Physiological Regulation of the Derepressible Phosphate Transporter in Saccharomyces cerevisiae

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Orthophosphate plays a pivotal role in cell functioning, being involved in most metabolic energy transductions, serving as an intermediate in the biosynthesis of numerous metabolites. Regulation of the phosphate uptake process represents a common biological strategy for modulation of and response to phosphate metabolism and cellular activities (18). The phosphate transport process in Saccharomyces cerevisiae is characterized by a high-affinity transport system operative at low (μM) concentrations of phosphate and a low-affinity transport system operative at high concentrations (mM) of phosphate. The low-affinity system, with a K_m for phosphate of approximately 1 mM at its proposed optimum of pH 4.5, is considered to be a constitutively expressed P/H+ cotransporter (16, 25). In contrast, the high-affinity system (K_m 1 to 15 μM) is derepressible by phosphate starvation during aerobic and anaerobic cell growth. Of the proteins responsible for the high-affinity transport of phosphate into the cell, one consists of a P/H+ cotransporter (Pho84p) with a pH optimum for phosphate uptake similar to that of the constitutive low-affinity system (1, 2, 4), and the other is a P/Na+ cotransporter with an alkaline pH optimum, being largely inactive at pH 4.5 (21). The identities of the genes encoding the proposed constitutively expressed low-affinity P/H+ and the high-affinity P/Na+ transporters have not yet been published.

The signal on the level of extracellular phosphate is known to be conveyed through the so-called PHO regulon (12, 18, 23). Although significant insight into the genetic regulation of phosphate signalling has been gained, the complex nature of the mechanisms underlying the physiological regulation of derepressible H+-coupled high-affinity Pi transport. The S. cerevisiae CW04 strain (Mat ade2 his3 leu2 trp1 ura3 can1) was used. Cells were routinely grown in shaking Erlenmeyer flasks at 30°C in low-phosphate (LP) medium prepared according to the method of Kaneko et al. (13). One liter of YEP (1% yeast extract, 2% Bacto Peptone) medium was supplemented with 10 ml of 1 M MgSO4 and 10 ml of 25% NH4 solution with stirring, allowed to stand at 25°C for 1 h to precipitate phosphate, and filtered through a Munktell no. 3 filter. The pH of the clear filtrate was adjusted to pH 4.5 with HCl, and 2% glucose was added. In some control experiments 0.2% KH2PO4 was used in high-phosphate (HP) medium. Growth was monitored by the change in optical density at 590 nm (OD590). At specified time points, samples of the culture were aseptically withdrawn, centrifuged at 2,300 × g for 10 min, and washed either once with ice-cold 25 mM Tris-succinate buffer (pH 4.5) (for P uptake assays) or twice with ice-cold distilled water (for 31P nuclear magnetic resonance [NMR] and respiratory rate analysis). The supernatants were subjected to glucose and phosphate concentration measurements.

In the phosphate uptake studies 1 μl of [32P]orthophosphate (0.18 Ci/μmol; 1 mCi = 37 MBq; Amersham) was added to aliquots (30 μl, 0.546 mg of dry weight) of cell suspension in 25 mM Tris-succinate buffer, pH 4.5, supplemented with 3% glucose, to a final concentration of 0.11 mM. The suspension was incubated for 1 min at 25°C. Phosphate uptake was terminated by adding 3 ml of ice-cold Tris-succinate dilution buffer. The sample was filtered immediately, the filter (Whatman GF/F) was washed once with the same cold solution, and the radioactivity retained on the filters was determined by liquid scintillation spectrometry. The maximum rate of phosphate transport catalyzed by the cells, estimated as the initial activity during the first minute of uptake per mg of cells (dry weight), is shown.

For sodium dodecyl sulfate-polyacrylamide gel electrophoresis and immunoblotting, the membrane fraction of S. cerevisiae cells was prepared as described by Ljungdahl et al. (15). Samples containing 20 mg of plasma membrane protein were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis by using a 12% polyacrylamide and bispolyacrylamide gel system (14). The electrophoresed proteins

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were transferred onto polyvinylidene difluoride membranes (Immobilon polyvinylidene difluoride; Millipore) according to the Amersham Western blotting protocol. Immunological detection was accomplished by using affinity-purified Pho84 anti-C terminal antibody (6) and anti-rabbit immunoglobulin donkey antibody-conjugated horseradish peroxidase (Amersham). After a short incubation with enhanced chemiluminescent substrate the blot was exposed to film for 2 min.

In the Northern analysis total RNA (15 μg) was isolated from CW04 cells grown in HP, and LP, media as described elsewhere (24), separated by electrophoresis on 1.5% agarose gels containing 2.2 M formaldehyde, blotted onto Hybond-N membranes (Amersham) according to the manufacturer's instructions, and hybridized under high-stringency conditions in accordance with standard procedures (22). The probes used were a 32P-labeled 0.7-kbp NdeI-KpnI PHO84 gene fragment contained in pUC19 (1) and the 1.65-kbp BamHI-HindIII ACTI gene (7) as a loading control. The probes were labeled by the random primer technique by using an oligolabeling kit (Pharmacia) according to the instructions of the manufacturer. Filters were exposed to film at −80°C.

All NMR experiments were conducted on a Varian Unity Plus 400 instrument. Aliquots (3.5 ml) of CW04 cell suspensions of 0.5 g (wet weight)/ml in 25 mM Tris-succinate buffer, pH 4.5, were subjected to 31P NMR analyses. A broad-band probe designed for 10-mm sample tubes was used. The spectral width was 10,000 Hz, centered on the 85% phosphoric acid peak at 0 ppm in a separate experiment. The pulse delay was 2 s, and 512 scans of 2,048 complex data points were collected during an experimental time range of approximately 20 min. The 90°C excitation pulse length was determined to be 21 μs.

No deuterium frequency lock or proton decoupling was used during the experiments. Experiments performed on one sample with pulse delays of 1, 2, and 4 s revealed no systematic changes of intensities, indicating that the 31P longitudinal relaxation rates are rapid. The relative contributions of different 31P-containing molecules could thus be calculated from the corresponding integrated intensities in the 31P NMR spectra. The NMR data were evaluated using the built-in VNMR software version 5.1 (Varian). The free induction decays were multiplied with an exponential window of 10 Hz, zero filled to 8,192 complex points, and Fourier transformed. The frequency domain spectra were baseline corrected, and the intensities and integrals were obtained using standard techniques. The assignment of the 31P NMR peaks of intra- and extracellular orthophosphate, ATP, and nonterminal P_i of polyphosphate were obtained as previously described (11). The total amount of ATP was calculated from the β-P_i of the ATP peak since other phosphorous compounds concur with the α-P_i and γ-P_i of the ATP peaks.

Phosphate and glucose concentrations in the growth media were assayed spectrophotometrically at 850 nm essentially as described by Nyre ´n et al. (17) and determined polarographically with glucose peroxidase according to the protocol of Okuda and Miwa (19), respectively.

Under HP, growth conditions, the P_i transport activity of the S. cerevisiae cells withdrawn at different growth phases was very low, 0.5 nmol of P_i transported per min and mg of cells (dry mass), and unaffected by the prevailing growth phase (data not shown). In contrast, in cells grown in LP, medium (Fig. 1A), containing approximately 200 to 300 μM phosphate, phosphate transport changes with cell growth. The uptake rate increases along the exponential phase to reach its maximum rate (5.3 nmol of P_i transported per min and mg of dry mass) in mid- to late-exponential-growth phase (an OD590 of approximately 3) before rapidly declining. The cell growth was accompanied by an initial rapid rate of extracellular phosphate consumption, from approximately 275 to 70 μM in the first 4 h of growth, followed by a slower rate of utilization during the early-exponential-growth phase (an OD590 of approximately 1) (Fig. 1A). The highest transport activity was achieved when the extracellular P_i concentration was in the range of 50 to 70 μM.

Interestingly, the onset of the decline in transport activity coincided with a situation where the extracellular phosphate concentration was very low, close to the K_m of 10 μM for the transporter, while glucose was still abundant (approximately 10 g/liter). This observation suggests not only, in agreement with earlier proposals, that the derepression of the PHO84 is under control of the extracellular phosphate level (4) but also that its inactivation is subjected to the same control.
Western blot analyses were performed on cells grown in LPi, related with the phosphate transport activity, Northern and well as the amount of Pho84p in the plasma membrane corresponding stored in the form of polyphosphates, whereas only low levels of intracellular free Pi were maintained at a significant level during growth in LPi medium (samples at OD590 of 0.9, 3, and 7 (cf. Fig. 1A) as well as in cells harvested at an OD590 of 7 and incubated for 45 min in medium containing 17 g of glucose/liter and 50 µM Pi. The samples analyzed revealed significant variations in the intensity of the immunolabeled major band corresponding to Pho84p (65.4 kDa). The variations in intensity of the bands correlate well with the changes observed in the rate of Pi uptake (cf. Fig. 1A). The immunoreactive band corresponding to the 65.4-kDa Pho84p transporter was absent in cells grown for 10 h under phosphate starvation and in HPi medium. The Pi transport activities for the two conditions were similar (less than 1 nmol of Pi transported per min and mg of cells, dry mass), suggesting that the low-affinity system active under repressive growth conditions in HPi medium is also responsible for the transport determined in 10-h-old cells. The sharp immunoreactive band of approximately the same molecular size as the Pho84p band present in these samples probably reflects the immunoreactivity of another protein which comigrates with the Pho84p.

The results obtained (Fig. 1A) clearly indicate that cell growth during the first 3 to 4 h is supported by a rapid uptake of external phosphate after which the remaining low extracellular phosphate concentration is not sufficient to maintain further exponential cell growth. This implies that in the latter case internal phosphate pools are being utilized. In order to study the intracellular changes of phosphorous compounds and their putative role in the Pi-sensitive regulation of the Pi transporters, the intracellular amounts of Pi, polyphosphate, and ATP in cells grown in LPi and HPi media were measured by the 31P NMR technique. The samples analyzed were cells grown for 5 and 10 h, corresponding to the situations when there was a dramatic increase in the rate of phosphate consumption and when the extracellular phosphate concentration was close to zero, respectively. In a composite of a series of 31P NMR analyses (Fig. 1B), the changes of intracellular phosphorous compounds, such as free orthophosphate (panel I), polyphosphates (panel II), and ATP (panel III) are depicted. It can be seen that cells grown in LPi medium maintained much lower levels of free phosphate, polyphosphates, and ATP than HPi-grown cells. Remarkably, in cells grown in LPi medium under conditions of extracellular phosphate deprivation (10 h), the polyphosphate pool was diminished to almost zero, whereas it was unaffected in cells grown in HPi. In contrast, the amount of intracellular free Pi was maintained at a significant level during growth in LPi. Thus, it appears that under conditions when the cell meets no Pi limitations, free Pi is predominantly stored in the form of polyphosphates, whereas only low amounts of Pi reserves are maintained during Pi starvation, indicating that Pi, taken up by the high-affinity system must be used immediately by the cell in essential cellular functions. It is conceivable that the intracellular polyphosphate pool might be responsible for sustaining cell growth when the extracellular phosphate is exhausted.

To investigate whether the transcription level of PHO84 as well as the amount of Pho84p in the plasma membrane correlated with the phosphate transport activity, Northern and Western blot analyses were performed on cells grown in LPi, medium and harvested at different growth phases (Fig. 2). For comparison purposes, blot analyses were also performed for cells grown for 10 h in HPi medium. Figure 2A illustrates the result of a Western blot analysis of the presence of the Pho84 transporter in LPi-grown cells harvested at OD590 of 0.9, 3, and 7 (cf. Fig. 1A) as well as in cells harvested at an OD590 of 7 and incubated for 45 min in medium containing 17 g of glucose/liter and 50 µM Pi. The samples analyzed revealed significant variations in the intensity of the immunolabeled major band corresponding to Pho84p (65.4 kDa). The variations in intensity of the bands correlate well with the changes observed in the rate of Pi uptake (cf. Fig. 1A). The immunoreactive band corresponding to the 65.4-kDa Pho84p transporter was absent in cells grown for 10 h under phosphate starvation and in HPi medium. The Pi transport activities for the two conditions were similar (less than 1 nmol of Pi transported per min and mg of cells, dry mass), suggesting that the low-affinity system active under repressive growth conditions in HPi medium is also responsible for the transport determined in 10-h-old cells. The sharp immunoreactive band of approximately the same molecular size as the Pho84p band present in these samples probably reflects the immunoreactivity of another protein which comigrates with the Pho84p. These results clearly indicate a derepressive synthesis of the high-affinity carrier proportional with the initial decrease in the extracellular phosphate concentration and its rapid degradation upon extracellular phosphate and intracellular polyphosphate depletion. Protolysis of a plasma membrane protein could be accomplished by a direct breakdown of a selected protein at the level of the plasma membrane or by selective internalization and transport to the vacuole for nonspecific proteolysis.

The results obtained strongly suggest that derepression of the transporter is maintained by the availability of extracellular phosphate rather than the level of intracellular phosphate, which is affected only to a minor extent by cell growth for 10 h. Even under conditions when external phosphate is fully depleted after 10 h of growth the cells still contain a considerable amount of intracellular phosphate and a significant level of ATP (Fig. 1B). Part of the intracellular phosphate reserve is contained in the vacuoles, where it can be mobilized when phosphate in the medium is limiting (5, 8, 9). The fact that essentially no polyphosphate was found in the cells showing the highest PHO84 expression level, i.e., after 10 h of growth, suggests that the Pi-sensitive regulation possibly is mediated by the concentration of these phosphate polymers. Bostian and coworkers (3) also found in their studies of the expression of the repressible acid phosphatase (rAPase) that changes in intracellular Pi levels did not correlate with rAPase derepression and concluded that Pi, therefore may not serve as a corepressor. The same authors suggested that Pi, or low-molecular-weight...
polyphosphates may serve as a metabolic regulator controlling the rAPase expression (3).

In summary, the results presented in this work clearly reflect a derepressible synthesis of the Pho84p carrier proportional with the initial decrease in the extracellular phosphate concentration and its rapid degradation upon glucose, extracellular phosphate, and intracellular polyphosphate depletion. The activation of P_i uptake under P_i starvation is due to a derepression of the transcription of the PHO84 gene. The inactivation of this transporter by nutrient (P_i, glucose) depletion is due to a negative regulation by which the carrier is degraded and the PHO84 transcription turned off.

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