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Conditions allowing different states of ATP- and GDP-induced permeability in mitochondria from different strains of *Saccharomyces cerevisiae*

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Abstract

The effect of ATP and other nucleotides on the respiration of *Saccharomyces cerevisiae* mitochondria was investigated. It was observed that ATP induced a stimulation of the respiration rate only in the presence of a salt in mitochondria from the baker's yeast Yeast Foam, whereas an ATP-induced stimulation occurred even in the absence of salt in mitochondria from three different laboratory strains. In both cases, the stimulation was related to a collapse of the transmembrane potential, suggesting the opening of ion- and/or proton-conducting pathways. Not only ATP, but also GTP and CTP, induced these pathways. Moreover, a similar stimulation was obtained with GDP and its analog GDP-β-S. The fact that, as opposed to NTPs, GDP did not induce any non-specific anion channel, allowed us to use it to demonstrate unambiguously that a proton-conducting pathway was opened through the inner mitochondrial membrane of laboratory strains but not of Yeast Foam. Three additional aspects of this nucleotide-induced permeability were investigated. (i) The proton-conducting pathway was insensitive to Mg^{2+}, whereas the anion-conducting pathway was fully inhibited by 4 mM Mg^{2+}. (ii) The proton-conducting pathway of mitochondria isolated from laboratory strains was opened by the action of nucleotides outside the mitochondrion, since it was fully insensitive to (carboxy)atractyloside, and fully active in mitochondria isolated from op1 and Δanc strains. On the other hand, the cation-conducting pathway of Yeast Foam mitochondria was partly sensitive to (carboxy)atractyloside and insensitive to bongkrekic acid, suggesting a role of the conformational state of ANC in this activity. (iii) Both the proton and cation-conducting pathways were inhibited by very low concentrations of vanadate, under conditions where this oxyanion was polymerized to decavanadate, a competitor to nucleotide-binding sites on some enzymes.

Keywords: Yeast mitochondrion; Ionic permeability; Nucleotide; Transmembrane potential; Vanadate

1. Introduction

According to the chemio-osmotic theory [1], the energetic intermediate between substrate oxidation at the level of the mitochondrial respiratory chain and ATP synthesis, is the delocalized electrochemical proton gradient maintained by the respiratory complexes. A basal requirement to this hypothesis is the

Abbreviations: AMP-PCP, β,γ-methyleneadenosine 5′-triphosphate; ANC, adenine nucleotide carrier; Ap5A, P^1,P^5-di(adenosine) 5′-pentaphosphate; GDP-β-S, guanosine 5′-*O*-(2-thiodiphosphate); GMP-PNP, β,γ-imidoguanosine 5′-triphosphate

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intrinsic low proton permeability of the inner mitochondrial membrane, allowing the proton gradient to be used by the systems involved in ATP synthesis (i.e., FoF1-ATP synthase, phosphate/H^+ cotransport and adenine nucleotides carrier). This low permeability can also be extended to other cations (essentially K^+) and to anions (phosphate, TCA cycle intermediates...) whose uncontrolled movements down the transmembrane potential may be potentially harmful for mitochondrial metabolism and membrane structure. For this reason, the existence of electrogenic movements of cations and anions through the inner membrane has been for a long time considered of poor physiological significance (see [2] for a review of pioneering works).

However, the necessity of mitochondrial volume to be regulated and the action of physiological effectors (e.g., thyroid hormones) or pathological conditions (e.g., ischemia), drove many investigators to focus their interest on this problem [3,4]. The major part of these works was done on mammalian mitochondria in which a wide variety of electroneutral, anionic, cationic and unspecific transport pathways have been characterized (see Refs. [5–11] for reviews).

An unquestionable approach of the physiological significance of these systems should be the inactivation of the genes involved in these activities and the study of the consequences on the mitochondrial metabolism. But this is not easy to conduct in mammalian cells, whereas the facultative aerobic yeast *Saccharomyces cerevisiae*, with the powerful tools of molecular biology, could have been a useful model. However, studies done by us and other investigators showed that yeast mitochondria were, in this aspect, somewhat different from mammalian mitochondria.

The $K^+(Na^+)/H^+$ exchange of yeast mitochondria appeared to be different from the mammalian system, since it was not sensitive to Mg^{2+} [12,13] which is considered as the main physiological regulator in mammalian mitochondria. On the other hand, the yeast system was, as the mammalian system, sensitive to Zn^{2+} [12,14]. The usefulness of other classical inhibitors of this activity (amphiphilic amines, dicyclohexylcarbodiimide) [15,16] appeared to be limited in yeast mitochondria [17].

An uniport to Cl^-, sensitive to Mg^{2+}, was identified in yeast mitochondria [18,19] which, on the basis of its specificity and pharmacology, did not appear to be an equivalent of the Inner Membrane Anion Channel of mammalian mitochondria [3,6].

Large conductances, which were observed in the outer mitochondrial membranes [20,21], and identified as a channel sensitive to signal import peptides of mitochondrial proteins (PSC) [22,23] were recently observed in mitoplasts [24]. Other authors observed a large conductance, identified as an equivalent of the mammalian mitochondrial multiconductance channel (MCC) [25,26], also known as the mitochondrial megachannel (MMC), and which reflects the electrical activity of the permeability transition pore (PTP) [10,27]. However, this conductance also exhibited some similarities with the PSC and the main characteristics of the mammalian large conductance, namely cyclosporin A-sensitivity, was not assayed on yeast mitochondria. The existence of a large (nS range) conductance in the inner mitochondrial membrane of yeast is therefore not ascertained.

A potassium-uniport, opened by ATP depletion at high pH (> 7.4), was identified [19] which could be an equivalent of the ATP-sensitive K^+-uniport of mammalian mitochondria [28,29]. However, Ballarin and Sorgato [30], who identified an ATP-sensitive conductance in yeast mitochondria, showed that it was slightly anionic, whereas the ATP-sensitive conductance of mammalian mitochondria was clearly cationic and specific for K^+ over other cations [31].

Quite surprisingly, it was found that ATP could also induce a range of permeabilities in yeast mitochondria, whereas such a phenomenon have never been observed, to our knowledge, in mammalian mitochondria. ATP was first found to induce a decrease of the transmembrane potential driving to a marked stimulation of the respiration [32,33]. These authors interpreted this observation as the opening of a proton-conducting pathway, able to uncouple oxidative phosphorylation. This interpretation was questioned by the observation that ATP could open an unspecific channel [34]. This channel being mostly anionic, it was identified by Ballarin and Sorgato to a slightly anionic conductance of which the open state probability was increased by ATP [30]. By working on the same material as Guérin et al. [34], we identified an ATP-induced collapse of the transmembrane potential, which occurred only in the presence of K^+ and we interpreted this phenomenon as the opening of a K^+-conducting pathway [35].

The aim of the present paper was to reinvestigate, under different conditions and on different yeast strains, these ATP-induced permeabilities, in order to understand the discrepancies reported by Prieto et al. [32,33], Guérin et al. [34] and us [35]. We observed that the functioning of this system was dependent on the strain and on the respiratory substrate. In addition to ATP (and other nucleotides triphosphate), it could be induced by GDP and its analog GDP-β-S. It also appeared to be dependent on the conformational state of the adenine nucleotide carrier. It was sensitive to very low concentrations of sodium vanadate, but the efficient chemical species seemed to be decavanadate.

2. Material and methods

The characteristics of the industrial wild-type strain Yeast Foam (Gift from Professor P.P. Slonimski, Gif/Yvette), the laboratory wild-type diploid strain W303, the laboratory wild-type haploid strains W303-1A, D273-10B/A and AB1-4A/8 and the mutant strains 777-3A [36], JL1-3-1A [37] and DBY747/b5 [38] are given in Table 1.

Cells were grown aerobically at 28°C in a semi-synthetic medium containing 1% Yeast Extract (Gibco), 0.1% potassium phosphate, 0.12% ammonium sulfate, 2% D,L-lactate, adjusted at pH 5.0 with NaOH. 777-3A and JL1-3-1A were grown in a 1% Yeast Extract, 1% Bacto-peptone (Gibco), 2% galactose medium, pH 5.0. Control experiments were done on their respective wild-types grown on the same medium. Cells were harvested in the mid-exponential growth phase ($A_{550nm} = 3–6$) and mitochondria were isolated as described previously [39]. Mannitol and sorbitol used in preparation buffers are routinely deionized. Proteins were measured by the biuret method. It should be noted that certain experiments presented herein could be done on mitochondria frozen as small beads in liquid nitrogen, stored at −80°C, and quickly thawed. This did not qualitatively change the observations described in this paper but may change them quantitatively.

Respiration was monitored with a Clark electrode. Mitochondria were suspended at 28°C in a 10 mM Tris/maleate buffer containing 2 mM EGTA, 0.3% BSA and either 0.6 M mannitol, or 0.4 M mannitol and 0.1 M of a salt (see results).

Transmembrane potential ($\Delta\Psi$) was monitored as the red-shift of the absorbance spectrum of rhodamine 123 following energization. Mitochondria were suspended in the same buffers as above added with 1 μg/ml rhodamine 123 and the signal was recorded in a SLM-Aminco DW2000 spectrophotometer in Dual mode (516–495 nm).

Mitochondria swelling was recorded at 520 nm in a Secomam S1000 spectrophotometer.

Preparation of different forms of vanadate [40]: the stock solution refers to a solution of 0.1 M sodium orthovanadate. *Orthovanadate*: the stock solution was adjusted at pH 10.5 with sulfuric acid/NaOH, and boiled for three minutes. This solution was translucent, which is characteristic of the vanadate monomer. *Decavanadate*: the stock solution is adjusted to pH 6.0 with sulfuric acid: this solution had an orange color, which is characteristic of the vanadate decamer. *Metavanadate*: the stock solution was adjusted to pH 8.0 with sulfuric acid: it had a bright yellow color, which is characteristic of the vanadate tetramer. Boiling and alkaline pH hydrolyse polyvanadate species (meta and deca) to orthovanadate.

Table 1
List and characteristics of *Saccharomyces cerevisiae* strains

Yeast Foam	Baker's yeast; wild-type diploid
W303	wild-type diploid; MATa/α, ade2-1, leu3-112, his3-11, trp1-1, can1-100, ura3-1
W303-1A	wild-type haploid, MATa, ade2-1, leu3-112, his3-11, trp1-1, can1-100, ura3-1
D273-10B/A	wild-type haploid, MATα, met6
AB1-4A/8	wild-type haploid, MATa, his4
777-3A	mutant haploid, isogenic to AB1-4A/8, MATα, ade2, op1
JL1-3-1A	mutant haploid, constructed from W303-1A MATa, ade2-1, leu3-112, his3-11, trp1-1, can1-100, ura3-1, anc1::LEU2, anc2::HIS3, anc3::URA3
DBY747/b5	MATa, his3Δ1, leu2 3-12, ura3-52, trp1-289a, POR::URA3

These solutions were diluted in water (at 1 mM) and then assayed on the stimulation of the respiration or the collapse of the transmembrane potential by nucleotides.

All drugs and nucleotides were from Sigma/Aldrich except GDP-β-S and Ap5A (Boehringer). Stock solutions of nucleotides were adjusted at pH 7.0 with NaOH.

3. Results and discussion

3.1. ATP-induced stimulation of respiration in the presence of salt

When ATP is added to Yeast Foam mitochondria oxidizing ethanol in the presence of K^+, the respiration was stimulated. This effect never took place in the absence of salt, or with Li^+ or Na^+ instead of K^+. It was fully prevented by phosphate (>4 mM) and atractyloside [35].

Moreover, additional experiments showed that this effect was specific for ATP: indeed, CTP, GTP, GDP and non-hydrolyzable analogs of ATP (AMP-PNP and AMP-PCP) did not induce any stimulation.

When we attempted to reproduce these results by using NADH instead of ethanol as the respiratory substrate, results were quite different. We still did not observe any stimulation in the absence of salt, but Na^+, Li^+ and choline were as efficient as K^+ to promote the effect (Fig. 1a). The reason for this difference between both respiratory substrates is not known to date. A possible relationship with the localization of the dehydrogenase (external for NADH, internal for ethanol) or with the electron flow rate through the respiratory chain (about two times higher on NADH than on ethanol) is under investigation.

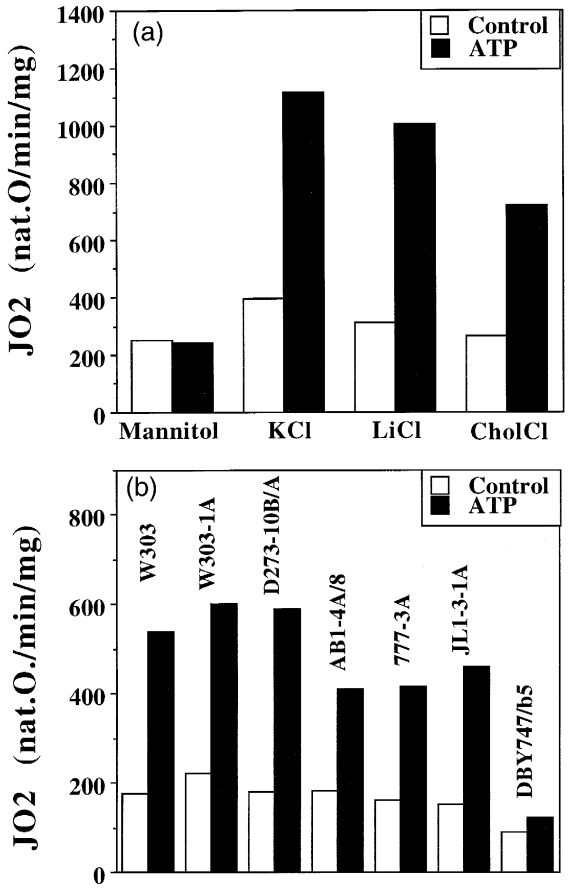

Fig. 1. Effect of nucleotides on the respiration of mitochondria isolated from Yeast Foam (a) and laboratory strains (b). Mitochondria (0.17 mg/ml) were suspended at 28°C in a 10 mM Tris/maleate buffer (pH 6.8) containing either 0.6 M mannitol (a, chart mannitol and b, throughout) or 0.4 M mannitol plus 0.1 M of the indicated salt (a, charts KCl, LiCl and CholCl), 2 mM EGTA, 0.3% BSA, 0.5 mM phosphate and 1.7 μg/ml oligomycin. The respiratory substrate was 2 mM NADH. ATP was added at a final concentration of 2 mM. Control refers to state 4 of respiration.

3.2. ATP-induced stimulation of the respiration in the absence of salt

When we repeated these experiments on mitochondria isolated not from Yeast Foam but from wild-type laboratory strains respiring on ethanol or NADH, we observed similar results as those reported by Prieto et al. [33]: ATP induced a marked stimulation of respiration rates (Fig. 1b). Similar results were observed as well on the diploid strain W303 (used by Prieto et al. [33]) as on the haploid strains W303-1A, AB1-4A/8 and D273-10B/A.

To ensure that the stimulation of respiration was actually related to a collapse of the transmembrane potential, and not to a possible kinetic effect on the respiratory chain, the effect of ATP was measured on the absorbance signal of rhodamine 123 (Fig. 2). It appeared clearly that ATP induced a marked decrease of the transmembrane potential maintained by the respiratory chain, and that this decrease was largely

reversed by the addition of a high phosphate concentration.

3.3. Effect of nucleotides triphosphate and analogs

In the following, the characteristics of the stimulation of NADH oxidation by nucleotides were determined on both types of material: Yeast Foam mitochondria in the presence of KCl and W303 mitochondria in the absence of salt.

Prieto et al. [33] reported that CTP or GTP induced the same decrease of the transmembrane potential as ATP. Similarly, Guérin et al. [34] reported that the anion unspecific channel was induced by CTP and GTP almost as well as by ATP.

Under our experimental conditions, and in both types of mitochondria, CTP or GTP induced a stimulation of respiration, although it was slightly lower than the stimulation by ATP (Fig. 3a).

Non-hydrolyzable analogs AMP-PCP, and GMP-PNP failed to stimulate the respiration; additionally, they partly prevented a further stimulation by ATP (Fig. 3a). These results clearly showed that the hydrolysis of NTP was required for the effect.

3.4. Effect of nucleotides diphosphate and analogs

In the presence of Ap5A to prevent any extramatricial synthesis of ATP from ADP by the adenylate kinase, ADP did partly prevent the stimulation of respiration by ATP (data not shown). It was verified that Ap5A did not have any effect on the stimulation by ATP (data not shown). The extent of the inhibition by ADP may vary from 50 to 100% depending on mitochondria preparation. Although quite high (IC_{50} = 0.25–1 mM), the inhibitory concentration was in the range of physiological concentration of ADP. The inhibition by ADP may therefore have a physiological significance.

The most surprising result we obtained was the fact that GDP induced a stimulation of respiration almost as high as the stimulation by ATP (Fig. 3b). This stimulation was also related to a collapse of the transmembrane potential, and was reversed by high phosphate concentrations (Fig. 2). It may be noted that the stimulation of the respiration by GDP was

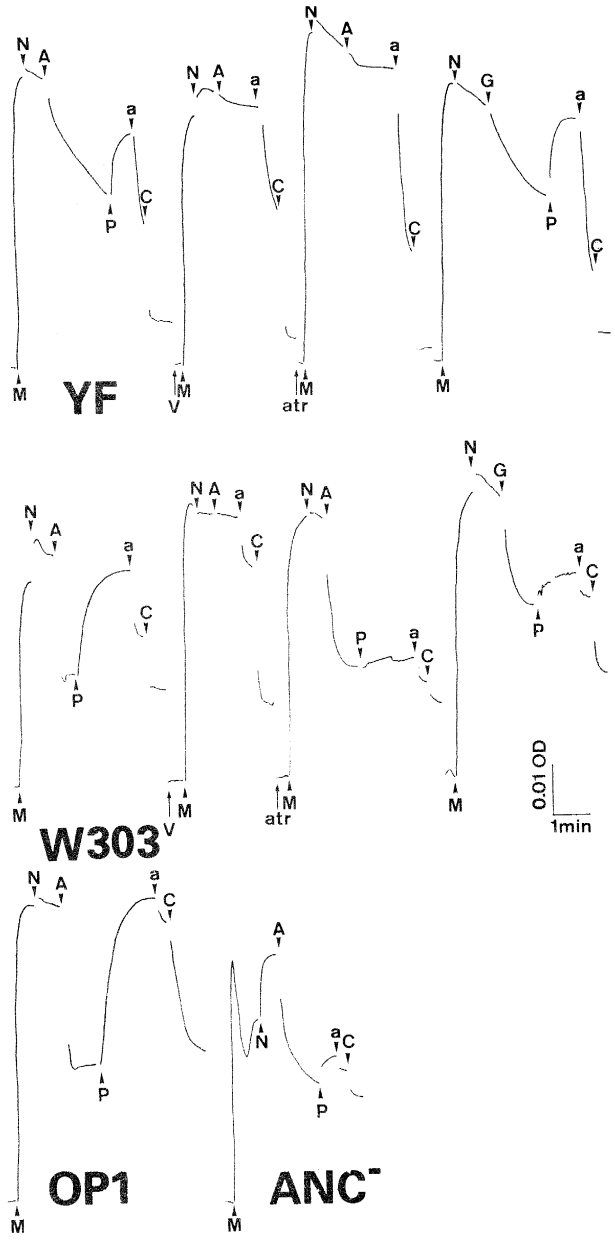

Fig. 2. Effect of ATP and GDP on the $\Delta\Psi$ maintained through the inner mitochondrial membrane. Mitochondria (0.2 mg/ml) were suspended under the same conditions as in Fig. 1, except that 1 μg/ml rhodamine 123 was added to the buffer. The difference of absorbance between 516 and 495 nm was measured in a DW2000 spectrophotometer. $\Delta\Psi$ of Yeast Foam mitochondria (first row) was measured in the presence of 0.4 M mannitol plus 0.1 M KCl; $\Delta\Psi$ of W303 mitochondria (second row) and from op1 (op1) and null (ANC^-) mutants of the adenine nucleotide carrier (third row) were measured in the presence of 0.6 M mannitol, without salt. Additions: M: mitochondria; N: NADH 2 mM; a: antimycin A 0.2 μg/ml; C: ClCCP 9 μM; A: ATP 2 mM; G: GDP 2 mM; P: Pi 10 mM; V: sodium decavanadate 10 μM (equivalent orthovanadate); Atractyloside 60 μM.

insensitive to Ap5A (not shown), and thus not related to a possible phosphorylation of GDP to GTP by the adenylate kinase.

Additionally, we observed a strong stimulatory effect of the non-phosphorylable GDP analog GDP-β-S (Fig. 3b). This compound induced a maximal stimulation of the respiration at a concentration 20-fold lower than the concentration of GDP and nucleotides triphosphate (100 μM instead of 2 mM) (Fig. 4).

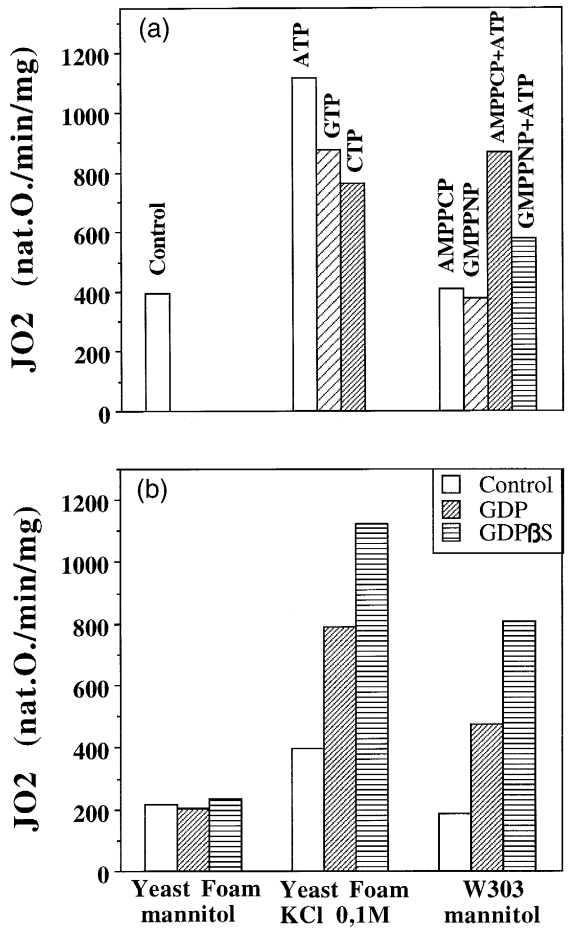

Fig. 3. Effect of nucleotides and nucleotides analogs on the respiration of mitochondria isolated from Yeast Foam and W303. Experimental conditions similar to Fig. 1. In (a), Yeast Foam mitochondria were suspended in the presence of 0.4 M mannitol plus 0.1 M KCl. In (b), 'mannitol' refers to 0.6 M mannitol and 'KCl' refers to 0.4 M mannitol plus 0.1 M KCl. Nucleotides were added at a final concentration of 2 mM, except for GDP-β-S (0.1 mM). Control refers to state 4 of respiration.

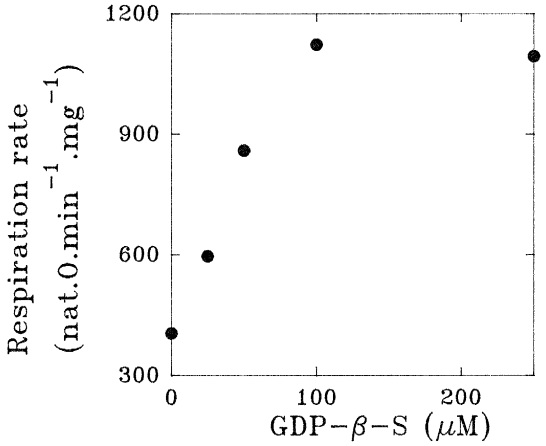

Fig. 4. Effect of GDP-β-S on the respiration of Yeast Foam mitochondria in the presence of KCl. Experimental conditions similar to Fig. 1 (0.4 M mannitol plus 0.1 M KCl).

3.5. Effect of nucleotides monophosphate

Nucleotides monophosphate AMP, GMP, cAMP and cGMP did not have any effect on respiration rates of Yeast Foam and W303. Moreover, they all partly prevented the stimulation of respiration by ATP, but the high concentrations required for this effect (over 2 mM) did not appear to be physiologically relevant (data not shown). It may also be noted that adenosine and adenine did not have any effect, stimulatory or inhibitory (data not shown).

3.6. Is the uncoupling observed in W303 mitochondria caused by the opening of a proton pathway?

The main point of discussion about the stimulation of respiration observed in W303 mitochondria was to know whether it was caused by the opening of a proton pathway, as proposed by Prieto et al. [32,33] or by the opening of the unspecific channel, as proposed by Guérin et al. [34]. The basis of this discussion was the observation by Prieto et al. [32] that ATP allowed a rapid swelling of W303 mitochondria suspended in isosmotic potassium acetate in the presence of valinomycin, thus supporting the hypothesis of an ATP-induced proton pathway allowing the electroneutral K^+/H^+ exchange schematized in Fig. 5a. This interpretation was questioned by Guérin et al. [34] who proposed that the acetate ion could be transported electrophoretically via the unspecific anion channel according to Fig. 5b.

To resolve this problem, we used the inability of GDP to open the unspecific anion channel described by Guérin et al. [34]. Fig. 6A shows the swelling of W303 mitochondria suspended in potassium gluconate in the presence of valinomycin. As reported by Guérin et al. [34], ATP induced a rapid swelling related to the opening of an anion channel. In this aspect, W303 therefore had the same behavior as Yeast Foam mitochondria. On the opposite, in the presence of valinomycin, GDP did not induce any swelling of W303 mitochondria (Fig. 6A), nor of Yeast Foam mitochondria (data not shown), that demonstrated its inability to open the anion channel. It was therefore possible to use the method reported by Prieto et al. [32], with GDP instead of ATP, in order to investigate the presence of a proton channel.

This experiment is reported in Fig. 6B. GDP did not induce any significant swelling of Yeast Foam mitochondria suspended in potassium acetate in the presence of valinomycin. This clearly demonstrated that Yeast Foam mitochondria did not support any proton-conducting pathway, and that the collapse of transmembrane potential induced by GDP reported above, was actually due to the opening of a cation transport pathway, as we already concluded for ATP induction [35].

On the opposite, GDP did induce a large and rapid swelling of W303 mitochondria suspended in potassium acetate in the presence of valinomycin, that

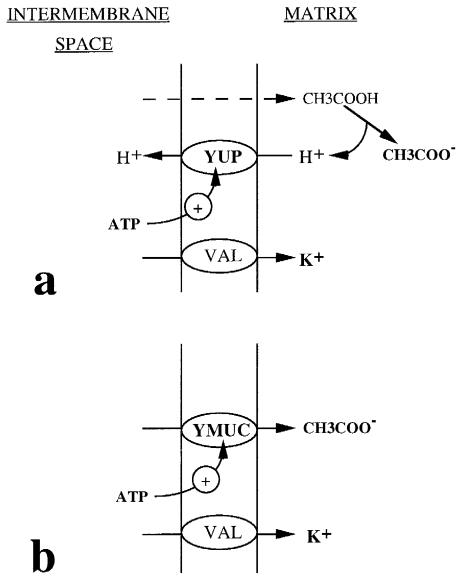

Fig. 5. Scheme of two possible mechanisms supporting a swelling of mitochondria suspended in isosmotic K-acetate. (a) The electroneutral diffusion of acetic acid and the electrophoretic entry of K^+ are electrically compensated by the exit of proton via an ATP-induced proton-conducting pathway [32]. (b) The electrophoretic entries of K^+ and of the acetate ion through the ATP-induced unspecific anion channel electrically compensate each other [34]. YUP stands for 'Yeast Uncoupling Pathway' [32] and YMUC stands for 'Yeast Mitochondria Unspecific Channel' (B. Guérin, personal communication).

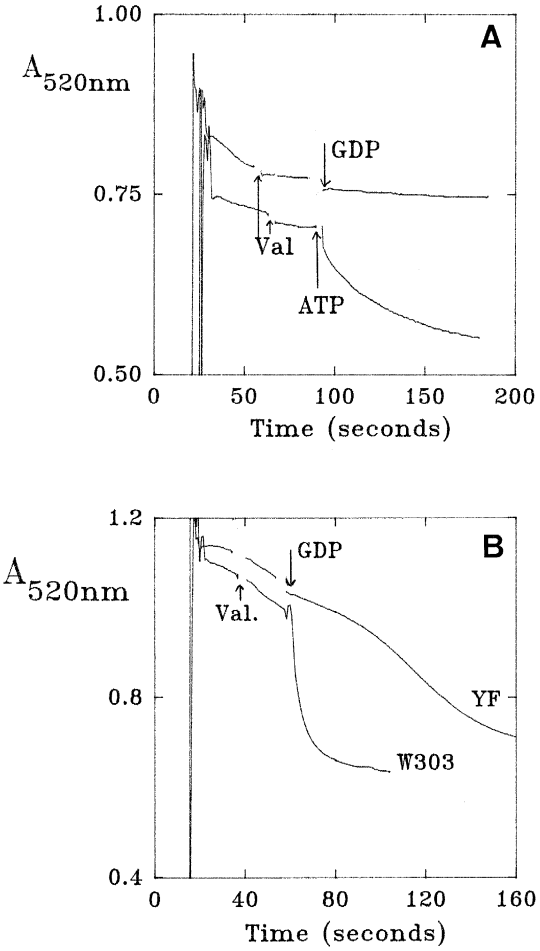

Fig. 6. Swelling of mitochondria suspended in isosmotic solutions of K-gluconate (A) and K-acetate (B). (A) W303 mitochondria (0.5 mg/ml) were suspended in a 0.3 M K-gluconate, 10 mM Tris/maleate buffer (pH 6.8) in the presence of antimycin A (0.125 μg/ml) and oligomycin (5 μg/ml). Valinomycin (0.2 μg/ml) and ATP (2 mM) or GDP (2 mM) were sequentially added. (B) Yeast Foam or W303 mitochondria (0.5 mg/ml) were suspended in a 0.3 M K-acetate, 10 mM Tris/maleate buffer (pH 6.8). Antimycin A, oligomycin, valinomycin and GDP were added as in (A).

demonstrated that GDP actually induced a proton-conducting pathway (Fig. 6B). It is relevant to note that, in the absence of valinomycin, GDP did not induce any swelling (data not shown), and thus did not induce any unspecific permeability, nor any stimulation of the endogenous K^+/H^+ exchange [12].

From this experiment, it was concluded that GDP actually opened a proton transport pathway in W303 mitochondria but not in Yeast Foam mitochondria. Can this conclusion be extended to ATP and other nucleotides triphosphate? We have no definitive arguments to conclude; but the similarity between the characteristics of the phenomena induced by GDP and ATP (see below) strongly argues for this hypothesis.

3.7. Effect of Mg^{2+}

An important aspect which was pointed out by Guérin et al. [34] and Prieto et al. [32,33] was the Mg^{2+}-sensitivity of the ATP-induced anion channel. From calculations of the respective concentrations of ATP^{4-} and $MgATP^{2-}$, it was concluded that ATP^{4-} was the active form to open the anion channel of Yeast Foam mitochondria [34].

The same behaviour was observed on W303 mitochondria (Fig. 7): the ATP-induced swelling in K-gluconate was almost fully inhibited by 4 mM $MgCl_2$.

On the contrary, we observed that the ATP-induced swelling of W303 mitochondria in K-acetate was totally insensitive to 4 mM $MgCl_2$ (Fig. 7) and raising the Mg^{2+} concentration up to 15 mM did not promote any inhibition (not shown). This observation was an additional evidence that the swelling in K-acetate did not reflect the same phenomenon than the swelling in K-gluconate and that a proton-conducting pathway, Mg^{2+}-insensitive, actually exists in W303 mitochondria.

3.8. Role of the adenine nucleotides carrier

In order to find the localization of the effect of nucleotides, we looked at the effect of the inhibitors of the adenine nucleotide carrier.

On W303 mitochondria in the absence of salt, we reproduced the observations by Prieto et al. [32,33]: the ATP-induction was fully insensitive to the competitive inhibitor atractyloside (Fig. 2) and to the

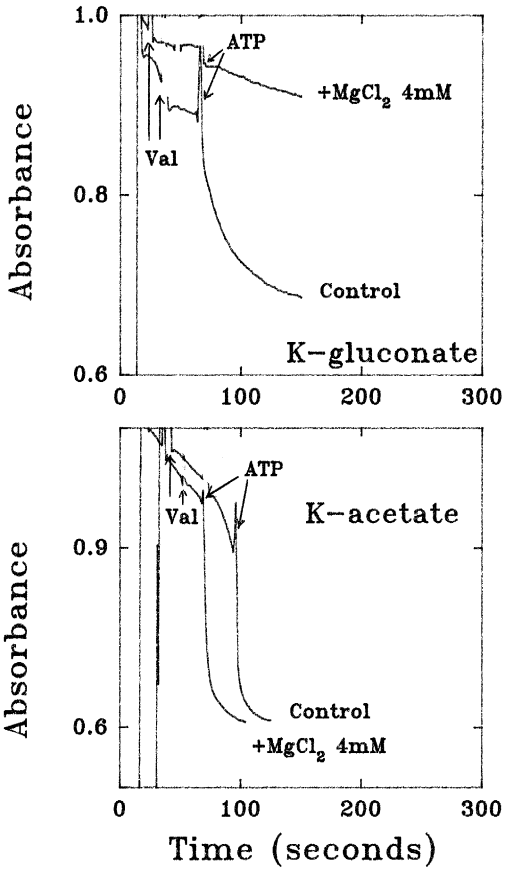

Fig. 7. Effect of $MgCl_2$ on the ATP-induced swellings of W303 mitochondria in K-gluconate or K-acetate. Experimental conditions similar to Fig. 6. Where indicated, 4 mM $MgCl_2$ were added after valinomycin and before ATP.

almost non-competitive inhibitor carboxyatractyloside (not shown). Additionally, we observed an ATP-induced stimulation of the respiration in mitochondria isolated from an op1 mutant (having an adenine nucleotide translocase with a dramatically reduced affinity for ADP and ATP [36,41]) and from a strain of which the three ANC genes were interrupted [37,42] (Figs. 1 and 2). These experiments clearly showed that, in the case of the induction of a proton-conducting pathway in W303 and other laboratory strains, ATP acted on the cytoplasmic side of mitochondria.

Very different results were obtained in the case of the cation-conducting pathway in Yeast Foam mitochondria. We observed that the ATP-induced stimulation of the respiration on NADH of Yeast Foam mitochondria in the presence of KCl was partially

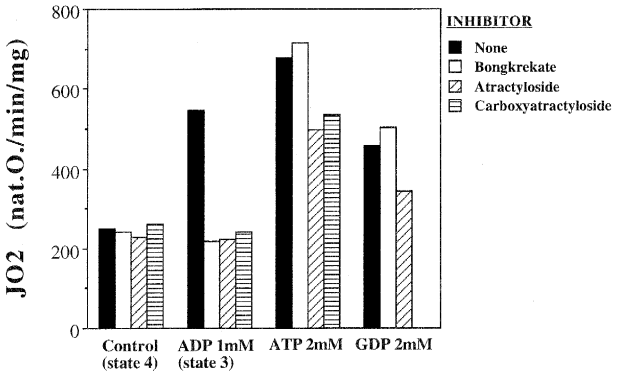

Fig. 8. Effect of the inhibitors of the adenine nucleotides carrier on the ATP-induced stimulation of the respiration in Yeast Foam mitochondria. Same experimental conditions as in Fig. 1 (0.4 M mannitol plus 0.1 M KCl). Bongkrekate, 36 μM; atractyloside, 60 μM; carboxyatractyloside, 0.26 μM.

sensitive to atractyloside (Fig. 8), as was the collapse of $\Delta\Psi$ (Fig. 2). The extent of the inhibition was not raised by using carboxyatractyloside, which has a much greater affinity than atractyloside. Additionally, another potent inhibitor of the adenine nucleotides carrier, bongkrekic acid, did not have any inhibitory effect.

It should be noted that both the stimulations by GTP (not shown) and GDP (Fig. 8) were also partially sensitive to atractyloside, as the stimulation by ATP.

The lack of inhibition by bongkrekic acid was obviously inconsistent with the hypothesis that ATP acted on the matricial side of the inner membrane. Moreover, atractyloside also partially inhibited the stimulation by the other nucleotides, which are not transported (and not even bound) by the adenine nucleotides carrier. It can be concluded that the effect of ATP and other nucleotides on Yeast Foam mitochondria occurred via an action on the external face of the inner membrane.

On the other hand, the (carboxy)atractyloside sensitivity is consistent with the hypothesis of an implication of the adenine nucleotides carrier itself in this phenomenon. Atractyloside and carboxyatractyloside block the transporter in the 'C' conformation whereas bongkrekic acid blocks it in the 'M' conformation [43–45] It can therefore be hypothesized that the conformational state of the adenine nucleotides carrier should be an element for the regulation of the ATP-induced stimulation of the respiration of Yeast Foam mitochondria in the presence of salt. In this hypothesis, the full inhibition of the ATP-induced stimulation on ethanol by atractyloside can now be understood as a full inactivation of this state of activity by the 'C' conformation of the adenine nucleotides carrier.

This dependence of the permeability of the inner mitochondrial membrane on the conformational state of the adenine nucleotides carrier has already been proposed for the regulation of the mammalian permeability transition pore [11,46,47]. In this latter system, however, the 'C' conformation appeared to induce the open state whereas the 'M' conformation seemed to induce the closed state, which is the contrary of the situation we observed.

3.9. Porin-less mitochondria

Some lines of evidences are available concerning the possible involvement of the outer membrane porin in the high-conductance channels depicted in mammalian mitochondria (see [27] for review). We tested the hypothesis that the outer membrane porin could be involved in the ATP/GDP induced permeability of yeast mitochondria by looking at their effect on porin-less mitochondria. Although the stimulation of respiration by ATP was much less marked than in other strains (essentially because of a low respiration rate on NADH [38]), it could also be reproducibly evidenced in porin-less mitochondria (Fig. 1b).

3.10. Effect of vanadate

From data presented above, it appeared that ATP-induced stimulation of the respiration is relevant from a complex regulation and the question is: by which way ATP is involved in those effects?

The lack of discrimination between ATP, CTP and GTP is a characteristic of most ATPases. But the inhibitory effect of Mg^{2+} [[34,35], this paper] together with the fact that the presence of EGTA or EDTA did not change our observations did not fit well with the hypothesis of the involvement of an ATPase. Nevertheless, we assayed a number of known inhibitors of ATPases.

All the experiments reported herein were done in the presence of oligomycin, showing that it did not

inhibit the induction. The same observation could be done for venturicidin, another specific inhibitor of the FoF1-ATPase. It might be noted that, on most mitochondria preparation, oligomycin and venturicidin potentialized the effect of ATP.

N-Ethylmaleimide, diethylstilbestrol, ouabain and bafilomycin A_1, which are inhibitors of different ionic ATPases, did not have any effect on the stimulation by ATP.

The orthovanadate ion is a potent inhibitor of enzymes (mostly ATPases) having an acylphosphate intermediate, since its trigonal bipyramidal structure can mimic the one of phosphate (see Ref. [48] for a review). When orthovanadate was assayed on the stimulation of the respiration by ATP, it did not have any inhibitory effect (Table 2, treatment a).

In solution, chemistry of vanadate is complex because it can polymerize depending on the pH of the solution; the major polymeric forms (meta and deca), which can be potent inhibitors of phosphotransferases activities (see Ref. [48] for a review), were assayed. Decavanadate proved to be a powerful inhibitor of the stimulation of both W303 mitochondria in the absence of salt and Yeast Foam mitochondria in the presence of K^+ (Table 2, treatments b,d and Fig. 9A). This effect was clearly related to a maintaining of the transmembrane potential (Fig. 2, first row). Metavanadate did not have any inhibitory effect (Table 2, treatment c).

Table 2
Effect of treatment of Na_2VO_4 (NaVi) solutions on their inhibitory effect on ATP-induced stimulation of the respiration of W303 mitochondria

	Respiration rates in the presence of	
	10 μM NaVi	0.25 mM NaVi
Treatment a	2.98	2.57
Treatment b	1.17	nd
Treatment c	3.30	2.71
Treatment d	1.06	nd

Same experimental conditions as in Table 1 (0.6 M mannitol). The stock solutions of 0.1 M sodium orthovandate were treated as follows: adjustment at pH 8.0 (a) and then at pH 6.0 (b) with sulfuric acid; adjustment at pH 6.5 with sulfuric acid (d) and then adjustment at pH 10.5 with NaOH and boiling for 3 min (c). Values give the ratio of the respiration in the presence of 2 mM ATP over respiration in the absence of ATP. The control ratio (in the absence of NaVi) was 3.06.

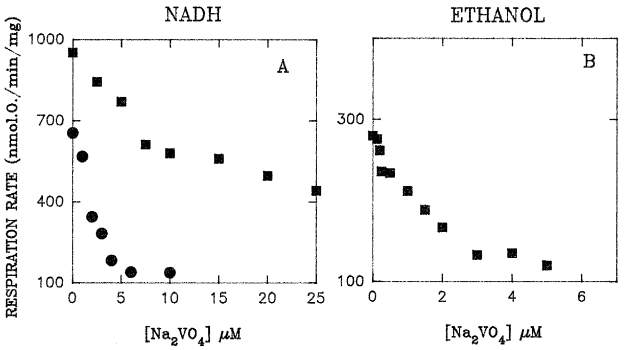

Fig. 9. Effect of Na_2VO_4 on the ATP-induced stimulation of the respiration in Yeast Foam and W303 mitochondria. Same experimental conditions as in Fig. 1. The stock solution of Na_2VO_4 was prepared at 0.1 M and adjusted at pH 6.5 with sulfuric acid. (A) Yeast Foam mitochondria in the presence of 0.4 M mannitol plus 0.1 M KCl (■) and W303 mitochondria in the presence of 0.6 M mannitol and in the absence of salt (●), respiring on NADH 2 mM. (B) Yeast Foam mitochondria respiring on ethanol 40 mM in the presence of 0.4 M mannitol plus 0.1 M KCl.

A possible effect of vanadyl, the reduced form of vanadate, was ruled out, since the addition of 20 μM vanadyl sulfate did not induce any inhibition (data not shown).

It was also observed that the K^+- and ATP-specific stimulation observed on Yeast Foam mitochondria respiring on ethanol [35] was also sensitive to sodium vanadate (Fig. 9B).

In summary, as concerned with the vanadate sensitivity, the three cases of ATP-induced stimulation of the respiration (Yeast Foam oxidizing NADH or ethanol in the presence of KCl, W303 oxidizing NADH in the absence of salt) were inhibited by the same low concentrations of sodium vanadate, under conditions where decavanadate was the mainly present chemical species.

Decavanadate inhibited as well the induction by GDP and GDP-β-S as the induction by NTPs (not shown), which is an additional evidence that the inhibition could not be due to the formation of a stable acylvanadate intermediate. Decavanadate is a known potent inhibitor of a limited number of nucleotides-binding enzymes such as skeletal muscle adenylate kinase ($K_i = 1$ μM) [49] and phosphofructokinase ($K_i = 45–550$ nM) [50]. In the case of adenylate kinase, crystallographic studies allowed to identify the decavanadate-binding site as the zone which binds the triphosphate part of ATP [51]. Deca-

vanadate also inhibits the reconstituted phosphorylase b by competing with both glucose-1-phosphate and phosphite [52]. This compound is therefore able to competitively inhibit certain enzymes at their (tri)phosphate binding site. It is therefore possible to propose that decavanadate should compete both with ATP and GDP at their respective binding sites.

4. Conclusions

4.1. The problem

The existence of (an) ATP-induced electrogenic transport pathway(s) the activity of which should be potentially able to have dramatic consequences on mitochondria function is of critical importance. In respiring yeast mitochondria, it was shown that ATP stimulated the respiratory chain. Prieto et al. [33] described an ATP-induced proton conducting pathway, while we evidenced an ATP-induced potassium electrophoretic pathway [35]: in both cases, ATP caused a collapse of the electrical transmembrane potential. Using the non energetic swelling technique, Guérin et al. [34] showed an ATP-induced unspecific channel, mostly anionic.

The central problem we tried to resolve in the present paper is to know whether the contradictory observations and interpretations described until now could find a common explanation.

4.2. Conditions of ATP-induced collapse of transmembrane potential depend on yeast strains

There are two main conclusions to this study. First, in Yeast Foam mitochondria, ATP induced a collapse of the transmembrane potential only in the presence of salt, suggesting that the nucleotide opened a cation electrophoretic pathway. A proton-conducting pathway could never be evidenced in this strain. On the other hand, in wild-type laboratory strains mitochondria, ATP induced a collapse of the transmembrane potential even in the absence of salt. The activation by GDP allowed to demonstrate that a proton-conducting pathway is actually present in these strains. This difference between these strains explain the discrepancies reported by Prieto et al. (on the strain W303) [32,33], and Guérin et al. [34] and us [35] (on Yeast Foam).

Second, the fact that we observed a sensitivity to micromolar decavanadate as well in Yeast Foam as in wild-type laboratory strains mitochondria, argues for the similarities of the molecular support responsible for these phenomena.

4.3. In what way do nucleotides act on the permeability?

This is not a trivial problem since the existence of a unique system of induction working with ATP, CTP, GTP, GDP and GDP-β-S but not with ADP and non-hydrolyzable analogs of ATP and GTP is unlikely. The fact that AMP-PCP and GMP-PNP did not induce any stimulation (and prevent a further stimulation by ATP) clearly argues for the necessity of an hydrolysis of ATP and GTP. But this is in contradiction with the inducing effect of GDP and the high efficiency of GDP-β-S. The only hypothesis which could take into account all these data should be the existence of two ways of induction: (i) a first system which bound and hydrolyzed nucleotides triphosphate and (ii) a second system which bound GDP and its analog GDP-β-S.

What could be the nature of these two systems?

Concerning the first system, the absence of a clear discrimination between ATP, GTP and CTP does not argue for the involvement of a protein kinase. Moreover, we assayed several inhibitors of protein kinases (staurosporin, 1-(5-isoquinolinylsulfonyl) 2-methylpiperazine, gennistein) which were without effect on ATP-induced stimulation. The existence of a P-type ATPase is also unlikely since the stimulation was not sensitive to high orthovanadate concentrations and to other classical inhibitors (NEM, diethylstilbestrol). Most of all, the absence of effect of chelators of divalent cations (EGTA, EDTA) and the inhibitory effect of Mg^{2+}, is clearly an argument for the substrate to be ATP and not MgATP. This is in accordance with the conclusion of Guérin et al. [34] and of Ballarin and Sorgato [30].

It is more likely to hypothesize that ATP hydrolysis should be required for a conformational change (e.g., chaperone-type ATPase, protease) allowing the opening of the system. In that case, the divalent cation required for (the slow and limited) ATP hydrolysis might be strongly bound to the protein responsible for the reaction.

The putative second mode of induction required GDP or its analog GDP-β-S which appears to be much more efficient. On the other hand, the non-hydrolyzable analog of GTP, GMP-PNP, was an inhibitor of the induction by both GDP and GDP-β-S. It is therefore possible that a protein having a guanydylic nucleotide binding sites should activate the system when GDP (GDP-β-S) is bound whereas it should inhibit it when GTP (GMP-PNP) is bound. An attractive hypothesis should be that of a G-protein-type system of which the inactivation (GDP/GDP-β-S binding) should open the ion-conducting pathway.

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