Biochimica et Biophysica Acta, 770 (1984) 40–46
Elsevier

BBA 72006

DEREPRESSION OF THE HIGH-AFFINITY PHOSPHATE UPTAKE IN THE YEAST *SACCHAROMYCES CEREVISIAE*

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(Received August 30th, 1983)

Key words: Phosphate transport; High-affinity phosphate uptake; Ion transport; Derepression; (S. cerevisiae)

Phosphate starvation derepresses a high-affinity phosphate uptake system in *Saccharomyces cerevisiae* strain A294, while in the same time the low-affinity phosphate uptake system disappears. The protein synthesis inhibitor cycloheximide prevents the derepression, but has no effect as soon as the high-affinity system is fully derepressed. Two other protein synthesis inhibitors, lomofungin and 8-hydroxyquinoline, were found to interfere also with the low-affinity system and with Rb^+ uptake. After incubation of the yeast cells in the presence of phosphate the high-affinity system is not derepressed, but the V_{max} of the low-affinity system has decreased for about 35%. Phosphate supplement after derepression causes the high-affinity system to disappear to a certain extent while in the meantime the low-affinity system reappears. The results are compared with those found in the yeast *Candida tropicalis* for phosphate uptake.

Introduction

In the yeast *Saccharomyces cerevisiae* there are three different systems involved in the uptake of inorganic phosphate (P_i). The so-called low-affinity transport system is considered to be a constitutive system with a dissociation constant in the order of 1 mM at pH 4.5 [1,2]. The two other systems are high-affinity systems that are derepressible. One consists of a H^+-phosphate cotransport with a K_m of approx. 10 μM at pH 4.5 [2,3], the other one is a Na^+-phosphate cotransport with a K_m for phosphate in the order of 1 μM at pH 7.2 [4]. We will call this the high-affinity system and the Na^+-phosphate cotransport, respectively. Derepression of these two processes occurs when yeast cells are incubated in a medium containing a suitable substrate but lacking in P_i, both under aerobic and anaerobic conditions [2,4,5]. The derepression of the high-affinity system has never been studied in detail. The rate of uptake via this system is maximal after approx. 2 h incubation [2,6], and the derepression can be impaired by adding an inhibitor of protein synthesis like cycloheximide [7].

In the yeast *Candida tropicalis* at least two phosphate transport systems do exist. One is constitutive and has a low affinity for phosphate (K_m: 1.2 mM). The other system is comparable with the high-affinity system of *S. cerevisiae*. It has a K_m of 4.5 μM and this system also appears only after phosphate starvation [8]. The latter system disappears quickly when the incubation medium of the yeast cells is supplemented with P_i. The protein synthesis inhibitors cycloheximide and 6-methylpurine block the derepression but have no effect as soon as the system is fully derepressed [9]. The authors explain the appearance and disappearance of the high-affinity system in this yeast as the result of a balance between continuous synthesis and degradation of the carrier involved. They assume a direct or indirect role of phosphate in this

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degradation [9]. The high-affinity system in *C. tropicalis* can be reduced drastically by osmotic shock [8] or protoplast formation [11] with a release of two binding proteins for inorganic phosphate [10,11]. These two binding proteins, located in the cell wall near the cell surface, should bind external P_i and could load a P_i-carrier present at the plasmalemma. It was hypothesized by Jeanjean et al. [11] that this membrane carrier, presumably a constitutive carrier, could also bind P_i directly without the mediation of the P_i-binding proteins though with a low affinity. This would result in low-affinity P_i-uptake which will predominate when the binding proteins are not derepressed by P_i starvation.

Although the high-affinity system in *S. cerevisiae* is osmotic shock sensitive [12] we have failed so far in isolating binding proteins from this yeast after osmotic shock as well as after protoplast formation [13]. Moreover, the disappearance of the high-affinity system after osmotic shock was found to be the result of severe damage of the plasmamembrane [13]. Information about the derepressible phosphate transport system in *S. cerevisiae* can therefore only be obtained by indirect means.

Materials and Methods

Yeast cells, *Saccharomyces cerevisiae* strain A294 (a non-flocculent brewing yeast obtained from Whitbread and Co., Ltd., Luton, U.K.) were grown as described in Ref. 14, until the early exponential phase of growth. After harvesting by centrifugation the cells were washed twice with 45 mM Tris buffer adjusted with succinic acid to pH 4.5 (Tris-succinate buffer), and finally resuspended to a density of 10 mg dry weight/ml in this buffer, provided with 3% glucose (w/v) and incubated for the indicated time at 25°C. The cells were kept anaerobic during this incubation by bubbling nitrogen through the suspension. Uptake of phosphate was performed as described in Ref. 4, uptake of Rb^+ was performed as described in Ref. 15. All uptakes were done in the Tris-succinate buffer provided with 3% glucose (w/v) at 25°C under anaerobic conditions. At low P_i concentrations (< 100 μM) the uptake is too fast at the above mentioned cell density and in those cases the cells were diluted to 2 mg dry weight per ml at the beginning of the uptakes. The results are presented according to Hofstee [16] by plotting the rate of uptake (v) against the quotient of the uptake rate and the phosphate concentration (v/s). The kinetical constants of the phosphate uptake were then obtained by fitting the results to a single hyperbola or, when deviations from simple Michaelis-Menten kinetics were found, to a double hyperbola with appropriate curve fitting programs. Cell pH was measured according to Ref. 17. Inorganic phosphate in the medium was estimated according to Ref. 18.

Cycloheximide (Sigma), lomofungin (a gift of Dr. H. Aelbers of Upjohn Nederland) and 8-hydroxyquinoline (Merck) were dissolved in ethanol. The control incubations received the same amount of ethanol (final concentrations always less or equal then 1% (v/v)). No effects were found if the inhibitors were only present during the uptakes itself and for this reason they were not removed after the respective incubations.

Results

Yeast cells of the strain A294 when harvested in the early exponential phase of growth and preincubated for 15 min with glucose exhibited a low-affinity phosphate uptake with a K_m of 1.65 mM. If cells were preincubated for 3 h with glucose, but without P_i, a high-affinity uptake system appeared with a K_m of 18.8 μM, while the low-affinity system had disappeared. However, for both systems approximately the same maximal velocity (V_{max}) was found (see also Table I). If cells were pre-incubated for an intermediate time (90 min) both systems were present and the total V_{max} under this condition was equal to the V_{max} of the high affinity system (Fig. 1).

This raised the question whether both systems share the same carrier, i.e. only the affinity for phosphate changed upon P_i starvation but not the carrier, as suggested by Jeanjean et al. [11], or that the low-affinity system disappeared by coincidence in the same time the high-affinity system appeared. The derepression of the high affinity system in *C. tropicalis* is dependent on protein synthesis as judged by the effect of cycloheximide [9]. Also in *S. cerevisiae* cycloheximide was found to impair derepression of phosphate uptake [7]. In

TABLE I

DEPENDENCE OF THE KINETIC PARAMETERS OF P_i UPTAKE IN *S. CEREVISIAE* UPON THE PRETREATMENT OF THE CELLS. EFFECT OF P_i STARVATION, P_i SUPPLEMENT AND CYCLOHEXIMIDE

Since the V_{max} values were found to vary between the different experiments (due to the use of different batches of cells) this parameter is expressed as percentage of the V_{max} of phosphate uptake in control cells after 3 h P_i starvation for each experiment. Where possible the values are presented as the mean, $\pm$S.E. V_{max} total is the sum of the percentage of the V_{max} of the low- and high-affinity system, respectively. P_i means 5 mM inorganic phosphate added, Mg^{2+} means 1 mM $MgCl_2$ added and cycloheximide means 50 μg/ml cycloheximide added. The cells were preincubated in the Tris-succinate buffer at 25°C, with 3% glucose (w/v).

Preincubation time before P_i uptake (min)	Addition	High affinity		Low affinity		V_{max} total (%)
		V_{max} (%)	K_m (μM)	V_{max} (%)	K_m (mM)	
15	–			87	1.65	87
90	–	60	19.5	44	1.65	104
180	–	100	16.0 ± 0.8			100
180	cycloheximide at $t = 0$	1 [a] (2)	10.1 [a]	53 [a] (106)	1.60	108
180	P_i at $t = 0$ until $t = 165$	1	10.3	34	1.70	35
180	$P_i + Mg^{2+}$ at $t = 0$ until $t = 165$	1	8.4	35	1.50	36
315	–	113 ± 4	17.5 ± 0.9			113
315	cycloheximide at $t = 180$	98	21.4			98
315	P_i at $t = 180$ until $t = 300$	11	10.8	29	1.65	40
315	$P_i + Mg^{2+}$ at $t = 180$ until $t = 300$	3	10.0	7	1.50	10

[a] The decrease in the K_m and the V_{max} of the high-affinity system and in the V_{max} of the low-affinity system is due to an effect of cycloheximide on the cell pH. The corrected values are shown within brackets (see also Results).

order to distinguish therefore between the two possibilities the derepression of the high-affinity system was blocked with inhibitors of protein synthesis like cycloheximide [19], 8-hydroxyquinoline [20] or lomofungin [20]. Since cycloheximide was found to inhibit to some extent respiration and fermentation rates [7] all compounds were tested over a concentration range for their effect on the high-affinity system, as well as the low affinity system and the monovalent cation uptake via the Rb^+ uptake, which is sensitive to impairment of metabolism [21]. The results are shown in Fig. 2.

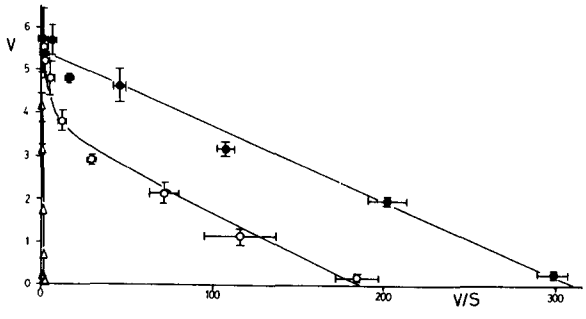

Fig. 1. P_i-uptake after 15 min, 90 min and 180 min P_i starvation, respectively. Cells were incubated with glucose for 15 min (△), 90 min (○) and 3 hours (●) without P_i. The lines represent linear and nonlinear regression of the data, used to calculate the values of V_{max} and K_m. The initial uptake rate v is expressed as mmol per kg dry weight per min and v/s as litre per kg dry weight per min. The bars denote standard deviations (duplicate measurements).

At 50 μg/ml cycloheximide fully inhibited the derepression of the high affinity system whereas 8-hydroxyquinoline gave 90% inhibition at 500 μg/ml. In contrast, the maximal inhibition found with lomofungin was only 42%. The effects of cycloheximide and lomofungin on the low-affinity system are quite similar. At the higher concentrations an increase of 20–30% was found. However, 8-hydroxyquinoline decreased phosphate uptake via the low-affinity system with 87% at 500 μg/ml. A stimulation of approximately 20% was found in the Rb^+ uptake with lomofungin at the higher concentrations. On the other hand both cycloheximide and 8-hydroxyquinoline were found to

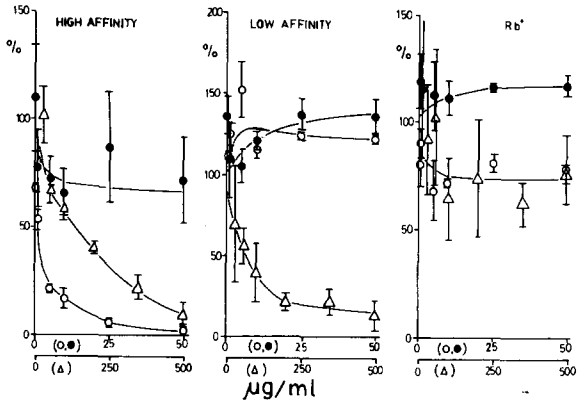

Fig. 2. Effect of protein synthesis inhibitors on P_i- and Rb^+ uptake. Cells were incubated with glucose for 15 min in case of the low-affinity P_i-uptake and the Rb^+-uptake and for 3 h in case of the high-affinity P_i-uptake. The respective inhibitors were added to the medium directly at the beginning of the incubation, and were not removed during the uptake measurements (see Materials and Methods). Uptake experiments were performed as described in Materials and Methods at a concentration of 1 μM (P_i) and 1 mM (Rb^+), respectively, and results are presented as percentage of the control. ○——○, cycloheximide; ●——●, lomofungin; Δ——Δ, 8-hydroxyquinoline. The results are means of duplicate measurements.

inhibit Rb^+ uptake for 20–30%. Since lomofungin did not give full inhibition of the derepression of the high-affinity system it is likely that this compound does not dissolve completely at the higher concentrations. 8-Hydroxyquinoline strongly inhibited phosphate uptake via the low-affinity system. To our opinion, therefore, cycloheximide is the best choice, although this compound interfered also to some extent with the low-affinity P_i-uptake and Rb^+-uptake. The effect of 50 μg/ml of this compound on the derepression is shown in Fig. 3.

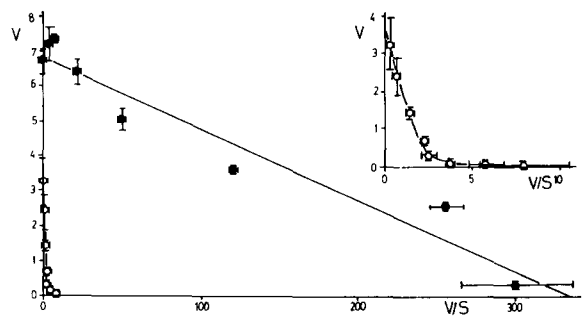

Fig. 3. Effect of 50 μg/ml cycloheximide on the derepression of the high-affinity P_i uptake. Cells were incubated with glucose for 3 h with (○) or without (●) 50 μg/ml cycloheximide. For further details see Fig. 1. Inset: Extended scale for the uptake after incubation with cycloheximide.

As expected, cycloheximide inhibited almost completely the derepression of the high-affinity system. Only 1% of the V_{max} of the control was found after pre-incubation with cycloheximide. The decrease in the V_{max} of the low-affinity system was probably due to the accompanying decrease in the cell pH, caused by incubation of the cells with cycloheximide. Lowering the cell pH of the yeast cell with 4 mM butyric acid caused the V_{max} of the low-affinity system to decrease with approx. 50% and the cell pH from 6.76 ± 0.02 (S.E.) to 6.39 ± 0.04, whereas the K_m remained constant. Since approximately the same decrease in cell pH was found with cycloheximide (6.27 ± 0.06) this will account for the decrease in V_{max} as found after incubation with cycloheximide. The decrease in cell pH may also account for the decrease in the K_m of the high-affinity system found after incubation of the cells with cycloheximide (10.1 μM compared to 20.4 μM). Since both K_m and V_{max} of the high-affinity system depend upon the cell pH [22], also the V_{max} of the high-affinity system should be higher namely 2% of the V_{max} of the control.

In *C. tropicalis* cycloheximide also blocks the derepression of the high-affinity system but has no effect as soon as the system is fully derepressed [9]. We have checked whether this is also true for *S. cerevisiae*. As found in *C. tropicalis* cycloheximide had no effect after derepression. The small increase in the V_{max} of the control found after 5 h pre-incubation compared to 3 h (6.98 vs. 5.71 $mmol \cdot min^{-1} \cdot kg^{-1}$ dry weight) showed that derepression was still not complete after 3 h. This small increase was prevented by cycloheximide. In *C. tropicalis* the same effect was found. Addition of cycloheximide during the derepression of the high-affinity system in this yeast arrested the uptake at the level attained then [9].

As mentioned in the introduction, the high-affinity system in *C. tropicalis* disappears quickly when the medium of the cells is supplemented with P_i. To test the effect of P_i on the derepression of the high-affinity system in *S. cerevisiae*, cells were incubated for 2.75 hours with or without phosphate, centrifuged, washed twice with fresh buffer

and resuspended in buffer with 3% glucose (w/v) for another 15 min. After this incubation the phosphate uptake was measured. The amounts of P_i remaining in the final incubation medium after the P_i treatment were of the order of magnitude of 10 μM (compared to 3 μM in the control). The results are presented in Fig. 4.

The presence of 5 mM P_i during the incubation almost completely prevented the derepression of the high-affinity system. Less than 1% of the V_{max} of the control was found. The V_{max} of the low-affinity system decreased to approx. 30%. Contrary to what was found with cycloheximide, this decrease could not be attributed to a decrease in the cell pH. No significant decrease in cell pH was found after the P_i treatment. We tested the effect of P_i on both phosphate transport systems after derepression of the high-affinity system. The same protocol was followed as in the experiment of Fig. 4 except that P_i was supplemented to the medium after 3 h incubation with glucose and was removed after another 2 h incubation, followed again by a final incubation of 15 min. The results are presented in Fig. 5.

Although the high-affinity system was fully derepressed after 3 h (control 3 h, Fig. 5) another 2 h incubation with P_i gave rise to a decrease in V_{max} of this system for approx. 90%. The low-affinity system, however, reappeared with a V_{max} of approx. 25% of the V_{max} of the control.

During prolonged phosphate accumulation by

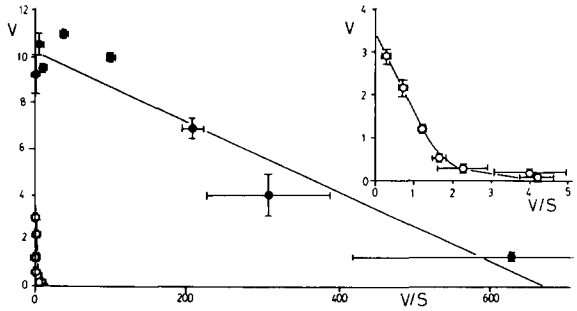

Fig. 4. Effect of 5 mM P_i on the derepression of the high-affinity P_i uptake. Cells were incubated with glucose for 2.75 h with (○) or without (●) 5 mM P_i. Afterwards the cells were washed and resuspended and phosphate uptake was measured after another 15 min incubation with glucose in the absence of phosphate. For further details see Fig. 1. Inset: Extended scale for the uptake after incubation with P_i.

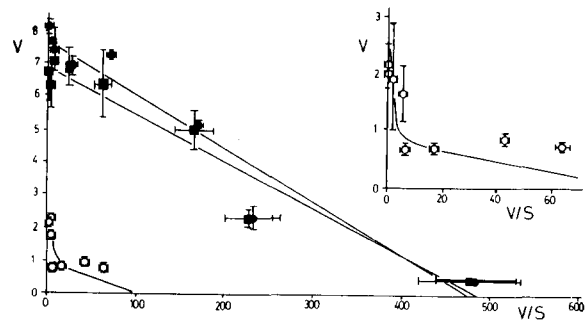

Fig. 5. Effect of 5 mM P_i on the P_i uptake after the derepression of the high-affinity system. After 3 h incubation with glucose, part of the cells was used to measure the P_i uptake (■), afterwards P_i (to a final concentration of 5 mM) was added to the medium of the remaining cells. 2 h later these cells were washed and resuspended as described in the text and after another 15 min incubation with glucose, P_i uptake was measured (○) and compared to control cells that received no P_i (●). Inset: Extended scale for the uptake in cells that received P_i after 3 h incubation.

yeast a decrease in the rate of phosphate absorption was found which was accompanied by a parallel decrease in glycolysis [23]. This was ascribed to be due to complexation of internal Mg^{2+} by phosphate compounds formed in the cell. The decrease in the rate of glycolysis could be come over by adding Mg^{2+} to the medium. These effects might well account for the decreased V_{max} of the low-affinity system found after phosphate accumulation in the experiments of Figs. 4 and 5 (and even for the lack of derepression of the high-affinity system), and therefore the experiments as described in Figs. 4 and 5 were repeated with 5 mM phosphate and 1 mM $MgCl_2$. It appeared, however, that also in the presence of Mg^{2+}, phosphate decreased the maximal rate of the low-affinity system and prevented derepression of the high-affinity system, see also Table I.

Discussion

According to earlier findings [2,4,5] starvation for inorganic phosphate led to the derepression of a high-affinity phosphate uptake system in the yeast *S. cerevisiae* as has been found in the yeast *C. tropicalis* [8] and in the related fungus *Neurospora crassa* [24]. The appearance of the high-affinity system was accompanied by the disappearance

of the low-affinity system. The sum of the maximal rates of uptake of the two transport systems appeared to be constant. When the high-affinity system was fully derepressed after 3 h P_i starvation, the low-affinity phosphate uptake system (present before P_i starvation) had disappeared. The results are not in accordance with the generally held view [25] that at least two distinct transport systems are involved in phosphate uptake in yeast, a constitutive low affinity system and a derepressible high-affinity system. In that case the low-affinity phosphate uptake should not decrease in activity, as was found by us. On the other hand the results are compatible with the hypothesis put forward by Jeanjean et al. [11], that the same (constitutive) carrier is involved in both the high-affinity and the low-affinity transport of phosphate. Apparently the state of the carrier changes during phosphate starvation turning from a low-affinity system to a high-affinity system. This change can be prevented by an inhibitor of protein synthesis like cycloheximide. Cycloheximide blocks protein synthesis by binding to the 60 S ribosomal subunit [19]. Although this compound was found to interfere with other cellular processes as shown by Reilly et al. [7], the results presented in this paper (Fig. 2) indicated only minor effects on the low-affinity phosphate uptake and the Rb^+ uptake and therefore it is likely that de novo protein synthesis is involved in the derepression of the high-affinity system.

The idea of a common carrier for both the low- and high-affinity system is supported by the fact that on adding P_i to yeast cells having developed the high-affinity system, the low-affinity system reappears again. It can be argued that the decrease in the total maximal rate of uptake may be traced to changes in the cellular phosphate concentration. As shown earlier, phosphate uptake via the high-affinity system can be accounted for by a transport system mediated by a mobile carrier [22]. The transport kinetics of a mobile carrier system are characterized by the fact that the kinetical parameters (K_m and V_{max}) are not constant anymore but depend upon the concentrations of the intracellular solutes, which also have affinity to the carrier [26]. For the high-affinity phosphate uptake system, a H^+-phosphate cotransport [3,27], these solutes are orthophosphate and protons. It has been shown experimentally that on varying the cell pH both the K_m and the V_{max} of this transport system change [22]. In this paper we have shown that the V_{max} of the low-affinity system also depends upon the cell pH in accordance with the view that a mobile carrier may be involved in the low-affinity transport, too, which should be expected according to the hypothesis of Jeanjean, mentioned above. The lack of detectable changes in the K_m for the low-affinity system in that case may be traced to the fact that, due to the already high K_m, small changes may be overlooked. That phosphate loading of the cells also affects the kinetical parameters of phosphate uptake, as theoretically expected, is indicated by the fact that the K_m of the high-affinity system is reduced by loading the yeast cells with phosphate, as shown in Table I. This K_m is in addition overestimated because of the presence of the low amounts of orthophosphate in the medium of the cells. Since changes in V_{max} and K_m are closely related for a mobile carrier system [22,26], it may be expected that loading of the cells with phosphate will lead to a reduction in the total maximal rate of uptake, too.

Our results did further show that, in accordance with the results of Blasco et al. [8] with *C. tropicalis,* loading with phosphate leads to a reduction in the high-affinity transport capacity for cells which have already developed the high-affinity system. These authors did not examine whether in *C. tropicalis* the low-affinity system also reappeared simultaneously, as was found by us for *S. cerevisiae.*

Acknowledgements

The strain A294 was a gift of Mr. D. Lawrence of Whitbread and Co., Ltd., Luton (U.K.). Lomofungin was a gift of Dr. H. Aelbers of Upjohn Nederland. The technical assistance of Mr. J. Dobbelmann and Mr. H. De Bont is gratefully acknowledged. This study was supported by a grant of the Netherlands Organization for Pure Research (Z.W.O.) under auspices of the Netherlands Foundation for Biophysics.

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