer [29] under continuous gassing with 100% O_2, stirring and 25°C. Tetraphenylphosphonium (TPP^+) distribution was determined after incubation of mitochondria with 2 μM [^{3}H]TPP^+ (100000 cpm/nmol) and centrifugation in a microfuge for 1 min. The matrix mitochondrial volume was assumed to be 1 μl/mg protein. The calculated values of the transmembrane potential using the Nernst equation were not corrected for TPP^+ bound to mitochondrial membranes.

Determination of the flux control coefficient (c_i). Titrations of the rate of state 3 respiration with different specific inhibitors were performed (see Fig. 1). According to the formulation proposed by Groen et al. [2], the initial slope of the titration curve (dF/dI) $[I] \rightarrow 0$ was related to the reference, non-inhibited point (initial flux, F_o) and to the kinetic parameters that depended on the inhibitor used. With an irreversible inhibitor, the kinetic parameter is the minimal amount of inhibitor required to obtain maximal flux inhibition (I_{max}); thus, $C_i = -I_{max}/F_o\ (dF/dI)\ [I] \rightarrow 0$ [2].

Chemicals. Rotenone, antimycin, oligomycin, carboxyatractyloside, and HQNO (2-heptyl-4-hydroxyquinoline *N*-oxide) were from Sigma and dissolved in ethanol. The concentrations of rotenone, antimycin and HQNO were verified spectrophotometrically as indicated elsewhere [30]. Fresh solutions of pyruvate, cyanide and mersalyl were used thoroughout this study.

Results

Heart mitochondria were incubated with a low concentration of pyruvate (0.5 mM) and 1 mM malate as oxidizable substrate. This concentration of pyruvate was in the range reported in heart [9,10] and liver [11] tissues. Determination of pyruvate at the end of a standard experiment (8–9 min) showed that its concentration diminished to 362 ± 27 μM ($n = 5$); this concentration still supported a steady-state rate of ADP-stimulated respiration. It is noted that before a steady-state rate of respiration was established, there was a delay of about 40–60 s after the addition of ADP with 0.5 mM pyruvate; with pyruvate (+malate) as oxidizable substrate such a delay was probably due to activa-

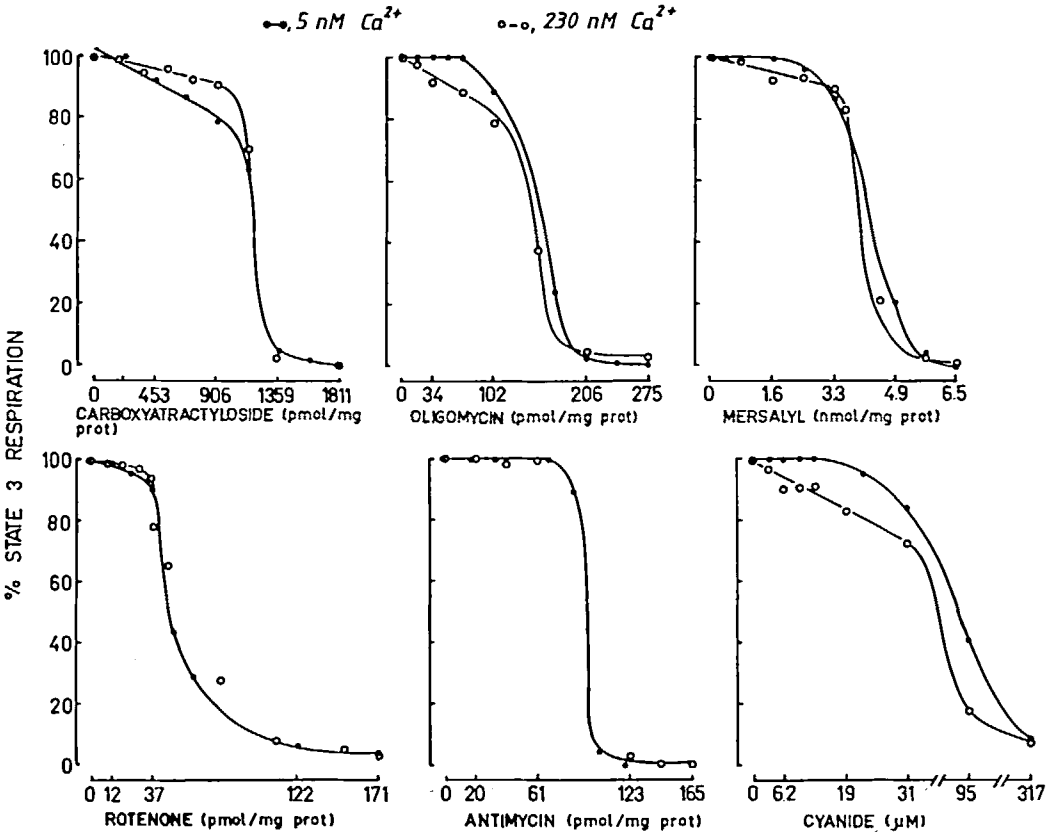

Fig. 1. Inhibition of the net rate of state 3 respiration by several specific inhibitors in heart mitochondria. Mitochondria (0.8–1.4 mg protein/ml) were incubated in 3 ml of standard medium containing 0.5 mM pyruvate + 1 mM malate and the indicated inhibitor concentrations for 5 min. The final free Ca^{2+} concentration was 5 nM (full circles). State 3 respiration was started by addition of 1–1.3 mM ADP. In another set of experiments a Ca/EGTA buffer (2.43 mM Ca^{2+}/3.75 mM in 0.1 M Mops (pH 7.25)) was added after 2 min of incubation to stabilize a free Ca^{2+} concentration of 230 nM (empty circles).

TABLE I

Flux control coefficients of various steps in oxidative phosphorylation from heart mitochondria oxidizing pyruvate

Mitochondria were incubated as described in Fig. 1 with the indicated pyruvate and Ca^{2+} concentrations. Flux control coefficients (C_i) were calculated as described under Methods using the data of Fig. 1 and other similar titration curves not shown. Except for cyanide, all other inhibitors used in Fig. 1 were considered irreversible inhibitors. The K_i used in the calculations were 2 μM for cyanide [38], 64 nM for HQNO [39], and 86 μM for azide (this was determined as described in Ref. 38). The data represent mean ± S.E., with the number of preparations assayed in parentheses. The rate of state 4 respiration was 26 ± 1 (n = 20) ngatom O_2/mg protein/min with 0.5 mM pyruvate + 5 nM Ca^{2+} and it was not significantly different from the other two conditions showed in the table. a, azide; b, cyanide; c, antimycin; d, HQNO.

	5 mM pyruvate +5 nM Ca^{2+}	0.5 mM pyruvate +5 nM Ca^{2+}	0.5 mM pyruvate +230 nM Ca^{2+}
% NAD(P)H in State 3	43 ± 2 (4)	30 ± 4 (5)	45 ± 7 (5)
Rate of state 3 respiration (ngatom O_2/mg protein per min)	291 ± 13 (9)	208 ± 9 (20)	301 ± 14 (18)
Step	C_i		
ATP/ADP carrier	0.05 ± 0.05 (3)	0.37 ± 0.06 (4)	0.24 ± 0.02 (3)
ATP synthase	0.32 ± 0.02 (3)	0.0 ± 0.0 (3)	0.34 ± 0.06 (4)
P_i carrier	0.27 ± 0.07 (3)	0.04 ± 0.02 (3)	0.27 ± 0.05 (4)
Site 1	0.13 ± 0.03 (3)	0.28 ± 0.03 (3)	0.15 ± 0.02 (3)
Site 2	0.00	0.00	0.01 [c], 0.03 [d]
Site 3	0.06 [a], 0.02 [b]	0.00 [b]	0.11 [b] (2)
ΣC_i =	0.83	0.69	1.12

tion of pyruvate dehydrogenase as well as isocitrate and 2-oxoglutarate dehydrogenases by ADP and a lower NADH/NAD ratio (see Refs. 12, 13 for reviews). Activation of these three NAD-linked dehydrogenases by increasing the extramitochondrial free Ca^{2+} concentration ($[Ca^{2+}]_{ex}$) from 5 to 230 nM (inferred from the rise in the level of NAD(P)H, see Table I), abolished the aforementioned lag period. This increase in $[Ca^{2+}]_{ex}$ was used in a previous work (Ref. 16, see also Ref. 17) in the absence of Mg^{2+} to generate a near half-maximal activation of pyruvate dehydrogenase in heart mitochondria.

Since at the steady state the rate of all the partial reactions are equal [31], the rate of state 3 respiration may be considered to reflect the rate of ATP synthesis, in the consideration that the H^+ leakage and slippage are relatively low and the ADP/O ratios remain constant with the different interventions used in this study. Thus the contribution of the O_2 uptake not associated to ATP synthesis (state 4) can be discarded when net rates of ADP-stimulated respiration are considered.

The titration curves of the net rate of steady state 3 (ADP-stimulated) respiration with specific inhibitors of several enzymes of oxidative phosphorylation are shown in Fig. 1. It is noted that mersalyl was considered a specific inhibitor of the phosphate (P_i) carrier in the range of concentrations used in this work (0.2–6 μM). This was concluded from experiments where inhibition of the rate of arsenate- and uncoupler-stimulated respiration by mersalyl was evaluated. The plots of the rates of both activities versus mitochondrial protein concentration [32] showed that arsenate-stimulated respiration was irreversibly blocked, whereas uncoupler-stimulated respiration was unaffected. Considering that arsenate-stimulated respiration requires the transport of arsenate through the P_i carrier (see Ref. 6 and references therein) and the transport of oxidizable substrate through the pyruvate and dicarboxylate carriers [33], and that uncoupler-stimulated respiration depends only on the transport of oxidizable substrate, the data indicate that under these conditions mersalyl acts as a specific inhibitor of the P_i carrier.

The flux-control coefficients calculated from experiments similar to those shown in Fig. 1 are shown in Table I. The amounts of inhibitors required to obtain maximal flux inhibition (I_{max}) were (per mg protein): 1360 ± 46 pmol carboxyatractyloside ($n = 13$); 216 ± 14 pmol oligomycin ($n = 15$), 5.7 ± 0.3 nmol mersalyl ($n = 10$); 172 ± 10 pmol rotenone ($n = 13$); and 127 ± 23 pmol antimycin ($n = 5$). The ADP/O ratios were 3.2 ± 0.05 ($n = 5$) for mitochondria oxidizing 5 mM pyruvate; 3.17 ± 0.08 ($n = 6$) for 0.5 mM pyruvate + 5 nM Ca^{2+}; and 3.09 ± 0.05 ($n = 8$) for 0.5 mM pyruvate + 230 nM Ca^{2+}. This indicates that the interventions used in this work did not alter the degree of coupling between the respiratory chain and ATP synthesis.

The predominant controlling steps at low dehydrogenase activity (low pyruvate, low Ca^{2+}) were the ATP/ADP carrier and Site 1 of the respiratory chain (see Table I, middle column). Increase in flux by dehydrogenase activation with either high pyruvate (left column) or high Ca^{2+} (right column) concentrations diminished the control exerted by the ATP/ADP carrier and Site 1; this was accompanied by a parallel increase in the control exerted by the ATP synthase and the P_i carrier. A high flux control coefficient for the ATP synthase and a low flux control coefficient for the ATP/ADP carrier were previously reported for heart mitochondria oxidizing 7 mM pyruvate + 0.7 mM malate [5]. The control exerted by sites 2 and 3 was negligible in all conditions (see Table I). The control exerted by the pyruvate carrier was also small. This was determined from titration with the specific, non-competitive ($K_i = 6.3$ μM) inhibitor α-cyano-4-hydroxycinnamate [34]: $c_i = 0.08 \pm 0.03$ ($n = 3$) for 0.5 mM pyruvate + 5 nM Ca^{2+}, and $c_i = 0.02$ ($n = 2$) for 0.5 mM pyruvate + 230 nM Ca^{2+}.

The contribution of the NAD-linked dehydrogenases to the control of flux may be estimated by applying the summation theorem of the control analysis [35–37]. The theorem indicates that the sum of the flux-control coefficients of the steps involved in a pathway must be unity. Under conditions where NAD-linked dehydrogenase activity was low, the sum of the flux control coefficients was below 1.0 ($\Sigma c_i = 0.69$); this implies that the dehydrogenases (plus substrate transport) exerted a significant control on oxidative phosphorylation when mitochondria were incubated with low pyruvate and low Ca^{2+} concentrations, i.e., the estimated flux control coefficient for the NAD-linked dehydrogenases, particularly pyruvate dehydrogenase as well as isocitrate and 2-oxoglutarate dehydrogenases, was 0.31. On the other hand, under conditions in which the dehydrogenases were activated the sum of flux control coefficients was close to one; this indicates that at high pyruvate or high Ca^{2+} the dehydrogenases did not exert significant control on phosphorylating flux ($c_i = 0$). According to previous reports [2,3,6], the flux control coefficient of H^+ permeability, C_{H^+}, determined from the titration of state 3 respiration with the uncoupler CCCP [2], was negligible ($C_{H^+} = 0$) with 0.5 mM pyruvate and 5 or 230 nM Ca^{2+}. Also, C_{H^+} in state 4 conditions was 0.87 ± 0.07 ($n = 3$) and 0.92 ± 0.08 ($n = 3$) for 0.5 mM pyruvate and 5 or 230 nM Ca^{2+}, respectively.

The values of the flux control coefficients calculated from experiments made with kidney mitochondria showed that activation of dehydrogenases by increasing $[Ca^{2+}]_{ex}$ resulted in an increase in flux when mitochondria were incubated with a low pyruvate concentration (Table II). However, in this case, it was necessary to include ATP in the incubation medium to

TABLE II

Flux control coefficients of various steps in oxidative phosphorylation from kidney mitochondria oxidizing pyruvate

Mitochondria (1.2–2 mg protein/ml) were incubated for 5 min in 3 ml of standard medium which contained additionally 1 mM ATP, 1 mM EDTA, 0.5 mM pyruvate, 1 mM ma late and the indicated Ca^{2+} concentration as stabilized with a EGTA buffer. Other experimental details are given in Table I. The rates of State 4 respiration were 31 ± 2 ($n = 14$) and 25 ± 2 ($n = 12$) ngatom O_2/mg protein per min for 5 and 200 nM Ca^{2+} respectively. The amounts of inhibitors required for maximal inhibition were (per mg protein): 613 ± 40 pmol carboxyatractyloside ($n = 5$); 178 ± 11 pmol oligomycin ($n = 6$); 7.1 ± 0.5 nmol mersalyl ($n = 5$); and 86 ± 6 pmol rotenone ($n = 6$).

	5 nM Ca^{2+}	200 nM Ca^{2+}
% NAD(P)H in state 3	31 (2)	37 (2)
Rate of state 3 respiration (ngatom O_2/mg protein/min)	98 ± 7 (14)	150 ± 10 (12)
Step	C_i	
ATP/ADP carrier	0.15 ± 0.07 (5)	0.0 ± 0.0 (3)
ATP synthase	0.0 ± 0.0 (3)	0.32 ± 0.06 (4)
P_i carrier	0.28 ± 0.07 (3)	0.43 ± 0.15 (5)
Site 1	0.05 ± 0.08 (3)	0.31 ± 0.08 (5)
$\Sigma C_i =$	0.48	1.06

promote inactivation of dehydrogenases, particularly pyruvate dehydrogenase [16]; EDTA was also included in order to prevent ATP hydrolysis by contaminating ATPases. The predominant controlling step at low dehydrogenase activity (low Ca^{2+}) was the P_i carrier with a moderate contribution of the ATP/ADP carrier (Table II). Similarly to heart mitochondria, Ca^{2+} also induced a reciprocal change in the control exerted by the ATP/ADP carrier and the ATP synthase in kidney mitochondria. By applying the summation theorem, it became apparent that the dehydrogenases would exert a significant control at low ($\Sigma C_i = 0.45$), but not at high Ca^{2+} concentrations ($\Sigma C_i \approx 1.0$).

In an attempt to elucidate the molecular mechanisms underlying the reciprocal changes in control exerted by the ATP/ADP carrier and the ATP synthase, the variation in the transmembrane potential and in the intramitochondrial ATP/ADP ratio was explored. It was considered that these two potential modulators could affect differentially the carrier and synthase activities.

Measurement of changes in the transmembrane potential can be followed with the semiquantitative probe safranine [29]. Traces of the absorbance difference of safranine in suspensions of heart mitochondria incubated with different pyruvate and Ca^{2+} concentrations are shown in Fig. 2. Addition of ADP induced a transient diminution in the signal which depended, but slightly, on the concentrations of pyruvate (Fig. 2A) and Ca^{2+} (Fig. 2B). Measurement of TPP^+ distribution across the inner membrane showed that ADP induced a small, but significant diminution in the transmembrane potential: 167 ± 1 mV (mean $\pm$ S.E.) in state 4 vs. 159 ± 3 mV in state 3 ($n = 4$; $P < 0.01$) with 0.5 mM pyruvate + 1 mM malate as substrate. The increase of $[Ca^{2+}]_{ex}$ from 5 to 230 nM did not modify the level of the transmembrane potential in either state 4 or state 3: 163 ± 1 mV in state 4 and 158 ± 5 mV in state 3 ($n = 4$). Thus, the diminution in transmembrane potential that could be induced by an increase in flux was balanced by the Ca^{2+} activation of NAD-linked dehydrogenases [12–14]. Interestingly, in experiments

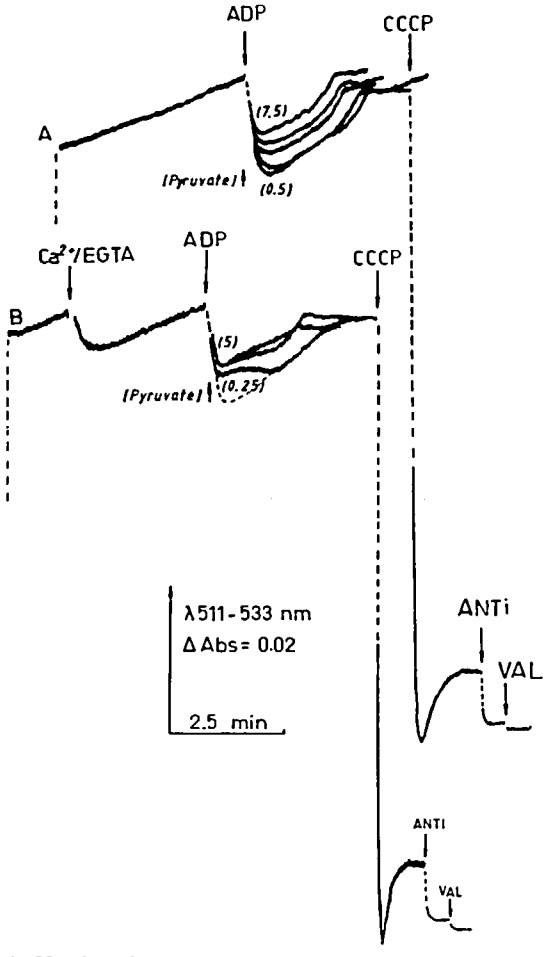

Fig. 2. Uptake of safranine in heart mitochondria. Mitochondria (1.6–1.9 mg protein/ml) were incubated in 2 ml of standard medium containing 10 μM safranine plus 1 mM malate and the following pyruvate concentrations: for the upper set of traces the concentrations were 0.5, 0.75, 1.25, 5 and 7.5 mM; for the lower set the concentrations were 0.25, 0.5 and 5 mM. The absorbance difference at 511–533 nm was measured as described under Methods. In (B) a Ca/EGTA buffer was added to give a $[Ca^{2+}]$ of 230 nM; the dashed line represents the result obtained with 0.5 mM pyruvate but not Ca^{2+}/EGTA buffer added. ADP was 1.3 mM, CCCP was 1 μM; antimcyin (Anti) 12.5 μM; valinomycin (VAL) 0.45 μM. The traces of the different experiments were superimposed using as reference the signal of zero transmembrane potential generated by addition of CCCP + antimycin + valinomycin. The results shown in the figure are representative of three different mitochondrial preparations.

with succinate as substrate [7], an increase in flux by increasing the P_i concentration induced a diminution of the transmembrane potential.

The redox state of cytochrome *c* in state 3 with 0.5 mM pyruvate as substrate was maintained at the same reduction level when $[Ca^{2+}]_{ex}$ was increased: $20 \pm 1\%$ cytochrome c^{2+} with 5 nM Ca^{2+} versus $22 \pm 2\%$ cytochrome c^{2+} with 230 nM Ca^{2+} ($n = 5$; means ± S.E.). The redox potential from NADH to cytochrome *c* in state 3 was of 579 mV with 5 nM Ca^{2+} and 585 mV with 230 mM Ca^{2+}. These values are comparable to those reported in blowfly muscle mitochondria oxidizing 2.5 mM pyruvate [28] and in rat heart mitochondria oxidizing 0.5 mM 2-oxoglutarate [40]. In addition, a lack of effect on the redox potential from NADH to cytrochrome *c* by Ca^{2+} has been reported [40]. Therefore, when an increase in phosphorylating flux is imposed, the activation of NAD-linked dehydrogenase by Ca^{2+} maintains the transmembrane potential at high levels [12–14]. Hence, the stability in the transmembrane potential and redox potential suggests that these parameters do not affect the control exerted by the ATP/ADP carrier and the ATP synthase at different flux levels.

The enzymatic determination of intramitochondrial adenine nucleotides showed that there were no significant differences in the ATP/ADP ratio with different pyruvate and Ca^{2+} concentrations: 0.66 ± 0.05 ($n = 5$) for 0.5 mM pyruvate + 5 nM Ca^{2+}; 0.76 ± 0.05 ($n = 5$) for 0.5 mM pyruvate + 230 nM Ca^{2+}; and 0.82 ± 0.04 ($n = 6$) for 5 mM pyruvate. In consequence, it would appear that the changes in activity of the carrier or the synthase cannot be ascribed to differences in the intramitochondrial ATP/ADP ratio, at least as reflected by determinations of total intramitochondrial adenine nucleotides. A more definitive answer should proceed from determinations of free and Mg-complex species, since the ATP/ADP carrier catalyzes the exchange of free ADP and ATP [41], whereas the ATP synthase uses Mg^{2+} complexes of adenine nucleotides [42].

The distribution of control of oxidative phosphorylation supported by 0.5 mM pyruvate oxidation under a less active phosphorylation state was also studied. A rate of 50–60% of the maximal state 3 respiration was established by addition of 0.4 mM Mg-ATP to mitochondria incubated with 1 unit hexokinase + 10 mM glucose. The predominant controlling step was the ADP-regenerating system ($c_i = 0.46$, $n = 2$), in agreement with data reported for liver mitochondria oxidizing succinate [2,43,44]. The flux control coefficients for the ATP/ADP and P_i carriers were 0.13 ($n = 2$) and 0.08, respectively, whereas the control exerted by the ATP synthase and the respiratory chain was negligible ($c_i = 0$). Then, using the summation theorem, the contribution of the NAD-linked dehydrogenases at 50–60% state 3 respiration would be estimated to be 0.33.

The control distribution of oxidative phosphorylation with 2-oxoglutarate (2-OG) as substrate in heart mitochondria is shown in Table III. In these conditions only 2-oxoglutarate dehydrogenase (OGDH) was activated by Ca^{2+}. A specific activation of isocitrate dehydrogenase by using isocitrate as oxidizable substrate was not feasible since the activity of the tricarboxylic acids carrier is very low in heart mitochondria [33]. When the OGDH activity was low (1 mM 2-OG, 230 nM Ca^{2+}), control was exerted by the phosphorylating system (see Table III, middle column). Activation of OGDH by increasing 2-OG concentration did not

TABLE III

Flux control coefficients of various steps in oxidative phosphorylation from heart mitochondria oxidizing 2-oxoglutarate

Mitochondria (0.9–1.4 mg protein/ml) were incubated as described under Methods with the indicated 2-oxoglutarate (2-OG) and Ca^{2+} concentrations. Further experimental details are given in the legend of Table I. The rates of State 4 respiration were 24 ± 3 ($n = 10$), 21 ± 3 ($n = 10$), and 27 ± 2 ($n = 10$) ngatom O_2/mg protein per min for 10 mM 2-OG, 1 mM 2-OG + 5 nM Ca^{2+}, and 1 mM 2-OG + 230 nM Ca^{2+} respectively.

	10 mM 2-OG +5 nM Ca^{2+}	1 mM 2-OG +5 nM Ca^{2+}	1 mM 2-OG +230 nM Ca^{2+}
% NAD(P)H in state 3	53 ± 1 (3)	31 ± 3 (4)	37 ± 1 (4)
Rate of state 3 respiration (ngatom O_2/mg protein per min)	180 ± 19 (10)	139 ± 14 (10)	204 ± 18 (10)
Step	C_i		
ATP/ADP carrier	0.32 ± 0.1 (4)	0.29 ± 0.05 (4)	0.02 ± 0.01 (4)
ATP synthase	0.22 ± 0.03 (3)	0.17 ± 0.02 (2)	0.14 ± 0.02 (3)
P_i carrier	0.25 (2)	0.31 (2)	0.02 ± 0.01 (4)
Site 1	0.03 ± 0.03 (3)	0.0 (2)	0.0
Site 2	0.04 (2)	0.05	0.22 (2)
Site 3	0.02	0.06	0.13 (2)
$\Sigma C_i =$	0.88	0.88	0.53

change the distribution of control of the six steps evaluated (Table III, left column). Activation of OGDH by Ca^{2+} resulted in a significant decrease in the control exerted by the ATP/ADP carrier and the P_i carrier; this was accompanied by an increase in the control exerted by the sites 2 and 3 of the respiratory chain (Table III, right column). In agreement with a previous work [26], addition of Ca^{2+} to mitochondria oxidizing a low 2-OG concentration induced an increase in flux that paralleled a change in mitochondrial NAD(P)H that was smaller than that induced by a high substrate concentration. This suggests that, in addition to the OGDH activation, Ca^{2+} may be activating some other sites, probably the respiratory chain [45,46] or the phosphorylating system [6,26,27]. Activation of OGDH by Ca^{2+} was better observed in state 4: $41 \pm 4\%$ NAD(P)H at 5 nM Ca^{2+} versus $68 \pm 2\%$ NAD(P)H at 230 nM Ca^{2+}.

An interrelation between the ATP/ADP and P_i carriers with Ca^{2+} fluxes has been suggested in experiments with high Ca^{2+} concentrations (Refs. 45, 47, 48; see Ref. 49 for a review). Application of the summation theorem to data of Table III indicated that the contribution of 2-OG dehydrogenation and transport would be low at 1 or 10 mM 2-OG, but high at high Ca^{2+} concentrations. Then, the diminution in the control exerted by the ATP/ADP and P_i carriers when Ca^{2+} is increased suggests an activating effect of Ca^{2+} on the phosphorylating system rather than on the respiratory chain.

Discussion

Recent studies on the relation between the rate of state 3 respiration and the level of NAD(P)H showed that at low substrate concentrations, dehydrogenase activity exerted a limitation of flux [25,26]. In this work a more quantitative approach was used in the evaluation of the role of the NAD-linked dehydrogenases. From the determination of the flux control coefficients with specific inhibitors and using the summation theorem of control analysis [34–36], an estimation of the control exerted by the dehydrogenases in different experimental conditions was made. When NAD-linked dehydrogenases activity was low (low pyruvate and Ca^{2+} and therefore low percentage NAD(P)H and flux) the sum of the flux control coefficients was 0.69 (see Table I); this indicated that the dehydrogenases control coefficient (plus substrate transport) would be 0.31. Stimulation of dehydrogenase activity by pyruvate or Ca^{2+} increased the value to nearly 1; this suggested that in these conditions the dehydrogenases control coefficient would be close to zero. The data of Tables II and III showed that, when the substrate or the type of mitochondrion is changed, significant control by dehydrogenases was also exerted in conditions that led to a low dehydrogenases activity, i.e., low substrate and Ca^{2+} concentrations. Due to a lack of specific inhibitors for the Ca^{2+}-sensitive dehydrogenases, this seems to be the only appropiate approach to determine the control exerted by the NAD-linked dehydrogenases. However, the values of the flux control coefficients derived from the summation theorem may represent a relative measurement since, in some conditions, the sum of the flux control coefficients may be greater than one (see Tables I and II). A sum of flux control coefficients higher than one has been reported for state 3 respiration supported by succinate oxidation [6]. Thus the summation theorem may require the following considerations when applied to oxidative phosphorylation:

(1) A possible partial channelling of the H^+ gradient between the respiratory chain and the ATP synthase [50]. In this case, the sum of the flux control coefficients in a pathway where metabolites are channelled between a few enzymes can be greater than 1, depending upon the degree of channelling [38,51].

(2) Some non-evaluated reactions could drain the main flux. These reactions would have negative flux control coefficient values [52], making the sum of the positive flux control coefficients larger than one. An obvious candidate in our system is the membrane H^+ permeability; this would drain the phosphorylating flux and in consequence increase the rate of respiration. To circumvent this problem, the net rates of state 3 respiration instead of absolute rates were used in the calculations. The rational is that state 4 O_2 uptake reflects passive H^+ leakage, whereas the ADP-stimulated O_2 uptake corresponds to phosphorylation rates. Indeed, the flux control coefficient of H^+ permeability for state 3 respiration was found to be negligible.

(3) The inhibitors used in the calculation of the flux control coefficients were considered irreversible. However, none of them follows a strict irreversible inhibition pattern [30,41,53,54]. These inhibitors correspond more to the tightly-bound type, where the dissociation constant has a value of a magnitude similar to the enzyme concentration (nanomolar range). The plot of [enzyme] versus rate in the presence of a tightly-bound inhibitor shows that there is remaining activity when [I] > [enzyme] [55]; this was the case for mersalyl and oligomycin (data not shown). Hence the minimal amount of inhibitor neccessary to attain maximal inhibition (I_{max}) would overestimate the amount of active enzyme. The flux control coefficient derived from a titration curve with a tightly-bound inhibitor would be also overestimated as well as the sum of the flux control coefficients. There is no simple way to calculate I_{max} when tightly-bound inhibitors are used; however, the tangent of the plot [I] versus rate at [I] = 0 when extrapolated to the abscissa gives a good approximation [55]. In our case, it was not possible to use this

type of plot, as titration of flux generated sigmoidal curves (see Fig. 1).

Despite these caveats, our data indicate that, because the contribution of the NAD-linked dehydrogenases to the control of phosphorylation flux is subject to changes induced by specific modulators (ATP/ADP, NADH/NAD, Ca^{2+}), the distribution of control and the pathway flux change according to the dehydrogenases activity.

Interestingly, in conditions where saturating concentrations of ADP (1 mM) and P_i (5 mM), and non-saturating concentrations of oxidizable substrates (0.5 mM pyruvate, 1 mM 2-OG) were used, the ATP/ADP carrier still contributed to a significant extent in the control of phosphorylation flux (see Tables I–III). Even though the cytosolic free ADP concentration may not change during active cardiac work, as determined by ^{31}P-NMR experiments [18,19], the results of this work indicate that the phosphorylating system, together with the NAD-linked dehydrogenases, share the control of oxidative phosphorylation, at least in conditions where the dehydrogenases activity can be modulated.

A similar pattern in the distribution of control was attained when flux was increased either by high pyruvate or high Ca^{2+} (Table I), but not when flux was increased by high 2-OG as compared to high Ca^{2+} (Table III). If both substrates and Ca^{2+} stimulate at the same point in the pathway, according to the summation theorem [35–37], a diminution in the flux control coefficient of the stimulated enzyme, and an increase in the flux control coefficient of the rest of the enzymes would take place. However, a complication in this analysis arises when the stimulation occurs at multiple sites, i.e., in mitochondria oxidizing pyruvate, Ca^{2+} activates three enzymes, whereas in mitochondria oxidizing 2-OG, Ca^{2+} activates only the 2-OG dehydrogenase; in consequence, the NADH supply to the respiratory chain, flux, and the transmembrane potential in state 3 are different in these conditions (see Tables I and III, and Fig. 2). In addition, Ca^{2+} may stimulate the respiratory chain [45,46] and the phosphorylating system [6,26,27]. In the light of these considerations, the pattern in the distribution of control would appear difficult to predict when flux is increased by two different substrates and compared to that obtained when flux is increased by Ca^{2+}.

The reciprocal change in control exerted by the ATP/ADP carrier and the ATP synthase, observed in heart and kidney mitochondria oxidizing pyruvate, a general phenomenon is probable in which a common mechanism modulates the activity of these two proteins. In fact, a similar reciprocal change in control has been determined in liver mitochondria oxidizing succinate [6,7]. However, in the latter case the flux control coefficient of the ATP/ADP carrier increased and that of the synthase decreased with the increase in flux; this was accompanied by a small but significant diminution of the transmembrane potential of about 10 mV [7]. In contrast, the transmembrane potential remained rather constant at different levels of flux with 0.5 mM pyruvate as substrate. In our experiments the changes in control exerted by the carrier and the synthase could involve slight variations of the transmembrane potential. Alternatively, with pyruvate as substrate, the maintenance of a constant value of the transmembrane potential when flux was increased can be explained in terms of a higher NADH accessibility to the respiratory chain induced by Ca^{2+} activation of dehydrogenases [12–14]; this situation is not found with succinate as substrate, where increase in flux by increasing P_i or Ca^{2+} concentrations [6,7] cannot cause NAD-linked dehydrogenase activation.

There are two possible common effectors for these proteins: $[Ca^{2+}]_m$ and the intramitochondrial ATP/ADP ratio. The total intramitochondrial ATP/ADP ratio was found to be of a similar magnitude in heart mitochondria oxidizing pyruvate or 2-OG. As a large fraction of intramitochondrial ADP is in the bound form [56], and the carrier exchanges free ADP by free ATP whereas the synthase uses the Mg-complex species, it would be more adequate to determine the relative concentrations of the free and Mg-species instead of total concentrations.

There is evidence for an activating effect of Ca^{2+} on the hydrolytic activity of the ATP synthase, either through a diminution in its interaction with an ATPase natural inhibitor protein [57], or to changes in the kinetic properties of the enzyme [58]. Even though the former effect of Ca^{2+} remains to be shown for the synthetic activity of the enzyme at different free Ca^{2+} concentrations, the data of the present work do not support such a mechanism, i.e., the flux control coefficient of the ATP synthase was increased by an increase in Ca^{2+} concentration with pyruvate as substrate (Tables I, II), whereas it was not modified with 2-OG (Table III); an activating effect of Ca^{2+} on the ATP synthase should have induced a diminution in its flux control coefficient.

A stimulation of the ATP/ADP carrier activity by Ca^{2+} would seem a more appropiate explanation, since the control exerted by this carrier was decreased by Ca^{2+} (Tables I–III). This would be consistent with the lack of effect of Ca^{2+} on oxidative arsenylation, a process that does not require flux through the ATP/ADP carrier [6]. An interaction between the ATP/ADP carrier and Ca^{2+} fluxes has been suggested [45,47–49]; furthermore an activation of the reconstituted carrier by Ca^{2+} has been reported [59]. However, the significance of such observations to the present studies remains to be explored, since the Ca^{2+} concentrations previously used were 2–3 orders of magnitude higher.

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