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EFFECTS OF PHYSIOLOGICAL MANIPULATION ON THE KINETICS OF MITOCHONDRIAL PHOSPHATE TRANSPORT IN *SACCHAROMYCES CEREVISIAE*

MICHEL RIGOULET, MARTINE GUERIN and BERNARD GUERIN

Laboratoire de Biochimie, UER de Biochimie et Biologie Cellulaire, Université de Bordeaux II, 351 cours de la Libération, 33405 Talence (France)

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Summary

The kinetics of [^{32}P] phosphate uptake has been studied in different types of *Saccharomyces cerevisiae* mitochondria. Mitochondria were isolated from yeast grown aerobically on 2% lactate (Lac-mitochondria), 2% galactose (Gal-mitochondria), 5.4% glucose (Glu-mitochondria) or from yeast grown anaerobically on 2% galactose (Promitochondria). The effect of chloramphenicol was alsc studied by adding it to the growth medium of yeast grown aerobically on 2% galactose (chloramphenicol-mitochondria).

[^{32}P]Phosphate uptake followed an oscillatory pattern in Lac, Gal-mitochondria and Promitochondria.

Saturation kinetics were detected in fully differenciated mitochondria and in Promitochondria, but not in chloramphenicol-mitochondria.

Glu-mitochondria did not translocate phosphate as shown both by lack of [^{32}P]phosphate uptake and lack of swelling in isoosmotic potassium solution.

Repressed yeast cells were incubated in a resting cell medium and mitochondria were isolated at different times of incubation. The rate of respiration and the oligomycin-sensitive ATPase increased during the course of the incubation. After 2 h, a mitochondrial mersalyl-sensitive swelling in an isoosmotic potassium phosphate solution was detected.

As expected, no increase of the rate of respiration was observed when chloramphenicol was added in the derepression medium. But the oligomycin-sensitive ATPase decreased. Chloramphenicol did not affect the phosphate transport activity as measured by the swelling of mitochondria, but the [^{32}P]phosphate uptake did not follow saturation kinetics. A complete derepression of the inorganic phosphate-carrier activity was achieved by a 4 h incubation of the repressed cells in the presence of chloramphenicol, followed by a 6 h incubation in presence of cycloheximide.

Abbreviations: CCCP, Carbonyl cyanide *m*-chlorophenylhydrazone; EGTA, ethyleneglycol-bis-(β-amino-ethylether)*N*,*N*'-tetraacetic acid; P_i, inorganic phosphate.

These data strongly suggest that the mitochondrial protein-synthesis system is required for the normal function of the inorganic phosphate-carrier.

Introduction

Mitochondria from mammalian tissues are believed to possess two phosphate transport systems catalyzing either a phosphate/hydroxyl (phosphate carrier) or a phosphate/dicarboxylate exchange (dicarboxylate carrier) [1,2]. Both were demonstrated by their differential sensitivity towards inhibitors: the phosphate carrier reacts with various thiol reagents (mercurials, maleimides) and the dicarboxylate carrier only with mercurials and butylmalonate (a dicarboxylate analog) [2—10]. Attempts have been carried out in various laboratories either to understand the exact role of the phosphate carrier in the mitochondrial metabolism (for instance ATP synthesis) [11—14] or to isolate it [15—17]. One of the essential difficulties of these studies is that manipulations can only be carried out on isolated mitochondria by means of inhibitors, since most of cells containing mitochondria will not tolerate any extensive modification of their mitochondrial apparatus.

However, yeast cells afford the possibility of phenotypic variations which can be easily obtained by physiological or genetic manipulations. Some of these variations have been used to study the origin of the protein components of the carrier systems (Krebs cycle intermediates [18] and ATP carriers (see ref. 19 for a review).

Swelling experiments in isoosmotic salts solutions have shown that the anionic permeability of yeast organelles is not basically different from that of mammalian mitochondria [20—23]. For instance, in phosphate solutions, a mersalyl-sensitive swelling was detected in Promitochondria (from anaerobically grown yeast) and in mitochondria isolated from yeast grown aerobically with or without chloramphenicol [22,23], an inhibitor of mitoribosomal protein synthesis [24]. But these experiments were not sufficient to conclude that the phosphate carrier was not modified by these physiological manipulations. In fact, swelling experiments were done in 0.2 M phosphate solutions and therefore, did not permit the detection of any modifications of the P_i-carrier, for instance the affinity for its substrate.

The objective of the present studies was to measure the kinetic parameters of the phosphate transport in yeast mitochondria in order to gain insight into the factors controlling the synthesis or/and the function of the P_i-carrier. For this purpose, effects of some physiological modifications were investigated in this system; these included catabolite repression and derepression, anaerobiosis and inhibition of the mitoribosomal protein synthesis by chloramphenicol.

Materials and Methods

Chemicals

Sorbitol and mannitol (Prolabo) were purified by stirring with Dowex 50 W (Fluka). Bovine serum albumin, 2-mercaptoethanol, EGTA, chloramphenicol, cycloheximide, CCCP, oligomycin, antimycin, valinomycin and mersalyl were

purchased from Sigma Chemical Co. Snail juice from *Helix pomatia* was obtained from Industrie Biologique Française (Gennevilliers, France). [^{32}P]Phosphate was from C.E.A. (Saclay).

Strain and growth conditions

Diploid wild strain *Saccharomyces cerevisiae* (Yeast Foam).

Aerobic cultures. Cells were grown in a New-Brunswick incubator at 28° C in a complete medium: 1% Yeast extract/0.1% potassium phosphate/0.12% ammonium sulfate, supplemented with either galactose 2% or lactate 2% or glucose 5.4% as carbon source. The cells were harvested in the logarithmic growth phase.

Aerobic culture in presence of chloramphenicol. 3 g/l of the antibiotic were added to the aerobic medium supplemented with galactose 2%.

Anaerobic culture. The aerobic medium (galactose 2%) was complemented with 0.34% Tween-80 and 0.0012% ergosterol; cultures were grown in 10-l bottles, from which the oxygen was removed by a nitrogen flow during 1 h (Azote R, Air Liquide, France). Before harvesting anaerobically grown yeast, cycloheximide (25 mg/l) was added, and centrifugation was performed under nitrogen by using a continuous flow system (Sorvall KSB).

Derepression conditions

Yeast cells grown aerobically on glucose were harvested early in the logarithmic growth phase (1 g dry weight of cells per l). After two washings with 0.1% galactose, cells were incubated at 28° C in the following derepression medium (2 g dry weight of cells per l): 0.13% potassium phosphate/0.12% ammonium sulfate/0.42% potassium citrate/0.34% Tween 80/0.0012% ergosterol/2% galactose, pH 4.5. When added, chloramphenicol was 4 g/l and cycloheximide 25 mg/l.

Spectrophotometric measurements

The cytochrome content of cells or mitochondria was measured at room temperature with a Perkin-Elmer 356 spectrophotometer. The following wavelength pairs and reduced-minus-oxidized extinction coefficients were used:

Cytochrome ($a + a_3$), 605—630 nm, $\Delta\epsilon$ mM = 13 $nmol^{-1} \cdot cm^{-1}$ [25],

Cytochrome ($c + c_1$), 550—540 nm, $\Delta\epsilon$ mM = 18 $nmol^{-1} \cdot cm^{-1}$ [26].

Full oxidation was achieved by addition of 10 µl of 10% H_2O_2 to 2.5 ml of yeast suspension, and full reduction by addition of sodium dithionite.

Isolation of mitochondria

Lac-mitochondria (from yeast grown aerobically on 2% lactate), Gal-mitochondria (from yeast grown aerobically on 2% galactose), Glu-mitochondria (from yeast grown aerobically on 5.4% glucose), chloramphenicol-mitochondria (from yeast grown aerobically on 2% galactose and in presence of chloramphenicol) and promitochondria (from yeast grown anaerobically on 2% galactose) were isolated from protoplasts following Kovac's procedure [20] slightly modified [23]. The preparation of protoplasts from anaerobic cells was carried out

under nitrogen atmosphere and in presence of cycloheximide in order to prevent respiratory adaptation. Mitochondrial protein was determined by the biuret method.

Measurements of [^{32}P]phosphate incorporation

Mitochondria (2 mg protein) were incubated for 1 min at 4°C in Eppendorf cups in 0.4 ml of the following incubation medium: 0.65 M mannitol/0.36 mM EGTA/10 mM KCl/5 mM $MgCl_2$/0.3% serum albumin (w/v)/10 μg oligomycin/0.5 μg antimycin/10 mM Tris/maleate, pH 6.7/5 mM butylmalonate (in order to block the dicarboxylate carrier). The reaction was initiated by addition of a [^{32}P]phosphate solution and stopped at different times by addition of mersalyl (final concentration 1 mM). Mitochondria were centrifuged immediately (Microcentrifuge 3200 Eppendorf). The pellet was suspended in 0.2 ml of distilled water and proteins were precipitated with 0.3 M trichloroacetic acid and centrifuged. The radioactivity of 0.1 ml of the supernatant was measured in 10 ml of water, using the Cerenkov effect [27] in an Intertechnique scintillation counter.

Other methods

The ATPase activity was measured according to Somlo et al. [28] at 30°C and pH 8.5.

Inorganic phosphate was measured according to Beremblum and Chain [29].

Respiration studies were carried out at 27°C in a 3 ml chamber equipped with a Clark oxygen electrode (Gilson) in the following basal medium: 0.65 M mannitol/10 mM KCl/5 mM potassium phosphate/0.3% serum albumin (w/v)/10 mM Tris/maleate, pH 6.7.

Swelling experiments were performed according to Arselin de Chateaubodeau et al. [23].

Results

Each sample of mitochondria (or mitochondria-like organelles) was controlled by various techniques. Particularly, the respiratory control, the ADP/O ratio and the ATPase activity were measured (Table I). It can be seen that both Lac- and Gal-mitochondria, isolated from aerobically grown yeast, presented a coupled respiration; however, the respiratory control was two times higher with Lac-mitochondria, but the ADP/O ratio was not significantly different in the two types of mitochondria. Also, the ATPase activity was slightly higher in Lac-mitochondria than in Gal-mitochondria (these values refer to the maximum activity, i.e. in presence of an uncoupler); in both organelles these activities were inhibited by oligomycin. These results were in agreement with the existence of a low catabolite repression by galactose. However, this catabolite repression was much more important when yeasts cells were grown on glucose 5.4% [28,30—32]: Respiration activity, respiratory control and oligomycin sensitive ATPase were low in Glu-mitochondria. Therefore, to study the effect of chloramphenicol or of anaerobiosis on the mitochondrial development, yeast cells were grown on galactose and not on glucose in order to prevent this high secondary effect of the catabolite repression.

Promitochondria were lacking in respiration [33,34] but an oligomycin-sen-

TABLE I

COMPARISON OF SOME MITOCHONDRIAL CHARACTERISTICS BETWEEN THE DIFFERENT TYPES OF ORGANELLES

For experimental conditions see Materials and Methods.

Types of organelles	Respiratory rate on ethanol + ADP (natom O · min^{-1} · mg^{-1})	Respiratory control	ADP/O	ATPase activity (nmol P_i min^{-1} mg^{-1} protein)	Oligomycin sensitivity (%)
Lac-mitochondria (12) *	600—700	5 —7	1.6	1800—2000	80
Gal-mitochondria (15)	500—600	2.5—3.5	1.4	1200—1400	80
Glu-mitochondria (7)	100	1.1	—	300— 650	50—60
Promitochondria (6)	0	—	—	200— 250	80
Chloramphenicol-mitochondria (8)	10— 20	1	—	300— 400	0

* Number of assays.

sitive ATPase was demonstrated according to Criddle et al. [34], although its specific activity was about ten times lower than in fully differentiated mitochondria. As expected, the ATPase of chloramphenicol-mitochondria was oligomycin insensitive [35]; respiration was low [36] and was not stimulated by ADP. However, the respiration rate on ethanol (or NADH) was stimulated twofold by CCCP (not shown).

Internal inorganic phosphate content

The concentration or inorganic phosphate in freshly isolated mitochondria (or mitochondria-like organelles) of the five cell types are compared in Table II.

TABLE II

INTERNAL P_i CONTENT IN FRESHLY ISOLATED MITOCHONDRIA AND EFFLUX OF P_i INDUCED BY CCCP AND VALINOMYCIN

Internal phosphate was measured before incubations (column 1). For P_i efflux experiments, mitochondria (4 mg) were incubated for 15 min at 4°C in 0.4 ml of the incubation medium (see Materials and Methods). As indicated, the additions were: 1 μM CCCP, 0.1 μg valinomycin. After centrifugation P_i was assayed in the supernatant (column 2).

Types of organelles	Internal P_i in the mitochondrial pellet (nmol P_i · mg^{-1} protein)	Release of internal P_i in the supernatant (nmol P_i · mg^{-1} protein)		
		None	CCCP	CCCP + Valinomycin
Lac-mitochondria (16)	15— 35	X	7—9	8—10
Gal-mitochondria (7)	30— 60	X	6—8	8—10
Glu-mitochondria (5)	4— 9	X	X	X
Chloramphenicol-mitochondria (34)	100—120	X	1—2	2— 3
Promitochondria (4)	20— 40	X	2—3	3— 4

X, undetectable: phosphate content < 0.2 nmol P_i · mg^{-1} protein.

The lowest content of phosphate was found for Glu-mitochondria and the highest for chloramphenicol-mitochondria. The internal phosphate content in Gal-mitochondria was two to three times higher than in Lac-mitochondria or Promitochondria.

It has been established that the phosphate accumulation inside mammalian mitochondria depends on the transmembrane pH gradient [37,38]. Thus, the addition of uncoupler or valinomycin plus uncoupler to a mitochondrial suspension, which collapses the ΔpH, induces a phosphate efflux. Table II shows that CCCP or valinomycin plus CCCP induced a release of internal phosphate with Lac-, Gal-, chloramphenicol- and Promitochondria. In all the cases this phosphate efflux was prevented by mersalyl (not shown). However, that release was rather low compared to the endogenous inorganic phosphate content (particularly with chloramphenicol-mitochondria).

[^{32}P]Phosphate uptake in the different types of mitochondria

A direct isotopic measurement of [^{32}P]phosphate uptake in Lac-mitochondria, in absence of an energy supply (antimycin and oligomycin were added), is shown in Fig. 1 (trace (a)). The expression of data in nmol of [^{32}P]phosphate incorporated was permitted since the concentration and the specific activity of external phosphate did not vary during the course of the experiment. However, an uptake of label can be due to two kinds of processes: a net uptake or an exchange of [^{32}P]phosphate with a portion of the internal phosphate pool. Therefore, it was important to discriminate between these two processes. Trace (a) of Fig. 1 shows that the maximum incorporation of [^{32}P]phosphate was comparable in size to the amount of endogenous phosphate released by uncoupler plus valinomycin addition (Table II). The label incorporation was con-

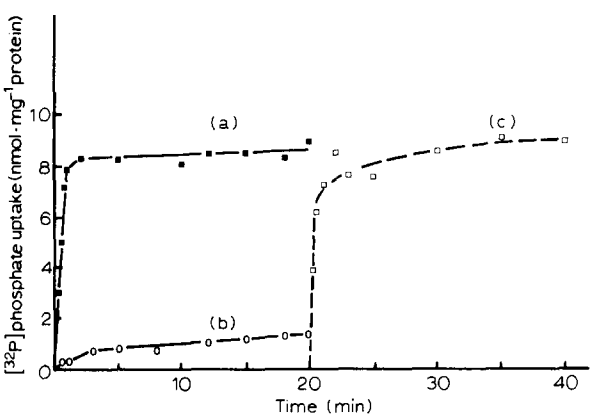

Fig. 1. [^{32}P]Phosphate uptake in Lac-mitochondria. Lac-mitochondria (2 mg protein) were incubated at 4°C in 0.4 ml of either the standard medium (see Materials and Methods) (a) or the medium supplemented with 1 μM CCCP (b). The incorporation was initiated by addition of 1 mM [^{32}P]phosphate and stopped at different times by 1 mM mersalyl. The radioactivity incorporated in mitochondria was corrected for the extra-matrix P_i (measured in assays where mersalyl was added before [^{32}P]phosphate). For back exchange experiments (c), mitochondria were incubated for 20 min in the standard medium containing 1 mM of unlabelled phosphate. The reaction was initiated by [^{32}P]phosphate addition in such a manner that the total external phosphate concentration was not significantly increased.

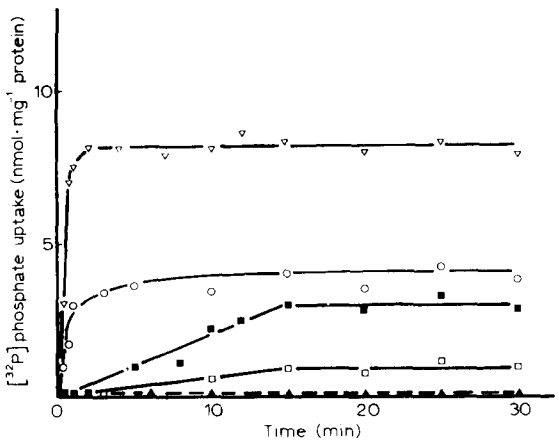

Fig. 2. [^{32}P]Phosphate uptake by different types of yeast mitochondria. Mitochondria were incubated at 4°C in 0.4 ml of the medium (as described in Materials and Methods). The incorporation was initiated by addition of 1 mM [^{32}P]phosphate and stopped at different times by 1 mM mersalyl. The radioactivity incorporated in mitochondria was corrected for the extra-matricial (cf. Fig. 1). ▽———▽, Gal-mitochondria; ○———○, promitochondria; ■———■, chloramphenicol-mitochondria; □———□, chloramphenicol-mitochondria incubated in presence of 1 μM CCCP; ▲———▲, Glu-mitochondria.

siderably decreased by uncoupler (trace (b) of Fig. 1). Besides, no net phosphate uptake was observed in assaying the internal inorganic phosphate content in the mitochondrial pellet under the experimental conditions of trace (a) of Fig. 1 (not shown here). These results strongly suggest that the [^{32}P]phosphate incorporation was essentially due to an exchange between the external inorganic phosphate and the part of internal phosphate dependent on the ΔpH. In another experiment (Fig. 1 trace (c)) [^{32}P]phosphate was added 20 min after the unlabelled inorganic phosphate in such a manner that the total external phosphate concentration was not significantly increased; under these conditions, only an exchange must be measured. It can be seen that the label incorporation paralleled the one depicted in trace (a).

In order to compare the efficiency of the different types of organelles in transporting phosphate, we used the direct isotopic method. As shown in Fig. 2, Gal-mitochondria and Promitochondria took up [^{32}P]phosphate very rapidly. Compared to these organelles, the label incorporation in chloramphenicol-mitochondria appeared to be very slow, but comparable in extent to the one observed in Promitochondria. In the three types of organelles, the [^{32}P]phosphate uptake was considerably decreased upon CCCP addition as for Lac-mitochondria (shown on Fig. 2 for chloramphenicol-mitochondria) suggesting that the internal pool of exchangeable phosphate was the one depending on the transmembrane pH gradient. In contrast to the other organelles, no mersalyl-sensitive [^{32}P]phosphate could be demonstrated in Glu-mitochondria. That result was in accordance with swelling experiments (see below).

Measurements of rates of transport

Oscillatory pattern of phosphate transport

In order to verify the validity of the stop inhibitor method, we compared

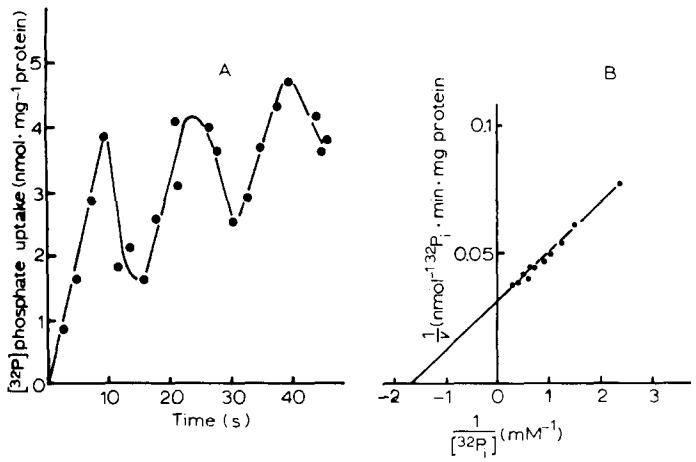

Fig. 3. Kinetics of [^{32}P]phosphate uptake in Lac-mitochondria. A. Time course of [^{32}P]phosphate uptake; external [^{32}P]phosphate concentration was 2 mM. B. Double reciprocal plot of the [^{32}P]phosphate uptake; the rates of [^{32}P]phosphate uptake were determined from the initial linear part of curve A during the first nine seconds. Experimental conditions are described in Materials and Methods and in the legend to Fig. 1 (a).

[^{32}P]phosphate incorporation in the mitochondrial pellet at time zero, under different conditions. Mersalyl was added to mitochondria either before [^{32}P]-phosphate (2 min, 1 min, 30 s), or at the same time as phosphate. In all cases, incorporations were equal; the precision of the stop inhibitor technique was approx. 5%.

The time course of the [^{32}P]phosphate uptake in Lac-mitochondria is shown in Fig. 3A. The curve was linear for about 10 s and then presented pronounced oscillations. The mean half period (for 12 experiments), defined as the time required to go from peak through was between 8 and 10 s for the first oscillation and then increased slightly; this increase, however, might be related to the dispersion of the experimental values during the course of the experiment, suggesting a loss of synchronisation of mitochondria, considered as individual oscillators. Similar curves were obtained with Gal-mitochondria and Promitochondria (not shown). Since during the first 10 s the [^{32}P]phosphate uptake was linear, we attempted to measure the apparent kinetic parameters in the different types of organelles.

Apparent kinetic parameters

Lac-mitochondria. The substrate concentration dependence on the rate of phosphate uptake was studied. Data of a typical experiment performed at pH 6.7 and at 4°C is shown in Fig. 3B as a double reciprocal plot. Only a straight line was obtained. On the basis of four determinations, the K_T value was 0.7 ± 0.2 mM and the V value 33 ± 7 nmol · min^{-1} · mg protein^{-1}.

Gal-mitochondria. Substrate concentration dependence on the rate of [^{32}P]-phosphate uptake in Gal-mitochondria is shown in Fig. 4A as a double reciprocal plot; an hyperbolic-like curve instead of a straight line was obtained. This result could suggest either the participation of two types of phosphate uptake in an homogenous population of mitochondria, or the existence of two popu-

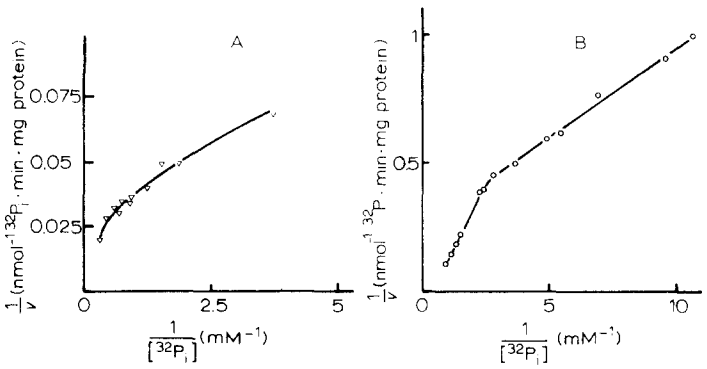

Fig. 4. Double reciprocal plot of the [^{32}P]phosphate uptake in Gal-mitochondria (A) and Promitochondria (B). Initial rates of phosphate exchange were measured as described in Fig. 3.

lations of organelles with a different phosphate system transport for each of them. However, it should be noted that the centrifugation of these organelles in a sucrose density gradient yielded only one band, pointing to a homogenous mitochondrial population (not shown).

Promitochondria. With this type of organelle the substrate concentration dependence on the rate of transport as a double reciprocal plot gave also a hyperbolic-like curve as for Gal-mitochondria (Fig. 4B). This result suggests that the anaerobic growth conditions did not affect the synthesis of the phosphate carrier system although the rate of phosphate exchange was lower in promitochondria than in Gal-mitochondria.

Chloramphenicol-mitochondria. It was shown in Fig. 2 that phosphate uptake in chloramphenicol-mitochondria was very low compared to the one observed in mitochondria of yeast grown in absence of chloramphenicol. Rates of [^{32}P]phosphate uptake as a function of the external phosphate concentration, are presented in Fig. 5. It can be inferred that the phosphate transport followed a non-saturation kinetics. Besides, the rate of phosphate transport, calculated for 1 mM of external phosphate, was 100 times lower than the one

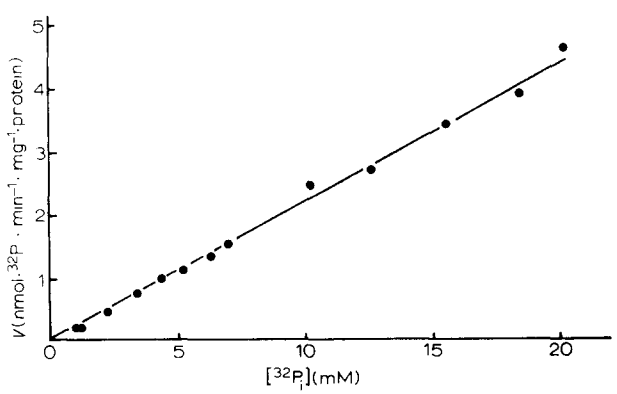

Fig. 5. Concentration-dependence of [^{32}P]phosphate uptake in chloramphenicol-mitochondria. Initial rates of phosphate exchange were measured as described in Fig. 3.

observed in the same conditions with Gal-mitochondria. Identical results were obtained with mitochondria isolated from a yeast "petite" mutant suggesting that the modifications of the [^{32}P]phosphate uptake kinetics are due to a lack in the mitoribosomal protein synthesis (not shown here).

Since the [^{32}P]phosphate uptake was fully inhibited in Glu-mitochondria (Fig. 2), we studied the phosphate transport in mitochondria from yeast undergoing glucose derepression [30,32,39,40]. Two techniques were used: (a) The swelling in isoosmotic salt solutions; (b) the uptake of [^{32}P]phosphate. The effects of chloramphenicol and cycloheximide on the phosphate transport derepression were tested.

Derepression of the mitochondrial system

Yeast grown aerobically on glucose was harvested early in the logarithmic growth phase and incubated in the derepression medium (cf. Materials and Methods). Galactose, instead of unfermentable substrate, was chosen as carbon source for the derepression experiments in order to prevent secondary effects due to a possible limitation in energy supply. This precaution was necessary when the inhibitors of protein synthesis were added into the medium.

Incubation of glucose-repressed cells in the derepression medium induced both quantitative and qualitative changes in their mitochondria. We compared the biogenesis of the mitochondrial apparatus in presence or absence of chloramphenicol. Under both conditions, the cell mass remained constant during the course of the incubation. However, a rapid enhancement of the mitochondrial mass, inhibited by chloramphenicol, was observed, as measured by the amount of mitochondrial protein isolated from 1 g dry weight of cells (Fig. 6). In agreement with previous reports [40,41], chloramphenicol inhibited the cytochrome ($a + a_3$) synthesis but not that of cytochrome ($c + c_1$). From the intracellular cytochrome content, it was possible to estimate the yield of the mitochondrial extraction and thus to validate the results presented in Fig. 6. For this, we compared the amount of cytochrome in 1 g dry weight of cells after 4 h of incuba-

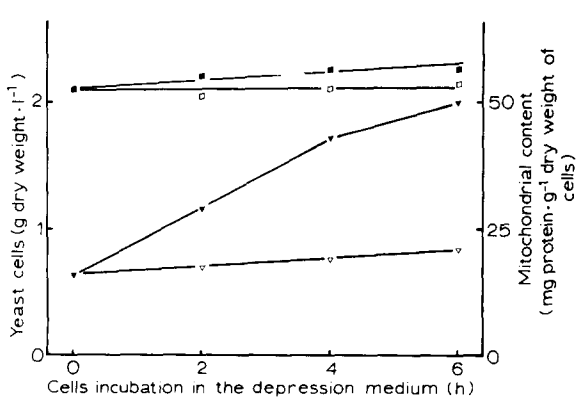

Fig. 6. Changes in mitochondrial content in yeast cells undergoing glucose derepression. Mitochondria isolated from 1 g dry weight of cells incubated in the control medium (▼———▼), or in presence of 4 g/l of chloramphenicol (▽———▽). Content in yeast cells (g dry weight/l) in the incubation medium during the course of the incubation: control (■———■) or in presence of chloramphenicol (□———□).

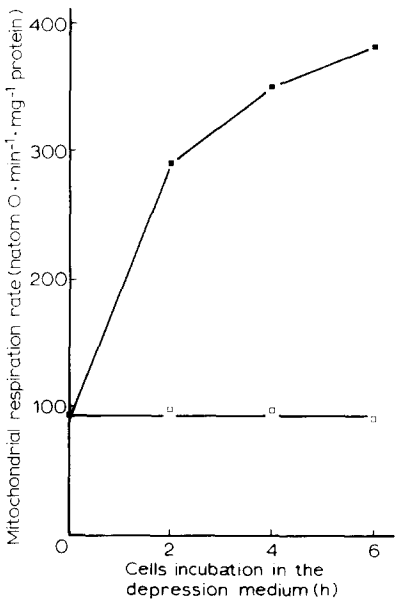

Fig. 7. Changes in respiration rates of mitochondria isolated from yeast undergoing glucose derepression. Mitochondria (0.8 mg protein) were suspended in 3 ml of 0.65 M mannitol/10 mM KCl/0.3% serum albumin/5 mM potassium phosphate/10 mM Tris-maleate (pH 6.7). Ethanol was 1% and ADP 0.1 mM, O_2 uptake was measured with a Clark electrode at 27°C. □———□, Mitochondria from cells incubated with chloramphenicol; ■———■, mitochondria from cells incubated without chloramphenicol.

tion and the amount recovered in the corresponding mitochondrial fraction. It was shown that the yield of mitochondrial extraction from cells incubated with or without chloramphenicol was approx. 80% in regard to cytochrome ($a + a_3$) content (not shown here).

Derepression conditions were also tested for the rate of mitochondrial respiration with ethanol as substrate (Fig. 7). A 6 h incubation of repressed cells in absence of chloramphenicol brought about a 4-fold increase in the rate of mitochondrial respiration; it reached 400 natoms O $min^{-1} \cdot mg^{-1}$ protein corresponding to approx. 70% of the respiratory rate of differentiated mitochondria (isolated from yeast grown strictly aerobically on galactose) (cf. Table I). This respiration increase was inhibited when the cells were incubated in presence of chloramphenicol. These data were in agreement with previously reports on derepression experiments performed on growning yeast [39—41].

The changes of the ATPase activity during the incubation of repressed cells revealed two phenomena: The ATPase activity increased during the course of incubation in absence of chloramphenicol but remained constant in presence of the inhibitor. As shown in Fig. 8A, after 6 h of derepression in the galactose medium, ATPase was 2.6 times increased; this agrees with the data of Somlo et al., who derepressed yeast in growth medium supplemented with ethanol [42]. When only the oligomycin-sensitive ATPase was measured, if this activity increased during the derepression in the control medium, in presence of chloramphenicol it decreased. The oligomycin sensitivity was lost after 4 h of incubation (Fig. 8B).

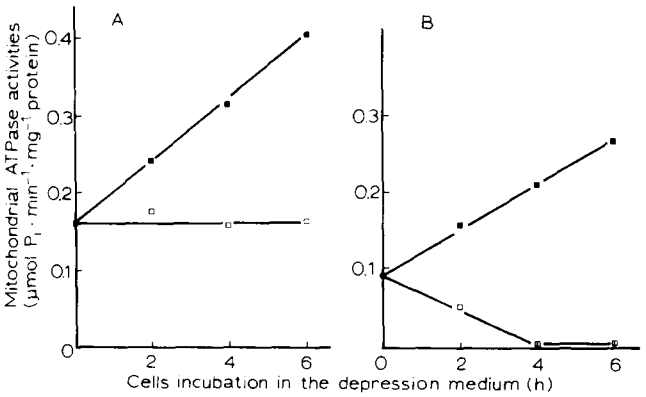

Fig. 8. Changes in ATPase activities of mitochondria isolated from yeast undergoing glucose derepression. A. Total ATPase activity was measured at pH 8.5 and at 30°C. B. Curves represent only the oligomycin sensitive ATPase: oligomycin 10 μg/mg prot. □———□, Mitochondria from cells incubated with chloramphenicol; ■———■, mitochondria from cells incubated without chloramphenicol.

Derepression of the phosphate transport system

The passive permeability properties of the inner membrane and the mechanism of the anionic diffusion can be inferred from swelling experiments of organelles in isoosmotic potassium salt solutions. For instance, it was previously described that mitochondria isolated from aerobically grown yeast swelled in potassium acetate or phosphate solutions only when nigericin or both valinomycin plus uncoupler were added, pointing to an electroneutral penetration of these anions (phosphate/OH^-, acetate/OH^- antiport or phosphate-H^+, acetate-H^+ symport); as in the case of mammalian mitochondria, phosphate swelling was inhibited by mersalyl [22,23]. The same qualitative results were obtained with organelles isolated from yeast grown aerobically in presence of chloramphenicol [23].

Swelling experiments with Glu-mitochondria gave different results (Fig. 9). When respiratory and ATPase activities were blocked, there organelles swelled spontaneously but very slowly and to a similar extent in potassium chloride, acetate or phosphate solutions. Nigericin induced a large swelling only in acetate and not in phosphate solutions. However, after 2 h derepression in galactose medium, with or without chloramphenicol, mitochondria swelled largely in potassium phosphate upon nigericin addition. The swelling rate became maximal after 4 h of cell incubation. Chloramphenicol did not affect this process although the rate of swelling was lower. In both cases the swelling was inhibited by mersalyl (Fig. 9).

The incubation of repressed cells in presence or absence of chloramphenicol did not modify the rate of swelling of mitochondria in acetate solutions.

The rate of [^{32}P]phosphate uptake was studied with mitochondria from cells incubated 4 h in the derepression medium in the presence of chloramphenicol. The substrate concentration dependence on the rate of transport as a double reciprocal plot shows that the transport followed a non-saturation kinetics (Fig. 10A) although the rates of label incorporation were significantly higher than in chloramphenicol-mitochondria.

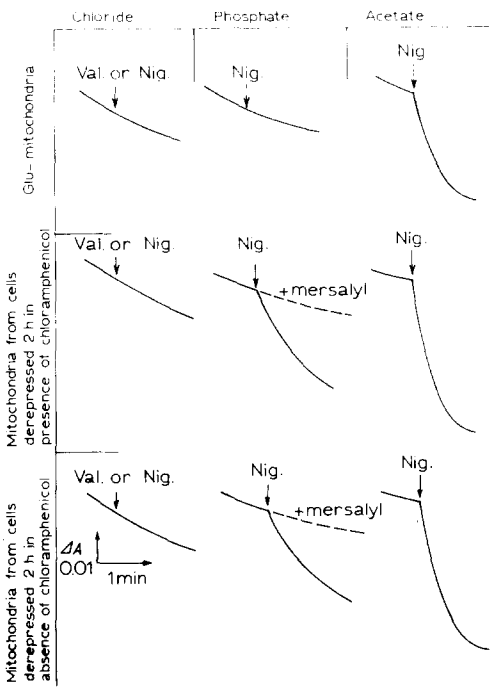

Fig. 9. Swelling in isoosmotic potassium salt solutions of Glu-mitochondria and mitochondria from yeast cells undergoing glucose derepression for 2 h in presence or absence of chloramphenicol. Mitochondria (1 mg protein) were suspended in 0.2 M potassium salt solutions containing 0.5 μg antimycin and 10 μg oligomycin, pH 6.8. As mentioned on the figure, additions were 0.01 μg valinomycin (Val.); 0.01 μg nigericin (Nig.); 1 mM mersalyl. Light scattering changes were measured at 546 nm and at 20° C using an Eppendorf spectrophotometer.

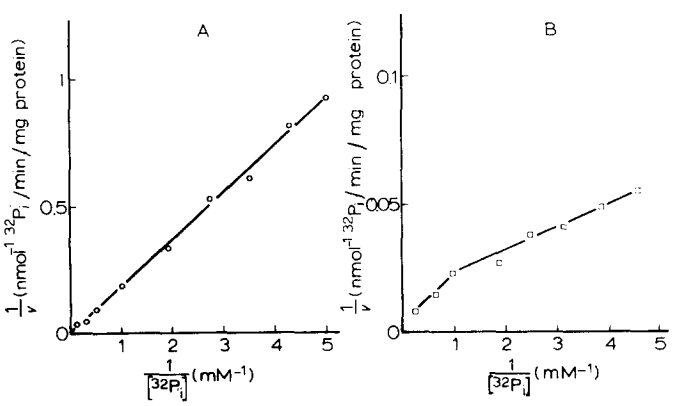

Fig. 10. Effect of addition of chloramphenicol and cycloheximide on [^{32}P]phosphate uptake in mitochondria from yeast undergoing glucose derepression. A. Glucose-repressed cells were incubated 4 h in the derepression medium in presence of 4 g/l chloramphenicol. B. Glucose-repressed cells were incubated 4 h in presence of chloramphenicol and then 6 h in presence of 25 mg/l cycloheximide. Initial rates were measured as described in Fig. 3.

Since the derepression transport system seemed to be inhibited by chloramphenicol, the effect of the cycloheximide, an inhibitor of the cytoplasmic protein synthesis was tested too. But Tzagoloff's experiments with yeast suggested that mitochondrial protein synthesis was dependent on some products of the cytoplasmic system and that the extent of synthesis may be proportional to the cytoplasmic products present in the cell [43]. Then, two inhibitors of protein synthesis, chloramphenicol and cycloheximide were added sequentially during the derepression of yeasts. After harvesting, yeast cells grown aerobically on glucose were first incubated for 4 h in the derepression medium containing 4 g/l of chloramphenicol. Then cells were harvested, washed in order to eliminate chloramphenicol and incubated again for 6 h in a fresh medium containing 25 mg/l of cycloheximide. Under these derepression conditions, an oligomycin sensitivity of the ATPase and a respiratory control were restored although the total ATPase activity and the Q 02 (in the uncoupled state) did not increase. The substrate concentration dependence on the rate of [^{32}P]phosphate uptake in these organelles is shown in Fig. 10B as a double reciprocal plot; a hyperbolic-like curve was obtained, as for Gal-mitochondria, pointing to a total derepression of the phosphate transport system in spite of cycloheximide addition.

Discussion

Two experimental approaches are generally used to study permeability and transport in mitochondrial membranes [44]: (a) The swelling of mitochondria in isoosmotic salts solutions; (b) the kinetics of the exchange.

The first method requires high concentrations of permeant substrates (0.2 M phosphate) so that the massive uptake of these substances down its concentration gradient is the cause of the swelling. The second method does not measure a net uptake although a disequilibrium of concentration of the substances between the intra and extra matrix spaces can exist. For instance, the distribution of the weak acids between the two phases is dependent on the ΔpH [37]. Therefore, since the two methods measure two different processes (net uptake or exchange) and in two different conditions (high or low external concentration) it was not implicit that the mechanism of the anion translocation was identical in both conditions.

Two types of modifications are described. (1) A full repression of the phosphate transport system by cells grown on high concentrations of glucose; (2) a transition from saturation kinetics of [^{32}P]phosphate uptake to a diffusion-like process by inhibition of the mitochondrial protein synthesis. The fact that mitochondrial products are required to obtain saturation kinetics for phosphate transport, is based on several lines of evidence: (a) The diffusion-like process for [^{32}P]phosphate uptake was observed in three types of organelles; chloramphenicol-mitochondria, mitochondria isolated from a "petite" mutant and mitochondria from yeast undergoing glucose derepression in presence of chloramphenicol. (b) The restoration of a saturation kinetics in mitochondria from glucose-repressed cells incubated in presence of cycloheximide after a prior incubation in presence of chloramphenicol.

In contrast with glucose repression, the lack of mitochondrial protein synthesis did not affect the mersalyl-sensitive swelling in isoosmotic phosphate

solution (see also ref. 23). To explain these results, two hypothesis can be proposed:

(1) The Pi cross the internal membrane by two independent ways; a carrier-mediated transport and a diffusion process. The Pi diffusion, however, depends on the integrity of the membrane since mersalyl inhibits that process. That interpretation is in accordance with the data obtained with Gal-mitochondria and Promitochondria since at least two mechanisms were detected (Fig. 4, A and B). However, in Lac-mitochondria only one carrier-mediated transport was observed (Fig. 3).

(2) There is only one system for Pi transport, but it can be modified in some types of organelles. Therefore, the phosphate carrier seems composed of at least two components; (a) the first component alone is sufficient to permit a mersalyl-sensitive phosphate uptake only when the external phosphate concentration is high (like-diffusion process). This component appeared to be damaged by the catabolite repression (Glu-mitochondria). It is possible that this component could be related with one of the thiol-reagents binding proteins isolated from mammalian mitochondria [15—17]. (b) A second component is required for the complete phosphate transport system (defined by the saturation kinetic). This component either binds phosphate or modifies the affinity of the first component. Since the ability of yeast mitochondria to catalyse a rapid phosphate exchange was inhibited by chloramphenicol and not by cycloheximide, the synthesis or the integration of the second component seems to depend on the mitoribosomal protein synthesis.

It is well known that mitochondrial products are insoluble species of protein which appear to have a high degree of hydrophobicity (see ref. 45 for review). Kadenbach and Hadvary were able to isolate from rat liver mitochondria a chloroform-soluble protein which binds phosphate specifically in chloroform solution [46]. The authors proposed that this protein could be the Pi-carrier. Therefore, it is possible that such a protein could be the component of the Pi-carrier synthesized on the mitochondrial ribosomes. Work is in progress in order to isolate chloroform-soluble proteins in the various types of yeast mitochondria [47].

Another point of view on this study is a comparison between the activity of the P_i-carrier and the other mitochondrial functions in order to establish a possible correlation between the Pi-carrier assembly and that of another mitochondrial enzyme or multienzyme complex. Promitochondria differ essentially from Gal-mitochondria since they are lacking in the respiratory chain [33,34] but both organelles possess an oligomycin-sensitive ATPase (ref. 34 and Table I) and at least one P_i-carrier system. This suggests that the assembly of P_i-carrier is dissociable from that of the respiratory chain. However, the P_i-transport is significantly modified in chloramphenicol-mitochondria which are lacking both in some cytochromes and in oligomycin-sensitive ATPase (refs. 35, 36 and Table I). However, an assembly relation between P_i-carrier and ATPase complex becomes more apparent from the derepression experiments. In fact, in presence of chloramphenicol, the ATPase loses its oligomycin sensitivity and the P_i-transport follows a diffusion like-process; the respiratory rate is not modified under these conditions. In the presence of cycloheximide, both P_i-carrier and ATPase complex are derepressed although any increase in the respiration rate is detected.

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