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ORIGINAL PAPER

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Novel method for the quantification of inorganic polyphosphate (iPoP) in *Saccharomyces cerevisiae* shows dependence of iPoP content on the growth phase

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Abstract Inorganic polyphosphate (iPoP)—linear chains of up to hundreds of phosphate residues—is ubiquitous in nature and appears to be involved in many different cellular processes. In *Saccharomyces cerevisiae*, iPoP has been detected in high concentrations, especially after transfer of phosphate-deprived cells to a high-phosphate medium. Here, the dynamics of iPoP synthesis in yeast as a function of the growth phase as well as glucose and phosphate availability have been investigated. To address this question, a simple, fast and novel method for the quantification of iPoP from yeast was developed. Both the iPoP content during growth and the iPoP "overplus" were highest towards the end of the exponential phase, when glucose became limiting. Accumulation of iPoP during growth required excess of free phosphate, while the iPoP "overplus" was only observed after the shift from low- to high-phosphate medium. The newly developed iPoP quantification method and the knowledge about the dynamics of iPoP content during growth made it possible to define specific growth conditions for the analysis of iPoP levels. These experimental procedures will be essential for the large-scale analysis of various mutant strains or the comparison of different growth conditions.

Keywords Inorganic polyphosphate · PolyP · *Saccharomyces cerevisiae* · Yeast · "Overplus," Growth phase · Diauxic shift · Polyphosphate quantification method

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Introduction

Inorganic polyphosphate (iPoP) consists of linear chains of few to several hundred phosphate residues linked by high-energy phosphoanhydride bonds. It is a widely distributed yet little studied polymer, which has been found in every organism studied so far (Kornberg et al. 1999). iPoP is involved in many functions and processes in prokaryotes (Kornberg et al. 1999; Ogawa et al. 2000; Rashid et al. 2000; Kuroda et al. 2001; Kim et al. 2002), but it has various functions in eukaryotes as well (for a review see Kulaev and Kulakovskaya 2000). Among these are the storage of phosphate (Martinez et al. 1998) and energy (Gomez-Garcia and Kornberg 2004), the role of a polyanionic counterion in fungal cell walls (Datema et al. 1977), the regulation of enzyme activity (Wang et al. 2003), the involvement in stress responses (Pick and Weiss 1991) and most probably the regulation of gene activity (Kulaev and Kulakovskaya 2000). Recently, a polymeric enzyme consisting of actin-related proteins was purified from the slime mold *Dictostelium discoideum* (DdPpk2) and shown to form polyphosphate chains during the polymerization of actin-like filaments (Gomez-Garcia and Kornberg 2004). Despite these proven functions and its ubiquitous distribution, the mechanisms governing iPoP accumulation are only partially understood, and enzymes responsible for iPoP synthesis in higher eukaryotes have not been characterized at all. *Saccharomyces cerevisiae* provides an appropriate model system for the study of the role of iPoP in eukaryotes, since iPoP has been detected in this organism in high concentrations under certain culture conditions. After transfer from a medium devoid of phosphate to a medium containing high concentrations of orthophosphate and an energy source, *S. cerevisiae* assimilates phosphate rapidly and condenses it to polyphosphate (Schmidt et al. 1946; Wiame 1947). This phenomenon has been termed the polyphosphate "overplus" (for a review see Harold 1966). It has also been shown that

the iPoP content of yeast cells depends strongly on the phosphate concentration in the medium: under conditions of extracellular phosphate deprivation, the intracellular iPoP pool diminishes to almost zero, whereas at the same time the intracellular levels of free phosphate remain constant (Martinez et al. 1998). This indicates a role of iPoP as a buffer in the homeostasis of intracellular phosphate concentrations (Martinez et al. 1998). The synthesis of iPoP also strongly depends on the availability and nature of an energy source. Fermentable energy sources, such as glucose, lead to much higher iPoP concentrations as compared to non-fermentable energy sources such as ethanol, suggesting that mainly energy derived from fermentation is used for the production of iPoP (Schuddemat et al. 1989). The finding that antimycin, a drug blocking respiration, had no effect on the synthesis of iPoP during "overplus" conditions supports this theory (Schuddemat et al. 1989).

In order to employ *S. cerevisiae* as a model system for the study of iPoP metabolism and function, it is essential to know the dynamics of iPoP synthesis and its dependence on culture conditions. However, comprehensive studies of iPoP content during growth with iPoP-specific methods are lacking and our initial attempts to quantify iPoP in yeast led to very inconsistent results. To address this problem, we first developed a simple, fast and novel method for the extraction, purification and quantification of iPoP from yeast, which is amenable for high-throughput analyses. This method was then used to study the influence of growth phase, glucose and phosphate on the iPoP accumulation of *S. cerevisiae*. Thereby, we were able to define specific culture conditions that lead to reproducible and predictable iPoP quantifications.

Materials and methods

Strains and culture conditions

Saccharomyces cerevisiae (strain BY4741 *Mat*a *his3Δ1 leu2Δ0 met15Δ0 ura3Δ0*) (Brachmann et al. 1998) was grown at 30°C under aerobic conditions in YPD medium (1% yeast extract–2% peptone–2% glucose). Growth was measured by determining the absorbance at 600 nm (OD_{600}). Low orthophosphate YPD medium (YPD-Pi) was made by precipitating free phosphate with ammonium as described (Kaneko et al. 1982), with the difference that the medium was filter-sterilized rather than autoclaved to prevent release of bound phosphate. Therefore, YPD-Pi medium contained more bound phosphate (1.5 mM) as compared to autoclaved YPD (0.7 mM). The free orthophosphate concentrations of YPD-Pi and YPD medium were 0.15 and 2 mM, respectively. To obtain media with different phosphate or glucose contents, appropriate volumes of 1 M sodium phosphate or 20% glucose, respectively, were added to 1.11 times concentrated YPD-Pi or YP (yeast extract–peptone).

To determine the iPoP content during growth, cells were grown in volumes of 0.5 ml in 96-well deep well plates. As an inoculum, an aliquot from cultures grown for 24 h in the corresponding medium was used. At every time point, cell densities, cellular iPoP contents and phosphate or glucose concentrations in the medium were determined. For each time point, three independent cultures were used.

To generate the iPoP "overplus," cells were grown in 5 ml YPD-Pi for 24 h to stationary phase and diluted with fresh YPD-Pi to a cell density of $OD_{600} = 1$ (in volumes of 5 ml). This primary culture was grown to an OD_{600} between 5 and 6. Cells were then diluted with normal YPD to an OD_{600} of 0.5. This secondary culture was split into volumes of 0.5 ml and grown in 96-well culture plates. To characterize the iPoP "overplus," cell density and iPoP contents were determined after 0, 1 and 3 h.

To test the ability of yeast cells in different growth stages to exhibit an iPoP "overplus," a primary culture was started in YPD-Pi by inoculation with cells grown for 24 h in YPD-Pi. Cells from this primary culture were tested after 2, 6, 10, 24 and 52 h for their ability to exhibit an iPoP "overplus" by diluting them with fresh YPD as described above. Cell densities and iPoP contents of these secondary cultures were again monitored for 25 h.

Exopolyphosphatase from *S. cerevisiae* (ScPpx1)

The gene encoding the exopolyphosphatase of *S. cerevisiae* (ScPpx1) (Wurst and Kornberg 1994) was amplified from isolated genomic DNA of the yeast strain BY4741 and cloned into the expression vector pKM263 (Melcher 2000) using standard methods. The enzyme was expressed in *E. coli* BL21 cells and purified with the help of an N-terminal histidine tag following recommended standard procedures (QIAGEN 2003).

Polyphosphate purification and quantification

Our iPoP purification protocol is based on the method of Ault-Riché and Kornberg (1999) with the most significant difference being the use of columns with silica-gel rather than glassmilk. iPoP from 0.5 or 1 OD_{600} units of harvested yeast cells was extracted for maximal 5 min at RT after resuspending the cells in 50 µl 1 M sulfuric acid, since iPoP is slowly hydrolyzed under acidic condition. The suspension was neutralized with 50 µl of 2 M NaOH and 100 µl of 1 M Tris pH 7.5 (or alternatively 1 M Tris/1 M malate pH 7.5), supplemented to 6% with a neutral red solution (0.1% neutral red in 70% ethanol) as a pH indicator. The pH indicator helped to assure that the pH was around 7, which was optimal for binding and

further purification. Samples too acidic (pink) or too basic (yellow) were corrected to the appropriate pH (orange–red) by addition of NaOH or sulfuric acid, respectively. Cell fragments were removed by centrifugation. After addition of 600 µl of 6 M NaI, the extracts were applied to either single Qiagen PCR purification columns or Qiagen 96 PCR purification plates. The columns were washed twice with wash buffer (10 mM Tris buffer pH 7.5, 50% ethanol, 1 mM EDTA and 100 mM NaCl). iPoP was eluted in 50 µl water. PCR purification columns or plates were regenerated by washing once with 0.2 M acetic acid and 50 mM EDTA (pH 8), and three times with water, each time using volumes of 750 µl. Performance of the columns/plates was monitored regularly to assure their quality of iPoP binding. iPoP was specifically digested by addition of 6×10^5 U (1 U corresponds to the release of 1 pmole Pi $\times$ min^{-1} at 37°C) of the exopolyphosphatase (ScPpx1) diluted in 50 µl of 0.1 M Tris (pH 7.2) and 10 mM $MgCl_2$, as described previously (Wurst et al. 1995). The reaction was carried out for 1 h at 37°C. To quantify released Pi, 86 µl of 28 mM ammonium heptamolybdate in 2.1 M H_2SO_4 and 64 µl of 0.76 mM malachite green in 0.35% polyvinyl alcohol were added. The OD_{595} was measured in a Dynatech MR 5000 Elisa Reader and compared with that of the phosphate standards (100 µl of 100, 80, 60, 40, 20, 10 and 5 µM Pi) that were included in each set of samples (Cogan et al. 1999).

Phosphate and glucose quantification

Pi in the medium was determined by a colorimetric assay as described in Cogan et al. (1999). Glucose content of the medium was measured after pelleting the cells using the glucose quantification kit from Megazyme (D-Glucose (GOPOD Format) Assay Kit).

Reproducibility and statistics

Each experiment was performed at least twice. Every point shown in the figures represents an average value obtained from three individually analysed yeast cultures, which were harvested at the same time. The error bars indicate the standard error derived from those three values. Error bars not visible were smaller than the symbols representing the average values.

Results

iPoP purification and quantification

The presence of highly active polyphosphatases in yeast is a problem for the quantification of iPoP in this organism. To overcome this difficulty, we used 1 M sulphuric acid to kill the yeast cells and to denature all proteins as fast as possible. To extract all iPoP the sulphuric acid suspension was neutralized by the addition of 2 M NaOH and 1 M Tris–malate buffer, which resulted in a combined acidic and high salt extraction. Repeated extractions recovered more iPoP from cells containing high iPoP concentrations. But both digestion of the remaining pellet with exopolyphosphatase and extraction of the pellet with NaOH released only very small amounts of iPoP that were proportional to the concentration measured with the standard extraction (not shown). It was therefore concluded that this iPoP corresponded to a remaining residue of the H_2SO_4/salt extraction and did not represent an additional insoluble fraction. iPoP was then purified by a method based on that of Ault-Riché and Kornberg (1999), who used glassmilk. Instead of glassmilk, we used Qiagen PCR purification columns containing silica-gel membranes to reliably purify and quantify iPoP. Just as for the purification of DNA, the optimal pH for binding of iPoP to the Qiagen columns was between 6.5 and 7.5 (not shown). The binding capacity of a column for iPoP exceeded 100 µg. Recovery of iPoP was about 80% and declined if less than 10 µg iPoP was applied to a column. Therefore, low iPoP levels are underestimated, but it was possible to detect iPoP amounts as low as 0.25 µg. Repeated regeneration and reuse of the columns did not affect the sensitivity of the iPoP measurements or reduce the binding capacity of the columns (not shown). Neither free phosphate nor repeated use of the columns caused a background without digestion with the iPoP-specific polyphosphatase. This method for the extraction, purification and quantification of iPoP proved to be reliable and fast enough to process several hundred samples per day.

Glucose and orthophosphate in the medium are required for iPoP accumulation

iPoP is a storage form not only for phosphate but also for energy, which is stored in the phosphoanhydride bonds linking the orthophosphate units. As a consequence, the production of iPoP is expected to depend on the type and amount of energy source that is provided. To verify this correlation, yeast was grown in YPD media containing different glucose concentrations, and cellular iPoP levels were determined at different time points during growth (Fig. 1a, c). High iPoP contents were found towards the end of the exponential phase, when the medium was supplemented with 1 and 2% glucose (Fig. 1c). Higher glucose concentrations led to the accumulation of more iPoP. During post-diauxic growth (for the definition of growth stages in *S. cerevisiae* see Stahl et al. 2004), iPoP contents decreased again to low levels (< 1 µg per OD_{600} unit). A comparison of iPoP and glucose concentrations showed that increased cellular iPoP contents (> 2 µg per OD_{600} unit) were

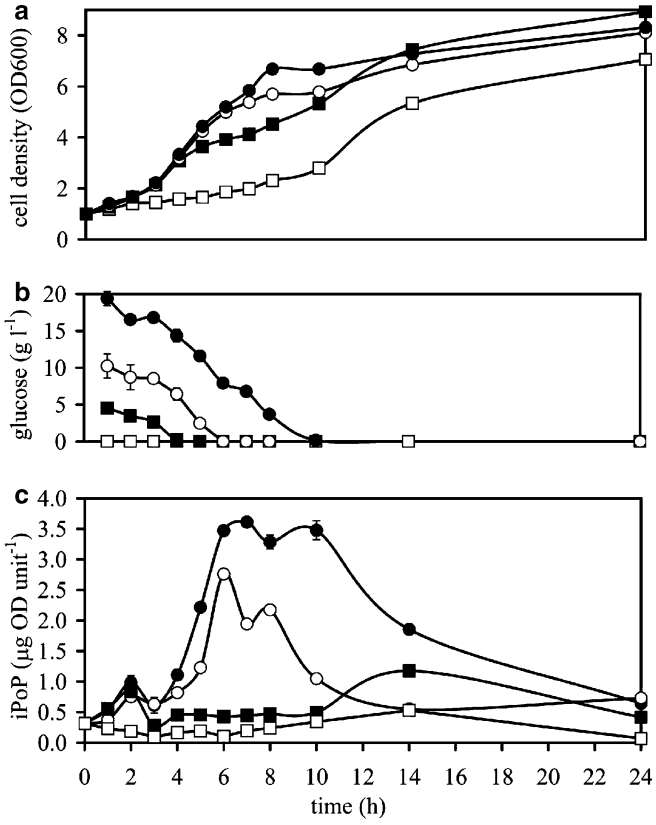

Fig. 1 Relationship between iPoP content, glucose availability and growth of *S. cerevisiae*. Cells cultivated for 24 h were diluted with media containing 0% (*open square*), 0.5% (*filled square*), 1% (*open circle*) and 2% (*filled circle*) glucose and grown for 1–24 h in YPD. **a** Cell density, **b** glucose concentration and **c** iPoP contents were determined at the indicated time points during growth

observed as long as the medium contained glucose (Fig. 1b).

The extent to which the availability of Pi in the medium affects iPoP accumulation is evident from a comparison of Figs. 2a and 3a, where iPoP contents of yeast cells were determined during growth in YPD and YPD-Pi. Cells grown under high-phosphate conditions accumulated up to 4 µg of iPoP per OD_{600} unit, whereas iPoP in cells cultured in YPD-Pi was hardly detectable. The iPoP concentration in yeast grown in YPD increased during exponential growth and declined thereafter (Fig. 2a). Despite the large difference in iPoP content, the growth rate of *S. cerevisiae* in YPD-Pi was indistinguishable from that in YPD.

iPoP "overplus" as a function of growth phase

To assess the iPoP synthesizing activity of yeast cells in vivo in different growth stages, cells were grown in YPD and YPD-Pi and tested for their ability to perform an iPoP "overplus" at different time points (Figs. 2 and 3). iPoP concentration in cells previously grown in YPD declined within the first hour after transfer to fresh medium (Fig. 2b–f). After the first hour, iPoP concentration rose again and followed a similar course as observed before for yeast cells in YPD. Only cells previously grown in YPD-Pi increased their iPoP concentrations within the first hour after dilution into fresh YPD and were thus able to exhibit an iPoP "overplus" (Fig. 3b–f). Cells tested at the late exponential phase exhibited the strongest "overplus": within 1 h, their cellular iPoP levels increased from an undetectable level to about 11 µg per OD_{600} unit (Fig. 3c). During the next 2 h, iPoP concentrations declined rapidly. Cells tested at an earlier stage during exponential growth and cells in the early post-diauxic phase reached only about half of the maximal iPoP levels during the iPoP "overplus" (Fig. 3b–d). Cells that already had reached the stationary phase produced no iPoP "overplus" and accumulated iPoP similarly to a culture that had not been deprived of phosphate (Fig. 3f). Seven hours after induction, or towards the end of exponential growth, all secondary cultures reached similar iPoP levels of 4–6 µg per OD_{600} unit. After the switch to the post-diauxic phase, iPoP contents declined in all cultures to low levels (< 1 µg per OD_{600} unit), as observed before.

Dependence of the iPoP "overplus" on phosphate deprivation and glucose availability

To study the degree of phosphate deprivation necessary for the induction of an iPoP "overplus," cells of *S. cerevisiae* were grown to late exponential phase in media with different Pi concentrations and tested for their ability to rapidly accumulate iPoP (Fig. 4a). This experiment showed a production of higher cellular iPoP concentrations after preculturing in media with lower orthophosphate content. A clear "overplus," defined by iPoP accumulation within the first hour of more than 6 µg per OD_{600} unit, was measured if the preculture contained ≤ 0.6 mM Pi. Cells grown in primary culture containing 2.4 or 4.28 mM Pi, respectively, had already accumulated more than 2 µg iPoP per OD_{600} at the time of dilution into fresh YPD and did not further increase their reserves of iPoP. Cells grown with 1.2 mM orthophosphate to late exponential phase can be considered to be on the threshold to an "overplus" since the accumulated iPoP during the first hour reached about 3 µg per OD_{600} unit, which did not exceed levels measured during normal growth in YPD.

Since the presence of glucose was essential for the accumulation of higher iPoP concentrations (Schuddemat et al. 1989, Fig. 1c), we assessed whether the rapid synthesis of iPoP during "overplus" conditions was also directly dependent on an external energy source. Thus, iPoP "overplus" experiments were carried out with different glucose concentrations in the secondary culture. The extent of the iPoP "overplus" was clearly dependent on the concentration of glucose in the medium (Fig. 4b). However, yeast cells transferred to the medium without additional glucose exhibited an "overplus" as well, albeit

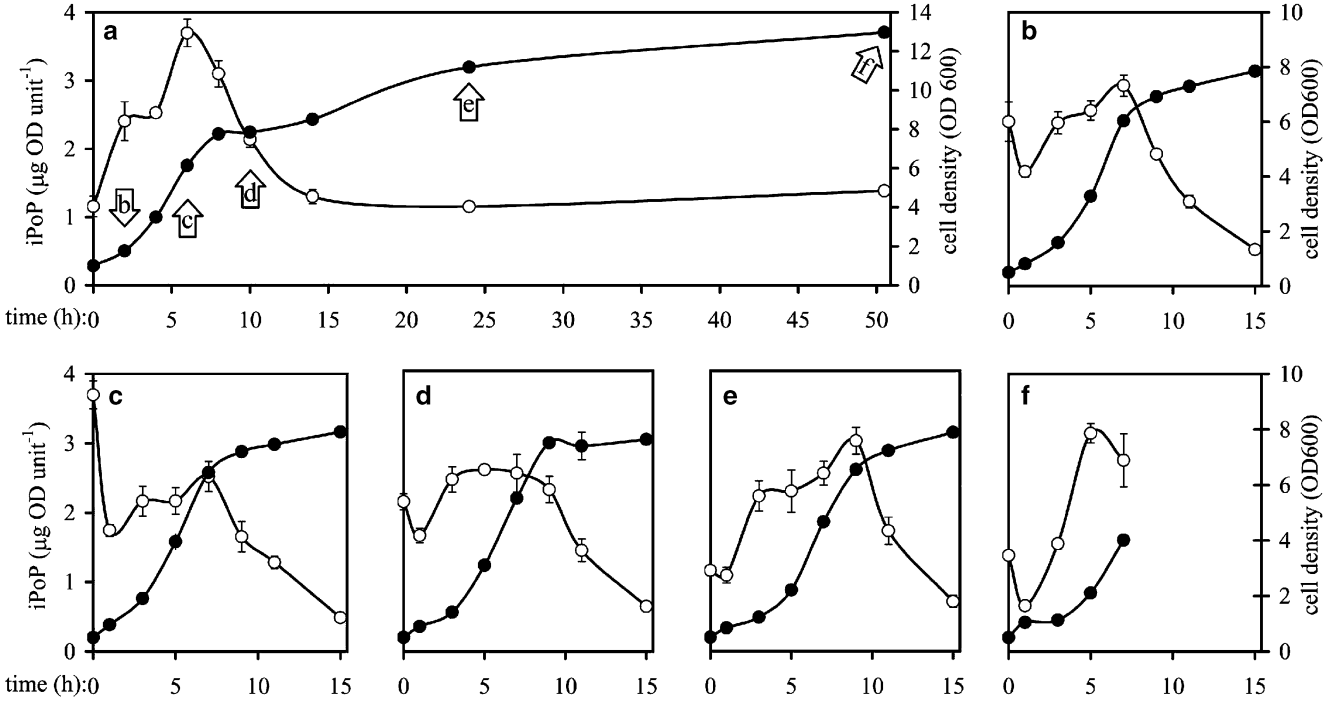

Fig. 2 Changes in iPoP levels in yeast cells upon dilution into fresh medium after preculturing to different growth stages. **a** Growth of *S. cerevisiae* in YPD. Cell density (*filled circle*) and iPoP concentrations (*open circle*) were monitored at different time points during growth. After 2, 6, 10, 24 and 52 h (indicated by *arrows b–f*), cells from this primary culture were transferred to fresh YPD. **b–f** Cell density (*filled circle*) and iPoP concentrations (*open circle*) of these secondary cultures were measured at time intervals of 15 h

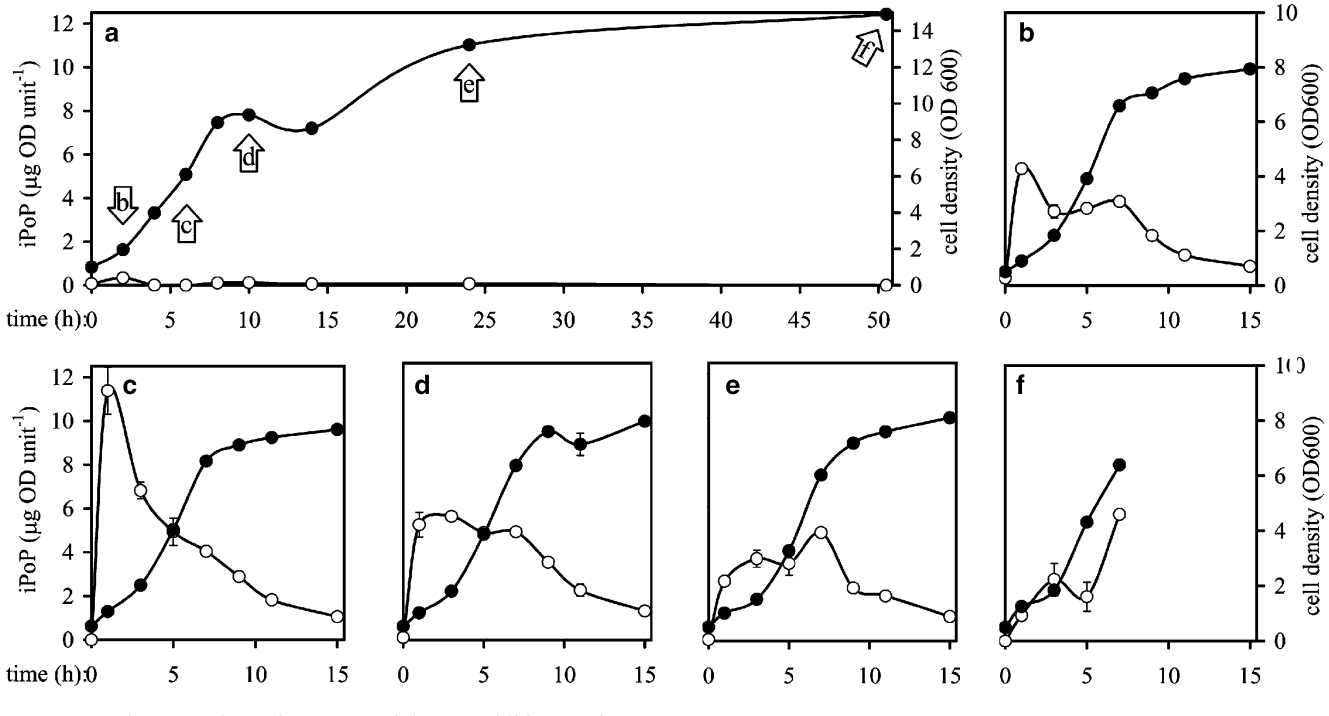

Fig. 3 Growth-stage dependent potential to exhibit an iPoP "overplus" of yeast cells deprived of phosphate. **a** Growth of *S. cerevisiae* in YPD-Pi. Cell density (*filled circle*) and iPoP contents (*open circle*) were monitored at different time points during growth. After 2, 6, 10, 24 and 52 h (indicated by *arrows b–f*), cells from this primary culture were transferred to YPD. **b–f** Growth of these secondary cultures was again monitored by measuring the cell density (*filled circle*) and intracellular iPoP (*open circle*) was quantified simultaneously

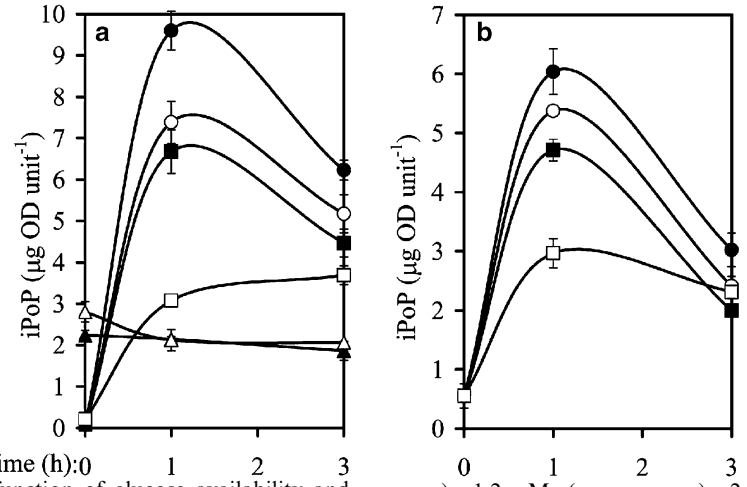

Fig. 4 iPoP "overplus" as a function of glucose availability and phosphate deprivation. The iPoP "overplus" was characterized by determining the iPoP content in cells at 0, 1 and 3 h after transfer to high-phosphate media. **a** The extent of iPoP "overplus" was measured after preculturing yeast cells for 6 h in media containing 0.15 mM (*filled circle*), 0.3 mM (*open circle*), 0.6 mM (*filled square*), 1.2 mM (*open square*), 2.4 mM (*filled triangle*) and 4.28 mM (*open triangle*) orthophosphate. **b** iPoP "overplus" was achieved by transferring phosphate-deprived cells to YP media containing 0% (*open square*), 0.5% (*filled square*), 1% (*open circle*) and 2% (*filled circle*) glucose

reduced to about 50% as compared to the iPoP level obtained with 2% glucose (Fig. 4b). In this case other extracellular energy sources present in YP medium are likely to be used for iPoP synthesis and effect a reduced, but clearly recognizable, iPoP "overplus" in the absence of glucose.

Discussion

For detailed studies of the synthesis and function of iPoP in yeast, exact and reliable knowledge of the temporal changes of the iPoP content in standard growth conditions are essential. The goal of this work was to address this problem and to define experimental conditions that allow reproducible and comparable iPoP quantification. For this purpose we first developed a reliable, fast and most importantly iPoP-specific method for the quantification of iPoP. iPoP was extracted with 1 M sulfuric acid, 2 M NaOH and 1 M Tris–malate buffer, which represents a combination of acid and high salt extractions as described previously (Langen and Liss 1958; Vagabov et al. 2000). After this extraction protocol we could recover significant additional iPoP amounts neither by a basic extraction nor by digestion of the remaining pellet with exopolyphosphatase. This agrees with the conclusion that the distinct acid-, high salt-soluble and acid-insoluble iPoP fractions, which are sometimes distinguished (Langen and Liss 1958; Vagabov et al. 1998; Kulaev et al. 1999; Vagabov et al. 2000; Kulakovskaya et al. 2003; Kulaev et al. 2004), are rather an artifact of the extraction procedure (Harold 1966; Indge 1968; Beever and Burns 1980). We therefore conclude that the iPoP, which is extracted by the presented protocol, corresponds to the total iPoP content of the collected yeast cells. The extracted iPoP was bound to silica-gel under high salt conditions, followed by elution with water, digestion with a specific recombinant polyphosphatase and quantification of the released phosphate by a colorimetric assay. This method gave accurate and reliable results with very small culture volumes, making it possible to analyse hundreds of samples per day.

To validate our iPoP extraction and quantification protocol we measured iPoP content during normal growth of *S. cerevisiae* and during "overplus" conditions in dependence of the free phosphate and glucose concentrations in the growth medium. These experiments confirmed previous findings of iPoP measurements at different time points and in different media (Katchman and Fetty 1955; Ehrenberg 1961; Liss and Langen 1962; Solimene et al. 1980; Schuddemat et al. 1989; Martinez et al. 1998; Vagabov et al. 1998, 2000; Kulaev et al. 1999, 2004; Kulakovskaya et al. 2003). But the much larger number of samples and time points analysed and in particular the experiment comprising the two consecutive time-courses revealed new insight into the dynamics of iPoP synthesis. Contrary to most previous descriptions, in our case iPoP content during "overplus" conditions reached a peak within 1 h and declined to normal level after another 3–5 h (Fig. 3b, c). Interestingly, it was not necessary to grow the yeast cells to stationary phase or to starve *S. cerevisiae* completely prior to the "overplus" experiment. The prerequisites for an "overplus" were only the reduction of the free phosphate concentration in the preculture medium and the correct growth phase of the cells as well as an excess of free phosphate in the fresh medium. Yeast cells harvested from the late exponential phase (from YPD-Pi medium) exhibited the highest iPoP "overplus" if transferred to YPD and *S. cerevisiae* grown with an excess of free phosphate had the highest iPoP concentration at the late

exponential phase as well. This strong dependence of the iPoP synthesis on the growth stage of the cells has been hypothesized (Kulakovskaya et al. 2003), but never been shown before and suggests a strict regulation of the synthesis of this polymer. This is also emphasized by the fact that iPoP content of yeast cells was maximal at the late exponential phase, when glucose became depleted. This growth stage, also called the diauxic shift, is characterized by the metabolic switch from fermentative growth to respiration (DeRisi et al. 1997; Stahl et al. 2004) and seems to be most supportive for iPoP synthesis. We therefore conclude that a growth-phase dependent mechanism is responsible for the regulation of iPoP synthesis, both during normal growth and during the iPoP "overplus."

In this work we present a new iPoP extraction and quantification method that is specific, accurate and reliable and that is amenable for large-scale analyses. This method was used to study the iPoP content in *S. cerevisiae* under different growth conditions and at various time points during growth. Since even the growth stage of the preculture from normal YPD medium did influence iPoP content, in the next culture we suggest to: (a) always use cells from stationary phase (after 24 h in YPD) as an inoculum of the culture to be quantified, (b) determine the iPoP content at the end of the exponential phase when the broad peak of the iPoP maximum is observed and (c) use cells grown in media with low free phosphate content from the late exponential phase to induce an iPoP "overplus." It remains to be tested if this hyperaccumulation of iPoP during the "overplus" or the accumulation during growth in high Pi media also has a direct physiological function. Although we have observed that the growth rate is not dependent on the synthesis of iPoP, cells containing high amounts of iPoP might be more stress resistant or competitive in their environment as has been observed in *E. coli* (Crooke et al. 1994).

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