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PHOSPHATE TRANSPORT IN *NEUROSPORA*

DEREPRESSION OF A HIGH-AFFINITY TRANSPORT SYSTEM DURING PHOSPHORUS STARVATION

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SUMMARY

In addition to the constitutive, low-affinity phosphate-transport system described previously, *Neurospora* possesses a second, high-affinity system which is derepressed during phosphorus starvation. At pH 5.8, System II has a $K_{1/2}$ of about 3μM and a J_{max} of 5.2 mmol/l cell water per min.

System II reaches maximal activity after about 2 h of growth in phosphorus-free minimal medium. Its formation is blocked by cycloheximide and, once made, it appears to turn over rapidly. Addition of cycloheximide to fully derepressed cultures results in the decay of System II with a $t_{1/2}$ of 14 min,very similar to the turnover rate previously reported (Wiley, W. R. and Matchett, W. H. (1968) J. Bacteriol. 95, 959–966) for tryptophan transport in *Neurospora*. Thus, these transport systems appear to be regulated by a balance between synthesis and breakdown, as affected by intracellular pools of substrate or related compounds.

INTRODUCTION

Work in a number of laboratories during the past few years has revealed a common pattern for the regulation of membrane transport in *Neurospora*. Transport systems for sugars [1, 2], amino acids [3–6], and SO_4^{2-} [7–9] are specifically repressed by their substrates or by related compounds.Thus, carbon-starved cells contain at least five sugar-transport systems; both a low-affinity and a high-affinity system for glucose, which have been extensively characterized [1, 2, 10], and separate systems for fructose, galactose, and lactose [1]. During growth in the presence of sucrose or glucose, however, the latter four systems are repressed and only low-affinity glucose transport remains. Similarly, cells grown in the absence of exogenous amino acids have a well-defined transport system for neutral amino acids [4, 11]; when the intracellular concentration of tryptophan or leucine (or presumably any other neutral

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amino acid) is raised, this system is rapidly repressed [3]. Finally, cells growing in minimal medium synthesize two distinct transport systems for SO_4^{2-}, one present in conidia and the other formed after germination [7–9]. Both systems are repressed in the presence of methionine, and two control genes have been identified which play a role in this regulatory process [12, 13].

During a study of phosphate transport in *Neurospora*, we became interested to see whether the same pattern of regulation holds. The first stage of the work established that normal exponential-phase cells, grown in Vogel's medium containing an excess of inorganic phosphate (37 mM), possess a phosphate-transport system with a J_{max} of about 1.5 mmol/l cell water per min and a $K_{1/2}$ which varies from 12 μM to 3.6 mM as the extracellular pH is varied from 4.0 to 7.3 (ref. 14). The present experiments reveal that, upon phosphorus starvation, a second phosphate-transport system is de-repressed.

A preliminary account of these results has already appeared [15].

METHODS

Wild-type strain RL21a of *Neurospora crassa* was used throughout this work. Cells were grown in Vogel's minimal medium (37 mM P_i) or, for phosphorus starvation, in P-free minimal medium [14]; in both cases, 2 % sucrose served as carbon source. Flux measurements were carried out with cells resuspended either in buffer (3,3-dimethylglutarate, pH 4.0–6.4, or *N*-2-hydroxypiperazine-*N*'-2-ethanesulfonate, pH 7.3) or in P-free minimal medium (see figure legends for details): no carbon source was added to the flux solutions [14].The methods for measuring $^{33}PO_4$ fluxes and computing results have been described previously [14].

To examine the role of protein synthesis in the increase in phosphate transport during P starvation, cycloheximide (2 μg/ml) was added to the growth medium. A control experiment showed that this concentration of cycloheximide was sufficient to inhibit protein synthesis almost completely: it decreased the incorporation of [^{14}C]-lysine (New England Nuclear) into trichloroacetic acid-insoluble material by 98 % but did not affect the initial rate of lysine uptake by the cells.

The osmotic shock procedure was similar to that of Wiley [16]. Cells were grown 15 h in Vogel's minimal medium + 2 % sucrose (normal) or 14 h in that medium followed by 3 h in P-free minimal medium + 2 % sucrose (P-starved). Cells were harvested, suspended at 30 °C in 30 % sucrose/33 mM Tris/1 mM EDTA, pH 8.1, and aerated vigorously for 15 min, then filtered and resuspended in distilled water at 0 °C and aerated for another 15 min, and finally filtered again, washed with cold distilled water, and resuspended in P-free minimal medium for the measurement of $^{33}P_i$ influx.

RESULTS

Stimulation of phosphate transport during phosphorus starvation

We have previously established that wild-type *Neurospora*, growing in Vogel's minimal medium (37 mM P_i), maintains a constant level of intracellular phosphorus (P_c; about 300 mmole/l cell water) during the entire exponential phase, from 8 to 20 h [14]. Fig. 1A illustrates the consequences of transferring the cells to P-free medium [14] at 14 h: when a carbon source (such as sucrose) is provided, growth continues for

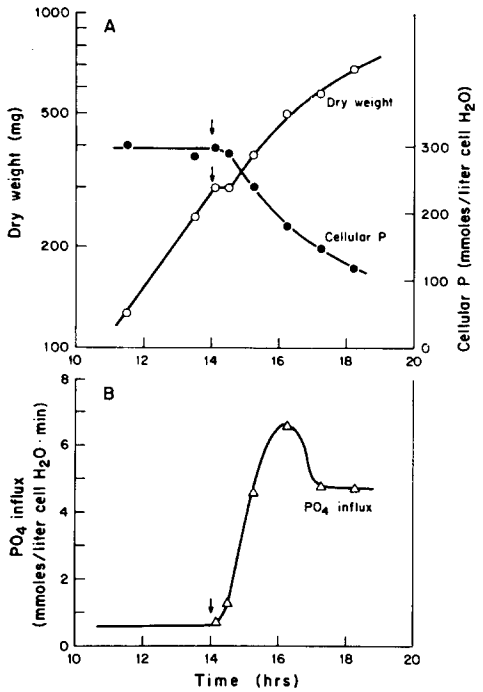

Fig. 1. Effect of P starvation on growth, cellular phosphorus, and P_i influx. Cells were grown in Vogel's minimal medium+2 % sucrose and, at 14 h, transferred to P-free minimal medium+2 % sucrose. At intervals, aliquots of the culture were removed for the determination of dry weight (mg/160 ml) and cellular P (see ref. 14), and also for the measurement of $^{33}PO_4$ influx (at a concentration of 0.05 mM P_i).

several hours, but more slowly than in the usual minimal medium and at the expense of intracellular phosphorus. In this experiment, P_c dropped to one-third of its normal value in 4 h.

At the same time, phosphate transport increased dramatically (Fig. 1B). The initial rate of transport, measured at 50 μM P_i, rose from 0.6 mmol/l cell water per min in normal cells to a maximal value of 6.5 mmol/l cell water per min after 2 h of starvation, and then stabilized at 4.7 mmol/l cell water per min, an 8-fold increase over the original rate.

Effect of cycloheximide

To determine whether protein synthesis is required for the increase in phosphate transport, cycloheximide (2 μg/ml, see Methods) was added to the culture, either at the time of resuspension in P-free medium or 1.25 h after resuspension, when the change in transport was well under way. The effects of cycloheximide on growth rate, cellular P and transport are illustrated in Fig 2A, B and C, respectively. It can be seen that early addition of cycloheximide slowed down growth and the depletion of cellular P and completely prevented any increase in P_i transport. When cycloheximide was added after 1.25 h, growth and depletion continued, although somewhat more slowly; not only did the P_i flux fail to increase further, but it actually dropped toward that of nor-

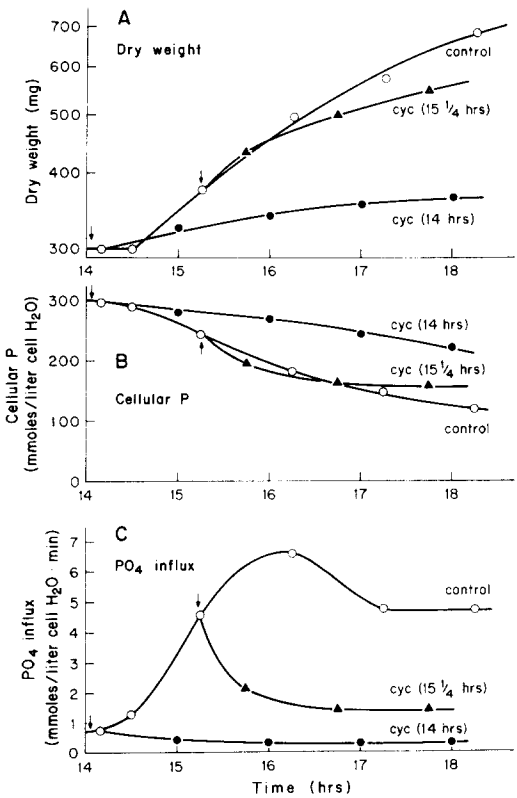

Fig. 2. Effect of cycloheximide on growth, cellular phosphorus, and P_i influx during P starvation. Cells were grown in Vogel's minimal medium+2 % sucrose for 14 h and then transferred to P-free minimal medium+2 % sucrose to begin P starvation. Cycloheximide (2 μg/ml) was added at 14 h or 15.25 h. Aliquots of the culture were removed at intervals for the measurement of dry weight (mg/160 ml), cellular P and $^{33}PO_4$ influx (at a concentration of 0.05 mM P_i), as described in the legend to Fig. 1 and in ref. 14.

mal cells, with a $t_{1/2}$ of about 14 min (a rough estimate, calculated from a semi-logarithmic plot of the data in Fig. 2C).

Kinetic analysis of transport in normal and P-starved cells

Studies of transport as a function of the external P_i concentration indicated that the increase in flux during P starvation results from the emergence of a new transport system. Lineweaver-Burk plots for four cases (normal cells, normal cells incubated in cycloheximide, P-starved cells and P-starved cells incubated in cycloheximide) are displayed in Fig. 3. The combined data were fitted by computer to the sum of two Michaelis functions:

$$J = \frac{J_{\max(\mathrm{I})} \cdot S}{K_{1/2(\mathrm{I})} + S} + \frac{J_{\max(\mathrm{II})} \cdot S}{K_{1/2(\mathrm{II})} + S}$$

holding $K_{1/2(\mathrm{I})}$ and $K_{1/2(\mathrm{II})}$ in common over the four cases and allowing $J_{\max(\mathrm{I})}$ and

TABLE I

KINETIC PARAMETERS OF PHOSPHATE INFLUX AS AFFECTED BY PHOSPHORUS STARVATION AND BY CYCLOHEXIMIDE

Parameters were estimated by joint computer fit of the data in Fig. 3 to the sum of two Michaelis functions, with the $K_{1/2}$ values of System I and System II held in common for the entire set of data and the J_{max} values allowed to vary. All values are given with confidence limits of ± 1 S.E.

Cells	System I		System II	
	$K_{1/2}$ (mM)	J_{max} (mmol/l cell water per min)	$K_{1/2}$ (mM)	J_{max} (mmol/l cell water per min)
Normal	0.166±0.051	1.52±0.26	0.0026±0.0008	0.16±0.18
Normal+cycloheximide		0.88±0.47		0.00±0.31
P-starved		1.19±0.40		5.19±0.30
P-starved+cycloheximide		2.45±0.23		0.33±0.20

$J_{max(II)}$ to vary. The resulting values are recorded in Table I, and have also been used to draw the lines in Fig. 3.

Inspection of the values in Table I reveals that normal cells, as reported previously [14], contain a low-affinity phosphate-transport system (System I) with a J_{max} of 1.52 mmol/l cell water per min and a $K_{1/2}$ (pH 5.8) of 166 μM, and in addition a very

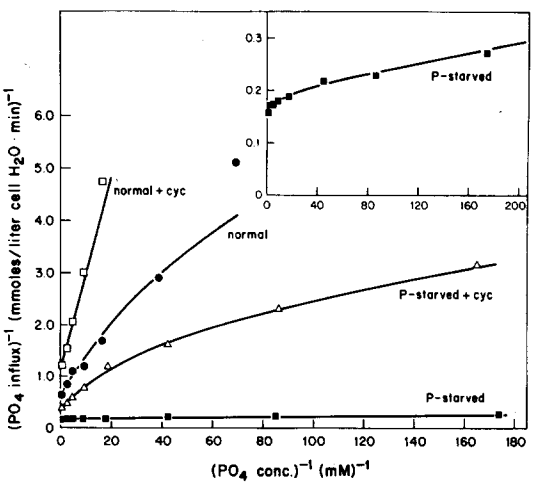

Fig. 3. Lineweaver-Burk plots describing P_i influx as a function of the extracellular P_i concentration. Cells were grown for 14–15 h in Vogel's minimal medium+2 % sucrose, subdivided into four batches, and incubated further as follows. Normal, no further treatment; normal+cycloheximide, 3 to 3.5 h in P-free minimal medium+2 % sucrose+cycloheximide (2 μg/ml); P-starved, 3 to 3.5 h in P-free minimal medium+2 % sucrose; P-starved+cycloheximide, 1 h in P-free minimal medium+2 % sucrose, followed by 2 to 2.5 h in the same medium+cycloheximide (2 μg/ml). Cells were then resuspended in P-free minimal medium (no sucrose) for measurement of $^{33}PO_4$ influx over a range of concentrations. The curves in Fig. 3 were generated by computer fit of the flux data to the sum of two Michaelis functions; parameter estimates are given in Table I.

TABLE II

KINETIC PARAMETERS OF SYSTEM I AND SYSTEM II AS A FUNCTION OF pH

Normal cells were grown 15 h in Vogel's minimal medium + 2 % sucrose. P-starved cells were grown 14 h in that medium followed by 3 h in P-free minimal medium + 2 % sucrose. For the measurement of $^{33}PO_4$ influxes, cells were resuspended in 20 mM buffer (3,3-dimethylglutarate, pH 4.0 and 5.8; *N*-hydroxypiperazine-*N'*-2-ethanesulfonate (HEPES), pH 7.3) + 20 mM KCl, or in P-free minimal medium + 20 mM buffer (dimethylglutarate, pH 4.0 and 5.8; HEPES, pH 7.3) (see ref. 14). At each pH, the flux data were fitted by computer to the sum of two Michaelis functions, with the $K_{1/2}$ values of System I and System II held in common and the J_{max} values allowed to vary. All values are reported with confidence limits of ±1. S. E. The apparent absence of System I in P-starved cells and of System II in normal cells at pH 4, as well as the large standard errors at this pH, reflect the fact that it is impossible to distinguish between two systems with very similar $K_{1/2}$ values; fitting of the data for each type of cells to a single Michaelis function (not shown) did not change the parameter estimates but reduced the standard errors by a factor of 10 to 100.

pH	$K_{1/2}$ (mM)	System I		System II		
		J_{max} (normal) (mmol/l cell water per min)	J_{max} (P-starved) (mmol/l cell water per min)	$K_{1/2}$ (mM)	J_{max} (normal) (mmol/l cell water per min)	J_{max} (P starved) (mmol/l cell water per min)
4.0	0.012±0.043	1.51±6.67	0.00±9.20	0.0048±0.0189	0.00±0.40	2.24±9.12
5.8	0.166±0.051	1.52±0.26	1.19±0.40	0.0026±0.0008	0.16±0.18	5.19±0.30
7.3	12.5±21.3	1.40±1.03	0.54±0.72	0.0064±0.0014	0.095±0.103	2.82±0.12

small amount of a second, high-affinity system (System II) with a J_{max} of 0.16 mmol/l cell water per min and a $K_{1/2}$ (pH 5.8) of 2.6 μM. During P starvation, System I remains essentially unchanged (its J_{max} drops slightly), but System II increases more than 30-fold, to a J_{max} of 5.19 mmol/l cell water per min.

The kinetic parameters of cells treated with cycloheximide are also listed in Table I. The most significant effect of cycloheximide is on System II which, in P-starved cells, declines nearly to the level found in normal cells (J_{max} = 0.33 mmol/l cell water per min). There are also small effects on System I: a 40 % decrease when normal cells are incubated in P-free minimal medium containing cycloheximide, and a 60 % increase in cells partially P-starved and then exposed to clycloheximide.

Effect of pH on System II

In an earlier characterization of phosphate transport in normal cells [14], we reported that System I showed a marked pH dependence, with the $K_{1/2}$ increasing from 12 μM at pH 4.0 to 3.6 mM at pH 7.3, and suggested models involving modification of a carrier by H^+ or OH^- or competitive inhibition between OH^- and P_i which could account for these results. It therefore seemed worth investigating the pH dependence of System II. Transport in P-starved cells was measured over a range of P_i concentrations at three pH values (4.0, 5.8 and 7.3). At each pH, the data from P-starved cells were compared with those from normal cells by computer fit to the sum of two Michaelis functions, again (as in the previous computation) holding $K_{1/2(I)}$ and $K_{1/2(II)}$ in common for the two kinds of cells but allowing $J_{max(I)}$ and $J_{max(II)}$ to vary. The resulting values are listed in Table II.

The most striking finding is that, while the $K_{1/2}$ of System I again showed a very large increase (1000-fold) as the pH increased from 4.0 to 7.3, the $K_{1/2}$ of System II remained essentially constant at 3–6 μM. For a phosphate-transport system which carries only $H_2PO_4^-$, one would expect to see as much as a 3-fold change in $K_{1/2}$ between pH 4.0 and pH 7.3, arising from a change in the relative concentrations of $H_2PO_4^-$ and HPO_4^{2-}. However, the standard errors in the $K_{1/2}$values of Table II (a reflection of the difficulties in working at extremely low P_i concentrations) do not permit a firm conclusion as to the specificity of System II for the two forms of phosphate.

Sensitivity of Systems I and II to osmotic shock

A fruitful approach to the study of certain bacterial transport systems, including one of the systems for P_i in *Escherichia coli* [17], has involved treatment of the cells by osmotic shock, which brings about the release of a class of substrate-binding proteins from the periplasmic space. The binding proteins can then be easily purified, and in several cases genetic studies have provided strong evidence for their role in transport. Much less is known about periplasmic proteins in the fungi. At least one case does exist, however, in which a binding protein is released by osmotic shock and has been tentatively correlated with transport; the tryptophan-binding protein of *Neurospora*, studied by Wiley [16] and postulated to play a role in amino acid-transport System I.

We therefore thought it useful to examine the response of phosphate-transport Systems I and II to osmotic shock. Normal cells and P-starved cells were treated by the procedure of Wiley [16] (see Methods), resuspended in P-free medium at 25 °C for 15 min with aeration, and then assayed for $^{33}PO_4$ uptake at a concentration of 2 mM (sufficient to saturate both Systems I and II). The results are given in Table III. In both types of cells, osmotic shock reduced P_i transport by more than 90 %. (By contrast, osmotic shock has a much smaller effect on at least one other transport process in *Neurospora*; net K^+ uptake [18] which is inhibited by only 30 to 50 % (Slayman, C. W., unpublished observations).)

TABLE III

EFFECT OF OSMOTIC SHOCK ON PHOSPHATE TRANSPORT VIA SYSTEM I AND SYSTEM II

Cells were subjected to osmotic shock as described in Methods, and then tested for phosphate influx at 2 mM P_i, a concentration sufficient to saturate both System I and System II. In normal cells, at 2 mM P_i, about 90 % of the influx is mediated by System I and 10 % by System II; in P-starved cells, about 82 % of the influx is mediated by System II and the remainder by System I.

	Normal cells		P-starved cells	
	mmol/l cell water per min	% control	mmol/l cell water per min	% control
Control	1.85	100	4.94	100
After osmotic shock	0.144	7.8	0.118	2.4

Although the data of Table III suggest that osmotic shock causes the release of some factor (or factors) essential to the activity of both phosphate-transport systems, efforts to detect a P_i-binding substance in the shock fluid have so far been unsuccessful.

DISCUSSION

The results presented in this paper serve to characterize a second phosphate-transport system, which appears in *Neurospora* under conditions of phosphorus starvation. In principle, the emergence of System II could be accounted for by a decrease in the intracellular concentration of an inhibitor of transport (either P_i itself or some related molecule), thereby activating transport sites which are already present, or by synthesis of new sites. The latter hypothesis is supported by the recent finding of Lehman et al. [19] that high-affinity P_i transport in *Neurospora* is one of a group of activities (along with alkaline phosphatase, acid phosphatase, and a transport system for phosphorylethanolamine and related zwitterionic substances) under the control of regulatory genes *nuc*-1, *nuc*-2, and *pcon*. We have confirmed that the transport activity studied by Lehman et al. is identical with System II (Lowendorf and Slayman, unpublished).

The effects of cycloheximide, although capable of several alternative interpretations when taken by themselves, are certainly consistent with the notion that System II is derepressed during phosphorus starvation. Addition of cycloheximide at the time of transfer to P-free medium inhibits the synthesis of System II, while addition of cycloheximide later (after 1.25 h of P_i starvation) not only inhibits further synthesis, but in addition reveals a decay process with a $t_{1/2}$ of 14 min (Fig. 2). Rapid decay has also been observed for tryptophan transport in the presence of cycloheximide ($t_{1/2}$ = 15 min, ref. 3); and somewhat slower decay, for mycelial SO_4^{2-} transport ($t_{1/2}$ = 2 h, ref. 8). The implication is that these transport systems, once derepressed, turn over continuously, a notion which is strengthened by the fact that blocking the synthesis of the mycelial SO_4^{2-} system in a totally independent way (by shifting to 37° C in a temperature-sensitive *cys*-3 regulatory mutant) also leads to a decay of transport [8].

The transport systems of *Neurospora* can thus be divided into three categories on the basis of their patterns of regulation. (1) Some (P_i System I, glucose System I [1, 2] and K^+,Na^+ transport [18]) are constitutive. (2) Others (glucose System II [1, 2], methionine transport [20] and conidial SO_4^{2-} transport [8]) are derepressible but, once formed, are stable under ordinary circumstances. The activity of these systems remains high in the presence of cycloheximide, although Schneider and Wiley [1] have shown that glucose System II is specifically degraded in the presence of glucose. (3) Finally, still other transport systems (P_i System II, tryptophan transport and mycelial SO_4^{2-} transport) are derepressible and in addition appear to turn over continuously, a process which at first sight seems wasteful but presumably allows the cell to adjust rapidly to changes in metabolic conditions.

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