

Regulation of Inorganic Phosphate Transport Systems in *Saccharomyces cerevisiae*

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A kinetic study of P_i transport with $^{32}P_i$ revealed that *Saccharomyces cerevisiae* has two systems of P_i transport, one with a low K_m value (8.2 μ M) for external P_i and the other with a high K_m value (770 μ M). The low- K_m system was derepressed by P_i starvation, and the activity was expressed under the control of a genetic system which regulates the repressible acid and alkaline phosphatases. The function of the *PHO2* gene, which is essential for the derepression of repressible acid phosphatase but not for the derepression of repressible alkaline phosphatase, was also indispensable for the derepression of the low- K_m system.

Saccharomyces cerevisiae has two species of acid phosphatase (EC 3.1.3.2) on the cell wall: one is coded for by the *PHO3* gene and is thought to be synthesized constitutively, and the other, repressible acid phosphatase (rAPase), a major fraction of which is coded for by the *PHO5* gene (8, 12), is derepressed upon P_i starvation (11). *S. cerevisiae* also has two species of an enzyme that hydrolyzes *p*-nitrophenylphosphate at an alkaline pH: specific *p*-nitrophenylphosphatase and nonspecific repressible alkaline phosphatase (rALPase; EC 3.1.3.1), which is coded for by the *PHO8* gene (6, 11, 15). Another gene, *PHO84*, may be involved in P_i transport (19). The transcription of *PHO8* is regulated in coordination with that of *PHO5* through signals indicating the presence or absence of P_i in the medium. The signals for external P_i are conveyed to the *PHO5* and *PHO8* genes by a regulatory system consisting of products of the *PHO4*, *PHO80*, *PHO81*, and *PHO85* genes (genetic symbols are reviewed in reference 11). The function of another regulator, the *PHO2* product, is indispensable for the transcription of *PHO5* but not for that of *PHO8*, whereas another gene, *PHO9*, is required for the expression of *PHO8* at the posttranscriptional stage (5) but not for that of *PHO5*.

A current model (5) proposes that a regulatory molecule (positive factor) specified by *PHO4* and produced constitutively is indispensable for the transcription of *PHO5* and *PHO8*. With a repressively high amount of P_i in the medium, a complex of the *PHO80* and *PHO85* gene products (negative factor) aggregates with the positive factor, preventing it from activating the transcription of *PHO5* and *PHO8*. Under derepressing conditions, i.e., when the P_i concentration in the medium is low enough, the *PHO81* product (mediator) reacts with the negative factor, thereby releasing the positive factor from the negative factor and allowing it to activate the transcription of the structural genes.

The presence of two P_i transport mechanisms in *S. cerevisiae* was suggested by Roomans et al. (13). Similar dual systems for P_i transport also exist in *Neurospora crassa*: one system has a low affinity for external P_i , and the other has a

high affinity (10). The high-affinity system is regulated by a genetic regulatory system which consists of the *nuc2*, *preg*, and *nuc1* genes and which regulates acid and alkaline phosphatases. A similar coordinate regulation of a P_i -binding protein with alkaline phosphatase was described in *Escherichia coli* (2, 18).

This communication describes the finding of two P_i transport systems in *S. cerevisiae*, one of which may have a high affinity for external P_i because it has a low apparent K_m value and the other of which may have a low affinity because it has a high apparent K_m value. The activity of the low- K_m system is coordinately regulated with the *PHO5* gene by external P_i through the *PHO* regulatory system, including the *PHO2* product.

Cells of the wild-type strain, P-28-24C (*MATa*, prototrophic), and various *pho2* mutants (all of which were constructed previously [15-17, 19, 20] and have the *pho3-1* genotype to eliminate constitutive acid phosphatase) were cultivated in synthetic low- P_i medium (17) by shaking at 30°C, except for a temperature-sensitive *pho2* mutant, P-1010-11D [*pho2*(Ts)], which was cultivated at 25 or 35°C. During the cultivation, rALPase activities were determined at appropriate intervals with dimethyl sulfoxide-permeabilized cells (1) as the enzyme source and *p*-nitrophenylphosphate as the substrate (Fig. 1). The contribution of specific *p*-nitrophenylphosphatase activity was negligible under these assay conditions (15). In the wild-type strain, rALPase activity was derepressed 6 to 8 h after inoculation of the cells into the low- P_i medium. In the *pho2* mutants, however [for example, P-73-3B (*pho2-1*)], the enzyme activity was derepressed from the initial phase of cell growth. The temperature-sensitive *pho2* mutant P-1010-11D [*pho2*(Ts)] had the same derepression kinetics as the *pho2* mutants when cultivated at 35°C but had the wild-type phenotype when cultivated at 25°C. Although detailed data are not shown, rALPase activity was not derepressed in high- P_i medium in any of the above strains, including all of the *pho2* mutants, and the enzyme levels (ca. 0.025 U/ml per unit of optical density at 660 nm [OD_{660}]) were essentially the same as those in the *pho2 pho4* and *pho2 pho81* mutants in low- P_i medium (Fig. 1) throughout the cultivation period. The *pho2-1 pho8-1* mutant (AL5-1C) had a lower enzyme level than the other mutants in high- P_i medium as well as low- P_i medium.

To confirm that the early derepression of rALPase was

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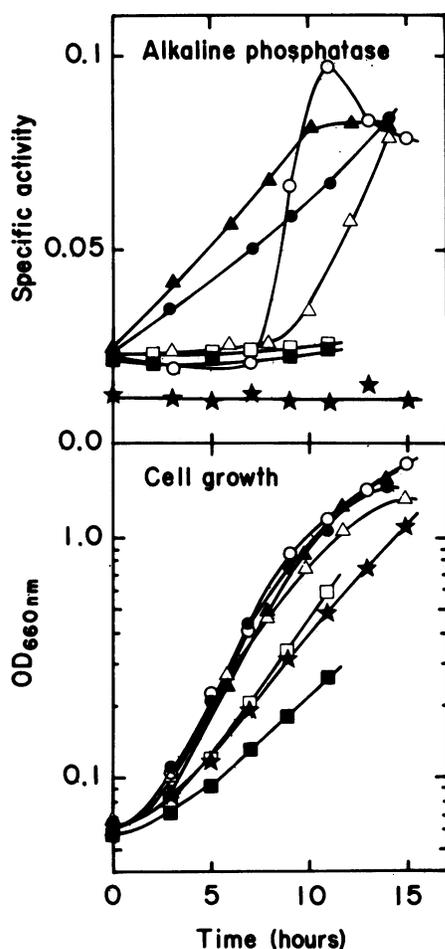


FIG. 1. Derepression of rALPase activity in various *pho* mutants. Cells grown at 30°C overnight in 2 ml of nutrient high- P_i medium (1.5 g of KH_2PO_4 per ml [17]) were washed and inoculated into 100 ml of synthetic low- P_i medium (Burkholder synthetic minimal medium in which KH_2PO_4 was reduced to 30 mg and 1.5 g KCl was added per liter [17]). The cultures were shaken at 30°C, except for the *pho2(Ts)* mutant, which was shaken at 25 or 35°C, and samples were taken at appropriate intervals. The cells were harvested, washed, and permeabilized to *p*-nitrophenylphosphate by suspension at 35°C for 30 min in 40% dimethyl sulfoxide solution in 10 mM succinate buffer (pH 4.2) as described by Adams (1). The cells were then washed with water, and their specific rALPase activity (U/ml per OD_{660} of the culture) was determined as described previously (15) with *p*-nitrophenylphosphate as the substrate. One unit of enzyme activity was defined as the amount of enzyme which liberated 1 μ mol of *p*-nitrophenol per min under the conditions. Symbols: \circ , P-28-24C (wild type); \bullet , P-73-3B (*pho2-1*); Δ and \blacktriangle , P-1010-11D [*pho2(Ts)*] at 25 and 35°C, respectively; \square , P-1288-2C [*pho2(Ts) pho81-1*] at 35°C; \blacksquare , P-1229-2D [*pho2(Ts) pho4-1*] at 35°C; and \star , AL5-1C (*pho2-1 pho8-1*).

caused by the *pho2* mutation, we isolated various revertants with rALPase activity from the *pho2-1* mutant P-73-3B (*MAT α pho2-1*) after mutagenesis with ethyl methanesulfonate (9) and discrimination of colonies with rALPase activity by staining through diazo coupling (16) on a low- P_i plate. These revertants were crossed with P-28-24C (*MAT α PHO2 $^+$*), and the resultant diploids were subjected to tetrad analysis. Some haploid segregants, derived from diploids showing

only a 4+ : 0- segregation of the rALPase phenotype in at least 10 asci, were cultivated to examine the time course of rALPase synthesis in low- P_i medium. All of them had the wild-type lag period (data not shown). Hence, we concluded that the early derepression was caused by the *pho2* mutation.

The finding that rALPase activity was not derepressed in a *pho2-1 pho8-1* double mutant (AL5-1C; Fig. 1) indicated that the enzyme derepressed from the early growth phase in the *pho2* mutants must be nonspecific rALPase, not specific *p*-nitrophenylphosphatase, and that it is under the control of the *PHO* regulatory system. This was confirmed with double mutants having the *pho2(Ts) pho81-1* (P-1288-2C) and *pho2(Ts) pho4-1* (P-1229-2D) genotypes by examining their time courses of rALPase derepression. The activity was not derepressed at 35°C (Fig. 1) or 25°C (data not shown).

We thought that the early derepression of rALPase in the *pho2* mutants might be due to a low intracellular concentration of P_i caused by their inability to derepress the P_i transport system and that the P_i transport system might be regulated by the *PHO* regulatory system, including the *PHO2* function. To examine these possibilities, we determined P_i transport with $^{32}P_i$ by a procedure described previously (19). The wild-type strain and various *pho* regulatory mutants were cultivated in low- P_i medium to derepress the possible P_i transport system, and cells collected in the growth phase were used to determine P_i uptake (Fig. 2A). The *pho2* mutants had a decreased level of P_i uptake of less than 5% of that of the wild-type strain, the same level as the *pho4* and *pho81* mutants, and a slightly higher level than the *pho84* mutant (19). The *pho2(Ts)* mutant P-1010-11D had the mutant level of P_i uptake at 35°C but the normal level at 25°C. These results indicate that all the mutants lacking the ability to synthesize rALPase were also defective in P_i transport activity.

Next, the kinetics of P_i transport in wild-type cells grown in minimal high- P_i medium were investigated as a function of the external P_i concentration. Lineweaver-Burk plots yielded a biphasic curve (Fig. 3), suggesting the participation of two P_i transport systems in *S. cerevisiae*, though the free or passive diffusion of P_i across the cell membrane could not be excluded. One of the P_i transport systems may have a low affinity for P_i because it had a high K_m value, whereas the other system may have a high affinity for P_i because it had a low K_m value and operated even with a low external P_i concentration. K_m and V_{max} values for the low- K_m system of high- P_i -grown cells were 8.2 μ M and 0.588 nmol/mg of dry cells per min, respectively. Using these kinetic constants, we determined the constants for the high- K_m system of the same cells by assuming that both of the systems were functioning. The calculated K_m and V_{max} values for the high- K_m system were 770 μ M and 3.14 nmol/mg of dry cells per min, respectively. The same wild-type cells cultivated in low- P_i medium had increased P_i transport activity, and the K_m and V_{max} values for the low- K_m system were 8.6 μ M and 37.0 nmol/mg of dry cells per min, respectively (Fig. 3). This K_m value was approximately the same as that of the repressed cells, whereas the V_{max} value of the derepressed cells was increased 60-fold. These findings indicate that the low- K_m system is repressed in high- P_i medium and derepressed by P_i starvation.

The pH dependence of the two transport systems was also determined under the above conditions with 10 mM citrate (pH 3.0), succinate (pH 4.0), and Tris-maleate (pH 5.0 to 8.0) buffers. The low- K_m system was maximal at pH 5.0, and the high- K_m system was maximal at pH 4.0 (data not shown).

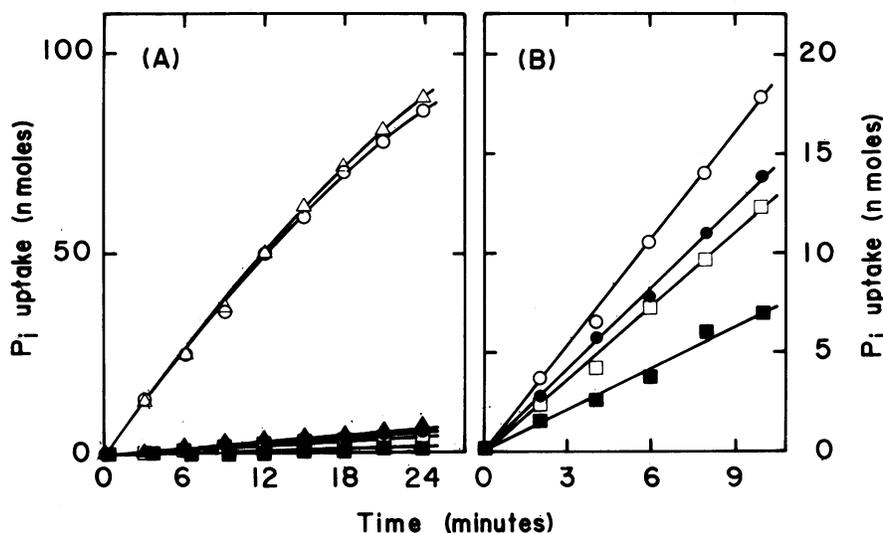


FIG. 2. P_i transport by various *pho* mutant cells. (A) Cells were precultured in synthetic low- P_i medium at 30°C, except for the *pho2*(Ts) mutant, which was cultivated at 25 or 35°C. The cells were collected when cell growth reached an OD_{660} of 0.8, washed, and inoculated into synthetic medium (in which the KH_2PO_4 concentration had been adjusted to 0.11 mM and an appropriate radioactivity with $^{32}P_i$) to give a cell concentration of ca. 0.1 OD_{660} . The cultures were shaken at 30°C or, for the *pho2*(Ts) mutant, at 25 or 35°C. (B) Cells precultured in minimal high- P_i medium at 30°C were inoculated (to give the same cell concentration as in panel A) into the same synthetic medium as in panel A, but the KH_2PO_4 concentration had been adjusted to 1.0 mM; the culture were then shaken at 30°C. Samples were taken at appropriate intervals and filtered through a nitrocellulose membrane filter. The amount of P_i absorbed by the cells was determined by counting the radioactivity on the filter as described previously (19) and was expressed in nanomoles of P_i per milliliter in cell suspensions with an OD_{660} of 0.1. Symbols: ○, P-28-24C (wild type); ●, P-73-3B (*pho2-1*); □, P-65-1D (*pho4-1*); ■, P-64-3D (*pho81-1*); △ and ▲, P-1010-11D [*pho2*(Ts)] at 25 and 35°C, respectively.

When the kinetic constants of the low- K_m system of the phosphatase-negative *pho2*, *pho4*, and *pho81* mutants were determined as a function of the external P_i concentration, the K_m values were approximately the same as that of the

wild-type cells, whereas the V_{max} values were significantly lower (Table 1). These data indicate that the mutants failed to derepress the low- K_m system. However, phosphatase-constitutive mutants having the *PHO81^c*, *pho80*, *pho85*, or

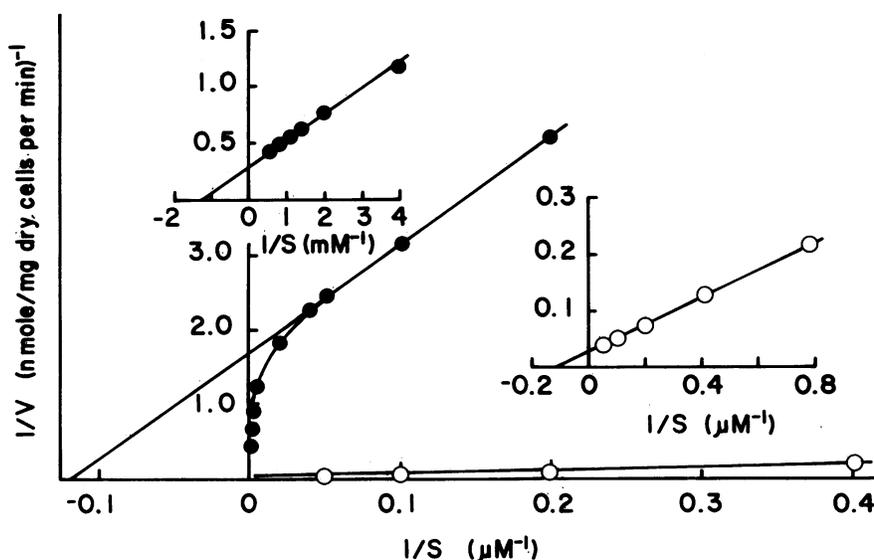


FIG. 3. Lineweaver-Burk plots of P_i transport activity in wild-type cells as a function of exogenous P_i concentration. Cells of strain P-28-24C cultivated in low- P_i or high- P_i medium were harvested at an OD_{660} of 0.8 and suspended in Burkholder synthetic minimal medium containing different P_i concentrations (17) and $^{32}P_i$ and adjusted to pH 5.0 with 10 mM Tris-maleate buffer. The amount of P_i absorbed by the cells was measured as described previously (19). Symbols: ●, P-28-24C cells cultivated in high- P_i medium; ○, P-28-24C cells cultivated in low- P_i medium.

TABLE 1. Michaelis-Menten parameters for the low- K_m system of P_i transport in various *pho* regulatory mutants

Strain	Genotype	Cell condition	K_m (μ M)	V_{max} (nmol/mg of dry cells per min)
P-28-24C	Wild type	Derepressed	8.62	37.0
P-73-3B	<i>pho2-1</i>	Derepressed	9.27	0.24
P-131-2B	<i>pho2-5</i>	Derepressed	8.75	0.26
P-65-1D	<i>pho4-1</i>	Derepressed	8.20	0.12
P-64-3D	<i>pho81-1</i>	Derepressed	8.07	0.10
P-28-24C	Wild type	Repressed	8.20	0.59
P-159-2C	<i>PHO4^c-1</i>	Repressed	8.00	2.34
P-32-2B	<i>pho80-1</i>	Repressed	8.13	7.80
P-211-4B	<i>pho85-3</i>	Repressed	8.13	8.36
P-131-7B	<i>PHO81^c-1</i>	Repressed	8.10	3.55

PHO4^c (i.e., the *PHO82* mutation [14]) genotype partially derepressed the low- K_m system in high- P_i medium (Table 1). Clearly, therefore, the low- K_m system appears to be under the control of the same regulatory system as *PHO5* expression. It is possible that the intracellular concentration of the effector (probably PP_i or a low-molecular-weight polyphosphate [3]) is quickly lowered to the derepressible level by cultivation of the cells in low- P_i medium. The P_i uptake activity of the high- K_m system in these mutants at a 1 mM P_i concentration, however, was almost the same as that in the wild-type cells (Fig. 2B). This suggests that the high- K_m system of P_i transport is expressed constitutively.

The defect in the low- K_m system of P_i transport accompanying the *pho2* mutation may affect the size of the internal P_i and polyphosphate pools. This was tested by measuring the intracellular levels of P_i and acid-soluble and acid-insoluble polyphosphate in the late log phase (at this stage the highest enzyme activity was observed in cells cultivated in low- P_i medium; Fig. 1). No measurable differences were found in the intracellular phosphate pools in the mutant cells as compared with the wild-type cells (Table 2).

In high- P_i medium, the *pho2* mutant could not derepress rALPase activity. The *pho84* mutant, which also has a defect in P_i transport and has the constitutive phenotype for rALPase synthesis in high- P_i medium (20), derepressed the rALPase

TABLE 2. Intracellular polyphosphate and P_i pools in the *pho2* mutant^a

Strain	Genotype	Growth medium	Phosphate concn (mg/100 mg of dry cells) in indicated fraction ^a		
			P_i	Polyphosphate	
				Acid soluble	Acid insoluble
P-28-24C	Wild type	High P_i	0.618	0.482	0.741
		Low P_i	0.023	0.044	0.044
P-73-3B	<i>pho2-1</i>	High P_i	0.521	0.493	0.596
		Low P_i	0.023	0.037	0.039

^a Cells were cultivated at 30°C in high- P_i or low- P_i medium and harvested when cell growth reached an OD_{660} of 0.8 to 1.0. Intracellular P_i and acid-soluble and acid-insoluble polyphosphate fractions were prepared by the method of Katchman and Fetty (7), and the amount of phosphate in each fraction was determined by the method of Chen et al. (4).

activity from the initial phase of cell growth in low- P_i medium, similarly to the *pho2* mutant, and partially derepressed the rALPase activity in high- P_i medium (data not shown). These facts suggest that the *pho2* mutant retained the basal activity of the low- K_m system, whereas P_i transport in the *pho84* mutant was more severely inactivated, as suggested by the P_i transport experiments (Fig. 2 and reference 19).

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