NMR-Observed Phosphate Trafficking and Polyphosphate Dynamics in Wild-Type and vph1-1 Mutant Saccharomyces cerevisiae in Response to Stresses

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The phosphagenic, osmotic, and metabolic roles of polyphosphate in chemostat-cultivated yeast were investigated with a new NMR cultivator. Wild-type yeast and a vacuolar vph1-1 mutant, which lacks polyphosphate, were subjected to different stimuli. Starved wild-type yeast exclusively directed phosphate to vacuoles despite other competing sinks. After DNP or iodoacetate exposure, which significantly affected cytosolic pH or ATP metabolism, polyphosphate hydrolysis did not occur, which casts doubt on the phosphagen function of vacuolar polyphosphate. It took about 1 h for Mn\(^{2+}\) to traffic to vacuoles, and some evidence was obtained for polyphosphate responding to osmotic challenges. Fast NMR scans show that rapid polyphosphate hydrolysis to small polymers follows alkalinization. The small polymers then degrade to orthophosphate, which coincides with sugar phosphates increasing and subsequent readicification. In contrast, when vph1-1 mutants were subjected to alkalinization, the absence of a vacuolar source of phosphate slowed readicification. Based on known yeast physiology and observed sugar phosphate dynamics, polyphosphate degradation may enable rapid glycogen mobilization to glycolysis for considerable acid and ATP production. Overall, maintaining both polyphosphate and carbohydrate reserves may endow yeast with the ability to rapidly manage the extracellular environment.

Introduction

Microorganisms ranging from bacteria to phytoplankton accumulate polyphosphates. Up to 20% of a yeast cell’s dry weight can be polyphosphate (Lusby and McLaughlin, 1980), a significant portion of which is vacuolar-localized and acid-soluble and has 2–10 residues (Kulaev et al., 1982). Many environmental stress-mediating roles have been proposed for polyphosphates. Micronutrient storage, for example, may enable the transition between growth phases (Solimene et al., 1980). Functioning as an energy reserve has also been proposed for polyphosphate because the bonds between its residues are energetically similar to those of ATP (Kulaev et al., 1982), and polyphosphate kinase activity has been isolated from Saccharomyces cerevisiae (Wood and Clark, 1988). Evidence consistent with polyphosphate being phosphagenic has been reported for isolated mitochondria (Beauvoit et al., 1989). Cellular extract studies, however, do not indicate that yeast polyphosphate can offset inhibited ATP production (Schuddenmat et al., 1989).

Maintaining cellular pH is another proposed function. After alkalinization, polyphosphate degrades to tri- and tetrapolyphosphate (Ludwig et al., 1977; Greenfield et al., 1987; Castro et al., 1995). As suggested for alga cells (Pick and Weiss, 1991), such hydrolysis may provide cytosolic pH buffering/restoration, which is consistent with the low pK\(_a\) of small polyphosphates (MacDonald and Mazurek, 1987).

The prospect of polyphosphate’s functions being integrated with other storage strategies has received less attention. For yeast, 16–20% and 6–10% of dry mass can also be glycogen and trehalose, respectively (Sols et al., 1973). Interestingly, it is well known that S. cerevisiae violates the widespread pattern of storing carbohydrate polymers when nitrogen is limiting and carbon is in excess. Indeed, at 30 °C and a specific growth rate of 0.05 h\(^{-1}\), glyogen content and glycogen phosphorylase activity are maximal under glucose limitation (Kuenzi and Fletcher, 1972). Using these stores during oscillations has been discussed (Satroutdinov et al., 1992). Yeasts are also used to produce a variety of products (e.g., Leaf et al., 1996; Fieschko et al., 1987), and sorting out the numerous roles proposed for polyphosphates and establishing whether integrated storage and utilization strategies exist might favorably impact current and future technological uses of yeast. Impact could conceivably occur through parlaying an improved understanding of C and P resource management to the development of metabolic engineering strategies that can better account for how modified or inserted subsystems will interact with the entire system. Additionally, insights may be gained that...
are relevant to understanding how acidic niches are formed and defended by yeasts and how inorganic nutrients are sequenced and cycled by microorganisms in natural and managed ecosystems (e.g., van Groenestijn et al., 1989).

To examine the roles of polyphosphate in yeast further, we used an NMR cultivator that allows for $^{31}$P NMR spectra to be directly acquired from chemostat cultures, which mimic flow ecosystems or technological production schemes. Controlled fluid flow through the radio frequency coil provides a low apparent spin-diffusion time (Suryan, 1951; Meehan et al., 1992). Consequently, NMR spectra can be acquired from relatively low to moderate volume percent (2–20%) cell cultures by using rapid pulsing. High signal-to-noise ratios can be achieved with time resolution ranging from 1 to 7 min, depending on cell concentration. Moreover, the chemostat facet allows for NMR studies to be performed on cells that are growing at a selected, constant specific growth rate. Magnetic field and signal calculations have recently led to modifications that improve spectral and temporal resolution (Castro, 1996).

The NMR cultivator was used to probe yeast cultures that were subjected to altered phosphate abundance, cation (Mn$^{2+}$) increase, osmolarity increase, alkalization, or blocked/decoupled energy metabolism under conditions of high glycogen accumulation. The wildtype’s intracellular dynamics were also contrasted to those of a vph1-1 mutant. The mutant has a defective ATPase subunit (Preston et al., 1989; Manolson et al., 1992). The resulting inability to energize the vacuole membrane and accumulate polyphosphate eliminates any potential vacuolar/polyphosphate-based contribution to readcification or ATP restoration, which, in turn, provides a control for interpreting the response of wild-type yeast. The results indicate that charging vacuoles with polyphosphate, like building glycogen reserves, can have high regulatory priority. Mn$^{2+}$ was observed to traffic through the cell over 1 h, and hyperosmolarity led to a decline in the polyphosphate signal. The sugar phosphate, polyphosphate, and orthophosphate dynamics during alkalization suggest that polyphosphate hydrolysation enables rapid glycogen mobilization for glycolytic ATP and organic acid production. Inhibiting ATP production with iodoacetate or dinitrophenol did not prompt polyphosphate hydrolysation.

**Materials and Methods**

**Yeast Strains and Cultivation.** Wild-type (diploid strain BJ 2935) and vph1-1 mutant (diploid strain BJ 4895) S. cerevisiae were obtained from Professor Beth Jones (Department of Biological Sciences, Carnegie Mellon University). The cultures were preserved in 40% glycerol tubes at −80°C to prevent reversion of mutant strains (Campbell, 1994). To grow a new culture, a small sample from the storage tube was plated out on YPD agar (Sigma, Y-1500) and incubated overnight at 30°C. An individual colony was then aseptically transferred to a shake flask containing 25 mL of batch media composed (in g/L) of glucose, 20; yeast extract (Sigma, Y-0375), 7.5; and ammonium sulfate, 5. Before inoculation with a cell colony, media components and the flask were autoclaved for 25 min at 121°C. Following inoculation, a Lab-Line Orbit Environmental-Shaker provided overnight incubation at 30°C. Samples were examined by microscope the following day to verify that there was no contamination.

To provide a viable, acclimated culture at a target concentration and not subject to excessive acid accumulation, fed-batch and chemostat cultivation were used to prepare cells for NMR experiments. Fed-batch cultivation began approximately 24 h after the start of batch growth or sooner. To begin the process, 0.1 mL of the culture was transferred to a sterilized, 1-L Applikon reactor which contained 500 mL of batch media and 0.1 mL of Fermax 27 (Union Carbide). Experiments (not included) indicated that 10 times higher Fermax 27 concentrations had no effect on yeast growth. A Cole-Parmer (model 5997-20) pH controller automatically added 5.0 N NaOH to maintain the culture’s pH at 5.75. The pH probe within the reactor was sterilized by immersion in either 6 N HCl or 1% (v/v) ROCAL detergent for a minimum of 1 day and then rinsed with sterile water. Culture temperature was kept at 30°C by using a water bath and heat exchanger. Aeration was provided through a 0.1-μm filter; to minimize evaporation from the cultivator, after filtration, the air was humidified by bubbling through a flask of sterile water. Agitation was provided at 500 rpm. The fed-batch medium and carbon balance-based, computer-controlled feeding strategy have been detailed elsewhere (Meehan et al., 1991, 1992). After the fed-batch interval, chemostat medium was added to the reactor at a rate of 40 mL/h until a total culture volume of 800 mL was achieved (Meehan et al., 1992). Peristaltic pumps (Masterflex) both supplied the feed at a fixed rate and drew off culture to maintain a constant amount. In general, the chemostat provided a steady supply of cells, and the fed-batch start-up increased the success and reproducibility of the chemostat start-ups. In all experiments reported, start-up and NMR chemostat cultivation used a dilution rate of 0.05 h$^{-1}$.

To measure cell mass concentrations, a calibration curve between mass concentration and absorbance was generated using a Bausch and Lomb Spectronic 2000 spectrophotometer. Cell suspensions were diluted to an optical density of less than 1; each point was the average of three samples. “High-density” and “low-density” suspensions refer to 30–50 dry g/L (or 10–16.7% v/v) and 4% by volume, respectively.

**Environmental Perturbations and Stresses.** NaOH solution was added to raise the extracellular pH; for all alkalinizations reported, the extracellular pH was increased from 5.75 to 8.5. The following amounts (in mL) of stock solutions were added to alter phosphate or Mn$^{2+}$ abundance: 0.5 (3.0 g of K$_2$HPO$_4$3.84 g of KH$_2$PO$_4$ in 150 mL of H$_2$O) or 5.4 (10 g of MnSO$_4$ in 1 L of H$_2$O). To decouple or inhibit energy metabolism, the following amounts (in mL) were added, respectively: 4.0 (75 mg of 2,4-dinitrophenol in 30 mL of H$_2$O) or 3.7 (2.794 g of iodoacetate in 34 mL of 0.412 N NaOH).

**$^{31}$P NMR Spectroscopy Preparation and Protocol.** A 7-T Bruker NMR spectrometer with a 12-cm-diameter horizontal bore was used. Cells used for an NMR experiment were obtained by transferring 250 mL from the start-up chemostat to the NMR cultivator. A temperature bath connected to the NMR cultivator’s interior heat exchanger kept the cell culture at 30°C. A syringe pump (Harvard Apparatus, Inc, Pump 22) provided fresh medium to establish a dilution rate of 0.05 h$^{-1}$.

The peristaltic pump maintained the culture’s volume at a fixed value by constantly removing fluid through a tube whose terminus was situated a specific height above the cultivator’s bottom. Pure oxygen was fed near the bottom of the reactor. Chemostat feed, matching that used to generate the cells, entered through a port in the reactor’s ceiling; the 1 N NaOH used to control pH was admitted through another inlet. When spectra obtained hours later...
from the preacclimated cells exhibited little variance, experiments were begun. Unless noted otherwise, scan parameters used were as follows: repetition time of 0.1 s, spectral width of 14 000 Hz, file size of 1K, acquisition time of 0.023 s, and pulse width of 50 μs. "High-density" and "low-density" cultures were scanned for on the order of 1 and 8 min, respectively. An oscilloscope or the WOBB function of the Bruker software was used for coil tuning. A methylene diphosphonic acid (MDP) standard, sealed with wax in a 1/16-in.-i.d. Teflon tube, was employed in some experiments. Because yeast extract contains some nonmetabolizable species (cyclic polynucleosides) that resonate far from interesting metabolic species, a standard is not always necessary.

Results

Wild-Type Saccharomyces cerevisiae. Phosphate Addition to Depleted and Acclimated Yeast. Figure 1 shows the response of a low-density yeast culture that was provided with a stepwise increase in orthophosphate. Prior to the increase, the culture was maintained for 10 h at 4 °C without nutrients or oxygenation. After these cells were placed in the NMR cultivator, provided with nutrients including 1.5 mL of phosphate stock and energy source for 1 h, all the phosphate in the medium was consumed, resulting in the large polyphosphate peak seen in the top spectrum. The second spectrum was acquired after addition of 0.25 mL of phosphate solution. Subsequent spectra reveal the exhaustion of orthophosphate from the medium and incorporation into polyphosphate. Interestingly, the extracellular phosphate appears to be almost as rapidly incorporated into polyphosphate as it is transported into the cell, based on the absence of a transient increase in other resonances. The response to increased phosphate for a long-term chemostat culture was quite different (not shown). These cells imported nil phosphate and did not increase vacuolar polyphosphate; the spectra were essentially invariant except for an increase in extracellular phosphate.

Cation Trafficking. To probe the time scale of cation trafficking and whether polyphosphate may play a role, yeasts were exposed to a stepwise increase in Mn2+. This cation was used because it is highly paramagnetic and phosphorus nuclei in its vicinity relax so rapidly that their resonances broaden into the baseline. Thus, cation trafficking within the cell can be followed by the disappearance of resonances particular to compartments.

Prior to addition of Mn2+, the cells had been growing on the yeast extract growth medium described in the Methods section and allowed to attain a steady state with a growth rate equal to 0.05 h−1. Mn2+ was provided via a syringe, and medium inflow was not interrupted over the course of the experiment. Immediately after addition (Figure 2), the divalent cation eliminated the right-most peaks in the 0 ppm region. Prior workers have assigned these peaks to extracellular phosphate and phospholipids such as glycerophosphoethanolamine and glycerophosphocholine (Beauvoit et al., 1989). Ascribing the origin of the unknowns to extracellular (or cell surface bound) species is thus verified by this experiment. Within 30 min, the α-NTP/NADH peak starts to broaden, indicating that Mn2+ is accumulating in the cytosol. Later (48–78

![Figure 1. Response to phosphate shift-up. Yeasts stored at 4 °C were provided phosphate. A second, smaller addition of phosphate was added just after the first 8-min scan; subsequent scans were acquired every 10 min. Most phosphate was again directed to vacuoles. Times for the scans (top to bottom convention used throughout) are 0, 10, 15, 20, 25, 30, and 40 min.](image1)

![Figure 2. Response to manganese addition. Times for the scans are 0, 4, 28, 48, 78, 94, 111, 134, and 159 min. In these and other spectra, CP, NTP, PP4, and SP denote cyclic nucleosides, nucleoside triphosphate (e.g., ATP), interior polyphosphate residues, and sugar phosphates. The paramagnetic metal ion "washes" out resonances as it permeates different cellular compartments.](image2)
min), the polyphosphate peak (PP4) begins to broaden, indicating that Mn^{2+} has reached the vacuole compartment. Some indication of partial recovery of the α- and γ-NTP peaks may exist in the final four spectra. Overall, intercompartment transport occurs, and considerable lags can exist for Mn^{2+} to traverse cellular compartments.

**Osmoregulation Changes.** The effect of increasing medium osmolarity on polyphosphate dynamics was investigated by subjecting a culture to a stepwise increase in NaCl. As was the case in the Mn^{2+} addition experiment, a steady state had been established on yeast extract medium with a growth rate of 0.05 h^{-1} prior to the perturbation. The spectra are shown in Figure 3. The NaCl addition approximately quadrupled the conductivity of the medium. Because of conductive losses, the rf coil was checked for tuning. Using the Bruker WOBB function showed that the coil remained tuned to the desired frequency of 121.5 MHz. The quality factor, however, decreased as should be expected; the decrease was equivalent to an approximately 21% reduction in signal-to-noise ratio. Postaddition spectra were thus vertically scaled with the cyclic polynucleoside (CP) peak to match the initial one. Negligible washout of the NaCl extract medium prior to the perturbation, and medium inflow was not interrupted. Figure 4 shows how the chemical shift (in ppm) of cytosolic inorganic phosphate, which is a measure of pH, changed with time, and how that change correlated with phosphate, sugar phosphate, γ-NTP/PP1 (PP1 denotes polyphosphate termini), and PP4 (polyphosphate interior residues) dynamics. The basic trends noted here were replicated more than three times. After alkalinization, the yeast restored the medium pH to 5.5–6.0 in 20 min. Cytosolic pH increased and then recovered over approximately 20 min as well (Figure 4a). The chemical shift of cytosolic phosphate appears to continue shifting even after recovery. This seemingly additional acidification results from the overlap between the cytosolic and vacuolar phosphate peaks. As more time elapses than shown, the two peaks split apart, where the cytosolic peak stops at the starting chemical shift and the vacuolar peak continues to its original shift (not shown).

Of special interest is what happens immediately after alkalinization, and what occurs when the cytosolic pH has been restored. After NaOH addition, phosphate increases after a short lag (Figure 4a), sugar phosphate rises (Figure 4b), the combined γ-NTP/PP1 peak increases (Figure 4c), and polyphosphate rapidly drops (Figure 4d). Upon readacidification of the cells’ interior, polyphosphate begins to rise and the other species decrease. In all the replicates, the combined γ-NTP/PP1 peak exhibited an early maximum and then another rise and fall which started when PP4 recovery began (e.g., at 210 min in Figure 4d).

**Effects of Metabolic Inhibitors.** Figure 5 presents the response of phosphorus metabolism to the addition of 2,4-dinitrophenol (DNP). DNP is a classic, lipid-soluble uncoupler of oxidative phosphorylation. Adding uncouplers allows respiration (electron transport) to occur or may even prompt an increase in whole cells or isolated mitochondria, but respiration-coupled phosphorylation of ADP to ATP can be decreased. It does not appear that DNP has an effect on the NTP or polyphosphate peaks, where the NTP peak has a significant ATP component. However, two aspects of the spectra do change with time. First, a peak emerges between the cytosolic phosphate and PLD peaks at a chemical shift of approximately 1 ppm. Second, the cytosolic phosphate peak tends to shift, indicating acidification throughout the experiment.

The alternate inhibition experiment entailed adding iodoacetate to steady-state cultures growing at 0.05 h^{-1} on yeast extract medium. Iodoacetate addition results in protein alkylation typically via the sulfhydryl moiety, which is present on dehydrogenase active sites. The iodoacetate-mediated reduction of glyceraldehyde-3-phosphate dehydrogenase activity in fermenting yeast with the concomitant accumulation of fructose 1,6-phosphate and triose phosphates was among the key experiments performed by Warburg and others that led to the elucidation of the glycolytic pathway. The results are shown in Figure 6. The NTP peaks decreased rapidly, suggesting that ATP production was decreased. Cytosolic phosphate also decreased, ultimately vanishing, while the sugar phosphate peak became one of the largest peaks in the spectra. Polyphosphate, in contrast, did not hydrolyze to a significant degree.

**Alkalinization Response of Mutant S. cerevisiae.** Figure 7 shows the spectra acquired when vph1-1 yeast cells were subjected to medium alkalinization after attaining a growth rate of 0.05 h^{-1} on yeast extract medium. No polyphosphate was detected, which is an expected outcome of impaired vacuolar membrane energization. However, the different relative abundance of metabolites in the mutant compared to that in the wild-type is interesting. Sugar phosphates and inorganic...
phosphate are more abundant in the mutant. Thus, while many aspects of the mutant's phenotype are comparable to the wild-type (e.g., growth rate on glucose for controlled pH; not shown), NMR-observable metabolic differences exist that may be magnified by stress.

Alkalinization caused the peak in the $\beta$-NTP region to vanish. Because little polyphosphate was present, there is no overlap or adjoining resonances to confound the interpretation of how the $\beta$-NTP resonance changes. Thus, this experiment clearly illustrates that alkalinization diminishes the NTP pool and presumably the ATP concentration. After alkalinization, the phosphate and sugar phosphate peaks shifted upfield, like before. Instead of immediate reacidification occurring, however, there was a 15-min lag, during which the sugar phosphate peak rose while the phosphate peak decreased. Once the sugar peak crested its maximum, the cells began reacidification. Imposing two additional, sequential alkalinizations on this culture produced the same sugar phosphate/phosphate patterns as well as the delay (not shown).

**Discussion**

**Phosphate Uptake, Storage, and Trafficking.** The spectra reveal that orthophosphate uptake and polyphosphate synthesis appear to be regulated in concert. Phosphate-starved yeast immediately transported orthophosphate and incorporated it into polyphosphate (Figure 1). In contrast, cells acclimated to chemostat growth at 0.05 h$^{-1}$ did not respond to increased orthophosphate availability, despite the abiotic concentration being only on the order of 1 mM. These findings parallel the prior descriptions of the “over(hyper)compensation” effect (Weimberg, 1975) and yeast impermeability to phosphate (Borst-Pauwels and Jager, 1969). However, the intracellular view of phosphate dynamics provided by NMR adds more detail. Phosphate is exclusively trafficked to vacuoles despite the many other metabolic processes that compete for phosphate. Regulation appears to assign a high priority to “charging” depleted vacuoles with polyphosphate. From a methodological standpoint, the different responses yeasts display indicates that caution should be exercised when preparing samples for NMR experiments. Prolonged storage prior to resuspension, even at low temperature, could affect metabolic observations if sufficient acclimation time is not provided.

**Cation Trafficking and Osmolarity Challenges.** Cytosolic accumulation of Mn$^{2+}$ required on the order of 30 min (Figure 2). About 1 h was required for the cation to enter the vacuolar compartment. While these time scales will be ion-dependent, the events are consistent with the implications of extraction studies: yeast vacuoles can accumulate cations due to the energized membrane and/or polyphosphate content (Lichko et al., 1982).

**Figure 4.** Response to alkalinization. A high-density yeast chemostat culture was subjected to alkalinization, and fast scans were acquired. Plots depict how (a) phosphate, (b) sugar phosphate, (c) penultimate phosphate (and $\gamma$-ATP), and (d) central polyphosphate levels correlate with intracellular pH dynamics.
Vacuolar metal accumulation, in turn, may endow cells with resistance to toxic metals (Keyhani et al., 1996), storage capability, etc. The possible recovery observed may reflect an alteration in ion distribution or new polyphosphate synthesis.

Within 15 min after NaCl addition, the polyphosphate peak broadened, while the remainder of the spectra was invariant when scaled to the CP resonance (Figure 2). Among microorganisms, yeasts are relatively halotolerant; some strains (e.g., Debaryomyces hansenii) can grow in seawater due to sodium transport mechanisms and glycerol synthesis (Hobot and Jennings, 1981). However, it is noteworthy that the PP4 response we observed for S. cerevisiae was also seen in NMR studies of the halotolerant organism, D. salina (Bental et al., 1990). It was suggested that osmotically driven water flux from vacuoles to cytosol increases polyphosphate chain packing, which due to reduced mobility will manifest as peak broadening and area decrease. Alternately, chain condensation occurs via metabolic reactions, as suggested by the contrast between low- and high-temperature responses (Bental et al., 1990). Other possible events linked to osmotic stress include the following: cell surface-bound polyphosphates are released by osmotic shock (e.g., Tijssen et al., 1983); polyphosphate enlargement maintains concentration when cells shrink (Bental et al., 1991); polyphosphate hydrolyzes in response to Na\(^+\) uptake and/or the action of Na\(^+\)/H\(^+\) antiporters (Weiss et al., 1991); and polyphosphate is used to supplement ATP production (Bental et al., 1990).

Concerning whether these mechanisms operate in yeast, severing polyphosphate from the cell surface would tend to increase mobility; hence, the PP4 peak should narrow, not broaden. No degradation products are evident in Figure 3, suggesting that mechanism may not be operative (or detectable) in our experiment. Nor does it appear that any change in inorganic phosphate level occurred. Finally, as discussed later, the responses to metabolic inhibitors suggest that vacuolar polyphosphate does not function as a phosphagen.

Water flow from vacuoles to the cytosol and/or chain condensation may conceivably operate in yeast. Both could lead to some polyphosphate precipitation as volutin-type granules, which have been isolated from yeast (e.g., Lichko et al., 1982), and account for the polyphosphate resonance broadening and area decreasing.

**Alkalization Response.** The data (Figure 4) appear to be inconsistent with the pH recovery mechanism
entailing the endohydrolysis of polyphosphate to produce tri- and tetrapolyphosphates and H⁺ (Pick et al., 1990; Pick and Weiss, 1991). Although over 50% of the polyphosphate hydrolyzes during the first 6 min (Figure 4d), the pH change is minimal. Another 30% of the polyphosphate hydrolyzes during the next 18 min, and most of the reacidification takes place during this time. It would be expected, however, that the amount of polyphosphate degradation should be proportional to the pH decrease if H⁺ generation from hydrolysis had a significant impact. Furthermore, because less H⁺ is required to drop pH from 8.5 to 7.5 than from pH 7.5 to 6.5, one would expect polyphosphate hydrolysis to have a greater impact on pH early in the experiment.

While the titration of metabolites and macromolecules may contribute, in part, to the inconsistency (e.g., Neurospora proteins; Sanders and Slayman, 1982), the post-alkalinization events appear to more readily conform to a central metabolic response. Sugar phosphates increase (Figure 4b), indicating that increased glycolysis occurs. The increased rate generates substrate-level ATP and acids (e.g., acetate); the latter contributes to cellular and environmental acidification. For glycolytic flux to increase, additional phosphate is required. This phosphate can be polyphosphate-derived as the NMR spectra indicate. Endopolyphosphatases can cut the phosphate polymers into tri- and tetrapolyphosphate. This step concurs with the first γ-NTP/PP1 maximum in Figure 4c and the common observation of tri- and tetrapolyphosphate production after alkalization. It is also consistent with the alkaline pH optimum of many phosphatases. The small polymers, in turn, provide more termini for exopolyphosphatases to excise orthophosphate, akin to the mixed enzyme activities that function during microbially mediated cellulose breakdown. This also explains the orthophosphate lag following alkalization (Figure 4a) and then the maximum. The glucose source can be glycerogen, which, as noted earlier, is accumulated along with glycerogen phosphorylase activity at the growth conditions used. Finally, as the cytosolic pH becomes restored, phosphate and sugar phosphates begin to decrease (Figure 4a and b). Short polyphosphate chains are remade, thus producing the second maximum in the γ-NTP/PP1 profile (Figure 4c). γ-NTP/PP1 ultimately decreases as the short chains lengthen or combine, which leads to PP4 increasing (Figure 4d).

**Phosphagen Potential. Dinitrophenol Addition.**

The spectra (Figure 5) reveal that neither NTP (i.e., ATP) nor polyphosphate changed appreciably after DNP addition. The lack of an acute effect on intact yeast has been reported before (Kaluzhin, 1990). It may reflect the activation of other stress-mediating mechanisms (Weltzel et al., 1985) or incomplete uncoupling of mitochondria under the conditions used. Thus, this experiment cannot provide solid conclusions on the phosphagen potential of polyphosphate. However, other facets of the experiment are interesting. During the experiment, a peak emerged between the phospholipid and cytosolic phosphate peaks. This new peak is attributed to extracellular phosphate. Its location and appearance are consistent with DNP’s inhibitory effect on phosphate uptake (Loomis and Lipmann, 1948; Borst-Pauwels and Jager, 1969). Despite the reduced phosphate influx, polyphosphate is not hydrolyzed.

Another interesting feature is cytosolic acidification. DNP-induced acidification has been observed before (Thevelein et al., 1987), and it is consistent with the ability of DNP to “short circuit” transmembrane H⁺ gradients. However, the cytosolic peak also seems to be oscillating, whereas the other peaks are stable. While this may be an artifact, it may be worth pursuing further because S. cerevisiae is well known for its ability to exhibit induced or spontaneous oscillations (e.g., Aon et al., 1991; Satroutdinov et al., 1992).

**Iodoacetate Addition.** After iodoacetate addition, sugar phosphates rise significantly within 10 min (Figure 6), which is consistent with the known inhibitory effect on glyceraldehyde-3-phosphate dehydrogenase. Thereafter, the sugar phosphate peak splits into several resonances. The increased glyceraldehyde-3-phosphate may be increasing other pathway fluxes via mass action (e.g., to glycerol via L-3-glycerol phosphate; den Hollander and Shulman, 1983) or upstream metabolites via feedback (e.g., hexose phosphates; Campbell-Burk et al., 1987). NTP and thus ATP also vanished, whereas the polyphosphate peak changed little. Therefore, it does not seem that polyphosphate is acting as an effective phosphagen for ATP restoration. Note that, because the mobile and abundant vacuolar pool of polyphosphate is NMR visible and dominates our observations, this conclusion does not rule out the phosphagenic capabilities of polyphosphates in other compartments (e.g., mitochondria). Evidence for an ADP–ATP shuttle or another vehicle that provides cytosolic ADP with access to vacuolar polyphosphate and polyphosphate kinase following an NTP pool collapse has not been provided by prior work (antimycin A addition; Schuddemat et al., 1989) or our iodoacetate addition experiment.

**vph1-1 Mutant Yeast Response to Alkalinization.**

Although the mutant’s phenotype is comparable to the wild-type in some cases (e.g., growth rate on glucose for controlled pH), its NMR spectrum is quite different, indicative of compensatory adaptations. The mutant dearly showed that alkalinization diminishes the NTP and presumably the ATP level. The drop may reflect less ATP production due to the decrease in the transmembrane mitochondrial H⁺ gradient. Unlike the wild-type yeast, the mutant displayed a delay before the onset of reacidification (Figure 7). The delay is explainable by the factors suggested to govern the wild-type’s response. To manage phosphorylation reactions, the mutant has only cytosolic phosphate, ATP, and what can be obtained from the extracellular environment at its disposal. Consequently, glycolytic flux cannot be increased by mobilizing glycogen with vacuolar-derived phosphate. Importing phosphate is problematic because the transport systems used are less active at alkaline pH (Cartwright et al., 1991). Additionally, ATP production is impaired, as is fructose diphosphate production. Reacidification is thus delayed because it takes longer to prime glycolysis. The lack of polyphosphate hydrolysis may also contribute somewhat if hydrolysis normally consumes some initial titration capacity.

In summary, the results from the NMR cultivator show that phosphate can be efficiently directed to vacuoles in starved cells despite other competing sinks. It can take on the order of 1 h for a cation to pass through all cellular compartments, and like alga, yeast polyphosphate may exhibit a response to hyperosmolarity. Vacular polyphosphate and glycogen reserves may work in tandem, as illustrated by the phosphate, polyphosphate, and sugar phosphate postalkalinization dynamics in wild-type and mutant yeast. The joint activities of two enzymes are needed, as opposed to assuming endophosphatase activity is dominant. The latter can be problematic because endo- and exopolyphosphatase activities have both been recovered from S. cerevisiae vacuolar membrane fractions (Klionsky et al., 1990). The value of producing tri- and
tetrapolyphosphoric acids for reacidification may still exist. However, the impact may be difficult to discern because the impact of all $\text{H}^+$-generating reactions may be initially masked by titration effects. Rapidly readying carbon reserves for central metabolism, however, simultaneously solves two problems: (substrate-level) ATP production and reacidification. Polyphosphate does not appear to be phosphagenic when iodoacetate inhibits ATP production. Finally, the results may provide some insights on yeast ecology. Yeasts acidify their niches, which provides one means to compete with bacteria. The investment in carbon and phosphorous storage compounds may ensure that the future ability exists to rapidly engage central metabolism for niche maintenance during environmental pH changes. These results also raise the question of whether polyphosphate oscillations accompany induced glycogen and trehalose oscillations (Sastroutdinov et al., 1992).

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References and Notes


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