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The Nature and Regulation of the ATP-Induced Anion Permeability in *Saccharomyces cerevisiae* Mitochondria¹

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ATP lowers the efficiency of oxidative phosphorylation in *Saccharomyces cerevisiae* mitochondria by a mechanism that involves the activation of cytochrome *c* oxidase and the increase in anion permeability of the mitochondrial inner membrane (S. Prieto, F. Bouillaud, and E. Rial (1995) *Biochem. J.* 307, 657–661). In this study, we have carried out experiments to determine the transport specificity of the ATP-induced permeability pathway and its regulation. The pathway allows permeation of anions such as Cl⁻ or Br⁻, while NO₃⁻, NO₂⁻, or Tes are not transported. Transport is activated by ATP, GTP, dATP, dGTP, and GDP, while ADP, AMP, GMP, and pyrimidine nucleotides are ineffective. Analysis of transport inhibition by ADP and phosphate suggests that ADP is a competitive inhibitor of ATP while phosphate inhibition is noncompetitive. These effectors are operative in the physiological range of concentrations. © 1996 Academic Press, Inc.

Key Words: *S. cerevisiae*; mitochondria; permeability; transport; regulation.

We have previously reported that cytosolic ATP triggers swelling when *Saccharomyces cerevisiae* mitochondria are suspended in potassium acetate plus valinomycin. We postulated that ATP was inducing a proton permeability pathway, a hypothesis that also agreed with the observed stimulation of respiration by ATP (1). Subsequent studies (2) revealed that the ATP action on mitochondrial oxidative phosphorylation was exerted in two steps. First, at low ATP/Pi ratios, ATP increases respiratory activity probably by altering the

kinetic properties of cytochrome *c* oxidase. This observation agreed with the reported interaction of ATP with solubilized yeast cytochrome oxidase that stimulates its activity (3, 4) and with the effect of external ATP on the control that cytochrome *c* oxidase exerts on the activity of the respiratory chain (5). Second, at higher ATP/Pi ratios, an increase in membrane permeability leads to a collapse in membrane potential (2). The studies by Guérin *et al.* (6) showing that external ATP opens a channel that allows swelling under an additional variety of conditions led to the proposal that the channel was unspecific. Two antagonists of the ATP action have been described so far: phosphate (1, 2, 6) and ADP (6). The effect of phosphate explains early observations (7) which considered it a “coupling factor” in yeast mitochondria.

The uncoupling of respiration described above is caused by external ATP since the effects are detected in the presence of atractylate (1, 2). There are, however, two recent articles (8, 9) that describe ATP effects on the inner membrane permeability exerted from the matrix side. Roucou *et al.* have reported the ATP-induced opening of a K⁺ pathway that can be inhibited by phosphate, propranolol, quinine, Zn²⁺, and Mg²⁺ (8). Ballarin and Sorgato have observed with the patch clamp technique two channels whose activity can be modulated by ATP (9). The two channels display a preference for anions, but while one is blocked by ATP, the other one gets locked in the open conformation. It is not possible to establish at present if there is a relation between the ATP-modulated channels revealed by patch clamping and the ATP-induced permeability changes observed with the swelling technique. However, a relation of any of these activities to either the inner membrane anion channel (6) or to VDAC (voltage-dependent anion channel) has been dismissed (9).

The reported lack of a transport specificity of the ATP-induced permeability pathway (6) prompted us to further investigate its activity. We report here that the pathway does show selectivity since the transport of

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anions such as NO_3^- or Tes is not stimulated by ATP. We have also investigated the inhibition of the path by ADP and Pi and data suggest that ADP is a competitive inhibitor of ATP while Pi inhibition is noncompetitive. The physiological implications of these findings are also discussed.

EXPERIMENTAL PROCEDURES

The diploid strain of *S. cerevisiae* W303 was grown aerobically at 28°C in 1% yeast extract, 2% bacto-peptone, and 3% lactate as carbon source. Yeast cells were harvested in logarithmic growth phase, and mitochondria prepared from protoplasts as previously described (1). Mitochondria (25–30 mg protein/ml) were suspended in 0.65 M mannitol, 2 mg/ml albumin, 2 mM EGTA, 0.5 mM phosphate, 10 mM Tris-maleate, pH 6.8. Protein was determined by the biuret method using albumin as standard.

Ion permeabilities were determined from the rate of light scattering decrease at 550 nm of mitochondria (0.5 mg protein/ml) suspended in various potassium salts, as previously described (1). Osmotic swelling was induced by addition of 0.5 μM valinomycin and/or nucleotide. Swelling buffer contained 0.2 M potassium salt, 2 mM MgCl_2 , 1 mg/ml albumin, 0.5 $\mu\text{g/ml}$ antimycin A, 10 μM atractylate, 12.5 $\mu\text{g/ml}$ oligomycin, 10 mM Tris-maleate, pH 6.8. Other additions are described in figure legends.

Growth media were from Difco. Cytohelicase was from Bio-Septra. Potassium gluconate and KBr were from Aldrich. Nucleotides (sodium salts) and the rest of reagents were from Sigma or the highest purity available. Nucleotide solutions were prepared fresh everyday in Tris-maleate 100 mM and carefully put to pH 6.8 with NaOH. The use of fresh solutions throughout was essential for the reproducibility of the results.

RESULTS AND DISCUSSION

The permeability properties of S. cerevisiae mitochondria. Guérin *et al.* and others have reported that yeast mitochondria swell spontaneously in potassium acetate as a result of the entry of acetate in its protonated form and the activity of the K^+/H^+ antiporter (6, 10, 11). Figure 1 shows the observations made in our laboratory with their experimental conditions. When yeast mitochondria are suspended in the buffer described in Ref. (6), mitochondria swell spontaneously although at a much slower rate than that reported by Guérin *et al.* (6): in their case, swelling was complete after 4–5 min, while with our mitochondrial preparation they have swollen to 50% of the total extent after 7 min (Fig. 1, trace a). However, under our standard experimental conditions (1), with 1 mg/ml albumin and 2 mM MgCl_2 in the swelling buffer, mitochondria can be incubated for longer periods with an extremely slow swelling rate and thus after 7 min they have swollen by 15% (Fig. 1, trace b). These lower swelling rates can be understood since removal by albumin of endogenous free fatty acids and lysoderivatives should contribute to preserve the integrity of the inner membrane and thus lower ion permeabilities. In addition, Mg^{2+} should inhibit both the K^+ uniporter (12) and the K^+/H^+ exchange (10, 11).

Therefore, we can consider that when, under our

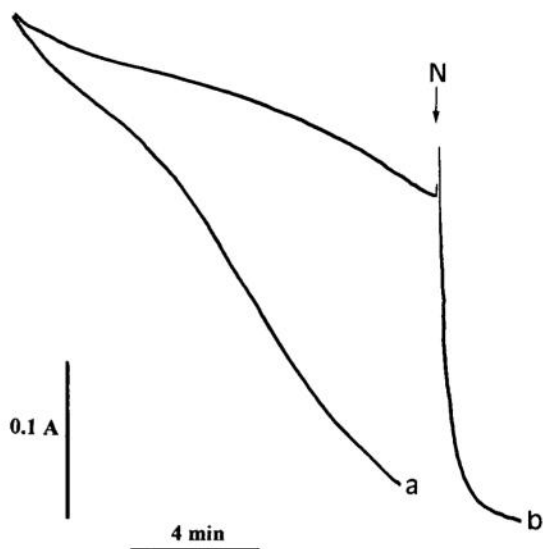


FIG. 1. Swelling of yeast mitochondria in potassium acetate. Mitochondria (0.5 mg/ml) were suspended in 0.2 M potassium acetate, 10 μM atractylate, 0.5 $\mu\text{g/ml}$ antimycin A, 12.5 $\mu\text{g/ml}$ oligomycin, and 10 mM Tris-maleate, pH 6.8. Light scattering changes were monitored from the decrease in absorbance at 550 nm. Vertical bar indicates 0.1 absorbance units. In trace b swelling buffer also contained 2 mM MgCl_2 and 16 μM albumin. Addition: N, 0.5 μM nigericin.

standard conditions, mitochondria are suspended in potassium acetate in the presence of valinomycin, the rate of swelling will reflect the endogenous permeability to $\text{H}^+(\text{OH}^-)$ and/or to the acetate ion (13, 14). In order to investigate the anion permeability properties of the mitochondrial inner membrane we have also monitored the mitochondrial swelling in the following salts: KSCN, KCl, KBr, KNO_3 , KNO_2 , and potassium salts of Tes and gluconate. In the presence of valinomycin, the rate of swelling in potassium salts will depend on the membrane anion permeability. Figure 2 reveals that, as expected, swelling is fast in KSCN since SCN^- can permeate efficiently through the membrane. Mitochondrial swelling is slower in KNO_2 , KNO_3 , and potassium acetate. When mitochondria are suspended in KCl or KBr there is an initial fast swelling rate that accounts for 15–20% of the total amplitude although after 30–45 s the rate markedly decreases. Finally, in Tes and gluconate, valinomycin does not elicit significant swelling, thus indicating that the inner membrane is virtually impermeable to these anions.

Anion specificity of the ATP-induced permeability in the mitochondrial inner membrane from S. cerevisiae. We have previously reported (1, 2) that when ATP is added to a buffer containing potassium acetate plus valinomycin there is fast swelling (see also Fig. 3). Guérin *et al.* have recently shown (6) that, in the presence of valinomycin, ATP also induces swelling in potassium gluconate, potassium glutamate, and KCl and there-

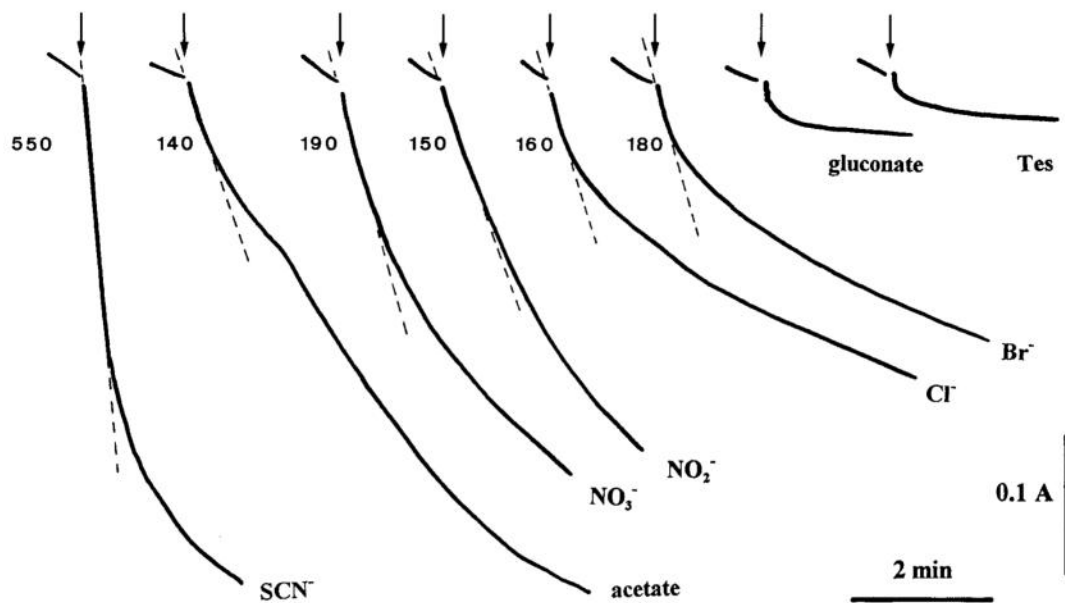


FIG. 2. Anion permeability of yeast mitochondria. Mitochondria (0.5 mg/ml) were suspended in buffers containing 0.2 M potassium salt, 2 mM $MgCl_2$, 16 μM albumin, 10 μM atractylate, 0.5 $\mu g/ml$ antimycin A, 12.5 $\mu g/ml$ oligomycin, and 10 mM Tris-maleate, pH 6.8. The salt anion is indicated next to the corresponding swelling trace. Swelling was initiated by addition of valinomycin 0.5 μM as indicated by the arrows. Traces shown are from representative experiments. Swelling rates are expressed in milliabsorbance units per minute and the slopes taken where indicated by the dotted lines. Rate variability is of the same order than in figures where error bars are shown (see for example Fig. 5).

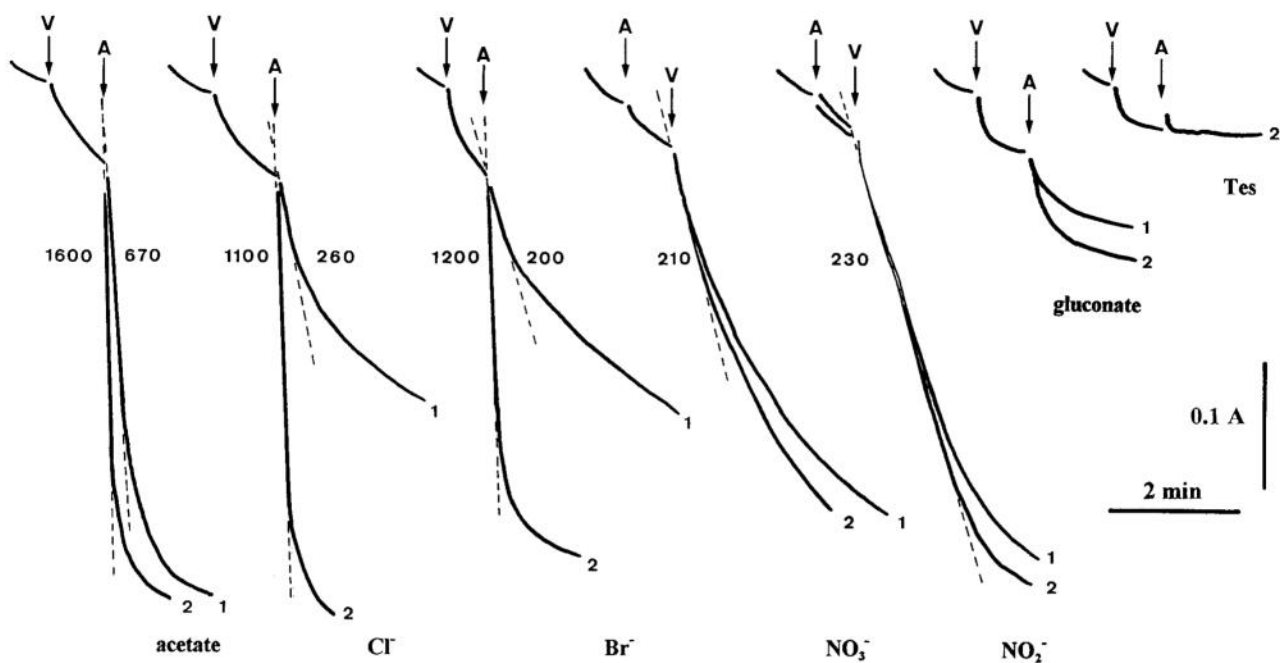


FIG. 3. Effect of ATP on the anion permeability of yeast mitochondria. Mitochondria were suspended in the medium described in the legend of Fig. 2. The salt anion is indicated next to the corresponding trace. Additions: V, 0.5 μM valinomycin. The effect of the addition of either 1 or 2 mM ATP is indicated at the bottom of each trace with the numbers 1 and 2, respectively. Swelling rates are expressed and calculated as indicated in the legend of Fig. 2.

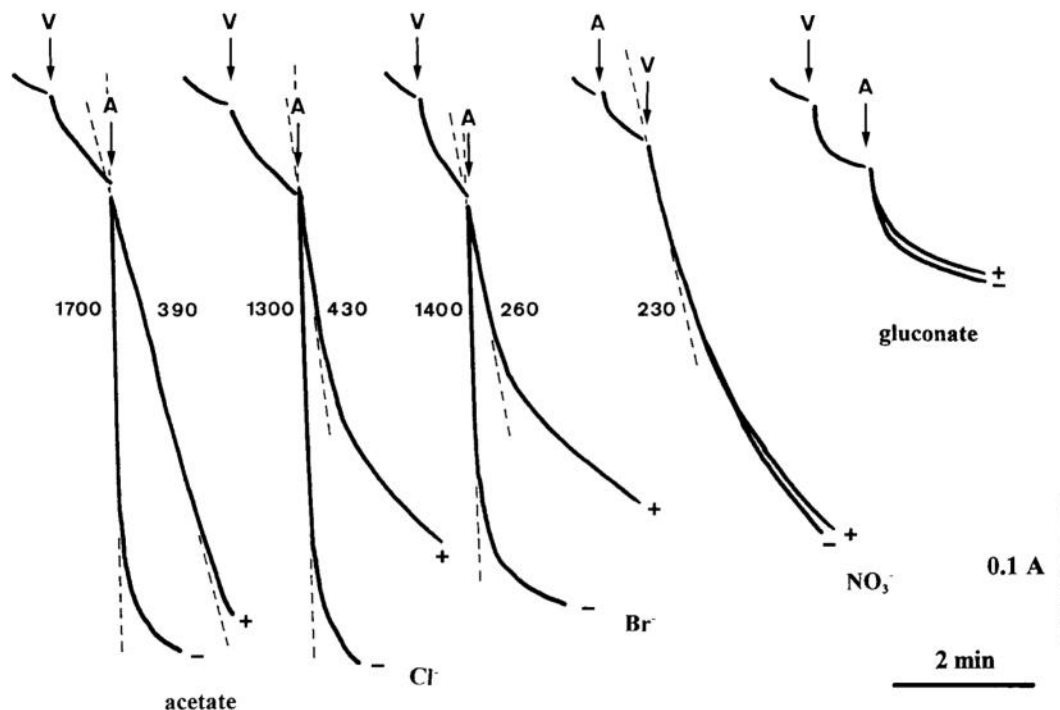


FIG. 4. Effect of phosphate on the mitochondrial swelling induced by ATP. Mitochondria were incubated in the medium described in the legend of Fig. 2: potassium acetate, KCl, KBr, KNO_3 , or potassium gluconate as indicated at the bottom of the traces. Swelling traces were obtained in the presence or absence of 1 mM phosphate; this is indicated next to the traces with the plus or minus signs, respectively. Swelling rates are expressed and calculated as indicated in the legend of Fig. 2. Additions: V, $0.5 \mu\text{M}$ valinomycin; A, 2 mM ATP.

fore proposed that the ATP-induced channel was un- specific. Figure 3 shows that ATP does not elicit swelling in all potassium salts. Thus, ATP markedly stimulates swelling: acetate, Cl^- , and Br^- , although for the halide salts a higher nucleotide concentration is required to reach the rates obtained with acetate. On the opposite side are NO_3^- , NO_2^- , and Tes, where ATP does not influence swelling rates. Since swelling in NO_3^- and NO_2^- is initiated by valinomycin (Fig. 2), ATP was added before the ionophore and then rates plus or minus ATP were compared. Finally, ATP induces fast swelling in gluconate although of low amplitude (some 30% for 1 mM ATP). Increasing ATP concentrations increase the amplitude although they never reach the extent observed with the rest of the salts.

We have also reported that millimolar phosphate inhibits the ATP-induced swelling in potassium acetate plus valinomycin (see (1, 2) and below) and for example, 1 mM phosphate inhibits by 80% the rate of swelling induced by 2 mM ATP (Figs. 4 and 8). A similar degree of inhibition is observed in KCl and KBr (Fig. 4). However, swelling in NO_3^- , NO_2^- , gluconate, and Tes are not affected by the presence of phosphate. This is illustrated in Fig. 4 with the swelling in NO_3^- and gluconate. The possibility remained that the lack of ATP effect in this group of salts could be due to an inhibitory action of the anion, thus analogous to that exerted by phos-

phate. We studied the effect of 1–5 mM NO_3^- on the ATP-induced swelling in potassium acetate and no inhibition was found (data not shown).

We can conclude that ATP opens a path for an electrical uniport that allows entry of anions such as Cl^- and Br^- and that it is inhibited by phosphate. Inhibition by phosphate of anion transport occurred only with species whose transport is markedly increased by ATP addition, thus suggesting that the partial swelling observed with gluconate is probably not related to the same transport pathway. The screening with potential substrates has revealed a transport specificity since anions such as NO_3^- , NO_2^- , and Tes do not permeate through the ATP-induced pathway. ATP-induced swelling in potassium acetate, in the presence of valinomycin, can occur in two modes: either acetate is transported through the ATP-induced pathway or acetic acid enters through the lipid bilayer with a compensating entry of OH^- through the path. Since we have previously described (2) that in respiring mitochondria high cytosolic ATP levels collapse the proton electrochemical potential gradient in the absence of permeant anions, we must assume that under the two conditions, passive swelling and respiration, the ATP-induced pathway acts as an anion transporter catalyzing the movement of OH^- .

Guérin *et al.* (6) compared this pathway with the

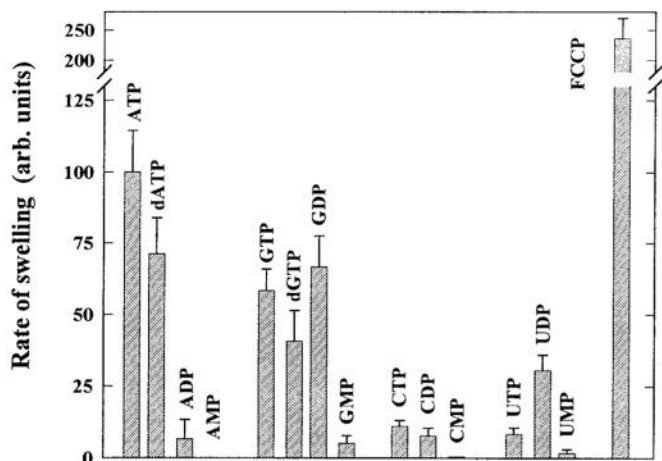


FIG. 5. Nucleotide specificity for the induction of potassium acetate swelling in the presence of valinomycin. Experiments were performed in the medium described in the legend of Fig. 2. Swelling was initiated by addition of $0.5 \mu\text{M}$ valinomycin followed by 1 mM nucleotide. Rates in the presence of valinomycin were subtracted from those after nucleotide addition. The effect of $3.3 \mu\text{M}$ FCCP is included as a comparison. Error bars indicate the SE of 3–5 independent determinations performed in triplicate. Rates are expressed in arbitrary units, taking the swelling induced by 1 mM ATP as 100.

inner membrane anion channel (15) due to the apparent lack of specificity and its sensitivity to propranolol and DCCD. However, these authors concluded that the pathway could be distinguished from the inner membrane anion channel since their experiments showed no evidence for a regulation of the activity by endogenous Mg^{2+} . The new data presented here on the specificity of the pathway reinforce the distinction with the inner membrane anion channel.

The specificity of the pathway for the nucleotides. It has been previously reported that the ATP effects on yeast mitochondria swelling can be mimicked by nucleotides such as CTP and GTP, while UTP, ITP, ADP, and AMP were ineffective (6). We have reported that ADP could not induce acetate swelling (1) and that ATP, dATP, and GTP but not UTP caused the activation of respiration and the collapse of membrane potential (2). Figure 5 shows the effect of a wider range of nucleotides on the swelling in potassium acetate. It is apparent that at 1 mM nucleotide, ATP is the most effective one and that dATP, dGTP, GTP, and GDP are also potent activators of the permeability. We have also shown that the kinetics of the activation by ATP is sigmoidal (1, 2). Figure 6 presents a comparison of the activation kinetics for GDP and GTP. It is striking that the two triphosphate nucleosides display sigmoidal kinetics while GDP presents a Michaelis–Menten type curve. Thus at low nucleotide concentrations GDP is the most effective one. Figure 7 shows the effect of P_i on the nucleotide-induced swelling and reveals that the

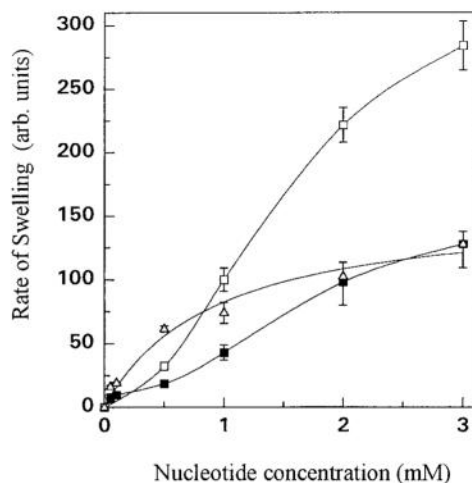


FIG. 6. Kinetics of the induction of potassium acetate swelling with ATP, GTP, and GDP. Determinations were performed as described in the legend of Fig. 5. Rates in the presence of valinomycin were subtracted from those after nucleotide addition. Nucleotides: \square , ATP; \blacksquare , GTP; \triangle , GDP. Error bars indicate the SE of 3–5 independent determinations performed in triplicate.

effects of GDP and GTP are also inhibited by millimolar concentrations. Interestingly, for 1 mM nucleotide, IC_{50} for P_i is close to $300 \mu\text{M}$ with GDP while those for ATP and GTP are around $75 \mu\text{M}$, thus reflecting the higher GDP binding affinity.

The regulation by phosphate and ADP of the ATP-induced swelling in potassium acetate. Figures 8 and 9 summarize the effect that increasing P_i and ADP concentrations have on the activation by ATP of potas-

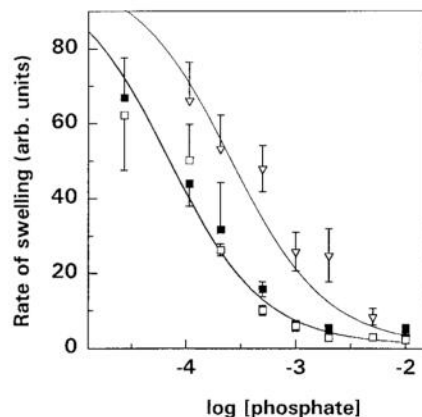


FIG. 7. Inhibition by phosphate of the potassium acetate induced by ATP, GTP, and GDP. Experiments were performed as described in the legend of Fig. 6. Nucleotide concentration was 1 mM . Rates in the presence of valinomycin were subtracted from those after nucleotide addition. Swelling rates in the absence of phosphate were considered as 100. Nucleotides: \square , ATP; \blacksquare , GTP; ∇ , GDP. Error bars indicate the SE of 3–5 independent determinations performed in triplicate.

sium acetate swelling. Pi has been reported to inhibit the effects that ATP exerts both on mitochondrial respiration and membrane permeability (1, 2, 6). It has also been previously described that ADP does not stimulate acetate swelling (1) and that it inhibits ATP-induced swelling in gluconate (6). Observation of Figs. 8 and 9 confirms the inhibitory action of the two effectors but suggests a different behavior of the two inhibitors. When curves are analyzed using the Hill equation (data not shown) it is revealed that Pi cause a decrease in affinity and V_{max} and therefore implies that it is a non-competitive inhibitor, while ADP does not affect the V_{max} while it decreases the ATP affinity and thus suggests that it acts like a competitive inhibitor.

Concluding remarks. In the present paper we have further characterized the specificity of the ATP-induced permeability pathway of *S. cerevisiae* mitochondria, both with respect to the transported species and to the regulation. A previous report (6) found no selectivity in the channel but now a wider screening of molecules reveals that, in the presence of valinomycin, ATP only stimulates markedly the transport of some anions and that in these cases Pi inhibits the ATP action. It should be emphasized that the ATP effects reported here are due to cytosolic ATP since all experiments were performed in the presence of atractylate (1, 2) and therefore are distinct from those that may be exerted by matricial ATP (8, 9).

When yeasts are grown under aerobic conditions, the

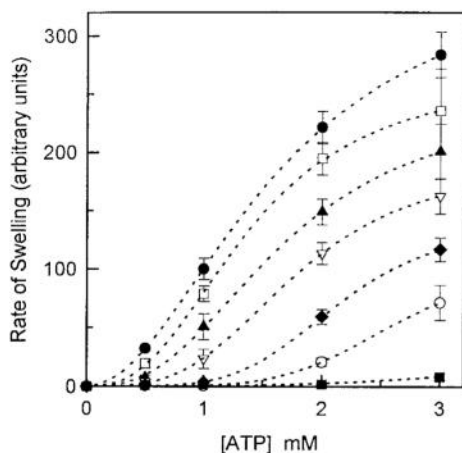


FIG. 8. Kinetics of the inhibition by phosphate of the ATP-induced swelling of yeast mitochondria suspended in potassium acetate. Experiments were performed as described in the legend of Fig. 6 but in the presence of varying concentrations of phosphate: 0, 0.02, 0.2, 0.5, 1, 2, and 10 mM (curves from top to bottom). Swelling was initiated by addition of valinomycin and varying concentrations of ATP (0.5–3 mM). Data points are averages of 3–5 independent determinations performed in triplicate. Error bars indicate the SE of 3–5 independent determinations performed in triplicate. Rates in the presence of valinomycin were subtracted from those after nucleotide addition.

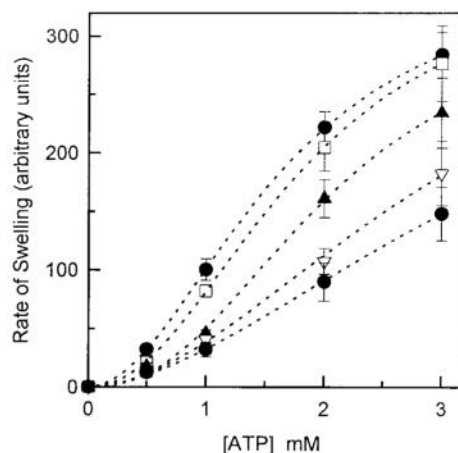


FIG. 9. Kinetics of the inhibition by ADP of the ATP-induced swelling of yeast mitochondria suspended in potassium acetate. Experiments were performed as described in the legend of Fig. 6 but in the presence of varying concentrations of ADP (0, 0.1, 0.5, 1, and 2 mM, curves from top to bottom). Further conditions and details described in the legend to Fig. 8.

growth yield depends on the nature of the substrate utilized (16–19). Thus, depending on the degree of reduction of the substrate the culture may be either carbon-limited (carbon supply limits growth while energy generation occurs in excess) or energy-limited (substrate too oxidized) (19). When growth is carbon-limited, redox balance must be maintained by eliminating the excess NAD(P)H in oxidative processes which are non-energy-conserving (18, 20). Indeed, a correlation has been found between the energetic efficiency and the cytosolic ATP concentration (21). In line with these observations, we have shown (2) that ATP activates respiration by a combination of two effects, first acting on cytochrome *c* oxidase increasing its activity and second, increasing the membrane permeability. We have therefore proposed that this uncoupling pathway could operate as an energy-dissipating mechanism (1, 2). In the present paper we demonstrate that the ATP-induced permeability is regulated by Pi and ADP, thus the *in vivo* membrane permeability will depend on the ATP/ADP + Pi ratio. A recent publication (22) has shown that, when *S. cerevisiae* is in stationary phase, the ATP concentration oscillates just above 3 mM, ADP is close to 0.5 mM, and Pi around 3.6 mM. These data indicate that *in vivo* changes in ATP levels could affect the efficiency of oxidative phosphorylation.

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