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Control of oxidative phosphorylations in yeast mitochondria. Role of the phosphate carrier

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This work describes the control of ATP synthesis and O_2 consumption as a function of external inorganic phosphate (P_i) concentration at steady states which are characterised by a high external (ADP/ATP) ratio. As a function of external P_i concentration both fluxes vary closely with each other and have a biphasic behaviour characterised by a rapid increase below 2 mM and a slow increase above this concentration. Nevertheless, the control of the fluxes is not always the same. The adenine nucleotide carrier exhibits no control in either flux whatever the external P_i concentration is. Cytochrome *c* oxidase is always a controlling step in both fluxes. At low P_i concentration, the proton leak controls both fluxes, but at high P_i concentration P_i transport takes the place of proton reentry as the other main controlling step. The steep threshold in external P_i concentration for which the exchange between the controlling steps occurs strongly depends on the proton permeability of the membrane. In ATP synthesis flux, proton permeability exerts a negative control (branched pathway) although the control is positive on the O_2 consumption flux. In accordance with the summation theorem, this property implies that the controls must be very different at some other steps of the two fluxes.

Introduction

The problem of the control of oxidative phosphorylations has been the object of a great deal of discussion over the last few years. There is indeed a popular belief that a few enzymes (if not a single one) are preeminent in bringing about the rate of metabolism fluxes and that most of the others can be ignored. Keeping this idea in mind, many authors have been looking for 'the rate-limiting step' in the control of oxidative phosphorylations in mitochondria. Adenine nucleotide translocator (e.g., Refs. 1–3) and cytochrome *c* oxidase (for a review, see Ref. 4) have been discussed as candidates for such a role. The behaviour of the cytochrome *c* oxidase as the unique limiting step was deduced from the observation of a near-equilibrium between the redox potential of the first two coupling sites and the external phosphate potential [4].

The application of Kacser and Burns's [5] and Heinrich and Rapoport's [6] theory of metabolic control by Groen et al. [7] afforded a beautiful solution to this long-standing riddle.

One of the most important predictions of the theory, repeatedly confirmed experimentally, is that control is shared between many steps within a given pathway. This is exactly one of the experimental conclusions of Groen et al.: using the glucose-hexokinase system to stimulate State 3 respiration in liver mitochondria, they have shown

Abbreviations: CCCP, carbonyl cyanide-*m*-chlorophenylhydrazone; TMPD, tetramethyl-*p*-phenylenediamine; P_i, inorganic phosphate; AdN, adenine nucleotides.

that the control of the oxygen-consumption rate is shared by the adenine nucleotide translocator, dicarboxylate carrier and cytochrome *c* oxidase.

Another prediction of the theory is that the distribution of control, as measured by the control coefficient (for the official terminology, see Refs. 8 and 9), can vary according to the different steady states. This predication has also been confirmed by Tager et al. [10], who have studied the control coefficient dependence of different steps as a function of respiration rate. Gellerich et al. [11] and Doussière et al. [12] have also shown that the control exerted by the adenine nucleotide translocator depends not only on the respiration rate, but also on the complexity of the metabolic network and on the mode of ATP utilization.

All these data have been obtained studying the respiratory flux. A relevant flux for the cell is the ATP regenerating flux, i.e., the rate of ATP synthesis. According to the chemiosmotic theory [13], it is quite widely accepted that in mitochondria the ATP synthesis flux is coupled to the respiration flux through the proton-motive force $\Delta\mu_{H^+}$. Nevertheless, this statement does not imply that these fluxes are identical, nor that their control is the same.

One of the aims of this paper is to compare the control of various steps on both fluxes in two different steady states. These two steady states are obtained by varying the inorganic phosphate concentration. Previous studies in our laboratory [14,15] have shown that P_i transport and ATP synthesis rates appear biphasic as a function of P_i concentration. Although this result is not surprising when considering proteins embedded within the membrane it allows for the study of these two fluxes over two separated ranges of P_i concentration (approx. below 1 mM and above 2 mM), where the ATP synthesis steady state seems to be different. As the P_i carrier mediates a co-transport H^+/P_i, it appeared worthwhile to study the control by P_i transport and H^+ leak of ATP synthesis and O_2 consumption as a function of external P_i concentration.

Materials and Methods

Chemicals

Sorbitol and mannitol (Prolabo) were purified by stirring with Dowex 50 W (Fluka). CCCP, Mersalyl and carboxyatractylate were purchased from Sigma Chemical Co., Snail gut juice from *Helix pomatia* was obtained from Industrie Biologique Française (Gennevilliers, France). [^{32}P] phosphate was from C.E.A. (Saclay, France).

Mitochondria preparation

Diploid *Saccharomyces cerivisiae* strain Yeast Foam cells were grown aerobically with 2% lactate as the carbon source in a complete medium containing 1% yeast extract/0.1% potassium phosphate/0.12% ammonium sulfate (pH 4.5). The cells were harvested in the logarithmic growth phase. Mitochondria were prepared as in Refs. 16 and 17.

Rates of respiration and ATP synthesis measurements

The oxygen consumption rate was measured polarographically at 27°C using a Clark electrode connected to a computer which gave an on-line display of rate values. The rate of ATP was measured by P_i incorporation as in Ref. 15. 0.2 mg/ml mitochondria were incubated in the following basal medium: 10 mM Tris maleate/0.65 M mannitol (pH 6.7), comprising 0.5 mM ADP/1 mM NADH. The kinetics were started by the addition of various concentrations of P_i (or $^{32}P_i$) as indicated. The external ATP concentration at this initial time is zero. O_2 consumption and ATP synthesis were always checked for linearity in order to ensure that the steady state was maintained during measurements. Inhibitors were incubated 1 min before P_i addition. NADH can be used as the respiratory substrate, since there is an NADH dehydrogenase on the external side of the inner membrane in yeast mitochondria [18]. Under these conditions, there is no limitation (no control) in the inflow of NADH. For most of the experiments described in this paper, both measurements were carried out simultaneously, the aliquot for labelled ATP evaluation being taken in the Clark oxygen electrode chamber. The protein concentration was measured by the biuret method using bovine serum albumin as a standard.

Determination of control coefficients

The control coefficients of various steps in-

volved in the oxidative phosphorylations were determined with specific inhibitors of these steps according to the definition

$$C_i = \left. \frac{\frac{\partial \ln J}{\partial I}(I=0)}{\frac{\partial \ln v_i}{\partial I}(I=0)} \right|_{\text{steady state}}$$

i.e., the ratio of the initial slope of the whole inhibition curve $J(I)$ over the initial slope of the inhibition curve of the isolated step $v_i(I)$ in the same conditions as in the pathway. Details concerning the measurement of individual control coefficients will be discussed in the Results section.

Result

Dependence of oxidative phosphorylation on P_i concentration

The dependence of respiration J_{resp} and ATP synthesis J_{ATP} on P_i concentration is shown in Fig. 1. Experimental points are phenomenologically fitted with a sum of two hyperbolas, whereas a fit with a single hyperbola gives a much less accurate fit. The estimated half-effects are in both cases close together ($K_1 = 0.16$–0.3 mM and $K_2 \approx 10$ mM), in accordance with the coupling of the two phenomena. The existence of these two hyperbolas can be an indication of two kind of steady states involving different controlling steps. In order to check this point, we chose 0.5 mM and 7.7 mM as external P_i concentrations representative of these two different steady states.

Control of oxidative phosphorylations

Cytochrome c oxidase

A direct approach to calculate the control coefficient of a given step within a pathway is to measure experimentally the initial slope of the inhibition curves of both the whole flux and the isolated step. This was done for the cytochrome *c* oxidase activity using its specific inhibitor potassium cyanide. This step was isolated from the respiratory chain by means of antimycin, with TMPD + ascorbate as the electron donor system. The rate was followed by oxygen consumption (Fig. 2C) and the initial slope (i.e., for no KCN

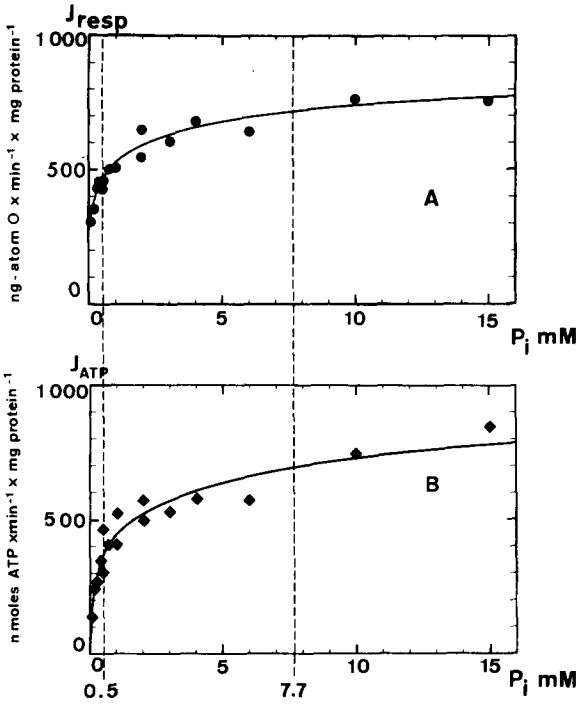
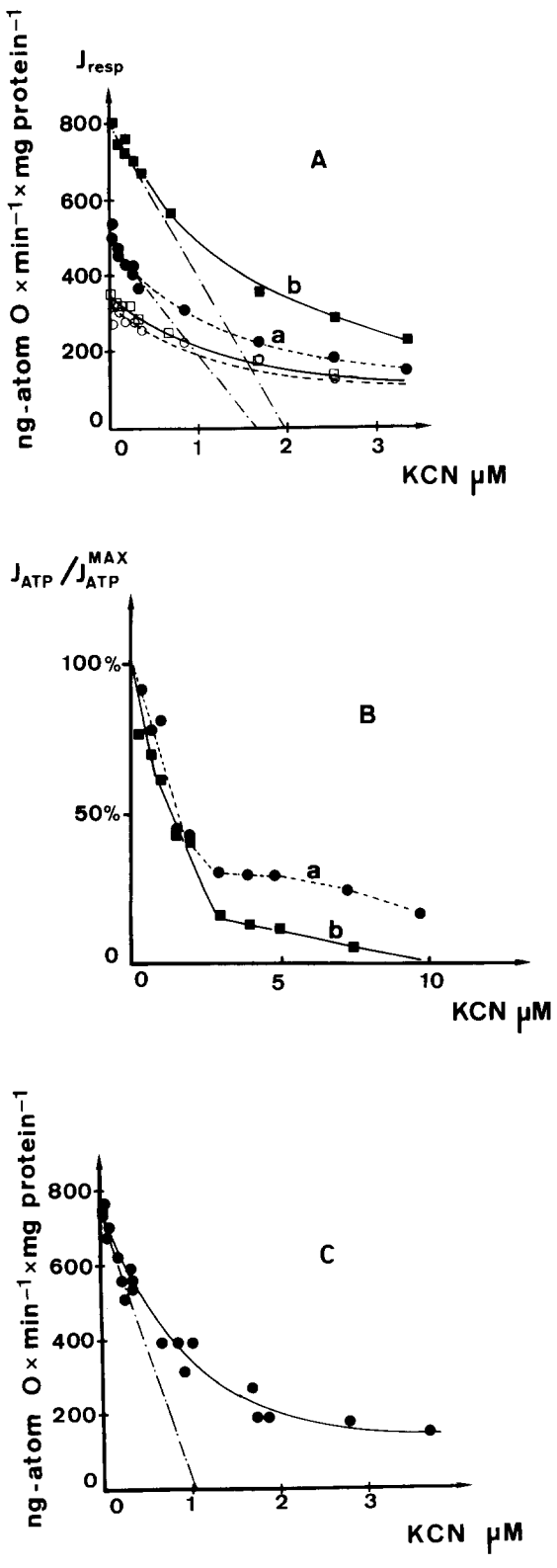


Fig. 1. Rate of O_2 consumption (A) and of ATP synthesis (B) as a function of P_i concentration. 0.5 mM and 7.7 mM are the P_i concentrations of the studied steady states. The experimental conditions are described in Materials and Methods.

present) found to intercept the *X*-axis at 1 μM KCN. This value has to be compared with the initial slopes of the whole fluxes J_{resp} and J_{ATP} at the two chosen steady states (0.5 mM and 7.7 mM P_i) (Fig. 2A and B). The intersection is then around 2 μM KCN. This shows that cytochrome *c* oxidase always controls both fluxes to a great extent (see Table I).

Adenine-nucleotide transport

It is not always necessary to measure the initial slope of an isolated step, since cases exist for which the theoretical inhibition expression is known (such as competitive, non-competitive, irreversible inhibitions) as pointed out by Groen et al. [7]. For instance, inhibition of the adenine nucleotide carrier by carboxyatractylate is known to be an irreversible process. Groen et al. derived a control-coefficient theoretical expression for such a case (Eqn. 5 in Ref. 7). Fig. 3 shows that the AdN translocator control is always zero, except



perhaps for J_{ATP} at $[P_i] = 7.7$ mM. Fig. 3B-b illustrates the difficulty of control-coefficient determination. Depending on the number of experimental points taken for the slope determination, the actual value lies between 0 and 0.4. This situation is probably due to the fact that under these conditions the adenine nucleotide translocator is just on the point of controlling (see Discussion).

Proton leak

In order to determine the control coefficient of J_{resp} and J_{ATP} by the proton leak step, we used the method described in Ref. 7 by Groen et al. They measured the respiration rate increase on the addition of an uncoupler, in the presence of oligomycin to block the phosphorylation step. They showed that over a wide range of uncoupler concentrations, the curve is a straight line which enables them to give an analytical expression for the control coefficient of this step (Fig. 3 in Ref. 7). We also observed a linear increase in the respiration rate as a function of the amount of uncoupler in the presence of oligomycin (Fig. 4). Fig. 5A and B show the effect of CCCP on the two fluxes. We find again the now classical results that proton leak is a controlling step at low respiration rates ($[P_i] = 0.5$ mM) and hence low ATP synthesis (Fig. 5A-a and 5B-a). At $[P_i] = 7.7$ mM, when the respiration rate reaches almost a maximum, no control is exerted by the proton leak. It must be pointed out that the proton-leak control coefficient of ATP synthesis flux is negative (branched pathway [19], see Table I).

P_i transport

The control of the P_i transport step was evaluated with the P_i carrier inhibitor mersalyl.

Fig. 2. Inhibition by KCN of O_2 consumption flux (A), ATP synthesis flux (B) and isolated cytochrome *c* oxidase activity (C). For (A) and (B) the conditions are given in Materials and Methods: (a) $[P_i] = 0.5$ mM (●); (b) $[P_i] = 7.7$ mM (■). The effect of KCN was also measured on the respiration without ADP: $[P_i] = 0.5$ mM (○); $[P_i] = 7.7$ mM (□). For the isolated cytochrome *c* oxidase activity measurements, mitochondria (0.2 mg protein per ml) were incubated in the basal medium containing 1 mM NADH, 0.1 μg per ml antimycin. 1 μM CCCP and various concentrations of KCN. The reaction was started by simultaneous addition of 0.7 mM ascorbate and 0.7 mM TMPD.

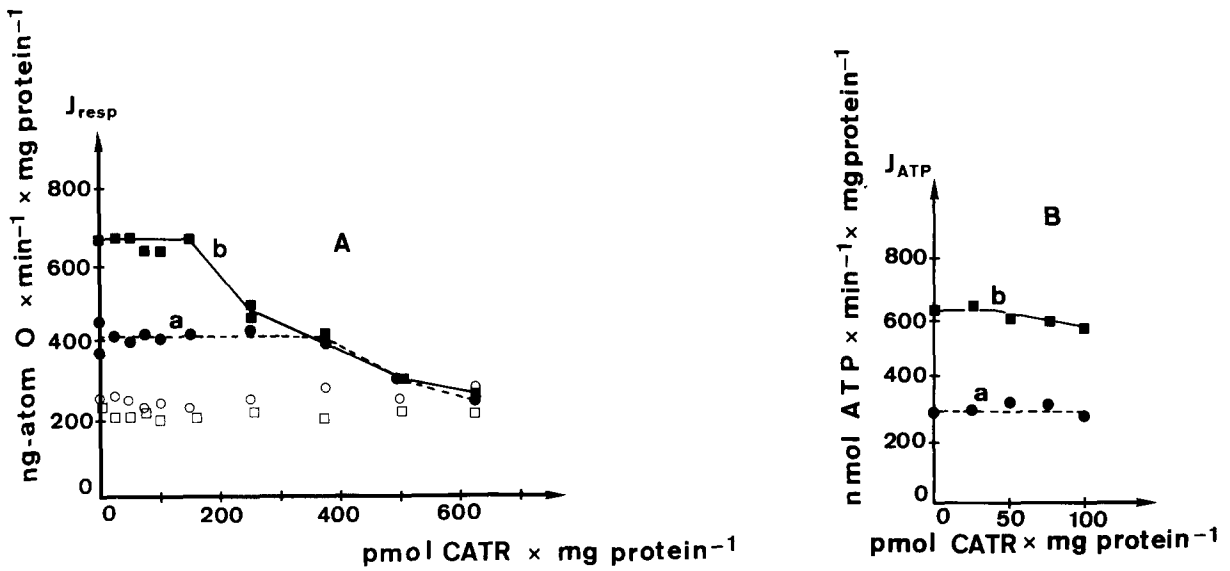


Fig. 3. Carboxyatractylate (CATR) effect on O_2 consumption flux (A) and ATP synthesis flux (B). (a) P_i = 0.5 mM (●); (b) P_i 7.7 mM (■); P_i = 0.5 mM without ADP (○); P_i = 7.7 mM without ADP (□). The other conditions are described in Materials and Methods.

The reference initial slope was obtained from the inhibition of swelling by mersalyl [20] that gave a value of 1 mg protein per 15 nmol Mersalyl. Fig. 6 shows that P_i transport exerts no control either on oxygen consumption or on ATP synthesis at the low P_i concentration (Fig. 6A-a and 6B-a). Nevertheless, at high P_i concentration, P_i transport becomes a controlling step (Fig. 6A-b and 6B-b). This last phenomenon required a more complete study, at least to compare it to the biphasic curve in Fig. 1. We then evaluated the control coefficients of both fluxes over a continuous range of P_i concentration (Fig. 7 for ATP synthesis flux). For both fluxes, a transition from zero to a high control coefficient (> 0.5) is observed with increasing P_i concentration. The threshold value of P_i concentration is around 2 mM and corresponds rather well with the transition between the two phenomenological hyperbolas in Fig. 1. The use of different mitochondrial preparations suggested to us that there was a relationship between the P_i concentration at the threshold and the degree of coupling. That prompted us to investigate the CCCP effect on the control of ATP synthesis by P_i carrier. If this relationship does exist, then the presence of an uncoupler at a low P_i concentration will trigger the control by the P_i transport step on the phosphorylation flux. As it turns out, Fig. 7 shows that at $[P_i] = 2$ mM there is no control on the ATP synthesis in the absence of CCCP, but a control coefficient of 0.7 for ATP synthesis in the presence of 0.4 μM CCCP (this amount of CCCP decreases the ATP synthesis rate by approx. 50% and does

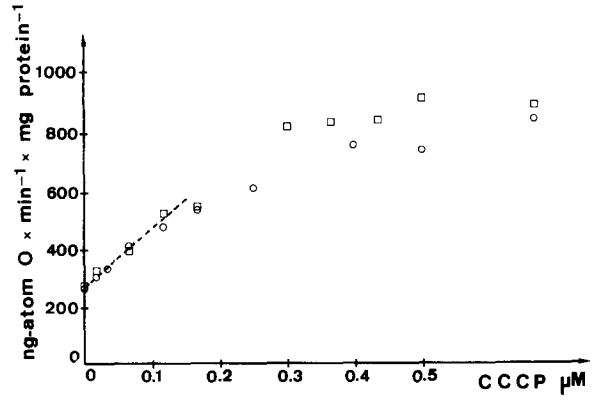


Fig. 4. CCCP effect on oligomycin-treated mitochondria. Mitochondria (0.2 mg protein per ml) were incubated in the basal medium containing 5 μg per ml oligomycin, 1 mM NADH and 0.5 mM P_i (○) or 7.7 mM P_i (□) and different CCP concentrations.

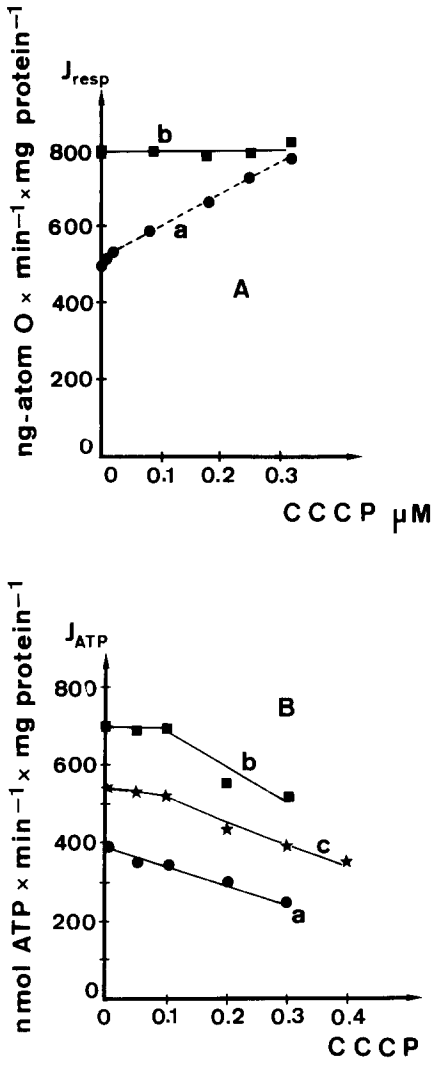


Fig. 5. CCCP Effect on O_2 consumption flux (A) and on ATP synthesis flux (B). (a) $[P_i] = 0.5$ mM (■); (b) $[P_i] = 7.7$ mM (■); (c) $[P_i] = 2$ mM (★). The other conditions are described in Materials and Methods.

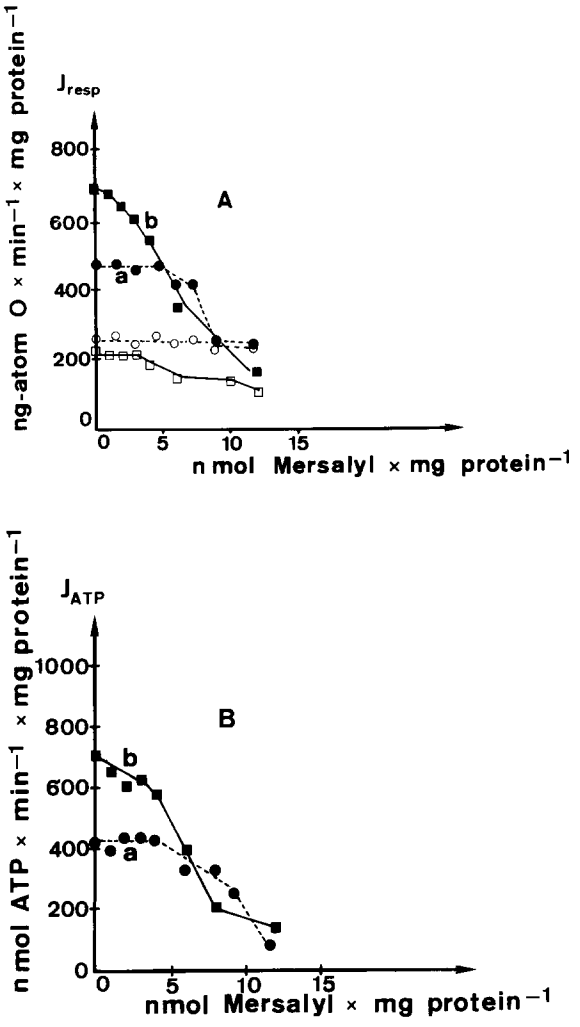


Fig. 6. Mersalyl effect on O_2 consumption flux (A) and ATP synthesis flux (B); (a) $[P_i] = 0.5$ mM (●); (b) $[P_i] = 7.7$ mM (■); $[P_i] = 0.5$ mM without ADP (○); $[P_i] = 7.7$ mM without ADP (□). The other conditions are described in Materials and Methods

not change the initial slope of the inhibition curve of swelling by mersalyl).

Discussion

Table I summarises the control coefficient values determined in this work for the two fluxes: O_2 consumption (J_{resp}) and ATP synthesis (J_{ATP}) at the two steady states according to the P_i concentrations: $[P_i] = 0.5$ mM and $[P_i] = 7.7$ mM. The values in Table I give the range of control coefficients determined from several experiments.

The control of cytochrome *c* oxidase is rather high in all cases ($0.3 < C < 0.6$). It is now well established that this step is indeed a controlling one in mitochondria from various origins. With respect to the proton leak we find the same result as Groen et al. [7], i.e., this step plays a role when the oxidative phosphorylation rate is low.

The adenine-nucleotide carrier exerts no control

TABLE I

Control coefficients of the different steps are listed in column (1). The specific inhibitors used are indicated in brackets (CATR, carboxyatractylate). Columns (2) and (3) concern the respiratory flux, columns (4) and (5) the ATP synthesis flux. In columns (2) and (4) the steady state is obtained at $[P_i] = 0.5$ mM and in columns (3) and (5) at $[P_i] = 7.7$ mM. The intervals given here correspond to the value of the control coefficients determined from several experiments. Control coefficients for ATP synthase are not determined (see text).

Step	Control coefficients			
	J_{resp}		J_{ATP}	
	$[P_i] = 0.5$ mM	$[P_i] = 7.7$ mM	$[P_i] = 0.5$ mM	$[P_i] = 7.7$ mM
(1)	(2)	(3)	(4)	(5)
AdN carrier (CATR)	0–0.2	0	0	0–0.4
Proton leak (CCCP)	0.3–0.4	0	(−0.2)–(−0.4)	0
P_i carrier (Mersalyl)	0	0.4–0.6	0	0.4–0.6
Cytochrome *c* oxidase (KCN)	0.5–0.6	0.5–0.6	0.3–0.4	0.3–0.4

except perhaps on J_{ATP} at $[P_i] = 7.7$ mM. In these conditions, the adenine translocator seems to be at the threshold where it passes from a non-controlling region to a controlling one. In the threshold transition, the determination of the control coefficient is very sensitive to many external parameters including the preparation conditions of mitochondria.

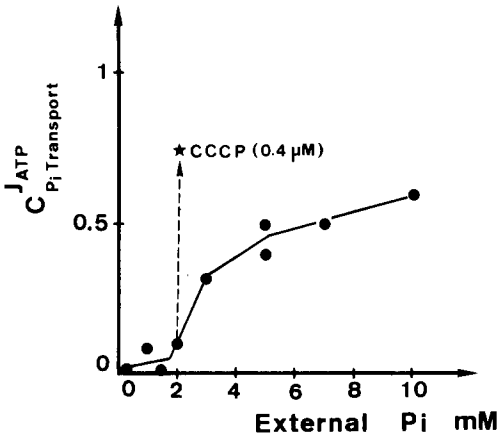


Fig. 7. Control coefficient of P_i transport on ATP synthesis flux as a function of P_i concentration (●). In order to calculate the control coefficients we use the initial slope of the inhibition curve of J(ATP) by mersalyl at each P_i concentration as in Fig. 6 and the initial slope reference value of 1 mg protein per 15 nmol Mersalyl [20] using the relationship given in Materials and Method. Control coefficient of P_i transport on ATP synthesis flux at $[P_i] = 2$ mM in the presence of 0.4 μM CCCP (★).

Control by P_i carrier

A control by the P_i carrier could appear surprising if we remember the kinetic parameters of rat liver mitochondria: a V_{max} of 3000 nmol/min per mg protein in non-respiring conditions and of 2700 nmol/min per mg protein in respiring ones [24]. Three properties must be taken into account.

(1) The P_i transport only exerts a control at high P_i concentration (Fig. 6 and 7), i.e., in a range where $\Delta\mu_{H^+}$ is decreased (210 mV at $P_i = 0.5$ mM and 170 mV at $P_i = 7.7$ mM) and ΔpH is low (0.3 units at $P_i = 7.7$ mM instead of 0.7 units at $[P_i] = 0.5$ mM [21]). As the P_i transport is a co-transport H^+/P_i, a decrease in ΔpH will limit the maximal rate of P_i carrier in the same way as in a classical two substrates reaction, i.e., the limitation by one substrate decreases the V_{max} for the other substrate. This interpretation is confirmed by the increase of control by the P_i carrier at a low P_i concentration in the presence of an uncoupler (Fig. 7). Under these conditions no control exists in the absence of the uncoupler. This result is in accordance with that of Baggetto et al. on newborn rat liver mitochondria, on which they find a greater permeability to protons and a marked increase in sensitivity to mersalyl [22].

(2) At high P_i and ADP concentrations, the flux of ATP synthesis is around 700 nmol ATP · min^{-1} · mg^{-1}. This value is only 4-times less than the V_{max} of the unidirectional P_i transport in respiring rat liver mitochondria [24].

(3) The V_{max} which have been measured are the V_{max} of the unidirectional transports. However, at steady state, the relevant parameter which must be taken into account is the net flux, i.e., the difference between P_i influx and efflux. This difference is indeed lower than each unidirectional flux.

All these characteristics can explain a control by the P_i carrier at high P_i concentration or at low P_i concentration in the case of partial uncoupling.

It should be pointed out that the threshold transition between no control and a high P_i carrier control coefficient occurs at an external P_i concentration around 1–2 mM, which is exactly the same as the P_i concentration where the transition between the two phenomenological hyperbolas occurs in Fig. 1. Since this transition occurs in a physiological range of the P_i concentration, this result could reflect a regulatory role on ATP synthesis played by the P_i carrier through the H^+/P_i co-transport mechanism. In yeast, this kind of control should be particularly relevant because of the multiplicity of physiological steady states.

Comparison between respiratory rate and ATP synthesis

One of the aims of this work was to compare the fluxes of O_2 consumption and of ATP synthesis and the control on both of these fluxes.

Fig. 1 shows that the two fluxes vary in a similar fashion as a function of P_i concentration with identical apparent K_m. Fig. 6 also emphasises that the control by P_i transport as a function of P_i concentration on both fluxes varies in a similar way. Nevertheless, not all the control coefficients are similar on both fluxes. The greater difference is observed for the control by an uncoupler at low respiratory rate or low ATP synthesis ($[P_i] = 0.5$ mM). One is positive and the other one is negative (see Table I). The negative value of this control coefficient has another important consequence: in order to fulfil the summation theorem for both fluxes, some of the other control coefficients must be very different. One step can exhibit a great difference in its control towards both fluxes: the ATP-synthase itself. Our measurements with oligomycin (not shown) are not easy to interpret mainly because we lack the reference inhibition curve on an 'isolated' ATP synthase under the same conditions. We are presently looking for other inhibitors in order to overcome this problem.

The main reason that differences are observed between the two fluxes can be explained as follows: the proton flux generated by the respiratory chain $J^{H^+}_{resp}$ breaks up into (i) the flux of ATP synthesis, (ii) the flux of transports (e.g., P_i and AdN transport), (iii) the proton leak.

$$J^{H^+}_{resp} = J^{H^+}_{ATP} + J^{H^+}_{Transp} + J^{H^+}_{leak}$$

For various steady states the distribution of $J^{H^+}_{resp}$ between the three fluxes of the protons' reentry will be different, and thus there will be no simple relationship between $J^{H^+}_{resp}$ proportional to the respiratory rate and $J^{H^+}_{ATP}$ related to the rate of ATP synthesis. Consequently, there is no reason for the control coefficient to be the same in all cases on the respiratory rate and the ATP synthesis rate.

Summation theorem. Difficulties in the control measurements by the inhibitor method

One of the predictions of the theory is that the sum of all the control coefficients of a given flux is equal to 1. Table I indicates that the summation theorem is nearly verified, except for the flux of ATP synthesis J_{ATP} at $[P_i] = 0.5$ mM. There are several reasons why the sum of the control coefficients can differ from one: (1) not all the steps have been studied, e.g., the NADH-dehydrogenase step due to the lack of a specific inhibitor; (2) limitations in the determination of the control coefficients (for a review, see Ref. 23).

In our work, we encountered some other difficulties. The main difficulty arose from the determination of the reference slope value deduced from the inhibition curve of the 'isolated' steps which are catalyzed by enzymes embedded in a membrane and in some cases involving the membrane itself as a required barrier (e.g., titration of the ATP synthase 'isolated' step by oligomycin). Another difficulty in line with the previous one was to maintain the 'same conditions' for the isolated step. For example, in the case of the proton leak step, $\Delta\mu_{H^+}$ must be set at the desired value.

The last difficulty we were faced with was that of determining the initial slopes. In some cases a little shoulder seemed to appear which was very

difficult to estimate. This situation seemed to arise at the passage from a non-controlling region to a controlling one. The inhibition curves in the first region exhibited an initial plateau, the length of which decreases as the steady state entered the second region. The role of CCCP on J_{ATP} as a function of P_i concentration (Fig. 5B-c) illustrates this situation: at $[P_i] = 2$ mM it is very difficult to decide whether or not the initial slope is zero.

We also encountered this situation with the adenine nucleotide translocator for J_{ATP} at $P_i = 7.7$ mM and perhaps at $P_i = 0.5$ mM for J_{resp}.

These difficulties should not diminish the interest that lies in evaluating control coefficients especially in the determination of controlling steps and their evolution when steady states vary.

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